Allograft inflammatory factor-1 (Aif-1), a 17-kDa protein bearing an EF-hand Ca$^{2+}$ binding motif, increases markedly in monocytes and macrophages participating in allo- and autoimmune reactions, including the perivascular inflammation in transplanted hearts, microglial infiltrates in experimental autoimmune neuritis, and the inflamed pancreas of prediabetic BB rats. To investigate the mechanism of this regulation, we isolated the mouse aif-1 gene and determined its genomic organization. The gene has six exons distributed over 1.6 kilobases, an interferon γ-inducible DNase I-hypersensitive site near −900, and flanking sequences on either side predicted to associate with nuclear matrix. Reporter gene analyses identified sequences between −902 and −789, including consensus Ets and interferon regulatory factor elements, required for macrophage-specific and interferon γ-inducible transcriptional activity. Pu.1 bound to the Ets site in electromobility shift assay and forced expression of Pu.1 activated the aif-1 promoter in 3T3 fibroblasts, in which it is normally inactive. However, the transcriptional activity of a concatamer of the Ets site alone did not increase with interferon γ treatment. Cooperation between Pu.1 and proteins binding to the interferon regulatory factor element appears to be necessary for both macrophage-specific and interferon γ-inducible expression of the aif-1 gene.

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EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—RAW264.7 cells and 3T3 fibroblasts (American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 mg/ml). Cells were seeded at a density of 0.2–0.3 × 10$^6$ cells/ml. Cytokines were stored at −80 °C and used within 2 weeks of thawing.
reconstituted or diluted as recommended by the suppliers. Recombinant murine IFN-γ was purchased from Invitrogen. Recombinant human platelet-derived growth factor BB, IL-1β, IL-4, and IL-10 were obtained from Collaborative Biomedical, recombinant macrophage colony-stimulating factor was from Calbiochem, and recombinant human tumor necrosis factor-α from BioLegend. The 32P-labeled 2,5-O-dideoxy glucose was purchased from Sigma. Concentrations are indicated in the figure legends.

Isolation of Genomic Clones—A 489-bp aif-1 CDNA fragment was generated from murine total RNA derived from RAW264.7 cells by the reverse transcriptase PCR (8). Primer sequences, derived from the sequence found under GenBank™ accession number AF074959 were 5′-CCACGCTTAAGAACCCAGC-3′ (forward primer) and 5′-ACATCTCACCCTCAATCAG-3′ (reverse primer). This fragment was radiolabeled with [32P]dCTP and used to screen a mouse genomic DNA phage library in the vector AFIIIX (Stratagene) as described (9). Hybridizing clones were isolated and purified, and phage DNA was prepared according to standard procedures (9).

RNA Blot Hybridization—Total RNA was obtained from cultured cells by guanidinium isothiocyanate extraction followed by centrifugation through cesium chloride (8). The RNA was fractionated on 1.2% formaldehyde-agarose gels and transferred to nitrocellulose filters, which were then hybridized at 68 °C for 2 h to the random-primed, [32P]-labeled mouse aif-1 cDNA probe (10 000 cpm/ml) in QuikHyb solution (Stratagene). Filters were washed with 2× saline sodium citrate, 0.1% SDS at 55 °C and autoradiographed with Kodak XAR film for 48 h at −80 °C. A [32P]-end-labeled oligonucleotide (10) complementary to 28 S ribosomal RNA was hybridized to the filters to correct for differences in RNA loading. Phosphor screens were scanned, and radioactivity signal intensity was determined with the ImageQuant v1.1 software analysis program (Molecular Dynamics).

Primer Extension Analysis—Primer extension analysis was performed as described (8). A synthetic oligonucleotide primer (5′-ATCCTCGTTTGGCTCAT-3′) complementary to the 5′ end of the mouse aif-1 cDNA was end-labeled with [γ-32P]ATP and hybridized to 10 μg of each RNA sample; annealed samples were subjected to reverse transcription. Extension products were analyzed by electrophoresis on an 8% denaturing polyacrylamide gel. The same primer, unlabeled, was used in a sequencing reaction with [32P]-labeled dideoxynucleotide termination (Thermosequenase, Amersham Biosciences) to display the genomic sequence corresponding to the end of the primer extension product. Rapid amplification of 5′ ends (Invitrogen) was performed according to the manufacturer’s protocol, and the resultant cDNA clones were sequenced by 5′-labeled dideoxynucleotide chain termination.

DNase I Hypersensitivity Analysis—Cultured cells were grown to near confluence, washed with phosphate-buffered saline, and collected by scraping. Cells were treated with lysis buffer (10 mM Tris, 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40), and recovered nuclei were exposed to DNase I (Roche Molecular Biochemicals) at concentrations of 0, 0.1, 0.3, 0.5, 0.7, and 1 μg/ml at 37 °C for 10 min. Partially digested DNA was recovered and extracted with phenol-chloroform, dialyzed with XbaI overnight, and used in Southern analysis with a 5′-labeled genomic DNA probe encoding bases −2132 to −1641 relative to the transcription start site. The hybridized filter was washed in 30 mM sodium chloride, 3 mM sodium citrate, 0.1% SDS at 65 °C before autoradiography.

In Silico Analysis of the aif-1 Locus—To determine the likelihood of interaction of DNA in the vicinity of the aif-1 locus with the nuclear matrix, the genomic sequence (under GenBank™ accession number AF109719) from −6382 to +6257 relative to the transcription start site was analyzed using the MAR-finder program (15) (Dr. Gautam B. Singh, www.futuresoft.org). Analysis of the 5′-flanking sequence for transcription factor binding sites was performed via the TESS server at the University of Pennsylvania (www.cbil.upenn.edu/tess) using the Transfac data base (11).

Genetic Reporter Studies—RAW264.7 and 3T3 cells (50,000/well) were transfected with FuGENE 6 reagent (Roche Molecular Biochemicals) on 24-well plates according to the manufacturer’s protocol. In brief, up to 1 μg/well total plasmid DNA was used in the experiments, including 0.1 μg of a cytomegalovirus-β-galactosidase reporter (CLONTECH) for normalization of transfection efficiency. Transfected cells were harvested 24 h later, and luciferase and β-galactosidase activities were determined. Luciferase activity for each well was corrected for variable transfection efficiency by dividing by the respective β-galactosidase activity. All transfections were performed in triplicate in at least three independent experiments.

Plasmids—To determine promoter activity of genomic sequences from the aif-1 locus, a 3.1-kb genomic DNA fragment was amplified by PCR using KlenTaq (CLONTECH), forward primer (−2994 to −2970) 5′-GGTATAGGTGCTGGCTTGGAGA-3′, and reverse primer (−labeled to +65) 5′-ATTGGCCATGGCTCTCAAGGCG-3′, in which an NcoI site was introduced in-frame with the translation start site by a single base pair change of G (C) to T. The PCR product was digested with NcoI, and the fragment from −902 to +75 was cloned into the NcoI site in the pGLO3 basic luciferase reporter (Promega). This construct was digested with HindIII and PvuII, and the 5′ end was extended by ligation of the −3.4-kb HindIII/PvuII genomic fragment (bases −4081 to −569) to generate the aif-1 −4082/−75 luciferase reporter. Additional constructs were generated by digestion of this plasmid with restriction enzymes to yield aif-1 −1876/−76, aif-1 −1286/−75, and aif-1 −569/−75 luciferase.

The Ets mutant in aif-1 −902/−75 luciferase was generated by site-directed mutagenesis (QuikChange, Stratagene) using primers with forward and reverse complements of the sequence 5′-TGGGGAGACCTGGTTGTGCTGTCCTACGTTG-3′, in which the underlined bases replace the wild-type bases GAA to disrupt the Ets consensus binding site (AGGA). The STAT mutant was produced similarly using forward primer 5′-CTGTTTCTGCAAGTGTCTCTTCAAC-3′ and reverse primer 5′-ACACCTTCGACGAAAGAAATGGA-3′; the underlined bases replace the wild-type bases TCTC to disrupt the STAT consensus binding site (TTTCCTCAGA). The IRF mutant was generated with forward primer 5′-CTACTGCTCTTATCAGCTCCTTCTC-3′ and reverse primer 5′-GGATGTGCTGCTCCTACGTTG-3′. This construct replaced the wild-type bases TCT to disrupt the IRF binding site (CCTTCTCAGT). The aif-1 −902/−789 luciferase construct was generated by digestion of cloned aif-1 genomic DNA with NcoI, fill-in with Klenow enzyme, digestion with BglII, and ligation of the resultant fragment into the pGLO3 promoter vector cut with Smal and BgIII. The aif-1 −902/−1505 luciferase reporter was produced by the addition of an NcoI site between bases +1505 and +1510 by site-directed mutagenesis, digestion with NcoI, and ligation into NcoI-digested pGLO3 basic. The Ets 3X luciferase concatamer was produced by annealing primer TGGTGGGGACACAGGAGATCTGCTTG-CTGTTGCGGACGAAAGATGTC-CTCTGCTGCTGCTCCTACGTTG to its reverse complement followed by digestion with NcoI and XbaI to cut at restriction sites located at each end of the Ets binding site (Promega). Underlined bases denote the putative Ets binding sites. All constructs were confirmed by restriction digests and DNA sequencing. The following investigators generously provided plasmids: J. Leiden (Ets-1, Ets-2, Pu.1), N. Xu (STAT1), D. Levy (IRF-9), K. Ozato (IRF-8), and B.-Z. Levy (IRF-4).

Electromobility Shift Assays—Electromobility shift assays were performed as described (10) with modifications. The sense strand sequence of the DNA probe for the aif-1-Ets binding site (−894 to −880) is GGGACAGGAGTACG; the predicted consensus binding site is underlined. The forward sequence of the Pu.1 consensus probe is GGCGCGT-TGGAGAATTAAGAT. The sense strand sequence of the probe for the IRF site (−866 to −847) is TCTTGTGCCTGCACTTCTG. These probes and their reverse complements were end-labeled with [32P]dCTP and annealed to one another. Typical binding reactions contained double-stranded DNA probe (20,000 cpm), 1 μg of poly(dI-dC)poly(dI-dC), 50 mM NaCl, 50 mM Tris HCl, pH 7.5, 1 mM MgCl₂, 4% glycerol, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 4 μg of nuclear extract in a final volume of 20 μl. The specificity of binding interactions was assessed by competition with a 100-fold excess of unlabeled double-stranded oligonucleotide of identical sequence or the Pu.1 consensus binding site sequence. The antibodies specific for Pu.1 and Sp1 were purchased from Santa Cruz Biotechnology.

Data Analysis—Quantitative results presented are representative of findings from at least three independent experiments. Comparisons among groups were made by factorial analysis of variance followed by a Bonferroni/Dunn post-hoc analysis. Statistical significance was accepted for a p value <0.05.

RESULTS

aif-1 mRNA Is Robustly Induced by IFN-γ in Murine Macrophages—To identify cell types with different levels of aif-1 gene transcription, we assessed aif-1 expression by Northern analysis in rat aortic smooth muscle cells, 3T3 fibroblasts, and RAW264.7 murine macrophage-like cells. The smooth muscle cell and fibroblast samples showed no signal (Fig. 1A); stimulation of smooth muscle cells with a panel of growth factors and cytokines likewise yielded no evidence of aif-1 expression (data...
Regulation of the aif-1 Gene

A DNase I Hypersensitive Site in Stimulated Macrophages Lies −0.9 kb Upstream of the Transcription Start Site—We then performed DNase I hypersensitivity analysis (8) of the aif-1 locus to identify potential changes in chromatin conformation linked to its characteristic expression pattern. We analyzed 3T3 cells, which have little or no aif-1 expression (Fig. 1A), RAW264.7 cells, which have moderate expression, and RAW264.7 cells stimulated with IFN-γ, which robustly express aif-1 mRNA (Fig. 1B). As shown in Fig. 1B, the bands identified in the 3T3 DNA indicated that the entire aif-1 locus was not accessible to DNase I in our test conditions, as the size of band II was consistent with a hypersensitive site distal to the 3' end of the aif-1 gene. In contrast, analysis of the RAW264.7 cells showed a specific band (band III) that corresponds to the location of the aif-1 transcription start site. Moreover, after stimulation with IFN-γ, which increases aif-1 expression, a faster migrating band (band IV) appeared, consistent with the presence of an inducible hypersensitive site −0.9 kb upstream of the transcription start site.

We also analyzed −11 kb of sequence from the aif-1 locus in silico for potential association with the nuclear matrix using the MAR-finder program (Ref. 15, see also “Experimental Procedures”). The results of this analysis are presented in Fig. 3C. The exon-intron structure and DNase I hypersensitive sites described above are indicated as points of reference. This analysis provides an estimate of the probability of DNA association with the nuclear matrix based on the combinations of characteristic patterns in the sequence. The aif-1 locus, including the hypersensitive site near −0.9 kb and all 6 exons and 5 introns, coincides with an area of low probability of nuclear matrix association extending over −4 kb, which is flanked on both sides by regions of high matrix association potential. The relatively low probability of nuclear matrix association of the aif-1 locus, including the regions of DNase I hypersensitivity (bands III and IV), may facilitate access by the RNA polymerase II and associated proteins for transcription, thus contributing to its inducibility.

Positive Transcriptional Activity Localizes to Sequences between −902 and −789—Although matrix association probability indicated potential accessibility of sequences between −1.8 kb and +2.5 kb, the DNase I hypersensitivity analysis suggested the regulatory importance of sequences near −0.9 kb in the aif-1 locus. We analyzed the 5′-flanking sequences for potential consensus binding sites for transcription factors that
might contribute to its inducible expression in macrophages. As shown in Fig. 4, these sequences contain multiple sites that might interact with transcription factors associated with activation-associated and macrophage-specific gene expression, including an AP1 site near −920, a Pu.1 site near −888, a STAT site near −840, multiple Ets sites near −892, a Pu.1 site near −888, a STAT site near −892, and IRF-1 sites near −860 and −989. Sequences upstream of this area have predominately repressive effects, whereas sequences between −383 to −902 resulted in increased activity (Fig. 5). Taken together, testing of the 5′ sequences between −383 to −902 Contain Most of the Macrophage-specific Activity of the aif-1 Promoter.

In addition to sites upstream of the transcription start site, many genes contain important regulatory elements within introns. Our DNase I hypersensitivity analysis suggested that this was not the case with aif-1. To corroborate this, we also tested a reporter construct bearing the aif-1 locus from −902 to +1505 containing the first 5 exons, all 5 introns, and the coding portion of exon 6. As expected (Fig. 5C), the activity of this plasmid was similar to that of aif-1−902/+79, suggesting that there is little intronic contribution to regulation of aif-1 expression.

Sequences between −902 and −789 Contain Most of the Macrophage-specific Activity of the aif-1 Promoter—Removal of repressive regulatory elements may lead to a loss of cell-type specificity of a promoter. With this in mind, we tested the reporter constructs in 3T3 fibroblasts and compared their activities to that obtained in RAW264.7 macrophages. As shown in Fig. 6A, the activity of the 5′ deletion constructs in fibro-

Fig. 2. Determination of the aif-1 genomic organization. Panel A, primer extension mapping of the 5′ end of the mouse aif-1 transcript. Total RNA (50 μg) was used as template for reverse transcription reaction with a 32P-labeled aif-1-specific primer (+26 to +43). The same primer (unlabeled) was used with 32P-dideoxynucleotide termination to generate the accompanying (antisense) genomic sequence. RNAs tested were 3T3 (lane 1), mouse macrophage RAW264.7 (lane 2), and mouse macrophage RAW264.7 stimulated for 24 h with IFN-γ (300 μg/ml) (lane 3). Two thymidine residues corresponding to 5′ end of the major extension products are indicated as capitals, with the dominant residue in bold. Panel B, exon-intron structure of the mouse aif-1 gene. The transcription start site 78 bases upstream of the initiation methionine codon is set as +1 and is shown as a bold A. Exons are underlined and numbered I–VI, nucleotides are numbered on the left, and predicted amino acids are numbered on the right. Within the protein sequence, the putative EF-hand-like motifs (amino acids 58–69 and 95–106) are indicated in bold. An asterisk marks the termination codon.
blasts was close to or less than that of the promoterless pGL3 basic vector; in RAW264.7 cells, activity of the /H11002 902/79 construct was nearly 15-fold above baseline. Sequences between /H11002 902 and /H11002 789 increased the reporter gene activity only slightly in fibroblasts, whereas in the macrophage cells these sequences conferred activity again close to 15-fold greater than the control vector. These findings suggest that the aif-1 promoter is regulated primarily by the interaction of positive transcriptional regulatory factors present in macrophages with sequences between /H11002 902 and /H11002 789. These promoter elements are not active in 3T3 cells, suggesting that these factors are not expressed in the fibroblasts or that their activity is subject to repression by additional factors present in this cell type.

The Ets Site at /H11002 891 to /H11002 883 Is Required for Basal and IFN-/H9253/-inducible Promoter Activity in Macrophages and Binds Pu.1—These findings indicated the importance of cis-acting elements between /H11002 902 and /H11002 789 in the aif-1 promoter. Inspection of the sequence in this region revealed three potentially important elements, consensus binding sites for Ets (/H11002 891 to /H11002 883), IRF (/H11002 864 to /H11002 851), and STAT (-846 to /H11002 838). We anticipated that transcription factors binding these sites would work in a coordinate fashion to confer macrophage-specific and IFN-/H9253/-inducible activity on the promoter. To assess their functional significance, we mutated these sequences in the context of the /H11002 902 /H11001 79 reporter. As shown in Fig. 7A (upper panel), mutation of either the Ets site or the IRF site markedly impaired the activity of the promoter. In addition, whereas the wild-type aif-1 /H11002 902 /H11001 79 reporter was induced nearly 10-fold by IFN-/H9253 treatment, disruption of the Ets site or the IRF site completely eliminated this response. Contrary to our expectations, mutation of the consensus STAT site at /H11002 846 to /H11002 838 had only a slight effect on the overall promoter activity.

**Fig. 3.** DNase I hypersensitivity analysis and matrix association potential of the aif-1 locus. Panel A, cellular nuclei were exposed at 37 °C for 10 min to increasing amounts of DNase I. DNA was extracted, digested overnight with XbaI, electrophoresed through 0.8% agarose, transferred to a nylon filter, hybridized with a /32P-labeled DNA probe, and washed to high stringency. Panel B, schematic location of XbaI restriction and DNase I hypersensitive sites flanking the aif-1 locus. Hypersensitive sites corresponding to bands II, III, and IV and the position of the DNA probe (~2132 to ~1641) are indicated. Panel C, matrix association potential of the aif-1 locus. Genomic sequence from ~6382 to ~6257 relative to the aif-1 transcription start site was analyzed using the MAR-finder program (see “Experimental Procedures”). The aif-1 gene exon-intron structure (Fig. 2) and the location of DNase I hypersensitive sites (A and B) are depicted along the length of the sequence on the x axis. The predicted matrix association potential, on a scale of 0–1.0, is plotted on the y axis. bp, base pair.

**Fig. 4.** Aif-1 5'-flanking sequence, indicating cis-acting elements associated with macrophage-specific and inflammatory activation. Sequences between ~2085 and ~1000 are not shown, as indicated by the ellipsis. Consensus cis-acting elements are underlined, with putative interacting factors are denoted above the sequence. The transcription start site is in bold and labeled as /H11001 1.

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**Fig. 5.** Regulation of the aif-1 Gene
Regulation of the aif-1 Gene

FIG. 5. Reporter gene analysis of aif-1 promoter activity. RAW264.7 cells were transfected with a series of deletion constructs containing sequences from the aif-1 locus-driving expression of the luciferase reporter gene in the pGL3 basic and promoter plasmids. Luciferase activity was determined 24 h after transfection, corrected for variation in transfection efficiency by reference to the activity of a co-transfected β-galactosidase plasmid, and normalized relative to the activity of the relevant pGL3 vector containing no aif-1 sequences. Panel A, aif-1 deletions from the 5′ direction in the pGL3 basic reporter; panel B, aif-1 deletions from the 3′ direction in the pGL3 promoter reporter; panel C, aif-1 construct containing 0.9 kb of 5′-flanking sequences and 1.5 kb of exon-intron sequences in the pGL3 basic reporter.

and did not diminish its responsiveness to IFN-γ. Moreover, the Ets site alone was not sufficient to confer the response to IFN-γ, as activity of a concatamer of the Ets site actually decreased with IFN-γ treatment (Fig. 7A, lower panel). Thus in the context of the aif-1 −902/+79 promoter, the Ets and IRF sites are essential for both basal and IFN-γ-stimulated activity.

To identify the proteins binding to the Ets sites at −891 to −883 and the IRF site at −864 to −851, we performed electromobility shift assays with nuclear proteins extracted from 3T3 and RAW264.7 cells. Although the radiolabeled Ets probe was only 15 bases in length, we found multiple bands in both cell types, suggesting that this sequence is capable of interacting with multiple nuclear protein species. A prominent band generated by macrophage extracts (Fig. 7B, lanes 3 and 4, arrow) and, to a much lower degree, by the 3T3 extract (lane 1, arrow) was specifically inhibited by an excess of both unlabeled identical competitor and by an unlabeled Pu.1 consensus competitor (lanes 5 and 6, arrow). Incubation of the binding reactions with an antisem specific for Pu.1 resulted in a supershifted band in RAW264.7 cells (lane 7, asterisk). An antisem specific for the transcription factor Sp1, used here as a control, caused no change in nucleoprotein-probe migration. The identity of the supershifted band in RAW264.7 extracts with Pu.1 was confirmed by supershift analysis of 3T3 cells transfected with a Pu.1 cDNA expression plasmid (Fig. 7B, lanes 9 and 10).

The result of electromobility shift analysis with the probe derived from the aif-1 IRF site is shown in Fig. 7C. The slowest migrating band (band a) is specific, as indicated by its disappearance in the presence of unlabeled competing oligonucleotide, but is present in both 3T3 and RAW264.7 extracts. However, two faster migrating, specific bands are also seen; band b is present only in the RAW264.7 extracts (lanes 3 and 4), with slight increase in intensity with IFN-γ treatment (lane 4), and band c is seen only in the fibroblast extract (lane 2). We tested several antibodies directed against candidate IRF family proteins (IRF-1, IRF-4, IRF-8, and IRF-9), but these failed to yield a supershifted band (data not shown).

FIG. 6. Comparative activity of aif-1 promoter constructs in 3T3 and RAW264.7 cells. Aif-1 deletion constructs in the pGL3 basic and promoter plasmids were transfected into 3T3 and RAW264.7 cells, and reporter gene activities were determined after 24 h of incubation, as described for Fig. 5. Panel A, aif-1 deletions from the 5′ direction in the pGL3 basic vector; panel B, the minimal aif-1 −902/+79 construct in the pGL3 promoter vector.

Ecogenous Pu.1 Is Sufficient to Activate the Promoter in Both Macrophage and Non-macrophage Cells—To test the ability of candidate transcription factors to interact with the aif-1 promoter, we performed transient assays with expression plasmids for Ets-1, Ets-2, STAT1, and Pu.1. Ets-1 and -2 activated the aif-1 promoter modestly in RAW264.7 cells, whereas Pu.1 alone was sufficient to activate the promoter, analogous to its effect in the macrophage cell line. Consistent with the limited effect of mutation of the consensus STAT site, co-transfection of a STAT1 expression plasmid did not activate the promoter and had only a limited effect on the aif-1 promoter in these cells, whereas Pu.1 alone was sufficient to activate the promotor, analogous to its effect in the macrophage cell line. Consistent with the limited effect of mutation of the consensus STAT site, co-transfection of a STAT1 expression plasmid did not activate the promoter significantly in either RAW264.7 or 3T3 cells. Along with the promoter mutation studies (Fig. 7), these findings further support a mechanism in which Pu.1 acts together with a factor binding to the nearby IRF site. We then assessed the ability of candidate IRF proteins to drive the aif-1 promoter alone and together with Pu.1. Consistent with the supershift analysis described above and in contrast to our findings with Pu.1, IRF-1, IRF-4, IRF-8, and IRF-9 both singly and in combination did not activate the promoter and had only a limited effect when added with Pu.1.

DISCUSSION

Although Aif-1 has been identified in several different experimental systems (2–4, 7), it is selectively expressed by cells of
monocyte/macrophage lineage. aif-1 mRNA has been found by Northern analysis of samples from spleen (2, 3), testis (2, 3), and brain (2–4), with faint signals also present in lung and kidney (3). Because immunohistochemical analyses have typically localized Aif-1 expression to cells of the monocyte/macrophage lineage, its presence in these tissues is likely attributable to monocytes in residual blood or macrophages in tissue. One exception to this may occur in the testis, in which its expression by differentiating germ cells has been described (2). Because Aif-1 is selectively expressed in monocyte/macrophage cells and is specifically up-regulated in several disease models, characterization of the aif-1 gene, including transcriptional regulatory elements, may lead to better understanding of molecular mechanisms controlling macrophage-specific gene expression in both basal and activated states.

To define the organization of the aif-1 gene, we first mapped its transcription start site. Consistent with other genes preferentially expressed in macrophages, such as macrosialin (12) and FcγRIIIA (13), we found some variability in the location of transcription initiation (Fig. 2A), with the major start site occurring 78 bases upstream of the translation start site. Alignment of the aif-1 cDNA to genomic sequences from the aif-1 locus indicated a relatively compact gene, with 6 exons distributed over ~1.6 kb. This transcription start site, identified by primer extension and 5′-rapid amplification of 5′ ends and supported by RNase protection (data not shown), applies to the transcript published as aif-1, but it is likely that additional 5′ exons exist for some related transcripts, as BLAST searches using the 5′ sequence unique to iba-1 cDNA show partial alignment with genomic sequences ~0.7, 3.4, and 3.6 kb upstream of the aif-1 transcription start site we have defined.2 The assessment of matrix association potential (Fig. 3C) indicates that this entire aif-1 locus, including the upstream regulatory elements defined by DNase I hypersensitivity (Fig. 3, A and B) and reporter gene studies (Figs. 5–7), fits within a single uninterrupted region unlikely to associate with the nuclear matrix; additional 5′ exons in the iba-1 transcript from near 3.4 and 3.6 kb upstream would fall between the peaks of matrix association potential at 2.3 and 4.1 kb upstream of the aif-1 start site. How this potential for nuclear matrix association relates to the tissue-specific and cytokine-mediated regulation of aif-1 expression is not precisely understood at present.

The 5′-flanking sequence of the aif-1 gene has multiple consensus binding sites for transcription factors associated with both macrophage-specific and inflammatory expression, including NFκB, AP1, CCAAT/enhancer binding protein, IRF-1, and Ets. Identification of a DNase I hypersensitive site near −0.9 kb focused our attention on this region; the importance of this hypersensitive site was supported by 5′ and 3′ deletion analyses (Fig. 5), which localized positive transcriptional activity to sequences between −902 and −789. This region contained consensus binding sites for Ets (−883 to −891), IRF

2 N. Sibinga and M. Jain, unpublished observation.
Regulation of the aif-1 Gene

16209

(−851 to −864), and STAT (−838 to −846) proteins; we anticipated that the Ets site would be important in controlling macrophage specificity and that the IRF or STAT sites would support IFN-γ inducibility of the promoter.

Selective mutation of the Ets, IRF, and STAT sites between −883 and −838 in the aif-1 promoter demonstrated that the Ets and the IRF binding sites were required for both basal and IFN-γ-stimulated promoter function, whereas the STAT site was not important for either activity (Fig. 7). The interaction of the Ets family protein Pu.1 with the Ets site was supported by electromobility shift analysis (Fig. 7B), and co-transfection experiments (Fig. 8) indicated that Pu.1 and not Ets-1 or Ets-2 was sufficient to drive expression of the aif-1 −902/+79 promoter in both macrophage and non-macrophage cell types. Pu.1 (16), initially characterized by virtue of its interaction with the purine-rich 5′-GAGGAA-3′ site in the murine MHC class II I-Aβ gene (17), has important regulatory functions in expression of genes in the lymphoid and myeloid lineages. When Pu.1 is disrupted by gene targeting, no mature lymphocytes or macrophages develop, and neutrophils that survive show incomplete and aberrant maturation (18). This maturation defect is cell-autonomous, because expression of mature macrophage markers can be rescued by re-introduction of Pu.1 into Pu.1−/− hematopoietic precursors (19). Putative Pu.1 target genes expressed in macrophages include the colony stimulating factor 1 receptor (20), FcγRIIa (13), Mac1/CD11b (21, 24), and the macrophage scavenger receptor (22).

An intact IRF site was required, like the Ets site, for promoter activity in both basal and IFN-γ-stimulated conditions (Fig. 7A). Because Pu.1 activated the aif-1 promoter in both 3T3 fibroblasts and RAW264.7 macrophage cells, expression of the factor or factors binding to the IRF site is probably not restricted to the macrophage. Moreover, the Ets site in isolation was not sufficient (Fig. 7A, lower panel) to mediate the IFN-γ responsiveness characteristic of the larger promoter constructs. Taken together, these results suggest that endogenous Pu.1 in macrophages and exogenous Pu.1 forcibly expressed in 3T3 cells interacts with more broadly expressed transcription factors binding to the nearby IRF site. Full activity of other Pu.1-dependent promoters has been found in several cases to depend on additional factors, including members of the AP1 (22), E2A, and IRF (23) families, which interact either directly or through formation of a DNA-dependent ternary complex with Pu.1.

The IRF site at bases −854 to −861 fits the consensus binding sites for IRF, IFN-γ, and IFN-β. Electromobility shift analysis using a probe based on the IRF site sequence (Fig. 7C) identified three bands between the two cell types examined, one present in both 3T3 and RAW264.7 cells and one unique to each cell type. These band patterns are consistent with three different hypothetical mechanisms that could account for the aif-1 promoter regulation we have observed; they are 1) a negative regulatory activity present in both 3T3 and RAW264.7 cells (band a), which might be overcome specifically in macrophages by a positive activity restricted to macrophages and binding to a site not included in the IRF probe used here; 2) a positive regulatory binding activity restricted to RAW264.7 extracts that increases slightly in response to IFN-γ treatment (band b); and 3), a negative regulatory binding activity present in 3T3 cells but not in RAW264.7 (band c). Pu.1 is typically a positive regulator of transcription and with its restricted pattern of expression, could supply the positive activity required in the first of these mechanisms if band a in fact reflects the binding of a transcriptional repressor.

Of the candidate IRF proteins listed above, IRF-1 and IRF-9 are transcriptional activators and are expressed in many cell types (25, 26). IRF-8, on the other hand, is typically a repressor with expression restricted to cells of lymphoid and myeloid lineages (27). The related factor IRF-4 can function both as a repressor and activator, has been identified in myeloid as well as lymphoid cells, and has been shown to synergize with Pu.1 in activation of the IL-1β promoter (28). Thus, with the possible exception of IRF-4, which might act as a macrophage-restricted transcriptional activator along with Pu.1, these factors do not fit well with the mechanisms suggested by the electromobility shift results with the aif-1 IRF site probe.

We were not able to identify these IRF family members in cellular extracts that interacted with the site in electromobility supershift assays (data not shown). Although this finding might reflect technical limitations of the anti-IRF antibodies tested in electromobility shift assays or could stem from the requirement of some IRF proteins, including IRF-4 and IRF-8, for interaction with other proteins to bind DNA efficiently (23), it is consistent with the results of our functional assessments. As mentioned above, evaluation of expression plasmids for IRF proteins IRF-1, IRF-4, IRF-8, and IRF-9 by cotransfection with Pu.1 did not add significantly to the level of activation attained by Pu.1 alone.

Comparison of the aif-1 sequence encompassing the Pu.1 and IRF binding sites with previously described composite Pu.1/IRF motifs (28) reveals differences that may be significant; the aif-1 sites are separated by 21 bases, whereas the composite motifs have only 2 bases between the core elements. In addition, the orientation of the sites in the aif-1 sequence relative to one another differs from that in the composite elements. These characteristics of spacing and orientation may underlie the functional differences we have found in the responsiveness of the aif-1 sequences to added IRF proteins. The possibility that additional, perhaps novel or less well characterized members of the IRF family might be involved in the regulation of the aif-1 gene must also be considered. Further studies will be required to identify definitively the additional proteins that act together with Pu.1 to control transcription of the aif-1 gene by macrophages in inflammatory settings.

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