Biochimica et Biophysica Acta 1849 (2015) 955–965

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagrm

A novel chromatin insulator regulates the chicken folate receptor gene from the influence of nearby constitutive heterochromatin and the β-globin locus

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ARTICLE INFO

Article history:
Received 6 March 2015
Received in revised form 22 May 2015
Accepted 25 May 2015
Available online 14 June 2015

Keywords:
Folate receptor and globin genes
Transcription
Heterochromatin
Chromatin insulator
DNA methylation
CTCF

ABSTRACT

The three-dimensional architecture of genomes provides new insights about genome organization and function, but many aspects remain unsolved at the local genomic scale. Here we investigate the regulation of two erythroid-specific loci, a folate receptor gene (*FOLR1*) and the β-globin gene cluster, which are separated by 16 kb of constitutive heterochromatin. We found that in early erythroid differentiation the *FOLR1* gene presents a permissive chromatin configuration that allows its expression. Once the transition to the next differentiation state occurs, the heterochromatin spreads into the *FOLR1* domain, concomitant with the dissociation of CTCF from a novel binding site, thereby resulting in irreversible silencing of the *FOLR1* gene. We demonstrate that the sequences surrounding the CTCF-binding site possess classical insulator properties *in vitro* and *in vivo*. In contrast, the chicken cHS4 β-globin insulator present on the other side of the heterochromatic segment is in a constitutive open chromatin configuration, with CTCF constantly bound from the early stages of erythroid differentiation. Therefore, this study demonstrates that the 16 kb of constitutive heterochromatin contributes to silencing of the *FOLR1* gene during erythroid differentiation.

1. Introduction

Chromatin domains are defined by an alternation of genomic regions enriched in repressive or active chromatin structures [1,2]. Based on the chromosome conformation capture technique (also known as 3C) and its derivatives, many large-scale genomic features have been proposed, including Topological Associated Domains or TADs [3,4]. These genome-wide studies have also contributed to a better understanding of the three-dimensional organization of the genome inside the nucleus but they involve considerably more distance than the local scale. In such lower-scale genomic resolution studies there are still many aspects to be understood, particularly from the perspective of how the genome is partitioned and regulated into transcriptionally active domains. Here we analyze the organization and dynamics of a paradigmatic group of chromatin domains associated with the chicken β-globin locus.

Historically, this locus attracted the attention of several research groups due to its differential regulation of gene expression but, more recently, by the early description of a transition between DNase I-resistant and hypoacetylated histones [5]. We now know that such a transition coincides with the location of the chicken cHS4 β-globin insulator [6–8]. Based on those seminal works, the early concept of transcriptionally active domains was initially exemplified by the description of the chicken folate receptor (*FOLR1*) gene, followed by approximately 16 kilobases (kb) of condensed chromatin and the β-globin locus separated by the cHS4 boundary element [9] (Fig. 1A). Interestingly, this compacted 16 kb genomic region possesses a significant amount of CR1-type repetitive sequences, histone repressive marks and notably, the 16 kb region is DNA-hypermethylated, which allows it to be recovered by sucrose gradient sedimentation when digested *in situ* with the HpaII restriction enzyme [9–11]. Furthermore, as additional evidence of its heterochromatic features, this region is transcribed and acquires a closed conformation in a Dicer-dependent manner [12]. Together, these observations strongly suggest that the 16 kb region of heterochromatin, which is located between the erythroid-specific folate receptor gene and the β-globin locus, represents a compact and very well-defined unit of constitutive heterochromatin (Fig. 1A).

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http://dx.doi.org/10.1016/j.bbagrm.2015.05.011
1874-9399/

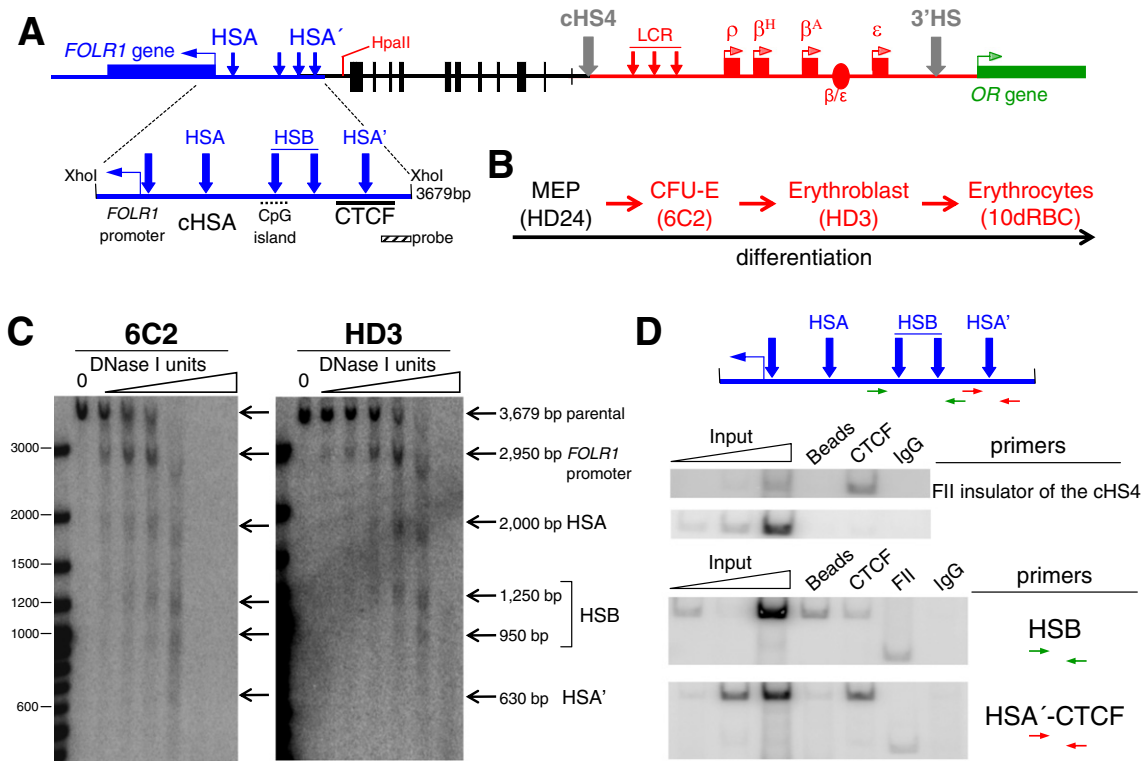

Fig. 1. Folate receptor gene expression and associated chromatin structure is restricted to an early erythroid differentiation stage. (A) Scheme of the genomic region encompassing the chicken folate receptor (*FOLR1*) gene and regulatory-related DNase I hypersensitive sites (HSA, HSB and HSA′); the 16 kb of constitutive heterochromatin with vertical black lines representing CR1-type repetitive sequences and the DNA methylated HpaII site (shown in red) correspond to the 5′ margin to the heterochromatic region [11]; the β-globin locus shielded by two chromatin insulators, cHS4 and 3′HS bound by CTCF [8,11], and the 3′-most domain represented by the brain-specific olfactory receptor gene [43]. A detailed map of the intergenic region (with the distribution of the DHSs) located between the *FOLR1* gene and the 16 kb of heterochromatin is shown. (B) Erythroid cell-types used in this study and their differentiation stages are shown. In black (HD24 cells) correspond to a myeloid/erythroid progenitor cell line and in red, erythroid cell lines at different stages of differentiation. (C) Comparative DNase I hypersensitive assays using nuclei isolated from 6C2 and HD3 cells. The DNase I units used for nuclei digestion are: 0.3; 0.7; 1.25; 2.5; 5 and 10. The probe location used for hybridization is shown in A. (D) Chromatin immunoprecipitation (ChIP) assay showing the *in vivo* binding of CTCF using, as a positive control, primers from the cHS4 β-globin insulator centered at the FII CTCF-binding site. Primers used to define the binding of CTCF at the HSA′ site are described in Supplementary Fig. S3. Note the lack of enrichment at the HSB site, a result that is in concordance with previously published data [29]. This is a representative set of results from four independent ChIP assays.

From the literature several lines of evidence have shown that constitutive heterochromatin can have a negative effect on the expression of genes that are translocated in its proximity, through a phenomenon known as position effect variegation [13]. It has been mechanistically demonstrated that certain histone repressive marks, like histone H3 lysine 9 trimethylation (H3K9me3) or even DNA methylation, can spread from a heterochromatic source over several kilo bases [14]. Therefore, it has been proposed that the chicken cHS4 insulator possesses a barrier function to counteract the spreading of the heterochromatin over the β-globin locus [15–17]. Here we asked whether, as in the case of the β-globin locus (Fig. 1A), the folate receptor gene, as an independent domain, needs to be protected by a barrier element against the silencing effects of the nearby heterochromatin.

This locus encodes an erythroid-specific folate receptor with a different and early program of gene expression compared to the chicken β-globin group of genes [9]. The chicken folate receptor gene is transcribed in early erythroid precursor cells and its expression is repressed during terminal erythroid differentiation [9]. In other words, the folate receptor pattern of gene expression precedes globin gene activation, and when the globin genes are transcriptionally activated, the folate receptor gene is already silenced [9,10]. A strong DNase I-hypersensitive site (DHS) designated HSA shows an apparent enhancer activity over the *FOLR1* gene, with an activity over a reporter gene that is copy-number dependent and independent of the integration site in stably transformed erythroid cells [9]. With all this in mind we asked if a chromatin insulator is present between the HSA enhancer and the delimiting HpaII site which represents the 5′ boundary of the 16 kb of heterochromatin (Fig. 1A). At this point it is important to outline that the previously reported work has described the existence of a boundary at the 5′ end of the condensed chromatin region, protecting against the encroachment of histone modifications in particular histone mono-ubiquitination [18]. We hypothesized that such an insulator may allow the *FOLR1* gene to be expressed based on an optimal chromatin configuration, and in an erythroid-specific stage of cellular differentiation.

To address this aim, a comparative DNase I hypersensitivity assay showed different and unobserved sites between the *FOLR1* HSA and the boundary of the 16 kb of heterochromatin. We found a novel CTCF binding site with chromatin insulator properties. Interestingly, when we compared the two erythroid stages represented by 6C2 and HD3 cells, we found a differential pattern of DNA methylation and binding of CTCF. Together, these results support the presence of CTCF and regulated boundary activity when the folate receptor gene needs to be expressed. Therefore, we have characterized a novel boundary element that acts in response to different stages of erythroid differentiation.

2. Materials and methods

2.1. Plasmid constructs

DNA fragments containing the HSB–HSA′ insulator was amplified by PCR from genomic DNA from 10-day-old chicken embryonic erythrocytes with the following primers: Forward: 5′-CCAGACACACACTGCT CCCAC-3′ and Reverse: 5′-GGCATCCATGGGAAAAGGCTGC-3′, then cloned into pGα^{D}3 at the EcoRI 5′ site and NheI–MluI at the 3′ site flanking the α^{D} promoter and EGFP cDNA, respectively. A DNA subfragment containing 6.7 kb of the 16 kb heterochromatin region was