Chimeric DNA methyltransferases target DNA methylation to specific DNA sequences and repress expression of target genes

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ABSTRACT

Gene silencing by targeted DNA methylation has potential applications in basic research and therapy. To establish targeted methylation in human cell lines, the catalytic domains (CDs) of mouse Dnmt3a and Dnmt3b DNA methyltransferases (MTases) were fused to different DNA binding domains (DBD) of GAL4 and an engineered Cys2His2 zinc finger domain. We demonstrated that (i) Dense DNA methylation can be targeted to specific regions in gene promoters using chimeric DNA MTases. (ii) Site-specific methylation leads to repression of genes controlled by various cellular or viral promoters. (iii) Mutations affecting any of the DBD, MTase or target DNA sequences reduce targeted methylation and gene silencing. (iv) Targeted DNA methylation is effective in repressing Herpes Simplex Virus type 1 (HSV-1) infection in cell culture with the viral titer reduced by at least 18-fold in the presence of an MTase fused to an engineered zinc finger DBD, which binds a single site in the promoter of HSV-1 gene IE175k. In short, we show here that it is possible to direct DNA MTase activity to predetermined sites in DNA, achieve targeted gene silencing in mammalian cell lines and interfere with HSV-1 propagation.

INTRODUCTION

Methylation of cytosine residues at CG dinucleotide sites in DNA is an important epigenetic modification (1–4) that, in general, leads to gene silencing (5–8). Aberrant changes in DNA methylation often contribute to tumorigenesis (9,10) and etiology of other diseases (11). Gene repression by DNA methylation is mediated by methyl-cytosine binding proteins assembled on methylated CGs (3). These proteins recruit corepressors like mSin3 or Mi2-NuRD and histone deacetylase and trigger the formation of condensed, repressive chromatin, which leads to stable inactivation of gene expression (12). Another repressive mechanism of DNA methylation is to interfere with the DNA binding of transcription factors (13,14).

DNA methylation is established by *de novo* DNA methyltransferases (MTases), Dnmt3a and Dnmt3b, during early embryogenesis and later maintained by Dnmt1 (2,6,15). The C-terminal catalytic domains (CDs) of Dnmt3a and 3b are active in the absence of their N-terminal part (16,17). They can be fused to a heterologous DNA binding domain (DBD) in order to target methylation activity to a predetermined DNA sequence (18–20). Targeted DNA methylation was observed *in vitro* using a fusion protein consisting of the DBD of Zif268 and the prokaryotic CG methytransferase M.SssI (18). In addition, in yeast cells, Carvin *et al*. demonstrated targeted methylation, after fusing the transcriptional factor PHO5 to the viral M.CviPI MTase as well as fusing the DBDs of zinc-finger proteins based on Zif268 with M.CviPI and M.SssI (19,21). Targeted methylation also has been observed using the bacterial Dam adenine-N6 MTase and it has been applied to map the DNA binding sites of transcription factors (20). However, so far gene silencing by targeted methylation has not been demonstrated.

In this study, we fused DBDs of transcription factors or engineered zinc-finger proteins to the CDs of either the Dnmt3a or Dnmt3b DNA MTases in order to achieve targeted DNA methylation and selective silencing of gene expression. These fusion proteins were directed to the specific target sites by the DBDs, such that DNA methylation only occurs in the vicinity of these target sites. We here report targeted DNA methylation as well as efficient gene silencing in three different reporter systems with presumably very different natural

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gene regulatory elements. This includes the TK (Human herpesvirus 1 thymidylate kinase) promoter with added binding sequence for GAL4 (UAS), the human c-Ha-ras gene promoter with added UAS and the immediate early IE175k promoter of the Herpes Simplex Virus type 1 (HSV-1). We also show that methylation-mediated gene silencing is effective in repressing infection with a wild type HSV-1. This opens possibilities of using this strategy as an anti-viral treatment against other, more serious human pathogens.

MATERIALS AND METHODS

Vectors construction

Construction of reporter genes. Minimal TK promoter (158 bp) containing five repeats of the GAL4 binding sequence (UAS) upstream of the promoter (22) was amplified by PCR and cloned into pGL3-basic luciferase reporter gene vector (Promega) to obtain pGL-5×UAS-Luc. TK promoter alone was also inserted into pGL3-basic as a non-targeted reporter gene control, named pGL-TK-Luc. Both promoter-reporter fusions were subsequently cloned into pcDNA3.1-neo as BglII–NotI fragments to construct 5×UAS-TK-Luc and TK-Luc that were used in all the co-transfection experiments. In addition, pcDNA-UAS-luciferase containing one UAS upstream of the TK was constructed (UAS-TK-Luc).

Human c-Ha-ras promoter (GenBank entry number: M13221.1) was amplified from HEK293T genomic DNA, and cloned into MluI and HindIII sites of pGL3-basic. A single GAL4 binding sequence (1×UAS) was introduced upstream of the promoter to generate UAS-ras-Luc. The entire IE175k promoter region (positions from −380 to +30) (23) of HSV-1 was amplified from the plasmid pPO13 (gifted by P. O'Hare), and cloned into pGL3-basic to obtain IE175-Luc. The binding site for B1 and 6F6 zinc-finger proteins (5′-GATCGGGCGGTAATGAGAT-3′) was deleted from IE175k promoter by PCR-based mutagenesis to generate IE175mut-Luc.

Construction of targeting methyltransferases. The GAL4 DNA binding domain (GBD, 1–147 amino acid of P04386) containing a 30 amino acid linker including a myc-tag was amplified by PCR from pGBKT7 (BD Biosciences) and equipped with a nuclear localization signal (PKKKKRK) at the C-terminus of GBD. The GBD was cloned into pcDNA3.1-neo (Invitrogen), to generate pcDNA-GBD. The CD of Dnmt3a (CD-Dnmt3a, 598–908 amino acid of o88508) and Dnmt3b (CD-Dnmt3b, 557–859 amino acid of NP_001003961) were cloned into pcDNA-GBD in frame with GBD to generate GBD-3a and GBD-3b. Specific mutations were introduced into the active site ENV motif of CD-Dnmt3a and CD-Dnmt3b, by changing the ENV sequence to ANV, and into coenzyme binding motif II (E74A) of CD-Dnmt3a. (The ENV to ANV mutation corresponds to E752A in full-length Dnmt3a and E703A in full-length Dnmt3b. The CD-Dnmt3a-E74A mutation corresponds to E660A in full-length Dnmt3a.) The CD-Dnmt3a-ENV, CD-Dnmt3b-ENV and CD-Dnmt3a-E74A variants were shown to be catalytically inactive (17,24). Also, two point mutations (L32P and C38G) were introduced to GAL4 domain to create mutGBD-3a and mutGBD-3b. These mutations were

previously demonstrated to abolish the binding of GAL4 to its target sequence completely (25,26). DNA fragments coding for zinc-finger proteins 6F6 (containing six zinc fingers and binding to −272 to −253 of HSV-1 IE175k promoter) and B1 (containing three zinc fingers and binding to −262 to −253 of HSV-1 IE175k promoter) (23) were amplified by high-fidelity PCR and cloned into pcDNA4-myc-His-A (Invitrogen) at HindIII and BamHI sites. The CDs of Dnmt3a, Dnmt3b and their mutants (described above) were cloned at BamHI and XbaI sites in frame with the zinc-finger domains and the C-terminal myc-His tag. However, no expression of the 6F6 and B1 fusion proteins containing Dnmt3b CD was detectable. Therefore, for further experiments with 6F6 and B1 only the Dnmt3a CD fusions were used. The plasmid RG50 containing the HSV-1 VP16 gene under the control of a CMV promoter, used for IE175k promoter activation studies, was kindly provided by P. O'Hare. All plasmids used for transfections with Transfast (Promega) were prepared using Maxiprep Kit (Qiagen) while plasmids used for electroporation with Amaxa system (Amaxa) were purified using Qiafilter Midi Kit (Qiagen).

Cell culture and reporter gene assays

HEK293T, HCT-116 and HeLa cells were gifted by the Institute of Genetics, Justus-Liebig University of Giessen while COS-7 cells were provided by M. Stocks of MRC LMB, Cambridge, UK. Cells were cultured in DMEM (PAA laboratories GmbH) supplemented with 10% fetal calf serum (FCS) (PAA laboratories GmbH). Briefly, the HEK293T cells were seeded in 24-well plates coated with polylysine, at the density of $3–5 \times 10^4$ cells/well. Plasmids for co-transfection, which included MTase gene vector (100–250 ng/well), GFP reporter gene (200 ng/well), *Renilla* luciferase reporter gene controlled by cytomegalovirus (CMV) (5 ng/well) (Promega) and target firefly luciferase reporter gene (5–50 ng), were diluted with serum free DMEM culture medium (200 μl/well) and mixed with 1 μl Transfast™ reagent (Promega). The transfection was performed as recommended by the supplier. The efficiency of transfection was monitored by green fluorescent protein (GFP) signal count under fluorescence microscope. Four days after transfection, the culture medium was removed and cells were lysed by adding 300 μl lysis buffer from *Renilla* Luciferase Assay System (Promega, Cat. E2810) to each well. Samples of 100 and 20 μl crude cell lysate were transferred to different wells of a non-transparent micro well plate (Packard) for firefly and *Renilla* luciferase activity assay, respectively. The luciferase activity (luminescence signal) was determined by Topcount®NXT™ Microplate Scintillation & Luminescence Counter (Packard). In all transfection experiments transfection yield and cell number was normalized by co-transfection with a construct expressing *Renilla* luciferase under the control of a CMV promoter. Expression data refer to the ratio of firefly and *Renilla* luciferase expression. Luciferase activity is given as average and standard deviation of at least three independent experiments at different days and using different batches of cells.

Western blot analysis of protein expression

The expression of recombinant proteins and HSV-1 antigens was monitored by western blot. Total cell lysates were

subjected to SDS–PAGE and electroblotted onto nitrocellulose membranes. In the case of GBD fusion proteins the expression was detected using anti-GBD antibody (Roche) while c-myc epitope tagged proteins were detected using mAb 9E10 (Santa Cruz). Viral proteins were detected using LP1 mAb agaist VP16 (kindly donated by S. Efstathiou) and rabbit polyclonal antibody against IE110k r191 (kindly donated by R. Everett). The anti-GAPDH antibody (Abcam) was used to verify equal loading of total cell lysates. The signal was detected by a secondary antibody fused to horseradish peroxidase (HRP) (Roche) and visualized using ECL detection system (Amersham Pharmacia). The same membrane was stripped and re-blotted up to three times.

HSV-1 infection of transiently transfected cells

COS-7 cells have been grown to the confluency of ∼75% in DMEM 10% FCS, harvested with Trypsin and electroporated using Amaxa Nucleofector (Amaxa Biosystems) according to the manufacturers protocol optimized for this cell line. Plasmid DNA used for these experiments included described above constructs p6F6-3a, p6F6-3aE74A, pB1-3a and also pmaxGFP (Amaxa) and p6F6KOX (23). Cells intended for HSV-1 infection were seeded in 24-well cluster dishes at the density of 0.5×10^6 cells/well and grown in DMEM 10% FCS at 37°C. The efficiency of transfection was estimated at 20–24 h after electroporation by counting a number of GFP positive cells in a sample of pmaxGFP (Amaxa)-transfected cells and varied between 60 and 72%. At 20–22 h after transfection, the cells were washed with phosphate-buffered saline (PBS). Following incubation in fresh medium supplemented with 10% FCS for ∼30 min, cells were infected with HSV-1 strain 17 at the multiplicity of infection (m.o.i.) of 0.05 p.f.u./cell in DMEM 2% FCS at 37°C. After 1 h of incubation HSV-1 suspension was removed and cells were washed and incubated in 0.5 ml DMEM 2% FCS per well at 37°C for up to 45 h. At various time points, post infection (p.i.) medium containing progeny virus was harvested and used for plaque assays (see below) while cells were washed in PBS and either harvested for western analysis in 0.1 ml of protein loading buffer heated to ∼80°C or harvested for DNA extraction with DNeasy Tissue Kit (Qiagen) and subsequent methylation studies.

Plaque assay

Medium samples containing HSV-1 have been serially diluted in DMEM 2% FCS and used to infect a confluent monolayer of COS-7 cells. Infection was performed in 6-well cluster dishes containing 2×10^6 cells/well in a volume of 1 ml per well. After 1 h incubation at 37°C, the virus suspension was removed and cells were overlaid with DMEM 2% FCS supplemented with 1% CMC. Following incubation for up to 96 h at 37°C, cells were washed in PBS and fixed with 4% formaldehyde for 15 min. HSV-1 plaques were visualized by incubation with 0.1% Toluidine Blue for 15 min at room temperature and scored.

Bisulfite sequencing

To analyze targeted DNA methylation in the reporter systems HEK293T cells were seeded in 6 cm plates, grown to 70–80% confluency and transfected using Transfast™ reagent (Promega) with constructs encoding targeting MTases mixed with target reporter gene at the ratio of 5:1 (GBD-3a or GBD-3b: 5×UAS-TK-Luc = 2 μg and 0.4 μg) or 20:1 (6F6-3a or B1-3a: pGL3-IE175 = 2 μg and 0.1 μg). After transfection for 4 to 5 days, cells were harvested and washed with PBS four times. Episomal DNA was isolated with modified method as described (27) by Qiagen miniprep kit for plasmids. To analyze methylation of HSV-1 DNA, COS-7 cells were transfected and infected as described above. At 30 h p.i. cells were harvested and total cellular DNA (also containing HSV-1 DNA) was isolated using DNeasy Tissue Kit (Qiagen). Purified DNA was digested by SalI for 4 h, purified by Qiagen PCR purification Kit. Bisulfite conversion was carried out as the standard procedure as described (28). The converted DNA was amplified by PCR with primers specific for the bisulfite converted template. The amplified fragments were cloned into TOPO-TA vectors (Invitrogen Life Technology Inc.) and individual clones were used for sequencing.

Statistical analysis

All statistical analysis have been performed using two flanked *t*-tests.

RESULTS

De novo DNA methylation targeted to UAS promoter region by GAL4 DBD-MTase fusion constructs

To target DNA methylation to a predetermined site in human cells, we fused the CDs of the *de novo* DNA MTases Dnmt3a or Dnmt3b with the GBD. The resulting constructs were named GBD-3a and GBD-3b, respectively (Figure 1A). The GBD-3a and GBD-3b fusion proteins and all their variants were transiently expressed at comparable levels in HEK293T cells (Supplementary Figure 1). Targeted DNA methylation was studied using the 5×UAS-TK-Luc reporter plasmid that contains five repeats of the GAL4 binding sequence (upstream activator sequence, UAS) inserted upstream of the minimal TK promoter, which controls the firefly luciferase reporter gene (Figure 1A). The reporter plasmid was co-transfected with the GBD-3a or GBD-3b construct into HEK293T and 4–5 days after transfection, the reporter DNA was retrieved and its methylation status analyzed by bisulfite sequencing. In agreement with earlier observations (29) no endogenous *de novo* methylation was detected either on the TK promoter or the five UAS repeats when the reporter construct was co-transfected with the vector expressing GBD in the absence of the MTase domain (Figure 1B). In contrast, when the reporter construct was co-transfected with GBD-3a or GBD-3b, dense methylation on both TK promoter and the UAS sequence, including its upstream flanking sequence, was detected in the majority of the clones (Figure 1C). In the case of GBD-3b, the methylation extends up to 80 bp into the 5′ part of the TK promoter adjacent to the UAS sequence. Altogether GBD-3b methylated DNA within a region of ∼230 bp containing 22 CG sites, which were methylated to 41%. In the case of GBD-3a, the targeted methylation extended over the longer region comprising 330 bp and 37 CG sites methylated to 56% on average.
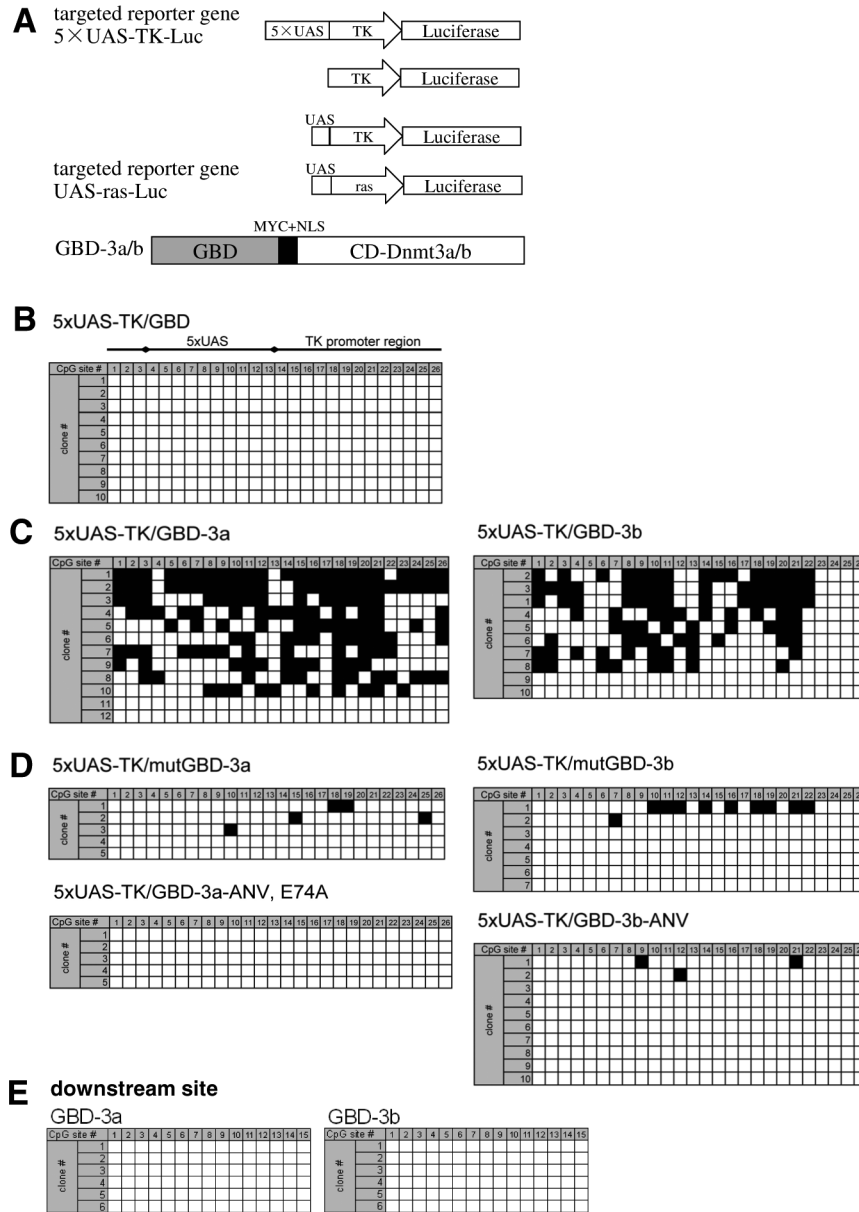
Figure 1. Targeted methylation at the 5×UAS-TK promoter region in HEK293T cells analyzed by bisulfite sequencing. (**A**) Schematic representation of the reporter constructs encoding the TK promoter fused to the luciferase firefly gene constructs as well as the constructs coding for targeted DBD-MTase fusion proteins. Targeted promoters contain one or five copies of the GAL4 binding sites (UAS). (**B–D**) Bisulfite sequencing of the promoter regions of the reporter constructs. Each line represents one individual clone sequenced. The empty squares denote for unmethylated CG sites, the filled ones for methylated sites. The location of the CG sites (1–26) within the 5×UAS-TK promoter region is schematically shown in (B). (B) Co-transfection of the 5×UAS-TK-Luc reporter construct with GBD only. (C) Co-transfection of 5×UAS-TK-Luc with GBD-3a or GBD-3b. The totally unmethylated clones #11 and 12 for 5×UAS-TK/GBD-3a and #9 and 10 for 5×UAS-TK/GBD-3b probably were PCR amplified from plasmids that were not taken up by the cells during the transfection procedure. (D) Co-transfection of 5×UAS-TK-Luc with the mutGBD-3a, mutGBD-3b, GBD-3a-ANV/E74A or GBD-3b-ANV variants. The mutGBD-3a and 3b variants carry an inactivated GBD domain. The MTase domain of the GBD-3a-ANV/E74A and GBD-3b-ANV is inactivated by mutations. (**E**) DNA methylation within the luciferase gene ~1 kb downstream of the TK promoter after co-transfecion of the 5×UAS-TK-Luc reporter gene with GBD-3a and GBD-3b.

To confirm that the methylation of the 5×UAS-TK depended on the specific DNA binding activity of GBD, a double mutation (L32P and C38G) was introduced into GBD that abolishes its DNA binding (25,26). To study dependence of the targeted methylation on the catalytic activity of the MTase domains, specific mutations were introduced into catalytic motifs VI and II of Dnmt3a (ENV to ANV and E74A, respectively) or motif VI of Dnmt3b (ENV to ANV) that were known to inactivate the enzymes (17,24). When

the reporter gene was co-transfected with fusion proteins containing the inactive MTase domain or the mutated GBD, little or no methylation was observed (Figure 1D) although the fusion constructs were expressed at the levels comparable to the levels of GBD-3a and GBD-3b (Supplementary Figure 1). Additionally, no methylation was observed within the firefly luciferase coding sequence located >1000 bp downstream of the target site, after co-transfection of 5×UAS-TK-Luc with GBD-3a or GBD-3b (Figure 1E). These results demonstrate
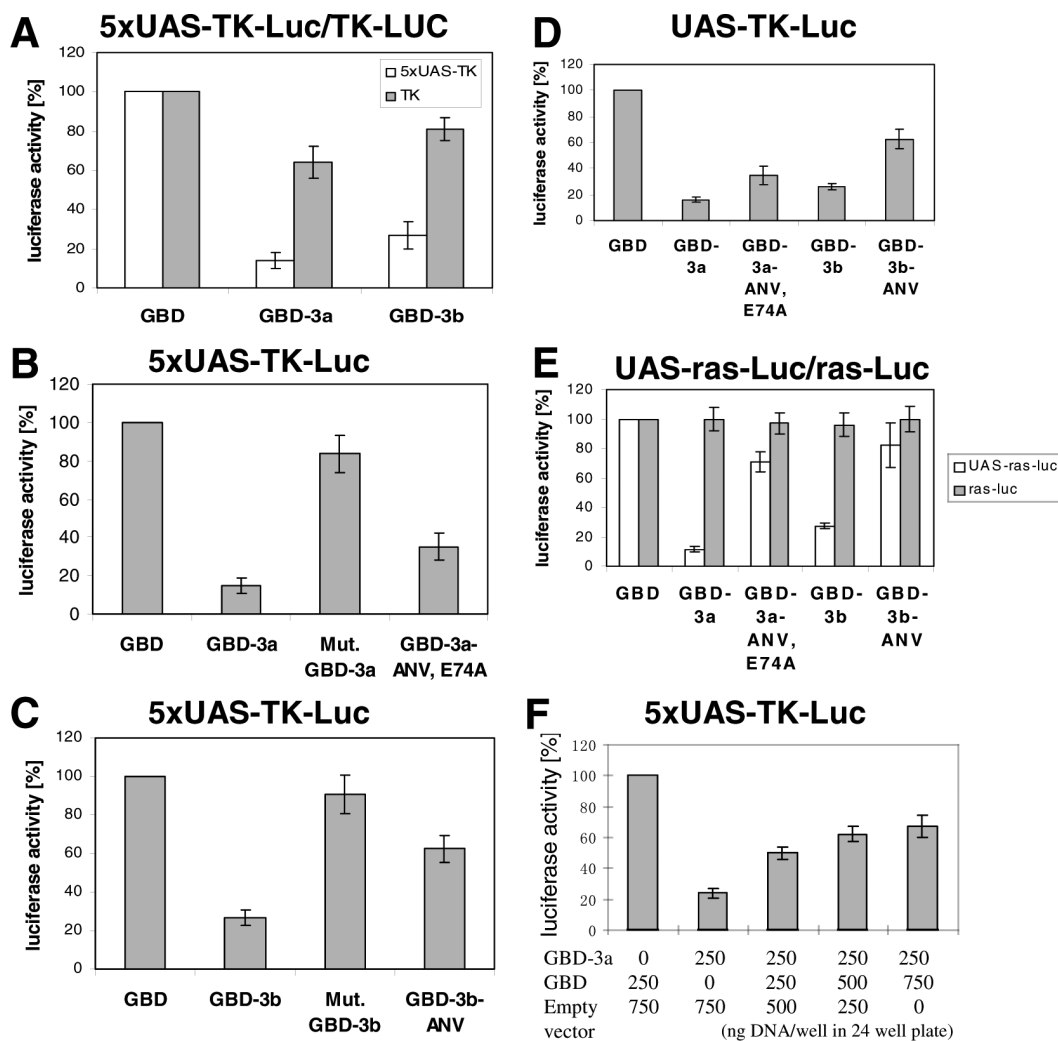
Figure 2. Specific repression of the luciferase reporter gene by GBD-MTase fusion constructs. (**A**) GBD-3a, GBD-3b and GBD expression constructs were co-transfected with firefly luciferase reporter plasmids 5×UAS-TK-Luc or TK-Luc into HEK293T cells and the reporter gene activity determined. (**B**) Co-transfection of the 5×UAS-TK-Luc reporter plasmid with GBD, GBD-3a, mutGBD-3a and GBD-3a-ANV/E74A expression vectors into HEK293T cells. (**C**) Co-transfection of the 5×UAS-TK-Luc reporter plasmid with GBD, GBD-3b, mutGBD-3b and GBD-3b-ANV expression vectors into HEK293T cells. (**D**) Co-transfection of the UAS-TK-Luc reporter plasmid with GBD, GBD-3a, GBD-3a-ANV/E74A, GBD-3b and GBD-3b-ANV into HEK293T cells. (**E**) Co-transfection of UAS-ras-Luc and ras-Luc reporter plasmids with the GBD, GBD-3a, GBD-3a-ANV/E74A, GBD-3b and GBD-3b-ANV into HEK293T cells. In each case the error bars give the standard deviations of at least three independent experiments. (**F**) Competition experiment of GBD-3a and GBD. To investigate the targeting function of the Zinc finger, GBD-3a or GBD-3b were co-transfected with the 5×UAS-TK-Luc reporter plasmid construct together with increasing amounts of a construct that expresses GBD. The total amount of DNA for transfection was normalized by empty vector. The numbers below the axis indicate the amount of DNA of each construct (ng/well in 24-well plate) used in each transfection.

that the GBD-3a and GBD-3b fusion proteins specifically methylate DNA in the neighborhood of their UAS target sequence.

Targeted DNA methylation leads to specific repression of gene expression

The influence of targeted DNA methylation on gene expression was investigated using 5×UAS-TK-Luc and TK-Luc (without UAS) as a control. The firefly luciferase gene was expressed at similar levels from both 5×UAS-TK-Luc and TK-Luc in HEK293T cells (Supplmentary Figure 5). The expression of the luciferase gene was strongly silenced after *in vitro* methylation of the reporter plasmids by M.SssI, which demonstrated the sensitivity of this promoter to DNA

methylation (Supplementary Figure 2). Co-transfection assays showed that GBD-3a and GBD-3b repress the 5×UAS-TK reporter gene expression by 6- and 3-fold, respectively (Figure 2A), co-transfection with GBD expressing vector did not influence expression (Supplementary Figure 5). To confirm that the repression of the 5×UAS-TK depends on the specific DNA binding activity of the GBD, the mutant form of GBD was used that lacks specific DNA binding. The fusion proteins comprising GBD-variant and Dnmt3a or Dnmt3b were expressed at similar level, as the wild-type forms (Supplementary Figure 1) but they have lost the ability to repress the UAS-TK reporter gene almost completely (Figure 2B and C). This finding demonstrates that specific DNA binding by the GBD is necessary for targeted gene silencing to occur. To further confirm this conclusion,

5×UAS-TK-Luc and GBD-3a were co-transfected with different amounts of the vector expressing only GBD (total DNA content was equalized by adding an empty vector). In this experiment GBD competes with GBD-3a for binding to target sites but could not lead to methylation or gene silencing. As shown in Figure 2F GBD reduces gene silencing by GBD-3a in a concentration dependent manner. Similar data were obtained for GBD-3b (data not shown). These results confirm that the GBD-MTase fusion proteins were targeted by GBD to the DNA.

To investigate whether the MTase activity is necessary for the repression of the 5×UAS-TK reporter gene, the fusion proteins comprising GBD and catalytically inactive Dnmt3a or Dnmt3b domains were employed. We observed residual repression of the 5×UAS-TK reporter gene with both catalytically inactive variants (Figure 2B and C). However, in both cases the loss of MTase activity significantly reduced the level of repression of the 5×UAS-TK reporter (*P*-values: 1.3×10^{-2} for Dnmt3a and 1.2×10^{-3} for Dnmt3b). While repression was almost 5.9-fold with the GBD-3a, the catalytically inactive variant GBD-3a-AND-E74A only led to a 1.8-fold repression indicating that the efficiency of repression is reduced 3-fold (Figure 2B). In the case of GBD-3b, the level of repression was reduced from 2.7-fold with the active MTase domain to 0.6-fold with the catalytically inactive domain, which represents a 4.5-fold change in efficiency (Figure 2C). The residual repression observed when the catalytically inactive Dnmt3a and Dnmt3b domains were used, might be caused by the interaction of these enzymes with other proteins that repress gene expression (30–35). These results demonstrate that the repression of the 5×UAS-TK promoter by GBD-3a and GBD-3b was in part mediated by DNA methylation. It is in agreement with the general view that DNA methylation contributes to gene silencing, but it is not the only process involved.

To explore whether a single GAL4 binding site is sufficient to recruit GBD-3a or GBD-3b and repress the gene expression driven by the targeted promoter, the five UAS repeats upstream of TK were replaced by a single one. As shown in Figure 2D, the expression of the UAS-TK-Luc reporter gene was repressed efficiently by GBD fused MTases. Inactivation of the MTase domain reduced repression 2.8- and 4.8-fold for GBD-3a and GBD-3b, respectively, (*P*-values 2.9×10^{-2} and 3.7×10^{-4}) indicating that MTase activity was important for the repression. Additional co-transfection assays in HCT-116 and HeLa cells showed that the repression of the reporter gene by GBD-3b (Supplementary Figure 3) or GBD-3a (data not shown) was not cell type dependent.

Targeted gene silencing of the human c-Ha-ras promoter

To explore whether targeted methylation and gene silencing can be also achieved with promoter sequences other than TK, human Ha-ras promoter was used. The firefly luciferase reporter gene under the control of the ras promoter was fused to a single UAS sequence upstream of the promoter (UAS-ras-Luc). A ras-Luc construct without the UAS was used as a control. Expression levels of luciferase from both constructs were identical (Supplementary Figure 6). *In vitro* methylation of the UAS-ras promoter by M.SssI resulted in the complete

silencing of the promoter (Supplementary Figure 2). UAS-ras-Luc and ras-Luc reporter gene constructs were co-transfected into HEK293T cells with the plasmids encoding GBD-MTases. As shown in Figure 2E, the expression of ras-Luc was not affected by either GBD-3a or GBD-3b, while expression of UAS-ras-Luc was repressed by both GBD-3a and GBD-3b. The mutations in the CD of Dnmt3a and 3b resulted in an almost complete loss of repression (*P*-values $<10^{-5}$ and 9×10^{-4} for GBD-3a and GBD-3b) indicating that targeted DNA methylation causes the silencing of reporter gene expression.

Targeted methylation of HSV-1 IE175k promoter by synthetic zinc-finger DBDs fused to the CD of Dnmt3a

Having shown that (i) chimeric MTases that contain GBD are capable of targeting methylation to predetermined DNA sites in the cell and (ii) the site-specific methylation by GBD-methyltransferases leads to silencing of target promoters, we set to develop a more universal system for targeting MTases to any DNA site. Over the past decade, Cys_2His_2 zinc fingers have emerged as universal DBD, which can be engineered to bind potentially any given DNA sequence (36,37). To validate the feasibility of targeting DNA methylation with zinc finger DBDs to facilitate gene repression, two engineered zinc-finger proteins B1 and 6F6, previously shown to bind specifically within the same target sequence in the HSV-1 immediate early gene IE175k promoter (23), were fused to the CD of Dnmt3a (Figure 3A). B1 and 6F6 comprise three and six zinc fingers, respectively, and 6F6 binds to the target site with much higher affinity (23). The B1/6F6 binding site is one of the two sites in IE175k promoter, which during HSV-1 infection is bound by the transcriptional activation complex containing VP16 (38).

To determine whether these synthetic zinc-finger DBDs could direct the CD of Dnmt3a to the target DNA sequence and facilitate DNA methylation, the luciferase reporter gene was cloned under the control of the IE175k promoter (IE175-Luc in Figure 3A), and the reporter plasmid was co-transfected with B1-3a or 6F6-3a into HEK293T cells. Both 6F6-3a and B1-3a were expressed at comparable levels (data not shown). Four days after transfection, episomal DNA was extracted and DNA methylation analyzed by bisulfite sequencing. As shown in Figure 3B, almost no methylation was observed in IE175k promoter co-transfected with the 6F6 zinc-finger DBD alone (lacking MTase domain) while co-transfection with B1-3a resulted in 57 CGs within the IE175k sequence being methylated to 81% on average (Figure 3C). The two CG sites within the B1/6F6 binding site were methylated to 96%. In co-transfection of IE175k promoter with 6F6-3a, slightly lower levels of methylation were observed (the average methylation density was 53%) (Figure 3C). Our finding that the level of methylation by 6F6-3a is lower than in a case of B1-3a, despite 6F6 binding DNA with much higher affinity (23), could be explained by B1 exhibiting a higher k_{off} from the DNA, which might allow more efficient methylation of the DNA in the vicinity of the target site.

To study the specificity of targeting, the B1/6F6 zinc-finger binding sequence GATCGGGCGgTAATGAGAT was deleted from the IE175k promoter (IE175mut-Luc Figure 3A).
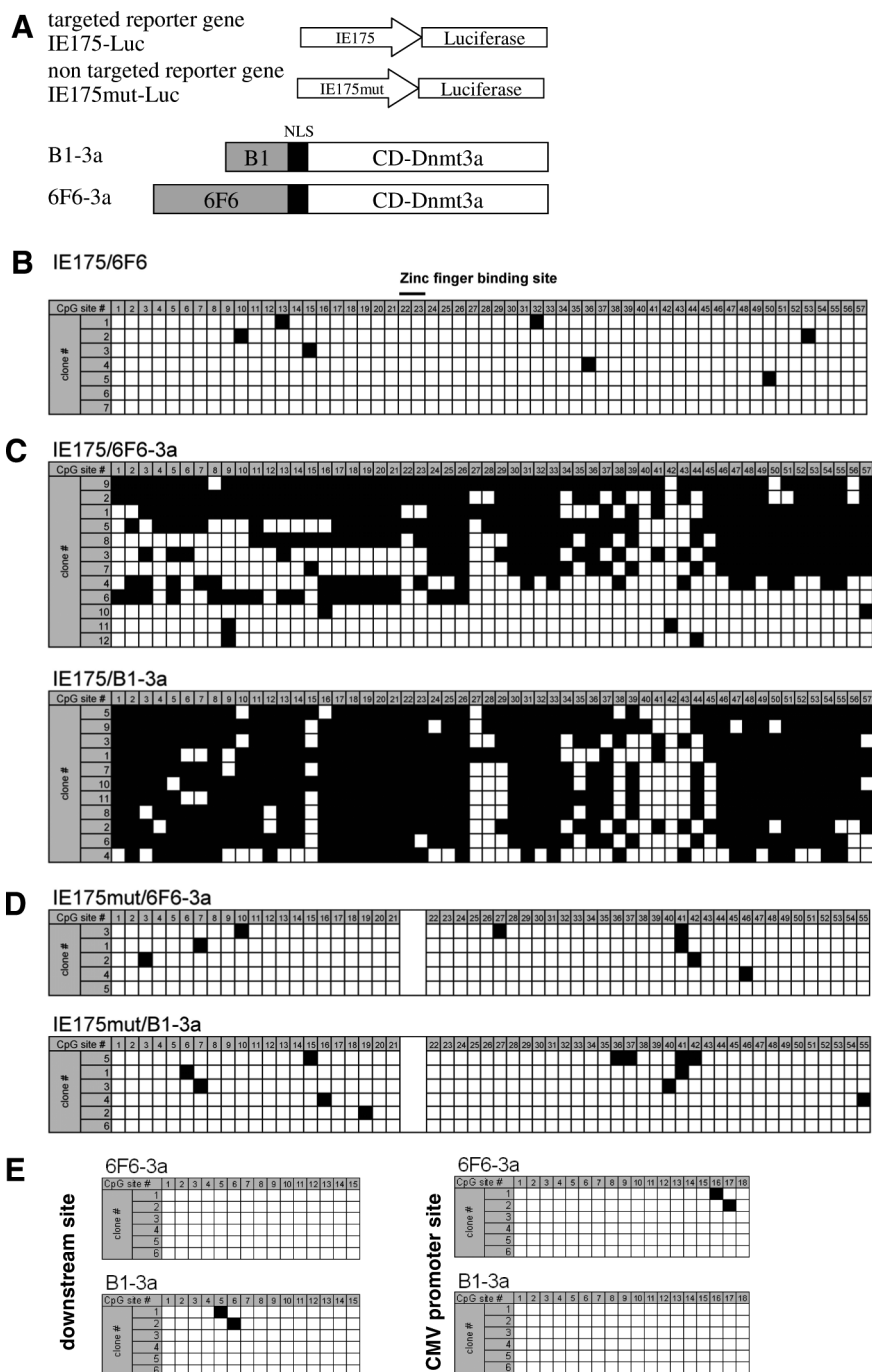
Figure 3. Targeted methylation of the IE175k promoter by 6F6-3a or B1-3a as analyzed by bisulfite sequencing. (**A**) Schematic representation of the reporter constructs comprising the wild-type IE175k promoter (IE175) or the IE175k promoter with deleted B1/6F6 site (IE175mut) fused to firefly luciferase gene and the constructs encoding targeted methyltransferase fusion proteins B1-3a and 6F6-3a. (**B–D**) DNA Methylation analysis of the IE175k promoter after co-transfection with MTase expression constructs into HEK293T cells. Each line represents an individual clone. The empty squares represent unmethylated CG sites, whereas the filled ones methylated sites. In (B) the IE175-Luc reporter gene is co-transfected with 6F6. In (C) IE175-Luc is co-transfected with 6F6-3a or B1-3a and in (D) the IE175mut-Luc construct is co-transfected with 6F6-3a or B1-3a. The B1/6F6 binding site is marked in panel B. (E) DNA methylation within the luciferase gene ~1 kb downstream of the target site (downstream site) in IE175-Luc and within the CMV promoter of the *Renilla* luciferase gene that was used for internal normalization in the presence of 6F6-3a and B1-3a.

As shown in Figure 3D, there was almost no methylation detected on the mutated IE175k promoter after co-transfection with B1-3a or 6F6-3a. In addition, no methylation was detected either in the region of the luciferase gene 1 kb downstream from the promoter or within the CMV promoter that drives expression of the *Renilla* luciferase gene, used for internal normalization of transfection efficiency (Figure 3E). Furthermore, we analyzed methylation at two CG islands in the unrelated promoter regions of the SON and TTC3 genes which are both unmethylated. After transfection with 6F6-3a

we did not observe major changes of the methylation level (Supplementary Figure 4). These results demonstrate that the B1-3a and 6F6-3a proteins cause a specific methylation at the IE175k target site. The catalytic activity of the Dnmt3a as well as the zinc-finger DBD and the intact zinc-finger binding site were all necessary for the targeted methylation.

Targeted methylation leads to specific repression of HSV-1 IE175k promoter

To determine the sensitivity of the IE175k promoter to DNA methylation, the IE175-Luc plasmid was methylated *in vitro* by M.SssI, and then transfected into HEK293T cells. The results indicated that the IE175k promoter is highly sensitive to DNA methylation both in the presence and in the absence of VP16, which is a HSV-1 encoded transcriptional activator of this promoter (Supplementary Figure 2).

In co-transfection reporter gene assays, the 6F6 zinc-finger protein was used as a control. The 6F6 itself did not repress the expression of IE175-Luc under these conditions (data not shown). At 72 h after co-transfection with IE175-Luc, both B1-3a and 6F6-3a caused repression of IE175-Luc by 2 to 3-fold (Figure 4A). The repressive effect of 6F6-3a on IE175k was reduced significantly when the catalytically inactive E74A variant of Dnmt3a was used (P-value: 3.5×10^{-3}). Residual repression with the inactive variant was only 0.4-fold, which corresponds to a 4.4-fold reduced efficiency of silencing in the absence of targeted DNA methylation. To study the specificity of repression, the B1/6F6 zinc-finger binding sequence was deleted from the promoter. The deletion itself increased the basal expression from this promoter (Supplementary Figure 7). Interestingly, both B1-3a and 6F6-3a lost the ability to repress the mutated IE175-Luc, indicating that the B1 and 6F6 zinc finger DBDs direct the activity of the Dnmt3a CD to their target sites. This conclusion was further confirmed by a competition experiment, which showed that the addition of 6F6 alone inhibits gene silencing mediated by 6F6-3a in a concentration dependent manner (Figure 4B).

Targeted methylation causes a reduction of HSV-1 titer during HSV-1 lytic infection

Having demonstrated that targeted methylation can be achieved using zinc fingers as DBD and that HSV-1 IE175k promoter can be silenced by such methylation in the reporter system, an attempt was made to determine if a similar effect can be observed during HSV-1 infection. The IE175k transcriptional regulator targeted here is one of the first viral genes to be expressed and it directly induces early and late genes (39). In order to test if 6F6-3a and B1-3a are capable of (i) accessing their binding sites in HSV-1 DNA during viral infection and (ii) inhibiting IE175k gene activity by targeted methylation of its promoter, COS-7 cells were firstly transfected with plasmids expressing either active chimeric Mtases (6F6-3a or B1-3a) or control proteins. The controls included constructs expressing zinc finger domain 6F6 fused to either mutant Dnmt3a (6F6-3a-E74A) or KRAB repression domain of KOX-1 (6F6-KOX) (23,40). The subsequent infection with wt HSV-1 strain 17 was carried out at a low m.o.i.
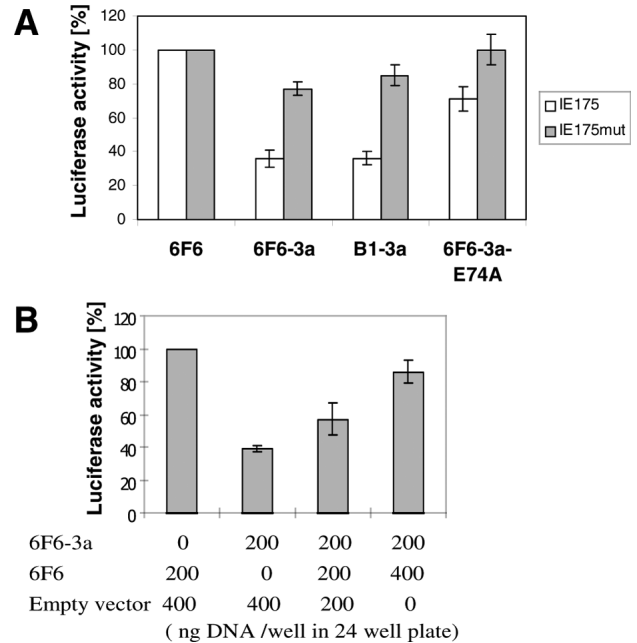

Figure 4. Targeted repression of the IE175k promoter by synthetic zinc-finger proteins fused to the CD of Dnmt3a. (**A**) The IE175-Luc or IE175mut-Luc reporter plasmids were co-transfected with 6F6, 6F6-3a, B1-3a or 6F6-3a-E74A into HEK293T cells. The IE175mut promoter does not contain the binding site for the engineered zinc finger protein B1/6F6. The Dnmt3a-E74A variant is catalytically inactive. The luciferase activity of reporter gene co-transfected with 6F6 was set to 100%. The error bars indicate the standard deviations of at least three independent experiments. (**B**) Competition between 6F6-3a and 6F6. To investigate the targeting function of the zinc-finger DBD, constant amounts of IE175-Luc and 6F6-3a were co-transfected with different concentrations of a construct expressing only 6F6, while the total amount of DNA in each transfection was normalized by adding an empty vector.

of 0.05 p.f.u./cell. At various times p.i. cells were harvested and used to isolate total DNA, which included HSV-1 DNA.

The methylation of the IE175k promoter was studied by bisulfite sequencing of viral DNA purified from infected cells expressing targeted MTases (Figure 5). As shown in Figure 5B after 30 h, most clones exhibited a low methylation of HSV-1 DNA of below 10% after correction for incomplete conversion, (which was between 5 and 10% in the various clones). The higher level of basal methylation observed here as compared to the other experiments, could be due to the different host cells used or a stimulation of the endogenous methylation activity by the viral infection. It has been shown that infection with several viruses including HIV-1, hepatitis B, adenovirus and Epstein–Barr virus up regulates DNA MTases (41–44). In addition, a Kaposi's sarcoma-associated herpes virus protein has been shown to recruit Dnmt3a to DNA (45).

The methylation levels of the 6F6-KOX and 6F6-3a-E74A samples were identical (P-value: 0.23). Significantly, when HSV-1 was propagated in cells expressing either the 6F6-KOX or 6F6-3a-E74A control proteins, no clones were methylated above 30%. However, in cells expressing 6F6-3a about one-third of all clones displayed a dense methylation of >30%. In cells expressing B1-3a, the fraction of highly methylated clones was lower. Using the 6F6-KOX and 6F6-3a-E74A data as reference a two flanked t-test indicates high significance of these changes (P-value <0.01). This
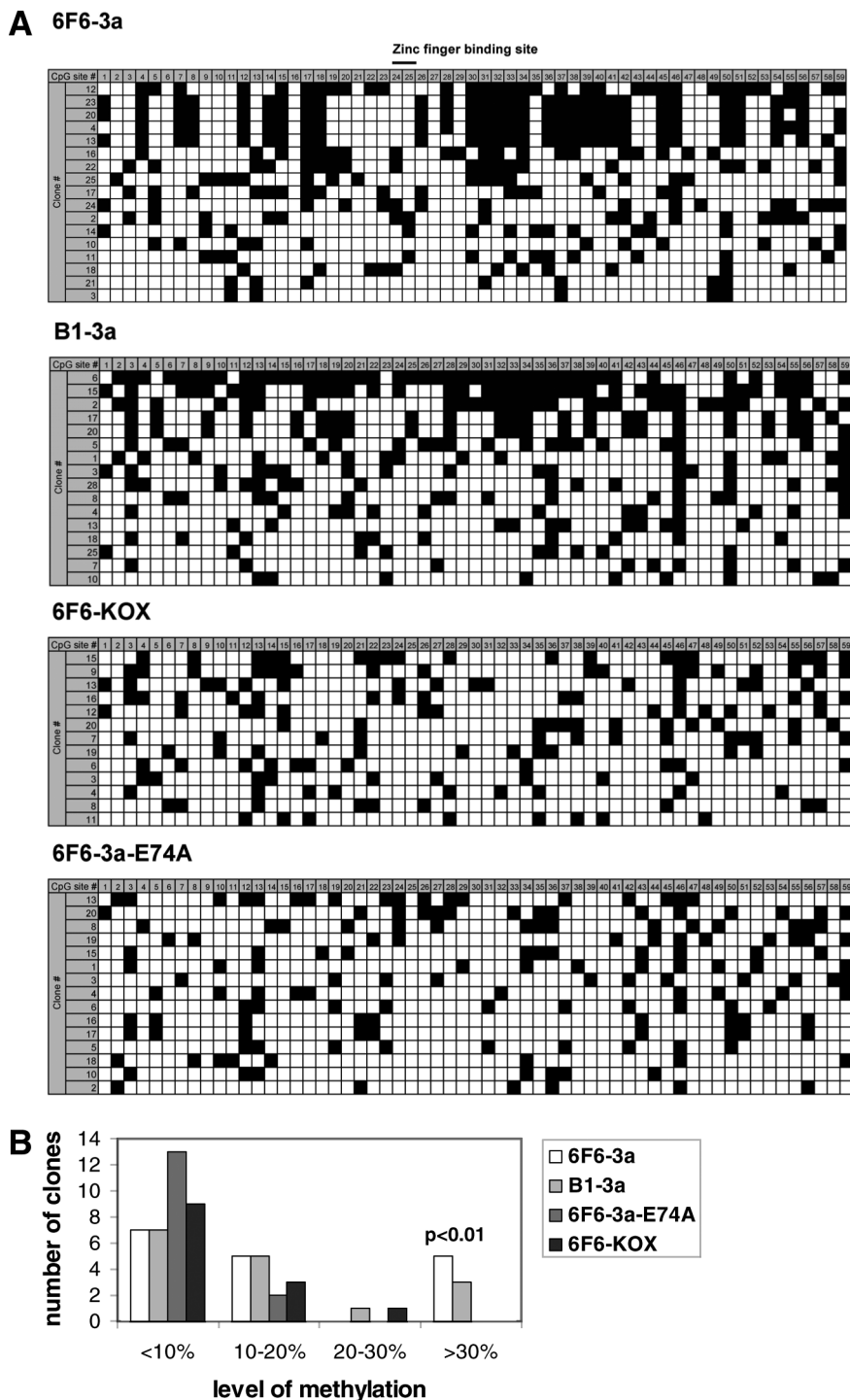
Figure 5. (**A**) Methylation status of HSV-1 DNA isolated during progressing viral infection. Analyses were performed at 30 h after infection of COS-7 cells transiently expressing targeted DNA Mtases (6F6-3a or B1-3a) or control proteins (6F6-KOX or catalytically inactive 6F6-3a-E74A). The analyzed region corresponds to IE175k promoter sequence containing the B1/6F6 binding site. Each line represents an individual clone. The empty squares represent unmethylated CG sites, whereas the filled ones represent methylated sites. (**B**) Comparison of the methylation levels of clones obtained in the bisulfite methylation analysis of the IE175k promoter region of HSV-1 DNA after infection of COS-7 cells expressing targeted DNA MTases [examples of the data are shown in (A)]. Methylation levels were corrected for the incomplete bisulfite conversion, which was between 2 and 10% in each case.

result indicates that the 6F6-3a construct is able to methylate HSV-1 DNA while the virus propagates in the cell. It has to be noted that potentially, methylation can occur on the initial as well as on the progeny HSV-1 DNA, during the progress of the lytic infection.

The lower level of methylation observed with the active DNA MTases as compared to the previous co-transfection experiments can be explained as follows (i) in these experiments targeted DNA methylation has to be introduced against the rapid DNA replication of the viral genome (ii) only DNA

isolated from the cells that were originally transfected with 6F6-3a or B1-3a constructs (~60%) could be specifically methylated while viral DNA isolated from untransfected cell (~40%) remains unaffected. In the previous experiments co-transfection of reporter gene and targeted MTase constructs ensured that in the majority of cells reporter plasmids co-existed with the MTase.

Further we examined the impact of methylation of the IE175k promoter on HSV-1 lytic cycle at low m.o.i. The HSV-1 virus was propagated in cells transiently expressing either one of the chimeric MTases (6F6-3a or B1-3a) or control zinc finger proteins (6F6-3a-E74A or 6F6-KOX) as described above. All these proteins were expected to have some inhibitory effect on HSV-1 propagation, since all of them contain engineered zinc finger DBDs designed to compete with HSV-1 activator VP16 for binding to the B1/6F6 site (23). At 30 and 45 h p.i. cells and corresponding culture media samples containing viral progeny, were harvested. The lysates of the cells that express different zinc finger fusion proteins after infection with wt HSV-1 were analyzed by western blots (Figure 6A). In the cells expressing the chimeric MTases 6F6-3a and B1-3a, HSV-1 infection cycle was progressing slower as indicated by lower levels of immediate early protein IE110k and late protein VP16 after 30 h, when compared with the cells expressing control fusion proteins and cells transfected with the empty vector (pcDNA 3.1), in which HSV-1 propagates the fastest.

The comparable amounts of viral proteins in 6F6-3a and control samples observed at 45 h p.i. indicate that at that late time point a similar fraction of surviving cells are infected. This suggests that the protection offered by the targeted MTase is less efficient during the later phases of the experiment, when secondary infection is expected to take place at higher m.o.i. This is in agreement with additional experiments carried out at higher initial m.o.i. (data not shown). In the control experiments (6F6-3a-E74A and 6F6-KOX) at 45 h many cells have already released progeny virus and have been lysed. Therefore, their viral protein content is no longer detected in Figure 6A.

Having shown that the presence of chimeric methylases can alter the levels of expression of HSV-1 proteins during the course of primary infection (30 h), we were interested to determine if methylation of HSV-1 DNA in the region of IE175k promoter affects the final titer of viable viral particles. To this end, the medium containing progeny virus particles was harvested at different times after infection of the cell expressing the active chimeric MTases (6F6-3A or B1-3a) or the 6F6-KOX and 6F6-3a-E74A control proteins, and the number of progeny virus particles was determined. As shown in Figure 6B, a significant reduction in the number of infectious particles was observed in the presence of 6F6-3a (P-values $<10^{-5}$). This effect is already detectable at 30 h post infection and it becomes very prominent at 45 h p.i. when the viral titer is more than 18-fold lower than the titer obtained from cells expressing 6F6-3a-E74A. A significant reduction was also observed when HSV-1 infection was carried out in the cells expressing B1-3a (P-values $<10^{-5}$).

From the comparison of Figure 6A and B it should be noticed that Figure 6A shows the amount of viral proteins in cells that are still alive, while Figure 6B shows the total
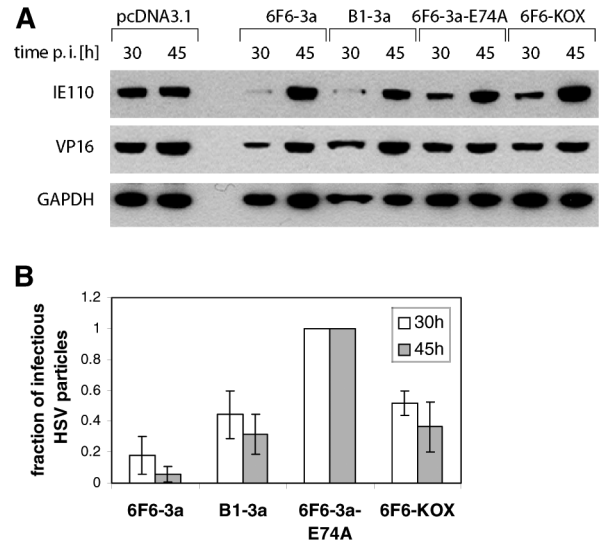

Figure 6. Inhibition of viral lytic cycle by zinc-finger MTase fusion constructs. (**A**) western blot analysis of the expression of HSV-1 antigens during the course of low multiplicity infection with HSV-1. Cells were transfected with zinc finger MTase or control constructs expressing 6F6-3a, B1-3a, 6F6-3a-E74A or 6F6-KOX (as indicted), infected with wt HSV-1 and harvested after 30 or 45 h p.i. Expression of HSV-1 VP16 was detected with mAb LP1 while expression of HSV-1 IE110k was detected using r191 antibody. Equal loading of the samples was verified using antibody against cellular protein GAPDH. (**B**) Inhibition of HSV-1 propagation by 6F6-3a and B1-3a. Culture medium samples harvested at 30 and 45 h after the infection with HSV-1 at low m.o.i were used for plaque assays on confluent monolayers of COS-7 cells, in 10-fold serial dilutions of the virus. The graph shows relative number of infectious HSV-1 particles released into the medium at indicated times p.i. from cells expressing active MTases (6F6-3a and B1-3a), catalytically inactive 6F6-3a-E74A mutant or 6F6-KOX fusion proteins. The error bars give the standard deviation of at least three independent experiments.

amount of infectious viral particles that accumulated throughout the complete experiment. In the time interval from 30 to 45 h p.i. the viral titer increased slower in the presence of 6F6-3a or B1-3a than in the control experiments (6F6-3a-E74A and 6F6-KOX), where in after 30 h the infection was already well advanced as indicated by the western blot results (Figure 6A). In contrast, in cells expressing 6F6-3a or B1-3a the infection was clearly delayed at 30 h, which accounts for fewer virus progeny produced in the time interval between 30 and 45 h p.i. This result indicates that expression of 6F6-3a, and to a lesser extent also B1-3a, can inhibit HSV-1 propagation in a cell culture system.

The 18-fold reduction in HSV-1 titer in the presence of 6F6-3a is noteworthy taking into account that (i) only the portion of transiently transfected cells (~60%) expresses 6F6-3a or other fusion proteins and (ii) methylation-mediated inhibition of HSV-1, in our system, is measured against a control where HSV-1 is grown in the presence of the catalytically inactive 6F6-3a-E74A mutant. This fusion protein, however, is still capable of binding to the B1/6F6 site and, therefore, competing with the VP16 activation complex. Therefore the overall inhibitory effect of 6F6-3a on HSV-1 propagation could be considered to be even greater, when viewed as a combination of effects exerted by targeted DNA methylation by Dmnt3a and promoter blocking by the 6F6 DBD.

DISCUSSION

In this study we have demonstrated that DNA methylation can be targeted to predetermined promoter regions and efficiently repress reporter gene expression. Targeted DNA methylation was achieved by fusing natural or engineered DBDs to the CD of mouse *de novo* DNA MTase Dnmt3a or 3b. DNA methylation and gene silencing were abolished when catalytically inactive DNA MTases were used, the DBD was mutated, or the target sequence in the promoter region was deleted. Therefore, it provides the evidence that targeted gene methylation can be directly responsible for gene silencing.

The stable silencing of gene expression by DNA methylation observed here suggests that targeted DNA methylation could provide a powerful technology for specific gene silencing. Unregulated gene expression is often a central mechanism of disease. For example viral infections are characterized by the preferential expression of viral genes, and in cancer, up-regulated expression of oncogenes is frequently observed. Silencing of such genes by transcriptional inactivation could be an approach for therapy of these diseases. Furthermore, gene silencing by targeted methylation that is specific and stable could have applications in basic research and could be eventually used to directly correct some epigenetic defects as observed in many cancers (46). However, before a therapeutic application of the targeted methylation approach presented here could be considered, a genome wide scan for non-target methylation is required.

While we have shown that targeted methylation is a feasible approach to repress the propagation of viral infections in cell culture it ought to be compared with the previous approaches, which used engineered zinc finger DBDs fused to repressor domains (23,47–49). The advantage of using targeted DNA methylation for gene silencing over the use of a gene repressor like the KRAB domain of KOX-1, is that the MTase has a direct enzymatic activity. Therefore, lower efficiency of targeting can lead to stable inhibition. Moreover, the DNA MTase introduces a permanent silencing imprint on DNA, which is propagated *in vivo* and remains stable even after dissociation of the modifying fusion protein. In agreement with this hypothesis we show here that 6F6-3a is significantly more efficient in gene silencing than 6F6-KOX (Figure 6B). In addition, B1-3a showed similar efficacy as 6F6-KOX in the present study, while B1-KOX was much less efficient than 6F6-KOX (23).

Evidence from various studies suggests that in the biological context Dnmt3a and 3b are targeted to specific genomic sites by interaction with other proteins, which bind either DNA or chromatin, such as RP58, HP1, c-myc, PU.1, SETDB1, Mbd3, Hdac1, Brg1 or p53 (31,32,50–54). We observed that targeted methylation induces dense methylation of DNA regions comprising up to 380 bp on both sides of the specific DNA binding site. In contrast, in their pioneering study conducted *in vitro*, Xu and Bestor have observed that a Zif268-M.SssI fusion protein methylates single CG sites 16–22 bp upstream of the Zif268 binding site (18). This difference suggests that the initial methylation by Dnmt3a or 3b, targeted to the DNA by a heterologous DBD, might serve as a trigger for an epigenetic response. For example, the targeted methylation could induce histone 3 lysine 9 methylation and

histone deacetylation (55–58). In turn, these responses could trigger additional DNA methylation (59–62). Alternatively, spreading of methylation could be mediated by Dnmt1, which could be recruited to DNA by Dnmt3a (63,64). Our data suggest that the CD of Dnmt3a and 3b are able to recruit endogenous gene silencing activities.

In summary, our study demonstrates for the first time in transient co-transfection experiments that, targeted DNA methylation can be employed for gene silencing in human cells. Using this approach we silenced different promoters including the human Ha-ras oncogene promoter and the viral IE175k promoter by targeted methylation and demonstrated that, targeted DNA methylation antagonizes viral infection in the cell culture model. Whether this approach is also applicable to endogenous genes in the context of natural chromatin needs further investigation. For anti-viral applications, fusion to a more active DNA MTase domain might provide a stronger and less transient protective effect, that might justify proceeding to animal studies. Furthermore, for future application of this technique, the problem of MTase delivery to the target tissue has to be solved.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

ACKNOWLEDGEMENTS

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