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Constitutive Expression of Murine Decay-Accelerating Factor 1 Is Controlled by the Transcription Factor Sp1

David M. Cauvi, Gabrielle Cauvi, and K. Michael Pollard

The complement regulatory protein decay-accelerating factor (DAF or CD55) protects host tissue from complement-mediated injury by inhibiting the classical and alternative complement pathways. Besides its role in complement regulation, DAF has also been shown to be a key player in T cell immunity. Modulation of DAF expression could therefore represent a critical regulatory mechanism in both innate and adaptive immune responses. To identify and characterize key transcriptional regulatory elements controlling mouse Daf1 expression, a 2.5-kb fragment corresponding to the 5′ flanking region of the mouse Daf1 gene was cloned. Sequence analysis showed that the mouse Daf1 promoter lacks conventional TATA and CCAAT boxes and displays a high guanine and cytosine content. RACE was used to identify one major and two minor transcription start sites 47, 20, and 17 bp upstream of the translational codon. Positive and negative regulatory regions were identified by transiently transfecting sequential 5′ deletion constructs of the 5′ flanking region into NIH/3T3, M12.4, and RAW264.7 cells. Mutational analyses of the promoter region combined with Sp1-specific ELISA showed that the transcription factor Sp1 is required for basal transcription and LPS-induced expression of the Daf1 gene. These findings provide new information on the regulation of the mouse Daf1 promoter and will facilitate further studies on the expression of Daf1 during immune responses. The Journal of Immunology, 2006, 177: 3837–3847.

DAF, decay-accelerating factor (DAF, CD55) is a member of the complement-regulatory protein family that protects cells from attack by autologous complement proteins (1). DAF functions to accelerate the dissociation of the preformed C3/C5 convertase complexes of both the classical and alternative pathways of complement, thus blocking complement activation (2). Two recent studies have shown that DAF also acts as a negative modulator of T cell immunity (3, 4) by limiting T cell hyperresponsiveness induced by alternative pathway C3 activation during T cell-APC interactions (4). Besides its major involvement in both the innate and adaptive immune systems, DAF also has noncomplement-related functions because it has been shown to be a receptor for several viral and bacterial pathogens (5), and more recently to be the cellular ligand for the leukocyte Ag CD97, a member of the epidermal growth factor family (6). The DAF-CD97 interaction appears to be involved in inflammatory responses (7, 8). DAF also has the capacity to act as a signal-transducing molecule in T cells (9) and monocytes (10) via interaction with src protein tyrosine kinases (11).

In humans, DAF is a GPI-anchored membrane glycoprotein encoded by a single gene which maps to q32 on chromosome 1 (12). It is widely expressed on the surface of all major circulating blood cells and numerous epithelial and endothelial cells (13, 14). The genomic structure of mouse DAF consists of two genes lying head-to-tail along chromosome 1 (15), with the Daf1 (Daf-GPI) gene located 5′ to the Daf2 (Daf-transmembrane) gene. Studies with Daf1 knockout mice have revealed that Daf1 is broadly expressed in most tissues, while Daf2 expression is restricted to the testis and CD11c-positive splenic dendritic cells (16, 17).

Expression of DAF is influenced in a number of ways. Constitutive expression can vary depending on tissue (16) and cell type (18). In the mouse, estrogen induces Daf1 expression in uterine tissue showing that the two mouse Daf genes can be independently regulated in a single tissue (19). In human cells, DAF expression is modulated by cytokines such as IL-1, IL-6, TNF-α, TGF-β1, and IFN-γ (20–22), prostaglandins like PGE2 (23), and tissue-specific factors (24). Although there is evidence that DAF mRNA stability can be affected by tissue-specific factors (24) and inflammation (25), a number of studies have suggested that the primary modulation of expression appears to be at the level of transcription (22–24, 26, 27).

The growing importance of DAF, not only as a key player in the innate immune system, but also as a critical component of T cell immunity, suggests that regulation of its expression may have significant effects on health and disease. The human DAF promoter has been identified, the transcription start site mapped, and regions of potential transcriptional regulation discussed (18, 28). In contrast, nothing is known regarding the promoter structure or mechanisms underlying the transcriptional regulation of murine Daf gene expression.

The aim of this report was to investigate the transcriptional regulation of the murine Daf1 promoter. To this end, a 2.5-kb genomic fragment of the Daf1 5′ flanking region was isolated, analyzed, and cloned into a luciferase-containing reporter vector. Transfection into murine embryo fibroblasts (NIH/3T3), mature B cells (M12.4), and macrophages (RAW264.7) demonstrated that this fragment contains a functional promoter capable of driving luciferase activity. Regions that positively and negatively regulate the Daf1 basal promoter activity were then identified using 5′ serial deletions of the 2.5-kb 5′ flanking region. The effect of sequence-specific mutations on transcription, as well as transcription...
factor binding to selected oligonucleotide sequences, were used to demonstrate that the transcription factor Sp1 plays a critical role in regulating the basal transcription of murine Daf1. Finally, Sp1 was shown to be important for LPS-enhanced expression of Daf1.

Materials and Methods

Cloning and sequence analysis

Genomic DNA was extracted from DBA/2 mice. PCRs were performed using KOI Hot start DNA polymerase (Novagen) under the following conditions: 94°C (30 s), 62°C (30 s), and 68°C (3 min). Primers were designed according to the mouse genome database (29): forward, 5′-GCATCTCGAGACACAAAACTCGCCAGCAC-3′ (XhoI site underlined) and reverse, 5′-CGATAAAGCTTACAGCAGACGAGACACG-3′ (HindIII site underlined). PCR products were separated using a 1.0% agarose gel, extracted, and then cloned into the pGEM3-basic vector (Promega). Sequencing, using an ABI PRISM 3100 sequencer, was performed in both directions with primers from the vector flanking sequence. DBA/2 genomic DNA sequence was identical to that of the C57BL/6 sequence (Ensembl gene ID no. ENSMUS00000026399). Genomatix’s ElDorado software (Genomatix Software) was used to identify the putative Daf1 promoter region and to search transcription factor databases to discover potential regulatory elements. Sequences showing over 85% similarity to reported transcription factor consensus sites were considered. BioEdit Sequence Alignment Editor Software (version 6.0.5) was used to align and compare the murine Daf1 sequence to those of rat, human, and chimpanzee.

Mapping of the transcription start site

Single-cell suspensions were obtained from DBA2/J spleen by mashing the organ in RPMI 1640 containing 10% FCS. Total RNA isolation was performed using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer’s protocol. RACE PCR was performed as directed in the GeneRacer kit protocol (Invitrogen Life Technologies). Reverse transcription was performed from 1 μg of total RNA using M-MLV reverse transcriptase (Tris-EDTA buffer; Ambion), and stored at −80°C. For real-time quantitative PCR, RNA was extracted with TRIzol reagent (Invitrogen Life Technologies). RNA was denatured at 65°C for 5 min, placed on ice, and reverse transcribed in a total volume of 20 μl using random hexamers, dNTPs, RNase inhibitor (RNAsenseOUT; Invitrogen Life Technologies), and 200 U of SuperScript III reverse transcriptase (Invitrogen Life Technologies). PCR was conducted under the following conditions: 94°C (30 s), 53°C (30 s), and 72°C (30 s) and products were separated using a 1.5% agarose gel and visualized by ethidium bromide. The following primers were used for amplification: Daf1 forward, 5′-CTTGAGCGCTTCTGAGATCT-3′ and Daf1 reverse, 5′-GTTAAGATCCTGTCGAGCCAGCAAACT-3′ and β-actin forward, 5′-GTTGAGCCCTTCATAGTCA-3′ and β-actin reverse, 5′-GTGATGAAA CCAGAGCTGTTCCAG-3′. For real-time quantitative PCR, RNA was extracted with TRIzol reagent (Invitrogen Life Technologies) from NIH/3T3, M12.4, and RAW 264.7 cells exposed to 10 μg/ml LPS (Sigma-Aldrich) or medium alone for 24 h. Reverse transcription was then performed from 1 μg of RNA as described above and the resulting cDNA was diluted 10-fold in 10 mM Tris (pH 8.0) containing 1 mM EDTA (Tris-EDTA buffer; Ambion), and stored at −80°C.

Promoter constructs

Various deletion constructs of the murine Daf1 promoter were generated from DBA/2 genomic DNA by PCR amplification using oligonucleotide primers (summarized in Table I) containing XhoI (forward) and HindIII (reverse) restriction enzyme sites for cloning purposes. PCR products were separated on agarose gels, digested with XhoI and HindIII enzymes, and then cloned into the pGEM3-basic vector (Promega). Substitution mutations were performed by using two rounds of PCR amplification. For example: Sp1-A mutant was produced by using the PCR product from oligonucleotides p(−619/+85)/RSp1-A mut as template in a second-stage PCR using primers p(−619/+85)/pSp1-A mut and pRev as template in a second-stage PCR using primers p(−619/+85)/pSp1-A mut and pRev. All mutants were prepared by using a similar strategy. All plasmids were purified using an endotoxin-free plasmid kit (Qiagen). The sequence of all constructs was confirmed using an ABI PRISM 3100 sequencer.

Table 1. Sequence of PCR oligonucleotides used in this study to generate deletion and mutant constructs*

| Name | Orientation | Construct | Oligonucleotide Sequence |
|------|-------------|-----------|--------------------------|
| p(−2408/+85) | Forward | Deletion | 5′-GCATCTCGAGCAACAAAACTCGCCAGCAC-3′ |
| p(−1746/+85) | Forward | Deletion | 5′-GCATCTCGAGGAATCTTGAACAGACAGAAG-3′ |
| p(−1104/+85) | Forward | Deletion | 5′-GCATCTCGAGACGCCACCAAAAG-3′ |
| p(−619/+85) | Forward | Deletion | 5′-GCATCTCGAGCTAAAGGATGACCTTCC-3′ |
| p(−337/+85) | Forward | Deletion | 5′-GCATCTCGAGCTACAGCAACACAAG-3′ |
| p(−179/+85) | Forward | Deletion | 5′-GCATCTCGAGCTTGAGTGAAGTGAAG-3′ |
| pRev | Reverse | Deletion | 5′-GCATCTCGAGCTTGGAGTGAAGTGAAG-3′ |
| pSp1-A mut | Forward | Mutation | 5′-GCTGAGGAAGGCAAGACATCGTACTTAAAGGGAGGAAG-3′ |
| RSp1-A mut | Reverse | Mutation | 5′-GCTGAGGAAGGCAAGACATCGTACTTAAAGGGAGGAAG-3′ |
| pSp1-B mut | Forward | Mutation | 5′-GCTGAGGAAGGCAAGACATCGTACTTAAAGGGAGGAAG-3′ |
| RSp1-B mut | Reverse | Mutation | 5′-GCTGAGGAAGGCAAGACATCGTACTTAAAGGGAGGAAG-3′ |
| pSp1-C mut | Forward | Mutation | 5′-GGGCTTCCTGGGTGCCATATGCCTACGCTGCGGAG-3′ |
| RSp1-C mut | Reverse | Mutation | 5′-GGGCTTCCTGGGTGCCATATGCCTACGCTGCGGAG-3′ |
| FC REB mut | Forward | Mutation | 5′-ATTTGAGACGTGGTGCAGATGGGATCGGAGGCCAGGAGGACTGC-3′ |
| RCREB mut | Reverse | Mutation | 5′-ATTTGAGACGTGGTGCAGATGGGATCGGAGGCCAGGAGGACTGC-3′ |
| FC REB/Spl mut | Forward | Mutation | 5′-GCAAGGAGCCGATGACATCCGCTTGGGGACAG-3′ |
| RCREB/Spl mut | Reverse | Mutation | 5′-GCAAGGAGCCGATGACATCCGCTTGGGGACAG-3′ |

* Oligonucleotide sequences corresponding to the template cDNA are represented in uppercase. Mutations are depicted in bold lowercase. XhoI and HindIII restriction enzyme sites are single or double underlined, respectively.
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Cells were plated the day before transfection in 12-well plates at 2.1 \times 10^5 cells/well for NIH/3T3 cells and in 24-well plates at 2.5 \times 10^5 cells/well for M12.4 and RAW 264.7 cells. Cells were washed twice with PBS and transfected with various firefly luciferase reporter vectors using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer’s instructions with a 10:1 Lipofectamine 2000-to-DNA ratio. pRL-TK vector (Promega) encoding Renilla luciferase was added in each transfection as an internal control plasmid. After 48 h of incubation, cells were harvested, lysed with Passive Lysis Buffer (Promega), and promoter activities were determined using the Dual-Luciferase Assay System (Promega).

When required, cells were stimulated 24 h posttransfection with 10 \mu M LPS. After an additional 24 h, cells were harvested and promoter activities were analyzed as described above. Luciferase activities were measured with the Clarity Luminescence Microplate Reader (Bio-Tek Instruments) and firefly luciferase activities were normalized to Renilla luciferase activities.

SL2 cells were plated the day before transfection at 2.5 \times 10^5 cells/well in 24-well plates and transfected as described above with various firefly luciferase reporter vectors along with different amounts of either the empty vector pPac or the Sp1-containing vector pPacSp1. Firefly and Renilla luciferase activities were measured as described above. Both pPac and pPacSp1 were provided by Prof. G. Suske (Philips University, Marburg, Germany).

Real-time quantitative PCR
DaF1 and cyclophilin A primers and probes were designed with Beacon designer 3.0 software (Premier Biosoft International). The primers used were as follows: DaF1 forward primer, 5'-CTTGGACATTGTTGCAAT GTGA-3'; DaF1 reverse primer, 5'-TCCATTCTCTTCTGACGAC TCT-3'; cyclophilin A forward primer, 5'-GGGCCGATGACGACGCCC-3'; cyclophilin A reverse primer, 5'-GTGGTTGGAATTGTTCGTC-3'. The following dual-labeled probes were obtained from Integrated DNA Technologies: DaF1, 5’-FAM-CTTGGACATTGTTGCAAT GTGA-BHQ1-3’; cyclophilin A, 5’-FAM-TGGGCCCAGCTTCTCTGAGBHQ1-3’. Cyclophilin A standards were cloned into pGEMTeasy (Promega) and a standard curve was generated for each experiment. All samples and standards were analyzed in triplicate. PCR amplification were performed in a total volume of 25 \mu l containing 1.0 mM Tris-HCl, 5 mM KCl, 200 \mu M dNTPs, 100 ng of forward and reverse primers, 4 mM MgCl₂, 0.625 U of AmpliTaq Gold (Applied Biosystems), and 2.5 \mu l DaF1 or cyclophilin A dual-labeled probes. The reactions conditions were 95°C for 10 min followed by 45 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C and conducted using the iCycler iQ (Bio-Rad).

Transcription factor ELISA
Transcription factor ELISA were conducted according to Hibma et al. (30) with some modifications. Both parent and complementary single-strand oligonucleotides encompassing the wild-type or mutated Sp1-binding site consensus sequences were obtained from Integrated DNA Technologies (Table II). Double-strand oligonucleotides were obtained by annealing either the biotinylated or the nonbiotinylated parent strand with the complementary strand by heating at 100°C for 10 min in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA (pH 7.8)) and gradual cooling to 25°C. Nuclear extracts were prepared from NIH/3T3, M12.4, and RAW264.7 cells using the NE-PER Extraction Reagents (Pierce) supplemented with the Halt Protease Inhibitor mixture (Pierce) according to the manufacturer’s instructions. Protein concentration of the nuclear extract was determined using Bio-Rad protein assay, rSp1 was purchased from Promega. Immulon 2B immunoassay plates (Thermo) were coated overnight at 37°C with 100 \mu l of a 10 \mu g/ml solution of ImmunoPure Streptavidin (Pierce) resuspended in distilled water. Plates were washed three times at room temperature (RT) for 5 min with PBST. Each well was blocked for 1 h with 3% BSA diluted in 12.5 mM Tris-HCl (pH 7.6), 1.25 mM MgCl₂, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT (freshly added), and 4% glycerol (binding buffer). Subsequently, 100 \mu l of a 1 \mu M solution of double-strand biotinylated wild-type or mutant oligonucleotides were added per well and allowed to attach for 30 min at 37°C on a shaking platform. Either nuclear extract or rSp1 diluted in the binding buffer supplemented with 0.05 mM double-strand poly(dI-dC) (Amersham Biosciences) were added and incubated for 1 h at RT. In competition assays, rSp1 was preincubated with increasing amounts of nonbiotinylated oligonucleotide for 45 min at RT and this mixture was then added to the well coated with the biotinylated wild-type oligonucleotide for 1 h at RT. Plates were washed three times at RT for 5 min with PBST. For detection, 100 ng/well of either anti-Sp1 (rabbit polyclonal; Upstate Biotechnology) Ab or an irrelevant Ab (rabbit polyclonal) diluted in binding buffer was added and incubated for 1 h at RT. After washing, an HRP-conjugated anti-rabbit Ab diluted in binding buffer was added and incubated for 45 min at RT. Wells were washed three times at RT for 5 min with PBST and, finally, 100 \mu l of ABTS substrate was added per well and color development was measured at 405 nm using a Vmax microplate reader (Molecular Devices).

Results
Cloning and analysis of the 5’-flanking region of the mouse DaF1 gene
A genomic fragment of ~2.4 kb from the ATG codon corresponding to the 5’-flanking region of the DaF1 gene was cloned. Genomatix’s El Dorado software was used to identify a 600 nt promoter region consisting of ~515 residues upstream and +85 residues downstream of the initiating ATG codon, as well as potentially important regulatory elements representing consensus-binding sites of known transcription factors (Fig. 1). Sequence analysis also revealed that the putative DaF1 promoter lacked conventional TATA and CCAAT boxes. The guanine and cytosine (GC) content of the −2408 to +85 bp sequence was 46% but increased to 58% for the −644 to +85 bp sequence and reached 65% for the −300 to +85 bp sequence. Four potential Sp1-binding sites, at positions −153 to −147, −109 to −103, −84 to −76, and +10 to +16 bp, were found embedded within this latter GC-rich region (Fig. 1).

Identification of mouse DaF1 transcription start site
TATA-less GC-rich promoters often contain multiple transcriptional start sites (31). To characterize the DaF1 transcription start site(s), we performed RACE on total cellular RNA from mouse splenocytes. The resulting PCR product (Fig. 2A) was cloned into a pCR4-TOPO vector and sequenced. Ten of 12 clones ended in the sequence AAAACAG (47 bp upstream of the ATG start codon) and consequently the terminal nucleotide A was designated as the major transcriptional start site (Fig. 2B). The two other clones terminated with the sequence GTTCTCT and CTCTTCT.

Table II. Sequence of oligonucleotides used in transcription factor ELISA

| Oligonucleotide | Parent     | Nonbiotinylated  | Complementary | Biotinylated  |
|-----------------|------------|------------------|---------------|--------------|
| wtSp1-B         | Parent     | Nonbiotinylated  | 5’-CCAGGCTCCTGCTCCCCACCCAGGTTGCAAGAG-3’ | 5’-Bior-CCAGGCTCCTGCTCCCCACCCAGGTTGCAAGAG-3’ |
| wtSp1-B         | Complementary | Nonbiotinylated  | 5’-CTGTCACCTCAGGCTGATGGCAGACCTGTCG-3’ | 5’-Bior-CTGTCACCTCAGGCTGATGGCAGACCTGTCG-3’ |
| mutSp1-B        | Parent     | Nonbiotinylated  | 5’-AGGAGCCCTGCTCCCCACCCAGGTTGCAAGAG-3’ | 5’-Bior-AGGAGCCCTGCTCCCCACCCAGGTTGCAAGAG-3’ |
| mutSp1-B        | Complementary | Nonbiotinylated  | 5’-GCTGTGCGAGGGCCGAGGAGCCCAAGC-3’ | 5’-Bior-GCTGTGCGAGGGCCGAGGAGCCCAAGC-3’ |

* Oligonucleotide sequences corresponding to the template cDNA are represented in uppercase. Mutations are depicted in bold lowercase.
(-20 and -17 bp from the ATG codon, respectively). These findings differ from a previous study (15) which identified the Daf1 transcription start 15 bp upstream of the translational ATG codon.

Identification of transcriptional regulatory regions of mouse Daf1 gene

Although DAF is expressed in virtually all cells, the level of expression can vary and this property has been used in transcriptional activity studies of the human DAF promoter (18). To help identify possible differences in transcriptional activity of Daf1 expression, we used three murine cell lines with different levels of expression (Fig. 3 A). The highest level of Daf1 transcript was observed in NIH/3T3 cells, followed by M12.4 cells, with RAW264.7 cells having the lowest expression.

To determine whether the 2.5-kb fragment of the 5’ flanking region of mouse Daf1 (Fig. 1) contained the Daf1 promoter, we cloned the fragment, designated p(2408/85)Luc, into the promoterless pGL3 reporter vector encoding the firefly luciferase. Either this construct or the control pGL3 vector were transfected in NIH/3T3, M12.4, and RAW264.7 cells along with a vector encoding the Renilla luciferase as a transfection internal control. In all three cell lines, the 2493-bp DNA fragment was fully capable of inducing luciferase activity (Fig. 3 B), indicating that the Daf1 basal promoter activity is contained within this fragment. To further define the region influencing Daf1 gene expression, a series of 5’-deletion constructs of the p(-2408/+85)Luc was generated (Fig. 4A), and expressed as percent activity relative to the p(-2408/+85)Luc luciferase activity (Fig. 4B). Deletion of 660 bp (p(-1746/+85)Luc) or 1302 bp (p(-1104/+85)Luc) did not produce any significant changes in relative luciferase activities. Further, deletion to position -619 (p(-619/+85)Luc) resulted in an increase of the promoter activity in all three cell lines suggesting the presence of negative regulatory element(s) within the -1104- to -619-bp region. The magnitude of the increase was much larger in RAW264.7 cells (193.5%) than in NIH/3T3 or M12.4 cells (74 and 109.4%, respectively). Additional deletions of 282 bp (p(-337/+85)Luc) and 440 bp (p(-179/+85)Luc) resulted in a gradual decrease of the promoter activity to a level comparable to the full-length construct, p(-2408/+85)Luc, indicating that the region between -619 and -179 bp contains positive regulatory element(s) involved in Daf1 promoter activity. However, the relative luciferase activity obtained with the p(-179/+85)Luc construct was still elevated, suggesting that regulatory elements involved in basal transcription of the Daf1 promoter were likely to be contained within this region. This was confirmed with the next deletion to position -18 (p(-18/+85)Luc), as the relative luciferase activities observed with this construct and the control pGL3 vector were almost indistinguishable (Fig. 4B) in all cell lines tested.

Alignment of the -179- to +85-bp murine sequence with that of the rat, human, and chimpanzee was done using the BioEdit Sequence Alignment Editor Software (version 6.0.5) (Fig. 4C). This sequence contains one Ikaros 1 site (-137 to -128), one CREB site (-98 to -91), and four Sp1-binding sites at positions
transcription was performed using random primers. Nested PCR was also performed using a forward GeneRacer 5' primer and a reverse Daf1-specific primer. Total RNA was extracted from mouse spleen tissue and reverse transcription was performed using random primers. Twelve clones were then sequenced and aligned with the sequence retrieved in Fig. 1. B. Transcription starts are represented by broken arrows and the number of clones is shown in parentheses.

Basal murine Daf1 gene expression is regulated through two Sp1-binding sites

The transcription factor Sp1 is often involved in the basal transcription of TATA-less genes and consequently has been shown to be a key element in the transcription of many housekeeping genes (32, 33). To determine whether Sp1 was a key cis-acting transcription factor in the regulation of Daf1 gene transcription, we used D. melanogaster Schneider SL2 cells, known to be deficient in endogenous Sp factors (34). SL2 were transfected with pGL3, p(-18/+85)Luc, p(-179/+85)Luc, p(-619/+85)Luc, and the full-length p(-2408/+85)Luc vectors along with the Renilla luciferase containing vector pRL-TK. Interestingly, none of the promoter 5'-deletion constructs produced luciferase activity, indicating that Sp transcription factors are required for Daf1 promoter activity (Fig. 5). The same Daf1 luciferase reporter vectors were then cotransfected with increasing amounts of pPacSp1, a vector encoding the Sp1 protein. Cotransfection of p(-18/+85)Luc with pPacSp1 failed to produce luciferase activity above the threshold observed in the absence of pPacSp1 (Fig. 5), confirming that the Sp1 site D located at position +10 to +16 is not involved in basal transcription of Daf1. In contrast, when p(-179/+85)Luc was cotransfected with 100 ng of pPacSp1, the relative luciferase activity increased by 265% (Fig. 5), showing that Sp1 binding to one or more of the three Sp1 sites located between -179 and -18 bp (Fig. 4C) is required for basal Daf1 gene transcription. Similar results were obtained with the p(-619/+85)Luc and p(-2408/+85)Luc constructs, displaying increases of 32% and 331%, respectively, when cotransfected with 100 ng of pPacSp1 (Fig. 5).

To further investigate the contribution of each Sp1-binding site to Daf1 transcriptional regulation, we performed mutational analysis of the p(-619/+85)Luc construct in which one, two, or all three Sp1-binding sites were mutated. Mutation of the Sp1-binding sites A or B, or double mutation of both sites A and B (Sp1-A/B mutant) did not produce significant change in luciferase activity compared with the wild-type p(-619/+85)Luc construct in all three cell lines (Fig. 6A). However, mutation within the Sp1-C site significantly reduced the luciferase activity by 46.1% in NIH/3T3, 41.6% in M12.4 and 52.7% in RAW264.7 (all three cell lines, p < 0.01) compared with the p(-619/+85)Luc construct suggesting that Sp1 binding to site C contributes significantly to basal transcription of Daf1. Similar reduction in the luciferase activities was observed with the Sp1-A/C double mutant. A more dramatic decrease in the promoter activity was detected using the Sp1-B/C double mutant as the luciferase activities obtained with this construct only corresponded to 14.52% in NIH/3T3, 15.27% in M12.4 and 52.7% in RAW264.7 (all three cell lines, p < 0.01) compared with the p(-619/+85)Luc construct suggesting that Sp1 binding to site C contributes significantly to basal transcription of Daf1. Similar reduction in the luciferase activities was observed with the Sp1-A/C double mutant. A more dramatic decrease in the promoter activity was detected using the Sp1-B/C double mutant as the luciferase activities obtained with this construct only corresponded to 14.52% in NIH/3T3, 15.27% in M12.4, and 19% in RAW264.7 of the luciferase activity observed with the p(-619/+85)Luc construct. Finally, minimal luciferase activities were obtained in all three cell lines using the triple mutant Sp1-A/B/C, although the difference between this construct and the Sp1-B/C double mutant was only significant in the NIH/3T3 and M12.4 (p < 0.01 and p = 0.023, respectively) but not in RAW264.7 (p = 0.093).

To confirm these findings, we transiently transfected the same deletion mutant constructs into Sp1-deficient Schneider SL2 cells
with or without cotransfection with the Sp1-containing vector, pPacSp1. The luciferase activity of the p(-619/+85)Luc vector showed an ~3-fold increase when cotransfected with the pPacSp1 plasmid, while luciferase activities of both pGL3 control vector and p(-619/+85)Luc construct remained unchanged (Fig. 6B). As expected, when cotransfected with pPacSp1, the increase obtained with Sp1-A mutant was very similar to that observed with p(-619/+85)Luc vector while it was reduced with both Sp1-C and Sp1-A/C mutants and nearly abolished by either Sp1-B/C or Sp1-A/B/C mutants. However, mutations in Sp1-B and Sp1-A/A/C mutants significantly decreased the Sp1 effect ($p < 0.01$ and $p = 0.0175$, respectively) compared with the p(-619/+85)Luc vector indicating that mutations in Sp1-binding site B might also affect Dafl promoter activity. This result differs from that obtained in Fig. 6A because, in all the cell lines tested, Sp1-B by itself did not appear to be involved in the Dafl promoter activity. Taken together, this result indicates that the basal Dafl promoter activity is mainly regulated by Sp1-binding sites B and C acting in a synergistic manner.

The transcription factor Sp1 associates with both B and C Sp1-binding sites

To investigate whether Sp1 physically binds to the Sp1-binding sites B ($-109$ to $-103$) and C ($-84$ to $-76$), we performed a transcription factor ELISA (TF-ELISA) using biotinylated dsDNA containing either the Sp1-binding sites B (Oligo B, Fig. 7A) or C (Oligo C, Fig. 7B). Representative TF-ELISA results depicted in Fig. 7, A and B, showed that Sp1 binding to both Sp1-binding sites B and C increased proportionally with the amount of nuclear extract from all three cell lines. For both oligo B and C, the OD405 obtained when using nuclear extract from NIH/3T3 cells was always higher than the OD405 recorded with nuclear extracts from M12.4 and RAW264.7 cells suggesting the presence of a higher amount of Sp1 in NIH/3T3 cells (Fig. 7, A and B). Dose response assays were also conducted using increasing amount of rSp1 and, similar to the results obtained with the nuclear extracts, Sp1-DNA
binding increased proportionally with the amount of rSp1 added (Fig. 7C). Moreover, binding to Sp1 site C was always higher than Sp1 site B for all amounts of rSp1 tested suggesting that Sp1 shows a higher affinity for the Sp1-binding site C (−84 to −76).

To confirm the specificity of Sp1 binding, we performed TF-ELISA using mutated Sp1-binding oligonucleotides and showed that mutation of both the Sp1-binding sites B and C greatly reduced the Sp1-binding affinity compared with the wild-type oligonucleotides (Fig. 7D). We also performed competition experiments in which a gradual decrease in Sp1-binding activity was observed with the addition of increasing amounts of competitor oligonucleotides (Fig. 7E). Taken together, these data demonstrate that the transcription factor Sp1 is capable of binding to the Sp1-binding sites B and C, which are located at the transcriptional start site of mouse Daf1 (Fig. 1). This CREB-binding site showed a high degree of sequence conservation between human and rodent species (Fig. 4C). Therefore, to evaluate the importance of the CREB-binding sequence in the murine Daf1 promoter activity, we prepared p(−619/+85)Luc mutant constructs in which the CREB-binding site was either modified alone or in conjunction with all three Sp1-binding sites. Mutations within the CREB-binding site reduced luciferase activity by 46.7, 44.3, and 40.7%, respectively, in NIH/3T3, M12.4, and RAW264.7 cells relative to the wild-type p(−619/+85)Luc construct (Fig. 8). When the CREB-binding site was mutated together with all three Sp1-binding sites (Sp1-A/B/C), a significant decrease in promoter activity, relative to the Sp1-A/B/C construct \((p < 0.01)\) for all three cell lines, was observed suggesting a functional cooperation between Sp1 and CREB transcription factors.

**LPS effect on Daf1 gene expression is abrogated by mutations within Sp1-binding sites**

Several previous studies have shown that Daf1 mRNA can be up-regulated by LPS (36–38), however, little is known about the regulation of Daf1 gene by LPS in the murine system. NIH/3T3, M12.4, and RAW264.7 cells were cultured with 10 μg/ml LPS for 24 h and Daf1 determined by quantitative real-time PCR. As expected, the level of Daf1 mRNA in untreated (control) NIH/3T3 cells was much higher than in M12.4 and RAW264.7 cells (data not shown) confirming the PCR results shown in Fig. 3A. In NIH/3T3 cells, LPS treatment did not significantly change the level of Daf1 mRNA while it increased the level by 1.85- and 2.32-fold in M12.4 and RAW264.7 cells, respectively (Fig. 9A). It has been
shown previously that NIH/3T3 cells lack an intact LPS-signaling pathway (39) which may explain why LPS did not affect Daf1 gene expression in these cells. We then transiently transfected the p(–2408/+85)Luc vector into the three cell lines and 24 h later cells were treated for an additional 24 h with 10 μg/ml LPS followed by a dual luciferase assay. The normalized luciferase activity was not affected by LPS treatment in NIH/3T3 cells but was increased in both M12.4 and RAW264.7 cells by 1.97- and 1.85-fold, respectively, matching the data obtained by real-time PCR on the Daf1 mRNA levels (Fig. 9B). To investigate whether the LPS effect was dependent on the presence of functional Sp1-binding sites, we transfected either the wild-type p(–619/+85)Luc construct or the corresponding Sp1-B/C double and Sp1-A/B/C triple mutants into all three cell lines for 24 h followed by an additional 24 h stimulation with 10 μg/ml LPS. In accordance with the data presented in Fig. 9, A and B, no LPS effect was detected for all the indicated constructs in the NIH/3T3 cell line. However, in M12.4 and RAW264.7 cells transfected with the p(–619/+85)Luc construct, LPS treatment increased the normalized luciferase activities by 1.82- and 1.95-fold, respectively, and because no LPS effect was observed in p(–18/+85)Luc-transfected cells (Fig. 9C), this would suggest that the region between –619 and –18 bp contains LPS-responsive element(s). Interestingly, inhibition of Sp1 binding by mutating either Sp1-binding sites B and C (Sp1-B/C) or A, B, and C (Sp1-A/B/C) completely abolished the LPS effect observed with the wild-type p(–619/+85)Luc construct suggesting that Sp1 binding to both Sp1-binding sites B (–109 to –103) and C (–84 to –76) is required for LPS-induced Daf1 transcription.

Discussion
To characterize key regulatory elements involved in the basal transcription of the mouse Daf1 gene, we cloned and analyzed a 2.5-kb genomic fragment corresponding to the 5′-flanking region of the Daf1 gene. Analysis of this fragment revealed that, like its human counterpart, the Daf1 promoter lacks conventional TATA and
M12.4, and RAW264.7 cells stimulated with 100 ng/ml LPS. Luciferase activities were then measured and normalized for transfection efficiency. Data (mean ± SD) from triplicate experiments is expressed as fold change over non-LPS treated controls. B, The full-length p(−2408/+85)Luc construct was transiently transfected into the indicated cell lines along with the transfection control vector pRL-TK and 24 h later cells were treated or not for an additional 24 h with 10 μg/ml LPS. Luciferase activities were then measured and normalized for transfection efficiency. Data (mean ± SD) are from triplicate transfections expressed as relative luciferase activity. C, NIH/3T3, M12.4, and RAW264.7 cells were transiently transfected with the indicated constructs and 24 h later, cells were treated or not for an additional 24 h with 10 μg/ml LPS. Luciferase activities were then measured and normalized for transfection efficiency. Data (mean ± SD) are from triplicate transfections expressed as fold change corresponding to the ratio of normalized luciferase activities obtained from LPS treated cells to the normalized luciferase activities recorded in nontreated cells.

CCAAT boxes (18). Absence of a consensus TATA box is a feature found in the promoters of other complement regulatory proteins including the membrane cofactor protein (CD46) (40), CD59 (41), CR1/CD35 (42), C1 inhibitor (43), C4b-binding protein (44), factor H (45), and factor I (46). We also found that the mouse Daf1 promoter contains a GC-rich domain which, in agreement with previous studies, shows that promoters lacking a TATA box often contain GC-rich regions proximal to their transcription start site (31, 47). Additionally, it has been shown that TATA-less GC-rich promoters are predominantly found in genes that are ubiquitously expressed, particularly those termed housekeeping genes (31). The mouse Daf1 gene is found to be widely expressed not only in hematopoietic cells but also in a wide variety of other tissues (16, 17). Previous reports have also indicated that the promoters of other members of the complement regulatory protein family, including CD46 (40), CD59 (41), and C1 inhibitor (43), are GC rich as well as being TATA-less.

It has been previously shown that GC-rich promoters that lack a TATA box display multiple transcription start sites (31). RACE analysis, performed on total RNA isolated from DBA/2 splenocytes, identified three different sites of transcription initiation situated 47, 20, and 17 bp upstream of the translational start codon. However, in 10 of 12 clones (~85%), transcription was initiated 47 bp upstream of the ATG codon thus defining the major transcription start site. A previous report described the transcription start of the murine Daf1 gene as 15 bp upstream of the ATG codon (15). The discrepancy between data presented here and the previous report may be explained by the origin of the total RNA which was, in the first study, isolated from the testes of C57BL/6J mice. In the human DAF gene, Ewulonu et al. (28) showed that the main transcription start site was located 82 bp upstream of the ATG initiation codon, while Thomas and Lublin (18) found multiple transcription start sites mapped in a stretch of 10 bp located 87 bp upstream of the translational start codon.

Deletion analysis of the 2.5-kb genomic fragment corresponding to the 5′-flanking region of the Daf1 gene revealed that gene expression is modulated by both negative and positive regulatory elements located between −1104 to −619 bp and −619 to −18 bp from the ATG codon, respectively. What transcription factor(s) exert a negative regulatory function on the mouse Daf1 promoter remains to be determined. An inhibitory sequence region, lying between −815 to −355 bp of the translation codon, has also been described in the human DAF gene promoter (28). Further deletions showed that the short region located between −179 and −18 bp upstream of the ATG codon was essential for constitutive Daf1 gene expression because deletion of this region abolished almost all promoter activity. Computational analysis of this core promoter region identified two putative GC boxes (CCCGGCC) located between −153 to −147 and −84 to −76 bp and one GT/CACCC box located between −109 to −103 bp upstream of the translational start codon. These GC-rich promoter elements have been previously described in many genes to be the binding site of the ubiquitously expressed transcription factor Sp1 found to be a key player in the basal transcription of many housekeeping genes (32, 33). To demonstrate that Sp1 was actually involved in the transcription of the mouse Daf1 gene, we used several approaches. First, we showed that the lack of promoter activity observed in Sp-deficient Drosophila SL2 cells transfected with Daf1 reporter constructs was reversed by the addition of exogenous Sp1. Second, we performed a mutational analysis of the Sp1-binding sites and demonstrated that the promoter activity was markedly reduced when both the GC box located −84 to −76 bp and the GT box located −109 to −103 bp from the ATG codon were mutated simultaneously, with mutation of the most proximal Sp1-binding site having the largest effect. Third, we confirmed by transcription factor ELISA that Sp1 is capable of binding to both Sp1-binding sites.

Previous studies have determined that Sp1-dependent transcriptional activation of TATA-less promoters is mediated by the interaction of the glutamine-rich domain of Sp1 and TATA-binding
protein-associated factors subunits of the RNA polymerase II basal transcription factor TFIIID (48, 49). Moreover, Ryu et al. (50) showed that the cofactor complex CRSP (cofactor required for Sp1 activation) was also required for efficient transcriptional activation by Sp1. Additionally, it has been demonstrated that the binding of Sp1 to multiple binding sites is often essential for significant transcription activity (51–53). This cooperative effect between Sp1-binding sites to achieve full gene expression has been shown to be mediated via the formation of Sp1-Sp1 complexes (54). However, in some cases, the most proximal Sp1-binding site seems to be more important, if not absolutely required, for transcription activity (31, 55, 56). We demonstrated in this study that the GC box located −37 to −29 bp upstream of the transcriptional start site was the major Sp1-binding site for transcription activity because mutation of this site reduced promoter activity by −50% in all the cell lines tested while mutations in the two other Sp1-binding sites did not significantly influence gene transcription. Consistent with this idea, we also demonstrated that the binding affinity of Sp1 for the most proximal Sp1-binding site was higher than for the one located −62 to −56 bp from the transcriptional start site. Furthermore, we showed that the increased promoter activity observed in LPS-treated cells was totally abolished when both Sp1 sites were mutated, indicating that functional Sp1 sites are required for the Daf1 promoter to function properly. Similarly, Tone et al. (57) showed that the LPS effect on CD40 gene expression was greatly reduced by introducing mutations into Sp1-binding sites.

In the human DAF gene, Thomas and Lublin (18) have identified three separate regions controlling DAF promoter activity. The first region, located between −54 and −34 bp from the transcription start, was shown to be necessary for low level transcription and to contain an Sp1-binding site. A second region, between −77 and −54 bp, encompassing both CREB- and AP-1-binding sites, was capable of up-regulating transcriptional activity. The same CREB-binding site was shown to be likely involved in Camp-mediated up-regulation of the promoter activity (28). In a comparative sequence analysis, we noted that the region containing the CREB-binding site, as well as both Sp1-binding sites, exhibits high sequence homology between human and mouse DAF. To assess the role of the CREB-binding site in the murine promoter, we generated constructs in which the CREB-binding site was mutated and showed that this mutant construct displayed a 40% decrease in promoter activity indicating that, as in human, the CREB-binding site is involved in the up-regulation of basal transcriptional activity of murine Daf1 gene. This supports previous studies which have revealed that functional cooperation between Sp1 and CREB is critical in driving gene expression (58, 59). Interestingly, the constitutive activation domain of CREB has been shown to interact with the TATA-binding protein-associated factor subunit and to mediate the recruitment of the RNA polymerase II complex (60). Finally, a third region located between −206 and −77 bp and containing potential enhancer element(s) was also identified in the human DAF gene. Interestingly, this region contains a GTCACC box consensus sequence perfectly conserved with that described in this study, and shown to be significantly involved in the transcription of the murine Daf1 gene.

In summary, our data clearly show that the transcriptional activity of the mouse Daf1 promoter requires the functional cooperation of two Sp1-binding sites, a feature frequently observed in mammalian GC-rich, TATA-less promoters. Sp1 also contributes to the induction of Daf1 transcription by LPS. Furthermore, CREB appears to be an important modulator of transcriptional activity by enhancing the basal transcription activity supported by Sp1. These findings not only identify the mechanism that drives basal expression but also provide the framework to explain the molecular basis for the regulation of Daf1 gene expression. These initial observations should facilitate future studies aimed at examining the regulation of Daf1 expression in immune responses.

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Disclosures
The authors have no financial conflict of interest.

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