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Epigenetic silencing of the *c-fms* locus during B-lymphopoiesis occurs in discrete steps and is reversible

Hiromi Tagoh[1], Alexandra Schebesta[2], Pascal Lefevre[1], Nicola Wilson[1], David Hume[3], Meinrad Busslinger[2] and Constanze Bonifer[1,*]

[1]Molecular Medicine Unit, St James's University Hospital, University of Leeds, Leeds, UK, [2]Research Institute of Molecular Pathology, Vienna Biocenter, Vienna, Austria and [3]Institute for Molecular Bioscience, University of Queensland, Queensland, Australia

The murine *c-fms* (*Csf1r*) gene encodes the macrophage colony-stimulating factor receptor, which is essential for macrophage development. It is expressed at a low level in haematopoietic stem cells and is switched off in all non-macrophage cell types. To examine the role of chromatin structure in this process we studied epigenetic silencing of *c-fms* during B-lymphopoiesis. *c-fms* chromatin in stem cells and multipotent progenitors is in the active conformation and bound by transcription factors. A similar result was obtained with specified common myeloid and lymphoid progenitor cells. In developing B cells, *c-fms* chromatin is silenced in distinct steps, whereby first the binding of transcription factors and RNA expression is lost, followed by a loss of nuclease accessibility. Interestingly, regions of *de novo* DNA methylation in B cells overlap with an intronic antisense transcription unit that is differently regulated during lymphopoiesis. However, even at mature B cell stages, *c-fms* chromatin is still in a poised conformation and *c-fms* expression can be re-activated by conditional deletion of the transcription factor Pax5.

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Introduction

During haematopoietic differentiation, haematopoietic stem cells (HSCs) become gradually restricted in their differentiation potential. The balanced formation of the different blood cell types therefore requires the activation of genes in appropriate cells as well as the silencing of genes in cells in which expression is undesired (Hu *et al*, 1997; Miyamoto *et al*, 2002; Smale, 2003). Blood cell growth and differentiation are regulated by specific cytokines, which act on cells expressing particular combinations of lineage-specific cytokine receptors. Because the cytokine requirement for precursor cells is different from that of mature cells, it is important to render cells of one lineage unresponsive to the cytokines regulating alternative lineage cells. Cell type-specific inhibitors of cytokine receptor signalling have been characterized (reviewed in Fujimoto and Naka, 2003), but little is known about how cytokine receptor gene expression is silenced at the epigenetic level.

Once a cell is committed to differentiate towards a particular lineage, it responds to lineage-specific signals and generally cannot alter its cell fate. However, certain types of apparently committed cells still have the potential to differentiate into cells of another lineage (reviewed in Graf, 2002). The importance of cytokine signals in lineage determination was emphasized by a series of experiments using transgenic mice expressing the human interleukin (IL)-2 receptor β or granulocyte–macrophage colony-stimulating factor (GM-CSF) receptor in haematopoietic progenitors (Kondo *et al*, 2000; Iwasaki-Arai *et al*, 2003). These experiments showed that purified common lymphoid progenitors (CLPs) and pro-T cells from these mice can be converted by alternative cytokine signalling into myeloid cells. Lineage switching was also found in murine pro-B cells ectopically expressing the human macrophage colony-stimulating factor (CSF-1) receptor (*c-fms*) gene (Borzillo *et al*, 1990). Such manipulated cells can differentiate to macrophages in response to CSF-1. This alternative differentiation is suppressed by IL-7 signalling, which suggests that signals through the CSF-1 and IL-7 receptors can play an instructive role in myeloid and lymphoid differentiation, respectively.

Expression of the *c-fms* gene is tightly controlled. *c-fms* mRNA is detected in HSCs at a low level and is upregulated during macrophage differentiation. Receptor protein expression on the surface of the cells is only found on committed macrophage precursors (Tagoh *et al*, 2002). Tissue-specific mRNA expression of *c-fms* is regulated by well-studied promoter and intronic enhancer elements (Figure 1). The promoter used in macrophages is a TATA-less myeloid promoter, with multiple purine-rich elements bound by Ets family transcription factors, notably PU.1 (Yue *et al*, 1993; Ross *et al*, 1998). Tissue-restricted expression of the gene is dependent on the *c-fms* intron regulatory element or FIRE (Himes *et al*, 2001; Sasmono *et al*, 2003). We have previously examined the mechanism of upregulation of *c-fms* expression during macrophage differentiation. We showed that the *c-fms* promoter is already occupied by transcription factors at the stage of common myeloid progenitors (CMPs) where only a low level of transcripts is detected. During macrophage differentiation, *c-fms* expression is regulated by the coordinated assembly and disassembly of transcription factor complexes on FIRE (Tagoh *et al*, 2002). *c-fms cis*-elements in macrophages show a high level of histone acetylation, but associate with both positive and negative chromatin modification activities (Follows *et al*, 2003).

*Corresponding author. Molecular Medicine Unit, St James's University Hospital, University of Leeds, Leeds LS9 7TF, UK.
Tel.: +44 113 206 5676; Fax: +44 113 244 4475;
E-mail: c.bonifer@leeds.ac.uk

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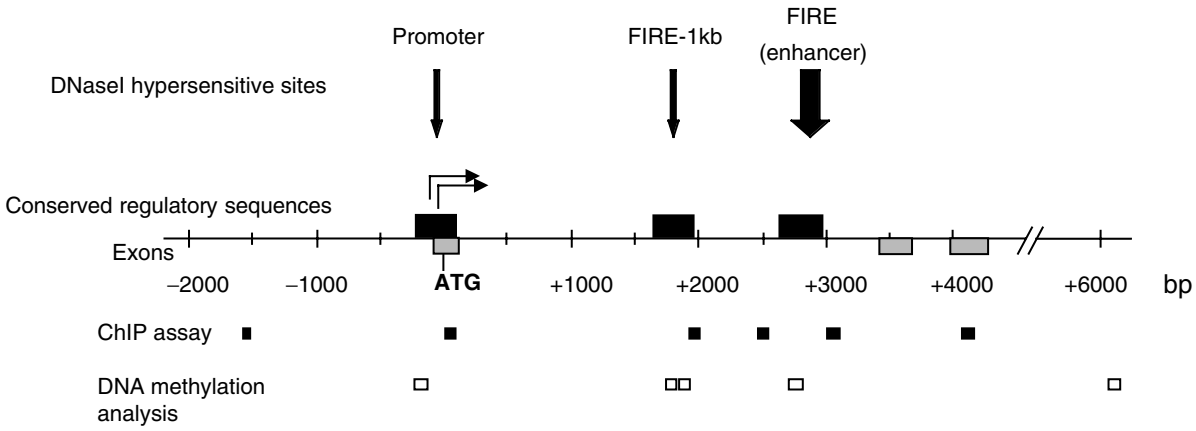

Figure 1 Map of the *c-fms* regulatory region. DHSs present in macrophages are shown as vertical arrows. Numbers indicate the nucleotide position relative to the ATG start codon. Grey boxes represent exons, and black boxes show regions conserved between human and mouse. The main transcription start sites are indicated as horizontal arrows. Amplicons for ChIP assays and DNA methylation analysis are indicated.

c-fms expression is silenced in non-macrophage cells. Silencing of *c-fms* mRNA in B lymphocytes is dependent on the presence of the transcription factor Pax5 (Nutt *et al*, 1999). Interestingly, conditional inactivation of *Pax5* in committed B cell precursors leads to derepression of the *c-fms* gene (Mikkola *et al*, 2002). This is also observed after overexpression of the myeloid transcription factors C/EBP α and β in B cells (Xie *et al*, 2004). However, the chromatin structure of *c-fms* in B cells with which these transcription factors interact and the mechanism of derepression are essentially unknown. Silencing of macrophage-specific genes during B-lymphopoiesis is not well understood and may not be straightforward, as B cells and macrophages share a number of different transcription factors such as PU.1. To address this issue, we have examined the chromatin alterations occurring during the silencing of *c-fms* expression in B cell development. We show that the chromatin at *cis*-regulatory regions of the *c-fms* locus is in an active conformation and is bound by transcription factors in stem cells and early precursor cells. Epigenetic silencing of *c-fms* during B-lymphopoiesis occurs in distinct steps, but even in mature B cells *c-fms* chromatin is still in a poised conformation. This is confirmed by our finding that *de novo* DNA methylation is increased in T cells, but is delayed in B cell development whereby the promoter and FIRE remain unmethylated throughout. We show that such a poised chromatin conformation correlates with the potential to re-activate *c-fms* expression even in purified mature B cells by conditional deletion of *Pax5*. An important finding of this study is that regions of *de novo* DNA methylation in lymphoid cells overlap with an intronic antisense (AS) transcription unit that is active in committed B cells and macrophages, but not in cells where *c-fms* is completely shut down.

Results

The c-fms promoter and FIRE bind transcription factors in pluripotent stem cells and common lymphoid progenitor cells but not in differentiated B cell populations

Expression of *c-fms* mRNA is detectable in HSCs and CMPs (Miyamoto *et al*, 2002; Tagoh *et al*, 2002), but is absent in pro-B cells (Nutt *et al*, 1999). To define the first steps in *c-fms* silencing, we asked whether transcription factor occupancy on *c-fms* in stem cells is complete and thus indicative of a primed chromatin structure. Secondly, because restricted lymphoid progenitor cells (CLPs) were shown to possess latent myeloid differentiation potential (Kondo *et al*, 2000; Iwasaki-Arai *et al*, 2003), we examined whether *c-fms* was still occupied by transcription factors. We addressed these questions by studying purified $Lin^{-}Sca1^{+}c\text{-}Kit^{hi}$ (LSK) cells, which were highly enriched for HSCs and short-term reconstituting stem cells (Geiger *et al*, 1998; Adolfsson *et al*, 2001), CLPs (Kondo *et al*, 1997) and CMPs (Akashi *et al*, 2000; Tagoh *et al*, 2002). As a control, we examined purified pro-B cells from the bone marrow of $RAG2^{-/-}$ mice and mature bone marrow-derived macrophages. The purity of each population was confirmed by surface marker analysis, Giemsa staining, colony assays and mRNA expression analysis (Figure 2 and Supplementary Figures 1 and 2; data not shown). Purified cells displayed distinct *in vitro* differentiation potentials (Figure 2A). As described previously, LSKs and CMPs mostly generated myeloid colonies (Akashi *et al*, 2000). CLPs generated lymphoid colonies containing B and NK cells but no myeloid cells, as confirmed by FACS analysis of CD19, NK1.1 and CD11b expression (data not shown). The CLP fraction formed in average one GM colony in 1000 cells, which was probably derived from a low-level contamination with myeloid precursors. Clonogenicity of CLPs under lymphoid assay conditions was much lower than that of myeloid progenitors under myeloid assay conditions, the latter regularly produced at least 250 colonies from 1000 cells (Tagoh *et al*, 2002 and this study). The expression profile of selected genes in purified restricted progenitors confirmed the identity of these cells (Akashi *et al*, 2000; DeKoter and Singh 2000; Supplementary Figure 1). mRNA for the myeloid-specific *lysozyme M* gene was only detected at trace levels in CLPs (Supplementary Figure 3A), confirming that this cell population had negligible myeloid contamination.

The result of our *c-fms* expression studies is shown in Figure 2B. *c-fms* transcripts were found around the detection limit in purified primary pro-B cells. In contrast, all other precursor cell types (LSKs, CLPs and CMPs) expressed low but clearly detectable levels of *c-fms* RNA. The low level of

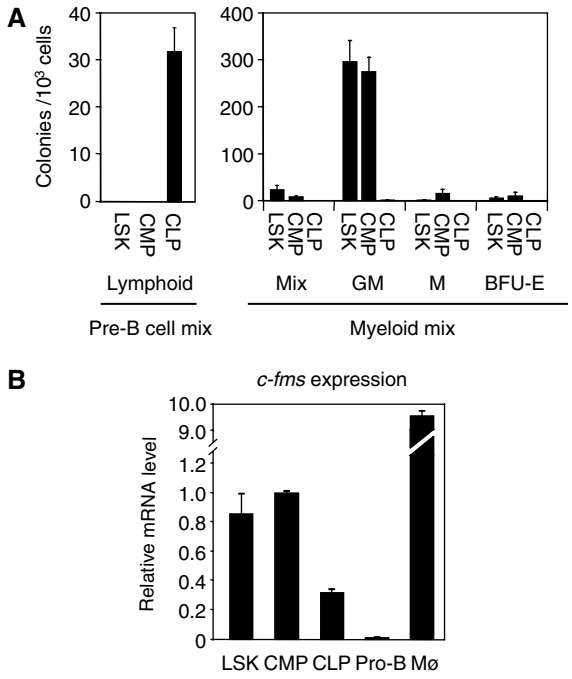

Figure 2 Characterization of purified LSKs, CLPs and CMPs. (**A**) Colony-forming activities of LSKs, CLPs and CMPs. Sorted cells were seeded at a density of 1000 cells/ml in methylcellulose medium containing IL-7, SCF and VEGF for lymphoid assays (pre-B cell mix), and IL-3, IL-6, SCF and Epo for myelo-erythroid assays (GM, M, Mix, BFU-E). Colonies were scored at days 8–10. The results are represented as the mean value of four independent experiments. (**B**) Expression of *c-fms* mRNA in purified LSKs, CLPs, CMPs, pro-B cells and macrophages (Mø). Gene expression was measured by RT–PCR. Arbitrary units were calculated relative to the expression level in CMPs. The bars represent the mean value of two independent experiments.

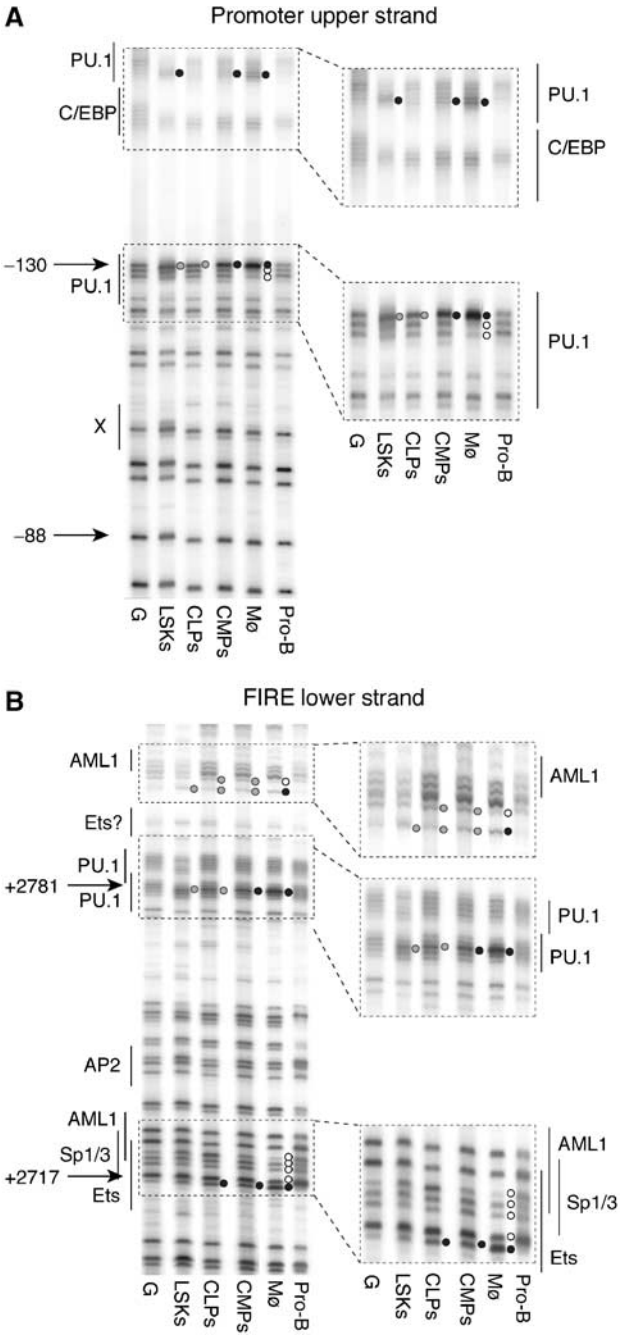

Figure 3 Transcription factor binding to the *c-fms* promoter and FIRE is lost in pro-B cells. DMS footprinting analysis of promoter (upper strand) (**A**) and FIRE (lower strand) (**B**). The numbers on the left indicate the nucleotide position relative to the ATG codon. Transcription factor-binding sites and their nature as determined by ChIP assays and *in vitro* DNA–protein interaction studies (Tagoh *et al*, 2002; Follows *et al*, 2003) are indicated. Black circles indicate hypermethylated guanines, and open circles indicate hypomethylated guanines compared with DMS-treated naked DNA (G). Grey circles indicate weaker footprints. From left to right: DMS-treated naked DNA (G), purified cells (LSKs, CLPs, CMPs, bone marrow macrophages (Mø), freshly purified pro-B cells from *RAG2*$^{-/-}$ mice).

GM colony formation of the CLP fraction (less than 1% compared with CMPs) was not reflected at the level of *c-fms* expression (20% of that of CMPs). These results were mirrored by transcription factor-binding site occupancy as revealed by *in vivo* DMS footprinting analysis with these cell populations. As shown in Figure 3, the PU.1 consensus sequences within the promoter and FIRE were occupied in LSKs, CLPs and CMPs. The footprints seen at the promoter were weaker in CLPs than in LSKs, CMPs or macrophages, whereas the footprints at FIRE were similar in both CLPs and CMPs. In contrast, the Ets/AML1/Sp1 cluster in FIRE was only occupied in CLPs, CMPs and mature myeloid cells. To further rule out the possibility of myeloid contamination, we performed a DMS footprinting experiment on a sample consisting of a mixture of 95% wild-type pro-B cells (lacking footprints) and 5% monocytic cells (with fully occupied transcription factor-binding sites). RNA prepared from this cell mixture contained a much higher level of *lysozyme M* RNA than CLPs (Supplementary Figure 3A), but showed no footprints at the promoter and FIRE (Supplementary Figure 3B and C). This confirms that the threshold for detecting alterations of DMS reactivity in mixed cell populations is significantly higher than any possible myeloid contamination. In summary, although purified CLPs were functionally distinct from CMPs and could not generate myeloid cells, *c-fms* chromatin in these cells is accessible to transcription factor binding, resulting in low level but clearly detectable *c-fms* mRNA expression. No footprints on any of the *c-fms cis*-regulatory elements were seen in pro-B cells, confirming that the loss of steady-state mRNA is accompanied by a loss of stable transcription factor binding in the majority of cells.

c-fms chromatin in B cells is still in a poised conformation

Experiments from our laboratory showed that genes destined for activation undergo a number of defined chromatin alterations during cell differentiation, which start long before the onset of gene expression (Kontaraki *et al*, 2000; Tagoh *et al*, 2004). If such a stepwise process could be linked to a specific chromatin fine structure of given genes during gene silencing, this would enable the definition of windows of opportunity for the reversion of specific gene expression programmes. We therefore examined different chromatin features of *c-fms* during B-lymphopoiesis as well as in other non-myeloid cell types and compared them with those of myeloid cells.

A hallmark of active chromatin is its enhanced accessibility to digestion with nucleases. We measured differential nuclease accessibility in various cell types using DNaseI and micrococcal nuclease (MNase). Digestion products were visualized at single nucleotide resolution by using two different types of ligation-mediated PCR (LM-PCR). DNaseI digestion followed by amplifying single-strand molecules measures the number and position of nicks in one DNA strand, thus assaying both DNA accessibility and DNA topology. MNase digestion was followed by the selective amplification of double-strand breaks, which usually occur in nucleosomal linker regions. The appearance of specific bands in such an assay is interpreted as an indication for a specifically positioned nucleosome. To perform these assays we isolated $B220^+$ bone marrow B cells, consisting mainly of early B cell precursors and $CD19^+$ splenic B cells consisting mostly of mature B cells, as indicated in Supplementary Figure 3. In addition, we assayed primary embryonic fibroblasts, mature macrophages and purified T cells (Supplementary Figure 3).

Figure 4A shows an MNase digestion experiment examining nuclease accessibility at the *c-fms* promoter. To control for equal digestion efficiency, we also examined the *GAPDH* promoter, which is active in every cell type. With fibroblasts we obtained a rather diffuse cleavage pattern except for one slightly stronger band upstream of the PU.1-binding sites at −130 bp just upstream of the main transcription start sites. Macrophages showed a different digestion pattern. Here we observed a strong MNase hypersensitive site (MNase HS) downstream of the main transcription start

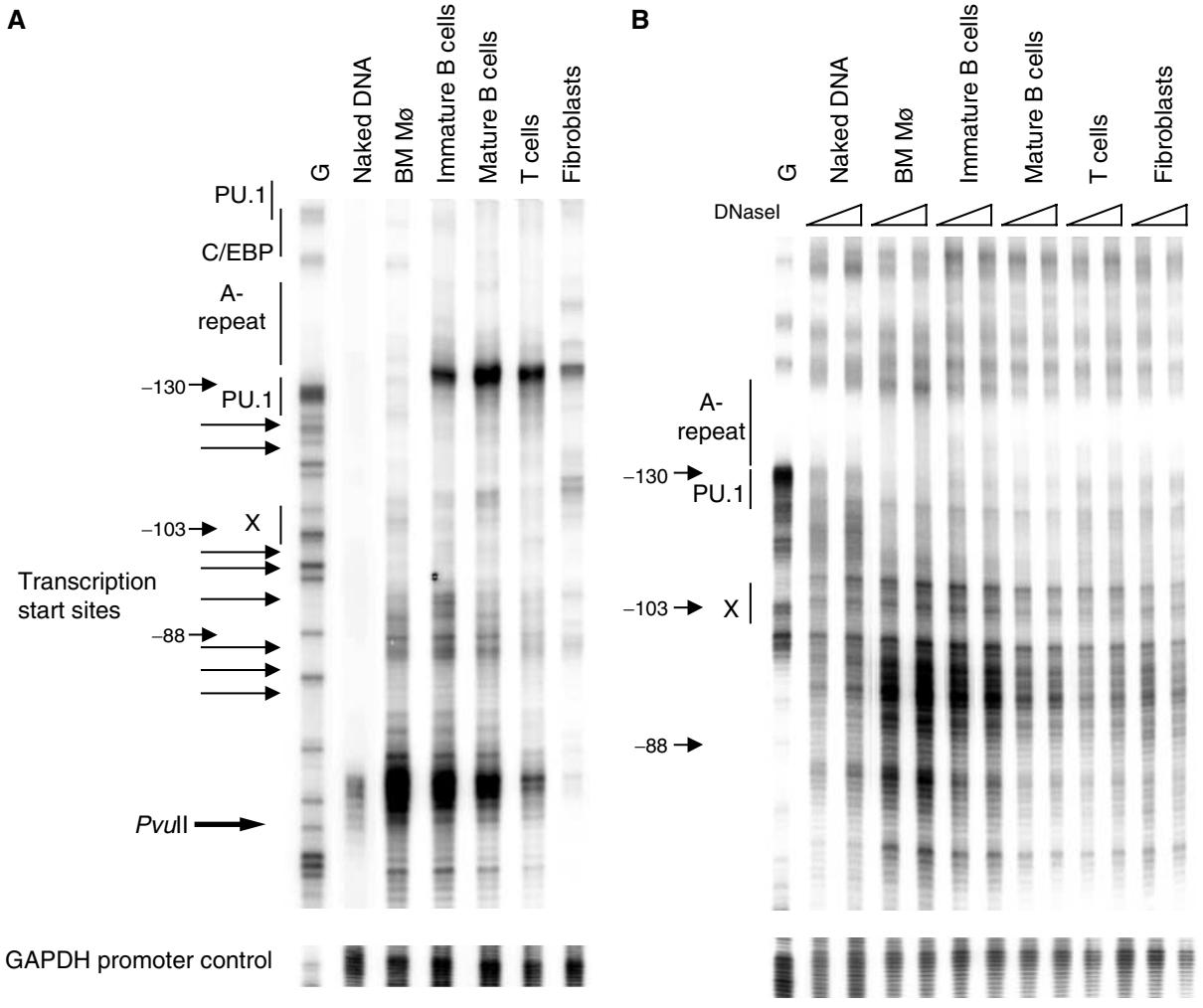

Figure 4 Chromatin at the *c-fms* promoter is in a partially active conformation in B cells. (**A**) From left to right: *In vivo* MNase footprinting experiment with naked DNA and chromatin prepared from the indicated cell populations using primers specific for the *c-fms* promoter (upper panel) or the *GAPDH* promoter (lower panel). Horizontal arrows indicate the position of transcription start sites. The *Pvu*II site is at position −66 bp. (**B**) *In vivo* DNaseI footprinting experiment with naked DNA and the indicated cell populations using increasing amounts of DNaseI (20 and 40 U) G: G-reaction of naked DNA. For further description, see Figure 3.

sites. Interestingly, we saw the same MNase HS in immature B cells, although the upstream band was also present. The same was true for mature B cells, although the intensity of the MNase HS was reduced compared to the upstream band. In contrast, the pattern in T cells resembled more that of fibroblasts, and the MNase HS had almost disappeared. However, as the naked DNA control showed some preferential digestion of the MNase HS sequence, we confirmed by restriction enzyme accessibility assay using *Pvu*II that this region was indeed differentially accessible to nuclease digestion in macrophage and fibroblast nuclei (Himes *et al*, 2001; data not shown).

In DNaseI hypersensitive site (DHS) mapping experiments, which assay double-strand cuts produced by DNaseI, we have previously shown that the *c-fms* promoter is strongly DNaseI hypersensitive in macrophages but not in fibroblasts (Himes *et al*, 2001). More importantly, no DHS was seen in an IL-7-dependent pro-B cell line (data not shown). Figure 4B shows the result of a high-resolution DNaseI digestion experiment with the *GAPDH* promoter as control. *c-fms* chromatin in macrophages was highly accessible across the entire promoter region, and a pattern was generated that was similar but not identical to that generated by naked DNA. DNaseI accessibility in immature B cells was only slightly reduced as compared to macrophages and was progressively reduced in mature B cells, T cells and fibroblasts.

Taken together, our experiments demonstrate that although transcription factors are no longer stably bound, the chromatin structure at the *c-fms* promoter in immature B cells is very similar to that of myeloid cells. Moreover, even in mature B cells, the region downstream of the main transcription start sites was still highly MNase accessible. No cell population exhibited a DNaseI hypersensitive region at the exact position of the MNase HS, which therefore most likely marks the position of linker DNA. A reasonable hypothesis is therefore that the *c-fms* promoter is occupied by a nucleosome that adopts alternative average positions in *c-fms*-expressing and nonexpressing cells. In B cells, this nucleosome maintains a position identical to that of myeloid cells and is only destabilized later in B cell development, along with gradually increasing chromatin compaction.

Histone modifications at the c-fms locus are altered in a complex fashion during B-lymphopoiesis

Chromatin activation and silencing are regulated by different enzymatic activities that modify the N-terminal tails of histones (Fischle *et al*, 2003). Histone acetylation is a hallmark of active chromatin, whereas the methylation of histone H3 lysine 9 (K9) is indicative of inactive chromatin. Moreover, transcription leaves a trail of histone modification (histone H3 lysine 4 (K4) trimethylation) behind, which has been interpreted as a 'memory' of recent transcriptional events and can be maintained through several cell generations (Ng *et al*, 2003). We examined the level of histone H3 modification at *c-fms* by chromatin immunoprecipitation (ChIP) assays using crosslinked chromatin digested with MNase (Figure 5). Each amplicon was represented at equal levels in the input DNA (data not shown). We have previously shown that the activation of *c-fms* expression in macrophages is accompanied by hyperacetylation of histone H3 at each regulatory region (Follows *et al*, 2003). These data were obtained with sonicated chromatin and could be reproduced by the experiment shown in Figure 5A, thus validating our experimental approach. The analysis includes examination of a second conserved region of the *c-fms* intron, which is annotated as FIRE-1kb. This sequence is also DNaseI hypersensitive in macrophages and has enhancer activity in transient transfection assays (Himes *et al*, 2001). An elevated level of H3K9 acetylation was only seen in macrophages and monocytes, whereas in all other cell types acetylation levels were low, but were still slightly elevated in immature B cells. H3K9 dimethylation levels across *c-fms* were high in fibroblasts, but lower in all other cell types (Figure 5B). However, the *c-fms* intronic region but not the promoter and downstream genomic regions showed increased H3K9 dimethylation levels in B-lineage cells as compared to myeloid cells. The result of the analysis of H3K4 trimethylation was surprising (Figure 5C). Here we saw a high signal at the promoter

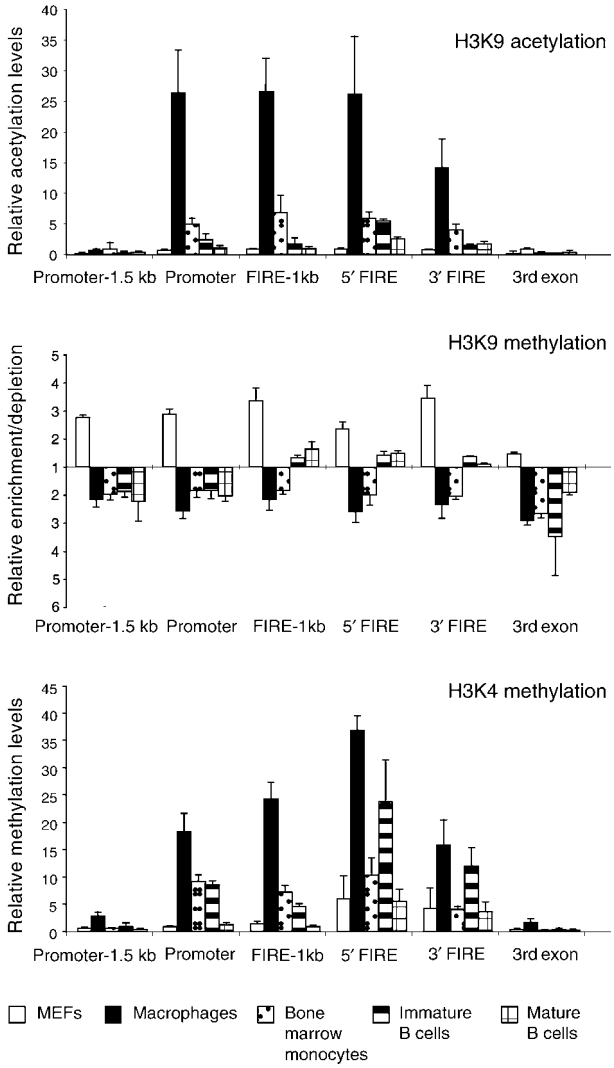

Figure 5 Alterations of histone H3 modifications inside and outside the *c-fms* regulatory region during B-lymphopoiesis. ChIP assays using antibodies specific for acetylated H3K9 (**A**), dimethylated H3K9 (**B**) and trimethylated H3K4 (**C**). The region-specific enrichment by ChIP was examined by real-time PCR using primers indicated in Figure 1. DNA enrichment was calculated as described in Materials and methods. Bars represent the mean ± s.d. of quantifications from two to four separate immunoprecipitations analysed in duplicate.

and downstream regulatory regions not only in macrophages and monocytes but also in B cell precursors. The H3K4 trimethylation level in the intronic region was as high as in macrophages.

CpGs at c-fms cis-regulatory regions are unmethylated in early progenitors and myeloid cells and are differentially methylated during B- and T-lymphocyte differentiation

Methylation of CpGs is a hallmark of silent chromatin (Bird, 2002). We therefore examined the DNA methylation status of selected CpG motifs centrally located within each of the *c-fms cis*-elements at the different stages of myeloid cell and B cell differentiation by using methylation-sensitive restriction enzymes. This analysis was performed with LSKs, CMPs, CLPs pro-B cells, sorted splenic B and thymic T cells as well as embryonic fibroblasts (Figure 6B and Supplementary Figure 4). The *c-fms* regions investigated were the promoter, FIRE-1kb, FIRE and a downstream region ((C); Figure 6A), which did not harbour a DHS and is not conserved between human and mouse. The promoter of the silent *α-fetoprotein* gene served as additional control. Both genomic regions were fully methylated in every cell type examined (Figure 6B).

The CpGs at all *c-fms cis*-elements analysed were highly methylated in embryonic fibroblasts and completely unmethylated in macrophages (Figure 6B). CpGs at the promoter (site 1) and FIRE (site 4) showed a similarly low methylation level in LSKs, CMPs and CLPs. However, the methylation level of CpGs at the intronic FIRE-1kb (sites 2 and 3) was elevated in CLPs and was further increased with progressing B cell differentiation. CpG methylation at the promoter and FIRE remained at low levels during B cell development. In contrast, CpG methylation levels at the promoter and FIRE were increased in T cells.

Regions of DNA methylation and increased histone H3K9 dimethylation overlap with a differentially regulated antisense transcription unit

As shown above, levels of H3K4 trimethylation were elevated throughout the intronic regulatory region in B cells. Two

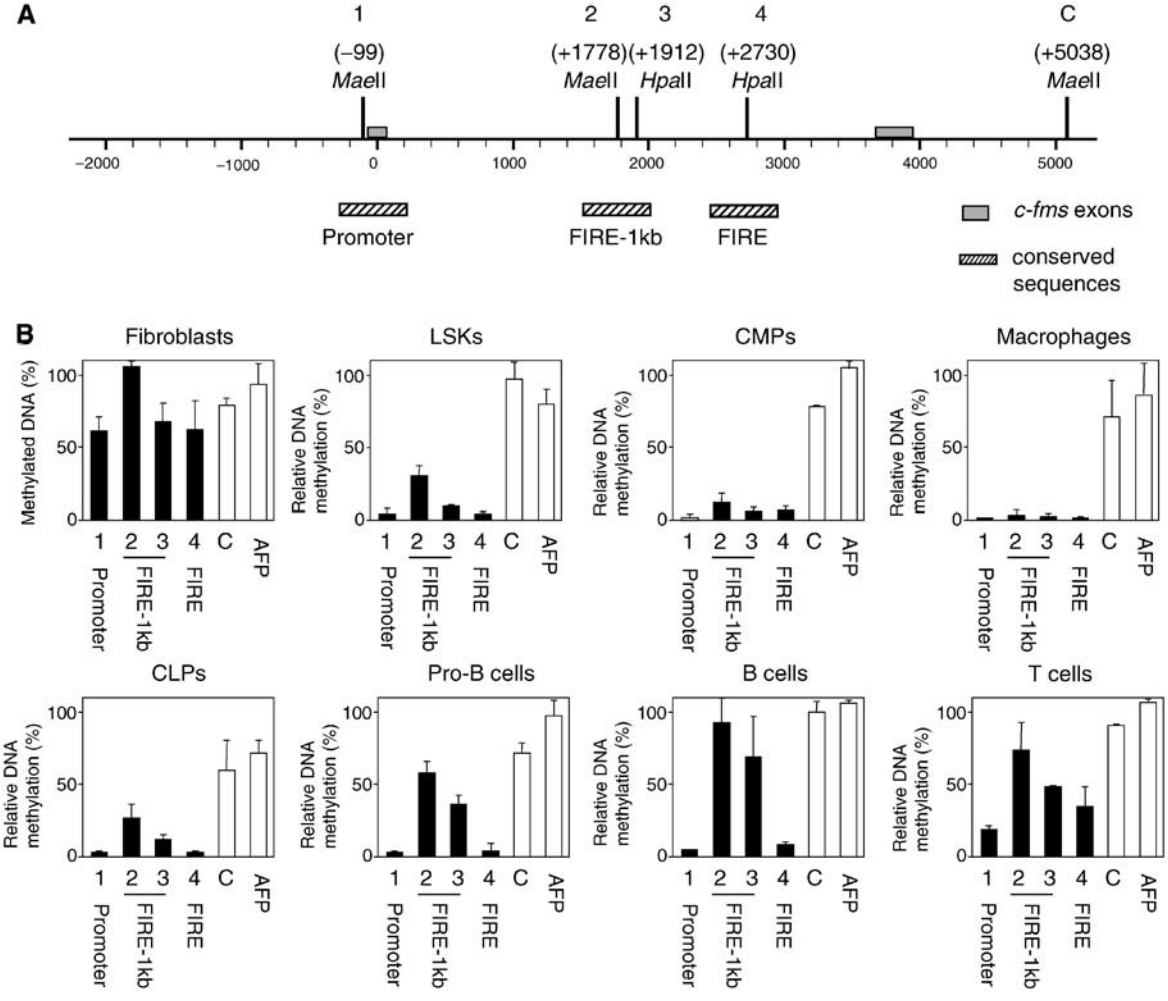

Figure 6 DNA methylation at specific *c-fms cis*-regulatory elements during haematopoietic differentiation. (**A**) Schematic representation of the position of the recognition sites of the differentially methylation-sensitive restriction enzymes *Mae*II and *Hpa*II in the *c-fms* promoter and first intron (1–4: regulatory regions; C: downstream control region). Relevant DNA sequences and the position of transcription factor-binding sites are depicted in Supplementary Figure 4. (**B**) DNA methylation status at specific *c-fms cis*-regulatory (black bars) and control regions (white bars) in the indicated cell types. After *Hpa*II or *Mae*II digestion of genomic DNA from each cell type, the amount of undigested DNA was measured by real-time PCR. The bars represent the mean value ± s.d. of two to four independent experiments analysed in duplicate. AFP: α-fetoprotein promoter.

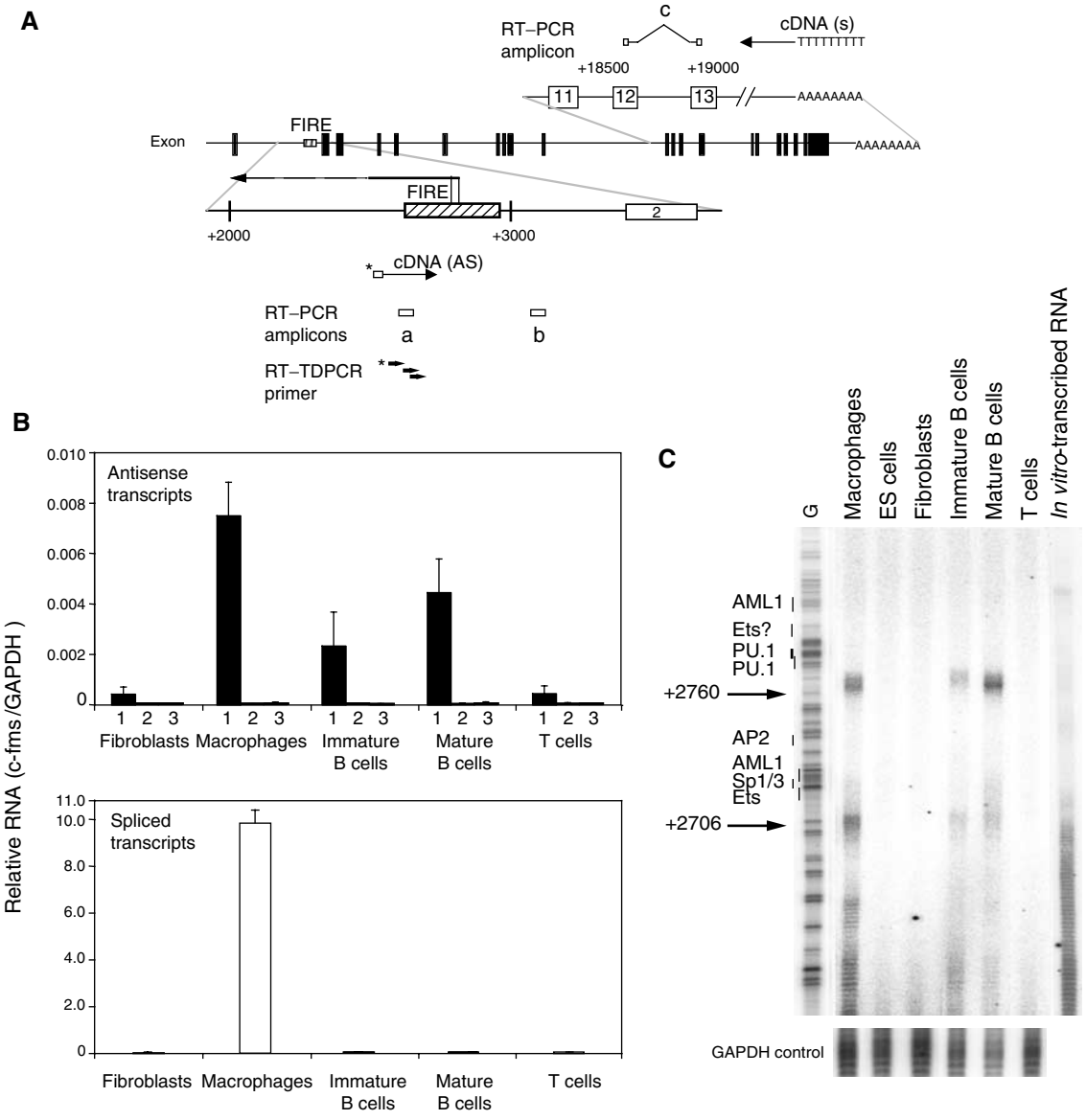

Figure 7 Antisense (AS) RNA starting at FIRE is expressed in macrophages and B cells. (**A**) Schematic representation of the position of primers used to carry out cDNA synthesis as well as real-time PCR amplicons. cDNA specific for AS RNA was synthesized from a biotinylated primer (indicated below the map), whereas an oligo (dT) primer was used to detect spliced transcripts (indicated above the map). Primer sets (a) (FIRE06) and (b) (3′-FIRE) were used to detect AS RNA transcribed from FIRE and from downstream of FIRE, respectively. Primer set (a) (*c-fms* QPCR), which is located between exons 12 and 13, was used to detect spliced sense transcripts. Black boxes in the map and numbered white boxes in the top and bottom map represent *c-fms* exons. Dashed boxes in the bottom row represent FIRE sequences. The numbers indicate the nucleotide position relative to the ATG start codon. The localization of the AS transcript is indicated as a horizontal arrow and the two AS transcription start sites mapped in (C) are indicated. (**B**) Expression of sense and AS RNA at the *c-fms* locus as assayed by real-time PCR. The top panel represents signals obtained with primer pair (a) measuring AS RNA originating from FIRE (1), signals from genomic DNA contamination (2) (assays with primer pair (a) but without reverse transcriptase) and signals obtained with primer pair (b) (3). The bottom panel represents spliced sense transcripts detected by primer pair (c). Bars represent mean value ± s.d. of two to four independent experiments analysed in duplicate. (**C**) Determination of the start site of the AS RNA within FIRE. RT–TDPCR was performed as described in Materials and methods using RNA prepared from the indicated cell types and from *in vitro*-synthesized RNA as a control for cDNA synthesis and amplification artefacts. An RT–TDPCR reaction examining the *GAPDH* gene was performed as internal control. A sequence reaction was run on the gel alongside the RT–TDPCR samples and the positions of transcription factor-binding sites within FIRE are indicated. This result was confirmed in two independently performed experiments.

explanations were possible for our findings. One was that H3K4 trimethylation was highly stable and was still present in B cells, although transcription had ceased. However, H3K4 trimethylation levels at some positions of the *c-fms* locus were almost as high in B cells as in macrophages. An alternative explanation was that the *c-fms* intronic region was subject to intragenic transcription. Small RNA molecules and in particular AS RNAs have been implied in gene silencing and the initiation of heterochromatin formation (Grewal and Moazed, 2003). To this end, we examined the different cell types for the presence of AS *c-fms* RNA using real-time RT–PCR analysis (Figure 7). To ensure that only AS RNA

molecules were detected, we used biotinylated primers for cDNA synthesis, bound the reaction products to magnetic beads and removed excess RNA and contaminating genomic DNA. To get a first idea of the position of a putative AS transcription unit and to quantify accurately RNA levels, we amplified specific regions by real-time PCR using different sets of primers. All cDNA synthesis reactions were further controlled by (i) including *GAPDH* primers to test for RNA quality, (ii) performing real-time PCR experiments on samples without reverse transcriptase (−RT) and (iii) verifying the correct fragment size by gel electrophoresis (data not shown). AS transcription was only found in macrophages and B cells, but not in fibroblasts and T cells. The level of AS RNA was low, but similar in macrophages and B cells, whereas significant levels of spliced sense transcripts were only detected in macrophages (Figure 7B). This indicates that AS transcription in B cells is genuine and does not result from contamination with myeloid cells. AS RNA was only found around FIRE. To confirm this finding, the start site of the AS transcript at FIRE was determined by reverse transcriptase-terminal transferase-dependent PCR (RT–TDPCR). This method uses terminal transferase tailing and linker ligation to amplify cDNA and to identify the start site of low abundant transcripts (Chen *et al*, 2000). Two major transcription start sites within FIRE at +2760 and +2706 were detected in macrophages and immature as well as mature B cells, but not in any other cell types tested (Figure 7C).

Epigenetic c-fms silencing is reversible throughout B-lymphopoiesis

Our observation of a poised chromatin structure and incomplete DNA methylation of *c-fms* in mature B cells raised the possibility that *c-fms* chromatin could be reprogrammed into the active state in mature B cells. Conditional deletion of the B cell commitment gene *Pax5* was previously shown to re-activate *c-fms* expression in pro-B cells (Mikkola *et al*, 2002). However, it is not known whether the loss of Pax5 at later stages of B cell development could also lead to derepression of the *c-fms* gene. To address this question, we have taken advantage of the *CD19-cre* line, which efficiently deletes a floxed (F) *Pax5* allele only in mature B cells (Horcher *et al*, 2001). Conditional *Pax5* inactivation in mature B cells of $Pax5^{F/-}$ *CD19-cre* mice leads to the loss of Pax5 function and concomitant upregulation of CD25 in contrast to the B cells of control $Pax5^{F/+}$*CD19-cre* mice (Horcher *et al*, 2001). We therefore sorted mature Pax5-deficient ($Pax5^{\Delta/-}$) B cells as $CD25^+IgM^+$ cells from the lymph nodes of $Pax5^{F/-}$*CD19-cre* mice after MACS depletion of non-B cells, whereas control $Pax5^{\Delta/+}$ B cells were isolated as IgM^+IgD^+ cells from $Pax5^{F/+}$*CD19-cre* mice (Supplementary Figure 5). RT–PCR analysis confirmed that the floxed *Pax5* allele was deleted in all sorted $Pax5^{\Delta/-}$ B cells, leading to the downregulation of the Pax5 target gene *CD19* (Figure 8A) as previously published (Horcher *et al*, 2001). Figure 8A clearly shows that the loss of Pax5 led to re-expression of the *c-fms* gene in mature $Pax5^{\Delta/-}$ B cells in contrast to the control $Pax5^{/+}$ B cells. Real-time PCR quantification furthermore revealed that the *c-fms* gene was re-activated to the same expression level seen in $Pax5^{-/-}$ bone marrow pro-B cells (Figure 8B). These results unequivocally demonstrate that *c-fms* silencing in B lymphocytes requires the continuous presence of Pax5 even at late stages of B cell differentiation.

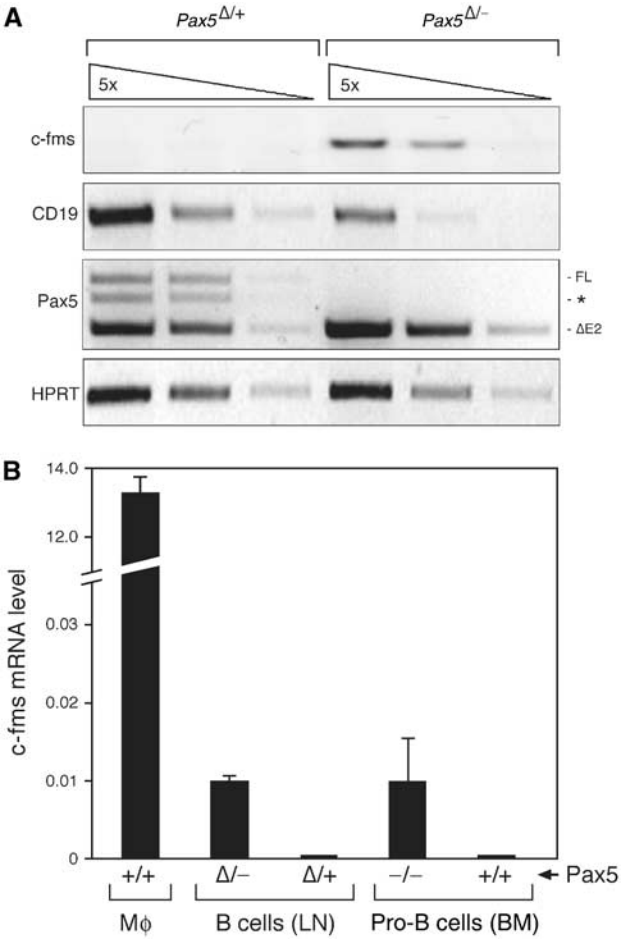

Figure 8 Epigenetic silencing of *c-fms* is reversible in mature B cells. (**A**) Mature control and Pax5-deficient B cells were isolated free of contaminating non-B cells from the lymph nodes (LN) of $Pax5^{F/+}$ *CD19-cre* mice (abbreviated as $Pax5^{\Delta/+}$) or $Pax5^{F/-}$*CD19-cre* ($Pax5^{\Delta/-}$) mice (Horcher *et al*, 2001) prior to RNA preparation and cDNA synthesis as described in Supplementary Figure 5 and Materials and methods. The cDNA of both cell types was normalized for equal expression of the *hypoxanthine phosphoribosyltransferase* (*HPRT*) gene followed by analysis of the indicated transcripts by semiquantitative RT–PCR of five-fold serial cDNA dilutions. *Pax5* transcripts were amplified from exons 1A to 5. ΔE2 denotes the truncated transcript of the $Pax5^{\Delta}$ allele lacking exon 2 in contrast to the full-length transcript (FL). A PCR artefact consisting of an FL/ΔE2 cDNA hybrid is indicated by an asterisk. All PCR fragments had the correct size, expect for the respective spliced mRNA. (**B**) Real-time PCR quantification of *c-fms* mRNA levels in macrophages (Mø), sorted lymph node (LN) B cells and *in vitro*-cultured bone marrow (BM) pro-B cells of the indicated *Pax5* genotypes. The *c-fms* mRNA level of the CMP was used as reference, which was arbitrarily assigned a value of 1.

Discussion

c-fms cis-regulatory elements are occupied in pluripotent and restricted haematopoietic precursor cells

In this paper, we present for the first time *in vivo* footprinting experiments with CLPs and with a cell population (LSKs) that was highly enriched for HSCs and short-term reconstituting stem cells. LSKs show basically the same degree of transcription factor occupancy at the *c-fms* promoter as CMPs, thus providing direct confirmation of the hypothesis that the chromatin of most, and not just a subset, of stem cells and

early progenitors is primed and accessible to transcription factor binding. Another important result of our study is that the *c-fms* gene is still expressed at low levels in CLPs. This is in apparent contrast to previous studies that have not detected *c-fms* mRNA expression in single CLPs that were purified by the same method (Miyamoto *et al*, 2002). This may be because the level of *c-fms* expression is below the detection limit of single-cell PCR. More convincingly, we observed that *c-fms* transcription factor-binding sites in CLPs were still occupied by transcription factors, although the signal was weaker than in CMPs and LSKs. We excluded the possibility that the signal originated from the small population of myeloid precursor cells in the sorted population or from a different level of transcription factors in these cells. Instead, we believe that transcription factor binding in CLPs is becoming destabilized, in contrast to cells which are functionally committed to the myeloid lineage. Support for the idea that transcription factor action can be dynamic comes from lineage tracing experiments in which mice carrying a Cre recombinase gene knocked into the myeloid-specific *lysozyme M* gene were crossed with ROSA26-EYFP reporter mice (Ye *et al*, 2003). These experiments revealed EYFP expression in non-myeloid cells, indicating that pluripotent cells giving rise to all myeloid and lymphoid cell types express the recombinase at a low level at some point in time. Transplantation experiments with sorted EYFP-negative cells from such mice showed restoration of EYFP activity, demonstrating that the expression of Cre recombinase under the control of the mouse *lysozyme M* gene is an infrequent but dynamic event.

Silent chromatin formation at the c-fms locus during B cell differentiation is a gradual process

Taken together, our results show that epigenetic silencing of the *c-fms* locus during lymphopoiesis is a slow process that begins at the CLP stage. *c-fms cis*-regulatory regions first lose transcription factor binding and DNaseI hypersensitivity. In parallel, mRNA expression from the *c-fms* promoter ceases and H3K9 hyperacetylation at the *c-fms* regulatory region is reduced. These processes are completed at the pro-B cell stage. However, at the same stage, not all *c-fms* chromatin features have reversed to an inactive state. In contrast to T cells, B cell precursors and even mature B cells showed certain chromatin features characteristic of an active locus. In immature B cells, DNaseI accessibility at the promoter was still high and levels of H3K4 trimethylation throughout the intronic regulatory region were elevated. In all B cell types studied, levels of H3K9 dimethylation were low compared to fibroblasts, and the region immediately downstream of the transcription start sites was still MNase hypersensitive. Our studies of DNA methylation confirmed this idea. Using a randomly integrated transgene, it was recently shown that DNA methylation is the last step in gene silencing and slowly increases during different cell generations (Mutskov and Felsenfeld, 2004). A similar observation was made with the mouse *Dntt* gene that is shut down during the maturation of immature thymocytes into mature T cells (Su *et al*, 2004). The situation at the *c-fms* locus appears to be more complex. *c-fms cis*-regulatory elements exhibited low DNA methylation levels in all precursor types and macrophages. However, although in T-lymphopoiesis DNA at all *c-fms* elements became significantly methylated, it was differentially methylated during B-lymphopoiesis. CpGs between FIRE and the promoter were progressively methylated, whereas CpGs at the cores of the *c-fms* promoter and FIRE stayed unmethylated throughout. Experiments from our laboratory indicate that CpGs located in the centre of critical transcription factor-binding sites but not in the flanking regions of myeloid-specific genes are the first to be demethylated during haematopoiesis (Tagoh *et al*, 2004). A possible role of transcription factors in protecting DNA from methylation has been reported and may be caused by an interference of these transcription factors with maintenance methylation after DNA synthesis (Mummaneni *et al*, 1998; Kress *et al*, 2001; Thomassin *et al*, 2001). Such transient interaction cannot be detected by DMS *in vivo* footprinting (Lefevre *et al*, 2003). Therefore, it is tempting to speculate that differential methylation of the *c-fms* promoter and FIRE in B cells and T cells may be caused by transcription factors (such as PU.1), which are expressed both in macrophages and B cells.

The c-fms locus contains a differentially regulated antisense transcription unit

Our experiments revealed the presence of an AS RNA within the coding region of the *c-fms* gene starting at the FIRE that is expressed at low levels in macrophages and B cells. AS RNAs have been shown to play a role in the regulation of X-inactivation and genomic imprinting (Lee *et al*, 1999; Sleutels *et al*, 2002). For the human α-globin locus, it was recently shown that the insertion of an AS promoter next to a CpG island leads to gene silencing and DNA methylation (Tufarelli *et al*, 2003). We do not yet know whether AS RNA expression is strictly required for *c-fms* silencing. However, our extensive analyses of *c-fms* chromatin and expression are fully consistent with a regulatory role of the AS transcription unit. We found that AS transcripts were upregulated by agonists (LPS, phorbol esters, CSF-1) that repress *c-fms* mRNA expression in macrophages (Himes and Hume, manuscript in preparation). Here, we show that the balance between sense and AS RNA expression in the *c-fms* intronic region is dynamically regulated in different cell types. AS RNA is expressed at similar levels in both macrophages and B cells, whereas sense RNA is only expressed in macrophages. The ratio of sense to AS expression therefore correlates with *c-fms* activity. This is in agreement with our previous observation that FIRE is a dynamic element, which differentially binds transcription factors in development and associates with histone acetylases as well as histone deacetylases (Tagoh *et al*, 2002; Follows *et al*, 2003). The data presented here show most convincingly that the AS transcription unit overlaps with a region of increased *de novo* DNA methylation as well as with areas of elevated H3K4 trimethylation and H3K9 dimethylation. This raises the interesting possibility that small double-stranded RNA molecules are formed that target gene silencing complexes to the *c-fms* intronic region, as shown in yeast and *Drosophila* (Schramke and Allshire, 2003; al-Bhadra *et al*, 2004; Verdel *et al*, 2004). The level of AS transcript was low as compared to the sense transcript. This is reminiscent of reports from intergenic transcripts within the β-globin locus that were found at similar low levels, as they were only expressed at a specific point within the cell cycle (Gribnau *et al*, 2000). AS transcription was absent in T cells and fibroblasts where *c-fms* chromatin was shut down. This could indicate a requirement for AS

transcription to initiate epigenetic silencing, but not for its maintenance.

c-fms silencing is reversible in B cells

Our chromatin structure studies exclude some possible mechanisms by which Pax5 represses *c-fms* in B cells. The presence of Pax5 in wild-type B-lineage cells does not cause remodelling of the promoter nucleosome into the inactive conformation, it does not induce locus-wide histone H3K9 hypermethylation and it does not induce competitive binding of Pax5 to *cis*-regulatory elements and other conserved regions of the *c-fms* gene (this study and unpublished observations). Our experiments point to a mechanism by which Pax5 shifts the balance between activating and repressing activities recruited to the *c-fms* locus, and it is likely that this balance needs to be continuously re-established during B-lymphopoiesis. In support of this idea, we show here that *c-fms* expression can be re-activated not only in pro-B cells (Mikkola *et al*, 2002) but also in mature B cells, indicating that the repression of *c-fms* requires the continuous presence of Pax5 throughout B cell differentiation. Our finding that the *c-fms* locus is not completely assembled into silent chromatin in mature B cells provides a molecular explanation for the *c-fms* re-activation in response to Pax5 loss and C/EBP overexpression (Xie *et al*, 2004). Importantly, the derepression of the *c-fms* gene in mature Pax5-deficient cells of the *Pax5F/-CD19-cre* genotype resulted in a similar level of *c-fms* mRNA expression as in $Pax5^{-/-}$ pro-B cells, strongly suggesting that re-activation events occur in the majority of the mature Pax5-deficient B cells.

Our data link the plasticity of the epigenetic state of the *c-fms* gene to a specific chromatin state that is depleted of activating histone marks (acetylated histones) and even contains partly methylated DNA, but is still in a poised conformation. Not only *c-fms* but also a number of other lineage-specific genes were activated upon the conditional inactivation of Pax5 in mature B cells (A Schebesta and M Busslinger, unpublished observations). It will be interesting to see whether these genes adopt similar intermediate epigenetic states in development.

Materials and methods

Purification and RT–PCR analysis of mature Pax5-deficient B cells

Lymph node cells from $Pax5^{F/+}$ *CD19-cre* or $Pax5^{F/-}$*CD19-cre* mice (Horcher *et al*, 2001) were stained with PE-anti-CD8 (53-6.7), CD4 (L3T4), DX5 (DX5), CD11c (HL3), Mac-1 (M1/70), Gr-1 (RB6-8C5), CD43 (S7) and Ter119 (TER119) antibodies, and non-B cells were eliminated by magnetic cell sorting with anti-PE MACS beads. Mature B cells of the $Pax5^{F/+}$ *CD19-cre* genotype were subsequently isolated by FACS sorting as IgM^+IgD^+ B cells, and Pax5-deficient mature B cells of the $Pax5^{F/-}$ *CD19-cre* genotype were sorted as IgM^+CD25^+ B cells (Horcher *et al*, 2001). Flow cytometric reanalysis indicated that the sorted cells were free of contaminating non-B cells (Supplementary Figure 5). RNA was prepared from the sorted cells and analysed by RT–PCR with previously described primers (Horcher *et al*, 2001) except for the *c-fms* primers.

Detection of antisense RNA

cDNA was synthesized from 2 μg of DNaseI-treated total RNA by using 400 U of M-MLV reverse transcriptase using biotinylated primers specific for *c-fms* (2 pmol) and *GAPDH* (0.2 pmol) in one reaction. Synthesized cDNA was immobilized on Dynabeads (Dynal, M-270). RNA and trace amounts of genomic DNA were removed by alkaline denaturation and serial washing (Chen *et al*, 2000). cDNA was eluted by heating the beads suspension in 0.1 × TE for 15 min at 99°C and was followed by real-time quantitative PCR. Relative activity was calculated using genomic DNA as a standard. Input and efficiency of cDNA synthesis was normalized against GAPDH signals. The amount of spliced transcripts was calculated using cDNA evaluated as equivalent amount to genomic DNA.

RT–TDPCR

RT–TDPCR reaction was performed as described previously (Chen *et al*, 2000). First strand cDNA was synthesized as described above. After the immobilization on Dynabeads, cDNA was tailed with three guanines and ligated to the linker, which contains three cytosines at the end. The ligated products were PCR-amplified using linker primer and nested gene-specific primer and visualized by primer extension reaction using a radiolabelled nested primer. *In vitro*-transcribed RNA was prepared from *c-fms* fragment (+849 to +3656) cloned into pBluescript™II KS+ using T7 RNA polymerase.

Further previously published methods can be found as Supplementary material. All primer sequences are described in Supplementary Table 1.

Supplementary data

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

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