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How transcriptional and epigenetic programmes are played out on an individual mammalian gene cluster during lineage commitment and differentiation

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Abstract

In the post-genomic era, a great deal of work has focused on understanding how DNA sequence is used to programme complex nuclear, cellular and tissue functions throughout differentiation and development. There are many approaches to these issues, but we have concentrated on understanding how a single mammalian gene cluster is activated or silenced as stem cells undergo lineage commitment, differentiation and maturation. In particular we have analysed the α globin cluster, which is expressed in a cell-type- and developmental stage-specific manner in the haemopoietic system. Our studies include analysis of the transcriptional programme that accompanies globin gene activation, focusing on the expression of relevant transcription factors and cofactors. Binding of these factors to the chromosomal domain containing the α globin cluster has been characterized by ChIP (chromatin immunoprecipitation). In addition, we have monitored the epigenetic modifications (e.g. nuclear position, timing of replication, chromatin modification, DNA methylation) that occur as the genes are activated (in erythroid cells) or silenced (e.g. in granulocytes) as haemopoiesis proceeds. Together, these observations provide a uniquely well-characterized model illustrating the mechanisms that regulate and

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memorize patterns of mammalian gene expression as stem cells undergo lineage specification, differentiation and terminal maturation.

Introduction

The process by which multipotent stem cells undergo lineage specification and differentiation to produce their highly specialized 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 transcriptional programmes of many different lineages [1,2]. 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 specialized cells.

Haemopoiesis provides an accessible mammalian system to address this question. Haemopoietic stem cells are self-renewing cells supporting the production of at least eight lineages of specialized, functionally and morphologically distinct blood cells [3]. The phenomenon of multilineage priming has been most clearly demonstrated in haemopoiesis [1,2]. The specific cellular pathway committing progenitors to form red blood cells (erythropoiesis) is well defined and results ultimately in highly specialized precursors (erythroblasts) which, in the later stages of differentiation, express almost exclusively the α- and β-like globin genes to synthesize haemoglobin [4]. 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.

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. In a series of ongoing experiments, we have used a variety of approaches to analyse the pattern of transcription factor binding and the associated epigenetic changes across the entire α globin domain, in both mouse and human, in cells representing different stages of the pathway from pluripotent cells to mature erythroblasts.

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

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Defining the α globin chromosomal domain and the key regulatory elements

Our laboratory concentrates on understanding how the α globin genes are activated during erythropoiesis. To understand fully how these genes are regulated in their natural chromosomal environment, we need to delimit the α globin regulatory domain, and 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.

It has frequently been suggested that mammalian chromosomes are subdivided into discrete structural and functional domains. This concept was originally based on the observation that chromatin within a nucleus is necessarily highly packaged and, at some level of compaction, is thought to be folded into topologically constrained loops tethered at their bases by nuclear attachment sites [5]. These models of structural organization have been extended to incorporate various aspects of function. For example, it has been suggested that structural loops contain independently regulated gene(s) and their *cis*-acting regulatory elements, protected from the influence of similar sequences in neighbouring domains by insulator elements, which may correspond to the physical boundaries of the structural loops. It has also been suggested that these structural/functional domains may be controlled independently in terms of their epigenetic modification (timing of replication, methylation, chromatin assembly and nuclear localization). The potential importance of these models is that, once identified, the genes within such domains should be correctly regulated regardless of their position in the genome.

Despite their attraction, no gene cluster conforms to all components or predictions of these models. Nevertheless, different aspects of the models are consistent with experimental observations on many genes. Therefore the models provide a reasonable starting point to address the question of whether there is a functional unit of a chromosome above the level of a gene.

Following completion of the Human Genome Project, there has been considerable interest in developing bioinformatic routines for annotating chromosomal domains, genes and their regulatory elements [6]. We have used such an approach, comparing the sequences of 22 species spanning 500 million years of evolution [7,8]. Together, these studies have defined a ~135 kb region of conserved synteny (Figure 1) that has been maintained in all 22 species. It seems reasonable to conclude that this region contains all of the *cis*-acting elements required to fully regulate expression of the α globin genes.

To identify *cis*-acting regulatory elements, we have used a variety of approaches. First, we have mapped all erythroid-specific and constitutive DNase 1-hypersensitive sites across this 135 kb region in human and mouse (Figures 1 and 2, and [9,10]). In addition, we have used three independent bioinformatics approaches, including the MCS protocol [11] and a newly developed algorithm (called GUMBY; S. Prabhakar, unpublished work) which, together, identified a total of 24 multi-species conserved sequences (denoted MCS in Figure 1) in a region of 238 kb. This defined seven erythroid-specific regulatory elements,

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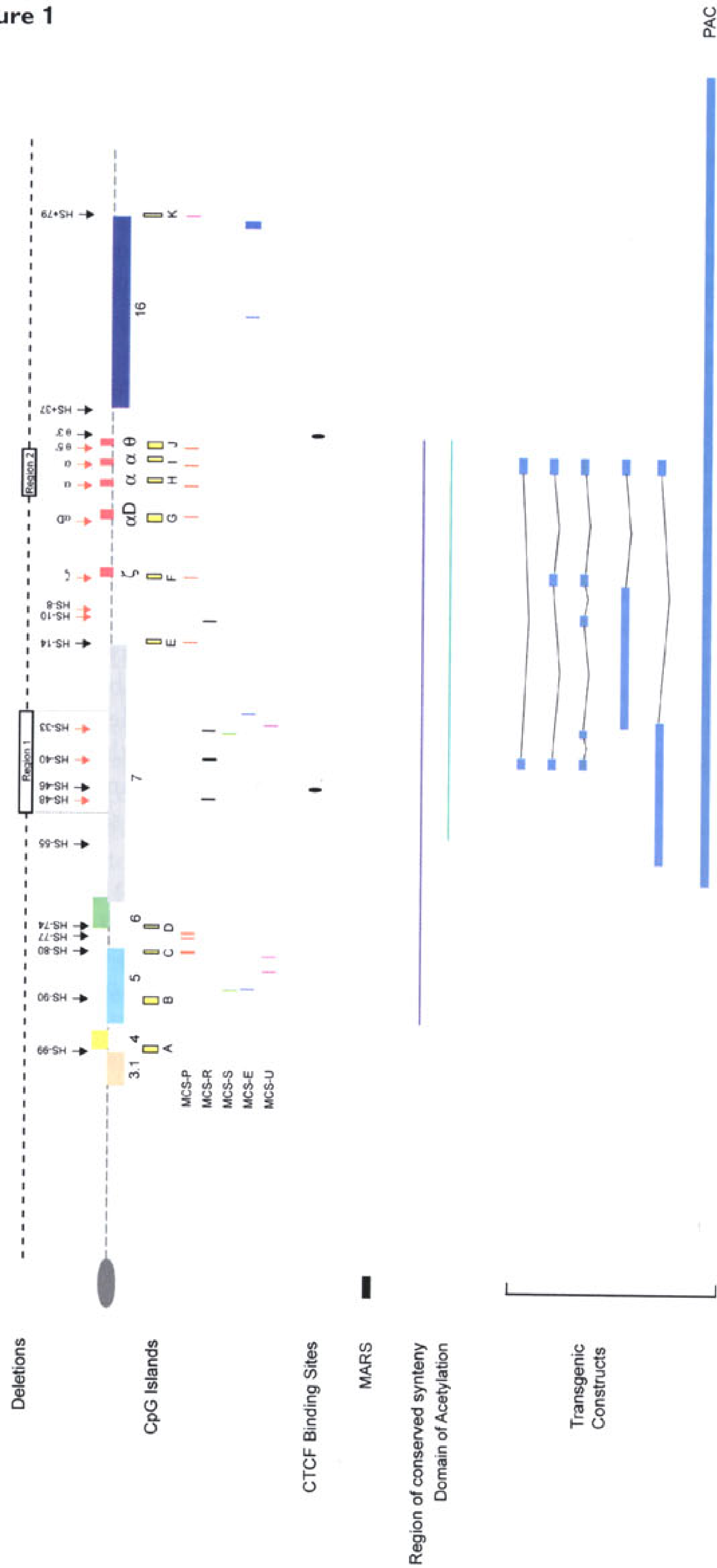
Figure 1



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including previously uncharacterized conserved non-coding sequences [8]. Close inspection of these small (~100–500 bp) conserved elements has revealed phylogenetically conserved motifs, many of which correspond to known haemopoietic transcription factor binding sites. In particular, we found that most elements contain highly conserved sites with the potential to bind the key erythroid transcription factor GATA-1.

These studies have thus defined a putative regulatory domain and the key *cis*-acting elements located within it.

Functional studies of the regulatory domain

Clearly, normal regulation of α globin expression requires the α genes themselves to be structurally present and normal. The inherited form of anaemia called α thalassaemia, in which α globin synthesis is down-regulated, most frequently results from deletion of the α globin genes (Figure 1, region 2, and [12]). In a series of experiments using stable transfectants [13], interspecific hybrids containing normal and abnormal copies of the human α globin cluster on chromosome 16 [14], transgenic mice [15] and mice in which the α globin cluster has been modified by homologous recombination [16], we have systematically analysed the role of upstream elements in regulating α globin expression *in vivo* (summarized in [17] and Figure 1). These experiments have been complemented by observations on rare patients with α thalassaemia in whom the α globin genes are intact but who harbour deletions of the upstream regulatory elements ([18]; and summarized in Figure 1, region 1). Together, these data show that fully regulated α globin expression depends on these elements (HS-48, HS-40, HS-33 and HS-10 in human; see Figure 1), and it appears (at least in human) that the major regulatory element is HS-40, the most highly conserved of all the elements. The role

Figure 1 The human α globin cluster. The telomere is represented as an oval. The annotated globin genes (red boxes) and flanking genes (coloured boxes) are shown. These genes are annotated as previously [7,31]: 3.1, *POLR3K*; 4, *C16orf33*; 5, *C16orf8*; 6, *MPG*; 7, *C16orf35*; 16, *LUC7L*. Above the line are DNase1-hypersensitive sites (black arrows for constitutive and red for erythroid-specific sites). The extent of previously described deletions is indicated by the black broken horizontal line, with a small box over the α genes (region 2; SRO2) representing many common deletions [12] that remove these gene(s). The shortest region of overlap (region 1; SRO1) of all upstream deletions [18] is shown. Below are CpG islands (yellow boxes), conserved promoter elements (MCS-P; red lines), conserved regulatory elements (MCS-R; black lines), conserved splicing intronic regulatory elements (MCS-S; green lines), conserved alternative exons (MCS-E; blue lines) and conserved elements of unknown function (MCS-U; pink lines). The region of conserved synteny is shown underneath as a purple line. The domain of acetylation is shown as a green line. Transgenic constructs are as described previously; boxes represent the segments of DNA joined together (lines) in each construct. The PAC construct which gives optimal expression is shown as a blue box at the bottom. CTCF, CCCTC-binding factor; MARS, mamx attachment regions.

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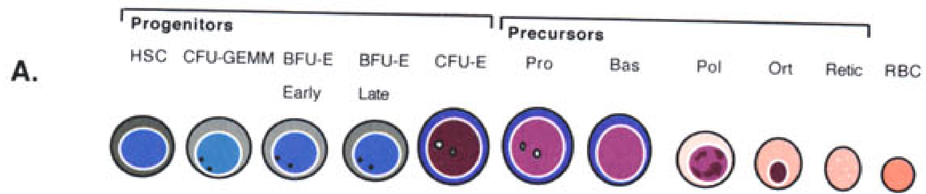

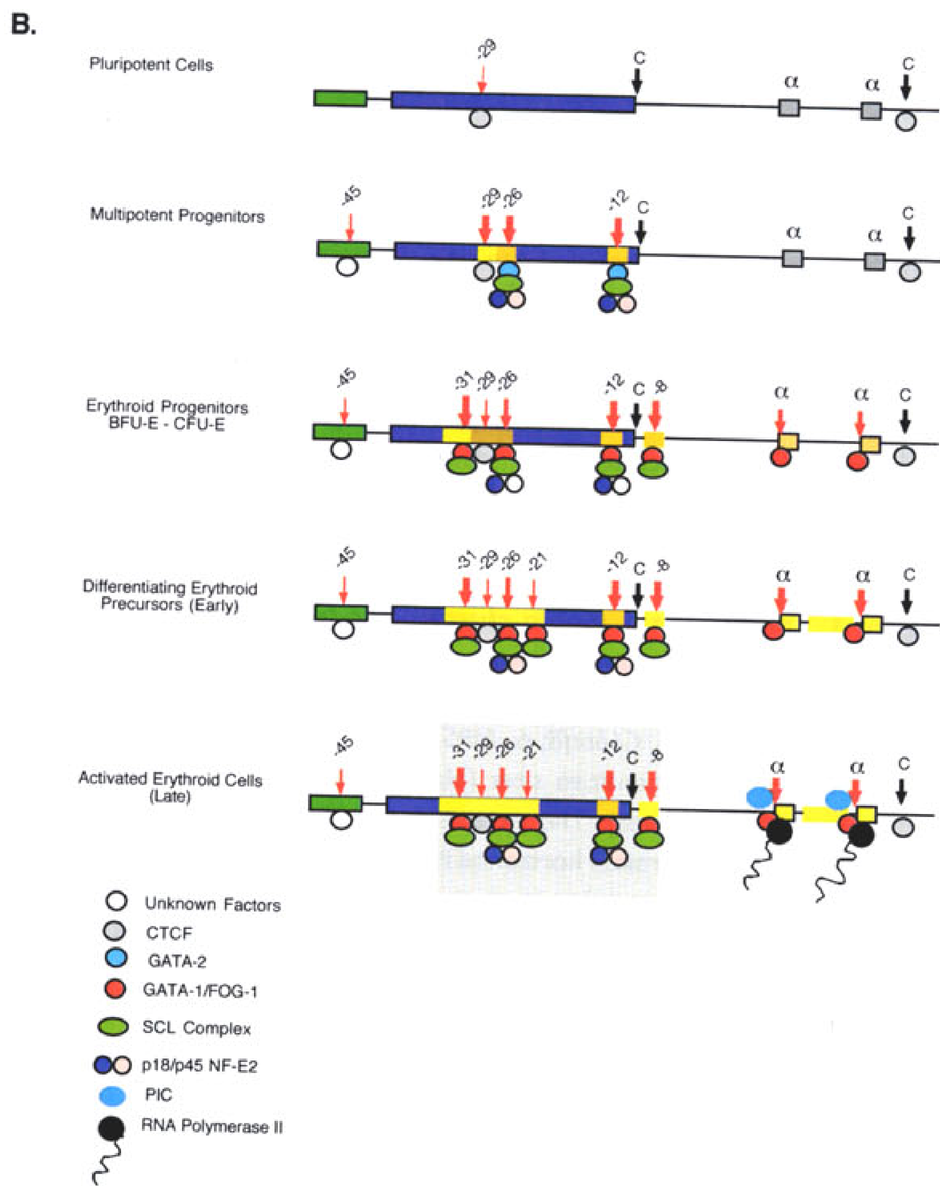

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of other regulatory elements and any potential redundancy is still under investigation, although we have noted that a large (120 kb) transgenic construct (Figure 1), including the entire cluster and all regulatory elements, is consistently expressed at higher levels than smaller transgenes [17].

Sequential binding of key haematopoietic transcription factors during lineage commitment

Many of the key factors that regulate globin gene expression have been well characterized [3] and their patterns of expression during erythropoiesis well documented (summarized in [10]). We have monitored when, during erythropoiesis, these factors bind the α globin cluster by characterizing the development of DNase 1-hypersensitive sites and by using ChIP, measured by quantitative PCR, across the entire mouse and human α globin domains ([10]; M. De Gobbi, unpublished work). The α globin gene cluster appears to be inert in pluripotent cells, but priming of expression begins in multipotent haemopoietic progenitors via GATA-2, which is expressed early in haematopoiesis [3]. 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 (stem cell leukaemia)/LMO2/E2A/Ldb-1 and NF-E2 (nuclear factor-E2). At this stage of erythroid commitment (see differentiating erythroid cells in Figure 2A), the entire α globin cluster appears to be poised for 'action', even though little or no globin mRNA is made.

Figure 2 Summary of α globin activation during erythroid differentiation. In pluripotent cells, the α globin cluster appears relatively inert; 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 non-coding 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 differs very little between cells expressing globin and those that do not. The exponential increase in globin mRNA synthesis would be consistent with co-operative interactions between proteins bound upstream of the cluster (shaded box) and multiprotein complexes, including Pol II at the α globin promoters. Abbreviations: HSC, haemopoietic stem cell; CFU-GEMM, colony-forming units, granulocyte, erythroid, megakaryocyte and monocyte; BFU-E, burst-forming units, erythroid; CFU-E, colony-forming units, erythroid; Pro, proerythroblast; Bas, basophillic erythroblast; Pol, polychromatic erythroblast; Ort, orthochromatic erythroblast; Retic, reticulocyte; RBC, red blood cell; CTCF, CCCTC-binding factor; FOG, friend of GATA.

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Epigenetic changes that occur during haematopoiesis

We have examined nuclear position [19], nuclear attachment [20], timing and pattern of replication [21], location with respect to chromosome territory (V. Buckle, unpublished work) and DNA methylation of CpG islands [9], and found no differences between erythroid and non-erythroid cells. Studies are currently under way to investigate these features during erythropoiesis (V. Buckle, unpublished work).

We have also compared activating and repressing histone modifications in erythroid and non-erythroid cells. Whereas we see a major difference in the activating modifications [H3ac (acetylated histone H3), H4ac and H3K4me2 (methylated histone H3)], as yet we have not identified any consistent repressing modifications in non-erythroid cells. Preliminary studies [22] demonstrated that a domain of acetylation, broadly corresponding to the region containing the conserved regulatory elements and genes, develops in differentiating erythroid cells of human, mouse and chicken. More recently, we examined this at high resolution [10]. As before, we found a low level of acetylation, greater than in heterochromatin, across the entire α globin cluster in non-erythroid cells. We found a similar pattern in pluripotent embryonic stem cells. A broad, erythroid-specific domain of histone acetylation (H3ac and H4ac) first appears in multipotent haematopoietic cells, where it initiates far upstream of the α globin cluster. The domain of acetylation extends during differentiation to become fully developed in mature erythroblasts (Figure 2). A similar pattern was seen for H3K4me2-modified chromatin. The broad domain of acetylation was punctuated by several distinct peaks of acetylation and H3K4me2 modification, which closely reflect the patterns of DNase 1-hypersensitive sites seen in corresponding cell types. At the resolution of these observations, it appears that histone modifications develop in parallel with the binding of transcription factors.

Recruitment of the PIC (pre-initiation complex) and Pol II (RNA polymerase II) during erythropoiesis

Over the past year, we have continued the description of globin gene activation by establishing when during erythropoiesis the PIC [including TFIIA (transcription factor IIA), TFIIB, TFIID, TFIIE, TFIIF and TFIIH, many of which are themselves multiprotein complexes] and Pol II associate with the α globin promoters (D. Vernimmen et al., unpublished work). Whether this occurs in a stepwise manner or involves interaction with a pre-formed holocomplex containing Pol II and at least some components of the PIC is not clear, although recent evidence suggests that active genes associate with pre-formed transcription factories [23].

Initially, we analysed the point during erythropoiesis at which Pol II first binds the complex. This appears to occur quite late in both mouse and human ([10]; M. De Gobbi, unpublished work), co-incident with the onset of globin gene transcription, as determined by *in situ* RNA analysis ([24]; V. Buckle, unpublished work). To look at this more specifically, we examined

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binding in uninduced MEL (mouse erythroleukaemia) cells (representing pro-erythroblasts) and induced MEL cells (representing intermediate and late erythroblasts). In uninduced cells there is little or no Pol II or PIC bound at the α globin promoters, even though, at this stage of erythropoiesis, all of the upstream elements appear fully occupied with the haemopoietic transcription factors (Figure 2). By contrast, in induced MEL cells there is a considerable enrichment of both Pol II and all tested components of the PIC (D. Vernimmen, unpublished work).

These observations suggest that the onset of globin gene transcription coincides with a *de novo* interaction between the α globin cluster, the PIC and Pol II, rather than enhanced initiation or elongation of a pre-bound PIC–Pol II complex.

Role of upstream elements in activating α globin expression

These findings suggest that upstream elements might play a role in facilitating or enhancing the interaction between the α globin complex and the PIC–Pol II complex. To examine this, we have analysed the binding of the PIC and Pol II in a single copy of human chromosome 16 in which the upstream elements (HS-48, HS-40 and HS-33) had been deleted. This chromosome was derived from a previously reported patient with α thalassaemia [25] and was transferred into an erythroid background (MEL cell) by cell fusion. When a normal copy of human chromosome 16 was transferred in this way, induction of the interspecific MEL hybrid greatly up-regulated both mouse and human α globin expression [14]. In the hybrid containing the abnormal copy of chromosome 16, although the endogenous mouse α globin was induced normally, the human α globin gene was not. This could be either because the upstream elements are normally required for the interaction between the α globin complex, the PIC and Pol II or that they are required to enhance initiation or elongation of a complex that forms independently of the upstream elements.

Preliminary data show that the association between the PIC, Pol II and the α globin promoters is significantly reduced in the absence of the upstream regulatory elements (D. Vernimmen, unpublished work), favouring a model in which the conserved, upstream sequences are required to initiate or maintain an interaction between the α globin promoters and the PIC–Pol II complex.

Physical interaction between key regulatory elements and transcription factories?

This raises the question of how the upstream elements, the α globin promoters and the PIC–Pol II complex interact with each other. There is increasing evidence from analysis of the β globin cluster [26–29] that regulatory elements interact physically by a looping mechanism mediated by protein–protein interactions involving complexes nucleated at the key sites. Furthermore, it has been suggested that active gene clusters are recruited to fixed transcription factories

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containing Pol II, whereas inactive clusters remain outside such factories (Figure 3 and [23]). Is there any evidence that the upstream elements and the α globin promoters interact simultaneously with a transcription factory?

We have noted that when the entire α globin cluster is profiled for binding of the PIC components and Pol II (D. Vernimmen, unpublished work), there are minor peaks of enrichment corresponding precisely to each of the upstream regulatory elements (HS-48, HS-40 and HS-33). It is possible that these sites, which lie within the introns of a widely expressed gene upstream of the α globin cluster, are cryptic promoters. However, it is equally likely that protein–DNA complexes assembled at these elements are physically in contact with each other, the α globin promoter and the PIC–Pol II complex within a transcription factory. In this way, ChIP assays using antibodies against either Pol II or components of the PIC may enrich both the promoter elements (directly) and the upstream regulatory elements (indirectly).

In this case, it should be possible to demonstrate a physical interaction between the upstream elements and the α globin promoters in erythroid cells (when the α globin genes are active), but not in non-erythroid cells (where the α genes are silenced). To address this, we and others [30] have used chromosome conformation capture. In preliminary experiments using oligonucleotide primers normally located close to the murine α globin regulatory element (HS-26) and the α globin promoter, which are normally ~35–55 kb apart, we have shown that PCR products are readily detectable in erythroid, but not in non-erythroid, cells (F. Iborra, unpublished work). This suggests that within the nucleus of erythroid cells there is frequent juxtaposition of the major α globin regulatory

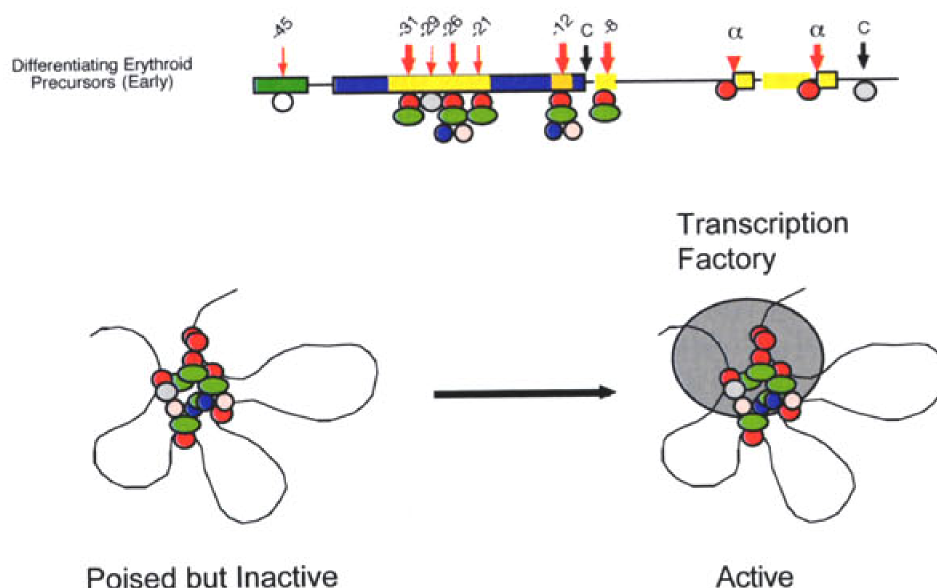

Figure 3 Model for α globin activation. This is based on the model proposed in [23]. Here the α globin cluster is recruited to a transcription factory when active, but lies outwith the factory when inactive, even though it is primed with the full repertoire of transcription factors. Annotation of transcription factors is as for Figure 2.

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element and α globin promoters. Further studies are under way to determine whether other regulatory elements also participate in this interaction.

Summary

Our studies on the α globin gene cluster, together with complementary data on the β globin cluster, have confirmed that the globin genes continue to provide one of the best characterized examples of how mammalian genes are regulated *in vivo*. The studies provide insight into the order of events and mechanisms by which mammalian genes are activated by key transcriptional regulators during lineage commitment, differentiation and development.

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