Regulation of eosinophil-specific gene expression by a C/EBP–Ets complex and GATA-1

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The EOS47 antigen is an early and specific marker of eosinophil differentiation in the chicken haematopoietic system. To elucidate the transcriptional events controlling commitment to the eosinophil lineage, we studied the regulation of the eosinophil-specific EOS47 promoter. This promoter is TATA-less, and binds transcription factors of the Ets, C/EBP, GATA and Myb families. These sites are contained within a 309 bp promoter fragment which is sufficient for specific high level transcription in an eosinophil cell line. Co-transfection experiments in Q2bn fibroblasts showed cooperative activation of the EOS47 proximal promoter by c-Myb, Ets-1/Fli-1, GATA-1 and C/EBP α . The Ets-1/Fli-1 and C/EBP α proteins were the most potent activators, and acted with high synergy through juxtaposed binding sites located ~60 bp upstream of the transcription start site. The Ets-1 and C/EBP α proteins were found to associate physically via their DNA-binding domains and to bind their combined binding site cooperatively. GATA-1 showed biphasic regulation of the EOS47 promoter, activating at low and repressing at high protein concentrations. These results demonstrate combinatorial activation of an eosinophil-specific promoter by ubiquitous and lineage-restricted haematopoietic transcription factors. They also indicate that direct interactions between C/EBPs and specific Ets family members, together with GATA-1, are important for eosinophil lineage determination.

Keywords: C/EBP/eosinophils/Ets-1/Fli-1/GATA-1

Introduction

The haematopoietic system consists of eight distinct cell lineages that are derived from common precursors in an ongoing process that involves the selective activation of gene expression programmes characteristic of individual cell types. Several transcriptional regulators have been shown to be involved in haematopoietic gene regulation. Phenotypes of c-Myb-, AML-1-, GATA-2- and SCL-1-deficient mice reveal a profound failure of definitive haematopoiesis (Mucenski *et al.*, 1991; Tsai *et al.*, 1994; Okuda *et al.*, 1996; Porcher *et al.*, 1996; Robb *et al.*, 1996). In contrast, GATA-1, PU.1, E2A, C/EBP α ,

C/EBP β and C/EBP ϵ knockouts have more selective phenotypes (Bain *et al.*, 1994; Scott *et al.*, 1994; Zhuang *et al.*, 1994; Screpanti *et al.*, 1995; Tanaka *et al.*, 1995; Fujiwara *et al.*, 1996; McKercher *et al.*, 1996; Yamanaka *et al.*, 1997; Zhang *et al.*, 1997). Thus, it appears that a combination of transcription factors is required for the correct execution of the haematopoietic differentiation programme. Typically, cell type-specific genes are regulated by a combination of these factors characteristic of the lineage. In the non-lymphoid compartment, erythroid-specific genes generally are regulated by combinations of GATA, Ets and Maf family transcription factors and granulocyte/macrophage-specific genes by combinations of AML1/CBF β , C/EBP, PU.1 and c-Myb. For example, the genes encoding the receptors for the myeloid growth factors granulocyte (G)-, macrophage (M)- and GM-colony-stimulating factor (CSF) all contain binding sites for C/EBP and PU.1 (and, in the case of M-CSF, a receptor for AML-1 also) (Hohaus *et al.*, 1995; Smith *et al.*, 1996; Zhang *et al.*, 1996). The neutrophil elastase (NE) and Mim-1 promoters are activated by C/EBPs in cooperation with Myb (and, for the NE promoter, also PU.1) (Ness *et al.*, 1993; Oelgeschläger *et al.*, 1996). Very little, however, is known about the regulation of eosinophil-specific genes. No eosinophil-specific promoters have been analysed at the molecular level, and no transcription factor knockout has been shown specifically to affect this lineage.

Previously we have shown that the E26 avian erythroleukaemia virus is able selectively to transform multipotent haematopoietic precursors. This virus encodes a tripartite fusion protein between the retroviral Gag protein and two cellular transcription factors c-Myb and c-Ets-1. Cells transformed by this virus spontaneously differentiate along erythroid and thrombocytic lineages and can be induced to differentiate along myelomonocytic and eosinophilic lineages by treatment with phorbol esters or by overexpression of kinase-type oncogenes (Graf *et al.*, 1992; Kraut *et al.*, 1994; Frampton *et al.*, 1995; Rossi *et al.*, 1996).

Using this *in vitro* differentiation system, we developed a monoclonal antibody, EOS47, which selectively identifies a cell surface antigen expressed by eosinophils but not by cells of any other haematopoietic lineage. Sequence analysis of the EOS47-encoding cDNA identified it as the avian homologue of the human oncofetal antigen, melanotransferrin (McNagny *et al.*, 1996). Within the haematopoietic system, we have shown that the onset of EOS47 expression on normal eosinophils occurs as cells commit to this lineage and precedes the expression of more mature eosinophil markers such as eosinophil peroxidase and the formation of mature granules (McNagny *et al.*, 1996; K.M.McNagny, unpublished). Likewise, it is one of the first genes to be activated when eosinophil differentiation is induced *in vitro*, either by ectopic GATA-1 expression in chicken myeloblasts or by activation

of a conditional C/EBP β allele in chicken multipotent precursors (Kulesa *et al.*, 1995; Müller *et al.*, 1995). Thus, EOS47 is the earliest and most specific marker of eosinophils described to date and is likely to be regulated by factors directly involved in eosinophil lineage determination.

To approach the molecular basis of eosinophil lineage determination, we therefore analysed the regulation of the EOS47 gene. We report here the identification of its eosinophil-specific promoter and the identification of the promoter elements critical for expression of EOS47 in eosinophils. Our data suggest that an interplay between Ets, C/EBP and GATA factors is required for restricted expression of this protein and probably for proper eosinophil lineage formation.

Results

Cloning and identification of the EOS47 promoter

To identify the site of transcription initiation of the EOS47 gene, RNase protection analysis was carried out using RNA from the 1A1 cell line, which expresses EOS47, and the HD57 and HD100 cell lines as non-expressing controls (Figure 1A). This identified the major sites of transcription initiation inside a TATA-less promoter sequence (Figure 1B), 154 and 157 bp upstream of the translation initiation codon, respectively. These positions accurately correspond to the 5' ends of three independent cDNA clones previously identified (McNagny *et al.*, 1996; K.M.McNagny, unpublished). When compared with the promoter of the melanotransferrin gene, which encodes the human homologue of EOS47, limited sequence homology was found, mainly consisting of conserved putative binding sites for proteins of the Myb, Ets and C/EBP families of transcription factors. As is the case for EOS47, the human melanotransferrin promoter (Duchange *et al.*, 1992) is TATA-less and the locations of the conserved transcription factor binding are in similar positions relative to the transcription start site (Figure 1C).

The EOS47 promoter is eosinophil specific

In order to determine whether the identified promoter was sufficient to direct eosinophil-specific transcription, promoter fragments containing the transcription start site and variable portions of 5' sequence were fused to the luciferase gene to generate reporter constructs (Figure 2A). These were transfected into the cell lines 1A1 (eosinophils) and HD57M (myeloblasts). Parallel experiments were carried out with the Mim-1 promoter. The Mim-1 gene is expressed at similar levels in eosinophils and myeloblasts (Kulesa *et al.*, 1995), and thus served as an additional indicator of equivalence of transfection efficiencies in the two cell types. As shown in Figure 2B, similar expression was seen from the Mim-1-LUC reporter in myeloid and eosinophilic cells. In contrast, all EOS47 reporter constructs were active preferentially in the 1A1 eosinophil cell line. The EOS47-770-LUC and EOS47-152-LUC reporters were expressed at similar high levels; the EOS47-87-LUC reporter had significantly lower activity. From this analysis, we conclude that the 309 bp of EOS47 promoter sequence contained in the EOS47-152-LUC reporter is sufficient for specific high level expression in

Fig. 1. Identification of the EOS47 promoter. (A) Polyacrylamide gel showing products from RNase protection analysis of transcripts from EOS47 + (1A1) and EOS47 - (HD57, HD100A, yeast) cells. '+' and '-' indicate protection using sense and antisense radiolabelled probes, respectively. '+1' and '+4' indicate major protected transcripts in the 1A1 cells. The sequencing ladder was generated using the same plasmid and primer as for the production of the radiolabelled probe. (B) Sequence of the EOS47 proximal promoter region. Numbers indicate base positions with respect to the major transcription initiation site identified by RNase protection and primer extension (McNagny *et al.*, 1996). Lines indicate consensus motifs for the indicated transcription factors. (C) Schematic representation of the relative positions of transcription factor-binding sites present in the chicken and human melanotransferrin promoters.

1A1 eosinophils. Further promoter analysis was therefore carried out using the EOS47-152-LUC construct.

The EOS47 promoter contains functional binding sites for Myb, Ets and C/EBP family proteins

We next tested the functionality of the putative transcription factor-binding sites in the EOS47 proximal promoter. Oligonucleotides containing the Myb, downstream Ets (dEts), and combined C/EBP-upstream Ets (C/EBP-uEts) binding sites were synthesized (for analysis of the GATA site, see Figure 9). The oligos were used as probes in bandshift experiments with nuclear extracts from 1A1 cells (as a source of C/EBP) and *Escherichia coli*-produced recombinant c-Myb, GST-PU.1 and GST-Ets-1 proteins, all containing the DNA-binding domains of the respective factors (Figure 3A-C).

As shown in Figure 3A, C/EBP activity was present in

Fig. 2. Identification of the promoter proximal sequences required for eosinophilic-specific gene expression. (A) Schematic diagram of luciferase reporter constructs fused to EOS47 promoter proximal sequences. (B) One μ g of reporter constructs + 0.5 μ g of RSV- β Gal reference plasmids were transfected into 5×10^5 1A1 eosinophils or HD57M cells and analysed for activity 48 h later. Fold-activation was normalized to β -galactosidase activity. The promoter-less pGL2-basic luciferase vector was given a reference value of 1. Error bars indicate deviation of two experiments.

nuclear extracts from 1A1 eosinophils as determined by gel shift with the C/EBP-uEts oligo. The binding activity was specific, as it could be competed out by excess cold C/EBP-uEts oligonucleotide (wild-type competitor), but not by an equal amount of an oligonucleotide containing a mutation in key residues in the C/EBP site (C/EBP M1 competitor). One concern was that the C/EBP site overlaps a TATA-like motif similar to that found in the SV40 early region, and that the RNase protection showed the presence of minor transcription initiation sites ~20 bp downstream of this element. To rule out the possibility that the M1 mutation of the C/EBP site interfered with promoter functions other than C/EBP binding, additional mutations of this site were made. The M2 mutation replaces the A/T-rich EOS47 C/EBP site with one derived from the murine C/EBP gene, which is G/C rich. This removes the TATA-like motif without affecting C/EBP. Conversely, the M3 mutation destroys C/EBP binding without affecting the TATA-like motif, allowing the importance of these elements to be assessed independently (see below; Figure 5E).

In order to determine which C/EBP isoforms were present in the 1A1 cells, gel retardations were performed in the presence of antibodies specific for the two isoforms characterized in chickens, C/EBP α and C/EBP β (Figure 3B). The antisera were titrated and maximal supershifting was observed in both cases at a concentration lower than the highest one used, indicating that the complexes containing the respective isoforms were supershifted quantitatively. This analysis showed that both isoforms were present, the β isoform being the more abundant. This is consistent with the presence of both mRNAs in the 1A1 cells (Nerlov *et al.*, 1998).

Fig. 3. Binding of C/EBP, Myb and Ets factors to the EOS47 promoter. (A) Nuclear extracts from 1A1 eosinophils (20 μ g protein/reaction) were used in a gel shift assay with a labelled oligonucleotide (5 fmol/reaction) covering the EOS47 promoter C/EBP-uEts site. The effect of mutation in the EOS47 C/EBP site on C/EBP binding was assayed by including excess cold oligonucleotide (0.3 and 1 pmol, left to right) containing the wild-type (WT) C/EBP-uEts site or the same oligonucleotide with the mutations (M1–3) shown below the figure. In the WT sequence, the C/EBP site is underlined; in the mutant sequences, deviations from the wild-type sequence are underlined. (B) The presence of C/EBP α and β in 1A1 nuclear extracts was tested by supershift analysis using C103 (anti-C/EBP α) and anti-NF-M (anti-C/EBP β) antisera (0.03, 0.1, 0.3, 1 and 3 μ l; left to right) as indicated. Specificity of binding was tested by addition of 1 pmol of unlabelled C/EBP-uEts oligo (WT) or the corresponding oligonucleotide carrying the M1 mutation in the C/EBP-binding site (M1). (C) Labelled oligonucleotides encompassing the downstream Ets (dEts), Myb and C/EBP-upstream Ets (C/EBP-uEts) were incubated with recombinant Ets-1, PU.1 and c-Myb DNA-binding domains (~0.2 and ~0.5 μ g protein, left to right), as indicated.

Binding studies with Myb and Ets proteins were performed using recombinant c-Myb DNA-binding domain and GST fusion proteins containing the Ets-1 and PU.1 DNA-binding domains (Figure 3C). We found that both PU.1 and Ets-1 were able to bind to the uEts and dEts sites. The binding was specific, as the Myb site-containing oligonucleotide did not bind either factor. Conversely, only the Myb site bound recombinant c-Myb DNA-binding domain. The two putative Ets-binding sites bound both Ets-1 and PU.1; uEts had a slightly higher affinity for Ets-1, dEts for PU.1.

Fig. 4. Distribution of Ets family proteins in chicken haematopoietic cell lines. RNA from HD3, HD4, HD37 (erythroid cells), HD50, HD57, HD100 (MEPs), HD57M (myeloblast), Bm2c2 (monoblast), HD11, MH2 (macrophage), HD13 (promyelocyte), HD50/4.8 1A1 (eosinophil), RPL12 (B cell), MSB-1 and NBP4 (T cells) chicken haematopoietic cell lines and CEF38 fibroblasts (20 µg/lane) was separated on a 1.2% formaldehyde-agarose gel and transferred to GeneScreen by capillary blotting. The membrane was hybridized sequentially to probes for chicken Ets-1, PU.1 and GAPDH (A), and Fli-1, Elf-1 and GAPDH (B). The Ets-1 probe was derived from the c-Ets-1 3' UTR and therefore does not hybridize to E26 v-Ets sequences.

Expression of Ets family genes in haematopoietic cell types

Identification of the endogenous factors for the Ets sites in 1A1 cells was complicated by the presence of the Gag-Myb-Ets fusion protein. However, by using a probe derived from the 3'-untranslated region (UTR) of Ets-1 (which does not hybridize with the v-Ets sequence present in the E26 virus), we found that Ets-1 mRNA is expressed in 1A1 eosinophils (Figure 4A). In contrast, PU.1 mRNA was not detected in 1A1 eosinophils but only, as expected, in myeloid cells and B cells (HD57M, HD11, HD13 and RPL12). Since probes for other chicken Ets family members were not available, we used probes for human Fli-1 and Elf-1 to assess the distribution of these transcripts by cross-species hybridization (Figure 4B). While Elf-1 showed a wide distribution (albeit at a relatively low level), Fli-1 mRNA was found to be particularly abundant in eosinophils (1A1 and 14-1 cell lines), multipotent progenitors (HD50, HD57) and the HD37 erythroid cell line.

C/EBPα cooperates with Ets-1 on the EOS47 promoter

The ability of the cognate transcription factors to activate the EOS47 promoter was tested in co-transfection experiments. Q2bn quail fibroblast cells were used as a recipient cell line, since these contain low to undetectable levels of Myb, C/EBP and Ets proteins. As can be seen from Figure 5B, combinations of C/EBPα, c-Myb, Ets-1 and PU.1 all activated the proximal EOS47 promoter with at least additive stimulation by all pairwise combinations [PU.1, although not expressed in 1A1 eosinophils, was included for comparison with Ets-1; see below (Figure 7)]. In particular, C/EBPα and Ets-1 synergized strongly on the EOS47 promoter. The omission of these two factors from a mixture of C/EBPα, PU.1, Ets-1 and c-Myb resulted in the most significant decrease in promoter activity. The other C/EBP isoform known to be expressed in chicken eosinophils, C/EBPβ, also activated the EOS47 promoter in cooperation with Ets-1, but somewhat less efficiently than C/EBPα (Nerlov *et al.*, 1998). As can be seen from Figure 5B, while PU.1 and Ets-1 were similarly active by themselves on the EOS47 promoter, Ets-1 clearly synergized more strongly with C/EBPα than PU.1.

To determine the relative contributions of the C/EBP, Myb and Ets sites, these were mutated individually in the context of the EOS47-152-LUC reporter. The ability of the mutations to impair protein binding was confirmed by gel shift analysis (Figure 3A and data not shown). When the mutant promoter constructs were assayed for promoter activity in 1A1 cells (Figure 5D), it was found that the combined C/EBP-uEts site was the most critical: abolition of this site led to an 80–85% decrease in promoter activity. However, all sites clearly contributed to promoter activity. Finally, the point-mutated promoter constructs were introduced into Q2bn cells in the presence of C/EBPα, Ets-1 and c-Myb. This was done to see if these factors reproduced the environment of the 1A1 cell line. As can be seen from Figure 5C, the effect of the introduced point mutations in transfected Q2bn cells was strikingly similar to that seen in 1A1 cells. These results are thus consistent with C/EBP and Ets-1 being major activators of the EOS47 promoter in chicken eosinophils, with c-Myb playing a smaller role.

Finally, we tested the possible role of the TATA-like motif overlapping the C/EBP site; the C/EBP M2 and M3 mutations were introduced into the promoter and their activity tested (Figure 5E). This showed no negative effect of disrupting the TATA-like motif while retaining C/EBP binding (M2 mutation), but a strong decrease in promoter activity upon mutation of the C/EBP site when leaving the TATA-like motif intact (M3 mutation).

Interaction between and cooperative DNA binding by C/EBPα and Ets-1

The most important contribution to EOS47 promoter activity came from the combined C/EBP-uEts site. Considering the high level of synergy between Ets-1 and C/EBPα in co-transfection experiments, this clearly suggested that these factors act through the combined binding site. Furthermore, since C/EBPα cooperated with Ets-1 more efficiently than with PU.1, a mechanism must exist to mediate this specificity. The close proximity of the

Fig. 5. Activation of the EOS47 promoter by Ets, Myb and C/EBP proteins. (A) The positions of binding sites for Ets, Myb, GATA and C/EBP proteins relative to the transcription start site (indicated by an arrow). The base pairs -152 to +157 are those included in the luciferase reporters used below. (B) Cooperative activation by Ets-1, c-Myb and C/EBP α . The EOS47/-152-LUC reporter was co-transfected with the pRSV- β Gal internal control plasmid (0.25 μ g) and expression vectors for C/EBP α (pCMV α ; 0.5 μ g), Ets-1 (pCRNCM-cEts-1; 0.5 μ g), PU.1 (pSG-PU.1; 0.5 μ g) and c-Myb (pCRNCM-cMyb; 100 ng) into Q2bn fibroblasts as indicated. After 48 h, luciferase and β -galactosidase activities were measured and their ratio calculated. The promoter activity in the absence of exogenous activator was arbitrarily assigned a value of 1. Four independent determinations were carried out for each effector combination. (C) The wild-type (WT) EOS47/-152-LUC construct and derivatives containing point mutations in the Myb, uEts, dEts and C/EBP sites as indicated, as well as the promoter-less pGL2-basic vector (-) (1 μ g each DNA), were transfected into Q2bn cells along with expression vectors for C/EBP α (pCMV α ; 10 ng), Ets-1 (pCRNCM-cEts-1; 100 ng), c-Myb (pCRNCM-cMyb; 100 ng) and pRSV- β Gal (0.5 μ g). Promoter activity is expressed relative to that obtained with the wild-type. (D) Similar analysis to that in (C), except that 1A1 cells were used as recipient, and no expression vectors were co-transfected. (E) Similar analysis to that in (D) using EOS47/-152-LUC and its derivatives containing the M2 and M3 mutations in the C/EBP site (see Figure 3A).

binding sites suggested that direct physical interaction could provide such a mechanism.

The ability of C/EBP α and β to interact with the C-terminal part of Ets-1 was therefore tested in a GST pull-down assay (Figure 6). This demonstrated a strong interaction between both C/EBPs and the Ets-1 C-terminus (Figure 6B). There are two blocks of significant sequence homology between C/EBP α and β : the basic region-leucine zipper (BR-LZ) domain and a region of homology in the transactivation domains of the proteins (known as boxA + boxB; Nerlov and Ziff, 1995; Figure 6A). We therefore separated these domains by deleting the C-terminal part of C/EBP α , including the BR-LZ, to generate C/EBP $\alpha\Delta$. This deletion abolished the interaction between C/EBP α and Ets-1, showing that the BR-LZ region of C/EBP α is required for interaction with Ets-1 (Figure 6C). Deletion analysis of the Ets-1 C-terminus (Figure 7A) showed the Ets domain itself is required for this interaction (Figure 7B). These results indicate that the interaction between Ets-1 and C/EBP α is mediated by the DNA-binding domains of the respective proteins. Consistent with this, we have found that the C/EBP α DNA-binding domain can bind to Ets-1 in the absence of the C/EBP α transactivation domain (data not shown). The specificity of the C/EBP-Ets interaction was assessed by testing the interaction of C/EBP α with various Ets family members (Figure 7C). This analysis showed that both Ets-1 and Fli-1 strongly interacted with C/EBP α , whereas Elf-1 and PU.1 showed only weak interaction. This sug-

gested that Fli-1, like Ets-1, could cooperate with C/EBP α on the EOS47 promoter. This was found to be the case, as a more than additive effect of the two factors was observed in transient transfection experiments (Figure 7D).

We next addressed the functional consequence of the C/EBP-Ets interaction by testing whether the presence of Ets-1 influences the ability of C/EBP α to (i) bind to and (ii) transactivate the EOS47 promoter. Using recombinant proteins, it was found that binding of the C/EBP α DNA-binding domain to the C/EBP-uEts site was stimulated in the presence of the Ets-1 DNA-binding domain (Figure 8A), in a manner dependent on both the C/EBP- and Ets-binding sites. In parallel, when the response of the EOS47 promoter to increasing levels of C/EBP α was assayed in the presence or absence of Ets-1, it was found that efficient EOS47 promoter activation by C/EBP α took place at a C/EBP concentration at least one order of magnitude lower when Ets-1 was present (Figure 8B). Indeed, saturation of the EOS47 promoter with C/EBP α was achieved readily in the presence, but not in the absence, of Ets-1. The activation of the EOS47 promoter by C/EBP α was dependent on the C/EBP site, both in the presence and in the absence of Ets-1, as point mutation of this site abolished C/EBP α stimulation in both cases. Taken together, these results show that Ets-1 and C/EBP α are able to cooperate on the EOS47 promoter at comparatively low C/EBP α concentrations, and that although Ets-independent activation by C/EBP α is possible, it requires significantly higher C/EBP concentrations.

Fig. 6. Interaction between Ets-1 and C/EBPs. (A) Homologous regions between the C/EBP α and β isoforms. The two regions of high homology are the C-terminal basic region-leucine zipper (BR-LZ; Cao *et al.*, 1991; Williams *et al.*, 1991) and the N-terminal transactivating domain containing boxA and boxB (Nerlov and Ziff, 1995). To generate C/EBP $\alpha\Delta$, the C-terminal part (amino acids 237–324) of C/EBP α was deleted by digestion with *Sac*II prior to *in vitro* transcription/translation. (B) 35 S-labelled *in vitro* translated C/EBP α and C/EBP β were used in a GST pull-down experiment with either GST or GST fused to the C-terminal part of Ets-1, including exon VII and the Ets domain (GST-Ets-1 106). One-third of the input C/EBP proteins, the C/EBP protein retained by GST alone, and that retained by GST-Ets-1 (left to right) were subjected to 12% SDS-PAGE and autoradiography. (C) GST pull-down as in (B) using 35 S-labelled, *in vitro* translated C/EBP α and the C-terminal deletion, C/EBP $\alpha\Delta$.

Biphasic regulation of the EOS47 promoter by GATA-1

The level of GATA-1 expression appears to play an important role in establishing the eosinophil phenotype (Kulesa *et al.*, 1995). The EOS47 promoter contains a consensus GATA site downstream of the transcription initiation site. We tested the functionality of the site by gel shift analysis using 1A1 nuclear extracts (Figure 9A; only the shifted and supershifted complexes shown). This demonstrated a specific complex formed on a labelled oligonucleotide probe containing the GATA sequence, which could be competed out by the wild-type GATA sequence, but not by a corresponding oligo containing a mutated GATA site (GATA to GTTA). The complex could be supershifted quantitatively by two independent anti-GATA-1 antisera (neither of which show detectable cross-reactivity with GATA-2 or -3; data not shown), but not with pre-immune serum. We concluded that the EOS47 promoter GATA site is functional, and is recognized by GATA-1 in 1A1 cells. The GATA-1 shift was not observed using nuclear extracts from HD50M myeloblasts, which do not express GATA-1 (Kulesa *et al.*, 1995). Previous experiments in our laboratory have shown that when GATA-1 is ectopically expressed in HD50M cells, low

Fig. 7. C/EBP α binds Ets-1 preferentially to PU.1, and interacts with the Ets domain. (A) GST fusions of PU.1 and Ets-1 contained the indicated amino acids of chick p68 c-Ets-1. The Ets domain homology is shown in black. (B) Mapping of the C/EBP interaction domain in Ets-1. The Ets domain and exon VII of Ets-1 were assayed by GST pull-down together (GST-Ets-1 106) and separately (GST-Ets-1 118 and 117, respectively) for interaction with 35 S-labelled, *in vitro* translated C/EBP α . (C) Specificity of C/EBP-Ets interaction. 35 S-labelled, *in vitro* translated C/EBP α was used in a pull-down assay with equal amounts of GST, GST-PU.1, GST-Fli-1, GST-Ets-1 118, GSF-Elf-1 (containing the entire human Elf-1 sequence fused to GST) and GST-Ets-1 FL (same for p54 cEts-1) as indicated. (D) Synergy between C/EBP α and Ets family members. One hundred ng of expression vectors for C/EBP α (pCMV- α), PU.1 (pSG-PU.1), Ets-1 (pCRNCM-cEts-1) and Fli-1 (pSV-Fli-1) were co-transfected with 1 μ g of the EOS47/-152-LUC reporter and 0.25 μ g of pRSV- β Gal into Q2bn fibroblasts. The promoter activity is expressed relative to that of the vector control (lane 1) which is set to 1.

levels of GATA-1 induces trans-differentiation into EOS47-expressing eosinophils, whereas high levels result in retro-differentiation into multipotent progenitors (MEPs) (which do not express EOS47) (Kulesa *et al.*, 1995). We therefore tested the dose-response of the EOS47 promoter to GATA-1. For this purpose, increasing amounts of GATA-1 expression vector were co-transfected with the EOS47/-152-LUC reporter into Q2bn cells, either in the presence or absence of a mixture of Ets-1, c-Myb and C/EBP α expression vectors (Figure 9B). Little effect of GATA-1 was observed on the EOS47 promoter in the

Fig. 8. C/EBPα and c-Ets-1 cooperate in binding and activating the EOS47 promoter. (A) The combined C/EBP-uEts-binding site oligonucleotide was incubated with C/EBPα DNA-binding domain (~30 ng; obtained by thrombin cleavage of a GST-αDBD fusion protein) in the absence or presence of ~30 ng of Ets-1 DNA-binding domain (expressed in bacteria and purified by His tag affinity chromatography) as indicated and subjected to gel shift analysis using radiolabelled probes containing the wild-type C/EBP-uEts sequence (WT) or the analogous probes with mutations in the Ets site (mut uEts) and C/EBP site (mut C/EBP). The positions of the free probe and the complexes obtained with Ets-1 alone (Ets-1 shift) and both C/EBPα and Ets-1 + C/EBPα (C/EBP shift) are indicated on the right. (B) Activation of the EOS47 promoter by C/EBPα in the presence or absence of Ets-1. The wild-type (WT)-152/+157-LUC reporter construct and the corresponding reporter containing a point mutation in the C/EBP site (C/EBP*) (1 μg each) were co-transfected with the pRSV-βGal internal control plasmid (250 ng) into Q2bn fibroblasts in the presence (+Ets-1) or absence of 100 ng of pCRNCM-Ets-1 expression vector and increasing amounts of the pCMV-α expression vector. The luciferase/β-galactosidase ratios were calculated and are shown as fold-activation (above co-transfection of empty control expression plasmids); they are the average values obtained from a representative duplicate experiment.

absence of co-transfected activators. In the presence of Ets-1, c-Myb and C/EBPα, low levels of GATA-1 stimulated the promoter moderately (~1.5- to 2-fold). This was followed by a significant repression (4- to 5-fold) at higher GATA-1 levels. The dosage effect of GATA-1 on the EOS47 promoter thus parallels its effect on the cellular phenotype, with intermediate levels of GATA-1 being optimal for eosinophil-specific gene expression. When the wild-type EOS47 promoter construct was compared with a construct carrying the mutant GATA site described above, it was found that the activation by GATA-1 was dependent on the intact GATA site, whereas subsequent repression was not (Figure 9C). Consistent with this observation, the promoter construct containing the mutated GATA-1 site had somewhat lower activity than the wild-type promoter in 1A1 cells (a 1.3-fold reduction; data not shown).

Fig. 9. GATA-1 binds to and biphasically regulates the EOS47 promoter. (A) A ³²P-labelled oligonucleotide spanning the GATA consensus motif of the EOS47 promoter (Table I) was incubated with nuclear extracts from 1A1 eosinophils (-). The specificity of complex formation was tested by competition with unlabelled GATA oligonucleotide (self-competition; 0.1, 0.3, 1 and 3 pmol, left to right) as well as supershift with anti-GATA-1 antiserum (0.03, 0.1, 0.3 and 1 μl; left to right) and a control non-immune serum (same amounts). The positions of the free probe, the GATA-1-shifted complex and the anti-GATA supershift are indicated (right side). (B) Increasing amounts of pSPCMV-GATA-1 expression vector (0, 1, 3, 10, 30, 100 and 300 ng; left to right) were co-transfected with -152/+157-LUC (1 μg) and pRSV-βGal (250 ng) either in the absence (vector) or presence (+C/EBPα, c-Myb, Ets-1) of 10 ng of pCMVα, 100 ng of pCRNCM-Ets-1 and 100 ng of pCRNCM-cMyb into Q2bn fibroblasts. Luciferase and β-galactosidase were measured, and the fold-stimulation over the basal level of -152/+157-LUC calculated. The results represent the average of four determinations; error bars indicate standard deviations. (C) Comparison of GATA-1 response of wild-type (WT) and GATA-1 site-mutated (GATA-1 MUT) -152/+157-LUC reporters. The reporter constructs were transfected into Q2bn cells as in (C) in the absence (no Act.) or presence (+C,M,E) of C/EBPα, cMyb and Ets-1 expression constructs. Increasing amounts of pSPCMV-GATA-1 expression vector were co-transfected (0, 1, 3, 10, 30, 100, 300 and 1000 ng; left to right) and promoter activity expressed relative to that seen in the presence of C/EBPα, cMyb and Ets-1 (=1).

Discussion

The EOS47 promoter: combinatorial activation of eosinophil-specific transcription

A recurrent theme in the regulation of genes specific for the myeloid lineage is that a combination of factors, such

as c-Myb, C/EBP, AML1/CBF β or PU.1, determines their lineage-specific expression, rather than one critical transcriptional activator. Indeed, ectopic expression of Myb proteins and C/EBPs has been shown to be sufficient to activate certain endogenous myeloid-specific genes, such as Mim-1 and lysozyme, in fibroblast cells (Ness *et al.*, 1993). Other examples include the NE promoter (regulated by C/EBP, Myb and PU.1; Oelgeschläger *et al.*, 1996) and the M-CSF receptor promoter (regulated by AML1/CBF β , C/EBP and PU.1; Zhang *et al.*, 1996). We now find a similar combinatorial activation of the eosinophil-specific EOS47 promoter, which is regulated by c-Myb, Ets-1/Fli-1, GATA-1 and C/EBP. As discussed above, the Ets-1/Fli-1 and C/EBP α proteins were the most potent activators in these experiments; however, both GATA-1 and c-Myb also contributed. Although more eosinophil-specific promoters will have to be analysed in detail to reach a firm conclusion, this observation indicates that myeloid and eosinophil gene expression specificities are achieved in similar fashions, namely through cooperation between factors generally expressed in the haematopoietic compartment (c-Myb, AML1/CBF β , Ets-1/Fli-1) and lineage-specific or lineage-restricted factors (GATA-1, PU.1, C/EBP). This allows for genes to be expressed specifically in either the myeloid (e.g. lysozyme) or eosinophilic (EOS47) lineage, or in both (Mim-1), depending on the specific combination of factors regulating the promoter. It should be noted that while several transcription factor-binding sites appear to be conserved between the human melanotransferrin and EOS47 promoters, the combined C/EBP–Ets and GATA sites are not. This may explain why melanotransferrin expression has not been observed in human eosinophils (S.Ackerman, personal communication).

GATA-1: both an activator and a repressor of the EOS47 promoter

The regulation of EOS47 by GATA-1 was found to be biphasic, in that low concentrations of GATA-1 activated the promoter in cooperation with C/EBP α , Ets-1 and c-Myb, whereas high concentrations were inhibitory. It is interesting to note that a parallel effect is seen when GATA-1 is ectopically expressed in myeloblasts (which do not express GATA-1). Here, low GATA-1 levels lead to trans-differentiation of myeloblasts into EOS47-expressing eosinophils, whereas higher GATA-1 levels result in an MEP phenotype, with no EOS47 expression (Kulesa *et al.*, 1995). Thus, levels of GATA-1 expression can specify lineage commitment. Our results show that GATA-1 can act both as a positive and negative regulator of the EOS47 gene. This is the first demonstration that GATA-1 can directly affect expression of a gene in opposite directions, depending on the concentration of the protein. While the mechanism underlying this effect currently is unclear, our data suggest that while the up-regulation of promoter activity at low GATA-1 concentrations is dependent on the EOS47 promoter GATA-1 site, the repression at high concentrations is not. The parallel to its effect on the cellular phenotype suggests that, in addition to its positive role in lineage-specific gene expression, an important function of GATA-1 may be to restrict expression of non-appropriate genes in lineages where GATA-1 levels are high (such as erythroid cells).

A similar, but opposing, role has been proposed for the MafB protein, which has a positive role in macrophage differentiation, and restricts the expression of erythroid genes (Sieweke *et al.*, 1996; M.Sieweke and T.Graf, unpublished), and it seems likely that many, if not most, lineage-restricted transcription factors will turn out to have dual roles in gene regulation.

When a GATA-1–oestrogen receptor fusion protein is activated in chicken myeloblasts, a rapid down-regulation of myeloid markers and induction of EOS47 mRNA expression is seen (E.Querfurth, C.Nerlov and T.Graf, unpublished). This suggests that GATA-1 expression is indeed the key difference between eosinophils and myeloid cells with respect to EOS47 promoter activity, and at present there seems to be no need to invoke other eosinophil-specific transcription factors to explain EOS47 regulation. The positive effect of GATA-1 seen here, while significant, is modest in comparison with the effect on the endogenous promoter. This suggests that not all regulatory functions of GATA-1 are reproduced in our simple co-transfection experiments. It is likely that features important to gene regulation by GATA-1, such as chromatin, are not reproduced accurately in our transient assays. Thus, the EOS47 promoter may be in a repressed state in myeloid cells, and GATA-1 may be necessary to obtain an open chromatin state on which C/EBPs and Ets-1, both of which are present in myeloid cells, subsequently can act. Experiments to test this possibility currently are under way.

Interaction between C/EBPs and Ets-1/Fli-1

The crucial regulatory feature of the EOS47 promoter seems to be its activation by C/EBP α and Ets-1 or Fli-1. This activation appears to be mediated by direct protein–protein interaction between these factors resulting in high-affinity binding to a combined binding site in the promoter. The interaction between Ets family proteins and BR-LZ-type transcription factors is a recurrent theme of many promoters. Thus, combined Ets–AP-1 elements have important functions in the polyoma virus and urokinase enhancers, as well as the collagenase promoter (Gutman and Wasyluk, 1990; Nerlov *et al.*, 1991). In this case, physical interaction between Ets-1 and Jun proteins has been demonstrated (Bassuk and Leiden, 1995). Similarly, a screen for PU.1-interacting proteins isolated C/EBP δ (Nagulapalli *et al.*, 1995). In these cases, cooperation in transactivation takes place on combined binding sites for the factors. In contrast, MafB is capable of binding to and inhibiting Ets-1 independently of MafB DNA binding (Sieweke *et al.*, 1996). The resulting repression may play a role in suppressing ectopic expression of erythroid genes. The C/EBP–Ets-1/Fli-1 interaction is a novel addition to this class of interactions. It is mediated by the BR-LZ domain of C/EBP α and the Ets domains of Ets-1 and Fli-1. Consistent with their ability to bind C/EBP δ , PU.1 and Elf-1 were also found to interact with C/EBP α , but more weakly than Ets-1 and Fli-1. This was reflected in a lower degree of synergy between C/EBP α and PU.1 compared with Ets-1, although PU.1 and Ets-1 activated the EOS47 promoter with similar efficiencies in the absence of C/EBP α . This suggests that the high affinity of the C/EBP–Ets interaction is important for the synergy between the two factors.

Table I. Sequences of oligonucleotides used for PCR, gel shift and mutagenesis

Oligonucleotide	Sequence
EOS47 genomic primers	
EOS47/-770	GGGGTACCTTCAGTAATGGAATTC
EOS47/-152	GGGGTACCTTACCCCTGTGAGTCA
EOS47/-87	GGGGTACCTCATTGGTGCAGTGAAT
EOS47/+157	GAAGATCTTTTCCAGCCTTCAGAACT
Gel shift and mutagenesis primers	
+GATA	AAGTAAGCAAGATAAATTGTTGAACAAAGT
- GATA	ACTTTGTTCAACAATTTATCTTGCTTACTT
+GATAmut	GCCAAAAGTAAGCAAGTTAAATTGTTGAACA
- GATAmut	TGTTCAACAATTTAACTTGCTTACTTT
+Myb	TGAGTCAGTGTAGCTGTTGATCTAAGTCT
- Myb	AGACTTAGATCAACAGCTACACTGACTCA
+Mybmut	GGGGTACCTTACCCCTGTGAGTCAGTGTGTCGATCT
- Mybmut	AGACTTAGATCGACAACACTACACTGACTCA
- dEts	AAGGAGCACACCAGGAAGAGCAGACTTGCA
+dEts	TGCAAGTCTGCTCTTCTGGTGTGCTCCTT
+dEtsmut	AAGGAGCACACCTGGTAGAGCAGACTTGCA
- dEtsmut	TGCAAGTCTGCTCTACCAGGTGTGCTCCTT
+C/EBP-uEts	TGAATATTTTGTAATTTTCTAGTCTTG
- C/EBP-uEts	CAAGACTAGGAAATTACAAAATATTCA
+C/EBP M1-uEts	TGAATAGTCTGTAATTTCTAGTCTTG
- C/EBP M1-uEts	CAAGACTAGGAAATTACAGACTATTCA
+C/EBP-uEtsmut	TGAATATTTTGTAATTTGGTAGTCTTG
- C/EBP-uEtsmut	CAAGACTACCAAATTACAAAATATTCA
+C/EBP M2-uEts	TGAATGTTGCGCCACTTCTAGTCTTG
- C/EBP M2-uEts	CAAGACTAGGAAGTGGCGCAACATTCA
+C/EBP M3-uEts	TGAATATTTGTACATTCCTAGTCTTG
- C/EBP M3-uEts	CAAGACTAGGAATGTACAAAATATTCA

+ indicates the upper strand, - the lower strand.

A role for C/EBPs and Ets-1/Fli-1 in eosinophil differentiation

The EOS47 gene is expressed exclusively in eosinophils within the chicken haematopoietic system, and is the earliest detectable marker of committed eosinophils (McNagny *et al.*, 1992, 1996). We found that the most crucial promoter element was composed of a combined C/EBP-Ets-binding site, and that Ets-1/Fli-1 and C/EBP α synergized to activate this promoter. This is the earliest eosinophil-specific promoter for which the crucial regulatory proteins have been identified, and our results raise the question of the general role of these transcription factors in eosinophil lineage commitment. We found Ets-1 and Fli-1 mRNAs to be expressed in both eosinophil cell lines and in MEPs (Figure 5), which are eosinophil precursors (Kulesa *et al.*, 1995). C/EBP α and C/EBP β , in contrast, are not expressed in MEP precursor cells (Katz *et al.*, 1993; Nerlov *et al.*, 1998). Based on these observations, it is likely that the induction of C/EBPs in MEPs is a critical event in their commitment to an eosinophil phenotype. This is supported by the observation that transient activation of a conditional C/EBP β allele in MEPs induces eosinophil lineage commitment (Nerlov *et al.*, 1998). In this scenario, Ets-1 and Fli-1 may provide a scaffold upon which C/EBPs can act. The ability of Ets-1 to cooperate with C/EBP α at C/EBP levels below those required for activation by C/EBP alone would make C/EBP-Ets co-regulated genes very sensitive monitors of C/EBP levels. We have found that the decision between myeloid and eosinophil differentiation is strongly influenced by ectopic C/EBP expression in MEPs, with high C/EBP activity favouring eosinophil differentiation

(Nerlov *et al.*, 1998). EOS47 may, therefore, be a paradigm for genes, highly sensitive to C/EBP, that are involved in early eosinophil differentiation. This in turn may explain why EOS47 is expressed at an early stage of differentiation, and suggests that other C/EBP-Ets co-regulated genes are involved in eosinophil commitment.

Consistent with a critical role for C/EBPs in the formation of the eosinophil lineage, it has been found recently that mice homozygous for a disrupted C/EBP α gene have no mature eosinophils (Zhang *et al.*, 1997); however, due to a lack of appropriate markers, it is unclear whether this is due to a failure of progenitors to commit to the eosinophil lineage or of committed cells to terminally differentiate. The lack of mature neutrophil granulocytes, but not myeloblast precursors, in *c/ebp α -/-* mice would be consistent with the latter possibility. However, if C/EBP α , as our results indicate, is an important regulator of early eosinophil gene expression, lack of this factor could lead to failure of eosinophil lineage commitment. No eosinophil lineage deficiency has been reported for C/EBP β knockout mice (Screpanti *et al.*, 1995; Tanaka *et al.*, 1995). This apparent preferential requirement for C/EBP α may reflect the regulation of the genes during the differentiation process. We have found that in the HL60 YY-1 pro-eosinophil cell line, C/EBP α , but not C/EBP β , is inducible by interleukin-5 (IL-5) (C.Nerlov and T.Graf, unpublished), which is an important regulator of eosinophil differentiation *in vivo* (Yoshida *et al.*, 1996). C/EBP β , while is able at least partially to fulfil the role of C/EBP α on most promoters, may therefore not be expressed in response to the appropriate signal (IL-5) at the time when eosinophil lineage commitment takes place.

This, in turn, may explain the specific requirement for C/EBP α in eosinophil lineage development. To resolve this question, it will be important to determine if differential regulation of the α and β C/EBP isoforms is responsible for the specific requirement for C/EBP α in the eosinophil lineage, or whether the two factors are functionally distinct with respect to induction of eosinophil lineage commitment.

Materials and methods

Genomic clones

EOS47 genomic clones were isolated from a λ FIX II library purchased from Stratagene and screened according to the recommendations of the manufacturer. Primary screening was performed using a radiolabelled 600 bp *Pst*I fragment containing 5'-untranslated and coding sequence from the EOS47 cDNA. Five positive clones were identified and purified to homogeneity.

Plasmid construction

EOS47 promoter constructs were generated by PCR from cloned genomic DNA, using *Pfu* polymerase (Stratagene) and the oligonucleotides listed in Table I. Amplified fragments were cloned into the pGL2-basic reporter construct (Promega) using the *Kpn*I (5' end) and *Bgl*II (3' end) restriction enzymes. Mutant promoter constructs were generated by overlapping PCR with EOS47/-152 and EOS47/+157 as outer primers and the appropriate pair of mutation-containing oligonucleotides (Table I), except for the myb site mutation, which was created using a modified 5' primer. The Mim-1 promoter luciferase plasmid has been described previously (Ness *et al.*, 1989).

The cytomegalovirus (CMV) promoter-based pSPCMV-GATA-1 expression plasmid, containing the chicken GATA-1 cDNA, and pSPCMV control vector were provided by Dr H.Kulesa. pSG-PU.1, containing a 1.4 kb *Eco*RI fragment encoding human PU.1 (Spi-1) in the pSG expression vector, and the pSV-Fli-1 vector, containing full-length human Fli-1, were kindly provided by Dr J.Ghysdael. pCRNCM-Ets-1 and pCRNCM-cMyb expression constructs, as well as the corresponding pCRNCM control vector, have been described (Lim *et al.*, 1992). The chicken C/EBP α (Calkhoven *et al.*, 1992) and C/EBP β (Katz *et al.*, 1993) cDNAs were cloned into pcDNA1 expression vector (Invitrogen) after introducing 5' *Bam*HI and 3' *Eco*RI sites by PCR. GST- α DBD, encoding a fusion between GST and chicken C/EBP α amino acids 226-324, was constructed by PCR amplification of the C/EBP α sequence followed by insertion into pGEX-4T-1 using the *Bam*HI and *Eco*RI sites.

Tissue culture and transfection analysis

The origins of the cell lines for transfection and as sources of RNA have been described previously: HD3 erythroblasts (Beug *et al.*, 1982); HD37 erythroblasts (Metz and Graf, 1991); HD11 macrophages (described earlier as LSCC-MC/MA1) (Beug *et al.*, 1979); HD13 granulocytes (Golay *et al.*, 1988; Kulesa *et al.*, 1995); HD57 multipotent cells (Metz and Graf, 1991); HD57M myeloblasts (Graf *et al.*, 1992); HD50 1A1 eosinophils (Kulesa *et al.*, 1995); MSB-1 T cells (Akiyama and Kato, 1974); NPB4 T cells (Beug *et al.*, 1981); RP-12 B cells (Siegfried and Olson, 1972); and HD100 progenitor/eosinophilic cells (McNagney *et al.*, 1996). HD100A cells are a subclone of HD100 which spontaneously lost expression of EOS47.

All cells were grown in blastoderm medium (Graf *et al.*, 1992) composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2.5% chicken serum, 0.15% NaHCO₃, 56 μ g/ml of conalbumin, 80 μ M 2-mercaptoethanol, 0.9 mg/ml insulin, and the standard complement of antibiotics at 37°C in 5% CO₂. Medium for HD50M and HD11 cells was supplemented with ~10 U/ml of recombinant chicken myelomonocytic growth factor (cMGF; Leutz *et al.*, 1989).

Q2bn fibroblasts were transfected and reporter assays performed as described (Frampton *et al.*, 1996). 1A1 and HD57M cells were transfected with 1 μ g of luciferase reporter and 0.5 μ g of Rous sarcoma virus (RSV)- β Gal as a reference using DMRIE-C at 2 μ l per 5 $\times$ 10⁵ cells, as described by the manufacturer (Gibco-BRL).

Protein expression and GST pull-downs

³⁵S-labelled proteins were obtained by *in vitro* translation of pcDNA1-based expression vectors, using T7 RNA polymerase and the TNT

translation system (Promega) according to the manufacturer's instructions. GST protein purification and GST pull-downs were performed as described (Nerlov and Ziff, 1995; Sieweke *et al.*, 1996). The expression vectors for GST-Ets-1 fusion proteins (Sieweke *et al.*, 1996), GST-PU.1, GST-Elf-1, GST-Fli-1, recombinant Ets-1 DNA-binding domain (Sieweke *et al.*, 1998) and recombinant c-Myb DNA-binding domain (Frampton *et al.*, 1995) have been described.

Oligonucleotides and gel shift analysis

Nuclear extracts of 1A1 cells were prepared (Dignam *et al.*, 1983), and gel shift analysis performed (Nerlov *et al.*, 1991) as described, using the double-stranded oligonucleotides obtained by annealing the single-stranded primers from Table I. Competitor (excess of unlabelled probe oligonucleotide or corresponding mutant oligonucleotide) and supershifting antibodies were included as described in the figure legends; antisera were polyclonal anti-GATA antisera obtained from Dr G.Goodwin (A antiserum) and Dr T.Evans (B antiserum), C103 polyclonal anti-rat C/EBP α (which is cross-reactive with chicken C/EBP α ; C.Nerlov, unpublished) obtained from Dr P.Rorth, and polyclonal anti-chicken C/EBP β antiserum (Katz *et al.*, 1993). For gel shifts with recombinant Ets and C/EBP proteins, the same conditions were used, except that 10 ng of poly[d(I-C)] was used per reaction.

RNA extraction and Northern blotting

Total cellular RNA was prepared according to Chomczynski and Sacchi (1987). After electrophoresis through a 1.2% formaldehyde-agarose gel, RNA was transferred to GeneScreen (Dupont) by capillary blotting and probed with cDNA labelled by random priming. The probes were an *Eco*RI fragment containing the 3' part of the chicken PU.1 coding sequence + 3' UTR (kindly provided by Dr J.Ghysdael), a 200 bp *Eco*RI fragment in pKH47 containing chicken c-Ets-1 3' UTR (obtained from Dr D.Leprince), an *Nci*-*Bam*HI fragment of pGST-Elf-1 encoding the human Elf-1 Ets domain, a *Pst*I-*Bam*HI fragment from pGST-Fli-1 encoding the human Fli-1 Ets domain (Sieweke *et al.*, 1998), and a chicken GAPDH cDNA (Dugaiczky, 1983).

RNase protection

Radiolabelled + and - strand RNA probes were produced from the EOS47/-770 promoter fragment cloned into PCRII in each orientation. Briefly, each plasmid was linearized with *Hind*III and labelled transcripts synthesized with T7 polymerase using a Maxiscript T7 kit (Ambion) and the conditions recommended by the manufacturer. Full-length probes were then gel purified and RNase protection was performed using a RPA II kit (Ambion), and protocols recommended by the manufacturer.

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References

- Akiyama, Y. and Kato, S. (1974) Two cell lines from lymphomas of Marek's disease. *Biken J.*, **17**, 105-116.
- Bain, G. *et al.* (1994) E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell*, **79**, 885-892.
- Bassuk, A.G. and Leiden, J.M. (1995) A direct physical association between ETS and AP-1 transcription factors in normal human T cells. *Immunity*, **3**, 223-237.
- Beug, H., von Kirchbach, A., Doederlein, G., Conscience, J.-F. and Graf, T. (1979) Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. *Cell*, **18**, 375-390.
- Beug, H., Müller, H., Grieser, S., Doederlein, G. and Graf, T. (1981) Hematopoietic cells transformed *in vitro* by REV-T avian reticuloendotheliosis virus express characteristics of very immature lymphoid cells. *Virology*, **115**, 295-309.

- Beug, H., Doederlein, G., Freudenstein, C. and Graf, T. (1982) Erythroblast cell lines transformed by a temperature sensitive mutant of avian erythroblastosis virus: a model system to study erythroid differentiation *in vitro*. *J. Cell. Physiol. Suppl.*, **1**, 195–207.
- Calkhoven, C.F., Geert, A.B. and Wijnholds, J. (1992) c/EBP, a chicken transcription factor of the leucine-zipper C/EBP family. *Nucleic Acids Res.*, **20**, 4093.
- Cao, Z., Umek, R.M. and McKnight, S.L. (1991) Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.*, **5**, 1538–1552.
- Chomczynski, P. and Sacchi, N. (1987) Single step isolation of RNA by acid guanidinium isothiocyanate method. *Anal. Biochem.*, **162**, 156–159.
- Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.*, **11**, 1475–1489.
- Duchange, N., Ochoa, A., Plowman, G.D., Roze, A., Amdjadi, M. and Zakin, M.M. (1992) Identification of an enhancer involved in the melanoma-specific expression of the tumor antigen melanotransferrin gene. *Nucleic Acids Res.*, **20**, 2853–2859.
- Dugaiczky, A. (1983) Cloning and sequencing of a deoxyribonucleic acid copy of glyceraldehyde-3-phosphate dehydrogenase messenger ribonucleic acid isolated from chicken muscle. *Biochemistry*, **22**, 1605–1613.
- Frampton, J., McNagny, K.M., Sieweke, M., Philip, A., Smith, G. and Graf, T. (1995) v-Myb DNA binding is required to block thrombocytic differentiation of Myb-Ets-transformed multipotent hematopoietic progenitors. *EMBO J.*, **14**, 2866–2875.
- Frampton, J., Ramqvist, T. and Graf, T. (1996) v-Myb of E26 leukemia virus up-regulates bcl-2 and suppresses apoptosis in myeloid cells. *Genes Dev.*, **10**, 2720–2731.
- Fujiwara, Y., Browne, C.P., Cuniff, K., Goff, S.C. and Orkin, S.H. (1996) Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proc. Natl Acad. Sci. USA*, **93**, 12355–12358.
- Golay, J., Introna, M. and Graf, T. (1988) A single point mutation in the v-ets oncogene affects both erythroid and myelomonocytic cell differentiation. *Cell*, **55**, 1147–1158.
- Graf, T., McNagny, K.M., Brady, G. and Frampton, J. (1992) Chicken 'erythroid' cells transformed by the Gag-Myb-Ets encoding E26 leukemia virus are multipotent. *Cell*, **70**, 201–213.
- Gutman, A. and Wasylyk, B. (1990) The collagenase promoter contains a TPA and oncogene responsive unit encompassing the PEA3 and AP-1 binding sites. *EMBO J.*, **9**, 2241–2246.
- Hohaus, S., Petrovick, M.S., Voso, M.T., Sun, Z., Zhang, D.-E. and Tenen, D.G. (1995) PU.1 (Spi-1) and C/EBP α regulate expression of the granulocyte-macrophage colony-stimulating factor receptor α gene. *Mol. Cell. Biol.*, **15**, 5830–5845.
- Katz, S., Kowenz-Leutz, E., Müller, C., Meese, K., Ness, S.A. and Leutz, A. (1993) The NF-M transcription factor is related to C/EBP β and plays a role in signal transduction, differentiation and leukemogenesis of avian myelomonocytic cells. *EMBO J.*, **12**, 1321–1332.
- Kraut, N., Frampton, J., McNagny, K.M. and Graf, T. (1994) A functional Ets DNA-binding domain is required to maintain multipotency of hematopoietic progenitors transformed by Myb-Ets. *Genes Dev.*, **8**, 33–44.
- Kulesha, H., Frampton, J. and Graf, T. (1995) GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thrombocytes and erythroblasts. *Genes Dev.*, **9**, 1250–1262.
- Leutz, A. *et al.* (1989) Molecular cloning of the chicken myelomonocytic growth factor (cMGF) reveals relationship to interleukin 6 and granulocyte colony stimulating factor. *EMBO J.*, **8**, 175–181.
- Lim, F., Kraut, N., Frampton, J. and Graf, T. (1992) DNA binding by c-Ets-1, but not v-Ets-1, is repressed by an intramolecular mechanism. *EMBO J.*, **11**, 643–652.
- McKercher, S.R. *et al.* (1996) Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J.*, **15**, 5647–5658.
- McNagny, K.M., Lim, F., Grieser, S. and Graf, T. (1992) Cell surface proteins of chicken hematopoietic progenitors, thrombocytes and eosinophils detected by novel monoclonal antibodies. *Leukemia*, **6**, 975–984.
- McNagny, K.M., Rossi, F., Smith, G. and Graf, T. (1996) The eosinophil-specific cell surface antigen, EOS47, is a chicken homologue of the oncofetal antigen melanotransferrin. *Blood*, **87**, 1343–1352.
- Metz, T. and Graf, T. (1991) v-myb and v-ets transform chicken erythroid cells and cooperate both *in trans* and *in cis* to induce distinct differentiation phenotypes. *Genes Dev.*, **5**, 369–380.
- Mucenski, M.I., McLain, K., Kier, A.B., Swerdlow, S.H., Schreiner, C.M., Miller, T.A., Pietryga, D.W., Scott, W.J. and Potter, S.S. (1991) A functional c-myc gene is required for normal mouse fetal hepatic hematopoiesis. *Cell*, **65**, 677–689.
- Müller, C., Kowenz-Leutz, E., Grieser-Ade, S., Graf, T. and Leutz, A. (1995) NF-M (chicken C/EBP β) induces eosinophilic differentiation and apoptosis in a hematopoietic progenitor cell line. *EMBO J.*, **14**, 6127–6135.
- Nagulapalli, S., Pongubala, J.M.R. and Atchison, M.L. (1995) Multiple proteins physically interact with PU.1. *J. Immunol.*, **155**, 4330–4338.
- Nerlov, C. and Ziff, E.B. (1995) CCAAT/enhancer binding protein- α amino acid motifs with dual TBP and TFIIB binding ability mediate transcriptional activation in both yeast and mammalian cells. *EMBO J.*, **14**, 4318–4328.
- Nerlov, C., Rörth, P., Blasi, F. and Johnsen, M. (1991) Essential AP-1 and PEA3 binding elements in the human urokinase enhancer display cell type-specific activity. *Oncogene*, **6**, 1583–1592.
- Nerlov, C., McNagny, K.M., Döderlein, G., Kowenz-Leutz, E. and Graf, T. (1998) Distinct C/EBP functions are required for eosinophil lineage commitment and maturation. *Genes Dev.*, in press.
- Ness, S.A., Marknell, Å. and Graf, T. (1989) The v-myb oncogene product binds to and activates the promyelocyte-specific *mim-1* gene. *Cell*, **59**, 1115–1125.
- Ness, S.A., Kowenz-Leutz, E., Casini, T., Graf, T. and Leutz, A. (1993) Myb and NF-M: combinatorial activators of myeloid genes in heterologous cell types. *Genes Dev.*, **7**, 749–759.
- Oelgeschläger, M., Nuchprayoon, I., Lüscher, B. and Friedman, A.D. (1996) C/EBP, c-Myb, and PU.1 cooperate to regulate the neutrophil elastase promoter. *Mol. Cell. Biol.*, **16**, 4717–4725.
- Okada, T., van Deursen, J., Hiebert, S.W., Grosveld, G. and Downing, J.R. (1996) AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell*, **84**, 321–330.
- Porcher, C., Swat, W., Rockwell, K., Fujiwara, Y., Alt, F.W. and Orkin, S.H. (1996) The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell*, **86**, 47–57.
- Robb, L., Elwood, N.J., Elefanti, A.G., Köntgen, F., Li, R., Barnett, L.D. and Begley, C.G. (1996) The *scl* gene product is required for the generation of all hematopoietic lineages in the adult mouse. *EMBO J.*, **15**, 4123–4129.
- Rossi, F., McNagny, K.M., Logie, C., Stewart, A.F. and Graf, T. (1996) Excision of Ets by an inducible site-specific recombinase causes differentiation of Myb-Ets-transformed hematopoietic progenitors. *Curr. Biol.*, **6**, 866–872.
- Scott, E.W., Simon, M.C., Anastasi, J. and Singh, H. (1994) Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science*, **265**, 1573–1577.
- Screpanti, I. *et al.* (1995) Lymphoproliferative disorder and imbalanced T-helper response in C/EBP β -deficient mice. *EMBO J.*, **14**, 1932–1941.
- Siegfried, L.M. and Olson, C. (1972) Characteristics of avian transmissible lymphoid tumor cells maintained in culture. *J. Natl Cancer Inst.*, **48**, 791–795.
- Sieweke, M.H., Tekotte, H., Frampton, J. and Graf, T. (1996) MafB is an interaction partner and repressor of Ets-1 that inhibits erythroid differentiation. *Cell*, **85**, 49–60.
- Sieweke, M., Tekotte, H., Jarosch, U. and Graf, T. (1998) Cooperative interaction of Ets-1 with USF-1 required for HIV-1 enhancer activity in T cells. *EMBO J.*, **17**, 1728–1739.
- Smith, L.T., Hohaus, S., Gonzalez, D.A., Dziennis, S.E. and Tenen, D.G. (1996) PU.1 (Spi-1) and C/EBP α regulate the granulocyte colony-stimulating factor receptor in myeloid cells. *Blood*, **88**, 1234–1247.
- Tanaka, T. *et al.* (1995) Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. *Cell*, **80**, 353–361.
- Tsai, F.-Y., Keller, G., Kuo, F.C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F.W. and Orkin, S.H. (1994) An early hematopoietic defect in mice lacking the transcription factor GATA-2. *Nature*, **371**, 221–226.
- Williams, S.C., Baer, M., Dilner, A.J. and Johnson, P.F. (1995) CRP2 (C/EBP β) contains a bipartite regulatory domain that controls transcriptional activation, DNA binding and cell specificity. *EMBO J.*, **14**, 3170–3183.
- Yamanaka, R., Barlow, C., Lektrom-Himes, J., Castilla, L.H., Liu, P.P., Eckhaus, M., Decker, T., Wynshaw-Boris, A. and Xanthopoulos, K.G. (1997) Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein ϵ -deficient mice. *Proc. Natl Acad. Sci. USA*, **94**, 13187–13192.

- Yoshida,T. *et al.* (1996) Defective B-1 cell development and impaired immunity against *Angiostrongylus cantonensis* in IL-5R α -deficient mice. *Immunity*, **4**, 483–494.
- Zhang,D.-E., Hetherington,C.J., Meyers,S., Rhoades,K.L., Larson,C.J., Chen,H.-M., Hiebert,S.W. and Tenen,D.G. (1996) CCAAT enhancer-binding protein (C/EBP) and AML1 (CBF α 2) synergistically activate the macrophage colony-stimulating factor receptor promoter. *Mol. Cell. Biol.*, **16**, 1231–1240.
- Zhang,D.-E., Zhang,P., Wang,N.-d., Hetherington,C.J., Darlington,G.J. and Tenen,D.G. (1997) Absence of granulocyte colony-stimulating factor signalling and neutrophil development in CCAAT enhancer binding protein α -deficient mice. *Proc. Natl Acad. Sci. USA*, **94**, 569–574.
- Zhuang,Y., Soriano,P. and Weintraub,H. (1994) The helix–loop–helix gene E2A is required for B cell formation. *Cell*, **79**, 875–884.

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