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

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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.

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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 KOD 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'-GCATCTCGAGACAAACACTCGCCGAC-3' (XhoI site underlined) and reverse, 5'-GGTACTTCTAGAGCGACAAGAGACAG-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 (version 6.0.5) was used to align and compare DNA sequence to those of rat, human, and chimpanzee.

Mapping of the transcription start site

Single-cell suspensions were obtained from DBA/2J 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 manufacturer’s protocol. RACE PCR was then performed using SuperScript III followed by PCR amplification of the cDNA 5' end using Platinum DNA Polymerase High Fidelity (Invitrogen Life Technologies). The following primers were used for amplification: Gene Racer forward primer and Daf1 reverse, 5'-CCGACGCTTGAACGAGACAG-3' (HindIII site underlined). PCR products were separated using a 1.0% agarose gel, extracted, and then cloned into the pG3L-basic vector (Promega). Se- quencing, 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 (version 6.0.5) was used to align and compare the murine Daf1 sequence to those of rat, human, and chimpanzee.

Cell culture

The NIH/3T3 embryo fibroblast cell line (ATCC CRL-1658) and RAW 264.7 murine macrophage cell line (ATCC TIB-71) were obtained from the ATCC, and maintained at 37°C in a humidified 5% CO2 atmosphere in high glucose DMEM with 2 mL-glutamine (Invitrogen Life Technologies). The NIH/3T3 embryo fibroblast cell line (ATCC CRL-1658) and RAW 264.7 murine macrophage cell line (ATCC TIB-71) were obtained from the ATCC, and maintained at 24°C in Schneider's Drosophila medium (Invitrogen Life Technologies) supplemented with 10% FBS (Omega Scientific).

RNA isolation and reverse transcription

Total RNA extraction from NIH/3T3, M12.4, and RAW 264.7 cell lines was performed 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 (RNaseOUT; 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'-CGTACAGCTTGATCATGTA-3' and Daf1 reverse, 5'-CTCATCTTCCTGGAGACAGTCT-3' and β-actin forward, 5'-TGGATCTTCCGCTCTTACAT-3' and β-actin reverse, 5'-TGGTAAACCCGCAGCTAAGTC-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 (RNaseOUT; Invitrogen Life Technologies), and 200 U of SuperScript III reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's protocol. RACE PCR was then performed using Superscript III followed by PCR amplification of the cDNA 5' end using Platinum DNA Polymerase High Fidelity (Invitrogen Life Technologies). The following primers were used for amplification: Gene Racer forward primer and Daf1 reverse, 5'-CCGACGCTTGAACGAGACAG-3'. The PCR product was separated using a 1.0% agarose gel, extracted, and inserted into the pCR4-TOPO plasmid vector. Twelve colonies were picked and submitted for sequencing using M13 Reverse and T7 Primers.

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Orientation</th>
<th>Construct</th>
<th>Oligonucleotide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p&lt;sup&gt;(−2408/+85)&lt;/sup&gt;</td>
<td>Forward</td>
<td>Deletion</td>
<td>5'-GCATCTCGAGACAAACACTCGCCGAC-3'</td>
</tr>
<tr>
<td>p&lt;sup&gt;(−1746/+85)&lt;/sup&gt;</td>
<td>Forward</td>
<td>Deletion</td>
<td>5'-GCATCTCGAGATGAATTAGTCACAGAAG-3'</td>
</tr>
<tr>
<td>p&lt;sup&gt;(−1104/+85)&lt;/sup&gt;</td>
<td>Forward</td>
<td>Deletion</td>
<td>5'-GCATCTCGAGACACCGACAGACAAAG-3'</td>
</tr>
<tr>
<td>p&lt;sup&gt;(−619/+85)&lt;/sup&gt;</td>
<td>Forward</td>
<td>Deletion</td>
<td>5'-GCATCTCGAGCTAAGAGGTAGTCCTCCTCA-3'</td>
</tr>
<tr>
<td>p&lt;sup&gt;(−337/+85)&lt;/sup&gt;</td>
<td>Forward</td>
<td>Deletion</td>
<td>5'-GCATCTCGAGACACACAGACACAGATTT-3'</td>
</tr>
<tr>
<td>p&lt;sup&gt;(−179/+85)&lt;/sup&gt;</td>
<td>Forward</td>
<td>Deletion</td>
<td>5'-GCATCTCGAGCTTGTTGGAAGTCAGTA-3'</td>
</tr>
<tr>
<td>p&lt;sup&gt;(−18/+85)&lt;/sup&gt;</td>
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<td>Deletion</td>
<td>5'-GCATCTCGAGTTTCACAGAAGAAGAG-3'</td>
</tr>
<tr>
<td>pRev</td>
<td>Reverse</td>
<td>Deletion</td>
<td>5'-GCATTACACACACACGAAACGAGAG-3'</td>
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<td>pFSp1-A mut</td>
<td>Forward</td>
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<td>5'-GCATCTCGAGTTCAATAGGAGGACAGAC-3'</td>
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<tr>
<td>pFSp1-B mut</td>
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<tr>
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<td>pRECRB/Spi mut</td>
<td>Reverse</td>
<td>Mutation</td>
<td>5'-GCATCTCGAGTTCAATAGGAGGACAGAC-3'</td>
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</tbody>
</table>

*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.*
Reprint requests: Richard F. Spade, 460 Park Avenue, 5th Floor, New York, NY 10022; e-mail: rvmny@aol.com.

Cells were plated the day before transfection in 12-well plates at 2.1 × 10^5 cells/well for NIH/3T3 cells and in 24-well plates at 2.5 × 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 μg/ml 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 × 10^3 cells/well in 24-well plates and transfected as described above with various firefly luciferase reporter vectors along with differing 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 3.01 software (Premier Biosoft International). The primers used were as follows: Daf1 forward primer, 5'-CTTTGACGCTTTGCTGTGA3'-3'; Daf1 reverse primer, 5'-TCCATCTCTTCTGAGCAGTCTC3'-3'; cyclophilin A forward primer, 5'-GGGCCATGCAGCAGCCC-3'; cyclophilin A reverse primer, 5'-GTGTTTTGAGTTTCTGTGC-3'. The following dual-labeled probes were obtained from Integrated DNA Technologies: Daf1, 5'-FAM-CTCTTCTCAGACACAGAAGATTCAGAGAGA BHQ1-3'; cyclophilin A, 5'-FAM-TGGCCCCTGTCCTCTGTGAGBHQ1-3'. Cyclophilin A standards were cloned into pGEMEase (Promega) and a standard curve was generated for each experiment. All samples and standards were analyzed in triplicate. PCR amplification was performed in a total volume of 25 μl containing 1.0 mM Tris-HCl, 5 mM KCl, 200 μM dNTPs, 100 ng of forward and reverse primers, 4 μM MgCl2, 0.625 μM AmpliTaq Gold (Applied Biosystems), and 2.5 μM 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. Each oligonucleotide was purified using PAGE purification and the 5' end of the parent oligonucleotide was either biotinylated or not by Integrated DNA Technologies (Table II). Double-strand oligonucleotides were obtained by annealing either the biotinylated or the non-biotinylated 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. Immunol 2B immunosassay plates (Thermo) were coated overnight at 37°C with 100 μl of a 10 μg/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 μl of a 1 μM 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(dl-dc) (Amershaw 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 μl 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. Genomix’s EDorado 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 GCTCTCT and CTCTCT

Table II. Sequence of oligonucleotides used in transcription factor ELISA

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<tr>
<th>Oligonucleotide</th>
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</tr>
</tbody>
</table>
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. 3A). 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. 3B), 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.

**FIGURE 1.** Nucleotide sequence of the 5′-flanking region of the Daf1 gene. Sequences that match consensus-binding sites of known transcription factors are indicated. Numbers designate the nucleotide position relative to the translational start site. The nucleotide A of the ATG codon is denoted +1. Oligonucleotide sequences used to generate 5′ deletion constructs shown in Fig. 4 are underlined by an arrow.

(−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.

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Identification of Daf1 transcription start site. Transcription start was performed using RACE. Total RNA was extracted from mouse spleen tissue and reverse transcription was performed using random primers (N<sub>6</sub>). RACE-ready cDNA was then amplified using a forward GeneRacer 5′ primer and a reverse Daf1-specific primer. Nested PCR was also performed using a forward GeneRacer 5′ nested primer and a reverse Daf1-specific primer. A, PCR products were separated on agarose gel, purified, and cloned into pCR4-TOPO vector. 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 co-transfected 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 322 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 activity 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/B 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 Daf1 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 Daf1 promoter activity. Taken together, this result indicates that the basal Daf1 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. 7A 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. 7A 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 (−109 to −103) and C (−84 to −76) and support the argument that this transcription factor is directly involved in the regulation of Daf1 gene expression.

Mutations in the CREB-binding site partially inhibit Daf1 promoter activity

CREB plays a pivotal role in the transcriptional activity of numerous genes (35) mediated by its binding to the cAMP-response element, a specialized region of genomic DNA that contains the consensus nucleotide sequence TGACGTCA. In the human DAF gene, two independent studies have demonstrated that the promoter region containing the cAMP-response element sequence is an important modulator of Daf1 gene transcription (18, 28). A CREB-binding site (−98 to −91) is located within 100 nt of 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 sites were 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 upregulated 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

FIGURE 7. Specific DNA-binding activity of Sp1 to the Sp1-binding sites B (−109/−103) and C (−84/−76) determined by transcription factor ELISA. Biotinylated oligonucleotides containing either Sp1-binding site B (oligo B) or C (oligo C) were generated and immobilized onto a 96-well plate precoated with streptavidin. Nuclear extract was then applied and DNA-Sp1 complexes were detected with an anti-Sp1 Ab followed by a HRP-conjugated secondary Ab. The ABTS substrate solution was then added and color development was measured with a 405 nm microplate reader. Omission of the Sp1-specific Ab and the use of an irrelevant Ab in combination with the highest amount of nuclear extract were used as negative controls. A, Dose-response assay using increasing amounts (0–40 μg) of nuclear extract of NIH/3T3, M12.4, and RAW264.7 cells to oligo B, B, Dose-response assay using increasing amounts (0–40 μg) of nuclear extract of NIH/3T3, M12.4, and RAW264.7 cells to oligo C, C, Dose-response assay using increasing amounts (0–10 ng) of rSp1 to both oligo B and C.

FIGURE 8. CREB-binding site is involved in the basal activity of Daf1 promoter. The p(−619/+85)Luc construct was used to generate mutant constructs in which the CREB-binding site was either modified alone or in conjunction with all the three Sp1-binding sites. These constructs were then transiently transfected together with the transfection control vector pRL-TK in the indicated cell lines and a dual-luciferase assay performed. Data (mean ± SD) are from duplicate transfections performed in quadruplicate and expressed as relative luciferase activity representing the percent activity relative to the p(−619/+85)Luc luciferase activity defined as 100%. Statistical significance was assigned based on a Student’s t test with * for p < 0.01.
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 hemopoietic 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 initiation 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 (CCCGCCC) located between −153 to −147 and −84 to −76 bp and one GT/CACCC box located between −109 to −103 bp upstream of the translation 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

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
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 (51–53). This cooperative effect between Sp1-binding sites by Sp1. Additionally, it has been demonstrated that the binding of the cofactor complex CRSP (cofactor required for Sp1 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 (51–53). This cooperative effect between Sp1-binding sites by Sp1. Additionally, it has been demonstrated that the binding of the cofactor complex CRSP (cofactor required for Sp1 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 (51–53). This cooperative effect between Sp1-binding sites by Sp1.


