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Journal of Cellular Biochemistry 105:301–312 (2008)

Journal of Cellular Biochemistry

Identification of Long Range Regulatory Elements of Mouse α-Globin Gene Cluster by Quantitative Associated Chromatin Trap (QACT)

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ABSTRACT

Chromatin from different regions of the genome frequently forms steady associations that play important roles in regulating gene expression. The widely used chromatin conformation capture (3C) assay allows determination of the in vivo structural organization of an active endogenous locus. However, unpredicted chromatin associations within a given genomic locus can not be identified by 3C. Here, we describe a new strategy, quantitative associated chromatin trap (QACT), which incorporates a modified 3C method and a quantitative assay tool, to capture and quantitatively analyzes all possible associated chromatin partners (ACPs) of a given chromatin fragment. Using QACT, we have analyzed the chromatin conformation of the mouse α-globin gene cluster and proved the extensive interaction between HS26 and α-globin genes. In addition, we have identified a candidate α1-globin gene specific silencer 475A8 which shows the differentiation-stage specific DNase I hypersensitivity. Functional analysis suggests that 475A8 may regulate the α1-globin gene during terminal differentiation of committed erythroid progenitor cells. ChIP (chromatin immunoprecipitation) and cotransfection assays demonstrate that GATA-1, a hemopoietic specific transcriptional factor, may increase α1-globin gene expression by suppressing the function of 475A8 in terminally differentiated erythroid cells. J. Cell. Biochem. 105: 301–312, 2008.

KEY WORDS: α-GLOBIN GENE CLUSTER; 475A8; QACT

In eukaryotic nuclei, DNA is packaged in the form of chromatin that controls gene transcription. An important question is how gene expression is regulated in the context of chromatin. Accumulating evidences suggest that chromatin is not randomly distributed but is organized as functional three dimensional (3D) structures [Chakalova et al., 2005; Fraser, 2006]. For example, *cis*-regulatory elements, including enhancers and locus control regions (LCRs), are thought to augment the activity of promoters by interactions through looping out of the intervening DNA. These regulatory elements may also act *in trans* [Spilianakis et al., 2005; Lomvardas et al., 2006]. For instance, physical and functional evidences have emerged for nonallelic interactions between chromosomes. An LCR in the interferon-γ locus can associate with interleukin-4 locus on a different chromosome in naïve T cells that are committed to differentiate into cells expressing only one of the two cytokines in a monoallelic manner [Spilianakis et al., 2005]. Similar nonallelic interchromosomal associations have been found in the imprinting control region of the *Igf2*/*H19* locus and the *Wsb1*/*Nf1* gene [Ling et al., 2006]. Moreover, Lomvardas et al. [2006] have demonstrated a specific association of an enhancer element H on chromosome 14 with multiple odorant receptor gene promoters on different chromosomes. It is likely that gene regulation through interchromosomal interactions may be a general phenomenon conserved in different regulatory systems. Thus, an in-depth analysis of all chromatin fragments that interact with a specific genomic locus is required to fully understand gene regulation.

However, the main problem is lacking of the appropriate method to analyze the unknown associations of one given chromatin region.

Li-Jun Di and Li Wang contributed equally to this work.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: National Natural Science Fundation of China; Grant numbers: 30393110, 30421003; Grant sponsor: "973" program of China; Grant number: 2005CB522402.

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Received 14 February 2008; Accepted 29 April 2008 • DOI 10.1002/jcb.21826 • 2008 Wiley-Liss, Inc.

Published online 24 July 2008 in Wiley InterScience (www.interscience.wiley.com).

For example, the 3C method is a technique commonly used for evaluating association frequency between two restrictive chromatin fragments which are far away from each other in their genomic locations on the same or even different chromosomes [Spilianakis et al., 2005]. Most of the known functional long range chromatin associations have been confirmed by the 3C. However, 3C is not suitable for identification of associations without prior knowledge of protein complex that may mediate such associations. Several new technologies have been introduced recently which can be applied to searching the unknown associated DNA regions [Dostie et al., 2006; Lomvardas et al., 2006; Simonis et al., 2006; Wurtele and Chartrand, 2006; Zhao et al., 2006]. However, these new technologies are either too complicated or too preliminary to be applied widely. For example, the associated chromatin trap (ACT) is developed based on 3C and theoretically could be used to capture all the associated chromatin fragments from other chromosomal regions. But this technology requires large amount of sequencing. Here, we report a revised method QACT that could be easily applied to quantitatively identify the most important ACPs genome wide.

The mouse alpha globin gene cluster is a good model system in studying the local chromatin associations. There are three functional members in this gene cluster, ζ, α1, and α2-globin genes. The ζ-globin gene mainly express in the embryonic and fetal stages, whereas the α1 and α2 globin genes begin to express after 12.5 dpc and play important roles through adulthood. The mouse α-globin locus is embedded in a GC-rich region neighbored by a cluster of housekeeping genes [Anguita et al., 2002]. Interestingly, its major regulatory element (MRE), HS26 (HS26, DNaseI-hyper-sensitive site 26, corresponding to HS40 in humans), is located in an intron within the housekeeping gene C16orf35 [Bouhassira et al., 1997]. Our previously study has proved the existence of αACH that represent an active chromatin organization status in terminal differentiated erythroid cells [Zhou et al., 2006]. The upstream regulatory elements and the active transcribed genes including the flanking housekeeping genes of α-globin gene cluster could interact to each other by looping out the intervening regions [Zhou et al., 2006]. In this report, we applied QACT to capture all the possible associated chromatin fragments of HS26 and proved that the HS26 interacts extensively with α-globin gene promoters. Additionally, a previously unknown candidate regulatory element 475A8 has also been identified in this assay. Analyzing of this regulatory element suggested a possibly new regulatory mechanism of α-globin genes in mouse.

MATERIALS AND METHODS

CHROMOSOMAL CONFORMATION CAPTURE (3C)

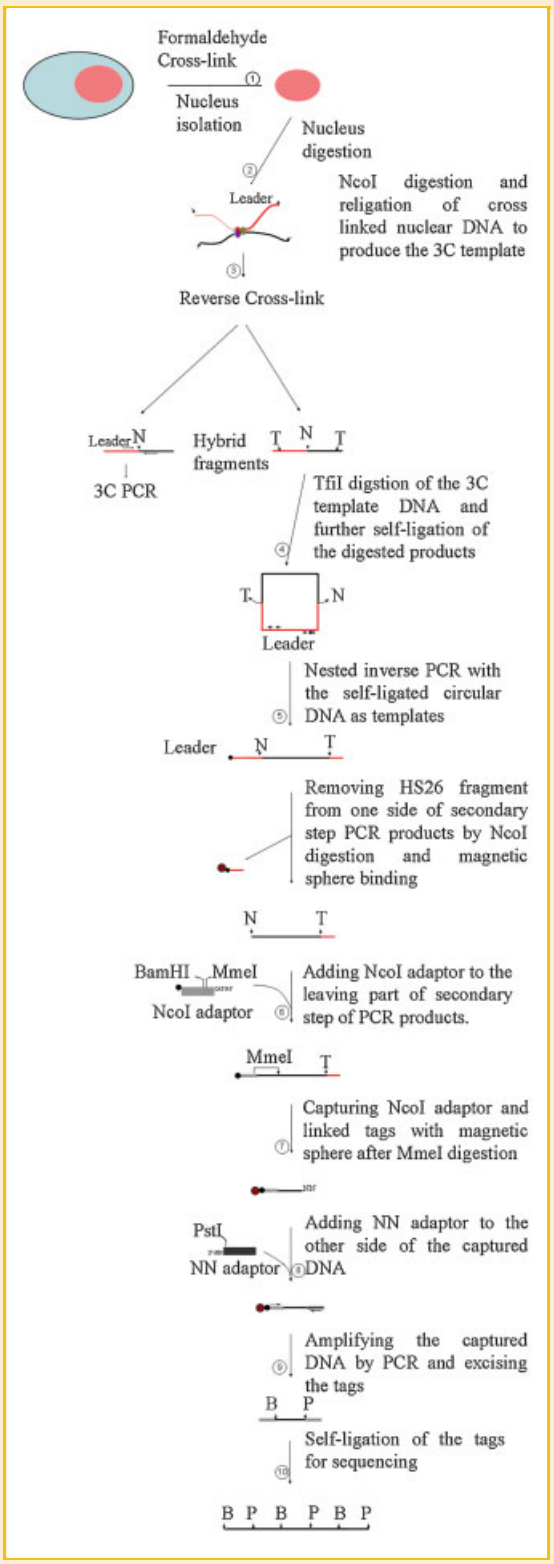
The 3C assay was performed as previously described [Dekker et al., 2002; Tolhuis et al., 2002] with a few modifications [Zhou et al., 2006]. We chose four representative sites at HS8, ζ, α1, and α2 RCF to perform the agarose gel electrophoresis, Southern blotting and semi-quantitative assay. Results show that the digestion efficiencies at different sites are similar (Supplementary Fig. 1).

The BAC clones used to correct for the PCR amplification efficiency of each set were 187-kb BAC (no. RP23-130H16; CHORI BACPAC Resources) and 214-kb BAC containing the 475A8 locus (no. RP23-475A8; CHORI BACPAC Resources). Primer sequences for the tested restriction fragments were provided in the supplementary data. Errc3 gene was used for correcting the template amount between fetal liver sample and MEL cell sample. Errc3 encodes a subunit of the basal transcription factor TFIIH and could be used to normalize the differences of DNA amounts between two samples [Palstra et al., 2003; Zhou et al., 2006]. All test primer pairs were verified by amplifying the control sample and sequencing the PCR products. All PCRs were carried out at least three times. The correction method was the same as that given in the work of Dekker et al. [2002] and Tolhuis et al. [2002]. The calculation gives a relative ligation frequency for each analyzed sample, since it corrects for the differences in PCR amplification efficiencies, amounts of templates, and sizes of PCR products.

QACT

QACT is designed on the basis of 3C [Dekker et al., 2002]. The prepared 3C templates were the starting material for QACT and 10 μg 3C templates DNA were completely digested by the secondary endonuclease TfiI to produce the cohesive end and then the digestion products were purified and self-ligated in 500 μl volume. This large volume ligation is inclined to raise the intramolecular ligation. The circular DNA, product of self-ligation can be used as the template for further inverse PCR. As a control, a parallel NcoI digested reverse cross linked non-ligated sample was also used for this self ligation in a large volume to perform the inverse PCR. For simplicity, the HS26 was considered located at the upstream or 5′ side of α-globin genes as illustrated in Figure 2. The QACT assay was designed to capture the ACPs that ligated to the downstream side or 3′ side of the leader RCF HS26. The primers used for nested inverse PCR were as follows: FHS26F, GTGATTACAGATACCTCCAAG; FHS26R, TTGGGAAGACTTTCTACATT; SHS26F, TCATAATAGGTGGGTGGAAT; SHS26R, TGTCTTGTTTGCTGCCTTCA. The 5′ side of SHS26R was modified with biotin. PCR reactions were performed as follows: one cycle at 94°C for 4 min; 25 cycles at 94°C for 30 s, 56°C for 40 s, and 72°C for 30 s; followed by one cycle at 72°C for 10 min. The first step PCR products were used as the templates of second step of inverse PCR after 100-fold dilution. The secondary PCR was performed as follows: one cycle at 94°C for 4 min; 30 cycles at 94°C for 30 s, 60°C for 40 s, and 72°C for 30 s; followed by one cycle at 72°C for 10 min. As illustrated in Figure 1 and described in supplementary data, the leader RCF was removed from the purified secondary step of PCR products and the *Nco*I adaptor was ligated to the leaving ACPs. NcoI adaptor was obtained by annealing the following primers: NcoIadF 5′-ATGATTACGCATTCGAGCTAGG-3′ and NcoIadR 5′-phosphate-GATCCCTAGCTCGAATGCGTAATCAT-3′. BamHI and MmeI restrictive recognition sites were included in the NcoI adaptor. Further MmeI digestion excised 19/20 bp tags from the ACPs and the tags could be captured by magnetic sphere through the NcoI adaptor and its biotin modification. The NN adaptor was ligated to the other side of the tags. NN adaptor was obtained by annealing of the following primers: NNadF 5′-ACGACGGCCAGTGCCAAGCTCTGCA-3′ and NNadR, 5′-phosphate-GAGCTTGGCACTGGCCGTCGT-3′. A PstI restrictive site was included in the NN adaptor. Tags were amplified by additional PCR reaction with NcoIadF and NNadF as primers. The

two sides of adaptors were removed by BamHI and PstI. Self-ligation of these tags produces concatemers including several to nearly 20 tags. This strategy is revised from Sabo et al. [2004b] and Velculescu et al. [1995]. The concatemers around 500 bp in length were cloned directly into the multi clonging site (MCS) of the vector pUC19. The inserted concatemers were sequenced. The sequences of the tags could be mapped to the mouse genome with the BLASTn tool.



CHIP ASSAY

ChIP analysis was carried out for DMSO induced (4 days) MEL cells and MEL cells. Briefly, isolated 1% formaldehyde-cross-linked cells were lysed in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1) containing protease inhibitors and sonicated on ice until cross-linked chromatin DNA was sheared to an average length of around 500 bp. Sonicated cell supernatant (1×10^7 cells per experiment) was diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl). The precleared chromatin using protein A-agarose (Upstate) was incubated overnight at 4°C with rat anti-GATA-1 (SC-265) antibody (Santa Cruz Biotechnology), anti-acetylated-H3 (06-599, Upstate), anti-acetylated-H4 (06-866, Upstate) or with pre-immune rat serum as the control. Immunoprecipitates were recovered by incubation with protein G-agarose (Upstate) at 4°C for 2 h, followed by low-speed centrifugation. Washed pellets were reverse cross-linked by incubation at 65°C for 6 h and digested with proteinase K (Roche) at 45°C for 12–14 h. The DNA was extracted with phenol-chloroform/isoamyl alcohol (25:24:1), precipitated with ethanol, and used for PCR analysis. Meanwhile, 1/10 of the sonicated cell supernatant was also reverse cross-linked, and the DNA was purified and used for input to correct for differences in PCR amplification efficiencies and DNA amounts. The primers used for ChIP analysis are available by request. The linear range of PCR amplification of ChIP sample and input were determined by serial dilution. An appropriate DNA amount was used for the quantitative PCR analysis. All PCRs were performed at least three times. All PCR products were resolved by 2% agarose gel electrophoresis, stained with ethidium bromide, and then quantified on an UVI.

TRANSFECTION ASSAY

Constructs used in transfection assay: pG is the abbreviations of pGL3-basic (Promega). pGαp is constructed by inserting the

Fig. 1. Schematic representation of QACT. 3C templates were generated by original 3C procedure and used for either 3C PCR assay or generating the QACT library through nested inverse PCR. Primers FHS26F and FHS26R were used for the first step of nested PCR. Primers SHS26F and SHS26R were used for the secondary step of the nested PCR. The 5′ side of SHS26R was modified with biotin (black filled circle). To obtain the sequence information of the RCFs ligated to HS26 RCF, HS26 fragment at one side of secondary step of PCR products were removed by NcoI digestion followed by magnetic sphere binding (brown filled circle). Another specifically designed NcoI adaptor was used to replace the removed part. There are both BamHI and MmeI recognition sites in the NcoI adaptor that facilitates the excision of 19/20 bp tags from the unknown RCFs. After digestion of the religated products by MmeI, a cohesive end with two non-specific nucleotide extrudings were produced which could be ligated to a NN adaptor. The sandwich fragment "NcoI adaptor-tag-NN adaptor" was then further amplified. The tags were excised with BamHI and PstI digestion. Self-ligation of tags generates the concatemers for high-throughput sequencing. N, T, B, P are abbreviations of NcoI, TfiI, BamHI, and PstI, respectively. Each step as indicated by the numbers is further described in supplementary data.

α1-globin gene promoter to the XhoI/HindIII site of the pG. The pG4αp and pG4αp' are constructed by inserting the 475A8 element to the XhoI site of pGαp with forward or reverse direction respectively. pG2αp is constructed by inserting HS26 element to the SalI/BamHI site of pGαp. pG24αp is constructed by inserting HS26 element to the SalI/BamHI site of pG4αp. The primers used to clone the 475A8 and α1 promoter are as following: αpF, 5′-CTCGAG-TTCTTCCCAAACGGCCATCA-3′; apR, 5′-AAGCTTTCCTGAGTCTGT-CAGAATZAGAA-3′; 475A8F, 5′-ACTCTCGAGCTGGGTGCCAAGC-TCTGA-3′; 475A8R, 5′-ACTCTCGAGTGGCCTCATCTTCAGCTTCA-3′.

Transfection to all DMSO induced MEL cells were performed after MEL cells were cultured in DMEM (10% FCS, fetal calf serum) medium (Gibco) supplemented with 2% DMSO for 48 h. Increased α-globin genes expression after DMSO induction is shown as in Supplementary Figure 2. Transfection was carried out according to the procedure provided along with the transfection reagent Lipofectamine™ 2000 (Invitrogen). Detection of reporter gene expression was according to the manual provided together with the Dual Luciferase Assay System (Promega Cat E1910). Each transfection experiment was triplicated and averaged, and then standard deviation was calculated. All the firefly luciferase signals have been normalized to the Renilla luciferase signals before being used to generate the columns.

DNase I SENSITIVITY ASSAY

The DNase I sensitivity assay was according to reference [McArthur et al., 2001]. Briefly, a standard amplification curve of each pair of primer was generated by using gradually diluted genomic DNA as templates. The remaining DNA templates of each sample after digestion by increasing amount of DNase I was calculated through the standard curve. A 400 bp fragment of NF-M was used to standardize the amount of DNA templates. The remaining DNA templates and the amount of DNase I used were used to generate the plot that showed the DNase I sensitivity of each fragment. The primers used in DNase I assay are as following: Nf-Mr, 5′-GCTG-GGTGATGCTTACGACC-3′; Nf-Mf, 5′-GCGGCATTTGAACCACTCTT-3′; 475A8F, 5′-TGCCAGAGCCTTGTGACCTT-3′; 475A8R, 5′-TTCCT-AGTCCTTACCTGGCCC-3′; HS26F, 5′-TCAGATTACCACTAGGCTCC-TGG-3′; HS26R, 5′-CCAACTGCAGGTGTACAAGGT-3′.

RESULTS

OUTLINE OF QACT

We applied QACT technology to characterize the genomic environment of the mouse α-globin gene cluster which is embedded in a GC-rich region neighbored by a cluster of housekeeping genes in 14.5 dpc mouse fetal liver (FL) cells [Higgs et al., 1998]. By applying the 3C assay, we have reported recently that an erythroid-specific α-like active chromain hub (α-ACH) is created through the formation of looping structures that brings together the upstream regulatory elements and developmentally activated α1 and α2 genes. In 3C experiments, formaldehyde is used to crosslink protein/DNA interactions in intact nuclei. The crosslinked chromatin is then digested by a restriction enzyme, followed by ligation. If there is apposition between a remote regulatory sequence and a promoter, new, hybrid fragments containing these two elements are generated and carefully designed PCR reactions can be used to detect and quantify hybrid fragments. This approach could be used to analyze physical interactions between cis-acting chromosomal elements.

In this QACT assay, we chose a restrictive chromatin fragment (RCF) that contains the major regulatory element (MRE), HS26, as the leader fragment. An outline of the QACT procedure is presented in Figure 1. A detailed procedure of QACT is described in Materials and Methods Section. As we previously reported in comparative 3C assay of α-globin gene cluster [Zhou et al., 2006], NcoI was used to digest the cross-linked DNA-protein complexes in the QACT assay, because the NcoI digestion can separate the important α-globin gene regulatory elements and promoters to individual restrictive fragment. We checked the digestion efficiencies of the cross-linked chromatin from FL cells and MEL cells with several different methods, including electrophoresis (Supplementary Fig. 1A), southern blotting (Supplementary Fig. 1B) and semi-quantitative PCR (Supplementary Fig. 1C). All these analyses demonstrated that similar digestion efficiencies could be obtained at different sites in FL cells and MEL cells. Then, the 3C template DNA from FL cells was prepared. A secondary restrictive enzyme TfiI was used to further digest the 3C template DNA to facilitate the self-circularization. TfiI is an endonuclease that recognize 5 nt with the middle nucleotide either A or T. TfiI has more recognition sites than NcoI in the genome. So, most of the ACPs will be digested by TfiI at the position just near the NcoI site. In addition, there is a TfiI recognition site just upstream of HS26 located NcoI fragment. So TfiI digestion also produces the fragments that have the appropriate sizes for further self-ligation. The TfiI restrictive fragments were self-ligated at low concentration to facilitate intra-molecular ligation. At the same time, the reverse crosslinked non-ligated DNA was also self-ligated as the negative control. Nested inverse-PCR was performed with these circular DNA as templates. A smear can be observed with the sample but not the control. Because the nested inverse-PCR primers are designed at the leader fragment, all PCR products were composed of two parts: one is from the leader fragment HS26RCF (leader side) and the other is from the unknown ACPs (ACP side). These PCR products constitute the QACT library. A biotin modification at the 5′ side of the primer (SHS26R) used in secondary step PCR facilitated the removal of the fragment that comes from HS26 RCF after NcoI digestion of the PCR products. A specifically designed NcoI adaptor was ligated to the leaving part of the nested PCR products. Replacement of the fragment coming from HS26 with this NcoI adaptor facilitates the further excision of the short tags. BamHI and MmeI recognition sites were incorporated in the NcoI adaptor near its cohesive end. MmeI belongs to type II restrictive enzyme and there is a 19/20 bp distance between its recognition site and digestion site. Therefore, there is a 19/20 bp DNA sequence maintained at the NcoI adaptor side after the MmeI digestion. By means of the biotin modification at the non-cohesive end of NcoI adaptor, the NcoI adaptor and the linked 19/20 bp DNA were captured by the magnetic sphere. The proportion of each captured 19/20 bp DNA coming from different RCFs represented the ligation frequency of these RCFs to the leader RCF. To obtain the sequence information of these short tags and quantitatively evaluate their proportion in the entire tags pool, another NN adaptor containing a PstI recognition site was ligated to the compatible end of the

magnetic sphere captured DNA that was generated by MmeI digestion. The short tags were then amplified by PCR. This amplification did not affect the percentage of each kind of tag in the entire tags pool if the PCR is performed in the linear range. Short tags were excised from the PCR products with BamHI and PstI that were included in the adaptors. Tags could be self-ligated to generate the concatemers that are cloned for sequencing. The amount of different tags for sequencing was proportionally maintained from the beginning.

QACT ASSAY OF MOUSE α-GLOBIN GENE CLUSTER

Using HS26 RCF as the leader RCF, we performed the QACT assay in 14.5 dpc mouse fetal liver cells and obtained more than 300 tags' sequence. Among these tags, 218 tag sequences (a little more than 70% of total number of tags) could be mapped to unique genomic loci of the mouse genome. Among these tags, 98 tags were mapped to the proximal region of HS26 and will not be considered further. Among the rest of 120 tags, 28 tags were mapped to the 5′ end of either α1 or α2-globin gene RCFs (in each RCF, the promoter of either α1 or α2-globin gene was presented). Because the 5′ end sequences of α1 and α2-globin RCFs are identical, it is impossible to distinguish α1 and α2-globin genes in QACT assay. For simplicity, we assumed that α1 and α2-globin genes have similar ligation frequency to HS26 RCF. Compared with other long range associated RCFs, α1 and α2 RCFs showed the highest association frequencies with HS26 RCF (Fig. 2). This result reconfirmed our previously finding that HS26 associates with α1/α2-globin gene promoters in fetal liver cells [Zhou et al., 2006]. Moreover, the precise capturing of the α1/α2-globin gene promoter RCFs reflects the fact that HS26 regulates the expression of α1/α2-globin genes specifically by being brought into spatial proximity and activates the promoters via looping. The ζ globin gene RCF sequence was not found in this assay. This is consistent with the fact that the ζ-globin gene is not expressed in 14.5 dpc FL. Both HS21 and HS8 RCFs were found to be ligated to HS26 RCF in significantly higher frequency (Fig. 2), suggesting that all the three HSs (HS26, HS21, and HS8) work together to form an α-like ACH [Zhou et al., 2006]. Besides these two DNaseI-hypersensitive sites, none of the other known HSs have been detected from the sequenced tags. Consistent with our previous report, we also found that the 5′ and 3′ UTR RCFs that belong to several neighboring genes of α-globin gene cluster could be identified through QACT at a relatively low frequency (Fig. 2). In conclusion, the QACT results are consistent with our previously reported chromatin structure assay of α-globin gene cluster by 3C assay, suggesting that QACT is a reliable method in analyzing the higher order chromatin structure organization. In addition, most of the sequenced tags that correspond to other unique locus (often on other chromosomes) of the mouse genome are always at very low frequencies (only once or twice) in the whole tags pool. By designing primers at 10 RCFs that harboring the low frequency tags, the 3C signals between these RCFs and HS26 RCF were detected. Most of these RCFs produce very weak signals even after increasing the PCR cycles, suggesting that the interactions are non-specific (supplementary Table I). Therefore, these low-frequency tags were considered as "background" signals in the QACT assay. This result also supports a high signal to noise ratio of QACT assay.

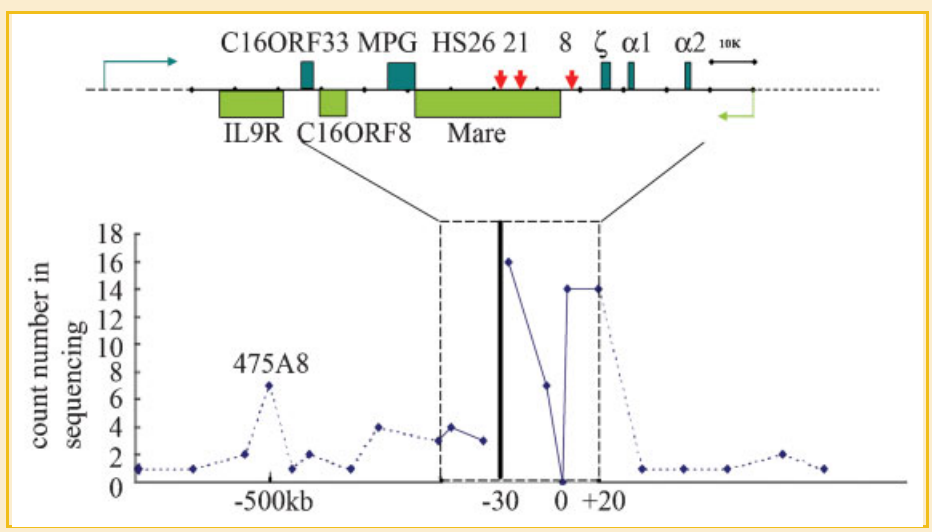


Fig. 2. QACT assay of mouse α-globin gene cluster with HS26 RCF as leader fragment. A schematic representation of the mouse α-globin locus and the flanking regions are shown at the top. Filled rectangles represent the genes and the vertical arrows represent the known erythroid specific hypersensitive sites. The transcriptional directions toward centromere (above the line) or telomere (below the line) are indicated by horizontal arrows. Dashed lines at the two sides of the solid line represent the remote region of α-globin gene cluster. The *Y*-axis value represents the observation times (count number) of each tag in sequencing. The dashed rectangle includes the region from C16orf33 to α2-globin gene. The vertical broad lines represent the position of the leader RCF. The tags that come from the broken line rectangle region mainly correspond to 5′UTR of C16orf33, 5′UTR of C16orf8, 5′UTR of MPG, HS21, HS8, ζ α1, and α2-globin gene promoters from left to right. The ζ globin gene has also been indicated, though there is no tags that correspond to the ζ-globin gene RCF. The *X*-axis values represent the distances of the analyzed RCFs away from ζ-globin mRNA cap site. Because of the long distance, the position of 475A8 RCF is not marked according to scale. The total observation times of α1 and α2 were assumed to be equally shared between α1 and α2 globin gene because the tags from α1 and α2 globin genes were completely identical. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

IDENTIFICATION OF 475A8 AS A α-GLOBIN GENE CLUSTER ASSOCIATING ELEMENT

We also observed one RCF located 500 kb upstream of HS26 that could associate with HS26 RCF at a significantly high frequency (Fig. 2). The size of this RCF is about 1.7 kb and it belongs to clone RP23-475A8 (the Genebank accession number AL713921). This clone is positioned at the 31,467K-31,732K region on mouse chromosome 11 (Supplementary Fig. 2). Analysis of this RCF using VISTA program identified two highly conserved regions which have close position and about 700 bp in length together [Couronne et al., 2003]. Here, we named these two conserved regions (700 bp fragment) together as 475A8.

To confirm the association between 475A8 RCF and HS26 RCF, we performed normal comparative 3C assay with HS26 RCF as the leader fragment in 14.5 dpc FL cells and MEL cells (Fig. 3A). MEL cell line is a malignant tumor cell line established from Friend virus induced mouse erythroleukemias which are blocked near the proerythroblast stage of differentiation [Marks et al., 1987]. Therefore, this cell line is appropriate to observe if the association between 475A8 and HS26 is differentiation stage specific. The ligation frequencies of 475A8, ζ, α1, α2, and MPG RCFs to the leader HS26 RCF show that 475A8 has similar ligation frequency to HS26 in FL cells and MEL cells. This ligation frequency is also consistent with the QACT assay. Because 475A8 locates far away from α-globin gene cluster, it is intriguing that 475A8 associates with α-globin gene locus in both kinds of cells where either a poised or activated chromatin structure has already formed [Vernimmen et al., 2007]. Therefore, the normal 3C assay with 475A8 RCF (Fig. 3B) as the leader RCFs was performed in both kinds of cells to investigate if 475A8 has other associating partners from α-globin gene cluster. The result showed a relatively higher ligation frequency of 475A8 RCF to α1 RCF than other RCFs in MEL cells. In FL cells, this tendency was not obvious. In the complimentary experiment using α1 RCF as the leader, the ligation frequency of 475A8 to α1 was the highest in MEL cells, though the ligation frequency between α1 and HS26 is also obvious. But in FL cells, it was HS26 RCF that had highest ligation frequency to α1 RCF (Fig. 3C). These results suggest that 475A8 could specifically associate with α1-globin gene RCF in MEL cells. It is interesting to note that a detectable ligation of 475A8 RCF to α1-globin gene promoter could still be observed when α1-globin gene was active (in FL cells), indicating that large scale chromosomal conformation could be maintained in erythroid cells, regardless of its differentiation stage.

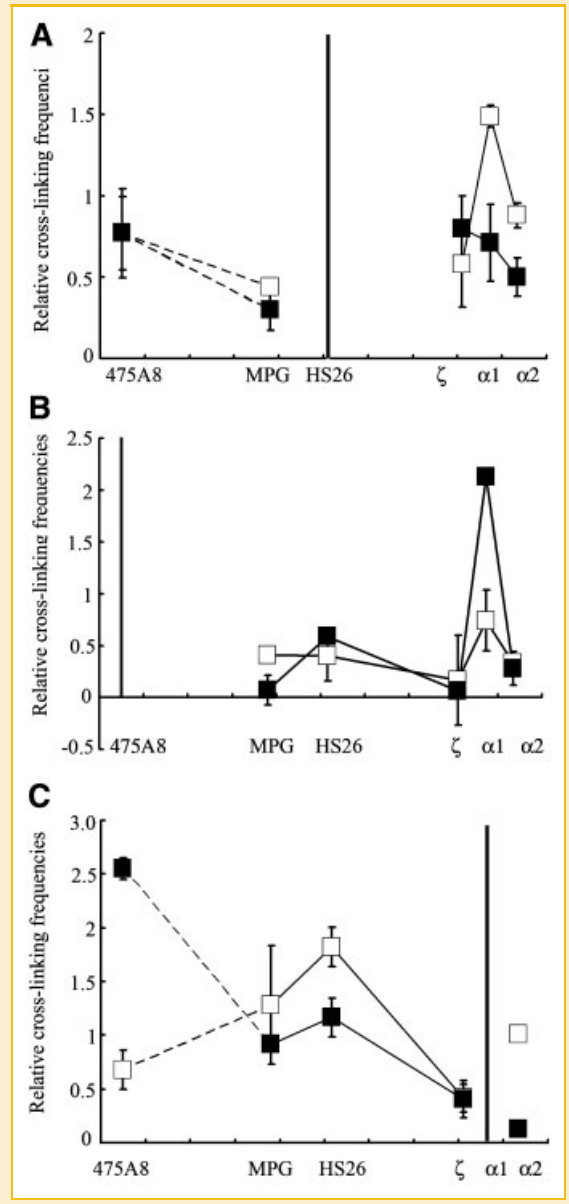


Fig. 3. 3C assay of the association between 475A8 and the elements of α-globin gene cluster. The cross-linking patterns of HS26 (A), 475A8 (B), and α1 (C) located RCFs to other analyzed RCFs indicated by the histograms are shown from top to bottom respectively in both 14.5 dpc mouse fetal liver (unfilled square) and MEL cells (filled square). The Y values of the histograms are the calibrated peak areas (determined with UVI scan) of PCR signals obtained with two-test fragment ligation products, which represent the ligation frequency of each pair of analyzed RCFs (see Materials and Methods Section for details). The vertical lines show the positions of leader RCFs. The PCR-amplified ligation product from Errc3 locus was used to correct for the amount of DNA (the 3C template DNA from FL cells and MEL cells) used in each PCR. Error bars represent the standard errors of the means from three separate experiments.

HISTONE ACETYLATION ASSAY AND DNaseI SENSITIVITY ASSAY OF 475A8

475A8 locates 500 kb away from α-globin gene cluster and the flanking region is rare of the genes but rich of repetitive sequences, suggesting that this region is relative inactive. The involvement of 475A8 in α-ACH establishment, however, suggests that 475A8 has an open chromatin structure. Because the chromatin structure of α-globin gene cluster is characterized by high level H3 and H4 acetylation, the ChIP assay was performed to analyze if 475A8 also has the same open chromatin structure. As expected, the result showed the high H3 and H4 acetylation of 475A8 that is comparable to both HS26 and α1-globin promoter in MEL cells (Fig. 4A). In addition, terminal differentiation of MEL cells could significantly increase the H3 and H4 acetylation of 475A8, which is consistent with the similar changes in HS26 and α1-globin promoter [Anguita et al., 2004]. To the flanking region of 475A8, the H3 and H4 acetylation is very low (data not shown).

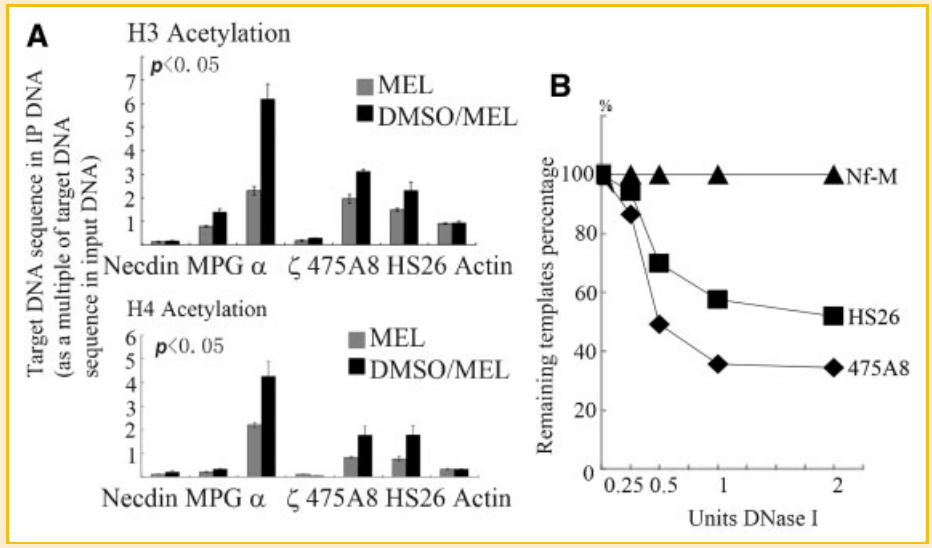


Fig. 4. Chromatin activity assay of 475A8 by detecting the histone acetylation status and DNaseI sensitivity. A: ChIP assay of H3 and H4 acetylation of important elements in α-globin gene cluster. The Necdin promoter served as the negative control and the β-Actin gene promoter served to correct the templates of the different samples. The input was used to correct the different amplification efficiency of the primers. The histograms represent the enrichment (Y value) of acetylated H3 or H4 in the analyzed regions (X value). Error bars represent the standard errors of the means from three repeats. Statistical significance was determined with *t*-test. *P*-value of less than 0.05 was considered statistically significant. B: DNaseI sensitivity assay of 475A8 in MEL cells by a real-time PCR based method. HS26 served as the positive control. Increasing amount of DNase I (0.25, 0.5, 1, 2 U) was used. The Y value represents the percentage of remaining DNA templates after DNase I digestion.

Because most of the *cis*-regulatory elements, no matter the enhancers or silencers, always show the high sensitivity to DNaseI digestion owing to the dynamic binding of the transcriptional factors, we also performed the DNaseI sensitivity assay of 475A8 using a real-time PCR based assay [McArthur et al., 2001]. In MEL cells, the well studied Nf-M gene which is known to be DNaseI insensitive in erythroid cells, was used as the control gene to correct the DNA template in real-time PCR assay. By applying different concentration of DNaseI from 0.25 to 2 U, the DNaseI sensitivity of HS26 and 475A8 was evaluated. The 475A8 was found to be more sensitive than HS26 in MEL cells (Fig. 4B). HS26 has already been proved to be an important DNaseI-hypersensitive site in both MEL cells and erythroblasts [Gourdon et al., 1995]. In addition, the DNaseI sensitivity of HS26 in MEL cells is relative weaker than that in erythroblast, which is consistent with the establishment of α-ACH in erythroblasts. Thus, this data support that 475A8 is a newly identified DNase I-hypersensitive site. Accordingly, we also observed that the DNase I sensitivity of 475A8 is less than HS26 after the induction of MEL cells (data not shown). Taken the above data together, it could be conclude that 475A8 is a spatially closing DNase I-hypersensitive element of α-globin gene cluster.

REGULATION OF α1-GLOBIN GENE PROMOTER BY 475A8

To further explore the function of 475A8 RCF, we constructed a series of luciferase reporter plasmids (Fig. 5A), which were transfected into MEL cells and DMSO induced MEL cells. The 700 bp fragment from 475A8 RCF, named 475A8, was cloned and inserted immediately upstream of the α1-globin promoter of pG4α/pG4α' and pG24α Figure 5A. At the same time, a 700 bp randomly chosen fragment was also inserted to the same position as 475A8 and acted as the negative control fragment (NCF). The position of 475A8 in the constructs was specifically designed considering the possible close proximity in space between 475A8 and α1-promoter observed in the 3C assay in MEL cells. Because HS26 could exert its function as an enhancer independent of its position in the constructs, insertion of 475A8 between HS26 and α1-promoter is presumed to have no influence on HS26s function if 475A8 was a non-functional sequence as the NCF. Because DMSO could induce the MEL cells to resume erythroid terminal differentiation and activate the expression of α1 and α2-globin genes, we chose DMSO induced MEL cells to simulate the 14.5 dpc FL cells for easy manipulation. We observed that the α1-globin gene promoter had equally high activity in both cell types in each separate transfection experiment when pGα was used for transfection (Fig. 5B–D). However, expression of the endogenous α1-globin gene in MEL cells was very low (Supplementary Fig. 4), indicating that the α1-globin gene promoter was repressed before the definitive erythroid progenitor cells undergoing terminal differentiation. The repression is possibly caused by lack of the activated chromatin structure [Vernimmen et al., 2007]. Transient transfection of pG4αp and pG4αp' into MEL cells showed that 475A8 significantly repressed α1-globin promoter activity regardless of the orientation of the 475A8 fragment, whereas the replacement of 475A8 with the NCF has no obvious effect on the reporter gene expression (Fig. 5B). The repressive activity of 475A8 nearly disappeared in DMSO induced MEL cells, suggesting that 475A8 might be a differentiation stage specific silencer of the α1-globin gene. When pG2α was transfected into MEL cells, the HS26 could mildly increase the activity of α1 globin gene promoter, whereas insertion of 475A8 (pG24α) between HS26 and α1-promoter could strongly reverse the effect, suggesting

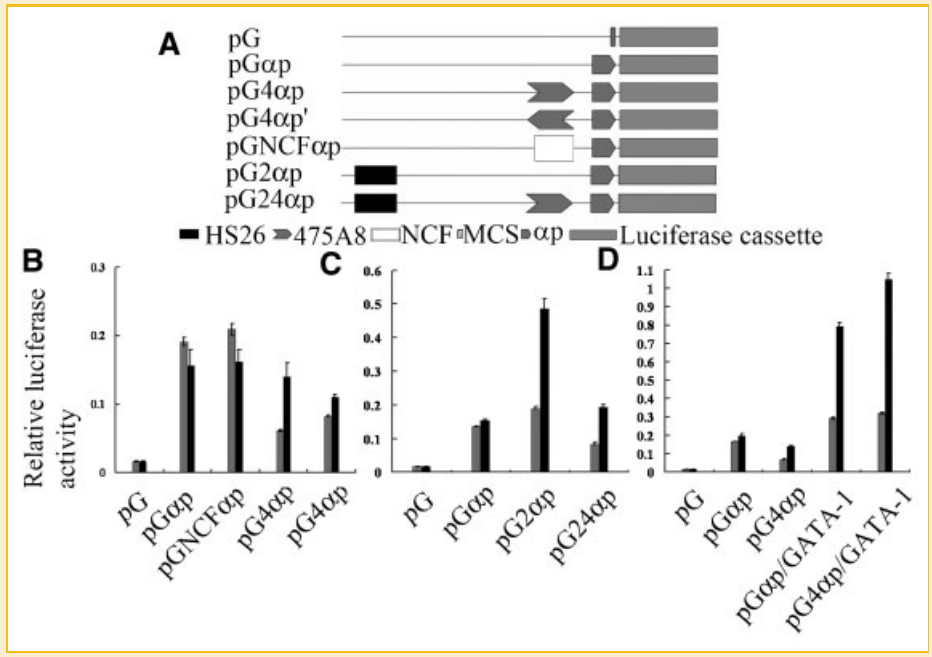


Fig. 5. Analysis of the function of 475A8 in regulating the α1-globin promoter activity through transient transfection assay. A: Diagram of the constructs used in transfection assay. pG is the abbreviation of pGL3-basic (Promega). The α1-globin gene promoter was inserted into the MCS (multi cloning sites) site of pG to form pGαp. The 475A8 fragment was also inserted into the MCS with both directions, just upstream of the α1-globin gene promoter to form pG4αp and pG4αp'. A randomly chosen fragment NCF was used to replace 475A8 in pG4αp that produce pNCFαp as the negative control. HS26 was inserted into the BamHI site downstream of luciferase cassette to form pG2αp and pG24αp. B–D: The bars in the histograms represent the relative expression level of the reporter gene after normalized to the Renilla luciferase in DMSO-induced MEL cells (black) and MEL cells (gray). *Y*-axis value represents the relative expression levels of the reporter gene. The plasmids used in each assay were indicated along the *X*-axis. D: pGαp/GATA-1 and pG4αp/GATA-1 represent the co-transfection of GATA-1 expression vector with pGαp or pG4αp. Confirmation of GATA-1 over expression is shown in Supplementary Figure 3.

that 475A8 still functioned independently to suppress the activity of α1 promoter in MEL cells (Fig. 5C). Additionally, 475A8 also strongly suppressed the reporter gene expression when pG24α was transfected into DMSO induced MEL cells (Fig. 5C). Because the repression effect of 475A8 to α1 promoter was alleviated or even abolished in pG4α transfected DMSO induced MEL cells, we conclude that 475A8 represses the reporter gene expression by inhibiting the enhancer function of HS26.

GATA-1 BINDING IS NECESSARY TO ABOLISH THE REPRESSION FUNCTION OF 475A8

What is the mechanism underlying 475A8 inhibition of HS26 when inserted between HS26 and the α1 promoter? One possibility is that 475A8 can compete with HS26 for the transcriptional factors accompanying the terminal differentiation of erythroid cells. In fact, when 475A8 RCF was analyzed with VISTA program several GATA-1 binding sites were predicted. GATA-1 is an erthyroid transcriptional factor essential for erythroid terminal differentiation. Over-expression of GATA-1 in uninduced MEL cells could induce the irreversible terminal differentiation of MEL cells [Choe et al., 2003]. GATA-1 could also promote expression of adult stage globin genes. Therefore, it is reasonable to suspect that the function of 475A8 is regulated by GATA-1 binding. Indeed, cotransfection of GATA-1 expression vector and either pGαp or pG4αp significantly increased the expression of the reporter gene in both MEL cells and DMSO induced MEL cells (Fig. 5D). It is possible that over-expression of GATA-1 triggers the terminal differentiation of MEL cells and promotes the direct binding of GATA-1 to 475A8 in both cells and thus, abolishes the repression function of 475A8, because 475A8 also lost its silencer function when pG4α is transfected into DMSO induced MEL cells (Fig. 5B). Therefore, ChIP assay was performed to analyze the natural variation of GATA-1 binding to 475A8 during the erythroid terminal differentiation. Results showed that the binding of GATA-1 to 475A8 increased significantly during terminal differentiation of the MEL cells (Fig. 6). DMSO induction could also mildly increase binding of GATA-1 to α-globin promoters which was consistent with a previous study [Anguita et al., 2004]. These data also indicate that increased GATA-1 binding to 475A8 is accompanied by the activation of α1-globin gene promoter. Therefore, the reason of the inhibition of HS26 function by 475A8 in the pG24α transfection assay in DMSO induced MEL cells is most likely caused by the strong competition for GATA-1 recruitment by 475A8 because the position of 475A8 is more adjacent to α1-globin promoter in our reporter construct. While in FL cells, though 475A8 present in the vicinity of α1-globin promoter, it can not affect α1-globin gene expression owing to the alteration of binding proteins. Taken together, these data suggest that 475A8 is a possible α1-globin gene specific silencer in the committed erythroid progenitor cells and GATA-1 binding to 475A8

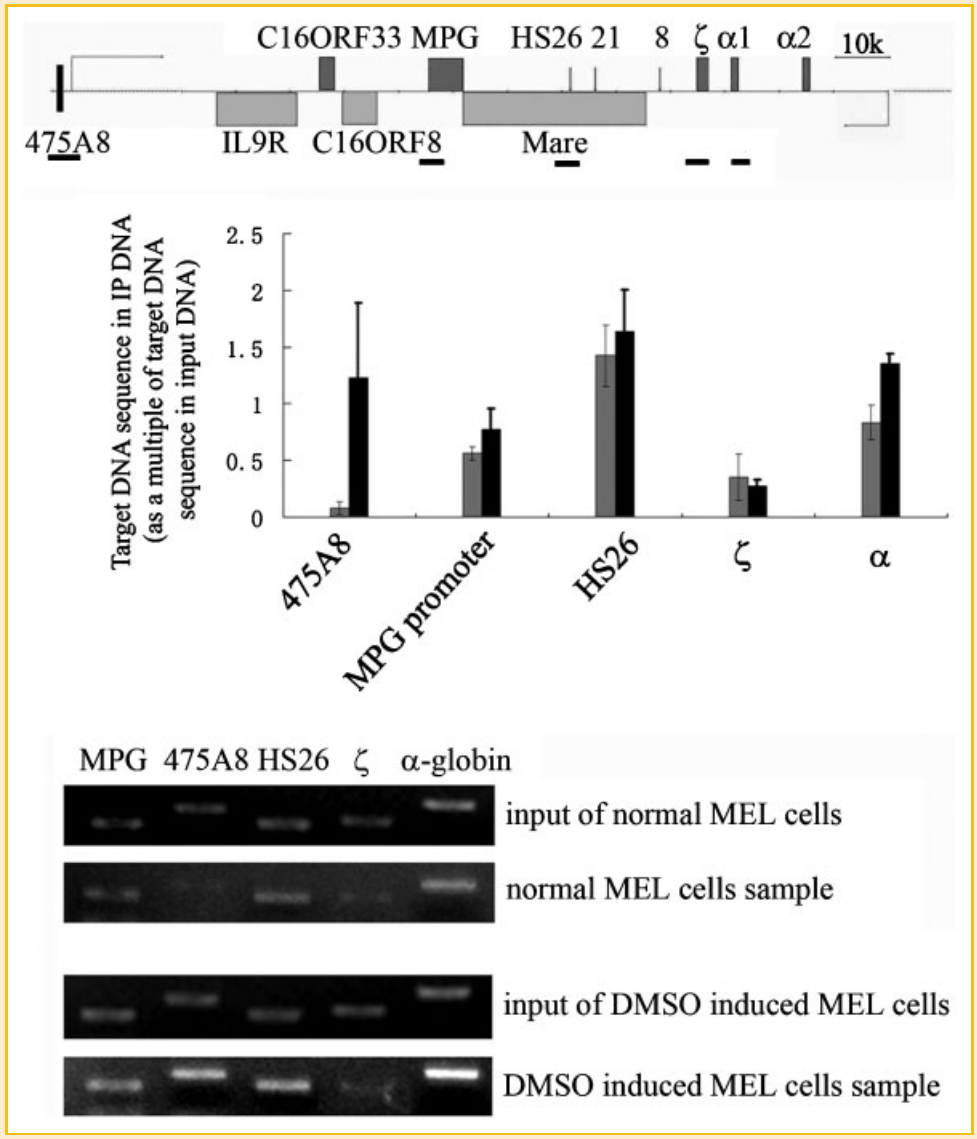


Fig. 6. ChIP assay of GATA-1 binding to 475A8 in DMSO induced MEL cells and MEL cells. The schematic representation of α-globin gene cluster is shown at the top. The horizontal bars represent the positions of the primer pairs used in the ChIP assay. *Y*-axis value of the histogram represents the calibrated peak area (determined with UVI scan) of PCR signals in MEL cells (gray) and DMSO induced (4 days) MEL cells (black). ζ-globin gene promoter serves as a negative control. Error bars represent the standard errors of the means from separate experiments. Gel electrophoresis samples are shown at the bottom.

could abolish its function as a silencer and release the α1-globin gene for activation.

DISCUSSION

The eukaryotic nucleus is a well organized structure. Chromatin movements in the nuclear space are relatively restricted [Gasser, 2002]. It implies that some genomic loci are more frequently found in close spatial proximity to a given locus than others. Such specific chromatin conformation is necessary in maintaining spatio-temporal patterns of specific gene expression [Tolhuis et al., 2002; Horike et al., 2005; Spilianakis et al., 2005; Ling et al., 2006]. Long range interactions of genomic loci include both intra-chromosomal and interchromosomal interactions that have been supposed to be the reason for forming gene locus specific chromatin conformation [Spilianakis and Flavell, 2004; Spilianakis et al., 2005]. Therefore, uncovering the possible chromatin associations of one given gene locus is necessary to elucidate its chromatin conformation and to understand the mechanism of gene regulation. Recently, we have reported the formation of the α-ACH (active chromatin harbor) structure when the α-globin genes express in the mouse fetal liver. However, the analyzed region spans only 130 kb flanking region of α-globin gene cluster owing to the technical restriction of the 3C method. Here, we describe a revised method named QACT that could facilitate the discovery of all potential associated chromatin partners with one given gene locus. Application of this method to α-globin major regulatory element HS26

demonstrates that HS26 could extensively associate with α1/α2 globin gene promoters. Additionally, nearly all the known RCFs that could associate with HS26 in 3C assay could be detected by QACT in similar frequency [Zhou et al., 2006]. This indicates that QACT is a reliable technique and could be widely applied in chromatin conformation analysis and in searching for long-range functional elements. However, we have yet to find any important inter-chromosomal associations in our study, suggesting that stable interchromosomal associations are neither universal nor stochastic in nucleus. In fact, QACT assay of the mouse α-globin gene cluster suggests that this gene cluster seems to locate in the interior of the chromosome territory (CT) 11 because the signals from other chromosomes are very limited. Interestingly, our result is contrary to a long-time speculation that some chromatin level coregulation may exist to coordinate the expression of α-globin genes and β-globin genes. A recent evidence to support this speculation is the observation by Osborne et al. [2004] that the 7% of the erythoid progenitor cells have co-localized RNA-FISH signal of α and β-globin genes. In addition, Brown et al. [2006] also reported that there is 13% of observed cells with all four globin loci transcribing having the α and β-globin gene juxtaposition or colocalization. However, this association only means the physical proximity but not physical interaction [Brown et al., 2006]. In fact, compared with the coregulated genes as observed by Osborn et al., this frequency is also not high enough to be considered as indication of stable association. Our QACT is based on 3C and only the physical interacted signal can be observed. So it is possible that such spatial proximity is out of the detect range of QACT. But a direct conclusion from these observation suggest that the supposed coregulation of α and β-globin gene cluster seems not depend on the spatial correlation. A possible reason for the coordinated expression of these two gene cluster is the sharing of common transcriptional factors. So this coordinated expression is indirect rather than direct. Our previous study also support that there is no direct correlation of human a and b-globin gene expression in transgenic mice [Huang et al., 2004].

Using QACT, we have also identified a previously unknown regulatory element of the α1-globin gene and showed it to be an important partner associated with HS26. Furthermore, 3C revealed that 475A8 extensively associates with the α1-globin gene promoter in adult erythroid progenitor cells (MEL cells). We have tried to detect the ligation between the RCFs adjacent to 475A8 RCF and the α-globin gene cluster RCFs, but we have failed to detect the appropriate PCR signals (data not shown), indicating that the association of 475A8 RCF with α1-globin gene cluster is exclusive. In addition, we have also demonstrated that 475A8 is a novel DNase I-hypersensitive site in MEL cells and has high level histone H3 and H4 acetylation. To further confirm the regulatory function of 475A8 RCF, a 700 bp fragment within 475A8 fragment, which includes two highly conserved regions, were identified and subcloned for transfection. The results suggested that 475A8 is a possible remote α1-globin gene specific silencer. Though the 475A8 is a highly conserved element, its corresponding conservative region in the human genome is located on chromosome 5 and has no obvious correlation with human α1-globin gene expression. Additionally, the human α-globin like genes could correctly switch on during development in BAC mediated transgenic mouse, suggesting that all the important regulatory elements of human α-globin genes have been included in the BAC [Feng et al., 2001]. Therefore, some differences may exist in the regulation of α-globin genes between human and mouse [Anguita et al., 2002]. The mouse α-globin gene cluster contains three genes of known functional significance: ζ, α1, and α2 [Higgs et al., 1998]. The expression of ζ globin gene is limited to the primitive erythroblasts in the embryonic yolk sac. In contrast, α-globin genes probably express at all developmental stages. However, α-globin genes could be selectively induced in concurrence with establishment of definitive erythropoesis in the fetal liver. Two attributes of α-cluster genes which define their developmental stage-specific expression are the reciprocal silencing of ζ-globin expression and induction of α-globin genes expression at the embryonic-to-fetal transition and the sustained high-level expression of the α-globin genes throughout fetal and adult life. The embryonic specific expression of ζ-gloibn gene seems mainly depending on its promoter and the expression level is enhancer associated [Sabath et al., 1993]. The α1-globin gene has higher expression level than α2-globin gene from fetal to adult and is more significant in hemoglobin production [Benz, 1980]. We have observed that the exogenous α1-globin gene promoter has similarly high activity in both DMSO induced MEL cells and MEL cells in the transfection assay, and the expression level of the endogenous α1-globin genes in MEL cells is almost undetectable. Therefore, we speculate that the repression of α1-globin gene expression in committed erythroid progenitor cells (corresponding to MEL cells) is due to the repression of its promoter. Here, our results indicate an efficient way of α1-globin gene regulation in its differentiation stage specific expression: repressing α1-globin gene expression by directly inactivating its promoter before terminal differentiation and gradually releasing the α1-globin gene promoters accompanied by the terminal differentiation initiation. The α-globin genes could then come to full expression in the adult stage. It has been reported that GATA-1 accumulates in the nucleus and along the α-globin gene locus accompanying the terminal differentiation of the MEL cells [Anguita et al., 2004]. Analyzing of GATA-1 binding to 475A8 also suggests that the repressive function of 475A8 could be abolished when GATA-1 binding increased. This result adds another layer of regulatory mechanism of GATA-1 in regulating the expression of α1-globin gene expression.

The newly developed chromatin association assay tool QACT has allowed the identification of 475A8. ACT method, as firstly described by Ling et al. [2006], determined CTCF mediated interchromosomal chromatin association and was later used by Wurtele and Chartrand [2006] to analyze *Hox*B1 gene associated chromatin partners. However, application of ACT to different gene loci produces distinct results. It is interesting that only three chromatin associations were found at the CTCF binding site upstream of H19 gene locus (among these three associations, only one has been verified) and more than hundreds of chromatin associations were found at the *Hox*B1 gene locus. Another study called "4C" clarified that numerous chromatin associations involving different chromosomes were found at the same *H19* gene locus [Zhao et al., 2006], suggesting that increasing the resolution of the detection method could increase the chances of identifying novel chromatin associations. One aim of these different methods is

to determine the high frequency associations. These methods require the sequences of the ACPs for evaluating the relevant frequencies of these ACPs. To overcome this limitation, a study called "4C" was developed. But the genomic chip used in this "4C" assay is a tailored microarray that contains probes each locates <100 bp away from a different HindIII restriction end in the genome [Simonis et al., 2006], that prevent the wide application of this strategy. Compared with these techniques, QACT avoids direct sequencing of ACPs and provides an effective and quantitative method to enrich and identify the target ACPs. In the QACT assay, the ACT products, ie, the inverse PCR products are analyzed by a high-throughput strategy and only 19–20 bp short tags were used to map these fragments in the genome. These 19–20 bp tags are more easily to be further enriched and self-ligated as concatemers for sequencing. These concatemers economically provide the sequence information of ACPs. Generally, 19–20 bp sequence is enough to correctly locate the sequence on the genome [Sabo et al., 2004a].Therefore, this technology could be widely applied in analyzing the genomic environment of RCFs containing desired gene fragments.

ACKNOWLEDGMENTS

We thank Dr. Chongwoo A. Kim of UTHSCSA, Dr. Yi Zhang of UNC and Dr. Yi Rao of Northwestern University for the critical reading of our manuscript.

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