Epigenetic regulation of cytomegalovirus major immediate-early promoter activity in transgenic mice

Abhishek Kumar Mehta, Subeer S. Majumdar, Parwez Alam, Neerja Gulati, Vani Brahmachari

Dr. B. R. Ambedkar Center for Biomedical Research, University of Delhi, North Campus, Delhi-110007, India
National Institute of Immunology, Aruna Asaf Ali Marg, Delhi, India

The expression of genes in transgenic mice is known to be influenced by the site of integration even when they carry their own promoter elements and transcription factor binding sites. The cytomegalovirus (CMV) promoter, a strong promoter often used for transgene expression in mammalian cells in culture, is known to be silenced by DNA methylation and histone deacetylation but there is no report on the role of histone methylations in its regulation. We generated two transgenic lines carrying green florescence protein coding gene as reporter driven by cytomegalovirus major immediate-early promoter/enhancer. We observe that silencing of CMV promoter is dependent on the site of transgene integration, except in testis, and the nature of DNA and histone methylations strongly correlate with the expression status of the reporter. We find that silenced CMV promoter interacts in vivo with Methyl CpG binding protein 2 (MeCP2), a recruiter of histone deacetylases (HDACs) and histone (H3K9) methyl transferase. Histone H3K4methylated, the active chromatin mark, is also associated with silenced promoter, suggesting bivalent marking of the promoter and its susceptibility to reactivation on induction.

Article history:
Received 26 May 2008
Received in revised form 18 September 2008
Accepted 18 September 2008
Available online 10 October 2008

Keywords:
Histone methylation
Transgene silencing
DNA methylation
Integration site
MeCP2
Position effect

1. Introduction

The human cytomegalovirus (CMV) major immediate-early promoter/enhancer (HCMV MIEP/E) region (referred as CMV promoter hereafter), is among the strongest promoters in vitro, and thus is often used in expression and gene therapy vectors. In cells in culture and in differentiated neurons, it has been shown to drive strong expression of reporter gene (Van den Pol and Ghosh, 1998; Fritschy et al., 1996). However, CMV promoter driven expression in vivo is not predictable. There are reports of its reproducible pattern of expression in multiple tissues of mice while others have shown that it gets silenced within a few weeks after gene transfer (Schmidt et al., 1990; Villuendas et al., 2001; Fitzsimons et al., 2002). HCMV MIEP/E region has four types of repeat sequences, 17-, 18-, 19- and 21-bp repeats, and several transcription factor binding sites (Niller and Hennighausen, 1991). These repeat sequences, like YY1 and ERF do not lead to reactivation of the silenced promoter (Murphy et al., 2002). However, the deletion of 21 bp repeats which bind YY1 and ERF may further silence methylated promoter in vivo (Wright et al., 2005; Murphy et al., 2002). The promoter silencing is also associated with reduced histone tail acetylation in vivo (Murphy et al., 2002). However, the deletion of 21 bp repeats which bind YY1 and ERF does not lead to reactivation of the silenced promoter (Murphy et al., 2002), suggesting the role of some other HDAC recruiters or other histone modifications in CMV silencing. The role of epigenetic modifications in CMV promoter silencing is further substantiated by the activation of the promoter by inhibitors of DNA methylation and histone deacetylation (Grassi et al., 2003; Meier, 2001; Choi et al., 2005).

Methylation of various lysine residues on histone tails is also a well-established epigenetic modification, which influences chromatin state and transcription (Kouzarides, 2007). But there is no report on the role of histone tail methylation in CMV silencing and reactivation. In the present study, we show that the CMV promoter driven expression in
transgenic mice is dependent on site of integration and analyze the histone methylation marking on the silenced promoter. We observe that the silenced promoter is hypermethylated and is associated with MeCP2, which recruits HDACs and H3K9 methyltransferases to the methylated DNA. Detection of H3K4 methylation in our study correlates with the ability of the promoter to get reactivated under appropriate conditions.

2. Materials and methods

2.1. Materials

Vector pEGFP-N3 was from Clontech and pGEM-T easy was from Promega. Restriction enzymes were either from Fermentas or New England Labs (NEB). Oligonucleotides were from Microsynth (Switzerland). QIAquick gel extraction kit was from Qiagen GmbH, Germany. Taq DNA polymerase was from Bangalore Genei, India. TRIZOL was from Invitrogen life technologies, California. CDNA synthesis kit was from Fermentas, USA. DNA walking Speed Up premix kit was from Seegene, USA. EZ-DNA methylation Gold kit was from Zymoresearch, CA. Antibodies were from Abcam and Santacruz. Protein-A agarose was from Santacruz. DNA sequencing services of The Center for Genomic Applications, Delhi, India, were utilized.

2.2. Methods

2.2.1. The transgene construct

The various sequences in the plasmid pCTGFP were obtained as follows: the CMV MIEP/E, GFP coding region and SV40 polyadenylation signal was PCR amplified from pEGFP-N3 plasmid, using primers AGEF and ECO81IR (Table S1) and was cloned in pGEMT-easy vector. The SV40 ori/EPR and a part of DMPK upstream of CMV-GFP in SphI, ScI and BglII sites were included in the transgene to study the effect of CTG repeats or SV40 ori/EPR on the promoter. The transgene was sequenced using primers for screening, walking PCR, ChIP and methylation analysis.

2.2.2. PCR based screening of transgenic mice

DNA was isolated from tail tissue of mice as described earlier (Baskaran et al., 2002). The mice were screened for presence of the transgene using primers spanning different regions of the transgene. The primers used were: SCN6931f, SCN77r; SCN150f, SCNGr; CTF1032, CTR1630; CTF1784, CTR1960; and CTF2285, CTR2516 (Table S1). The PCR conditions used were: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, and extension at 72 °C for 45 s, then a final extension at 72 °C for 10 min. Primer annealing was for 45 s and appropriate temperature used for each primer set is given in Table S1 (supplementary data).

2.2.3. Expression analysis of transgenic mice

Total RNA was extracted from different tissues of mice with TRIZOL. The tissues used were liver, kidney, brain, spleen and testis. Six mice from each line were studied for expression analysis. One microgram of total RNA was taken for cDNA synthesis using First strand cDNA synthesis kit. The expression of GFP was analyzed using primers CTF1784 and CTR1960. GAPDH was used as expression control.

2.2.4. Analysis of site of integration of the transgene

DNA Walking Speed Up™ Premix Kit was used for determining the site of integration of the transgene. Transgene specific primers used were: CTF2285, SCNGf and CTF2510. The products of third round walking PCR were sequenced and analyzed by homology search using BLASTn for site of integration.

2.2.5. Methylation analysis of CMV promoter DNA

EZ DNA Methylation-Gold kit was used for bisulphite conversion of genomic DNA. Converted DNA was used for methylation analysis of promoter region with primers CTMF2 and CTMR2. The products were cloned in InstaClone PCR cloning kit and clones were sequenced and compared for methylation status. The number of clones sequenced for each sample is mentioned under results.

2.2.6. Chromatin immunoprecipitation

ChIP was performed as described by Kuo and Allis (1999). The antibodies against H3K9me2 (ab12220), H3K4me3 (ab8580) and H3K27me3 (ab6002) were from Abcam and that against MeCP2 (sc20700) was from Santa Cruz Biotechnology. Primers CTF1198 and CTR1304 were used to analyze the immunoprecipitated DNA.

3. Results

3.1. Generation of transgenic mice

The construct CTGFP, (Fig. 1) having enhanced green fluorescence protein (EGFP) gene under the control of CMV immediate early promoter/enhancer, from pEGFP-N3 vector cloned in pGEMT-easy vector, downstream of SV40 ori/EPR and (CTG)n repeats was microinjected into fertilized eggs from FVB/NJ mice. The SV40 ori and (CTG)n repeats were included in the transgene to study the epigenetic factors influencing repeat expansion (unpublished results). We have studied the expression of reporter GFP in this transgene. Four independent lines, C1, C3, C7 and C9, were generated from four founder mice.

3.2. Expression of GFP reporter

There was no drastic effect of CTG repeats or SV40 ori/EPR on the expression of GFP as fluorescence levels of GFP from pCTGFP and parent plasmid pEGFP-N3 was comparable in transfection assays in HEK cell line (Fig. S1). However, the four independent transgenic mice lines showed differences in the expression of GFP. Only line C7 expressed GFP in all tissues studied, namely liver, kidney, brain, testis and spleen (Fig. 2). On the other hand, in line C1 the expression was restricted to testis only (Fig. 2). In C3 and C9 lines expression was studied in liver, kidney and spleen tissues and was found to be absent.

Fig. 1. Diagrammatic representation of transgene CTGFP. Ori-SV40 origin of replication, DM—a part of 3’UTR of human DMPK gene having 22 CTG repeats. HCMV MIEP—HCMV major immediate early promoter/enhancer. GFP—EGFP gene from pEGFP-N3 plasmid. 1–10 are primers used for screening, 9, 11 and 12 were used in walking PCRs, 13 and 14 were used in methylation analysis and 15 and 16 for ChIP analysis. The bent arrow indicates transcription start site. Primer sequences are given in Table S1.
3.3. Effect of site of integration of transgene

The site of integration was determined by DNA walking. The integration in C1 is within an L1F LINE element. The chromosomal location of LINE L1F could not be deciphered due to the repetitive nature of these sequences, however it is known that L1F LINE 1 element is not an active element (Adey et al., 1991) thus suggesting position effect on CMV promoter activity, leading to silencing. In C7 line the integration is in an intergenic region. The flanking sequences are detected both on chromosomes 2 and 18, however the EST sequence corresponding to this region, maps on chromosome 18. Since we obtained a hit with significant match (4e-176) in the EST database, it appears that this region is transcriptionally active. We analyzed all these lines for rearrangements by sequencing the amplicons derived from the transgene. In C1, C7 and C9 lines, we detected two transgene copies integrated in tandem, while single copy integration in C3 by semi quantitative PCR and junction PCR (Fig. S2; semi quantitative PCR data not shown for C3 and C9). The position effect could be due to cis-elements and also the spreading of the epigenetic marking to the transgene. Therefore, we analyzed the DNA methylation and histone modifications of the transgene in the two lines.

3.4. Silent HCMV IE promoter is hypermethylated in vivo

We studied DNA methylation of the CMV promoter by bisulphite sequencing (Grunau et al., 2001) of a region containing 14 CpGs (−280 to −7 with transcription start site as +1) amplified by primers CTMF2 and CTMR2 (Fig. 1). Six animals from each line were used for methylation analysis. For each tissue, 8–10 clones were sequenced and analyzed for CpG methylation.

The promoter region of line C7, which shows GFP expression in all tissues, was either completely unmethylated or there was methylation at very few residues. While in C1, C3 and C9 lines the promoter was hypermethylated in somatic tissues (Figs. 3 and S3). This correlates with the lack of CMV promoter activity. In C1 and C7 lines, DNA from testis shows absence of CMV promoter methylation, concomitant with GFP expression.

Early reports have suggested the role of DNA methylation in silencing of CMV IE promoter/enhancer (Prosch et al., 1996; Brooks et al., 2004; Grassi et al., 2003; Meier, 2001; Choi et al., 2005). The effect of DNA methylation on promoter silencing is reinforced by inactivating modifications of histones through interaction of methylated CpG interacting protein. We analyzed the interaction of the transgene with MeCP2 and status of histone methylation in the active hypomethylated testis tissue and inactive hypermethylated somatic tissues in the C1 transgenic line.

3.5. MeCP2 is associated with methylated CMV promoter

MeCP2 is a methylated DNA binding protein and is also a recruiter of HDACs (Fuks et al., 2003). We analyzed MeCP2 recruitment on CMV promoter by chromatin immunoprecipitation with anti-MeCP2 antibody in liver, kidney and testes of C1 line mice. CMV promoter is methylated and is associated with MeCP2 in liver and kidney but in testis where it is unmethylated, it did not have any association with MeCP2 (Fig. 4). To compare the levels of MeCP2 association with CMV promoter between tissues, densitometric analysis was carried out and p values were calculated at 5% significance level by t-test as 2.7765. In comparison with the active promoter in testis, liver and kidney have significantly higher levels of MeCP2 (p-value=5.19, 5.67 respectively).

3.6. Histone modifications on CMV promoter region

We analyzed chromatin from different tissues of C1 for repressive chromatin marks, as MeCP2 is also a recruiter of H3K9 methyl transferase (Fuks et al., 2003). The inactive promoter in liver and kidney is associated with methylation of lysine 9 and 27 (H3K9me2 and H3K27me3), but the active promoter in testis lacks them (Fig. 4). To compare the levels of various histone modifications between tissues, densitometric analysis and p value calculations were carried out as in the case of MeCP2 at 5% significance level by t-test (p-value=2.7765). In the liver and kidney, the levels of H3K27me3 (p-value=3.92, 8.91 respectively) and HeK9me2 (p-value=22.02, 28.80 respectively) were significantly higher in comparison to that in the testis. The level of H3K4me3 is slightly lower in testis as compared...
3.7. Silenced CMV promoter is primed for reactivation

Earlier reports show that on induction by bacterial lipopolysaccharide (LPS) or phorbol esters (PHA and PMA), the CMV promoter is reactivated (Hunninghake et al., 1989; Kuramoto et al., 2006; Loser et al., 1998). These compounds are inducers of NF-κB and cAMP, the positive regulators of CMV promoter. The DNA methylation of CMV promoter however, counters its susceptibility to reactivation. We observed reactivation of CMV promoter in kidney and spleen, but not in liver in C1 line by intraperitoneal injection of LPS (Loser et al., 1998). The basis of the differential reactivation of CMV promoter is not clear and needs further analysis (Fig. S4).

The chromatin immunoprecipitation for activating mark shows that the CMV promoter in both active (testis) as well as inactive state (liver and kidney) is associated with trimethylation of lysine 4 on histone H3 (H3K4me3, Fig. 4). H3K4me3 marks not only the promoters of actively transcribing genes, but also the promoters of inactive genes primed for activation (Schneider et al., 2004; Koch et al., 2007). Thus, the presence of both H3K4me3 and H3K27me3 on silent CMV promoter shows that in silent state, the CMV promoter carries bivalent marking and is primed for reactivation on induction.

4. Discussion

CMV promoter is a very strong promoter in vitro. In mice, the CMV promoter exhibits varied expression profiles, it is active in neurons, testis and certain other tissues (Van den Pol and Ghosh, 1998; Fritschy et al., 1996; Schmidt et al., 1990; Villuendas et al., 2001), but there are other reports of it being silent in vivo (Brooks et al., 2004; Grassi et al., 2003; Meier 2001; Choi et al., 2005). Schmidt et al. (1990) have referred to it as a pan-active element, showing widespread expression in 24 out of 28 tissues tested in transgenic mice. However, in the same report they have shown it to be inactive in three other transgenic lines.

In the present study, we have presented epigenetic analysis of two transgenic lines with GFP reporter under the control of CMV promoter. One of the proposed reasons for transgene silencing is the high copy number tandem integration (Tang et al., 2007), which does not explain the differential expression we detect in C1 and C7, as only two copies of the transgene are present in both lines. But we observe that when the integration is within a permissive region as in C7 transgenic line, where the integration is in intergenic region it is actively transcribed (Yan and Boyd, 2006), while in line C1, where the integration is in an L1F element it is repressed. The L1 and other transposable elements are generally kept silent by DNA methylation in somatic tissues (Yoder et al., 1997). LINE1 methylation has been used as an indicator of global methylation status of the genome (Weisenberger et al., 2005). We observe that the transgene in C1 line is inactivated in all tissues except testis, suggesting position effect on CMV promoter activity. Schmidt et al. (1990) also observed that CMV promoter is active in two transgenic lines but inactive in other third though the same transgene is present in all the animals. Thus, site of integration influences the expression of transgene in mice. We observe that this position effect translates into multiple silencing epigenetic markings including DNA methylation and histone methylation, as shown in our analysis and histone deacetylation as shown earlier (Murphy et al., 2002). It is known that actively transcribing regions are depleted of nucleosome positioning elements and enriched in nucleosome excluding sequences (Ganapathi et al., 2007); moreover DNA and histone modifications distinctly vary across genomic regions, correlating with promoter activity (Koch et al., 2007).

We observe methylation of CMV promoter in C1 in tissues that lack promoter activity, while the promoter is under methylated in C7 line as well as testis of both C1 and C7 where it is active.

Differences in DNA methylation between somatic tissues and testes are well known wherein testes show hypomethylation at many regions (Oakes et al., 2007). Though sperm chromatin is compacted by protamines, a fraction of mouse sperm is organized as nucleosomal hypersensitive domains enriched in retroposon DNA (Pittoggi et al., 1999). L1 sequences are particularly abundant and are hypomethylated in these hypersensitive domains (Pittoggi et al., 2000). The hypomethylation of transgene in testis in line C1, due to its integration in to L1F element, indicates the effect of site of integration on promoter activity through epigenetic modifications.

It is known that DNA methylation is not always sufficient to repress transcription but interaction with transcription repressors and/or repressive chromatin organization is also required (Tsuikina et al., 2008). The silencing of HCMV has been shown to be associated with DNA methylation and deacetylation of histone H3 and H4 tails (Brooks et al., 2004; Murphy et al., 2002). The known recruiters of HDAC at the CMV promoter are ERF and YY1, which are established negative regulators (Wright et al., 2005; Murphy et al., 2002). However, CMV promoter containing vectors with deletion of ERF and YY1 binding sites, respond to treatment with deacetylase inhibitor TSA by activation, suggesting that other HDAC recruiters are also acting on HCMV promoter (Murphy et al., 2002). Our results suggest that McCP2 may be the alternative HDAC recruiter on CMV promoter. McCP2 is also a known recruiter of histone H3 K9 methyl transferase. Methylation of Lys 9 of histone H3 is a signal for recruiting HP1 protein (Bannister et al., 2001). Murphy et al. (2002) have shown that in the silent state CMV promoter is associated with HP1. Thus, silencing of CMV promoter is associated with repressive chromatin.
H3K4Me3 is associated with both transcriptionally active (in testis) and inactive (in liver and kidney) promoter. H3K4Me3 marks active genes as well as inactive genes primed for activation. Thus, the silent CMV promoter in somatic tissues of line C1 is epigenetically poised for activation on induction.

The level of H3K4Me3 on CMV promoter in GFP negative tissues, liver and kidney is higher than in GFP positive testis (Fig. 4). This could be due to the known depletion of nucleosomes at transcription start site of active promoters, leading to decrease in the ratio of modified to unmodified histones (Koch et al., 2007). Our results show that the silent HCMV MIEP is methylated in vivo and is associated with MeCP2, which may be involved in silencing through histone modifications.

Thus the position effect on gene expression can be attributed to the spreading of the epigenetic markings characteristic of the endogenous genomic region to the integrated or translocated DNA sequences. However in this case the bivalent histone methylation marks can direct silencing as well as activation of CMV promoter in vivo.

Acknowledgements

We thank Mr. Jaspal Singh (NII) for helping in animal maintenance. The project was supported through a grant to VB and SM from Council for Scientific and Industrial Research, India. AKM acknowledges CSIR and PA acknowledges UGC for research fellowships.

Appendix A. Supplementary data


References


