Molecular characterization of the constitutive expression of the plasma platelet-activating factor acetylhydrolase gene in macrophages

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Plasma platelet-activating factor acetylhydrolase (PAF-AH) is a phospholipase that inactivates platelet-activating factor (PAF) and PAF-like lipids to generate products with little or no biological activity. The levels of circulating PAF-AH correlate with several disease syndromes. We previously reported that mediators of inflammation regulate the expression of the human PAF-AH gene at the transcriptional level. In the present paper, we characterize the constitutive expression of plasma PAF-AH using the mouse gene as a model system, and we report comparative results obtained using human and mouse promoter constructs. We first cloned, sequenced and analysed the promoter region of the murine plasma PAF-AH (mPAF-AH) gene and found that this gene lacks a canonical TATA box. We demonstrated that the *cis*-elements required for basal transcription are localized within the –316 to –68 bp region. *In vitro* band-shift and supershift assays showed that Sp1 and Sp3 transcription factors from RAW264.7 and J774A.1 macrophage nuclear extracts bound strongly to a distal

GC-rich site within –278/–243 [specificity protein (Sp-A)] and to a proximal TC-rich motif within –150/–114 (Sp-B). In addition, we observed weak binding to a GA-rich site within –110/–82 (Sp-C). The regions containing Sp-B and Sp-C are highly conserved between the human and mouse genes. Forced expression of Sp1 or Sp3 in Sp-lacking *Drosophila* SL2 cells induced markedly the activity of the exogenous mPAF-AH promoter in a dose-dependent manner, and this induction was dependent on the presence of intact Sp-A and Sp-B. Interestingly, we found that the Sp1- and Sp3-associated DNA-binding activities increased during the maturation of primary human monocytes into macrophages in cell culture. These results demonstrate that Sp1 and Sp3 are key factors that contribute to the basal, constitutive transcription of the plasma PAF-AH gene in macrophages.

Key words: inflammation, macrophage, PAF-acetylhydrolase, platelet-activating factor (PAF), Sp1, transcriptional regulation.

INTRODUCTION

PAF (platelet-activating factor; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent pro-inflammatory phospholipid synthesized when endothelial cells, neutrophils and monocytes are stimulated by mediators of inflammation and thrombosis [1–3]. In addition to PAF, certain members of a large family of mediators produced as by-products of oxidative fragmentation of cellular phospholipids also act as ligands and agonists of the PAF receptor [3]. These compounds are collectively known as oxidatively fragmented phospholipids (OxPLs) or PAF-like lipids, as they share with PAF an almost identical spectrum of biological activities. It has been proposed that the systemic elevation of PAF and PAF-like lipids participates in physiological (or acute) inflammatory responses as well as in pathological (or chronic) conditions, such as arthritis, septic shock, reperfusion injury and atherogenesis [4].

The levels of PAF are controlled at two levels. First, the synthetic enzymes, cytosolic phospholipase A₂ (cPLA₂) and PAF acetyltransferase, are tightly regulated by phosphorylation [3]. Secondly, the rapid hydrolysis of PAF and PAF-like lipids serves as a key mechanism to down-regulate signals elicited by these molecules. PAF-AHs (PAF-acetylhydrolases) convert PAF into biologically inactive lyso-PAF and acetate [5]. PAF-AHs comprise groups VII and VIII of the PLA₂ superfamily of proteins [6,7]. PAF-AHs differ from other PLA₂s by their Ca²⁺-

independence and unique substrate specificities [5]. The secreted, plasma form of PAF-AH has anti-inflammatory properties *in vivo* [8], and it circulates in blood as a complex with lipoproteins [9]. Our current understanding of the function of PAF-AH indicates that this gene product is important for monocyte/macrophage effector functions that contribute to the resolution of inflammation. Altered expression of PAF-AH in the plasma correlates with inflammatory diseases [10,11]. The protective role of plasma PAF-AH in inflammation and atherogenesis has been demonstrated in a variety of *in vivo* experiments [8,12,13]. The plasma activity levels vary 6-fold within healthy Caucasians [14], suggesting that the basal enzymic activity is regulated by diverse factors among individuals.

Our previous studies have shown that macrophage-enriched tissues, such as lymph node, thyroid gland, spleen and placenta, are the major sources of plasma PAF-AH [8,15]. Consistent with this observation, the differentiation of peripheral blood monocytes into macrophages in culture results in the robust up-regulation of intracellular PAF-AH expression at the mRNA, protein and activity levels [8,16]. This process is accompanied by the secretion of PAF-AH into the medium [17]. Narahara et al. [18] observed that pro-monocytic HL-60 cells secrete increased levels of plasma PAF-AH during differentiation mediated by phorbol esters. Howard et al. [19] also reported that Küpffer cells, the hepatic macrophages, account for the *in vivo* pool of plasma PAF-AH transcript in the liver. In an analysis of Japanese subjects

Abbreviations used: Ab, antibody; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EKLF, erythroid krüppel-like factor; EMMPRIN, extracellular matrix metalloproteinase inhibitor; EMSA, electrophoretic mobility-shift assay; IL-10, interleukin-10; PAF, platelet-activating factor; PAF-AH, PAF-acetylhydrolase; mPAF-AH, murine plasma PAF-AH; PLA₂, phospholipase A₂; RT, reverse transcriptase; SFM, serum-free medium; SL2, Schneider's *Drosophila* line 2 cells; Sp, specificity protein; SV40, simian virus 40; ZBP-89, zinc-finger binding protein-89.

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Table 1 Oligonucleotide primers for PCR

Name	Sequence¶	Strand	Location**
mAH35F*	5'-GGATCTGACTCGCTCTGGTG-3'	Sense	+ 16 ~ + 35
mAH68R*	5'-CTGGTCACTGCACGT ACTCCC-3'	Antisense	+ 86 ~ + 66
mAHRTF†	5'-GAGCGTCTTCGTGCGTTTGTACTA-3'	Sense	n.a.
mAHRTR†	5'-GGTCTCCATGT TCAATGCAAGAATC-3'	Antisense	n.a.
mbActinF†	5'-GTGGGCGCGCTCTAGGCACCAA-3'	Sense	n.a.
mbActinR†	5'-CTCTTTGATGTCACTACGATTTC-3'	Antisense	n.a.
M92R‡	5'-CAGCAATGCCACAGAGCGAGTC-3'	Antisense	+ 44 ~ + 22
+ 86R/BglII	5'-CGATTAGATCTGGTCACTGCAGTCC-3'	Antisense	+ 89 ~ + 67
- 1806/BglII§	5'-CGTAAGATCTGAAGGCAGTCATAGTCACAGG-3'	Sense	- 1806 ~ - 1786
- 487/BglII§	5'-CGTAAGATCTATCTCCTCAAGAGGTGAAGCG-3'	Sense	- 487 ~ - 467
- 316/BglII§	5'-TGTAAGATCTCTGATGAGAATCGGGTGTACG-3'	Sense	- 316 ~ - 296
- 179/BglII§	5'-ACTAAGATCTAGCCTGAGTCGCAGCAGCTT-3'	Sense	- 179 ~ - 160
- 68/BglII§	5'-TCAGAGATCTTACGAGGAGGCTGTATGCTC-3'	Sense	- 68 ~ - 49
M6F/XbaII	5'-AGGAGGCTGGGCCACACGCTTCTagaCTGTCCCTCTT-3'	Sense	- 150 ~ - 114
M6R/XbaII	5'-AAGAGGGACAGTctAGaAGCGTGTGGCCAGCCTCCT-3'	Antisense	- 114 ~ + 150
M7F/EcoRI	5'-CAGCAACCGGAGaAttcGAGGGTCGAGCC-3'	Sense	- 110 ~ - 82
M7R/EcoRI	5'-GGCTCGACCCTCgaaTtCTCCGGTTGCTG-3'	Antisense	- 82 ~ - 110
M2F/HindIII	5'-CCATCATCACCCCTCgaaGcttGCCAGGCACAGTCAG-3'	Sense	- 278 ~ - 243
M2R/HindIII	5'-CTGACTGTGCCCTGGcgaGcttGAGGGTGATGAGG-3'	Antisense	- 243 ~ - 278
pGL3F	5'-CTAGCAAAATAGGCTGTCCCGAGTGCAAGTGC-3'	Sense	n.a.
pGL3R	5'-CATGGTGGCTTTACCAACAGTACCAGGAATGC-3'	Antisense	n.a.

* Primers used for PCR-mediated P1 genomic screening.

† Primers used for RT-PCR.

‡ Primers used for primer extension.

§ Primers used for the generation of 5' deletion constructs.

|| Primers used for the generation of promoter reporter constructs with site-directed mutations.

¶ Sequence underlined represents the restriction enzyme site; sequence in lowercase denotes nucleotides targeted for mutation.

** Nucleotide positions are relative to the transcriptional start site (+ 1) of the murine PAF-AH gene. n.a. not applicable.

who received an allogeneic bone marrow transplant, Asano et al. [20] found that the plasma PAF-AH activity depended on the donor's, but not the recipient's, genotype. One recipient who was homozygous deficient for PAF-AH in leukocytes and homozygous wild-type in buccal mucosa had undetectable PAF-AH activity in the plasma. These studies suggest that most of the PAF-AH activity in human plasma originates from haematopoietic cells.

We previously cloned and analysed the 5' flanking region of the human plasma PAF-AH gene [15]. We found that the human PAF-AH promoter does not contain a TATA box; instead, the promoter region contains a set of GC-rich motifs surrounding the transcription start site, a feature characteristic of promoters that regulate the expression of housekeeping genes [21]. In the present study, we sought to identify the minimal promoter features required for basal expression of the PAF-AH gene. We reasoned that the identification of elements that are responsible for constitutive promoter activity would shed light into the mechanisms that regulate the expression of PAF-AH during the monocyte/macrophage differentiation process. Using cellular and biochemical analyses, we established that binding of the specificity protein transcription factors Sp1 and Sp3 to both canonical and non-canonical Sp-binding sites localized within the - 316 to - 68 bp region participate in the constitutive transcription of the murine plasma PAF-AH gene in macrophages. These findings significantly increase our understanding of the regulation of an important anti-inflammatory gene, and they underscore the participation of the transcription factors Sp1 and Sp3 in the differentiation of haematopoietic cells.

EXPERIMENTAL

Cell culture

Murine RAW264.7 and J774A.1 macrophage cells were purchased from American Type Culture Collection (ATCC, Manassas,

VA, U.S.A.) and were maintained in a 5% CO₂ atmosphere, at 37 °C in DMEM (Dulbecco's modified Eagle's medium; Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% (v/v) foetal bovine serum (HyClone, Logan, UT, U.S.A.). SFM (serum-free medium)-adapted SL2 (Schneider's *Drosophila* line 2) cells (Invitrogen) were maintained at 28 °C in *Drosophila*-SFM supplemented with 16.5 mM L-glutamine (Invitrogen). Primary human monocytes were isolated and cultured as described previously [15].

Isolation and sequencing of the upstream region of the murine PAF-AH gene

A mouse P1 genomic library (Incyte Genomics, St. Louis, MO, U.S.A.) was screened by PCR using a pair of primers (mAH35F and mAH68R; Table 1) that amplifies a 71-bp fragment of exon 1 of the mPAF-AH gene [22]. Two positive P1 clones were grown overnight in 50 ml of LB (Luria-Bertani) medium containing 25 µg/ml kanamycin. The P1 DNAs were isolated using a plasmid purification kit (Qiagen, Valencia, CA, U.S.A.) following the manufacturer's instructions. We then digested the P1 clones with *EcoRI*, *EcoRV* or *PstI*, and cloned the fragments into a pBluescript SKII vector. Positive colonies were identified by PCR and the plasmid DNA was sequenced using automated analyses. These studies resulted in the isolation of plasmids pMGS1 (11.8 kb) and pMGS2 (10.7 kb), which contain mouse genomic sequences upstream and downstream of exon 1 of the mouse PAF-AH gene respectively.

RT (reverse transcriptase)-PCR

Total RNA was isolated from cultured cells using TRIzol® (Invitrogen), according to the manufacturer's instructions. We used 5 µg of total RNA in reverse transcription reactions, performed using a commercially available cDNA synthesis kit

(Invitrogen). The mRNA levels of mPAF-AH and β -actin were determined by amplification using specific primers (Table 1) and the following parameters: β -actin: 30 cycles of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 120 s; mPAF-AH: 36 cycles of 94 °C for 45 s; 57 °C for 60 s and 72 °C for 90 s. The PCR products were then subjected to electrophoresis on 1.5 % (w/v) agarose gels and visualized using ethidium bromide.

Primer-extension analysis

An antisense oligonucleotide (M92R; Table 1) that started 33 bp downstream of the 5' end of the original mPAF-AH cDNA clone (GenBank® accession number U34277) was labelled at the 5' end using [γ -³²P]ATP and T4 polynucleotide kinase (Promega, Madison, WI, U.S.A.). Reverse transcription was carried out at 42 °C using AMV-reverse transcriptase (Promega) in the presence of 0.3–1 pmol of labelled M92R and 250 ng of murine spleen polyadenylated RNA (Ambion, Austin, TX, U.S.A.) or 32 μ g of J774A.1 total RNA. The reaction products were resolved on 8 % (w/v) denaturing polyacrylamide gels and were then subjected to autoradiography.

Plasmids

Eight regions of the mouse PAF-AH gene containing various lengths of the sequences immediately upstream of the transcription initiation site, were amplified using *Bgl*III-tagged primers (Table 1) and pMGS1 as template. The PCR products were digested with *Bgl*III and were then cloned into pGL3-Basic or pGL3-Enhancer (Promega) vectors to generate all the promoter-reporter constructs. DNA fragments containing mutated sites within region –316/–68 were generated by a two-step PCR procedure [23] using specific primers (Table 1), and were then inserted into pGL3-Basic or pGL3-Enhancer vectors to yield all the mutant constructs. We also cloned DNA fragments corresponding to nt –316 to –68 (both wild-type and mutated forms) into p β RGB (Promega) to generate the constructs used in transfection of SL2 cells. All the human PAF-AH promoter-luciferase constructs were generated as described previously [15]. The human construct (Sp-Mu) with a mutated Sp1-/Sp3-binding site was generated by two-step PCR. The Sp1 and Sp3 expression vectors including pPacSp1, pPacUSp3 and the empty vector pPac were kindly provided by Dr Guntram Suske (Institut für Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, Germany). The pBKCMV and pBKCMV-ZBP-89 (zinc-finger-binding protein-89) plasmids were gifts from Dr Juanita Merchant (Department of Internal Medicine, University of Michigan, Ann Arbor, MI, U.S.A.). All plasmid constructs were prepared using the Endo-free Plasmid Preparation kit (Qiagen) and were characterized by restriction and automated sequence analyses.

Transient transfection and promoter activity assays

RAW264.7 cells were transfected using LIPOFECTAMINE™-Plus reagents (Invitrogen). Briefly, 8×10^5 cells were co-transfected with 2 μ g of various promoter reporter constructs plus 0.2 μ g of pRL-TK (Promega) in serum-free DMEM for 4 h. Cells were harvested 24 h after transfection and firefly and *Renilla* luciferase activities were assayed using the Dual-luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. In some cases, the luciferase constructs were co-transfected with the normalizing vector pSV- β -gal (Promega) and the corresponding activities were measured using the Luciferase Assay System (Promega) and Galacto-Light Plus reagents (TROPIX, Bedford, MA, U.S.A.) respectively. SL2 cells were co-transfected with

promoter constructs driving *Renilla* luciferase expression and the Sp expression vectors, using LIPOFECTAMINE™ 2000 (Invitrogen). The total amount of transfected DNA was normalized to 4.5 μ g using the empty vector, pPac. After 48 h *Renilla* luciferase activity was determined relative to the total protein content in cell lysates using the *Renilla* Luciferase Assay System and the D₅ Protein Assay Reagent (Bio-Rad) respectively.

Preparation of nuclear extracts and electrophoretic mobility-shift assays (EMSAs)

Nuclear extracts were prepared as described previously [24], with modifications. Briefly, cells from 90 % confluent cultures were washed with PBS, and then resuspended in hypotonic lysis buffer [10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT (dithiothreitol) and 1 % (v/v) protease inhibitor cocktail (Sigma, product code P8340)]. Cells then were disrupted using 10–20 strokes of a glass homogenizer to achieve 80–90 % lysis (determined by Trypan-Blue staining). The nuclei were sedimented by centrifugation, resuspended in extraction buffer [20 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 25 % (v/v) glycerol, 1 mM DTT and 1 % (v/v) protease inhibitor cocktail] and incubated at 4 °C for 30 min with gentle shaking. The supernatant from the final centrifugation step (performed at 16000 *g* for 30 min) was collected, snap-frozen in liquid nitrogen, and stored at –70 °C until use.

To perform EMSAs, we pre-incubated 5 μ g of crude nuclear extract in binding buffer [20 mM Tris/HCl, pH 8.0, 4 mM MgCl₂, 50 mM KCl, 0.1 % (v/v) Nonidet P40, 10 % (v/v) glycerol, 1 mM DTT, 1 mM EDTA and 200 μ g/ml BSA] with 2 μ g of poly(dI-dC)·(dI-dC) (Roche Molecular Biochemicals), in the absence or presence of 20–100-fold molar excess of competitor probes for 10 min at 4 °C. We then added 0.1 pmol of ³²P-labelled oligonucleotide probe (62500–250000 d.p.m.) and continued the incubation for an additional 20 min at 4 °C. To perform supershift experiments, we pre-incubated nuclear extracts with 2 μ g of purified polyclonal antibodies (Abs) against Sp1, Sp2, Sp3, Sp4, EKLF (erythroid krüppel-like factor; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or murine ZBP-89 (a gift generously provided by Dr Satoshi Takaki, Division of Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, University of Tokyo, Tokyo, Japan) for 1 h at 4 °C before the addition of labelled probes. The binding mixtures were subjected to electrophoresis on 4.5 % (w/v) non-denaturing polyacrylamide gels and the protein–DNA adducts were visualized by autoradiography.

RESULTS

Cloning and characterization of the murine PAF-AH promoter

To characterize the mPAF-AH promoter, we isolated a P1 clone containing >16 kb of the genomic sequences flanking exon 1 of the mPAF-AH gene. For subsequent analyses, we obtained two genomic subclones harbouring 8.8 kb of the promoter (clone pMGS1) and 7.7 kb of exon I and a portion of intron 1 (clone pMGS2). We determined the expression levels of the plasma PAF-AH transcript in two mouse macrophage cell lines using semi-quantitative RT-PCR. PAF-AH mRNA was highly expressed in J774A.1 macrophages and, to a lesser extent, in RAW264.7 macrophages (results not shown). We next performed primer extension analyses to map the transcription start site (site +1) using an antisense oligonucleotide (M92R) that started 33 bp downstream of the 5' end of the previously isolated mouse cDNA clone [22] (GenBank® accession number U34277).

Figure 1 Cloning and characterization of the 5' regulatory region of the murine plasma PAF-AH gene

(A) A 32 P-labelled antisense oligonucleotide (M92R, see the Experimental section) was used at two levels (0.3 pmol, lanes 2 and 4; 1 pmol, lanes 3 and 5) in primer-extension analyses performed to reverse-transcribe total RNA from J774A.1 cells (32.5 μ g, lanes 2 and 3) or polyadenylated RNA from mouse spleen (250 ng, lanes 4 and 5). Lane 1, 32 P-labelled ϕ X174/HinfI DNA markers; lanes 6–9, PCR-sequencing products obtained using plasmid pMGS1 and M92R primer. The corresponding sequence is shown next to the sequencing ladder; the +1 site is indicated as a boxed 'A'. (B) A genomic subclone (pMGS1) was sequenced by PCR using an antisense primer derived from exon one. The numbering indicated is based on the location of the nucleotides relative to the transcription initiation site (+1, an underlined and bold-faced 'A'). Potential transcription-factor-binding sites (shown in underlined and bold-faced characters) were predicted using MatInspector 2.0. The sequence of the antisense primer for primer extension analysis (M92R) is shown in italics. The vertical arrow indicates the position of the 5' end of the original mPAF-AH cDNA clone (GenBank® accession number U34277). Sequences shown in lowercase within the three proximal putative Sp1-binding sites represent the consensus motifs recognized by Sp1.

Figure 1(A) shows that a DNA product of 44 nt was reverse-transcribed from both mouse spleen polyadenylated RNA and total RNA from J774A.1 macrophages (Figure 1A; lanes 2–5). Thus the site +1 (an underlined, bold-faced 'A' in Figure 1B) is localized 11 bp upstream of the 5' end of the mouse cDNA sequence previously reported [22]. The proximal 5' flanking region of the mPAF-AH gene is devoid of TATA or CCAAT

sequences and contains fewer GC-rich elements compared with the human PAF-AH promoter (Figure 1B) [15]. We examined the mouse promoter region using computational analyses of the 2320 bp fragment spanning the –2035/+285 region {TRANSFAC 4.0 (<http://transfac.gbf.de/TRANSFAC>) and MatInspector (<http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl>) [24a]} and identified several potential binding sites for members

of the Sp1 family of zinc-finger proteins. These putative Sp1-binding regions included a GA-rich region at position –102, a TC-rich motif at –131 and a GC-rich region at –265. In addition, we identified a potential STAT (signal transducer and activator of transcription) binding site at position –395 and a Pu.1/Ets binding site at –547 (Figure 1B).

DNA sequences within nt –316 and –68 of the mouse and nt –139 and –74 of the human promoters are essential for basal transcription of the PAF-AH gene

To identify the regulatory elements required for basal transcription of the mPAF-AH gene, we generated a series of reporter constructs in which the firefly luciferase gene was cloned downstream of various mouse genomic fragments. We chose RAW264.7 cells as our model system because these cells are more readily transfected than J774A.1 cells (results not shown). We co-transfected each construct into RAW264.7 macrophages in conjunction with a normalizing vector (pRL-TK), and then determined luciferase activity. We found that all the constructs showed low to moderate levels of promoter activity in RAW264.7 cells (Figure 2A); the highest activity (2.4-fold over pGL3-Basic) was observed when we used the construct harbouring 316 bp upstream of the transcription initiation site (Figure 2A; –316/+86). Inclusion of additional upstream sequences (–316 to –1400) led to a decrease in promoter activity, suggesting the presence of inhibitory elements in this region (Figure 2A). To increase the sensitivity of our assays, we examined the behaviour of reporter constructs in which an SV40 (simian virus 40) enhancer element was cloned downstream of the luciferase gene (Figure 2B). The results of these studies confirmed our initial observations and demonstrated further that nt –316 to –68 contain essential promoter elements that define the constitutive expression of PAF-AH in macrophages. In addition, our data demonstrate that sequences comprised within nt –1400 to –316 probably contain one or more negative regulatory element(s).

To examine the transcriptional regulation of the human PAF-AH gene in macrophages, we transfected RAW264.7 cells with the human PAF-AH promoter constructs reported in our previous studies [15]. We found that the essential promoter elements are located between nt –139 and –74 of the human gene. Inclusion of additional 5' sequences up to nt –3416 decreased promoter activity by nearly 75% (Figure 2C). These results are similar to those obtained with the mouse constructs (Figures 2A and 2B), suggesting that both genes are regulated in similar manners.

Identification of Sp1/Sp3/ZBP-89- and Sp1/Sp3-binding sites within nt –316/–68 of the murine PAF-AH promoter

Our next goal was to identify further transcription factors responsible for the basal expression of the PAF-AH gene in macrophages. To accomplish this, we synthesized 11 oligonucleotide probes spanning the region comprised between nt –316/–68 of the mouse gene (Figure 3A) and tested each probe in individual EMSAs, using nuclear extracts from RAW264.7 and J774A.1 macrophages. We found that P3 (–278/–243), P8 (–150/–114) and P10 (–110/–82) altered the electrophoretic pattern observed when nuclear extracts of both RAW264.7 (Figure 3B, lanes 3, 9 and 11) and J774A.1 macrophages (Figure 3B, lanes 6, 13 and 14) were used, demonstrating that these nuclear extracts contain proteins that bind to P3, P8 and P10 *in vitro*. The intensity of the signals obtained using P3 and P8 was much stronger than that observed when P10 was used (compare lane 11 with lanes 3 and 9 in Figure 3B). The differences may reflect the relative strength with which each probe binds to its target protein(s), the abundance of these proteins, or a combination of both effects.

Figure 2 Functional analyses of the murine and human PAF-AH gene promoters

RAW264.7 macrophages were transfected with a series of 5' deletion mutants of the mPAF-AH promoter sequences linked to the firefly luciferase gene cassette (A), mouse 5' deletion constructs in which the SV40 enhancer element was inserted immediately after the 3' end of the luciferase gene (B), and with human 5' deletion constructs (C). Cells were co-transfected with a normalizing vector (pRL-TK). The luciferase activity was measured and normalized as described in the Experimental section. The ordinates represent the ratio between the normalized luciferase activity of each promoter reporter construct to that of the backbone vector [pGL3-Basic in (A) and (C) or pGL3-Enhancer in (B)]. Each bar represents the mean $\pm$ S.D. of three independent transfection experiments.

To identify precisely the presence of transcription-factor-binding sites within each region, we focused on individual domains in a systematic manner. First, we analysed the region comprising nt –278/–243 using MatInspector. The results of this analysis predicted the presence of putative binding sites for several zinc-finger proteins including Sp1, EGR-1 (early growth response factor-1), ZBP-89, MZF-1 (myeloid zinc-finger protein-1) and EKLF (Figure 1B). To directly examine if ZBP-89, Sp1, Sp3 and EKLF-like factors bind to the PAF-AH promoter region comprised between nt –278 and –243, we performed supershift assays using specific Abs raised against these transcription factors. We initially observed that anti-Sp1 Ab caused the supershifting of complex 1 and to a lesser extent of complexes 2 and 4 (Figure 3C, lane 2). Treatment with anti-Sp3 Ab resulted in the loss of complexes 2 and 4 (Figure 3C, lane 3), and the combination of both Abs resulted in the supershifting of all species except 3 (Figure 3C, lane 4). In contrast, anti-EKLF Ab failed to alter

Figure 3 Three domains within $-316/-68$ bp bind nuclear proteins from murine macrophages *in vitro*

(A) Sequence and distribution of 11 double-stranded oligonucleotide probes (P1–P11, 5' $\rightarrow$ 3') that collectively span the mouse proximal promoter region ($-316/-68$ bp). **(B)** EMSAs using 32 P-labelled probes P1–P11 were carried out as described in the Experimental section. Lanes 1–5 and 7–12, RAW264.7 (R) nuclear extracts (N.E.); lanes 6, 13 and 14, J774A.1 (J) N.E. **(C)** Supershift assays using labelled P3 and RAW264.7 N.E. (all lanes) in the absence (lane 1) or presence of anti-Sp1 (lane 2), anti-Sp3 (lane 3), anti-Sp1 + anti-Sp3 (lane 4), anti-EKLF (lane 5), anti-ZBP-89 (lane 6), anti-Sp1 + anti-Sp3 + anti-ZBP-89 (lane 7) Abs or control IgG (lane 8). **(D)** Supershift assays using labelled P8 (all lanes) in the absence (lane 1) or presence of either anti-Sp1 (lane 2), anti-Sp3 (lane 3), or both anti-Sp1 and anti-Sp3 (lane 4) Abs. Nuclear extracts from RAW264.7 cells were included in all lanes. **(E)** Supershift assays were performed as in **(D)** except that labelled P10 was used instead of P8. SS, supershifted bands. Complexes designated as (1)–(4) are discussed in the text.

complex formation (Figure 3C, lane 5) suggesting the lack of participation of EKLF transcription factors in these interactions. The addition of anti-ZBP Ab caused the complete and exclusive disappearance of complex 3 (Figure 3C, compare lanes 1 and 6) confirming the participation of ZBP-89 in the formation of this complex. Finally, the inclusion of anti-Sp1, anti-Sp3 and anti-ZBP Abs resulted in the complete supershifting of all complexes

to higher-molecular-mass regions (Figure 3C, lane 7). Control IgG (Figure 3C, lane 8) and Abs against Sp2 and Sp4 (results not shown) did not supershift the complexes formed between P3 and nuclear protein components. These data provide key evidence that identifies Sp1, Sp3 and ZBP-89 as the transcription factors that bind to the mPAF-AH promoter region located between nt -278 and -243 .

Figure 4 Identification of nucleotides in probes P3, P8 and P10 necessary for nuclear-protein binding

(A) The sequences (5' → 3') of wild-type P3 and its derivatives (M1–M5). Mutated residues are shown in bold lower case. (B) EMSAs using labelled wild-type P3 (all lanes) in the absence (lane 1) or presence of unlabelled competitors: P3 (lanes 2 and 3), M1 (lanes 4 and 5), M2 (lanes 6 and 7), M3 (lanes 8 and 9), M4 (lanes 10 and 11) or M5 (lanes 12 and 13), at the indicated molar ratios. (C) The sequences (5' → 3') of wild-type P8 and P10 and their corresponding derivatives (M6 and M7). Mutated residues are shown in bold lower case. (D) EMSAs using labelled P8 (lanes 1–5) in the absence (lane 1) or presence of unlabelled competitors: P8 (lanes 2 and 3) or M6 (lanes 4 and 5), at the indicated molar ratios. The same competition assays were performed using labelled P10 (lanes 6–10) in the absence (lane 6) or presence of unlabelled competitors [P10 (lanes 7 and 8) or M7 (lanes 9 and 10)], at the indicated molar ratios. RAW264.7 nuclear extracts were included in all lanes in (B) and (D).

We next performed similar studies aimed at identifying the transcription factor(s) responsible for interactions with two additional regions within the PAF-AH promoter (Figure 3B). Individual analyses of the regions comprised between nt –150/–114 and nt –110/–82 using online resources (MatInspector) revealed the presence of putative binding sites for Sp1 transcription factors (Figure 1B). To examine this issue further, we performed supershift assays and tested the participation of Sp1 or Sp1-related proteins in the formation of DNA–protein complexes. The results of these studies are shown in Figures 3(D) and 3(E). Anti-Sp1 Ab mediated the supershifting of complex 1 and, to a lesser extent, complex 2. In addition, anti-Sp3 Ab resulted in the supershifting of complex 3 and, to a lesser extent, complex 2. Finally, the combined use of both Abs resulted in the loss of all three species formed (Figures 3D and 3E, compare lanes 1 with 4). Control IgG, anti-Sp2 and anti-Sp4 Abs failed to alter the band-shift patterns (results not shown). These experiments demonstrate that Sp1 and Sp3 bind to two discrete TC/GA-rich fragments within the proximal murine PAF-AH promoter.

Minimal sequence requirements for interaction with murine macrophage nuclear factors

Our next goal was to determine the structural features necessary for the binding of Sp1, Sp3 and ZBP-89 to probe P3. We designed five oligonucleotide probes with altered nucleotides at various locations within the P3 region (M1–M5; Figure 4A). We then asked if these probes competed with P3 for binding to nuclear proteins using EMSAs. Probe M1 competed with P3 for Sp1/Sp3 binding, but was not as effective in its ability to compete for ZBP-89 binding (Figure 4B, lanes 4 and 5). These results suggest that the region targeted for mutation in M1 contributes to the binding of P3 to ZBP-89, and that this region is not important for interaction with Sp1/Sp3. In contrast, M2 did not compete with P3 for binding of either Sp1/Sp3 or ZBP-89-like proteins (Figure 4B, compare lanes 1, 6 and 7). These results indicate that the sequence CCCGCCCC (–264/–257) includes the nucleotides required for interaction with both Sp1/Sp3 and ZBP-89 transcription factors. M3 and M4 competed with P3 for Sp1/Sp3 binding, but neither

probe was able to compete for ZBP-89 binding (Figure 4B, lanes 8–11). In contrast, M5 effectively competed with P3 for binding to both the Sp1/Sp3 and ZBP-89 domains (Figure 4B, lanes 12 and 13). The region comprising nt –264/–257 is 100% conserved in M5. In contrast, M4 contains a C-to-T mutation at nt –257. These results are consistent with the hypothesis that cytosine (–257) plays a key role in the interaction with ZBP-89-like factors. In summary, these results demonstrate that the sequence CCCGCCCC (–264/–257) consists of a core motif essential for P3 to form complexes with both Sp1/Sp3 and ZBP-89 transcription factors, and that the terminal C residue in this sequence is essential for interactions with the latter. We will refer to this Sp1/Sp3-binding site with the above sequence motif as Sp-A.

In the next series of experiments, we characterized the interaction between probes P8 and P10 with Sp1/Sp3 further. According to our sequence analyses, the Sp1/Sp3 sites are located within the domains represented by CGTCCTCCCCTG in P8 and GAGGGGAGGGTCGA in P10 (MatInspector). We synthesized two mutant probes (M6 and M7) that had sequences identical with P8 and P10, except for four nucleotides (Figure 4C). We tested the ability of M6 and M7 to compete with P8 and P10 for binding to nuclear proteins. We found that the mutations introduced in M6 and M7 prevented these probes from binding to nuclear proteins (Figure 4D, lanes 4, 5, 9 and 10). Thus the mutations introduced in M6 and M7 abolished the *in vitro* Sp1/Sp3 binding activities of the two proximal non-canonical sites. Our results suggest that the Sp1/Sp3-binding activities of probes P8 and P10 are largely due to the TC/GA-rich regions targeted for mutation. We will refer to these regions as Sp-B and Sp-C localized in P8 and P10 respectively.

Comparative role of the mouse Sp-binding sites in basal promoter activity

We next evaluated the relative contribution of each of the three Sp-binding sites within the proximal promoter region to the expression of the murine gene. Our previous studies demonstrated that the mutations introduced in M2, M6 and M7 disrupted the interaction of Sp-A, Sp-B and Sp-C with nuclear proteins (Figure 4). To test the effect of these genetic alterations in functional promoter assays, we generated constructs that incorporated one or more of these mutations within the region encompassing nt –316/+86 fused to a luciferase reporter (Figure 5). We then tested these constructs in transfection assays, using luciferase expression to report promoter activity. We found that mutation of Sp-A (M2) and Sp-B (M6) inhibited the basal promoter activity by 54% and 23% respectively (Figure 5A). Disruption of both Sp-A and Sp-B (M2/M6) resulted in additive inhibitory effects (approx. 80% decrease in basal promoter activity) suggesting that sites Sp-A and Sp-B act independently of each other. In contrast, mutation of Sp-C (M7) did not affect promoter activity. We also modified construct –316/+86–SV40 by introducing the same mutations into Sp-A/B or Sp-A/B/C and then tested the mutated constructs, as above. We found that mutations in Sp-A/B completely inhibited promoter activity despite the presence of a robust SV40 enhancer (Figure 5B). These combined results demonstrate that the basal promoter activity of mPAF-AH is dependent on two Sp1/Sp3-binding sites, Sp-A and Sp-B, that these sites act independently, and that engagement of the Sp-A site enhances transcription to a larger extent than activation of the Sp-B site.

Sp1 and Sp3 stimulate an exogenous mouse PAF-AH promoter in *Drosophila* SL2 cells

To investigate further the role of Sp1 and Sp3 in the transcriptional regulation of the mPAF-AH gene, we used an approach that

Figure 5 Relative contribution of Sp-A, Sp-B and Sp-C to the basal mPAF-AH promoter activity

(A) A wild-type promoter construct [–316(Wt)] or its derivatives harbouring a single mutation [–316(M2), –316(M6) and –316(M7)], double mutations [–316(M2/M6)] or triple mutations [–316(M2/M6/M7)] were transfected into RAW264.7 cells. Luciferase (Luc) activity assays then were performed as described in the Experimental section. (B) RAW264.7 cells were transfected with the wild-type promoter-reporter-enhancer construct [–316(Wt)–SV40] or one of the two mutant forms [–316(M2/M6)–SV40 and –316(M2/M6/M7)–SV40] and were then assayed for luciferase activity. The mutations present in M2, M6, and M7 are depicted in Figures 4(A) and 4(C). Open and closed circles represent wild-type and mutated Sp1/Sp3-binding sites (Sp-A, Sp-B and Sp-C) respectively. The normalized luciferase activity of the wild-type construct [–316(Wt)] in (A) or –316(Wt)–SV40 in (B) is arbitrarily set as 100%. Results are means $\pm$ S.D. of three independent experiments.

employed SL2 cells, which do not express members of the Sp family of transcription factors. To circumvent a high background problem encountered when we used pGL3 vectors, we screened other commercial vectors and found that phRGB (Promega) was satisfactory in this regard. We generated another promoter construct (–316/+86–R) in which the region spanning nt –316/+86 was cloned upstream of the synthetic *Renilla* luciferase gene. We next transfected this construct in combination with an Sp1-expression plasmid (pACSp1). We found that forced expression of Sp1 in SL2 cells robustly enhanced the promoter activity elicited by the promoter region comprising nt –316/+86 (Figure 6A). Sp1 also induced luciferase expression when the backbone vector (phRGB) was used, but this effect was negligible compared with the results obtained with the promoter construct (12-fold compared with 76-fold). These results demonstrate that the ability of Sp1 to mediate promoter activation was specific and dependent on the DNA elements present in the promoter construct. We conclude that the –316/+86 fragment contains *cis* elements required for binding of the transcription factor Sp1 to this region. In addition to Sp1, Sp3 also stimulated exogenous promoter activity in SL2 cells in a dose-dependent manner (Figure 6B), although the effects were less pronounced (48-fold stimulation above control).

Our next experiments were designed to assess further the participation of sites Sp-A and Sp-B on the induction mediated by Sp1 and Sp3. We transfected SL2 cells with a mutant construct [–316(M2/M6)/+86–R] containing altered Sp-A and Sp-B sites within the promoter region nt –316/+86 fused to the *Renilla* luciferase reporter. Disruption of both Sp-A and Sp-B strongly decreased induction of the exogenous mPAF-AH promoter activity by Sp1 and Sp3 (Figure 6C). In addition, co-transfection

Figure 6 Sp1 and Sp3 stimulate the activity of an exogenous mPAF-AH promoter when expressed in SL2 cells

(A) A wild-type mouse PAF-AH promoter construct driving *Renilla* luciferase expression [–316(Wt)–R] and the backbone vector (phRGB) were transfected separately into SL2 cells along with various amounts of an Sp1 expression vector (pPacSp1) as described in the Experimental section. The luciferase activity of each sample was measured and normalized for protein content. The ordinate represents the ratio between the normalized luciferase activity of each Sp1 transfectant and that of the mock transfectant. Solid bars, –316(Wt)–R; open bars, phRGB. (B) Same as in (A), except that an Sp3 expression vector (pPacUSp3) was used in the co-transfection experiments. (C) Co-transfection of SL2 cells with the wild-type [–316(Wt)–R, solid bars] or mutant [–316(M2/M6)–R, open bars] constructs with the indicated amount of pPacSp1 and/or pPacUSp3. (D) RAW264.7 cells were co-transfected with increasing amounts of a ZBP-89 expression vector (pCMV-ZBP-89) along with the promoter construct [–316(Wt)]. The total plasmid amount was maintained at 2 μg in each transfection, using the empty vector (pBK-CMV). Firefly luciferase activity of each sample was measured and normalized for protein content. The ordinate represents the ratio between the normalized luciferase activity of each ZBP-89 transfectant and that of the mock transfectant. All results are means ± S.D. of three independent experiments.

of both Sp1 and Sp3 had additive effects, suggesting that Sp1 and Sp3 activate this region of the PAF-AH gene independently of each other. We also compared the relative contributions of the Sp family members with that of ZBP-89 in the transcriptional regulation of the PAF-AH gene. We co-transfected RAW264.7 cells with the mPAF-AH promoter construct (–316/+86) and with an expression vector harbouring the ZBP-89 cDNA (pCMV-ZBP-89). We observed that expression of ZBP-89 moderately enhanced luciferase expression (>1.8-fold; Figure 6D), confirming our observation that ZBP-89 participates in the mechanisms that control constitutive expression of the mPAF-AH gene.

Characterization of an essential Sp1/Sp3-binding site in the human PAF-AH promoter

We next compared the sequences of the human and mouse proximal regions spanning Sp-A, Sp-B and Sp-C. We found that

a domain spanning Sp-B and Sp-C (but not Sp-A) is conserved between the two species (Figure 7A). The results shown in Figure 2(C) demonstrate that key elements of the human PAF-AH promoter reside between nt –139 and nt –74. This region contains a putative Sp-binding site (CCTCCC) identical with Sp-B in the mouse gene (Figure 7A). To examine if this sequence is important for promoter activity of the human gene, we designed a probe spanning nt –90/–66 and used it in EMSAs. We found that factors present in nuclear extracts from human macrophages bound to this probe and formed three complexes (1, 2 and 3; Figure 7B, lanes 2 and 3) similar to those previously characterized (Figure 3). To examine the identity of these complexes, we used anti-Sp1 and anti-Sp3 Abs and observed a supershifting of the complexes formed (Figure 7B, lanes 5–8), which revealed the presence of Sp1 and Sp3 in these complexes. To evaluate further the role of this Sp1/Sp3-binding site, we mutated the sequence of the human reporter construct by replacing the core motif CCTCCC

Figure 7 Characterization of an Sp1/Sp3-binding site in the human PAF-AH promoter

(A) Alignment of the 5' flanking regions of human (hAH, GenBank® accession number AF027357) and mouse PAF-AH (mAH) using program LALIGN. The regions corresponding to nt -297/-79 of the mouse PAF-AH and nt -255/-28 of the human PAF-AH sequences are shown. The individual sequences of probes P3, P8 and P10 are shown in open boxes. The core motifs of the three Sp1/Sp3-binding sites (Sp-A, Sp-B and Sp-C) within the mouse proximal promoter are shown in shaded boxes within P3, P8 and P10. (B) EMSAs using ³²P-labelled probe spanning nt -90/-66 of the human PAF-AH promoter (5'-CACACACGCTCTCCCGGTACCTC-3') in the absence (lanes 1-3 and 5) or presence of either anti-Sp1 (lane 6), anti-Sp3 (lane 7) or anti-Sp1 + anti-Sp3 (lane 8). Abs. An unlabelled probe (Mu, 5'-CACACACGCTAATCCCGGTACCTC-3') with the core Sp1/Sp3 motif (nt -79/-80) mutated was supplemented at a 100-fold molar excess (lane 4). Nuclear extracts from undifferentiated human monocytes (M), 6-day monocyte-derived macrophages (D6), and 10-day macrophages (D10) were included in lanes 1, 2 and 3-8 respectively. NS, non-specific band; SS, supershifted bands. Complexes designated (1)-(3) and that marked by an asterisk are discussed in the text. (C) A wild-type (WT) human promoter construct (-139/+206) or its derivative (Sp-Mu) were co-transfected into RAW264.7 cells with a normalizing vector pSV-β-gal. The luciferase level of each construct was normalized to that of β-galactosidase to yield the relative luciferase activity. Results are means ± S.D. of three independent transfections.

with AATCCC. This mutation prevented complex formation with macrophage nuclear proteins (no competition mediated by excess mutant probe; Figure 7B, lane 4) and also abolished basal promoter activity by approx. 90% (Figure 7C). These results establish a key role for this Sp1/Sp3-binding site in the basal promoter activity of the human PAF-AH gene.

Participation of the proximal Sp1/Sp3-binding site of the human PAF-AH promoter during maturation

We asked if the Sp1/Sp3 site characterized in the human proximal promoter is important for differential expression of PAF-AH during cellular maturation. To address this issue, we performed EMSAs using the probe spanning nt -90/-66 and compared complex formation using either primary human monocytes or macrophages differentiated in culture for 6-10 days. Nuclear

extracts from monocytes formed DNA-protein complexes that differed from those observed when macrophage nuclear extracts were used (Figure 7B, lanes 1-3). The main DNA-protein complex formed by monocyte nuclear extracts (identified by an asterisk in Figure 7B, lane 1) had a much higher electrophoretic mobility than the characteristic complexes 1-3 formed by proteins present in macrophage nuclear extracts (Figures 3B-3E and Figure 7B, lanes 2, 3, and 5). These results indicate that the Sp1/Sp3-binding site identified in the human PAF-AH promoter is likely to be involved in the differential expression of the PAF-AH gene during maturation of myeloid cells.

DISCUSSION

We previously reported the cloning of human PAF-AH genomic sequences located upstream of the transcription initiation site as an

initial step towards understanding the regulatory mechanisms that govern plasma PAF-AH expression. We characterized promoter activities associated with these sequences under both basal and stimulated conditions [15]. In the present study, we cloned, sequenced and functionally characterized the 5' flanking region of the murine PAF-AH gene, and found features that are both similar to, and distinct from, those of the human gene.

First, we precisely identified the transcription initiation site to an adenosine residue located 11 bases upstream of the 5' end of the cDNA construct previously reported. In addition, we established that the proximal mouse promoter region lacks a consensus TATA box upstream of the transcription initiation site, a feature shared with the human gene. The absence of a TATA box was initially thought to be characteristic of housekeeping genes; that is, genes that are expressed ubiquitously and at low levels in differentiated cells [21]. However, additional studies have demonstrated that TATA-less promoters have the ability to initiate transcription in a tissue-specific fashion, as is the case with PAF-AH [25]. The TATA-less PAF-AH promoters of mouse and human origin contain several potential Sp1/Sp3-binding sites in the proximal region, which is characteristic of a specific class of TATA-less gene promoters [26].

Our initial studies demonstrated that the region located between nt -316 and -68 relative to the transcription start site is critical for the constitutive activity of the mouse promoter. Computer analyses predicted the presence of several putative consensus binding sites for two Sp family transcription factors, Sp1 and Sp3. The data presented here demonstrate that these factors interact with a distal canonical GC-rich site (Sp-A), and two proximal non-canonical TC/GA sites (Sp-B and Sp-C) in the mouse gene promoter. A thorough analysis of sequences within Sp-A revealed that, in addition to the presence of Sp1/Sp3-recognition sequences, this domain contains a putative binding site for ZBP-89. ZBP-89, a krüppel-like zinc-finger protein can behave as either a transactivator or a repressor [27,28]. Our EMSA studies demonstrated that ZBP-89 interacts with Sp-A *in vitro* (Figure 3C). In functional studies, we found that ZBP-89 moderately stimulated mPAF-AH promoter activity when overexpressed in RAW264.7 cells (Figure 6D), and that Sp-A is crucial for the intact function of the mouse promoter (Figure 5A). The Sp-A domain is not conserved between the human and mouse promoter sequences (Figure 7A). In addition, the human proximal promoter has no putative binding sites for ZBP-89. These combined observations indicate that ZBP-89 probably has a role in the regulation of the mouse, but not the human, PAF-AH gene. A human promoter construct (-139/+206) that lacked the region equivalent to Sp-A in the mouse gene displayed full promoter activity (Figure 2C), indicating that regulatory mechanisms that rely on interactions with this region of the mouse promoter are likely to have only species-specific implications.

In contrast with Sp-A, the Sp-B and Sp-C regions of the mPAF-AH gene are highly homologous with the human cognate sequence (Figure 7A). These regions, however, differ quantitatively in their ability to regulate expression of the mouse and human PAF-AH genes. Our results are consistent with the hypothesis that the Sp-B domain plays a more important regulatory role than the Sp-C region. First, Sp-B has higher affinity for Sp1/Sp3 than Sp-C *in vitro* (Figure 3B). Secondly, the promoter activity of the mPAF-AH construct was compromised by mutagenesis of site Sp-B, but not by that of Sp-C (Figure 5A). Thirdly, in transfection studies, we found that inclusion of nt -139/-74, which contain the Sp-B site in the human promoter, was sufficient to confer maximal basal promoter activity (Figure 2C), and that destruction of this site severely impaired the human promoter activity (Figure 7C). These studies demonstrate that Sp-B is a critical regulatory region

for basal PAF-AH expression, and they indicate that the non-canonical Sp1/Sp3-binding site within this region is responsible for the observed effects.

Sp1 is widely regarded as a ubiquitously expressed nuclear factor that functions as a general transcriptional activator. However, the expression levels of Sp1 vary widely (more than 100-fold) among cell types and stages of development, both at the mRNA and protein levels [29]. Interestingly, Sp1 levels appear to be highest in developing haematopoietic cells, foetal cells and spermatids, suggesting that elevated Sp1 levels are associated with cellular differentiation [29]. These data indicate that Sp1 has a regulatory function and that it may participate in tissue- and cell-specific gene expression. For example, the expression of CD14 (a component of the lipopolysaccharide receptor complex) in monocyte/macrophages is mediated exclusively by Sp1 [30]. Sp1 also regulates the myeloid-restricted expression of the human HCK (haematopoietic cell kinase) gene [31]. Interestingly, the Sp1 sites within the proximal promoter regions of these genes are sufficient to confer higher promoter activity in myeloid versus non-myeloid cells [30,31]. Sp3 also participates in the cell-specific expression of many genes. Recent studies have shown that Sp1 and Sp3 activate the expression of two myeloid-restricted integrin genes, CD11b and CD11c in differentiating monocytes [32-34]. Sp1 and Sp3 also participate in the macrophage-specific expression of the EMMPRIN (extracellular matrix metalloproteinase inducer) gene [35]. Liang et al. [35] speculated that the higher levels of Sp1 and Sp3 in macrophages compared with monocytes accounts for the increase in EMMPRIN expression during monocytic differentiation. Our results are consistent with the hypothesis that Sp1/Sp3 participate in the differential expression of PAF-AH (Figure 7B), since we observed much higher levels of Sp1/Sp3 binding activity in macrophages compared with monocytes. Finally, the expression profile of Sp1 is consistent with its participation in the tissue-specific expression of PAF-AH, as Sp1 is expressed at high levels in many of the tissues in which PAF-AH expression is found [15].

Sp1 can interact directly with the basal transcription complex [36] and it can also determine the transcription start site in TATA-less promoters [37]. To this end, Sp1 is thought to recruit the TATA-binding protein to the transcription initiation site, thus facilitating assembly of the basal transcriptional machinery [38]. Unlike Sp1, Sp3 can either activate or repress gene expression [39,40]. Sp3 has been reported to antagonize the stimulatory effect of Sp1 by competing for the same binding site [41]. Thus the nuclear level of Sp3 and/or the Sp1/Sp3 ratio can have important consequences in gene activation and silencing during development and differentiation [42-46]. Our data show that Sp1 and Sp3 act independently, not synergistically, to regulate PAF-AH expression (Figure 6C). Each individual factor stimulated PAF-AH promoter activity in SL2 cells that lack endogenous Sp family members. Moreover, the activation by Sp1 was not repressed by co-expression of Sp3 (Figure 6C). Our results are reminiscent of studies in which the transcriptional regulation of IL-10 (interleukin-10), another anti-inflammatory gene, was analysed. Tone et al. [47] demonstrated that both Sp1 and Sp3 stimulate IL-10 promoter activity. Interestingly, the TC-rich motif critical for basal IL-10 promoter activity is highly homologous to the TC-rich sequence found in site Sp-B of the mouse and human PAF-AH promoters ([47], and the present work).

In summary, we have established a key role for members of the Sp family of transcription factors in the regulation of plasma PAF-AH expression in mammals. Our results indicate that the basal promoters of the human and mouse PAF-AH genes are regulated in similar, yet not identical, manners. The non-canonical Sp1/Sp3-binding site Sp-B is essential for both promoters to

function. For the mouse promoter, full basal promoter activity requires another canonical Sp-binding site (Sp-A), while the human promoter seems to only rely on site Sp-B. Finally, we have shown that the Sp family of transcription factors participates in the differential expression of PAF-AH during maturation of monocytes to macrophages.

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