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MBD2 contributes to developmental silencing of the human ε-globin gene☆,☆☆

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ABSTRACT

During erythroid development, the embryonic ε-globin gene becomes silenced as erythropoiesis shifts from the yolk sac to the fetal liver where γ-globin gene expression predominates. Previous studies have shown that the ε-globin gene is autonomously silenced through promoter proximal cis-acting sequences in adult erythroid cells. We have shown a role for the methylcytosine binding domain protein 2 (MBD2) in the developmental silencing of the avian embryonic ρ-globin and human fetal γ-globin genes. To determine the roles of MBD2 and DNA methylation in human ε-globin gene silencing, transgenic mice containing all sequences extending from the 5′ hypersensitive site 5 (HS5) of the β-globin locus LCR to the human γ-globin gene promoter were generated. These mice show correct developmental expression and autonomous silencing of the transgene. Either the absence of MBD2 or treatment with the DNA methyltransferase inhibitor 5-azacytidine increases ε-globin transgene expression by 15–20 fold in adult mice. Adult mice containing the entire human β-globin locus also show an increase in expression of both the ε-globin gene transgene and endogenous ε^{Y} and β^{H1} genes in the absence of MBD2. These results indicate that the human ε-globin gene is subject to multilayered silencing mediated in part by MBD2.

Introduction

The genes of the human β-globin locus are located on chromosome 11 in the order of their expression during development: 5′ ε, γ, δ, and β 3′. During development, a sequential switch occurs whereby the respective 5′ globin gene becomes silent and the adjacent downstream gene becomes transcriptionally active. The exact mechanisms of this process are not yet fully understood; however, it has been shown to involve multiple interactions between *cis* elements, erythroid specific *trans* factors, ubiquitous *trans* factors, and epigenetic signals [41,42,49,54]. Mice transgenic for human β-type globin genes have provided much insight into the mechanism(s) of developmental globin gene switching. Mice containing the entire β-globin locus as a yeast artificial chromosome (β-YAC) transgene show correct developmental expression and silencing of human globin genes [13,37]. In addition, mice transgenic for smaller β-globin gene locus constructs show similar developmental regulation [51]. These transgenic studies have led to the concept of both competitive and autonomous developmental silencing of β-type globin genes. High level expression of globin genes is mediated by a complex enhancer locus located 5′ of the human ε-globin gene termed the locus control region (LCR). In the competition model, the β-type globin genes compete for LCR enhancer activity with one gene being highly expressed at the expense of the others. According to this model, in the absence of an alternative globin gene, the less competitive gene will still be expressed throughout development. The human β-globin gene is believed to be silenced during embryonic and fetal stage erythropoiesis primarily if not exclusively by this mechanism [9,10,44]. According to the autonomous silencing model, a given β-type globin gene would not be expressed outside of its correct developmental stage even in the absence of other globin genes in the locus. The predominance of published evidence suggests that the human ε-globin gene is regulated in this manner. In the case of mice transgenic for only an ε-globin gene in a construct containing a so-called "mini-LCR" consisting of the major hypersensitive sites of the

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locus control region, the transgene is significantly but not completely silenced in the absence of other globin genes and it thus has been assumed to be autonomously silenced [39]. The fetal γ-globin gene has been shown to be regulated by a combination of autonomous silencing and competition for the LCR, such that transgenic mice with the γ-globin gene and the LCR in the absence of other globin gene exhibit decreased expression of the transgene during the transition from fetal to adult development, but it is not as completely silenced as the ε-globin gene [2,9,10]. Recently, the BCL11A gene product has been shown to exert a major effect on γ-globin developmental gene silencing in β-YAC transgenic mice [43].

The autonomous silencing of the ε-globin gene is mediated by several different known factors binding to disparate sites near the coding sequences of the gene. Both GATA-1 and YY1 have been shown to bind to distinct regions of the ε-globin gene 5′ flanking sequences and to mediate transcriptional repression [38]. In addition, a complex was identified and shown to bind to two inverted direct repeats in the region of the CCAAT box. These repeats contain a short motif analogous to DR-1 binding sites for non-steroid nuclear hormone receptors [53]. Mutation of these repeats leads to expression of the ε-globin gene in adult β-YAC transgenic mice [53]. A complex binding to this site, termed DRED, was found to contain the nuclear orphan receptors TR2 and TR4 [52]. More recent studies using transgenic β-YAC constructs have demonstrated that either additional flanking sequences of the ε-globin gene or competition for the LCR from downstream globin genes may contribute to its developmental silencing [30]. Thus, it appears that multiple factors contribute to the autonomous silencing of the human ε-globin gene during development in transgenic mice.

Vertebrate globin genes also have been shown to be regulated in part by DNA methylation. The first reports of an inverse correlation between transcription status and DNA methylation were from studies of globin genes in various species [26,27,45,55]. The compound 5-azacytidine inhibits the enzyme DNA methyl transferase-1 and leads to a decrease in DNA methylation levels. Treatment with 5-azacytidine induces the expression of silenced embryonic and fetal β-type globin genes in many different model systems as well as in human patients [3,7,15,22,32]. A family of proteins known as methylcytosine binding domain (MBD) proteins binds to densely methylated DNA and recruits transcriptional co-repressor complexes that include histone deactylases [11,18,29]. One member of this family, methylcytosine binding domain protein 2 (MBD2), has been shown to bind to a densely methylated chicken embryonic ρ-globin construct *in vitro* as a large complex containing histone deactylase 1 and chromatin remodeling proteins including Mi-2, MTA-1, and RbAp48 [20,48] and it also binds *in vivo* at the developmentally silenced and methylated ρ-globin gene in adult erythroid cells [20]. Loss of MBD2 results in failure of complete silencing of the human fetal γ- and avian embryonic ρ-globin genes in β-YAC transgenic mice and stably-transfected MEL cells, respectively [19,40]. Short chain fatty acids, compounds known to inhibit histone deactylases, among other functions, have been shown to induce the expression of embryonic and fetal β-type globin genes [5,6,15,23,25,33–35,50]. Thus, in addition to known trans-acting factors that mediate autonomous and competitive silencing, epigenetic modifications contribute to vertebrate globin gene silencing.

Because of the extremely large gradient of silencing of the embryonic globin genes in adult erythroid cells of vertebrates, it has been postulated that multiple, perhaps redundant, mechanisms may exist to enforce this process. Herein, we present evidence of a role for DNA methylation and MBD2 in mediating human ε-globin silencing in adult transgenic mice in the presence or absence of competing fetal γ and adult β-globin genes. Transgenic mice containing sequences 5′ of HS5 of the LCR and extending 8 kb downstream of the ε-globin gene polyA addition site correctly silence the transgene during erythroid development. When adult transgenic mice containing these constructs are treated with 5-azacytidine, they express the normally silenced ε-globin transgene at a 15–20 fold increased level compared to untreated animals. Furthermore, adult human ε-globin gene transgenic mice null for MBD2 also express the transgene at 15–20 fold higher levels than MBD2 wild type controls. Adult β-YAC transgenic mice containing the entire β-globin locus treated with 5-azacytidine or null for MBD2 also express significantly increased but relatively lower absolute levels of ε-globin mRNA. Thus, these findings confirm a role for MBD2 in the methylation mediated silencing of embryonic β-type globin genes of more than one vertebrate species.

Materials and methods

Generation of transgenic mice

Transgenic mice were generated using two different constructs derived from the cosmid cosLCRε (a generous gift from John Strouboulis and Frank Grosveld) that contains all the sequence from 5′ of HS5 of the LCR through 12 kb 3′ of the human ε-globin gene polyA site [51] (Fig. 1). A 35 kb construct was excised from cosLCRε by Nae I resulting in a 3′ sequence extending to position 33069 in GenBank access no. U01317.1 approximately 1 kb upstream from exon 1 of the ^{G}γ-globin gene. A 28 kb construct was generated by digesting cosLCRε with NotI and BstBI to release a fragment containing 4 kb of the 3′ sequence, extending to position 25412 of the GenBank sequence database access no. U01317.1 which is located ~8.5 kb 5′ upstream from exon 1 of the human ^{G}γ-globin gene. The 35 kb construct was run on low-melting agarose and purified with gelase and phenol/chloroform. The 28 kb construct was run on a low-melting 0.6% agarose gel and purified using the Bio 101 Gene Clean Spin Kit (Qbiogene, Irvine, California). Constructs were then injected into fertilized eggs and implanted into pseudopregnant mothers to generate transgenic mice. Founders were determined by performing PCR on tail snipped DNA. A total of four independent lines were established. One line of the 28 kb construct had a single copy integration in the Y-chromosome and baseline expression of the human ε-globin gene in this line was too low to reliably measure by real-time PCR. Whether this was due to the fact that there was a single copy integration or due to a previously described y-chromosome inactivation mechanism {{1687 De Bonis,M.L. 2006}} was not determined. β-YAC mice were a generous gift from Dr. Karin Gaensler. The A20 and A85 lines were used in this work [12,13].

β-YAC, 28 kb and 35 kb LCRε transgenic mice were bred with MBD2−/− (knockout) mice to generate hemizygous transgenic mice. These mice were bred with MBD2−/− mice to generate compound transgenic β-YAC, 35 kb LCRε and 28 kb LCRε/MBD2−/− mice. Mice were screened for the presence of the transgene and the absence of MBD2 by PCR with tail snip DNA. Copy number for each LCRε transgenic line was determined by Southern blot and qPCR. Expression levels of the human ε-globin gene were linearly correlated with copy number. All mice used for gene expression analyses were highly outbred crosses among FVB, C57BL6, and BALB/C strains to minimize inbred strain specific genetic modifier effects.

Expression analysis of transgenic mouse erythroid cells

For adult erythroid cell analyses, peripheral blood was collected from tail veins. RNA was isolated using Trizol (Invitrogen) as per the manufacturer's protocol.

The developmental regulation of the ε-globin transgene in mice was determined by analyzing expression of the respective transgene at different developmental stages. Timed matings were performed and RNA was extracted from 10.5 dpc yolk sacs, 16.5 dpc fetal livers, and peripheral blood from adult anemic mice.

For each expression analysis, RNA was reverse transcribed using iScript (Bio-Rad) and quantitated by using either Taqman or SYBR green chemistry on an Applied Biosystems 7300 Real-Time PCR system.

RNA levels from each independent transgenic line were corrected for gene copy number. At least 3 mice from each independent line