

Globin gene activation during haemopoiesis is driven by protein complexes nucleated by GATA-1 and GATA-2

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How does an emerging transcriptional programme regulate individual genes as stem cells undergo lineage commitment, differentiation and maturation? To answer this, we have analysed the dynamic protein/DNA interactions across 130 kb of chromatin containing the mouse α -globin cluster in cells representing all stages of differentiation from stem cells to mature erythroblasts. The α -gene cluster appears to be inert in pluripotent cells, but priming of expression begins in multipotent haemopoietic progenitors via GATA-2. In committed erythroid progenitors, GATA-2 is replaced by GATA-1 and binding is extended to additional sites including the α -globin promoters. Both GATA-1 and GATA-2 nucleate the binding of various protein complexes including SCL/LMO2/E2A/Ldb-1 and NF-E2. Changes in protein/DNA binding are accompanied by sequential alterations in long-range histone acetylation and methylation. The recruitment of polymerase II, which ultimately leads to a rapid increase in α -globin transcription, occurs late in maturation. These studies provide detailed evidence for the more general hypothesis that commitment and differentiation are primarily driven by the sequential appearance of key transcriptional factors, which bind chromatin at specific, high-affinity sites.

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Introduction

The process by which multipotent stem cells undergo lineage specification and differentiation to produce their highly specialised progeny is poorly understood. Transcriptional profiling of stem cells and the analysis of selected genes in single cells known to have multiple potential have led to the concept that such cells are 'primed' to express the transcrip-

tional programmes of many different lineages (Jimenez *et al*, 1992; Hu *et al*, 1997). Commitment and differentiation involve both consolidating expression of the chosen subset of lineage-affiliated genes and repressing the other primed programmes that are not part of the lineage in question: so-called multilineage priming. This term describes the global changes in transcription programmes, but the specific mechanisms underlying commitment and differentiation have not yet been fully explored. Therefore, to take this hypothesis forward, it is necessary to determine how an *individual* gene is switched on or off as multipotent cells differentiate into increasingly more specialised cells.

Haemopoiesis provides an accessible mammalian system to address this question. Haemopoietic stem cells (HSCs) are self-renewing cells supporting the production of at least eight lineages of specialised, functionally and morphologically distinct blood cells (Cantor and Orkin, 2002). The phenomenon of multilineage priming has been most clearly demonstrated in haemopoiesis (Jimenez *et al*, 1992; Hu *et al*, 1997). The specific cellular pathway committing progenitors to form red blood cells (erythropoiesis) is well defined and ultimately results in highly specialised precursors (erythroblasts), which, in the later stages of differentiation, express almost exclusively the α - and β -like globin genes to synthesise haemoglobin (Steinberg *et al*, 2001). Therefore, the globin genes, which are silent in early haemopoietic progenitors and expressed at high levels during terminal erythroid differentiation, provide ideal candidates for understanding the principles by which individual genes are activated during lineage commitment and differentiation.

The structure of the α -globin gene cluster has been highly conserved throughout 500 million years of evolution, and a region (135–155 kb of the human sequence) of conserved synteny containing the entire α cluster, its regulatory elements and several widely expressed genes lying upstream is present in 22 species analysed (Flint *et al*, 2001; Hughes, in preparation). The process of erythropoiesis is well defined in the mouse; primary haemopoietic cells are accessible and several murine cell lines that faithfully represent different stages of haemopoiesis are available (Figure 1). We therefore chose to study the mouse α -globin gene cluster, which includes an embryonic gene (ζ) and a duplicated set of three α -like genes 5'- ζ - $\psi\alpha 2$ - $\psi\alpha$ - $\alpha 1$ - θ - $\psi\alpha$ - $\alpha 2$ - θ -3' (Figure 2) (Flint *et al*, 2001). To understand fully how mammalian genes are regulated in their natural chromosomal environment during erythropoiesis, we ultimately need to identify all key regulatory elements, the factors and cofactors that bind to them *in vivo*, how the assembled nucleoprotein complexes interact and the epigenetic events that develop as the genes are activated or repressed. Many aspects of gene regulation have been extensively analysed in the globin gene system revealing a common repertoire of *cis* elements and *trans*-acting factors controlling α - and β -globin gene expression. By contrast, the epigenetic modifications that develop at the

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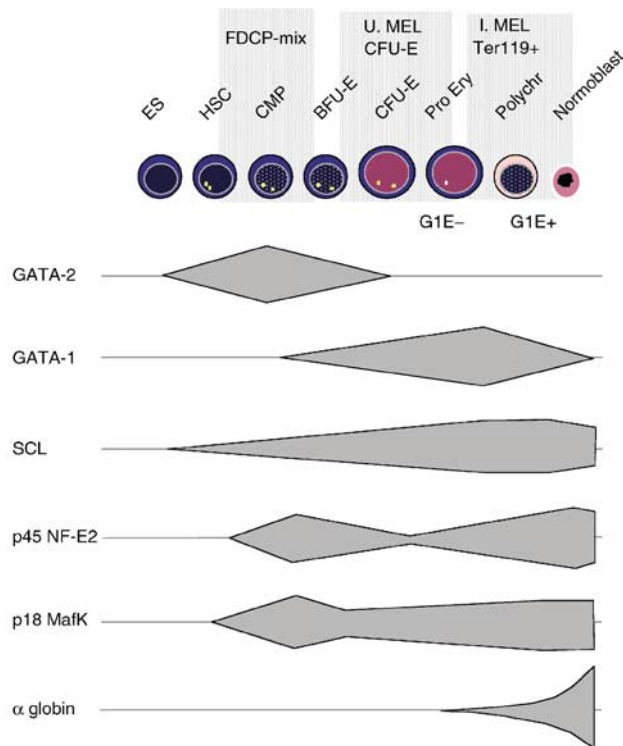


Figure 1 Expression of key transcription factors during erythropoiesis. Data were obtained from previously reported protein and mRNA analyses and observations reported in this study (see Supplementary data). ES, embryonic stem cell; HSC, haemopoietic stem cell; Pro Ery, proerythroblast; Polychr, polychromatic erythroblast; U. MEL, uninduced MEL cells; I. MEL, induced MEL cells; G1E⁻, G1E GATA-1-ER before estradiol treatment; G1E⁺, G1E GATA-1-ER after estradiol treatment. Although G1E cells were considered to be proerythroblasts recent data (Welch *et al*, submitted) suggests that they have some characteristics of BFU-E.

α and β clusters during haemopoiesis are surprisingly different, suggesting that despite their common evolutionary origins, the extant α and β clusters lie in quite different chromosomal environments (Higgs *et al*, 1998).

Most previous studies of mammalian globin gene expression have concentrated on fully committed erythroid cells. However, to further our understanding of how the globin genes are activated during erythropoiesis, we need to establish the hierarchy and order of events as commitment and differentiation proceed. Here we have used chromatin immunoprecipitation (ChIP) to analyse the pattern of transcription factor binding and the associated changes in chromatin across a region of ~ 130 kb including the entire mouse α -globin domain in cells representing different stages of the pathway from pluripotent cells to mature erythroblasts.

These studies thus define in detail the steps involved in the sequential activation of the α -globin genes during lineage commitment and differentiation, and provide a paradigm for the mechanism by which other mammalian genes may be similarly regulated.

Results

Primary cells and cell lines representing key stages of erythropoiesis

The cellular basis of erythropoiesis has been well defined (Steinberg *et al*, 2001; Cantor and Orkin, 2002) (Figure 1).

Primary cells from the early stages of haemopoiesis are generally too rare for standard genetic and epigenetic analyses and, even when purified, are inevitably heterogeneous. On the other hand, haemopoietic cell lines, although homogeneous, may not faithfully reflect all aspects of the corresponding cell *in vivo*. Therefore, where possible we have included analyses of both primary cells and representative cell lines (Figure 1).

As a source of pluripotent mouse cells, we analysed embryonic stem cells (ES, line E14Tg2a), which we have shown to retain erythroid capability *in vitro* as well as contributing to all mouse tissues *in vivo* (Anguita *et al*, 2002).

For multipotent haemopoietic progenitors, we studied factor-dependent cell Paterson (FDCP)-mix cells (subline A4), which were originally derived from long-term cultures of mouse bone marrow. These cells appear normal in that they do not have any karyotypic abnormalities and are not leukaemogenic *in vivo*. In early passage, they form CFU-S and are radioprotective when transplanted into irradiated animals. They can be maintained as self-renewing, undifferentiated blast-like cells in the presence of IL-3 and are thought to most closely resemble the common myeloid progenitor cell (CMP) (Sponcer *et al*, 1986). Clonal analysis of the FDCP-mix cells used in this study showed that, as previously described, they maintained the ability to differentiate with equal efficiency along neutrophil, erythroid and megakaryocytic lineages (data not shown).

Erythroid colony forming units (CFU-E) are fully committed erythroid progenitors (Figure 1), which can be readily isolated from anaemic spleen cells by FACS sorting CD71⁺ and Ter119⁻ cells. Primary, purified CFU-E ($\sim 1 \times 10^7$ from 2–3 phenylhydrazine-treated mice) were isolated and aliquots formed small erythroid colonies with high efficiency (>80%) in methylcellulose (data not shown). In addition, we analysed uninduced mouse erythroleukaemia cells (MEL), which are well-characterised, transformed erythroid cells that are blocked at the CFU-E or early pronormoblast stage of differentiation (Marks *et al*, 1987).

Induction of MEL cells by a variety of agents produced terminally differentiated, but still nucleated, erythroid cells. In addition, large numbers of primary erythroblasts were isolated from the spleens of phenylhydrazine-treated mice by selection of Ter119-positive cells (Spivak *et al*, 1973).

L929, a fibroblast cell line, was used as a source of differentiated murine non-erythroid cells.

Appearance of DNase1 HSS during lineage commitment and differentiation

Previous characterisation of evolutionarily conserved non-coding sequences (Flint *et al*, 2001), localisation of erythroid-restricted DNase1 hypersensitive sites (HSS) (Sheffery *et al*, 1984; Kielman *et al*, 1994, 1996; Anguita *et al*, 2002) together with functional studies (Gourdon *et al*, 1995; Bouhassira *et al*, 1997; Anguita *et al*, 2002; Loyd *et al*, 2003) have identified several potentially important regulatory elements in the mouse α -globin cluster (Figure 2). Since the analysis of DNase1 HSS represents a simple way of identifying changes in chromatin associated with the binding of transcription factors and/or chromatin-modifying factors *in vivo*, we evaluated such sites in the panel of cell lines and primary cells representing different stages of haemopoietic lineage commitment and differentiation (Figures 1 and 2A).

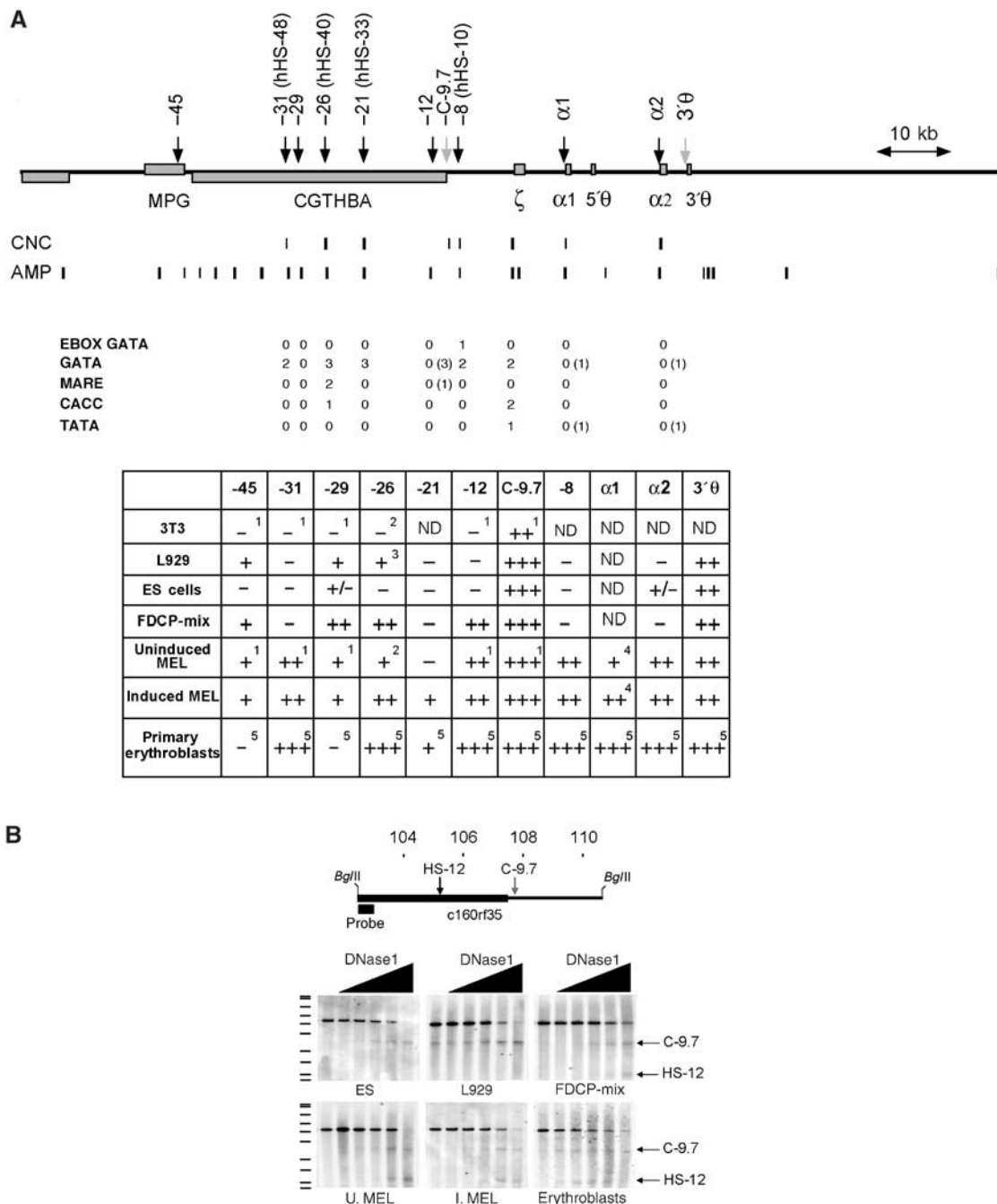


Figure 2 Erythroid-specific DNase1 HSs at the α -globin domain are mostly found at noncoding sequences that contain conserved transcription factor binding sites. (A) The mouse α -globin locus: erythroid-specific HSs (black arrows) and constitutive HSs C-9.7 and 3' θ (grey arrows) are shown. Coordinates relative to the ζ globin gene are indicated above the arrows. Coordinates of the human homologues are shown in parentheses. CNC, conserved noncoding sequences (Hughes *et al*, in preparation); AMP, amplicons used in real-time PCR. Below: The number of sequences for key transcription factor binding sites that are conserved throughout evolution are shown. Potential binding sites present only in mouse are shown in parentheses. The table summarises the results from DNase1 analysis in non-erythroid (L929 and 3T3, fibroblast cell lines) and erythroid cells (see text). ¹Kielman *et al* (1996) and this study; ²Kielman *et al* (1994) and this study; ³+/- RAG cells, Gourdon *et al* (1995); ⁴Sheffery *et al* (1984); ⁵Anguita *et al* (2002). (B) HS mapping exemplifying the dynamics of erythroid-specific DNase1 HSs during erythropoiesis. Above: The area analysed in this experiment, showing *Bgl*III sites and the probe used. This fragment includes a constitutive DNase1 HS (C-9.7) associated with the promoter of the gene *c160rf35* (CGTHBA), and the erythroid-specific site HS-12. Increasing concentrations of DNase1 (black triangles) demonstrate C-9.7 in all cell types. HS-12 can only be seen in FDCP-mix, MEL cells and primary erythroblasts. A faint band corresponding to C-9.7 is seen in L929 cells without added DNase1, probably due to endogenous nuclease activity. On the left of the autoradiograph is an ³⁵S-labelled DNA marker (Amersham, Pharmacia Biotech, UK). U. MEL, uninduced MEL cells; I. MEL, induced MEL cells.

In pluripotent ES cells, two constitutive sites (C-9.7 and 3' θ ; Kielman *et al*, 1996) within the α -globin cluster were clearly present (Figure 2A and B). All the haemopoietic HSs were either absent or weak.

Analysis of FDCP-mix cells, which correspond to early multipotent haemopoietic progenitors, demonstrated four HSs (HS-45, -29, -26 and -12) that have been associated with haemopoietic cells. Other regions containing putative

regulatory elements, including the α -globin promoters, remained insensitive to DNaseI.

In uninduced MEL cells, additional HSs (HS-31, -8 and α -gene promoters) corresponding to putative regulatory elements were seen. After induction, the pattern of HSs in MEL cells changed very little although one new site (HS-21) appeared. A similar pattern to that seen in induced MEL cells was seen in primary erythroblasts although sites at -45 and -29 were not readily detected.

Gene expression in the selected cells and cell lines

The presence of DNaseI HSs frequently corresponds to the binding of transcription factors. Detailed analysis of the evolutionarily conserved transcription factor binding sites in these hypersensitive regions (Figure 2; Hughes *et al*, in preparation) has demonstrated combinations of GATA (Ko and Engel, 1993; Merika and Orkin, 1993), MARE (Maf Recognition Element, Andrews *et al*, 1993a), CACC box (Hartzog and Myers, 1993), E box (Murre *et al*, 1989) and as yet unidentified factor binding sites (Figure 2). Therefore, we examined expression of these factors in cells representing the different stages of differentiation.

The changes in timing and levels of expression of the key transcription factors are summarised in Figure 1 and Supplementary data. Representative experiments are shown in Figure 3.

The pattern of histone modification during commitment and differentiation

Histone acetylation is a major modification of chromatin, which increases the accessibility of nucleosomal DNA, through structural transitions at the level of the nucleosome (Vettese-Dadey *et al*, 1996), and also by influencing higher order chromatin structure (Tse *et al*, 1998). We previously determined the pattern of histone acetylation across the entire α -globin locus in human, mouse and chicken, demonstrating a well-delimited domain of highly acetylated chromatin encompassing the α genes and their regulatory elements in the corresponding region of conserved synteny in all three species (Anguita *et al*, 2001). Here we have extended our original study by performing ChIP analysis at higher resolution, with greater sensitivity and considering a wider range of chromatin modifications: acetylated histone H4 (Ac-H4), acetylated histone H3 (Ac-H3) and histone H3 dimethylated at lysine 4 (H3-diMeK4), followed throughout erythropoiesis.

As before, we found a uniform, low level of acetylation, greater than in heterochromatin, across the entire α -globin cluster in non-erythroid cells (Supplementary Figure 3). We found a similar pattern in pluripotent ES cells. The previously characterised, broad, erythroid-specific domain of histone acetylation (H3 and H4) first appears in multipotent haemopoietic cells, where it initiates far upstream of the α cluster (HS-29). The domain of acetylation extends during differentiation to become fully developed in mature erythroblasts (induced MEL cells and erythroblasts; Figure 4). A similar pattern was seen for H3-diMeK4-modified chromatin (Figure 4). In particular, we noted that the overall level of histone acetylation and H3-diMeK4 methylation increases and extends between early committed erythroid precursors (CFU-E) and late, mature (Ter119-selected) erythroblasts.

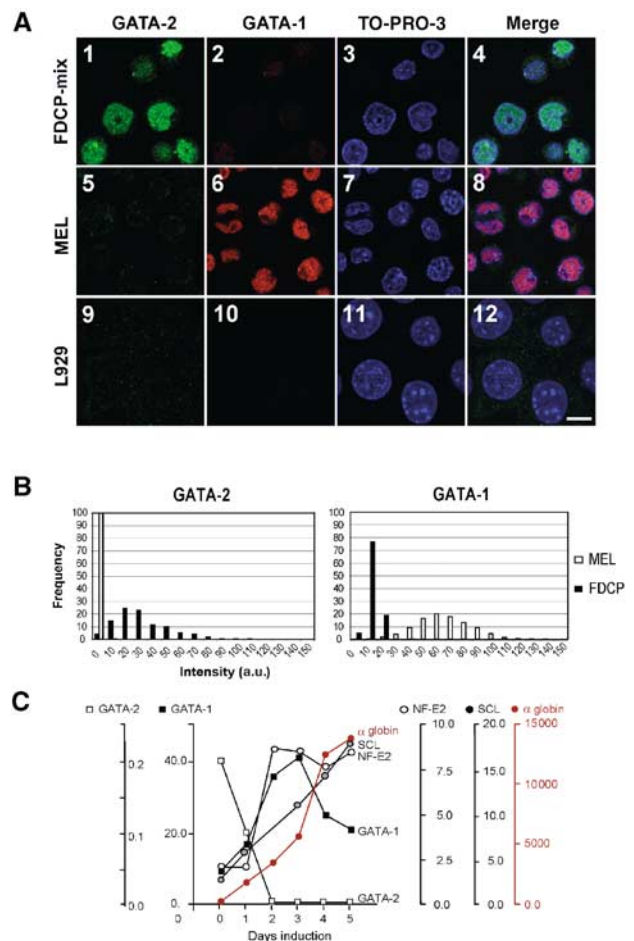


Figure 3 Changes in transcription factor expression during erythroid differentiation. **(A)** Immunofluorescence, illustrating the switch from GATA-2 (green) to GATA-1 (red) expression. (1–4) FDCP-mix cells express high levels of GATA-2 and low levels of GATA-1; (5–8) noninduced MEL cells show intense positivity for GATA-1; (9–12) L929 fibroblast cell line. The white bar represents $\sim 10 \mu\text{m}$. **(B)** Quantification of immunofluorescence. Left: The relative intensity of GATA-2 staining in FDCP-mix (black bars) compared to uninduced MEL cells (white bars); the y-axis represents the proportion of cells (%) in each category. L929 nuclear fluorescence was subtracted as background. a.u., arbitrary units. Right: Fluorescence quantification of GATA-1. These experiments show that almost all FDCP-mix cells express both GATA transcription factors. Most cells express more GATA-2 than GATA-1, but there is considerable variability. A small number of cells are strongly positive for GATA-1, and these have a weaker GATA-2 staining and could represent cells that have started to differentiate along the erythroid lineage. **(C)** mRNA analysis by nuclease protection assay of the key erythroid transcription factors and α globin throughout MEL cell differentiation.

The broad domain of acetylation was punctuated by several distinct peaks of acetylation and H3-diMeK4-modified histones, which closely reflect the patterns of HSs seen in corresponding cell types. In multipotent cells, H3 and H4 histone acetylation was predominantly seen at HS-29, a region containing no known erythroid-specific binding sites (Figures 2 and 4 and Supplementary 1). Somewhat lower levels of modification were detected at HS-31, -26, -12 and the α -globin promoters. Peaks of H3-diMeK4 modification were also seen in these cells but those at the α promoters were relatively more prominent.

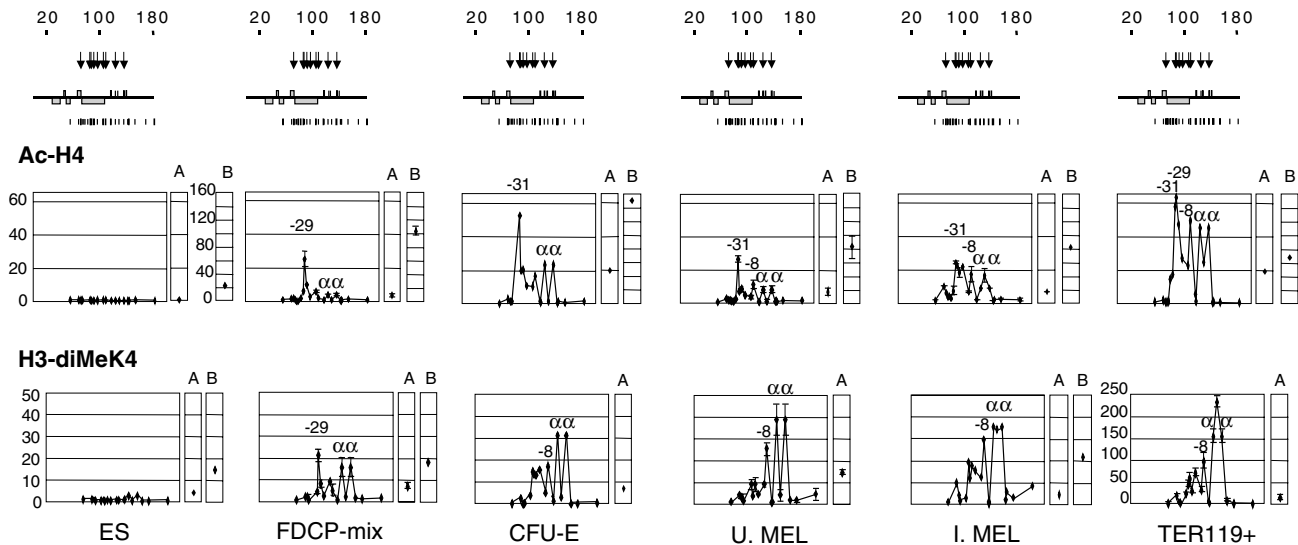


Figure 4 Histone modifications at the α -globin domain change during haemopoiesis. Above: Schematic representation of the α -globin locus as in Figure 2A, including erythroid-specific HSs (arrows) and real-time PCR amplicons (vertical lines). Below: Real-time PCR analysis of ChIP with antibodies against Ac-H4 and diMeK4. The y-axis represents enrichment over the GAPDH locus. The x-axis represents the coordinates at the α -globin locus. A, control sequence at HS2 of the β -globin LCR; B, control at the β -actin CpG island. The results from the different cells analysed are shown in the order of differentiation from left to right. Elements corresponding to the highest peaks of enrichment are indicated. Error bars correspond to ± 1 s.d. from two independent ChIPs. Note the different scale for Ac-H4 β -actin control.

At the next stage of erythropoiesis, the peak at HS-29 substantially decreased and now the highest peak of H3 and H4 acetylation in uninduced MEL cells and CFU-E occurred at HS-31 (Figure 4), which contains two evolutionarily conserved GATA binding sites. This was reflected by a change in the patterns of HSs in these cells (Figure 2). Less prominent peaks of acetylation were also seen at HS-29, -26, -8 and the α -globin promoters. No peak of H3-diMeK4 was seen at HS-31, but this modification was prominent at HS-8 and the α -globin promoters. In terminally differentiating erythroblasts, all of the corresponding peaks of acetylation and diMeK4 became further enriched and a newly modified region between α genes appeared. At all stages of erythropoiesis, the patterns of H3 and H4 acetylation appeared quite similar to each other (Supplementary Figure 1).

Role of GATA-1 and GATA-2

Nearly all of the putative regulatory elements associated with HSs and peaks of histone acetylation contain conserved GATA motifs (Figure 2). Such sequences have been found in the regulatory elements of virtually all erythroid genes and therefore it seemed likely that the two haemopoietic, zinc-finger DNA binding proteins GATA-2 and GATA-1 would bind to these elements *in vivo* (Orkin, 1992). GATA-2 is thought to be necessary for expansion and survival of early haemopoietic progenitors (Tsai *et al*, 1994) while inhibiting differentiation. By contrast, GATA-1 appears to exert a positive effect on both commitment and maturation of the erythroid lineage (Pevny *et al*, 1991).

We evaluated the binding of GATA-1 and GATA-2 *in vivo* using ChIP and the same set of amplicons (Figure 2) used to assess the histone modifications described above. In ES cells, neither GATA-1 nor GATA-2 bound the α -globin cluster. However, in FDCP-mix, in which GATA-2 is first expressed (Figure 3A), it bound specifically at HS-26 and -12 (Figure 5),

concurrent with the appearance of HSs and the small peaks of H4 acetylation and dimethylation of H3-K4. After commitment (CFU-E and MEL cells), GATA-2 was no longer expressed and was replaced at the α cluster by GATA-1.

Very low levels of GATA-1 binding (HS-31, -26 and -12) were detected in FDCP-mix cells and this could be explained by the small proportion of differentiated cells discussed in Figure 3. However, in fully committed early erythroid progenitors (uninduced MEL cells and CFU-E), in which GATA-1 levels are high but the α genes are not transcribed, all conserved elements containing GATA-1 sites (HS-31, -26, -21, -12, -8 and the α promoters) were bound *in vivo*. This pattern of GATA-1 binding appeared almost identical in induced MEL cells that have begun to make α -globin mRNA, suggesting that there are no readily detectable changes in GATA-1 binding associated with induction of globin synthesis. However, in more mature erythroblasts (Ter119-selected cells), GATA-1 binding was no longer detected.

Therefore, it appears that GATA factors play a key role, by binding to a very restricted set of highly conserved sites, in priming and initiating activation of the chromatin containing the α -globin cluster.

Binding of GATA-1 is influenced by friend of GATA (FOG-1)

The N-terminal fingers of GATA factors contact a coregulator called friend of GATA (FOG-1), which does not appear to bind DNA directly (Tsang *et al*, 1997). Mutations that abolish or downregulate FOG-1 expression perturb erythropoiesis in a very similar, albeit less severe, way to GATA-1 mutations (Tsang *et al*, 1998). It was recently shown that FOG-1 can bind at least one region of chromatin in the mouse α -globin cluster (HS-26) (Pal *et al*, 2004).

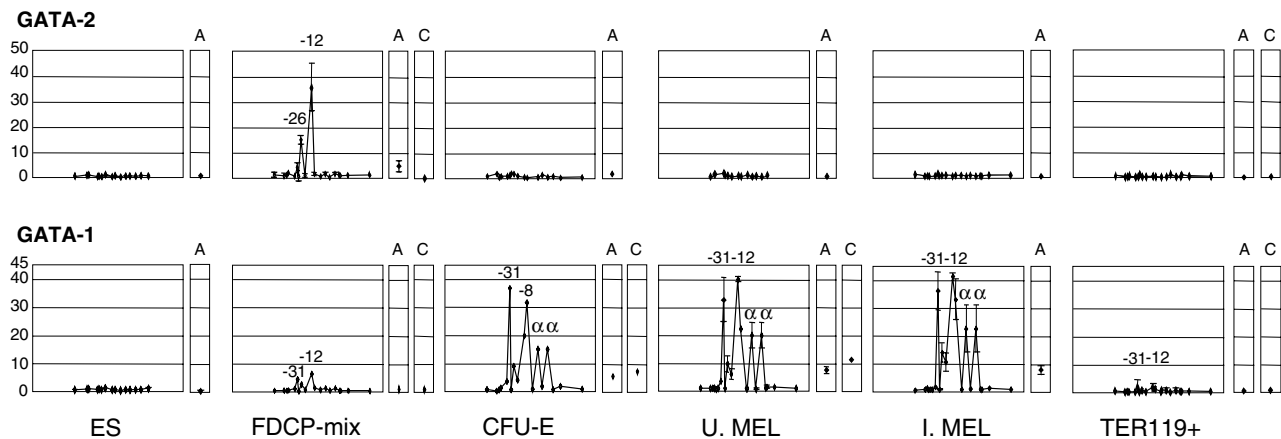


Figure 5 GATA-1 and GATA-2 bind to specific elements in the α -globin locus according to their expression pattern. Plots follow Figure 4. Above: GATA-2 binds only in FDCP-mix. Below: GATA-1 replaces GATA-2 as differentiation proceeds and binds most HSs, including the α -globin promoters. In late erythroblasts, no GATA binding is detected. A, HS2 of the β LCR; C, -2.8 region of the GATA-2 locus (Grass *et al*, 2003). Error bars represent ± 1 s.d. from two independent ChIPs.

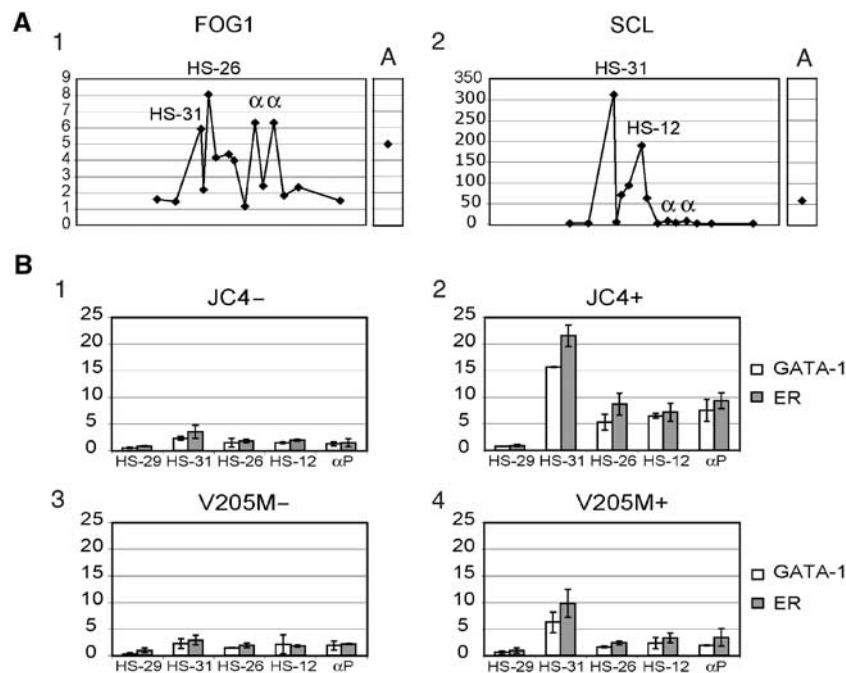


Figure 6 GATA-1 requires FOG-1 interaction for adequate DNA binding and/or recruitment to the α -globin locus. (A) (1) The pattern of FOG-1 binding to the α -globin cluster in U. MEL cells using newly established conditions for ChIP analysis. (2) As a control, SCL binding was analysed under the same conditions (compare with previously established conditions in Figure 7). A, HS2 of the β LCR. (B) GATA-1 ChIPs were analysed at GATA-1 binding sites (HS-31, -26, -12 and α -globin promoters) and at a site where GATA-1 does not bind (HS-29). ChIP analysis using anti-GATA-1 or anti-ER antibodies was performed with cells expressing wild-type GATA-1-ER (JC4) before (1) and after (2) estradiol induction, and in the V205M GATA-1-ER mutant, defective for FOG-1 binding, without (3) or with (4) estradiol treatment. The error bars represent ± 1 s.d. from two independent ChIPs. α P, α -globin promoters.

To detect binding of FOG-1, it was necessary to decrease the extent of sonication (shearing to < 1.5 kb) and adjust the crosslinking (1% formaldehyde for 15 min at room temperature). Under these new conditions, FOG-1 binding could now be detected at all sites where GATA-1 was found (Figure 6A1). The pattern of SCL binding using these conditions appeared identical to that seen using the previously established protocol (Figure 6A2).

We also analysed a GATA-1-deficient mouse erythroblast cell line (G1E) stably expressing either GATA-1 fused to the

ligand binding domain of the oestrogen receptor (GATA-1-ER cells) or GATA-1 (V205M)-ER cells in which the FOG-1/GATA-1 interaction is impaired due to a mutation (V205M) in the N-terminal finger of GATA-1 (Nichols *et al*, 2000). The V205M mutation does not affect DNA binding of GATA-1.

In GATA-1-ER cells, induction of the fusion protein with estradiol increased binding (~ 4 – 7 -fold) of GATA-1-ER to sites at which we had previously demonstrated GATA-1 binding in committed erythroid cells *in vivo* (-31 , -26 , -12 and the α -globin promoters) but not to HS-29, which does not naturally

bind GATA-1. By contrast, binding of GATA-1 in GATA-1 (V205M)-ER cells was significantly reduced at all sites (Figure 6), even though both cell lines express equal amounts of GATA-1 protein, presumably as a result of the impaired interaction between GATA-1 and FOG-1 in these cells. It is interesting that whereas GATA-1 replaces GATA-2 at HS-26 and -12, no prior binding of GATA-2 occurred at HS-31 or the α -globin promoters. Taken together, these data demonstrate that FOG-1 directly facilitates GATA-1 binding or recruitment to various degrees (Letting *et al*, 2004) at all sites studied and is not restricted to sites at which GATA-1 is exchanged for GATA-2 as observed for the elements studied by Pal *et al* (2004).

Recruitment of the GATA/SCL/E2A/LMO2/Ldb-1 erythroid holocomplex

SCL is predominantly, but not exclusively, expressed in haemopoietic cells where it clearly plays a fundamental role in forming complexes with other transcription factors. The best characterised of these is a pentameric complex containing SCL, E2A, Lim-only 2 (LMO2) and LIM domain binding protein 1 (Ldb-1) tethered to DNA via GATA-1, GATA-2 or SCL/E2A, either alone or in combination (Wadman *et al*, 1997; Lecuyer *et al*, 2002; Xu *et al*, 2003; Lahlil *et al*, 2004).

We found that neither SCL nor any of its partners bound the α -globin cluster in non-erythroid or pluripotent ES cells. However, in FDCP-mix, SCL bound HS-31, -26 and -12 in a similar pattern to that seen for GATA-2. We therefore looked for evidence that other members of the SCL/GATA complex bound to these regions and found almost identical ChIP profiles for E2A, LMO2 and Ldb-1 (Figure 7 and Supplementary Figure 2), demonstrating for the first time that the full pentameric SCL nucleoprotein complex had assembled on DNA *in vivo*. Later in erythropoiesis we found that the SCL complex was present with GATA-1 at HS-31, -26, -21, -12 and -8 (Figure 7), again suggesting that the multiprotein SCL complex including GATA-1 had assembled at these sites.

Although GATA-1 and FOG-1 clearly bind at the α -globin promoters in committed erythroid cells, SCL and its partners were not associated with these elements, suggesting that

GATA-1 binds the mouse α -globin promoters as part of a different complex.

Role of NF-E2

In addition to GATA-1, GATA-2 and SCL, previous work has also identified NF-E2 as an erythroid-specific transactivator. NF-E2 is made up of a b-zip protein (p45 NF-E2) (Andrews *et al*, 1993a) and a small Maf protein (p18 NF-E2 or MafK) (Andrews *et al*, 1993b). Both p18 and p45 are members of families of related proteins, which may have overlapping, redundant function (Motohashi *et al*, 2002).

None of the NF-E2 components bind the α cluster in ES cells. However, in FDCP-mix, both p18 and p45 NF-E2 were bound at HS-26 and -12, which both contain MAREs (consensus binding sites for the p18/p45 heterodimer), suggesting that NF-E2, like GATA-2, may play a role in priming the α cluster in multipotent progenitors. In CFU-E, little or no p45 was bound, whereas p18 bound to HS-26 and to a lesser degree to HS-31 and -12. In uninduced MEL cells, both p18 and p45 bound to HS-31, -26 and -12 but there was relatively more p18 bound than p45. Of interest, HS-31 does not contain any classical MARE site, suggesting that NF-E2 may bind indirectly as part of another protein complex. These findings suggest that in early erythroid progenitors MAREs may be predominantly occupied by inactive p18 homodimers (Motohashi *et al*, 2002) or heterodimers with Bach1 (a known transcriptional repressor) (Brand *et al*, 2004). We cannot exclude the possibility that NF-E2 p45 may reside in a different complex in these cells, which renders it less accessible to the antibody. In late erythroblasts and induced MEL cells, in which expression of p45 increases, equal, high levels of p18 and p45 were bound to HS-31, -26 and -12 but not the α promoters (Figure 8). These findings suggest that the binding of high levels of p18 and p45 NF-E2 normally plays an important role in activating α -globin expression during terminal erythroid differentiation.

Recruitment of polymerase II

The ultimate purpose of erythropoiesis is to activate fully the α - and β -globin genes to accumulate high levels of globin mRNA (10–20000 molecules per cell), synthesise massive

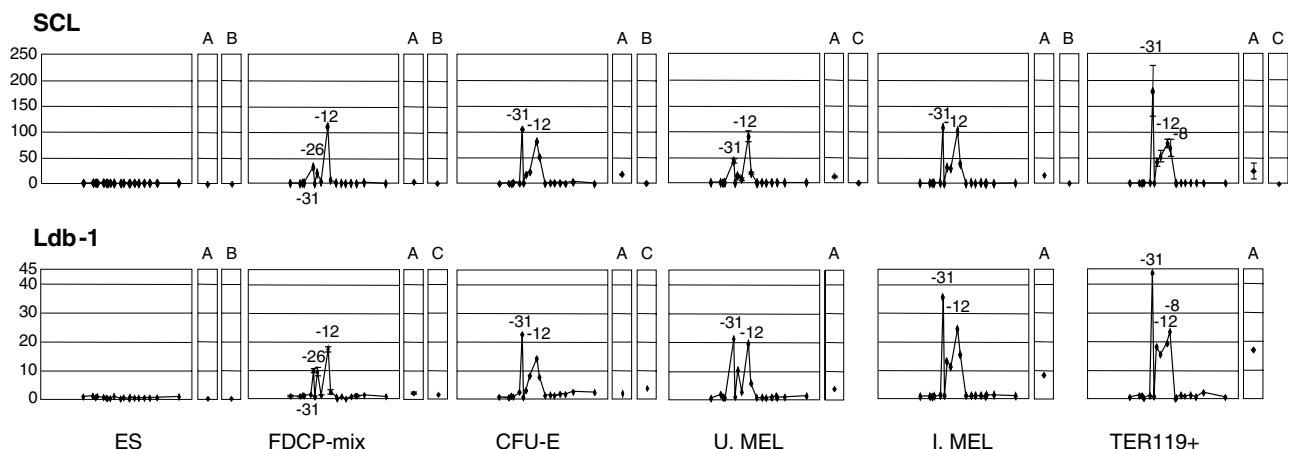
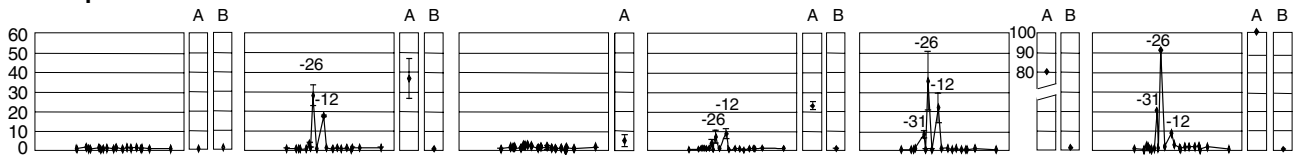


Figure 7 The SCL complex binds to the α globin upstream HSs in a similar pattern to GATA factors, but not to the α -globin promoters. ChIP analysis of SCL (top) and Ldb-1 (bottom) in cells representing different stages of erythropoiesis. Plots follow Figure 4. A, HS2 of the β LCR; B, β -actin CpG island; C, -2.8 region of the GATA-2 locus (Grass *et al*, 2003). The error bars represent ± 1 s.d. from two independent ChIPs.

NF-E2 p45



NF-E2 p18

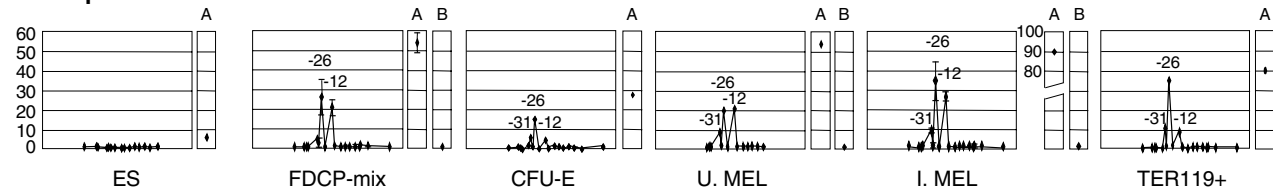


Figure 8 NF-E2 binds in a bimodal fashion priming the α -globin cluster in multipotent progenitors and increasing again when α globin is expressed. ChIP assay on NF-E2 p45 (top) and p18 (bottom). Both subunits first bind in FDCP-mix to HS-26 and -12 in the same proportions. Later in differentiation (CFU-E), p45 disappears and only recovers the equilibrium with p18 subunit at stages in which α globin is expressed. This disequilibrium in p18 and p45 binding may prevent or activate α -globin transcription at different stages of haemopoiesis. At the β -globin cluster, the HS2 (A) shows a similar pattern.

PolII

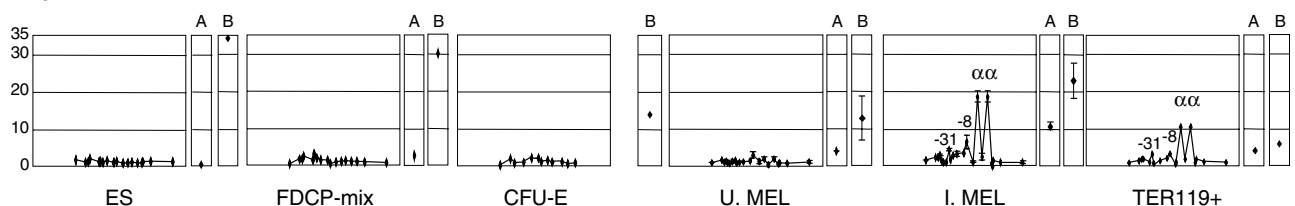


Figure 9 RNA PolII is only detectable at the α -globin promoters late in differentiation. ChIP assay shows no enrichment of PolII in cells not expressing α globin. By contrast, the β -actin promoter (B) binds PolII in these cells, decreasing later in erythroid differentiation as the nucleus becomes pyknotic and expression of many genes (but not globin) is diminished. In globin-expressing cells (I. MEL and Ter119-selected cells), PolII binds not only the α -globin promoters but also β -HS2 as previously described (Johnson *et al*, 2002) (A). Simultaneously, very small enrichment of PolII can be detected upstream the α genes.

amounts of haemoglobin (280×10^6 molecules per cell) and form mature red cells.

Carefully controlled ChIP assays across the entire α cluster showed that polymerase II (PolII) is not engaged at the α -globin promoters in ES cells, FDCP-mix cells, CFU-Es or uninduced MEL cells and only binds at the later stages of erythroid maturation as the binding of NF-E2 and histone acetylation increase (Figure 9). Therefore, it appears that all relevant transcription factors are already bound and many of the associated chromatin modifications including the HSs have substantially developed, before PolII is engaged. From these observations, it seems likely that the sequential binding of GATA-2, GATA-1, SCL complex and NF-E2, described above, precedes binding of PolII and plays a role in its recruitment or activation.

PolII is detectable at the α promoter in induced MEL cells when GATA-1 is also present. The continued presence of GATA-1 at the promoter may not be required to maintain PolII transcription since little or no GATA-1 was found at the later stages of erythropoiesis.

In addition to binding at the α -globin promoters, we also noted very small peaks of PolII bound to HS-31 and -8 in induced MEL cells and erythroblasts. The significance of this low level of binding is not clear.

Discussion

We have established in detail the order of events that occur as a chromosomal domain is 'set up' and the genes contained within it become fully activated during commitment and differentiation (Figure 10). In pluripotent ES cells, in which key haemopoietic transcription factors are not readily detectable, none of the conserved regulatory elements of the α -globin cluster are occupied and chromatin in this region, as determined by DNase1 HSs and histone modification, appears similar to that observed in non-erythroid cells. Therefore, we found no evidence for priming of haemopoiesis in pluripotent cells. By contrast, in multipotent haemopoietic cells maintained under conditions of self-renewal, the α cluster appears to be primed for globin gene expression at several upstream elements, even though these cells retain the potential to form cell types in which globin expression is ultimately silenced, providing further evidence for multilineage priming in these cells. ES cells may either be unprimed or may prime expression of early developmental genes but not haemopoietic genes; whichever, they can give rise to specialised stem cells primed specifically for haemopoiesis.

In multipotent cells, in which haemopoietic transcription factors are expressed, the chromatin in the region upstream

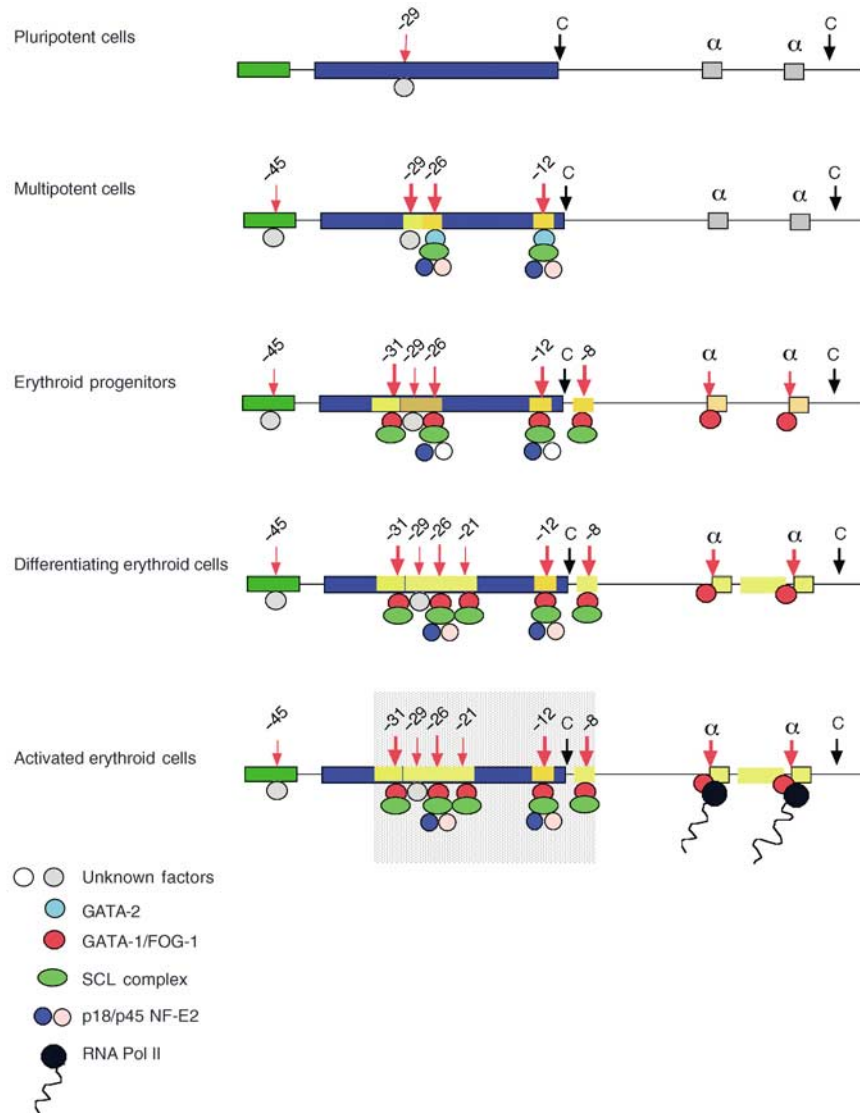


Figure 10 Summary of α -globin activation during erythroid differentiation. In pluripotent cells, the α cluster appears relatively inert, and only HS-29 appears to be bound and the significance of this is unknown. In multipotent cells, the cluster is primed in the upstream region by multiprotein complexes containing SCL and NF-E2 nucleated by GATA-2. HS-29 becomes very prominent and the associated histones are highly acetylated. Otherwise, only limited histone modifications (indicated by yellow boxes) are present. In committed erythroid progenitors, most HSs associated with conserved noncoding sequences are bound by multiprotein complexes containing various combinations of SCL and NF-E2, now nucleated by GATA-1. The α -globin promoters are also bound by GATA-1 either alone or as part of a different multiprotein complex. In differentiating erythroid cells, histone modifications extend throughout much of the locus, creating an erythroid-specific domain of histone hyperacetylation. The pattern of binding alters very little between cells expressing globin and those that do not. The exponential increase in globin mRNA synthesis would be consistent with cooperative interactions between proteins bound upstream of the cluster (shaded box) and multiprotein complexes, including PolII at the α -globin promoters.

of the α -globin cluster becomes hypersensitive to DNase1 at specific sites, namely HS-26 and -12. At these sites, the histones have become weakly acetylated and the DNA bound by GATA-2, NF-E2 and the SCL complex. We also noted a strong HS at -29, of unknown function, in these cells that becomes progressively weaker and loses acetylation during differentiation.

By the time the cell is fully committed as a unilineage erythroid progenitor (CFU-E and uninduced MEL cells), GATA-1 has replaced GATA-2 at HS -26 and -12 and new sites develop at HS -31, -8 and the α -gene promoters, which also bind GATA-1 or the GATA-1/SCL complex. As this occurs, histone acetylation appears to extend further throughout the entire region containing these elements, including the

α -globin promoters. At this stage of erythroid commitment, the entire α cluster appears to be 'poised' for action, even though little or no globin mRNA is made. In this poised state, the chromatin associated with the promoters is also highly enriched in H3-diMeK4 as noted for other poised promoters (Bottardi *et al*, 2003).

During terminal differentiation and maturation, the levels of GATA-1, p45 NF-E2 and SCL mRNA and binding increase and a new HS appears at -21. Concurrently, the levels of histone acetylation increase at the regulatory elements and throughout the domain, most notably in the region lying between the two α genes. However, it appears that most if not all changes reinforce existing patterns rather than develop new changes. The last event in activation involves

recruitment of PolII to the hyperacetylated α -globin promoters and transcription of the genes begins (Figures 3C and 9). Throughout erythropoiesis, regions flanking the globin domain remain uniformly acetylated at relatively low levels and at present it is not clear what confines chromatin activation to the α -globin region.

The initial stages of erythroid commitment, mediated through a common set of binding sites, are brought about by GATA-2, rather than the lineage-restricted factor GATA-1. Consistent with this, GATA-2 is abundantly expressed in multipotential cells, whereas GATA-1 is only upregulated at the later stages of commitment and differentiation. Thus, priming initiates via the less restricted factor (GATA-2) and subsequently full-scale transcriptional activation occurs as this is replaced by the lineage-specific factor (GATA-1), which consolidates or substitutes its function. These findings are consistent with a general model in which commitment and differentiation are substantially driven by development of the transcriptional programme.

Several important questions arise from these findings. The first is how the specific sites (e.g. GATA and MARE sites) associated with conserved regulatory elements differ from the thousands of similar sites within and surrounding the α -globin region so that only these very restricted, evolutionarily conserved sites are primed by their cognate transcription factors. One view is that such regions contain unique sequences and/or chromatin structures, which promote the cooperative binding of transcription factors and their cofactors to each other and to the site. Such nucleoprotein complexes have been referred to previously as enhanceosomes (Thanos and Maniatis, 1995). Presumably, the free energy required to form an enhanceosome is fine-tuned to the concentration and combinations of the relevant activators and coactivators at any particular stage of commitment or differentiation so that the site in question is greatly favoured for binding over all others. The various multiprotein combinations found binding specific conserved elements in this study (e.g. GATA-1/2-SCL, GATA-1/2-NF-E2, GATA-1) and the dependence of GATA-1 binding on FOG-1 would be consistent with the enhanceosome concept. Furthermore, it seems probable that the programme of erythropoiesis is primarily driven by the recruitment of GATA-2 and GATA-1. Extensive evolutionary analysis of the α cluster (Hughes *et al*, in preparation) shows that the WGATAR motif is by far the most consistently conserved at the sites bound in erythroid cells. Some MAREs are also conserved, but E boxes and CACCC boxes appear to be relatively poorly conserved. Therefore, it seems likely that the haemopoietic complexes identified here are nucleated in chromatin via sequential binding of GATA-2 and GATA-1.

Once assembled, how might enhanceosomes induce exponential increases in gene expression? It seems probable that these sites individually or as a large nucleoprotein complex might interact with the α -globin promoters. However, in contrast to the β promoters (Sawado *et al*, 2001; Johnson *et al*, 2002), NF-E2 could not be detected by ChIP at the α promoters and neither could the SCL complex, suggesting that any interaction between the upstream elements and the α promoter may be rapid and/or transitory.

Together with complementary data on the β cluster, the globin genes continue to provide the best-characterised examples of how mammalian genes are regulated *in vivo*.

The studies provide insight into the order of events and mechanism by which individual mammalian genes are activated by key regulators, in this case GATA-1 and GATA-2, throughout lineage commitment, differentiation and development.

Materials and methods

Primary cells and cell lines

MEL cell line 585 was grown and induced as described (Higgs *et al*, 1990). FDCP-mix A4 cells, derived from bone marrow cultures, were grown and assayed for differentiation (Spooncer *et al*, 1986). ES and GIE cells were grown as reported (Weiss *et al*, 1997; Anguita *et al*, 2002). GIE cells expressing GATA-1-ER or GATA-1 (V205M)-ER (Nichols *et al*, 2000) were induced with 1 μ M estradiol for 21 h. Primary CFU-E were obtained from mice as described (Nijhof *et al*, 1982; Miller *et al*, 2002). Mature primary erythroid cells were obtained from phenylhydrazine-treated adult mice as described (Spivak *et al*, 1973). TER119+ cells were magnetically sorted, after indirect labelling.

Chromatin analysis

DNase1 HSs were analysed as before (Higgs *et al*, 1990). For ChIP assay, protein-DNA crosslinking was performed by incubating $\sim 10^7$ cells with 0.4% formaldehyde for 10 min at room temperature. Glycine (0.125 M) was added to quench the reaction. Cells were washed twice in PBS with protease inhibitors (Complete, Roche). Nuclei were lysed in a solution of 1% SDS, 10 mM EDTA and 50 mM Tris-HCl (pH 8.1). The lysate was sheared by sonication to reduce the chromatin fragments to ~ 200 base pairs. GATA-1 ChIP assay was performed as described (Johnson *et al*, 2002). Other assays used the ChIP assay kit (Upstate Biotechnology).

Real-time PCR

Primers and 5'FAM-3'TAMRA labelled probes were selected from unique sequences in the murine α -globin locus and appropriate external controls using Primer Express (Supplementary Table). Input and immunoprecipitated material were analysed in duplicate using an ABI prism 7000 Sequence Detection System following PE Applied Biosystem's Taqman Universal PCR Master Mix protocol. The fold difference of a given target sequence precipitated by a specific antibody was determined by dividing the amount of this in the immunoprecipitated fraction by the amount of target sequence in input DNA (Litt *et al*, 2001). Results were related to a control sequence in the GAPDH gene (Supplementary Table).

Immunofluorescence

Cells adhered to coverslips coated with poly-L-lysine 0.01% (Sigma) or grown on coverslips (L929 and ES cells) were fixed with 4% v/v paraformaldehyde (Electron Microscopy Sciences) in PBS for 10 min. The cells were then permeabilised in 0.5% Triton for 10 min and blocked for 30 min with PBS + B: 3% w/v albumin bovine serum (Sigma), 5% v/v fetal calf serum (PAA Laboratories GmbH, Linz, Austria) in PBS. Antibodies were diluted 1:100 in PBS + B and incubated with the cells for 1 h. After three washes of 10 min in PBS, the cells were incubated with an appropriate secondary antibody diluted 1:200 in PBS + B for 1 h. After three washes of 10 min in PBS and fixation with 4% paraformaldehyde for 10 min, nuclei were stained with 200 nM TO-PRO-3 (Molecular Probes). Images were obtained using an Olympus BX 51 microscope with a confocal BioRad Radiance 2000 system and analysed using Metamorph 6.1. Images were imported into Photoshop and the only manipulation performed was contrast stretch.

Antibodies for immunofluorescence and ChIP

The antibodies used were anti-diacetylated histone 3 (06-599), anti-tetra-acetylated histone 4 (06-866), anti-dimethyl H3-K4 (07-030) (Upstate Biotechnology), RNA PolII (N-20), NF-E2 p45 (C-19), NF-E2 p18 (C-16), GATA-2 (H-116), GATA-1 (N6), E2A.E12 (V-18), FOG-1 (M-216) (last seven from Santa Cruz Biotechnology), SCL (Porcher *et al*, 1999), Ldb-1 and LMO2 (Visvader *et al*, 1997).

RNA analysis

RNA was prepared with TRI reagent (Sigma). RNase protection assay was performed using riboprobes for the mouse GATA-1, GATA-2, NF-E2 p45, SCL (subcloned from cDNA fragments provided by S Orkin) and α globin (Higgs *et al*, 1990).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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