

Recruitment of transcription complexes to the β -globin locus control region and transcription of hypersensitive site 3 prior to erythroid differentiation of murine embryonic stem cells

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Eukaryotic chromosomal DNA is densely packaged in the nucleus and organized into discrete domains of active and inactive chromatin. Gene loci that are activated during the process of cell differentiation undergo changes that result in modifications of specific histone tail residues and in loosening of chromatin structure. The β -globin genes are expressed exclusively in erythroid cells. High-level expression of these genes is mediated by a locus control region (LCR), a powerful DNA regulatory element composed of several DNase I hypersensitive (HS) sites and located far upstream of the β -globin genes. Here we show that RNA polymerase II and specific histone modifications that mark transcriptionally active chromatin domains are associated with the LCR core elements HS2 and HS3 in murine embryonic stem cells prior to differentiation along the erythroid lineage. At this stage HS3 is abundantly transcribed. After *in vitro* differentiation, RNA Polymerase II can also be detected at the embryonic ϵ - and adult β -globin genes. These results are consistent with the hypothesis that activation of the β -globin gene locus is initiated by protein complexes recruited to the LCR.

Multicellular organisms are composed of a variety of cell types, all derived from a common precursor and identified by different patterns of gene expression. It is the transcriptional profile of a specific cell type that determines its morphology and function. The establishment of expression patterns in terminally differentiated cells is mediated by various ubiquitously expressed and tissue-specific transcription activators and repressors, as well as nucleosome modifying and remodeling factors, whose activity results in the proper spatial and temporal expression of specific subsets of genes. The sequential silencing of genes involved in maintenance of pluripotent and multipotent states and the activa-

tion of those involved in differentiation is believed to be a dominant factor in the progression from multilineage precursors to that of specific cell types. The maintenance of this transcriptional state following cell division depends upon not only the direct action of *trans*-acting factors, but also the heritable epigenetic status they impart [1]. Data accumulated in recent years indicates that combinations of covalent histone modifications may constitute a 'histone code' that regulates the use of genetic information [2]. The manner in which the acquisition of various epigenetic states is regulated during development is only partially understood.

Abbreviations

AcH4, acetylated histone H4; ChIP, chromatin immunoprecipitation; ES cells, embryonic stem cells; ETCM, early transcription competence mark; HPC, hematopoietic progenitor cell; HS, hypersensitive; LCR, locus control region; LIF, leukemia inhibitory factor; Me₂K4H3, histone H3 dimethylated at lysine 4; MEF, mouse embryonic fibroblast; MEL, murine erythroleukemia; RNA Pol II, RNA polymerase II; RT-PCR, reverse transcription-polymerase chain reaction; TBP, TATA binding protein.

The vertebrate globin gene family has provided a model system to study the molecular basis of developmentally regulated differential gene expression [3–5]. It contains a number of tissue-specific genes that are coordinately regulated and whose expression changes during development of the hematopoietic system, a process termed ‘hemoglobin gene switching’ [6]. Epigenetic modifications have been shown to play an important role in the expression of the β -globin genes [7]. The chicken β -globin locus has been shown to reside in a domain of uniform histone hyperacetylation with the active genes being acetylated on lysine 9 of histone H3 and inactive genes exhibiting H3 lysine 9 methylation [8,9]. Differential acetylation has also been observed in the murine β -globin locus. Forsberg *et al.* [10] observed dynamic changes in histone acetylation of the globin genes during development, with the locus control region (LCR) and active genes marked by increased H3 and H4 acetylation. These observations suggest epigenetic modifications may be an important factor in the maintenance of an active gene locus, however, how and when these patterns are established is not entirely known. Bottardi *et al.* [11] investigated the epigenetic state of the human β -globin locus in hematopoietic progenitor cells (HPCs) and transgenic mice. They found that histone H3 at the β -promoter was hyperacetylated and dimethylated at lysine 4 in HPCs but deacetylated in mature erythroid cells. In contrast, the human γ -promoters lacked these modifications in HPCs and transgenic fetal liver cells. These results indicate acetylation plays a critical role in the transcriptional potentiation and developmental regulation of these genes in progenitor cells or cells that have yet to express the genes at physiologically relevant levels [11]. Chromatin structure modifications in uncommitted progenitor cells have also been observed for the murine β -globin locus [12,13]. Recent studies showed that RNA Pol II is recruited in a strictly localized fashion within the LCR and was only detected at the core regions [14,15]. Localization of RNA Pol II to the LCR was independent of active transcription elongation; the addition of the elongation inhibitor DRB did not affect recruitment [14]. Similar changes in chromatin structure that occur during the establishment of transcriptionally competent chromatin domains have also been made at other loci, such as at the lysozyme locus [16], c-fms [17], and the myeloperoxidase gene [18].

Understanding how epigenetic states are acquired during development and how they impact globally on gene expression is a critical step in the treatment of a number of diseases, ranging from birth defects to cancer [19]. A logical first step in this process would be to

determine the mechanisms involved in this process at the level of individual gene loci.

In this study, we investigate chromatin structure modifications and factor recruitment at the murine β -globin locus in uninduced embryonic stem cells (day 0), as well as that of primitive and definitive erythroid cells (days 5 and 12, respectively). Using chromatin immunoprecipitation (ChIP), we demonstrate that core elements of the LCR adopt a structure characteristic of transcriptionally active chromatin and recruit RNA polymerase II prior to erythroid differentiation in murine embryonic stem (ES) cells. Real-time PCR analysis indicates that the locus is first activated at the LCR and that this state is perpetuated to more distal regions as the process of differentiation proceeds. Histone modifications and factor recruitment corresponding to a transcriptionally permissive state appear to be acquired prior to gene expression.

Results

We began our studies by examining the association of RNA Pol II with the β -globin gene locus in murine erythroleukemia (MEL) cells using chromatin immunoprecipitation (ChIP, Fig. 1). We observed that RNA Pol II is associated with the active β major-globin gene but not with the repressed $\epsilon\gamma$ -globin gene. Importantly, we found that RNA Pol II is associated with the core of HS2 but not with a region located in between HS2 and HS3. As a negative control, we analyzed interactions of RNA Pol II with the necdin gene, which is not expressed in erythroid cells, and found that RNA Pol II is not associated with this gene in MEL cells. These results confirm previous findings by Johnson *et al.* [14]. We also analyzed the interaction of RNA Pol II with the β -globin gene locus in mouse embryonic fibroblasts (MEFs) and OP9 stromal cells (OP9). These cells were used in our subsequent studies to support the growth of undifferentiated and differentiated ES cells. The data in Fig. 1B show that RNA Pol II does not interact with the β -globin loci in these cells, while it efficiently binds to the positive control GAPDH gene. We next analyzed ongoing transcription by nuclear run-on in the LCR and the β major-globin gene in MEL cells. The data show that HS2 and the β major-globin gene are transcribed while a region upstream of HS5 is not.

Having established that LCR core elements recruit RNA Pol II, we were interested in examining whether recruitment of RNA Pol II and other factors associated with transcription to the LCR can be temporarily separated from the recruitment to the globin gene promoters. We thus analyzed recruitment of RNA Pol II,

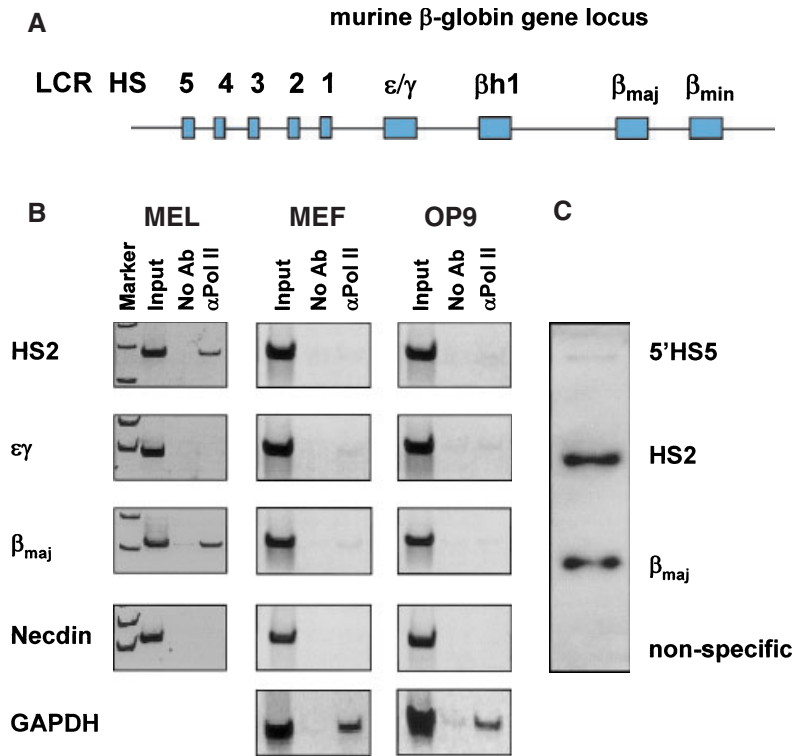


Fig. 1. RNA Pol II is recruited to active gene promoters and to the LCR of the murine β -globin gene locus in MEL cells. (A) Schematic representation of the murine β -globin gene locus. LCR hypersensitive sites and globin genes are shown as shaded boxes. (B) ChIP analysis of RNA Pol II associations with the murine β -globin gene locus in MEL, MEF, and OP9 cells as indicated. PCR amplification products were run on an acrylamide gel and stained with SYBR green. Antibodies and the regions amplified are shown at the top and right, respectively. (C) Nuclear run-on transcription analysis in specific regions of the β -globin locus. The RNA was hybridized to specific DNA fragments in the globin locus as indicated. The *nonspecific* lane shows hybridization to the negative control plasmid pK0916.

TPB, and specific histone modification marks to the β -globin gene locus during erythroid differentiation of murine ES cells *in vitro*. In these experiments we utilized the ES/OP9 cell *in vitro* differentiation system described by Kitayima *et al.* [20]. The ability of these cells to gen-

erate mice was not examined so their pluripotency was not directly confirmed, however, these cells express markers of early development, such as Rex-1, and do not express any of the globin genes (Fig. 2). Furthermore, we were able to generate cells of both hematopoi-

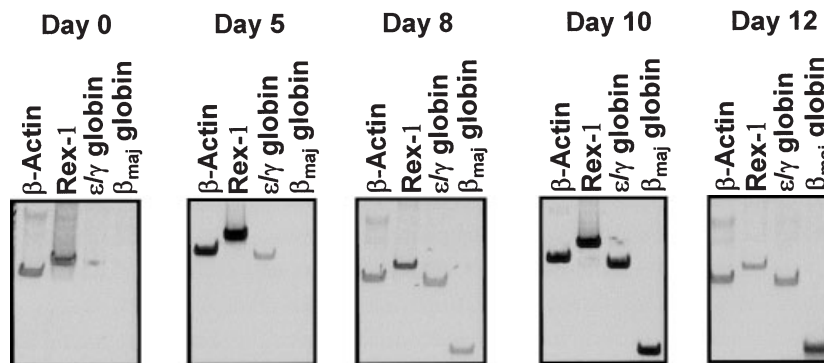


Fig. 2. Sequential activation of globin gene transcription during *in vitro* erythroid differentiation of murine embryonic stem cells. PCR analysis of DNase I treated and reverse-transcribed total RNA extracted from differentiating embryonic stem cells at the indicated time points. All primer sets span introns, with the exception of Rex-1, and the size of each RT-PCR product is as follows: Rex-1, ~600 bp; β -actin, 480 bp; ϵ/γ -globin, 400 bp; β_{maj} , 220 bp. None of the samples showed genomic DNA amplification (not shown).

etic and nervous systems *in vitro* (data not shown). Total RNA was isolated from ES/MEF and ES/OP9 cultures at specific time points following the start of induction and treated with DNase I to remove genomic DNA. Reverse-transcription polymerase chain reaction (RT-PCR) was used to examine the developmental progression of cell samples and in all cases except that of the *Rex-1* gene, primer sets span introns. Day 0 cells are composed of ES cells and MEF cells grown in ES media containing leukemia inhibitory factor (LIF). These cells express the *Rex-1* and β -actin genes but not the embryonic and adult globin genes (Fig. 2). Upon differentiation the embryonic- and adult-specific β -globin genes are sequentially activated. The $\epsilon\gamma$ -gene is activated first with transcripts appearing as early as day 5 of the time course. Expression of the adult-specific gene is first observed at low levels at day 8 and is then up-regulated upon the initiation of definitive erythropoiesis (days 10–12). Expression of *Rex-1* is reduced at day 12. The fact that *Rex-1* expression is still detectable at later stages of differentiation is most likely to be due to the presence of residual undifferentiated cells.

We next analyzed the interaction of RNA Pol II and TATA binding protein (TBP) as well as the appearance of modified histones within the globin locus during the course of differentiation using the ChIP assay (Fig. 3). We used antibodies specific for RNA Pol II, which recognize both phosphorylated and unphosphorylated RNA Pol II, TBP, acetylated histone H4 (AcH4), and histone H3 dimethylated at

lysine 4 (Me₂K4H3). Dimethylation of H3 at lysine 4 is associated with regions permissive for transcription [21]. Each antibody was used in at least two independent experiments.

The results show that RNA Pol II, TPB, and Me₂K4H3 are present at the core regions of the LCR (HS2 and HS3) but not at the $\epsilon\gamma$ - and β major-globin genes in undifferentiated ES cells (day 0, Fig. 3) indicating that dimethylation of H3K4 and recruitment of RNA Pol II and TBP to the LCR occurs before activation of any of the globin genes. The presence of H3 dimethylated at K4 indicates that these elements are permissible to active transcription. H3K4 dimethylation and recruitment of RNA Pol II is specific to the core regions of the HS sites; this mark is not detected in a region between the HS2 and 3 cores (3/2Flank). There is a low level of acetylated H4 detectable at the β -globin gene promoter but no dimethylated H3K4, consistent with our previous observation [15]. This suggests that the chromatin structure is somewhat open but not transcriptionally permissive in this region. The *Rex-1* gene is associated with a chromatin structure characteristic of an open, transcriptionally active domain [15]. Me₂K4H3 is detectable throughout the globin locus in both MEF as well as OP9 cells (data not shown). We do not believe that the low levels of Me₂K4H3 detected in MEF cells contribute significantly to this modification detected at LCR core elements in day 0 ES cells. First, the day 0 ES cell culture contains less than 10% MEF cells. Secondly,

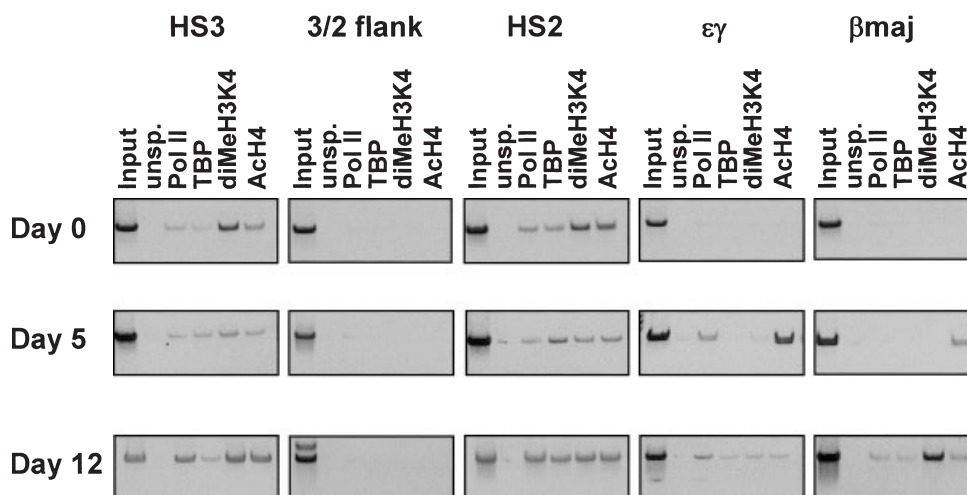


Fig. 3. Interaction of transcription factors and RNA polymerase II with the β -globin locus. Undifferentiated (day 0) and differentiated (day 5 and 12) ES cells were incubated in formaldehyde and the cross-linked chromatin was fragmented, isolated, and precipitated with antibodies specific for chicken anti-IgG (unspec.), RNA polymerase II (Pol II), TATA binding protein (TBP), di-methylated histone H3 lysine 4 (Me₂H3K4), and acetylated histone H4 (AcH4). DNA purified from the precipitate was analyzed by PCR with primers corresponding to regions in the murine β -globin locus as indicated.

Me₂K4H3 is detectable throughout the β -globin gene locus in MEF cells whereas the increase in Me₂K4H3 in day 0 ES cells is restricted to LCR core elements. Importantly, we did not detect associations of RNA Pol II (Fig. 1B) or TBP (data not shown) with the β -globin gene loci in MEF or OP9 cells.

In differentiated erythroid cell samples at day 5, we again observed association of RNA Pol II, TBP and dimethyl H3K4 with HS2 and HS3. At this time point RNA Pol II is also bound at the transcribed $\epsilon\gamma$ -globin gene promoter, which is now associated with acetylated H4 and weakly with dimethyl H3K4. We did not detect TBP at the $\epsilon\gamma$ -globin gene promoter, consistent with our previous findings [15]. Failure to detect TBP at the transcribed embryonic globin gene is likely due to the masking of the TBP epitope. However, the possibility that TBP is not bound at the promoter can not be ruled out. There is also an increase in the association of acetylated H4 present at the β major-globin gene promoter at day 5. At day 12, RNA Pol II and TBP are bound at HS2, HS3, as well as at the $\epsilon\gamma$ - and β major-globin gene promoters. At this time point, the LCR elements and the genes are associated with dimethyl H3K4 and acetylated H4. None of these marks are present in a region flanking HS2 and HS3 or in the murine necdin gene (data not shown).

We used real-time PCR for quantification of the DNA precipitated with antibodies against RNA Pol II, Me₂K4H3, and Ach4 and normalized the data to those obtained from the neuronal necdin gene (Fig. 4). The data show that RNA Pol II is recruited to HS2 but not to the β -globin gene at day 0 in undifferentiated ES cells. At day 12 RNA Pol II is also present at the β major-globin gene, but not at a region between HS2 and HS3. There is a four- to five-fold increase in RNA Pol II association with HS2 over the course of differentiation. The changes in the association of modified histones parallel that of RNA Pol II recruitment.

Our data show that RNA Pol II and dimethylated H3 lysine 4 are detectable at LCR elements HS2 and HS3 at day 0. We next examined whether recruitment of RNA Pol II to HS2 and HS3 is accompanied by transcription of these elements. The results are shown in Fig. 5A and demonstrate that HS3 is abundantly transcribed at this stage, while transcription in HS2 and HS4 is not as efficient at this time point. We also detect transcripts originating upstream of HS3 but not in between HS2 and HS3, or downstream of HS2. In contrast, after 12 days of differentiation transcription can be detected in HS3, HS2, and the β major-globin gene, but not in HS4, or in between HS2 and HS3. The transcripts originating in between HS4 and HS3 are strand-specific proceeding unidirectional toward

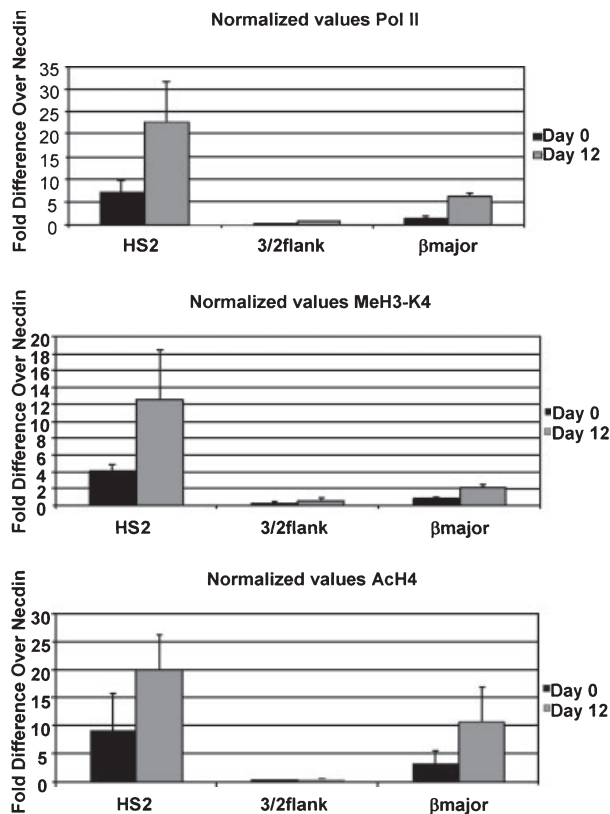


Fig. 4. Quantitative analysis of RNA Pol II recruitment and association of H3 dimethylated at K4 and acetylated H4 with the globin gene locus in undifferentiated and differentiated ES cells. ES cells were taken at day 0 or 12 days after induction of erythroid differentiation and subjected to ChIP and analyzed by RT-PCR using primers specific for mouse LCR HS2, a region between HS2 and HS3, the adult β major-globin gene, and the necdin gene, which served as an internal control. The data were normalized to those obtained from analyzing the necdin gene, which does not associate with RNA Pol II, H3 dimethylated at K4, or acetylated H4 in erythroid or undifferentiated ES cells ([15], and data not shown). The bars represent the average of three independent experiments. The changes in factor recruitment during differentiation were found to be significant ($P < 0.05$).

the globin genes. This was determined by strand-specific RT-PCR, in which the reverse transcription reaction was performed either with the upstream or downstream 5'HS3 primer (Fig. 5A).

To address the question of whether HS3-specific transcription is unique to the mouse embryonic stem cell system, we also analyzed transcription in the β -globin gene locus in human CD133+ hematopoietic progenitor cells, which are not yet committed to the erythroid lineage (Fig. 5B). Transcripts can be detected in the LCR HS3 core region and to a significantly lower degree in HS2 and the β -globin gene. It should be mentioned that CD133+ cells also include between

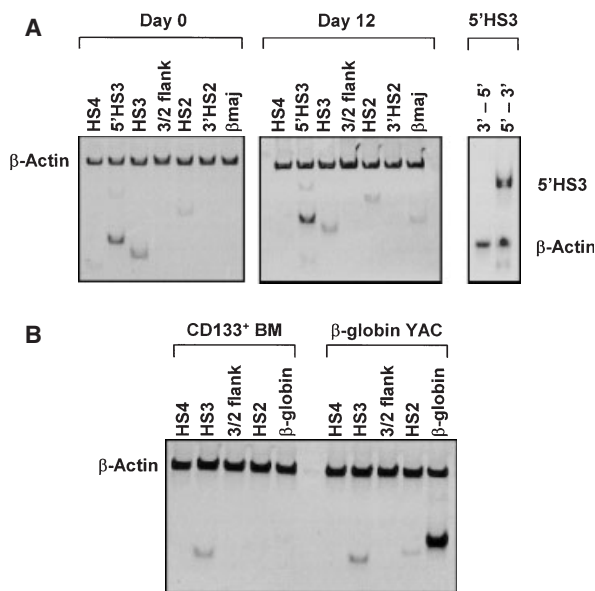


Fig. 5. Transcription of LCR hypersensitive site 3 in undifferentiated murine embryonic stem cells and in human CD 133+ bone marrow cells. (A) Transcription of LCR regions and the β_{maj} -globin gene during differentiation of erythroid cells from murine ES cells. RNA was isolated at the indicated time points, reverse transcribed and subjected to PCR using primers specific for the HS4 core enhancer (HS4), a region 5' to HS3 (5'HS3), the core of HS3 (HS3), a region flanking HS2 and HS3 (3/2 flank), the core of HS2 (HS2), a region downstream of HS2 (3'HS2), and the β_{maj} -globin gene (β_{maj}). The panel on the right shows that transcription 5' to HS3 is directional and proceeds towards the HS3 core enhancer. The RNA was isolated and reverse transcribed using a primer specific for the bottom strand (3'–5') or for the top strand (5'–3'). (B) Transcription in the human β -globin gene locus in CD133+ cells as well as in adult erythroid cells from β -globin yeast artificial chromosome transgenic mice (β -globin YAC, 27). Total RNA was reverse transcribed and analyzed by PCR with primers specific for HS4, HS3, the HS2/HS3 flanking region, HS2, and the adult β -globin gene.

20 and 30% of CD34+ cells, which are known to express low levels of the adult β -globin gene; this could explain the presence of HS2 and β -globin gene transcripts in these cells. HS3 transcription was analyzed with primers that detect transcripts originating from a start site that we previously mapped to within the core of HS3 [22]. This start site was later confirmed in transfection studies by Routledge *et al.* [23]. Using a 5' primer that hybridizes to the 5'-end of HS3 did not yield any PCR products (data not shown), suggesting that transcription starts within the core in human hematopoietic cells. This contrasts with transcripts that are detectable in the mouse LCR, which initiate upstream of HS3. We did not detect transcripts in HS2 with primers spanning the entire core. However, using an upstream primer that hybridizes just downstream of

the tandem maf recognition element (MARE) sequence in HS2, we detect transcripts. This is again consistent with our previous data showing that *in vitro* transcription initiates upstream of the tandem MARE sequence [22]. Taken together the results demonstrate that HS3 is abundantly transcribed in uninduced murine ES cells and in human hematopoietic progenitor cells.

Discussion

The commitment of pluripotent stem cells to successively less plastic progenitors and, finally, differentiated cells exhibiting stable expression patterns is thought to involve the reorganization of the chromatin environment of many lineage-specific genes. The timing of these changes, in many cases, has been shown to precede gene transcription [11,14,17]. In the present study, we have assessed the temporal nature and extent of covalent histone modifications and association of transcription complexes at the murine β -globin locus during the *in vitro* differentiation of murine embryonic stem cells. We observed that elements of the β -globin LCR are capable of recruiting RNA polymerase II and histone modifications compatible with transcription prior to lineage specification. We also observed transcription in the LCR in undifferentiated murine ES cells and in human hematopoietic progenitor cells. These results suggest that a domain in the β -globin locus already exists in a transcriptionally active state very early during differentiation. It appears that in the context of this system the locus remains so in a number of prehematopoietic precursor cell populations and undergoes a number of alterations in chromatin structure and factor recruitment as these cells progress towards hematopoietic commitment. Quantitative analysis shows that recruitment of transcription complexes and histone modifications are present in greater abundance at the LCR compared with the gene promoters. This is consistent with the idea that the LCR may be activated in a number of hematopoietic and prehematopoietic cell types, whereas the activation of the genes is restricted to that of the erythroid lineage. Whether or not this is a requirement for the proper stage-specific activation of the genes is not known.

Tuan *et al.* [24,25] described transcripts that initiate within the core enhancer of HS2 and proceed in a unidirectional manner toward the genes. The authors discussed the possibility that LCR-recruited RNA Pol II could track through the globin locus and that activation of the genes is regulated by this tracking process. Indeed, if the LCR is inverted, or if insulators or transcription terminators are placed between the LCR and the genes, globin gene expression is significantly

reduced [26–28]. During differentiation, the LCR is relocated from within inaccessible chromatin territories to the surface of these territories [29]. It has been proposed that transcription in the nucleus takes place in specific domains enriched for transcription complexes, often referred to as transcription factories [30]. It is possible that one of the early events in globin locus activation involves the association of the LCR with transcription factories. If the LCR remains somehow fixed at this location the process of intergenic transcription at later differentiation stages would reel the genes into this domain. A reeling mechanism of enhancer function has previously been discussed by Riggs [31] and more recently by Fraser and colleagues [32]. It should be noted that a transcriptionally inactive form of RNA polymerase II is recruited to the murine β -globin promoter in the absence of the LCR [33]. This result is consistent with the hypothesis that the LCR is required for recruiting active transcription complexes to the β -globin gene locus.

Recently, a study by Szutorisz *et al.* [34] produced similar observations for the B-cell specific VpreB1 and $\lambda 5$ genes. They characterize a *cis*-acting element in this locus marked by H3 acetylation, H3 lysine 4 di-methylation, and RNA Pol II recruitment in ES cells and show that these marks occur independently of the recruitment of any lineage-specific transcription factors such as PU.1. Furthermore, they observe the presence of components of the TFIID complex (TAF 10 and TBP) at this element in ES cells. They label these marks collectively as the early transcription competence mark (ETCM) and substantiate its importance by making light of the fact that subsequent, similar modifications appear to spread outward in both directions to the genes it controls. This is identical to the observed appearance of these marks at the LCR of the globin locus in ES cells followed by the genes in our ES/OP9 cultures. Anguita *et al.* [35] recently analyzed recruitment of factors to the α -globin gene locus during the differentiation of erythroid cells. The regulatory elements located upstream of the α -globin genes also appear to initiate the activation of the gene locus. However, in contrast to the β -globin LCR and the VpreB1 and $\lambda 5$ gene locus, the α -globin regulatory elements do not recruit RNA Pol II and it appears that recruitment of RNA Pol II to the α -globin gene promoters is a late event in the activation of this gene locus. This study demonstrates that the recruitment of transcription complexes to regulatory DNA elements is not necessarily a common feature of control mechanisms in multigene loci.

Our observation that HS3 is transcribed more efficiently than HS2 in undifferentiated cells, suggests

functional differences between these two elements during the establishment of permissive chromatin structure in the globin gene locus. Other studies have shown that although the HS sites function together in generating a fully functional LCR, they are not all redundant. For example, we have shown that while HS4 could be replaced by HS3 without impairing globin gene expression in β -globin YAC transgenic mice, replacing HS3 by HS4 had a deleterious effect on globin gene expression [36]. Our data suggest that transcription through HS3 could mark the globin locus for activation. Chromatin opening could then initiate in HS3 and spread along the globin gene locus. This is consistent with previous studies by Ellis *et al.* [37] demonstrating that HS3 harbors a dominant chromatin-opening activity. In other words, HS3 could maintain a small accessible region in the globin locus during the differentiation of hematopoietic stem cells to erythroid cells. Transcription of HS3 could be important in maintaining this accessible structure, particularly in light of the fact that RNA Pol II is known to associate with chromatin modifying activities, e.g. histone acetylases and methylases, which could establish a memory mark for subsequent cell divisions [38]. This would be similar to memory elements in drosophila, which are important for developmental stage-specific gene expression [39].

Experimental procedures

ES cell differentiation

Mouse ES cells were differentiated to generate cells of the hematopoietic lineage using the ES/OP9 method established and described by Kitajima *et al.* [20]. Briefly, ESD3 cells (ATCC, CRL-1934) were seeded onto a confluent monolayer of MEFs at a density of 10^5 cells/25 cm² in ES media [Dulbecco's modified Eagle's medium (DMEM), 4.5 g·L⁻¹ glucose, 1.5 g·L⁻¹ sodium bicarbonate, 15% fetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol and 10⁶U·mL LIF, grown for 2 days, then passaged (1 : 6) and grown for another day. An aliquot of the cells ($3\text{--}4 \times 10^7$) was taken at this time (day 0) and subjected to RT-PCR and ChIP analysis. The remaining day 0 cells were then seeded onto confluent OP9 stromal cells in OP9 media [α -modified Eagle's medium (MEM) with ribonucleosides and deoxyribonucleosides; 20% FBS] in the absence of LIF at a density of 10⁴ cells/well in six-well tissue culture dishes. At day 3, Epo or Epo and stem cell factor (SCF) was added (2 U·mL⁻¹ and 50 ng·mL⁻¹, respectively) for the remainder of the course of induction. On day five of induction, cells were passaged and reseeded onto fresh OP9 cultures at a

density of 10^5 cells-well⁻¹. The cells were passaged again and reseeded on day 8. On days 0, 5, 8, 10 and 12, cells were collected and subjected to RT-PCR and/or ChIP analysis.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described by Leach *et al.* [40]. The following DNA primers and antibodies were used in the experiments:

Primers

Mouse *βmajor*-globin: US 5'-AAGCCTGATTCCGTAGAGCCACAC-3' and DS 5'-CCCACAGGCAAGAGACAGCAGC-3'; mouse *εγ*-globin: US 5'-CAAAGAGAGTTT TTGTTGAAGGAGGAG-3' and DS 5'-AAAGTTCACCA TGATGGCAAGTCTGG-3'; mouse *HS3* core: US 5'-TG TTTCCCTGATGAGGATTCAATGG-3' and DS 5'-CCC ACACATGGTCATCTATCTGAGC-3'; mouse *HS2* core: US 5'-TTCCTACACATTAACGAGCCTCTGC-3' and DS 5'-AACATCTGGCCACACACCCTAAGC-3'; 3/2flank, US 5'-CTATTTGCTAACAGTCTGACAATAGAGTAG-3' and DS 5'-GTTACATATGCAGCTAAAGCCACAAATC-3'; mouse *Rex-1*: US 5'-AACTGCATCCTCTGCTTGTG-3' and DS 5'-TGCGCTCTATTTCTCTTG-3'; mouse *GAPDH*, US 5'-GATGATGGAGGACGTGATGG-3' and DS 5'-GGCTGCAGGAGAAGAAAATG-3'; mouse *Necdin*, US 5'-TTTACATAAGCCTAGTGGTACCCTTCC-3' and DS 5'-ATCGCTGTCTGCATCTCACAGTCG-3'.

Antibodies

TBP *sc-273*, (Santa Cruz Biotechnology, Santa Cruz, CA, USA), RNA Pol II *05-623*, histone H3 di-methylated at lysine 4 *07-030* and acetylated histone H4 *06-866* (Upstate Biotech, Charlottesville, VA, USA) were obtained from the suppliers indicated.

Nuclear run-on

The nuclear run-on experiments were performed as described by Greenberg and Bender [41]. Globin-specific DNA fragments serving as targets for labeled RNA in slot-blot experiments were generated by PCR. The following primers were used: mouseHS5 US: 5'-GGTACCTATATAGGT GACTTACATA-3' and DS: 5'-CACCTAAGACACTGTG GAAGAGCAG-3'; mouseHS2 US: 5'-GGGTCTCTCTA GGAGGAAGTCCACAGG-3' and DS: 5'-CAGATCTAAT GACCCTAACTCTAAC-3'; mouse *βmajor* US: 5'-GGT GCACCTGACTGATGCTGAGAAG-3' and DS: 5'-GTG GTACTTGTGAGCCAGGCGAGTG3'. We used pK0916 (Stratagene, La Jolla, CA, USA) as a negative control probe. Slot blot was performed as described by Kang *et al.* [42]. RNA was extracted using the RNeasy kit (Qiagen,

Valencia, CA, USA) according to the protocol provided by the manufacturer.

RT-PCR

RNA was isolated for RT-PCR using the Arum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Reverse Transcription was performed using 200–250 ng RNA and the iScript cDNA synthesis Kit (Bio-Rad) as described by the manufacturers' protocol. PCR amplification was performed using the Eppendorf PCR Mastermix (Eppendorf, Westbury, NY, USA) and primer sequences specific for mouse *β-actin* [43], *Rex-1* [44], mouseHS4RT2 US: 5'-GAGATCCTGCCAAGAC TCTG-3' and DS, 5'-GGGCTGTACAGACATCTAGG-3'; mouse5'HS3: US, 5'-GCCCCTCCTCTCATGAGCC-3' and DS, GATGGGGCAAGGGCCAAGGC-3'; mouseHS3RT US: 5'-GGAGCACAGGTTTCTAAGAC-3' and DS, 5'-CCCACACATGGTCATCTATCTGAGC-3'; mouse5'HS2: US 5'-TTAAAGCCTCATTATCTCAAACCA-3' and DS 5'-GTGTGCACTGGGTGGGTAGA-3'; mouseHS2RTB: US, 5'-GAGGCTTAGGGTGTGGGGCCA-3' and DS, 5'-GTCCCCTTTTCATTGTAATGC-3'; mouse3'HS2B: US, 5'-GGACCTGCCTTGCTGTGTG-3' and DS, 5'-GGAA ACAGGGTACCAGTGAATG-3'; mouse *βmajor*-globin: US, 5'-CACCTTTGCCAGCCTCAGTG-3', DS, 5'-GGTT TAGTGGTACTTGTGAGCC-3'; mouse *εγ* US, 5'-AACC CTCATCAATGGCCTGTGG-3', DS, 5'-TCAGTGGTA CTTGTGGGACAGC-3'; human *β-actin*: US, 5'-GGACG ACATGGAGAAGAT-3' and DS, 5'-ATCTCCTGCT CGAAGTCT-3'; humanHS4: US, 5'-GCTGTGACATGGA AACTATG-3' and DS, 5'-GGACTTTCTCAGTATGA CATG-3'; humanHS3RT: US, 5'-CCACCAGCTATCA GGGCCAG-3' and DS, 5'-GCTGCTATGCTGTGCCTC-3'; human5'HS2: US, 5'-TGGGGACTCGAAAATCAA AG-3' and DS, 5'-AGTAAGAAGCAAGGGCCACA-3'; humanHS2RT3: US, 5'-GAGTCATGCTGAGGCTTAG GG-3' and DS, 5'-GTCACATTCTGTCTCAGGCA-3'; human *β-globin*: US, 5'-ACACAACTGTGTTCACTAG CAACCTCA-3' and DS, 5'-GGTTGCCATAACAGCAT CAGGAGT-3'.

Real-time PCR

Real-time PCR analysis was carried out using the DyAmo HS SYBR green qPCR kit (MJ Research, Hercules, CA, USA) and the following primers: mouse *βmajor*-globin: US 5'-CAGGGAGAAATATGCTTGTCTATCA-3' and DS 5'-GTGAGCAGATTGGCCCTTACC-3'; mouse HS2core: US 5'-AGTCAATTCTACTCCCCACCCT-3' and DS 5'-ACTGCTGTGCTCAAGCCTGAT-3'; 3/2flank, US 5'-TT AAAGCCTCATTATCTCAAACCA-3' and DS 5'-GTG TGCATGGGTGGGTAGA-3'; mouse *necdin*: US 5'-AC TCTTCTGGCTTCCCAAC-3' and DS 5'-GGAGACCAG

CAGAGGAAG-3'. All reactions were carried out in duplicate with a 'no template' control. Final quantification analysis was performed using the relative standard curve method and results were normalized to the values for the internal control, the *necdin* gene.

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