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A Two-Step, PU.1-Dependent Mechanism for Developmentally Regulated Chromatin Remodeling and Transcription of the c-*fms* Gene▽

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Hematopoietic stem cells and multipotent progenitors exhibit low-level transcription and partial chromatin reorganization of myeloid cell-specific genes including the c-*fms* (*csf1R*) locus. Expression of the c-*fms* gene is dependent on the Ets family transcription factor PU.1 and is upregulated during myeloid differentiation, enabling committed macrophage precursors to respond to colony-stimulating factor 1. To analyze molecular mechanisms underlying the transcriptional priming and developmental upregulation of the c-*fms* gene, we have utilized myeloid progenitors lacking the transcription factor PU.1. PU.1 can bind to sites in both the c-*fms* promoter and the c-*fms* intronic regulatory element (FIRE enhancer). Unlike wild-type progenitors, the PU.1$^{-/-}$ cells are unable to express c-*fms* or initiate macrophage differentiation. When PU.1 was reexpressed in mutant progenitors, the chromatin structure of the c-*fms* promoter was rapidly reorganized. In contrast, assembly of transcription factors at FIRE, acquisition of active histone marks, and high levels of c-*fms* transcription occurred with significantly slower kinetics. We demonstrate that the reason for this differential activation was that PU.1 was required to promote induction and binding of a secondary transcription factor, Egr-2, which is important for FIRE enhancer activity. These data suggest that the c-*fms* promoter is maintained in a primed state by PU.1 in progenitor cells and that at FIRE PU.1 functions with another transcription factor to direct full activation of the c-*fms* locus in differentiated myeloid cells. The two-step mechanism of developmental gene activation that we describe here may be utilized to regulate gene activity in a variety of developmental pathways.

It is now well established that the chromatin of genes expressed in specific hematopoietic lineages is already partly reorganized towards an active state in hematopoietic stem cells (HSCs) and multipotent progenitors, and a number of such genes are expressed at a low level prior to lineage commitment (10, 12, 15, 18, 33). During progressive lineage restriction and cell fate specification this promiscuous gene expression program is then restricted by upregulation of lineage-appropriate genes and silencing of lineage-inappropriate genes (8, 24). These observations indicate that lineage-specific gene priming must occur at an early stage of HSC development. However, due to the low abundance of HSCs and multipotent progenitors little is known about the mechanistic details of how such priming events are achieved and how an active chromatin structure is established that supports high-level transcription later in development.

PU.1, a member of the Ets family of DNA-binding proteins, is a transcription factor that is critical for the development of myeloid lineages such as monocytes and granulocytes. Deletion of the PU.1 gene leads to defects in myelopoiesis, including loss of monocytes and macrophages (22, 28). Early myeloid progenitors are generated in PU.1-deficient mice, albeit at reduced numbers, but their differentiation is blocked (6). PU.1$^{-/-}$ myeloid progenitors fail to undergo macrophage differentiation and do not express the colony-stimulating factor 1 (CSF-1) receptor gene (c-*fms*), one of the most important genes regulating macrophage survival and proliferation. This gene is absolutely required for macrophage development (4). However, rescue of PU.1$^{-/-}$ myeloid progenitor cells with a c-*fms* expression vector restores macrophage progenitor growth and proliferation but not macrophage differentiation, indicating that PU.1 regulates a larger program of macrophage gene expression (6).

c-*fms* belongs to a class of myeloid genes which are already expressed at a low level in HSCs (24, 34). Tissue-specific expression of c-*fms* mRNA is regulated by well-defined promoter and intronic enhancer elements (Fig. 1). The promoter used in macrophages is a TATA-less promoter, with multiple purine-rich elements bound by Ets family transcription factors (26). Tissue-restricted high-level expression of the c-*fms* gene is dependent upon the c-*fms* intron regulatory element termed FIRE, within the first intron (11, 27). Both the promoter and FIRE are bound by PU.1 in macrophages (5, 6, 13, 36). We previously showed by in vivo footprinting that the c-*fms* locus is already partly occupied by transcription factors in HSCs and becomes fully occupied in committed myeloid progenitor cells

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(34). In contrast, cell surface expression of CSF-1 receptor protein and high levels of mRNA are readily detected only in committed macrophage precursors (31) and their progeny. An initial mechanistic explanation of why this was the case was provided by in vivo footprinting studies demonstrating that the increase in c-*fms* mRNA expression during macrophage differentiation correlates with a dynamic assembly and disassembly of transcription factor complexes on the FIRE enhancer (31). However, the molecular details of this dynamic behavior are unknown because the identities of the specific factors and cofactors recruited were not determined in the previous study. It is also not known whether other transcription factors can bind to c-*fms* in the absence of PU.1 and to what extent chromatin of c-*fms* is reorganized in PU.1$^{-/-}$ cells.

To address the above questions, we examined the chromatin fine structure of c-*fms* by performing in vivo footprinting experiments and chromatin immunoprecipitation (ChIP) assays. For a model system we employed a myeloid progenitor cell line derived from PU.1-deficient mice, which cannot differentiate into macrophages but can proliferate in the presence of interleukin-3. In contrast to wild-type myeloid progenitor cells, the c-*fms* locus was not occupied by any transcription factors in the PU.1$^{-/-}$ cells. To further study the role of PU.1 in the regulation of the c-*fms* locus, we employed a well-established derivative of the PU.1$^{-/-}$ cell line (PUER) that expresses an inducible form of PU.1 (36). Significantly, induction of PU.1 in PUER cells that resulted in restoration of macrophage differentiation led to in vivo transcription factor occupancy at the c-*fms* locus. The promoter was very rapidly occupied by transcription factors, whereas it took significantly longer for the same transcription factors to assemble at FIRE and for elevated levels of c-*fms* mRNA to be expressed. This delayed kinetics could be explained by our finding that formation of an active enhancer complex at FIRE required the induction of at least one secondary transcription factor, Egr-2, by PU.1. These observations suggest a two-step mechanism of c-*fms* activation which involves the promoter being active in early progenitor cells, thereby enabling low-level c-*fms* mRNA expression, whereas activation of FIRE occurs at a later developmental time during the course of macrophage differentiation. We suggest that this mechanism ensures that high levels of c-*fms* mRNA and CSF-1 receptor protein are expressed only in cells destined to be CSF-1 responsive.

MATERIALS AND METHODS

Cell culture. Generation of PU.1$^{-/-}$ and PUER cells has been described previously (36). Cells were cultured in phenol red-free Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, 50 μM β-mercaptoethanol, 100 units/ml penicillin, 100 units/ml streptomycin, and 5 ng/ml recombinant mouse interleukin-3 (Biosource). For 4-hydroxytamoxifen (OHT) treatment, cells were plated at 0.2×10^6 to 0.3×10^6 cells/ml in complete medium supplemented with 100 nM OHT (Sigma) and harvested at the indicated time points. RAW 264 and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin.

ChIP assays and real-time PCR analysis. The ChIP assay was performed essentially as described previously (21). If not stated otherwise, antibodies were purchased from Santa Cruz. Immunoprecipitation was performed overnight at 4°C on a rotating wheel with 5 μl of normal rabbit immunoglobulin G (IgG; Upstate Biotechnology) or anti-Krox-20/Egr-2 serum (Covance PRB-236P) or 5 μg of anti-PU.1 (sc-352X), anti-C/EBPβ (sc-150X), anti-RNA polymerase II (Pol II) (sc-900X), anti-Brg1 (sc-10768X), anti-TATA binding protein (anti-TBP) (sc-273), anti-trimethyllysine-4-histone H3 (Abcam 8580), and anti-acetyl histone H3 (Lys9) (Abcam 4441-50). The amount of precipitated DNA was measured by real-time quantitative PCR with an ABI Prism 7700 or 7900HT sequence detection system (Perkin-Elmer Life Sciences) using SYBR green as described in reference 20. Amounts of DNA precipitated were calculated using a standard curve obtained from amplification of serially diluted mouse genomic DNA. Signals observed with the specific antibody were divided by the signals obtained from the IgG control (nonspecific background). To correct for the efficiency of immunoprecipitation in different experiments, this relative PCR signal was then normalized to the signal from the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter primer set. Primers were designed using Primer Express 1.5 software, and their sequences were published in reference 34. Primers for FIRE (HpaII, 4) were used to amplify the FIRE region in transcription factor ChIP assays.

mRNA expression analysis. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. Contaminating genomic DNA was removed by treatment with DNase I. Two micrograms of total RNA was used in first-strand cDNA synthesis using an oligo(dT) 15-mer primer and Moloney murine leukemia virus reverse transcriptase. Real-time quantitative PCR was performed on an ABI Prism 7700 or 7900HT sequence detection system (Perkin-Elmer Life Sciences) using SYBR green. Relative expression was calculated as a ratio of the gene of interest to GAPDH. Primers were designed using Primer Express 1.5 software. Primer sequences for c-*fms* and GAPDH were described in reference 34. The remaining primer sequences were as follows: Egr-2 forward, GTG CCA GCT GCT ATC CAG AAG, and Egr-2 reverse, GGC TGT GGT TGA AGC TGG AG.

Flow cytometry. Cell surface expression of CSF-1 receptor was detected by staining with biotinylated monoclonal anti-mouse CD115 antibody (clone AFS98; eBioscience) followed by streptavidin *R*-phycoerythrin–Cy5 (Serotec). Flow cytometric analysis was performed on an Epics flow cytometer (Beckman Coulter).

Plasmid construction and reporter gene assays. Plasmids used in this study were described previously (32). The Egr-2 binding site in FIRE (see Fig. 4) was mutated using standard PCR techniques.

Transient transfections in RAW 264 cells were performed exactly as described previously (32). Cells were transfected with 0.13 pmol of reporter plasmid (pGL2 basic, pGL2 [simian virus 40 {SV40}] promoter, pGL2 SV40 promoter/FIRE), 7.7 fmol of effector plasmid (pCB6Egr-2), empty pCB6, or pBluescript (Stratagene) and 0.46 fmol of cytomegalovirus-driven *Renilla* plasmid. pCB6Egr-2 was a gift of J. Svaren, University of Wisconsin.

Electrophoretic mobility shift assay (EMSA) and in vivo footprinting analysis. The preparation of nuclear extracts was adapted from reference 7. Briefly, cells (approximately 10^8) were spun down and resuspended in sucrose buffer (0.32 M sucrose, 50 mM KCl, 20 mM NaCl, 3 mM $CaCl_2$, 2 mM magnesium acetate, 10 mM Tris [pH 8.0], 0.15 mM spermine, 0.5 mM spermidine, 10 mM NaF, 1 mM dithiothreitol [DTT], 0.5 μM phenylmethylsulfonyl fluoride [PMSF], and 0.1% protease inhibitor cocktail [Sigma]). An equal volume of sucrose buffer containing 0.2% NP-40 was added to lyse the cell membrane, and nuclei were pelleted by centrifugation. Nuclei were resuspended in sucrose buffer without NP-40 and repelleted. Pelleted nuclei were resuspended in low-salt buffer (10 mM HEPES, pH 7.9, 20% glycerol, 2 mM $MgCl_2$, 20 mM KCl, 10 mM NaF, 1 mM sodium pyrophosphate, 2 mM EGTA, 1 mM DTT, 0.5 μM PMSF, and 0.1% protease inhibitor cocktail). Nuclei were lysed by slowly adding an equal volume of high-salt buffer (low-salt buffer supplemented with 0.7 M KCl and 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS]) and incubating the mixture at 4°C for 20 min. Debris was removed by centrifugation, and the protein solution was dialyzed in a large volume of 20% glycerol, 20 mM HEPES, pH 7.9, 100 mM KCl, 2 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 0.5 mM PMSF, and 1 mM DTT.

Binding assays were performed as described previously (2). To detect the binding of protein to FIRE sequence-specific probes, 20 fmol of probe was incubated with nuclear extract in 18 μl binding buffer (15 mM HEPES, pH 7.9, 50 mM KCl, 50 mM NaCl, 10 μM $ZnCl_2$, 10% glycerol, 1 mM EDTA, 1 mM DTT) in the presence of 2 μg poly(dI-dC) for 30 min on ice. When a supershift assay was performed, 5 μl EGR-2 serum (Covance; PRB-236P) or 5 μg control IgG was added to the nuclear proteins before the probe was added. Protein-DNA complexes were resolved on a 4% polyacrylamide gel and exposed to a PhosphorImager screen.

Probes were as follows: FIRE Egr-2, ATGTGTTTCCGCCCACACAGGC; Egr consensus, TTTGCGGGGGCGTCTCTT; Sp1 consensus, TTTTGAGGG GCGGGGCTT; and Oct1, GATCCTAATTTGCATGATC.

DNase I treatment and ligation-mediated PCR were performed exactly as described previously (20). Primer sequences for the c-*fms* promoter and FIRE

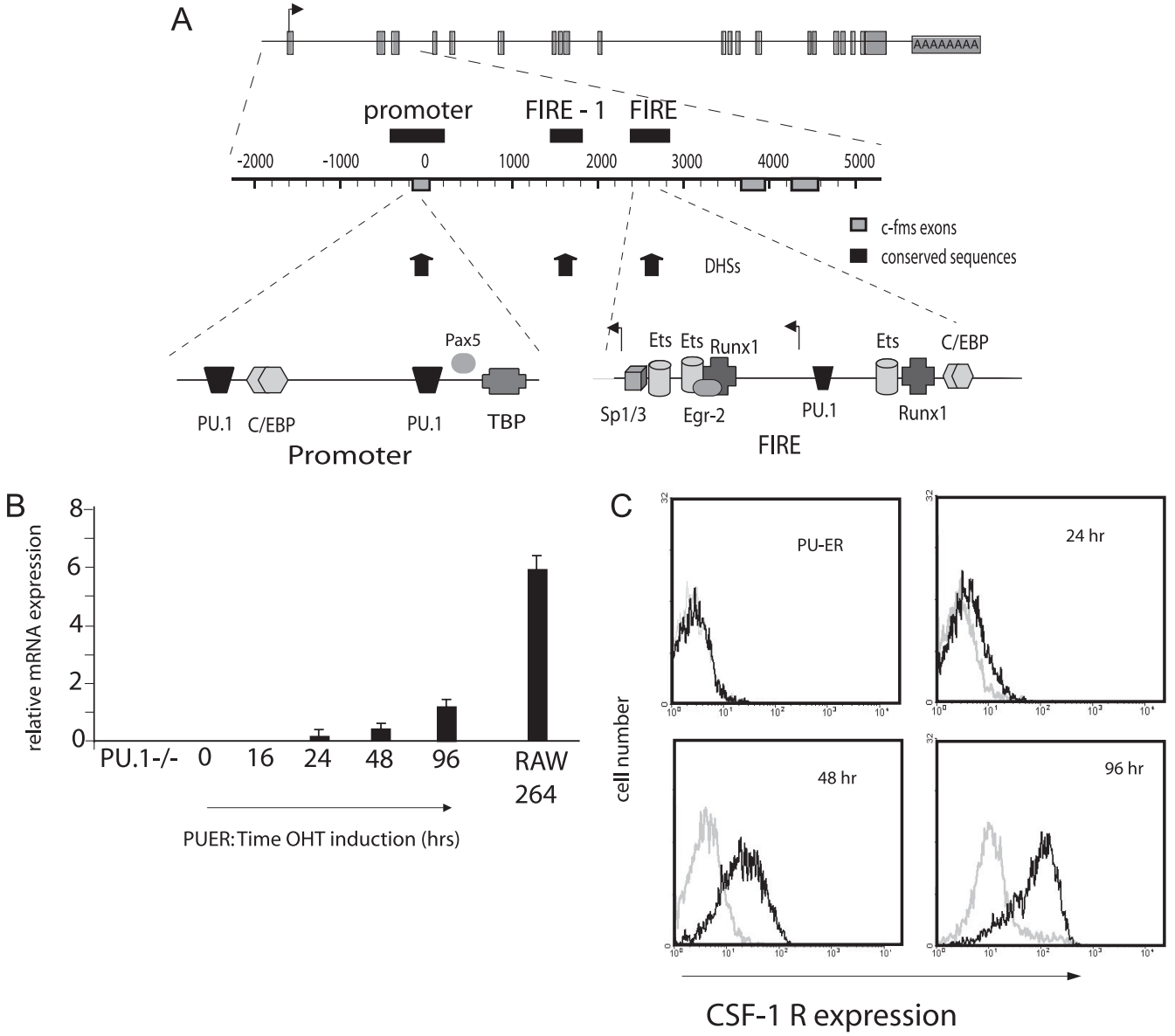

FIG. 1. Map of the mouse c-*fms* locus and induction of c-*fms* expression on addition of OHT. (A) Chromatin structure of mouse c-*fms* locus regulatory regions around the proximal promoter with indications of the transcription factor binding sites, the localization of DNase I-hypersensitive sites (DHSs; large black arrows), the transcription start site (small black arrow in top diagram), and the antisense RNA transcription start sites (small black arrows in FIRE in lower diagram). (B) Expression of c-*fms* mRNA in PU.1$^{-/-}$ parental cell line, OHT-inducible PUER cells, and control RAW 264 macrophage cell line. Following the addition of 100 nM OHT to the growth medium PUER+OHT cells start to transcribe detectable levels of c-*fms* mRNA after 24 h. Results are expressed relative to GAPDH expression. Results shown are representative of three independent induction experiments. (C) The expression of CSF-1 receptor protein on cell surface after treatment of PUER cells with OHT was determined by flow cytometry. Controls are indicated as light gray histograms.

were described previously (31, 32, 34). PCR products were labeled by primer extension using γ-^{32}P-labeled nested primers and were analyzed on 6% denaturing polyacrylamide gels.

RESULTS

Transcription factor assembly at the c-*fms* locus requires PU.1 and occurs with different kinetics at the c-*fms* promoter and FIRE. Previous studies had shown that both the c-*fms* promoter and FIRE contain binding sites for PU.1 (Fig. 1A). Based on in vivo footprinting experiments we have previously reported the presence of two PU.1 sites in FIRE (31). We have revisited this issue using EMSA and found that the downstream site is bound by another factor forming a complex that is not competed by a PU.1 consensus sequence (data not shown). FIRE thus contains only one functional PU.1 site. As expected, no c-*fms* mRNA was expressed in PU.1$^{-/-}$ cells (Fig. 1B) and in PU.1$^{-/-}$ cells carrying a PU.1-estrogen receptor fusion protein (PUER) in the absence of OHT. Induction of PU.1 in PUER cells (PUER+OHT) led to induction of c-*fms* mRNA expression, as previously observed (3, 36). The same

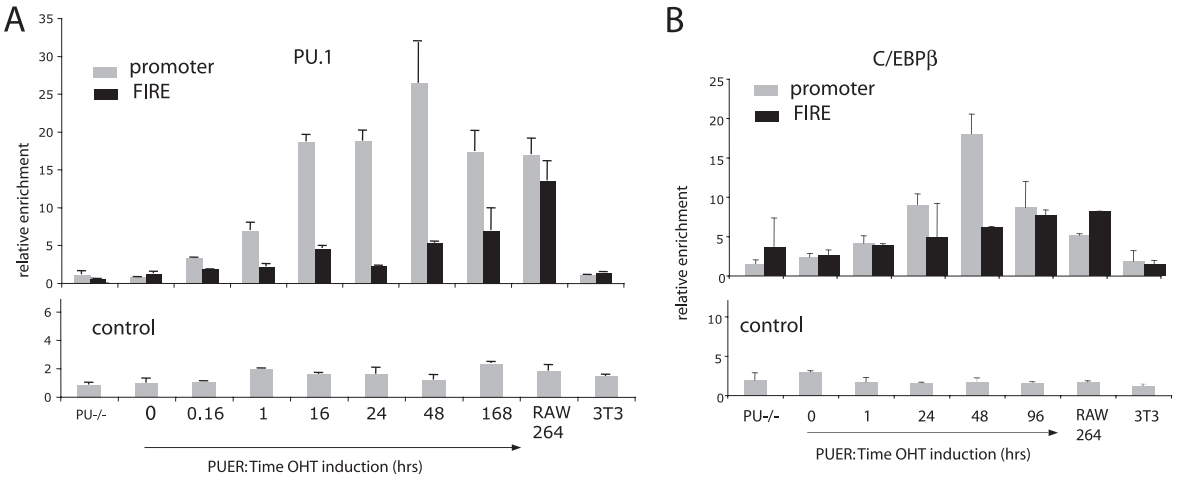

FIG. 2. Transcription factor binding to the mouse c-*fms* promoter and FIRE in the PUER cells is seen only after the induction of PU.1. (A) Time course of binding of PU.1 to the c-*fms* promoter and FIRE in PUER+OHT cells, showing rapid association of PU.1 with the promoter and slower kinetics at the FIRE (ChIP assay). (B) Time course of binding of C/EBPβ follows the pattern observed with PU.1 (ChIP assay). ChIP results are normalized to the GAPDH control amplicon and represent averages of two independent experiments analyzed in triplicate. An amplicon located in exon 3 was used as a control region.

previous studies also showed that in this cell line macrophage differentiation and the onset of a macrophage-specific gene expression program are strictly dependent on the presence of the PU.1 DNA-binding and transactivation domains in the PUER fusion protein (36). A low level of c-*fms* mRNA expression was detected after 24 h of OHT induction, with levels increasing during the 4-day incubation period. High-level surface expression of CSF-1 receptor protein on PUER+OHT cells was detected only after 48 h of induction, as measured by flow cytometry (Fig. 1C), thus confirming that the in vitro system recapitulates the events seen in primary cells (31).

We were interested to see whether c-*fms* regulatory elements were stably occupied by transcription factors already expressed in PU.1$^{-/-}$ cells in the absence of PU.1. Our previous experiments demonstrated that c-*fms* is also bound by C/EBPα and -β, which both bind to the promoter and FIRE (31) (data not shown) and are expressed in PU.1-deficient progenitor cells (reference 19 and data not shown). We therefore measured the kinetics of transcription factor assembly at the c-*fms* promoter and FIRE by chromatin immunoprecipitation assays using antibodies to PU.1 and C/EBPβ. Upon OHT treatment of the PUER cells, we detected PU.1 and C/EBPβ association with both the promoter and FIRE, but we observed an interesting difference in the kinetics of binding to these two elements. Already after 10 min of OHT induction, some PU.1 was associated with the promoter and association was complete after 16 h (Fig. 2A). This was confirmed also by dimethyl sulfate footprinting experiments demonstrating that complete PU.1 association was seen after 6 h (data not shown). In contrast, PU.1 association with FIRE occurred with a much slower kinetics (Fig. 2A). This differential association kinetics was also observed for C/EBPβ (Fig. 2B). Importantly C/EBPβ binding to the promoter and enhancer was dependent upon PU.1. Thus, PU.1 differentially associates with the c-*fms* gene during the course of macrophage differentiation and facilitates the binding of other transcription factors.

FIRE activity requires the induction of Egr-2 by PU.1. We next addressed the molecular basis of the differential binding of transcription factors to the c-*fms* promoter and FIRE. Aside from C/EBPβ, a number of other transcription factors involved in the regulation of c-*fms* expression, such as c-Jun and Runx1, are already expressed in multipotent myeloid progenitor cells and are also expressed in the absence of PU.1 (reference 19 and data not shown). The transcription factor Egr-2 is encoded by an early growth factor response gene that is upregulated during macrophage differentiation (16, 17). One of our laboratories has recently identified Egr-2 (Krox-20) as a gene strongly induced by PU.1 after OHT induction of PUER cells (19). In this study it was shown that RNA interference-mediated knockdown of Egr-2 in the PUER system led to an impediment of macrophage differentiation and an impaired induction of c-*fms* expression, without affecting the expression of c-Jun, which is important for PU.1-mediated c-*fms* activity in transient-transfection assays (1). These experiments indicated that Egr-2 could play an important role in c-*fms* regulation. Intriguingly, FIRE contains two putative binding sites for Egr-2, which overlap with Sp1 binding sites. We were therefore interested to see whether (i) c-*fms* was a direct target of Egr-2, (ii) the late onset of FIRE activation correlated with Egr-2 induction, and (iii) the Egr-2 binding site is required for FIRE activity. Little or no Egr-2 mRNA can be detected in noninduced PUER cells, and it took 12 to 24 h to be fully induced (Fig. 3A and data not shown); however, once induced, Egr-2 rapidly associated with FIRE (Fig. 3B). To identify which one of the two sites was a functional Egr-2 binding site, we performed EMSAs with nuclear extracts from PUER and PUER+OHT cells (Fig. 4A). Only the site downstream of +2717, which partially overlaps with the binding site for an as-yet-unknown Ets factor, could bind Egr-2 (Fig. 4B and data not shown). Consistent with the ChIP assay, no Egr-2 binding activity could be detected in PUER cells, whereas strong specific binding was seen in extracts prepared from PUER+OHT

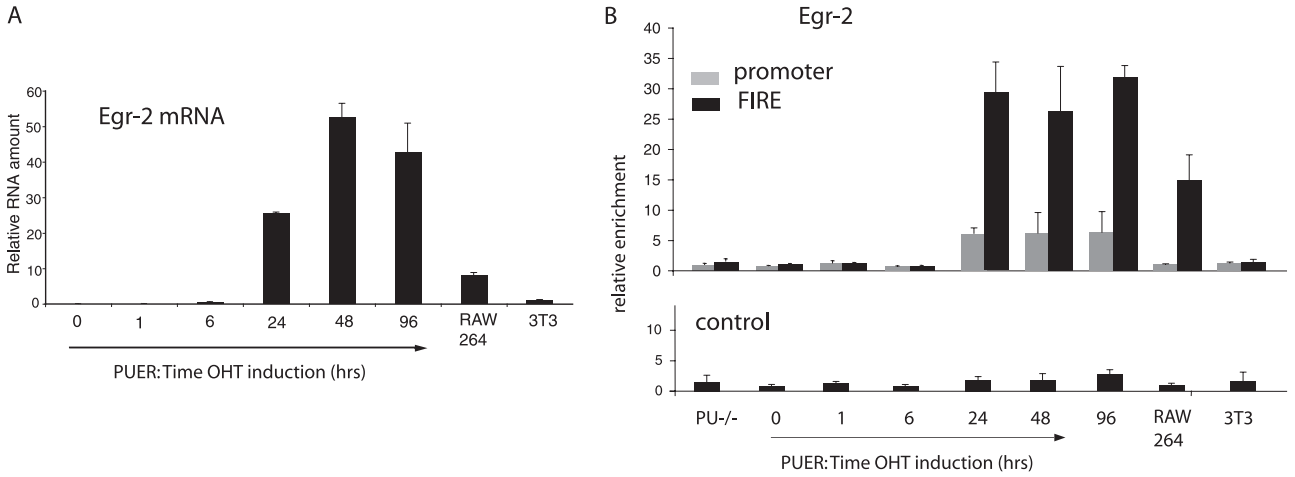

FIG. 3. Egr-2 is induced by PU.1 and binds to FIRE. (A) Time course of induction of Egr-2 mRNA as measured by real-time PCR analysis. (B) ChIP assay demonstrating the binding of Egr-2 to FIRE immediately after the induction of Egr-2 expression. ChIP results are normalized to the GAPDH control amplicon and represent averages of two (A) and three (B) independent experiments analyzed in triplicate. An amplicon located in exon 3 was used as a control region.

cells (Fig. 4A). An EMSA assaying the ubiquitously expressed transcription factor Oct1 was used as a control and demonstrated that extracts from PUER cells were of good quality (data not shown). We next transfected luciferase constructs in which FIRE was linked to the SV40 promoter into RAW 264 cells (Fig. 4C). FIRE stimulated promoter activity threefold over that of the promoter alone. Furthermore, FIRE activity was significantly enhanced by cotransfecting an Egr-2 expres-

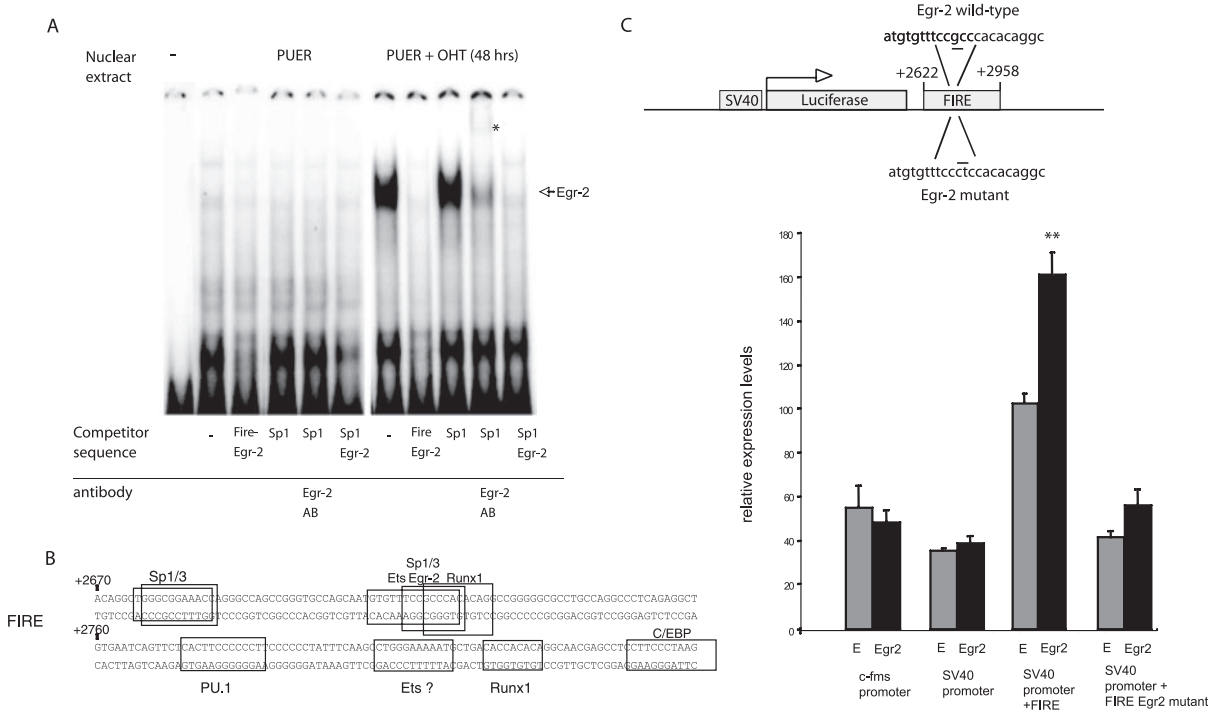

FIG. 4. Egr-2 binds to FIRE and is required for FIRE enhancer activity. (A) EMSA with nuclear extracts from PUER and PUER+OHT cells and a 100-fold excess of unlabeled competitor oligonucleotides (FIRE Egr-2, Egr-2 consensus, and Sp1 consensus oligonucleotides), demonstrating that (i) no specific complex is formed with the FIRE probe in PUER cells in contrast to PUER+OHT cells and (ii) the major binding activity on FIRE in PUER+OHT cells is for Egr-2 and not Sp1. Note a supershifted band marked by an asterisk; the supershift occurred when the reaction mixture was incubated with an Egr-2 antibody. (B) Sequence of FIRE with indicated transcription factor binding sites. (C) The upper panel depicts the basic outline of the wild-type and mutated constructs carrying FIRE inserted in sense orientation downstream of the luciferase gene. The indicated luciferase constructs plus a pGL2 basic luciferase vector were transfected into RAW 264 cells with expression vectors with (EGR-2) or without (E) Egr-2 cDNA. Expression levels were normalized to pGL2. The experiment represents the mean values of eight independent transfections. The asterisks indicate a statistically significant enhancement of FIRE activity by EGR-2.

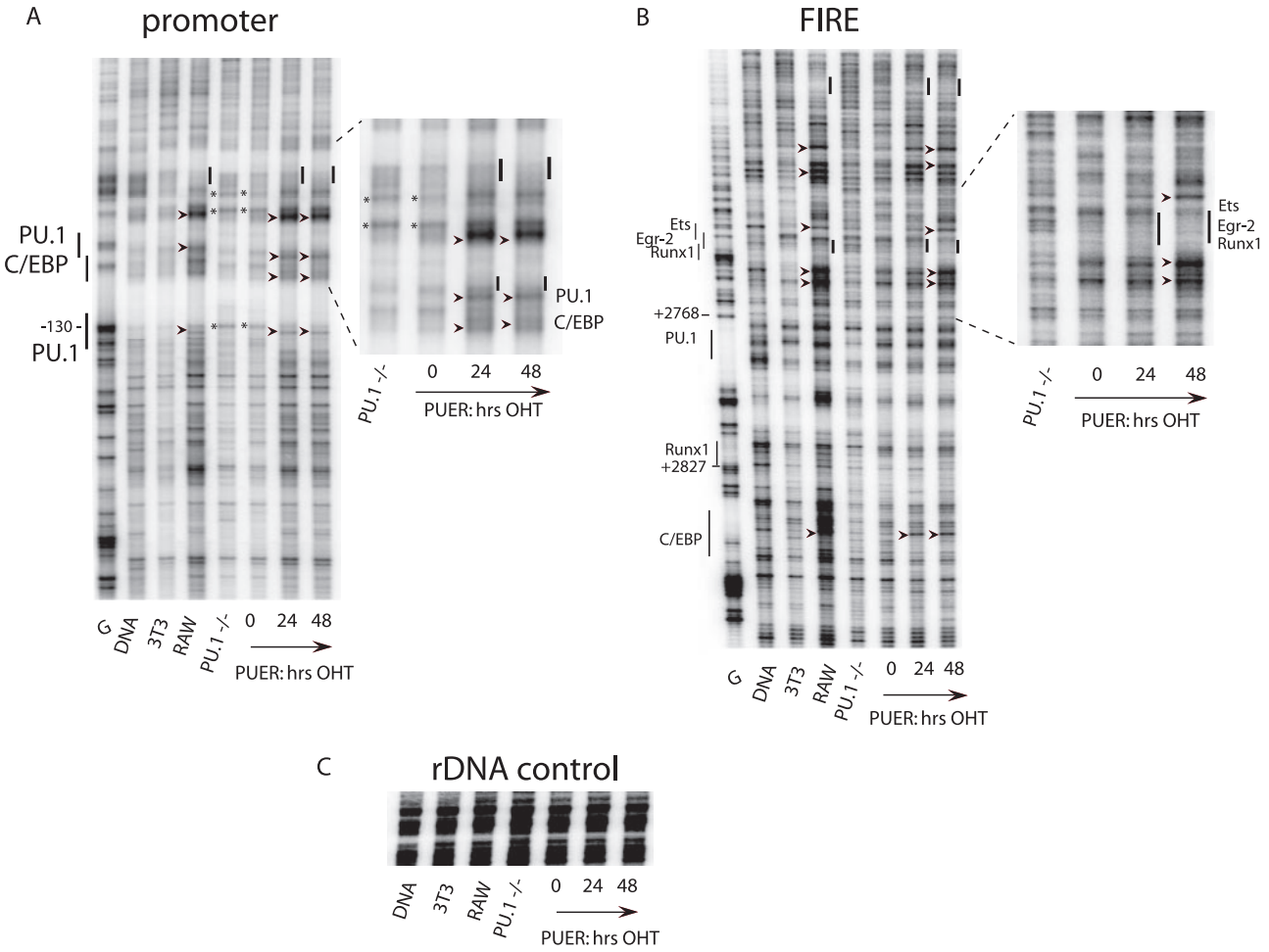

FIG. 5. DNase I in vivo footprinting experiment demonstrating a gradual reorganization of c-*fms* chromatin with increasing levels of PU.1. The indicated cells were treated with DNase I in situ, and DNA lesions were visualized by ligation-mediated PCR as described in Materials and Methods. Selected regions of DNase I hyperaccessibility at the c-*fms* promoter (upper strand) (A) and FIRE (upper strand) (B) are indicated by arrowheads; selected regions showing protection from DNase I digestion as a result of transcription factor binding are indicated by black bars. Cleavage sites seen only in PU.1$^{-/-}$ and PUER cells are marked with asterisks. Only changes that were seen in more than one experiment were considered. Selected regions of the gel are enlarged at the right of each panel. (C) Ribosomal DNA (rDNA) control demonstrating equal DNase I digestion in all samples.

sion construct ($P < 0.008$). More importantly, a mutation of the Egr-2 binding site that completely abolished Egr-2 binding in EMSAs with nuclear extracts from PUER+OHT cells (data not shown) also abolished FIRE enhancer activity. The c-*fms* promoter did not respond to Egr-2 overexpression. Taken together, these data suggest that Egr-2 is important for FIRE activity and that the delayed assembly of transcription factors on FIRE is likely to be due to the necessity of inducing Egr-2 by PU.1.

Chromatin remodeling at FIRE parallels Egr-2 induction. The rapid recruitment of the PUER fusion protein to the promoter suggested that c-*fms* chromatin was highly accessible. To further investigate the chromatin structure of the c-*fms* promoter and FIRE and the effect of PU.1 binding on chromatin remodeling, we performed a DNase I in vivo footprinting experiment (Fig. 5A and B). This is a powerful method to examine transcription factor binding as well as chromatin fine structure and accessibility. We examined the chromatin fine structure of c-*fms cis* elements in PU.1$^{-/-}$ cells, PUER cells, PUER cells during OHT induction, and control cells (NIH 3T3 fibroblasts and RAW 264 macrophages). To compare equal extents of overall digestion, reactions were controlled by amplifying the same material with primers specific for the ribosomal DNA locus (Fig. 5C). Interestingly, in PU.1$^{-/-}$ cells and uninduced PUER cells we observed regions of enhanced DNase I cleavage at the c-*fms* promoter that differed from NIH 3T3 cells and naked DNA (indicated by asterisks in Fig. 5A). A similar phenomenon was also seen at FIRE. This could indicate that some chromatin remodeling events had already taken place in the absence of PU.1. In contrast to NIH 3T3 cells, we also did not see elevated levels of histone H3K9 methylation at the c-*fms* locus in PU.1$^{-/-}$ and uninduced PUER cells and DNA at the promoter was already demethylated (reference 9 and data not shown), thus explaining the accessibility of the c-*fms* promoter.

In the fully active state in RAW 264 cells the c-*fms* locus showed a strongly elevated accessibility to DNase I digestion as indicated by an increased cleavage frequency manifesting itself

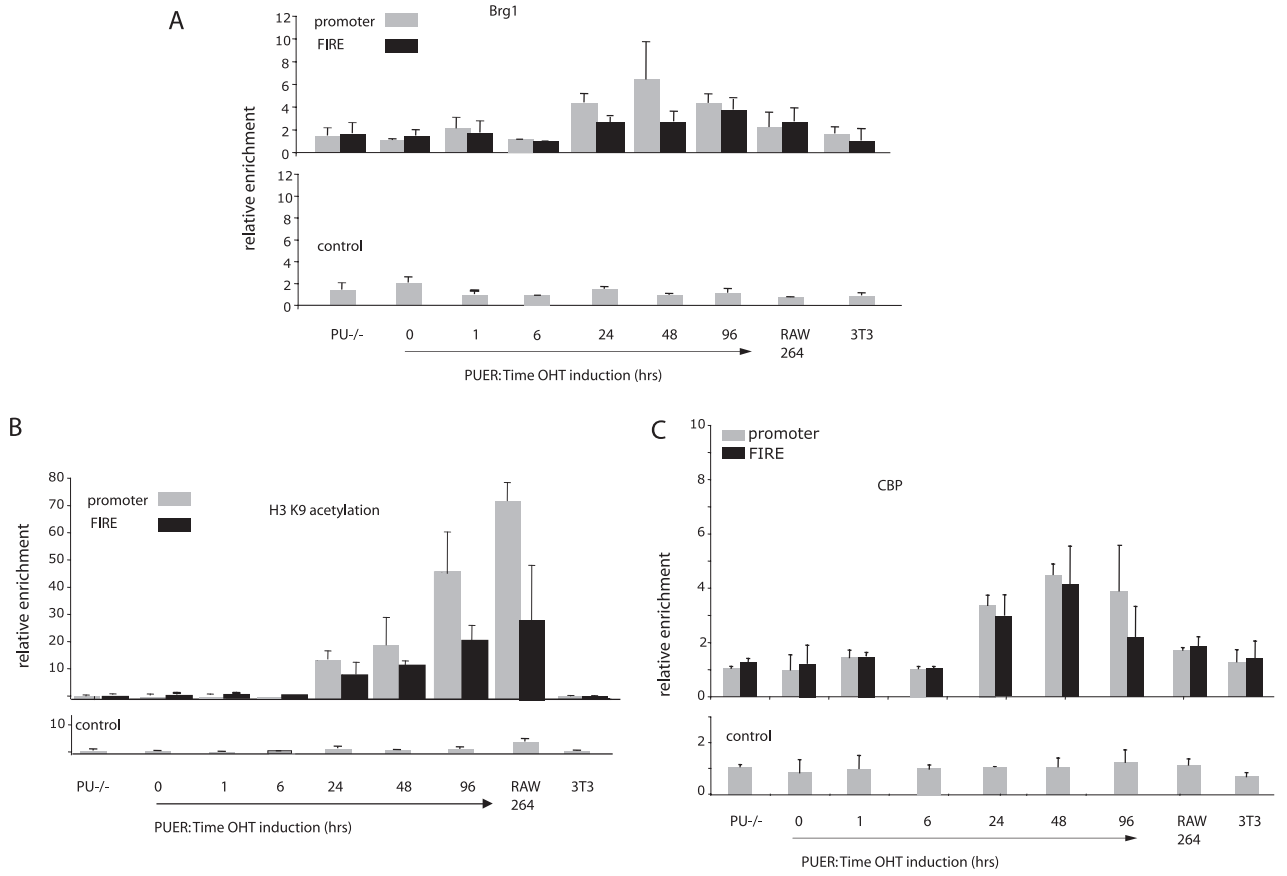

FIG. 6. Cofactor recruitment and histone modification after PU.1 induction. Shown are results of ChIP assays demonstrating the kinetics of recruitment of Brg1 (A), the kinetics of histone H3K9 acetylation (B), and the recruitment of CBP (C), after induction of PUER cells with OHT for the indicated lengths of time. ChIP results are normalized to the GAPDH promoter and represent averages of three independent experiments analyzed in triplicate. Exon 3 was used as the control region.

in an increase in band intensity. In addition, transcription factor binding and chromatin remodeling led to an altered cleavage pattern compared to NIH 3T3 cells and naked DNA. We observed the appearance of a number of DNase I-hypersensitive regions (some of which are indicated in Fig. 5 as arrowheads) as well as regions of protection from DNase I digestion at the position of transcription factor binding sites (some of which are indicated as bars in Fig. 5) (31). Importantly, in induced PUER cells promoter chromatin was already fully reorganized after 24 h of OHT treatment as indicated by an equal intensity of DNase I-hypersensitive sites and equal protection from digestion. This notion is illustrated in the enlargement of Fig. 5A, indicating the appearance of regions of DNase I hypersensitivity/protection upstream around the distal PU.1 site after PU.1 induction. In contrast, chromatin at FIRE was fully remodeled only after 48 h (Fig. 5B, enlargement). Here, full protection of cleavage by binding of Egr-2 with a concomitant increase in DNase I hypersensitivity downstream of the adjacent Ets site was seen only after 48 h. We note that the actual DNase I digestion pattern observed with fully induced PUER+OHT cells was highly similar to that seen in RAW 264 cells, thus confirming that our assay is capable of reproducibly detecting a macrophage-specific chromatin fine structure. These data confirm the kinetically distinct assembly of transcription factors and chromatin reorganization at the promoter and FIRE elements.

Our previous experiments demonstrated that c-*fms* is bound by a component of the nucleosome remodeling complex SWI/SNF, Brg1 (9). In order to correlate alterations in DNase I accessibility with chromatin remodeling, we measured the association of Brg1 in PUER+OHT cells during induction (Fig. 6A). Brg1 was not associated with c-*fms* in PU.1$^{-/-}$ and uninduced PUER cells. An association with the promoter was seen after 24 h of OHT induction, but association with FIRE was weak and reached elevated levels only after 96 h, correlating with the delay of increase in DNase I accessibility compared to the promoter.

We have previously shown that in macrophages all c-*fms cis* regulatory elements display a high level of histone acetylation (34). In order to investigate at which developmental stage this modification is established, we measured the level of H3 lysine 9 acetylation by ChIP (Fig. 6B). The data clearly show that, although the c-*fms* promoter was already fully occupied after 6 h of OHT treatment, acetylated histones were observed only at later differentiation stages. We next wanted to investigate why histone acetylation levels were low in PU.1$^{-/-}$ cells and in PUER cells at early time points of OHT induction. This was not due to the absence of nucleosomes, as shown by a ChIP

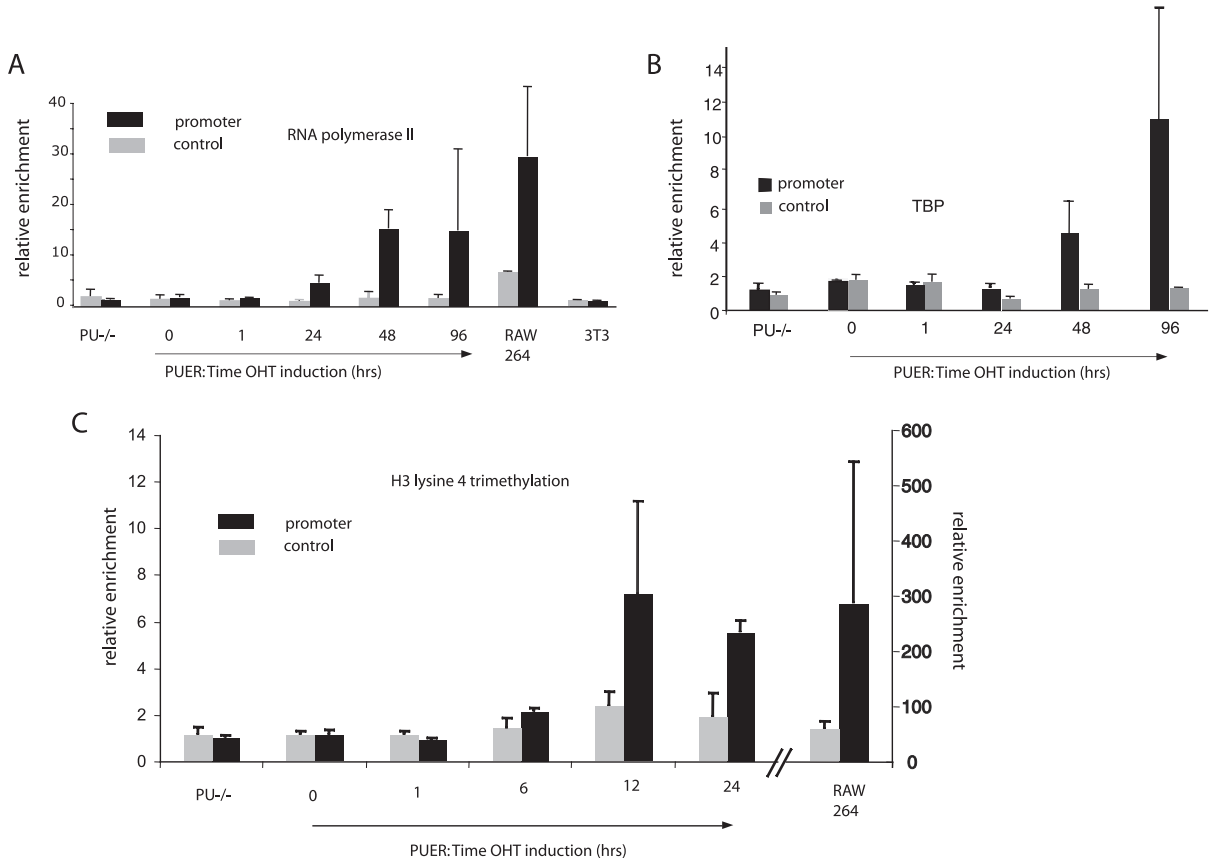

FIG. 7. RNA Pol II recruitment and histone H3K4 trimethylation after PU.1 induction. Shown are results of ChIP assays demonstrating the kinetics of recruitment of RNA polymerase II (A) and TBP (B) and the onset of histone H3K4 trimethylation (C), after induction of PUER cells with OHT for the indicated lengths of time. ChIP results are normalized to the GAPDH promoter and represent averages of two independent experiments analyzed in triplicate. An amplicon 1.5 kb upstream of the promoter was used as a control. The graphs depict the mean values of two independent experiments analyzed in triplicate.

assay with an antibody to the histone H3 C terminus (data not shown). C/EBPβ and PU.1 have previously been shown to interact with the histone acetylase CREB binding protein (CBP) (23, 37), and work from our lab demonstrated that active c-*fms* regulatory elements recruit CBP (9). It has been shown previously that the activity of CBP can be regulated as well (35); it was therefore possible that CBP was recruited but was inactive. Figure 6C demonstrates that little or no CBP recruitment was seen at early time points of induction. Interestingly, CBP was recruited at levels similar to those of the promoter and FIRE at later time points. Thus, chromatin remodeling factors are recruited to the c-*fms* gene at later time points correlating with assembly of transcription factors at the FIRE enhancer.

Elevated RNA Pol II and TBP recruitment along with chromatin modification parallels FIRE activation. We next wanted to know why the level of c-*fms* mRNA expression at early time points of OHT induction was low in spite of the clear evidence for association of PU.1 with the promoter. This could be due to a lack of RNA Pol II recruitment or the recruitment of an inactive form of the basal transcription machinery. We therefore performed ChIP assays with an antibody to RNA Pol II that recognizes all forms of the enzyme as well as with antibodies to TBP and TFIIE α (Fig. 7A and B and data not shown). RNA Pol II, TBP, and TFIIE α were recruited with kinetics similar to that of the promoter. We were unable to detect any RNA Pol II association before 24 h of OHT induction (Fig. 7A and data not shown). The question therefore arose whether there was any promoter activity at all at early time points of OHT induction. We therefore assayed histone H3 lysine 4 trimethylation at the c-*fms* promoter as a stable mark indicating current or recent transcriptional events (25) during early time points of PUER induction (Fig. 7C). We observed a small but reproducible increase of H3K4 trimethylation already after 6 h of induction, which, with some variability between different induction experiments, increased between 12 and 24 h. This indicates that at early differentiation stages low-level transcription does take place when the promoter is fully occupied but before FIRE is fully active.

In summary, our data show clearly that (i) the low transcription level at early time points of induction is indeed due to low Pol II recruitment and not to posttranscriptional events, (ii) we do not see a paused polymerase or a sole preinitiation complex in the absence of PU.1, and (iii) recruitment of high levels of the basal transcription machinery parallels the onset of transcription factor occupancy at FIRE.

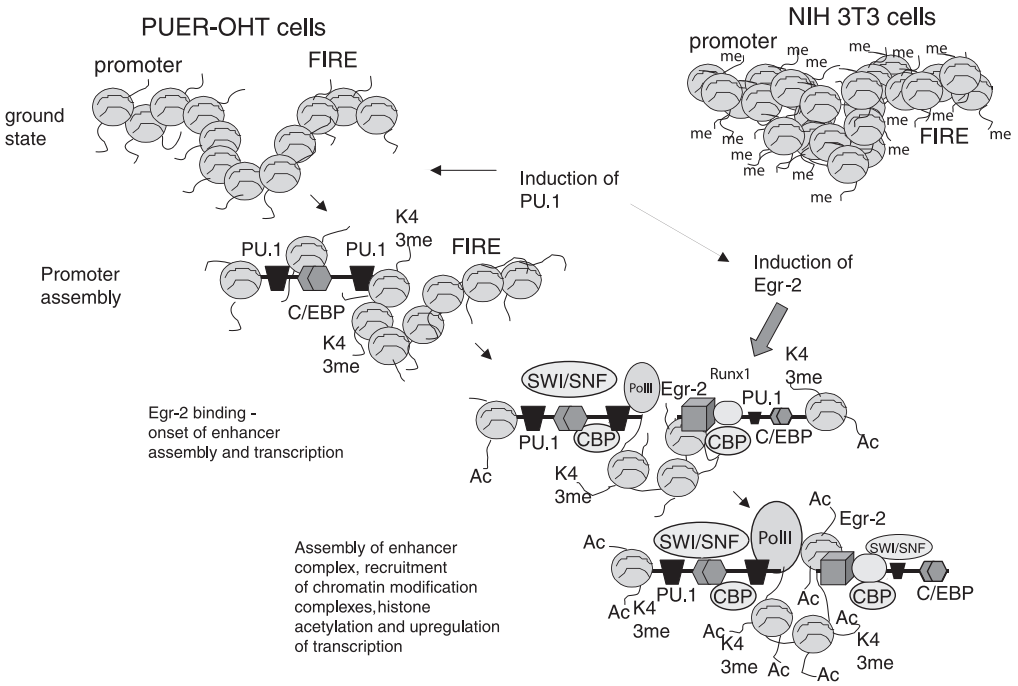

FIG. 8. Model of the order of events of chromatin reorganization and transcription factor assembly at the c-*fms* locus after PU.1 induction. Chromatin is depicted as DNA wrapped around nucleosomes with protruding histone tails, which are differently modified. In NIH 3T3 cells c-*fms* is organized in a compact chromatin structure symbolized by stacked nucleosomes and a high level of histone H3 lysine 9 methylation (9). The chromatin structure in PUER+OHT cells is more DNase I accessible, and nucleosomes are more loosely packed. After the induction of PU.1, transcription factor complexes consisting of the indicated sequence-specific transcription factors, RNA polymerase, and cofactors (depicted by differently shaded shapes) assemble firstly on the promoter and subsequently on FIRE.

DISCUSSION

The transcriptional activation of c-*fms* occurs in two stages. Our experiments with the PUER system clearly show that PU.1 is a rate-limiting factor for stable transcription factor assembly at the c-*fms* gene. Based on the analysis of a number of different genes it has been suggested that enhancer elements serve as nucleation centers for the establishment of active chromatin in stem cells (30). For example, after induction of GATA-1, transcription factor complexes on the β-globin locus are assembled in a stepwise fashion and associate first with the upstream locus control region and only later with the promoter (14). The same is true with the *VpreB1* and the λ*5* locus, where enhancer- but not promoter-bound transcription factor complexes early in development (29). However, our kinetic analysis shows that c-*fms* behaves differently and thus uses a different initiation mechanism. Figure 8 schematically illustrates the order of events by which PU.1 orchestrates the assembly of transcription factors at c-*fms cis* regulatory elements. Chromatin in PUER cells is already readily accessible to the binding of transcription factors and lacks inactive histone marks (H3K9 methylation) but does not contain active histone marks such as H3K9 acetylation and H3K4 trimethylation. After PU.1 induction, the promoter is the first *cis* element to bind transcription factors. However, even after transcription factor assembly at the promoter, histones are not hyperacetylated and mRNA expression levels are very low. Our data therefore suggest that in early progenitor cells and HSCs and in the absence of a fully assembled FIRE complex the c-*fms* promoter mediates a low level of mRNA transcription but is insufficient to induce high-level chromatin modification or transcription. This type of priming event at the promoter and the low levels of active as well as inactive histone marks were also seen in other c-*fms*-expressing multipotent progenitor cell types, such as Pax5$^{-/-}$ pro-B cells (32). Pax5 is required not only to activate a B-cell-specific gene expression program but also to repress c-*fms* during B lymphopoiesis. Our studies of the silencing of c-*fms* during B lymphopoiesis by Pax5 demonstrated that this factor targets mainly the c-*fms* promoter to restrict c-*fms* expression to the multipotent precursor compartment and to myeloid cells.

The second phase of c-*fms* activation requires the induction of a second transcription factor. Previous in vivo footprinting experiments from our lab analyzing primary cells have shown that although the c-*fms* promoter is fully occupied in multipotent precursor cells, full occupancy of FIRE is seen only in more mature macrophage precursors (31, 34). This includes the PU.1 sites at both elements. The experiments presented here now point to a mechanism of how this occurs and show that expression of PU.1 alone is not sufficient to induce full factor assembly at FIRE. This requires a PU.1-dependent differentiation step, which is defined as the alteration of a genetic program. In this case it is the PU.1-mediated induction of Egr-2. Only after Egr-2 binding do we see the stable assembly of other transcription factors such as PU.1 itself, C/EBPβ, and Runx1 (ChIP data not shown) on FIRE. While this complex is assembled, increased levels of RNA polymerase II are recruited to the promoter and we see the recruitment of CBP to both the promoter and the enhancer and progressive acquisition of histone H3 acetylation.

Taken together, our data indicate that the main element

required for establishing active transcription factor complexes and regulating high-level transcription is FIRE. The two-step activation mechanism that we describe ensures that although c-*fms* expression is already activated in stem cells, high levels of c-*fms* mRNA and CSF-1 receptor protein are expressed only in cells destined to be responsive to CSF-1 signaling. Such promoter-mediated transcriptional priming in progenitor cells and enhancer-dependent upregulation in differentiating precursors may be utilized to regulate gene activity in a variety of developmental pathways.

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