A conserved regulatory element located far downstream of the gls locus modulates gls expression through chromatin loop formation during myogenesis

Katsutoshi Yuasa, Shin’ichi Takeda, Takao Hijikata

Department of Anatomy and Cell Biology, Research Institute of Pharmaceutical Science, Faculty of Pharmacy, Musashino University, 1-1-20 Shinmachi Nishitokyo-shi, Nishitokyo, Tokyo 202-8585, Japan

Department of Molecular Therapy, National Institute of Neuroscience, NCNP, Tokyo 187-8502, Japan

Article info

Article history:
Received 6 April 2012
Revised 9 July 2012
Accepted 30 July 2012
Available online 9 August 2012
Edited by Ned Mantei

Keywords:
Chromatin loop
Chromosome conformation capture assay
Distant regulatory element
gls gene
Enhancer
Myogenesis

1. Introduction

Gene transcription in higher eukaryotes has recently been reported to be regulated by the 3-dimensional organization of chromatin [1], in addition to epigenetic modification of chromatin [2]. The spatial organization of chromatin is modulated remotely through distant regulatory elements. However, transcriptional regulation via distant regulatory elements has not yet been well characterized because the wide distribution of the regulatory elements throughout the genome complicates the identification of the genes controlled by particular regulatory elements and the analysis of the functional relationships of these genes with distant target promoters.

To circumvent these challenges, the chromosome conformation capture (3C) technique has recently been developed and has been used to detect physical association points between genomic loci [3]. This method is useful for examining the mechanism underlying transcriptional regulation by chromatin loops formed by direct association between distant regulatory elements and their target promoters [1,4]. Using 3C assays, long-range looping events have been detected in the β-globin [5], α-globin [6], and interleukin gene clusters [7] and in single genes such as FSHD region gene 1 (FRG1) [8], the cystic fibrosis transmembrane conductance regulator (CFTR) gene [9], and the myeloblastosis oncogene (Myb) [10]. Understanding the spatial organization of chromatin will help elucidate the mechanism underlying distant regulation of gene transcription.

Comparison of DNA sequences across species is an efficient approach to identifying putative functional regions in non-coding DNA [11]. In this study, using comparative analyses and bioinformatic approaches, we identified a conserved distant regulatory element in the region upstream of the signal transducer and activator of transcription 1 (STAT1) gene, the expression of which contributes to the proliferation of myoblasts and inhibition of differentiation into myotubes [12]. Using reporter assays, we showed that this regulatory element enhanced the transcriptional activity of the STAT1 promoter. In addition, by using 3C and reporter assays, we found that the distant regulatory element also enhanced the expression of the adjacent gls gene, which encodes kidney-type glutaminase (GLS). We here reported characterization of a novel distant regulatory element and its regulatory relevance to transcriptions of STAT1 and gls gene during myogenic differentiation.
2. Materials and methods

2.1. Plasmid construction

To generate pRL reporter vectors, PCR fragments were amplified from the mouse genome, and were then inserted into the pRL-null vector (Promega, WI, USA). The pGL–CMV vector was generated by replacing the SV40 promoter of the pGL3-control vector (Promega) with the CMV promoter. The primer sequences and procedures for each plasmid are shown in the Supplementary data.

2.2. Luciferase reporter assay

Mouse C2C12 myoblasts or RGB-3T3-5 fibroblasts were co-transfected with the pGL control vector, encoding firefly luciferase, and the pRL reporter vector, encoding Renilla luciferase (Rluc), at a molar ratio of 1:6.7 by using the Lipofectamine™ 2000 reagent (Invitrogen, CA, USA). The cells were then cultured in growth medium (GM, viz., DMEM with 20% fetal bovine serum) or differentiation medium (DM, viz., DMEM with 5% horse serum and 10 μg/ml insulin) for 2 days. Transcriptional activities were assessed using the Dual-Luciferase® Reporter Assay System (Promega).

2.3. Quantitative reverse transcription-polymerase chain reaction

C2C12 (4 x 10^5 or 8 x 10^4 cells) or RGB-3T3-5 cells (6 x 10^5 cells) were cultured on 12-well collagen-coated plates in GM or DM for 2 days. Total RNA was isolated using the ISOGEN reagent (Nippon Gene, Tokyo, Japan), and cDNA was synthesized using a High Capacity cDNA Reverse Transcription (RT) kit (Applied Biosystems, CA, USA). The cDNA was quantified on a LightCycler2.0 apparatus (Roche, Mannheim, Germany) by using SYBR Green. The expression levels of mRNA were normalized to that of 18S rRNA. The primer sequences are shown in the Supplementary data.

2.4. 3C assay

The 3C assay was performed according to a previously described procedure [13,14], with some adaptations for myogenic cells [8]. Briefly, nuclei were isolated, crosslinked by formaldehyde, and then digested with EcoRI. The mixtures were incubated with T4 DNA ligase, decrosslinked, and purified by proteinase K and phenol–chloroform treatments. An additional digestion with HindIII was performed for each sample. The 3C templates were

---

**Fig. 1.** Identification and characterization of a distant regulatory element (5.5URR). (A) Multiple sequence alignment of 5.5URRs across species. Using the TSEARCH program, each 5.5URR upstream of the human, chimpanzee, monkey, marmoset, pig, cattle, dog, rabbit, mouse, and rat *stat1* genes was identified. The 5.5URRs were aligned using CLUSTALW (http://www.genome.jp/tools/clustalw/). The asterisks indicate nucleotides conserved among these mammals. The putative TF-binding sites for NF-κB, IRF, STAT, MYC, and MAZ are underlined, and their consensus sequences are shown above each site. (B) Tracks depicting the chromatin state in the 5.5URR of human myoblasts (Mb) and myotubes (Mt). The epigenetic signals were obtained from the Chip-seq datasets of the Broad/MGH ENCODE group at the UCSC Genome Bioinformatics Site (http://genome.ucsc.edu/). The epigenetic modifications are indicated to the left of the tracks. The black bar and arrows on the top indicate the positions of the human 5.5URR and genes (stat4 and stat1), respectively.
quantified in the LightCycler by using hybridization probes. Standard curves were generated using the BAC clone RP23-164N3 (Invitrogen), which spans the stat1 and gls loci. Relative interaction frequencies were calculated as the ratio of the amount of cross-linked DNA template to that of non-crosslinked internal control (gapdh). The results were further normalized to the 3C amplification products of 2 EcoRI fragments, that is, fragments 1 and 2 (interaction frequency control). Detailed procedures, and the sequences of primers and hybridization probes are provided in the Supplementary data.

Fig. 2. The 5.5URR acts as an enhancer in myogenic cells. Transcriptional activities of the stat1 proximal promoters with a series of upstream regions (A), with 5.5URR and various intervals (B), or with mutated 5.5URR (C) were examined by reporter assays. In (C), all TF-binding sites between the NF-κB and MAZ motifs were deleted. A schematic of the region upstream of the mouse stat1 promoter is indicated at the top. The individual promoter constructs and their relative expression levels are shown on the left and right, respectively. Nucleotide numbering is shown relative to the transcriptional start site (TSS, +1) of the stat1 gene. The genes and exons are designated as arrows and boxes, respectively. C2C12 myoblasts were co-transfected with the pRL-STAT1 reporter and pGL3 control vectors and then cultured in DM for 2 days. (D) Expression of stat1 and myogenin (myogenic differentiation marker) transcripts during myogenic differentiation. C2C12 myoblasts were cultured in GM or DM and harvested at different stages of cell proliferation such as the pre-confluent (P) or fully confluent (C) state. The mRNAs were quantified by real-time RT-PCR. The data shown represent 3 (A–C) or 4 (D) independent experiments, and the error bars represent the S.D. of the mean. Statistical significance was evaluated by the Student’s t-test. $P < 0.05$ vs. pRL-STAT1 (2.6 kb); $^\#P < 0.05$ vs. pRL-STAT1 (0.6 + 2.6 kb) WT; $^{##}P < 0.01$. 
3. Results

3.1. Identification and characterization of a distant regulatory element

We found a putative regulatory region, including several transcription factor (TF)-binding sites, when we searched for regulatory elements of the stat1 gene by using the TFSEARCH program (http://mbs.cbrc.jp/research/db/TFSEARCH.html). This regulatory region was located 5.5 kb upstream of the mouse stat1 gene and was therefore designated as the 5.5 kb upstream regulatory region (5.5URR). The 5.5URR was a small (~180 bp) cluster, consisting of 5 TF-binding motifs for the NF-κB, IRF, STAT, MYC, and MAZ proteins. Interestingly, similar clusters of these five binding motifs were found in the regions upstream of the stat1 genes of various mammals such as humans, chimpanzees, monkeys, marmosets, pigs, cattle, dogs, rabbits, mice, and rats, indicating that the 5.5URR is conserved among mammals (Fig. 1A).

Next, we examined whether the 5.5URR could act as a regulatory element in myogenic cells by utilizing ChIP-Seq datasets that are publicly available at the UCSC Genome Browser (http://genome.ucsc.edu/), because epigenetic signatures enabled us to identify functional regulatory elements, including promoters and enhancers [15]. Several types of histone modifications, representative of promoter and regulatory elements [16,17], such as H2A.Z, H3K4me2, H3K4me3, H3K9ac, and H3K27ac, were enriched in the human 5.5URR of both myoblasts and myotubes (Fig. 1B), strongly suggesting that the 5.5URR can act as a functional regulatory element.

3.2. Regulatory function of the 5.5URR in myogenic cells

The 5.5URR was assumed to be a regulatory element of the stat1 gene because of the active epigenetic features shared between the 5.5URR and the stat1 promoter in human myogenic cells (Fig. 1B) and their proximity within the genome. On the basis of this assumption, we examined the transcriptional activities of the stat1 proximal promoters in the presence or absence of the 5.5URR in C2C12 cells by using reporter assays. As shown in Fig. 2A and B, all stat1 promoter constructs containing the 5.5URR exhibited greater reporter activities than the stat1 proximal promoter alone or stat1 promoters lacking the 5.5URR did. A mutated construct containing a deletion between the NF-κB- and MAZ-binding motifs showed complete loss of the enhancer effect of the 5.5URR (Fig. 2C). These results indicated that the 5.5URR functions as a transcriptional enhancer in C2C12 cells.

Next, to examine whether the 5.5URR act as a transcriptional enhancer during myogenic differentiation, we measured the level of stat1 transcripts during the proliferative or differentiated stages of C2C12 cells by quantitative real-time RT-PCR. We found that stat1 transcript levels were similar during myogenic differentiation (Fig. 2D).

---

Fig. 3. Long-range interactions between the 5.5URR and gls promoter during myogenic differentiation. (A) Schematic representation of the mouse 5.5URR-stat1-gls locus. The bold arrows and vertical lines at the top indicate genes and their exons, respectively. Nucleotide numbering is shown relative to the TSS of the stat1 gene. EcoRI sites are shown under the line, with restriction fragments numbered from 1 to 29. The 5.5URR and gls promoters were present within EcoRI fragments 1 and 25, respectively. The small arrows under the line indicate the relative positions of constant and test primers used in 3C assays. The constant primer within the EcoRI fragment 1 was used as an anchor and was paired with each test primer within EcoRI fragments 2–29 across the stat1 and gls loci. (B, C) Quantitative 3C analyses of C2C12 myoblasts (B) and myotubes (C). In these experiments, myoblasts (Mb) and myotubes (Mt) were cultured under the conditions of GM-P and DM-C (cf. Figs. 2D and 4A), respectively. The graphs represent the relative interaction frequencies between the 5.5URR and each EcoRI fragment on the vertical y-axes. The x-axes indicate the position, in kb, relative to the TSS of the stat1 gene.
3.3. Long-range interaction between the 5.5URR and gls promoter

A single distant regulatory element can associate with multiple genes through multiple long-range interactions [5–7]. To assess whether this was true for the 5.5URR, we examined whether the 5.5URR could associate with other genes adjacent to the stat1 gene. On searching for epigenetic features of genes upstream or downstream of the stat1 locus on publicly available ChIP-Seq datasets, we observed similar epigenetic signatures between the 5.5URR and the gls promoter, but not the stat4 promoter, in myogenic cells (Supplementary Fig. 1), suggesting the possibility of interactions between the 5.5URR and the gls promoter. Therefore, we focused on the gls gene located downstream of the stat1 gene.

To clarify whether the 5.5URR could physically interact with the gls proximal promoter located at a distance of 120 kb from the 5.5URR (Fig. 3A), we performed a quantitative 3C assay using DNA specimens prepared from C2C12 myoblasts. As shown in Fig. 3B, a local increase was found in interaction frequencies at the position of EcoRI fragment 25, indicating that the 5.5URR could physically interact with EcoRI fragment 25, which contains the gls proximal promoter.

To assess whether the physical interaction between the 5.5URR and the gls promoter was maintained during myogenic differentiation, we further analyzed the interaction frequencies in DNA specimens obtained from differentiated myotubes. However, the local increase in interaction frequencies, observed at the position of EcoRI fragment 25 in myoblasts, was negligible in myotubes (Fig. 3C), suggesting a loss of interaction between the 5.5URR and the gls proximal promoter in differentiated myotubes. This further indicates dynamic changes of the interaction during myogenic differentiation.

3.4. Regulation of the gls gene by the 5.5URR

To assess the functional significance of the dynamic changes in the chromatin loop during myogenesis, we examined the expression of the gls gene in myoblasts and myotubes by quantitative real-time RT-PCR. The gls transcripts were reduced in myotubes than in myoblasts (Fig. 4A). A similar reduction in the expression of gls transcripts was observed early in cardiotoxin-induced muscle regeneration in mouse skeletal muscles (Supplementary Fig. 2). These results, together with those of the 3C assay, suggested that the expression of gls was regulated by switching off the interaction during in vitro and in vivo myogenesis. In myoblasts, the physical interaction between the 5.5URR and the gls promoter appears to enhance the expression of gls transcripts, whereas the interaction was abrogated to reduce this expression in myotubes.

Next, we generated 2 reporter constructs of the gls promoter, with or without the 5.5URR upstream of the luciferase gene, to mimic interaction or non-interaction between the 5.5URR and the gls promoter, and then determined the reporter activities in myoblasts and myotubes (Fig. 4B–D). We first compared the reporter activity of the gls promoter with the 5.5URR, in myoblasts, to that of the gls promoter alone, in myotubes, on the basis of our 3C results indicating that the gls promoter interacted with the 5.5URR in myoblasts, but not in myotubes. The gls promoter with the 5.5URR in myoblasts showed a higher reporter activity than the gls promoter alone in myotubes. This was consistent with the results of real-time RT-PCR analysis. However, comparison between myoblasts and myotubes with respect to the reporter activities of each construct revealed significantly more activity in myotubes than in myoblasts, which was inconsistent with the real-time PCR results.
Myogenic differentiation was promoted by switching to a medium with low serum content; this may act as an environmental stress that induces abrogation of the interaction. To address this possibility, RGB-3T3-5 fibroblast cells were cultured in GM or DM, and the expression of the gls gene and the interactions between the 5.5URR and the gls promoter were examined by qPCR and 3C assays, respectively (Fig. 5). In fibroblasts, gls transcripts were expressed at similar levels in both GM and DM. Reporter assays using the gls promoter alone or with the 5.5URR showed similar reporter activities under both culture conditions. In 3C assays, local interactions between the 5.5URR and the gls promoter were detected at the position of EcoRI fragment 25, which includes the gls promoter, in cells cultured in both GM and DM. These results indicated constant interaction between the 5.5URR and the gls promoter in fibroblasts and suggested that low serum content may not be a major factor abrogating the interaction.

4. Discussion

In this study, we identified a novel distant regulatory element, the 5.5URR, which was well conserved among mammals and was epigenetically modified. We investigated the regulatory relevance of the 5.5URR for stat1 and gls expression in myogenic cells and found that the 5.5URR enhanced the transcriptional activity of the stat1 and gls promoters in reporter assays. In addition, the 5.5URR differentially regulated the transcription of the gls gene during myogenic differentiation. The 5.5URR and the gls promoter, located 120 kb from each other, physically interacted, leading to an increase in the gls transcript levels in myoblasts; however, this interaction did not occur in differentiated myotubes, and the transcript levels were lower in these cells. These findings suggested that the 5.5URR is an active enhancer and that differential control of gls expression during myogenesis is enabled by a chromatin loop involving the 5.5URR.

Chromatin loops, resulting in physical interaction between the 5.5URR and the gls promoter, were formed prior to myogenic differentiation but were subsequently abrogated. Similar dynamic changes in chromatin loops have been reported in skeletal muscles and erythrocytes during differentiation. For instance, myogenic differentiation accompanied an almost complete loss of the chromatin loops formed between a distant regulatory element and the FRT1 promoter [8]. Similarly, during erythroid differentiation, chromatin loops observed between a distant regulatory element and the Myb promoter were lost [10], while the locus control region (LCR), located almost 60 kb from the β-globin genes, seemed to modify the substructures of chromatin loops to coordinate the temporal expression of the β-globin gene family [5, 18]. This differentiation-dependent formation or loss of chromatin loops may be attributable to the presence or absence of protein complexes epigenetically modifying regulatory and/or promoter regions. However, the molecular mechanisms underlying the formation of chromatin loops and the formation of physical interactions between chromatin loci are not yet clear.

The 5.5URR could function as an active enhancer in myogenic cells and fibroblasts, as indicated by ChIP-Seq datasets from the UCSC Genome Browser. In both cell types, histone modifications of the human 5.5URR, such as H3K4me3 and H3K9ac, but not H3K27me3, were present [Fig. 1, Supplementary Figs. 1 and 3]. Active promoters and enhancers have been associated with H3K4me3 and H3K9ac, while repressed promoters have been reported to be marked by H3K27me3 [16, 17]. In particular, H3K4me3 enrichment in active enhancers has been correlated with the accumulation of RNA polymerase II [19].

In addition to the 5.5URR, epigenetic modifications of target promoters are important for active gene regulation. Interestingly, the epigenetic signatures of the stat1 and gls promoters were similar to those of the 5.5URR in myogenic cells and fibroblasts (Supplementary Figs 1 and 3), suggesting active associations between the 5.5URR and each promoter.

Although the gls gene was a target of the 5.5URR, the stat1 gene could also be a target gene in myogenic cells. This was supported by following findings: (i) the 5.5URR and the stat1 promoter shared the same epigenetic features in human myogenic cells (Fig. 1B), and (ii) the longest stat1 promoter construct (6.6 kb), encompassing the genomic region that includes the stat1 promoter, exhibited much higher reporter activity than the stat1 proximal promoter alone (Fig. 2B). Under this assumption, it is plausible that the 5.5URR could constantly enhance the transcriptional activity of the stat1 promoter throughout myogenic differentiation.

In glutamine metabolism, GLS is an enzyme that converts glutamine to glutamate, which is, in turn, converted to α-ketoglutarate and then further metabolized in the TCA cycle to produce ATP. Thus, GLS contributes to the energy supply in many types of mammalian cells, especially in actively dividing cells and highly proliferative neoplastic cells [20]. This property of GLS may account for why myoblasts exhibited higher levels of gls expression than myotubes, as actively proliferating myoblasts would require more energy than differentiated myotubes without contraction; this energy could be provided through upregulation of gls expression via interaction between the 5.5URR enhancer and the gls promoter.
In conclusion, we identified a functional distant regulatory element—the 5.5URR—located far downstream of the gls gene. The element enhanced promoter activity in reporter assays. In addition, it physically interacted with the gls promoter in myoblasts, leading to abundant expression of gls transcripts, but not in myotubes showing lower levels of gls expression. These results provide novel insights into the mechanism of transcriptional regulation of the gls genes during myogenesis.

Acknowledgements

This work was supported by a general Grant-in-Aid from Musashino University and an Intramural Research Grant (22-5) for Neurological and Psychiatric Disorders of NCNP.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.07.074.

References