Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 4675–4679, June 1991
Immunology

Characterization of the decay-accelerating factor gene promoter region

(complement/cAMP/phorbol ester)

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Communicated by Oscar D. Ratnoff, January 14, 1991

ABSTRACT **Decay-accelerating factor (DAF) expression modulates susceptibility of cells to autologous complement attack. To characterize the regulatory region controlling DAF gene transcription, genomic DNA extending from 815 base pairs (bp) upstream to ≈4 kilobases downstream of DAF's AUG codon (designated +1) was cloned and sequenced. The 5′ flanking sequence showed 59–76% G+C content (−355 to +1), at least one GC box(es) (−135 to −131), and variable length sequences (from −629 to −285) conforming to the motifs TCCTCC and TC_n. Nuclease S1 digestions and primer extensions localized a major transcriptional start site to −82/−81, 38 bp downstream of a possible TATA variant, (A)TTTAA. In COS cell transfections, the sequence encompassing −815 to −67 functioned 2.5% as efficiently as the Rous sarcoma virus 3′ long terminal repeat, but following deletion upstream of −355 its activity increased ≈4-fold. Two octanucleotides exhibiting partial homology to phorbol 12-myristate 13-acetate (PMA) and cAMP responsive elements (PREs and CREs, respectively) were detected, and the respective modulators enhanced transcriptional efficiency 2- and ≈10-fold, respectively. Thus, the DAF gene promoter (*i*) exhibits sequences resembling both conventional and unconventional transcriptional control elements, (*ii*) possesses a region with negative regulatory activity, and (*iii*) responds to PMA and cAMP induction presumably via PRE- and CRE-like enhancer elements.**

Surface constituents on host tissues contain free hydroxyl and amino groups that can condense covalently with nascent C4b and C3b (reviewed in ref. 1). Because these complement activation fragments, once bound, can serve as sites for assembly of autologous C3 convertases, which can amplify activation on host cells, their activities must be strictly controlled. Important in this control is an ≈70-kDa regulatory protein termed decay-accelerating factor (DAF) (reviewed in ref. 2). The interaction of this molecule with convertase complexes (wherever deposited C4b or C3b fragments initiate their assembly) destabilizes the enzymes before they can function and thereby prevents amplification of C3b deposition. This DAF activity is essential physiologically because its deficiency in paroxysmal nocturnal hemoglobinuria, an acquired hemolytic disorder (2), leads to exaggerated C3b uptake by affected cells *in vivo*, rendering them susceptible to lysis.

DAF is expressed on all blood and vascular endothelial cells in contact with C4 and C3 in plasma (2). High level expression on leukocytes and endothelium (≈6×10^4 and ≈2×10^5 molecules per cell, respectively) is thought to safeguard against locally augmented C3 activation that can occur with inflammation. DAF is also found on epithelial surfaces lining extracellular compartments and in soluble form in adjacent fluids (2). Elevated expression in some of these sites—e.g., the corneal surface (>1×10^5 molecules per cell) (3)—has suggested that C3b deposition may pose a problem in these locations as well as in the vascular space.

Starting at its N terminus, DAF is composed of four contiguous 60- to 70-amino-acid-long homologous sequences (4, 5) found in other complement regulatory proteins and termed short consensus repeats (SCRs) (reviewed in ref. 6). The four SCRs (thought to mediate C4b and C3b interactions) are attached to a 70-amino-acid-long threonine- and serine-rich stretch (which may appropriately position the functional SCR domains above the cell surface). DAF mRNA predicts a C-terminal hydrophobic membrane-spanning segment, which is replaced (7) immediately posttranslationally by a glycosylplasmanylinositol anchoring structure. DAF mRNA derives from a single ≈35-kilobase (kb)-long structural gene (8, 9) localized at band q32 of chromosome 1 (9). The DAF gene is flanked upstream by the genes for complement receptors CR1 and CR2 and downstream by the gene for C4 binding protein, other C3/C4 regulatory proteins that exhibit similar SCR-based structures.

The present study was undertaken to characterize the DAF gene's 5′ regulatory region and to analyze its initial coding intron–exon organization.†

MATERIALS AND METHODS

Reagents and Labeling. The Charon 21 library containing human chromosome 1 DNA was obtained from the American Type Culture Collection. Composite DF13:2 cDNA and oligonucleotides P1–12 were prepared as described (8). CAT/SVO and RSV·CAT/SVO containing the Rous sarcoma virus (RSV) long terminal repeat were obtained from B. Howard (10). Oligonucleotides P-2 [5′-CAGCAGCCGGGGCAGCTCCCCGAGGAGGGGCAGCGCCGCGGGCACGCT-3′ (4)], P-4.3 [5′-GGCAGGTCACTGAGTCCTTC-3′ (4)], P-4.6 [5′-GTTGGCACCTCGCAGCTACG-3′ (4)], P-SVO [5′-GACTACGCCATAAAAGAGGA-3′ (10)], and P-chloramphenicol acetyltransferase (CAT) [5′-GGGATATATCAACGGTGGTA-3′ (11)] (see Fig. 2) were synthesized in an Applied Biosystems model 380A DNA synthesizer (8). For library screening, DF13:2 cDNA was ^{32}P-labeled by nick-translation and, for DNA blot analyses, by the random hexanucleotide primer method. Oligonucleotides were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase.

Cloning, Southern Analyses, and Nested Deletions. Screening was performed on *Escherichia coli* LE 392. Colony/Plaque Screen filters were hybridized with ^{32}P-labeled DF13:2 cDNA as described (4). Genomic inserts were subcloned into *Eco*RI sites of pBluescript (BT) (Stratagene). DNA transfers on GeneScreen*Plus* and hybridizations were

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Abbreviations: DAF, decay-accelerating factor; SCR, short consensus repeat; BT, pBluescript; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase; PMA, phorbol 12-myristate 13-acetate; PRE, PMA response element; CRE, cAMP response element.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M64356).

carried out as described (8). Exo III/mung bean nuclease (Stratagene) deletions were made in BT.

Primer Extensions and Nuclease S1 Digestions. Poly(A)$^+$ RNA was isolated from HeLa cells as described (4, 8). DF13:2 cDNA transcribed RNA was synthesized with *Cla* I-digested plasmid, the T7 primer, and T7 RNA polymerase. RNA (10 μg) hybridized at 55°C for 45 min to oligomers (0.1 pmol) in 100 mM KCl/50 mM Tris·HCl, pH 8.3, was incubated at 50°C for 45 min with avian myeloblastosis virus reverse transcriptase, and products were analyzed by 8 M urea/6% PAGE autoradiography.

Nuclease S1 mapping of DAF sequence was carried out (12) with 0.01 μg of 5′-end-labeled DNA, 18 hr of hybridization at 60°C with 10 μg of HeLa poly(A)$^+$ RNA, and 1 hr of incubation at 30°C with 200 units of nuclease S1. For mapping of the DAF·CAT sequence (see below) 0.1 μg of chimeric DAF·CAT DNA and 50 μg of transfectant poly(A)$^+$ RNA were substituted.

Expression Constructs and Transfections. The DAF 5′ flanking region mobilized with (Klenow filled-in) *Eag* I and *Eco*RV was inserted into the filled-in *Hin*dIII site of CAT·SVO [pSVO·CAT (11)]. Deleted −355 and −286 DAF·CAT/SVO constructs were prepared by *Xba* I and *Sac* I secondary cleavage (see Fig. 1) of the 5300-base-pair (bp) fragment of a *Bal* I partial digest of DAF·CAT/SVO and religation of appropriate blunt-ended products. The −242 DAF·CAT/SVO construct was prepared by *Sma* I digestion of DAF·CAT/SVO and religation. The −159 DAF·CAT/SVO constructs were prepared by *Hga* I digestion, Klenow fill-in, secondary *Bam*HI cleavage, and ligation of the *Bam*HI/*Hga* I fragment into DAF·CAT/SVO or CAT/SVO (prepared with *Bam*HI/*Sma* I or *Bam*HI/filled-in *Hin*dIII, respectively). All constructs were verified by sequencing.

DNA (12 μg) preincubated for 10 min at 20°C with lipofectant (30 μl) (Bethesda Research Laboratories) in Dulbecco's modified Eagle's medium (2 ml) was added to 60-mm plates inoculated 12 hr earlier with 1.5×10^6 COS cells. Cells were harvested after 48 hr at 37°C. CAT activity was assayed by incubating cell extracts (25 μg) kinetically, counting acetylated and unacetylated species (10), and calculating pmol of acetylchloramphenicol generated per hr per mg of protein.

Sequencing. Inserts were sequenced by dideoxynucleotide chain termination (13) using Sequenase (United States Biochemical) in both single- and double-stranded protocols. The region upstream of −67 was resequenced in both directions in DAF·CAT/SVO constructs with P-SVO and P-CAT (see above) used as primers. Sequence analyses and homology searches were performed using DNASIS, DNASTAR, and GenBank.

RESULTS

Isolation of DAF Genomic Clones. Based on prior chromosomal assignment of the DAF gene (9), a chromosome 1-specific library (in Charon 21) was used. Screening with ^{32}P-labeled DF13:2 cDNA (8) yielded 14 putative clones designated λDAF1–14. Southern analyses of *Eco*RI digests hybridized with ^{32}P-labeled P1–P12 revealed that λDAF5 (Fig. 1) encompassed 5′ sequences extending from the 5′ end of DF13 cDNA to the third SCR of DAF (4).

Due to loss of the λDAF5 3′ *Eco*RI site, the use of a downstream λ *Bam*HI site necessitated carry-over into BT of 1134 bp of λ sequence together with ≈5 kb of DAF5 sequence. The resulting DAF5/BT plasmid (Fig. 1) contained ≈0.8 kb of insert sequence upstream of the 5′ end of DF13. In Southern analyses (data not shown) a 500-bp *Sma* I/*Sma* I fragment from the 5′ end of this sequence and the 300-bp fragment upstream of the *Hin*dIII site of DF13:2 cDNA (8) hybridized to common genomic fragments, verifying that DAF5 corresponded to authentic DAF sequence.

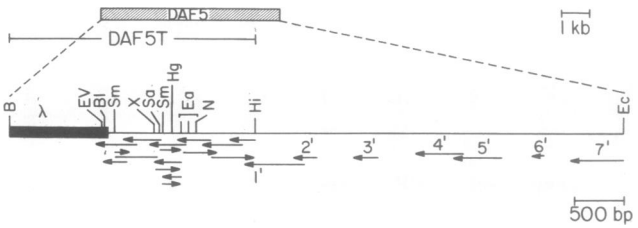

FIG. 1. Characteristics of genomic clone λDAF5. The expansion shows relevant restriction endonuclease sites in the DAF5 insert and its DAF5T derivative used in Southern analyses and expression constructs. EV, *Eco*RV; B, *Bam*HI; Bl, *Bal* I; Sm, *Sma* I; X, *Xba* I; Sa, *Sac* I; Hg, *Hga* I; Ea, *Eag* I; N, *Nae* I; Hi, *Hin*dIII; Ec, *Eco*RI. Regions sequenced are designated by arrows. Numbers 1′–7′ correspond to the similarly designated sequences in Fig. 2.

Characterization of DAF Gene 5′ Flanking Sequence. To facilitate analyses, DAF5T/BT [in which sequences downstream of *Hin*dIII were deleted (see Fig. 1)] was subjected to Exo III/mung bean nuclease deletion. Sequencing of products (Fig. 2) revealed a 480-bp intron beginning 1 bp downstream of the 3′ end of DAF's prepeptide and ending 2 bp upstream of codons for DAF's first SCR. Further downstream sequencing showed a second intron beginning immediately after the first SCR of DAF and restriction mapping identified a third intron immediately following the second SCR of DAF. In all cases, exon–intron junctions fell between the first and second nucleotides of codons and exhibited characteristic GT–AC splice sequences.

Initial analyses of the DAF5T sequence upstream of the AUG codon failed to identify typical CAAT or TATA consensuses. The region, however, exhibited up to 76% G+C content (Fig. 2), possessed at least one GC box, and contained sequences with the motifs TCCTCC and TC$_n$. Placing the AUG codon of DAF at position +1, the most prominent of the former mapped to positions −135 to −131 and of the latter mapped adjacently, and to positions −285 to −266. The analyses also disclosed two octanucleotides (TGAGTCCA and TGACGCAG) approximating PREs and CREs (reviewed in ref. 14) at positions −311 to −304 and −159 to −152, respectively, and three other sequences exhibiting partial homology at positions −236 to −229, −220 to −213, and −208 to −201. Comparisons with the promoter regions of other PRE/CRE genes (Fig. 3) showed that the sequence at −159 to −152 aligned with similar elements in the human collagenase (15) and rat stromelysin (16) genes. The comparisons revealed multiple additional homologies between DAF5 and these promoters, including ACTC immediately flanking their TATA consensus sequences.

Localization of Transcriptional Start Site(s) of DAF. To identify the position(s) of the DAF transcriptional start site(s), primer extension and nuclease S1 digestion analyses were performed with HeLa cell poly(A)$^+$ RNA (4). For the former assays, P-2, P-4.3, and P-4.6 (see *Materials and Methods* and ref. 8) were prepared. Reverse transcriptions with each oligonucleotide, shown for P-4.6 in Fig. 4*A*, yielded major and minor products corresponding to transcripts initiated 82 and 234 bp upstream of the AUG codon of DAF. Control studies substituting DAF RNA transcribed *in vitro* from DF13:2/BT or oligomer hIIICS 3′ and growth hormone poly(A)$^+$ RNA from bovine pituitary tissue (17) yielded predicted products. For the latter assays, a segment of DAF5T was prepared. As shown in Fig. 4*B*, nuclease S1 digestions, after hybridizing with HeLa poly(A)$^+$ RNA, yielded 171- and 170-bp products corresponding to transcripts initiating at −82 and −81 bp upstream of DAF's AUG, respectively.

Transfection Studies Using the 5′ Flanking Region of DAF. The DAF sequence in DAF5T/BT (extending 30 bp upstream

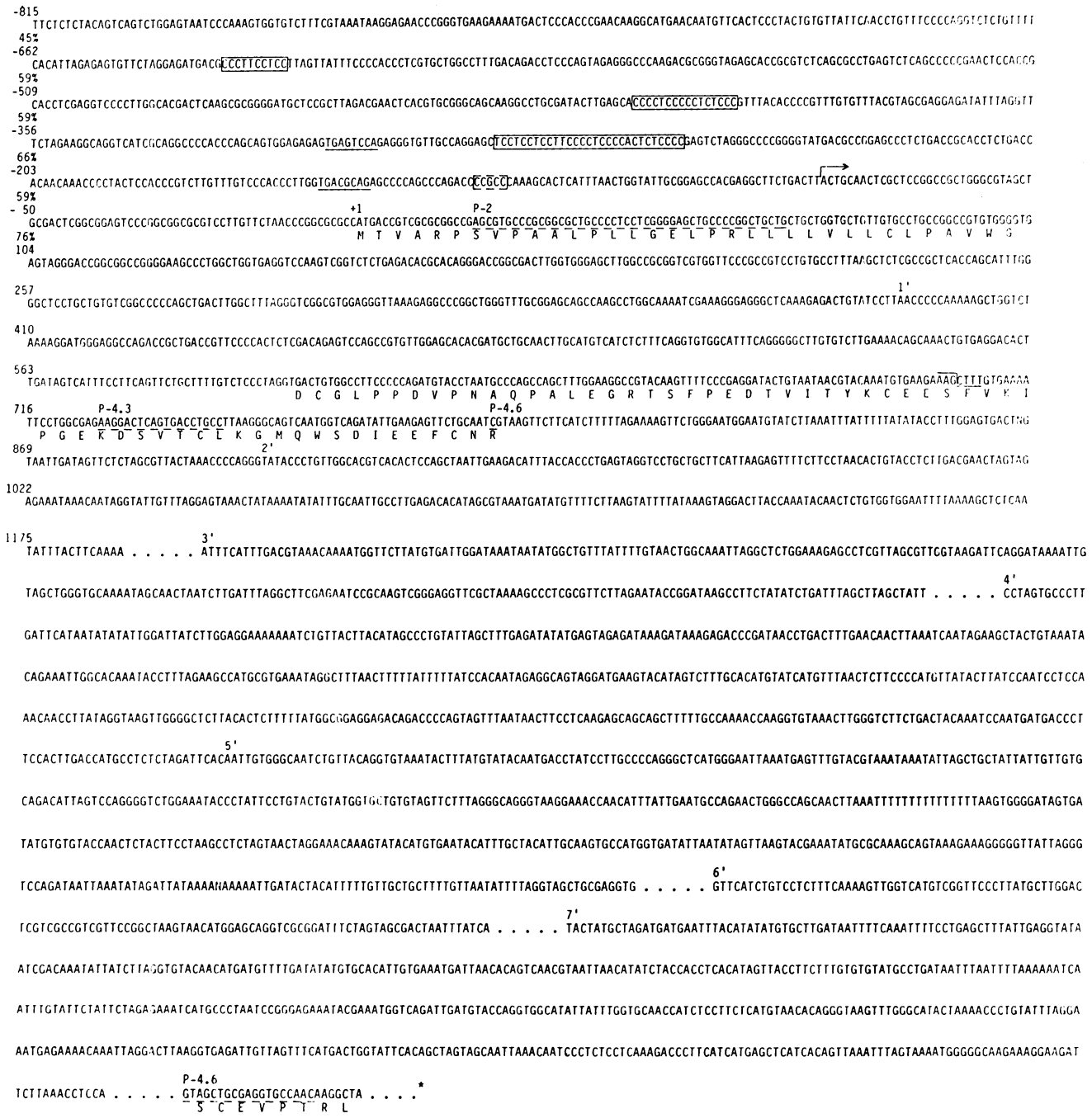

FIG. 2. Nucleotide sequence of DAF5. In the sequence upstream of the AUG codon of DAF, GC homologies and sequences with the motifs TCCTCC and TC_n are boxed with broken and solid lines, respectively. The GC contents of selected regions are indicated. In the sequence downstream of the DAF AUG codon, the position of the *Hin*dIII site in DAF's first SCR (⌐└) and of downstream sequences designated 1′–7′ in Fig. 1 is shown. Sequences corresponding to oligonucleotides P-2, P-4.3, and P-4.6 used in primer-extension assays (see Fig. 4) are shown by broken underlining. *, cDNA sequence taken from ref. 4. Sequences with homology to phorbol 12-myristate 13-acetate (PMA)/cAMP response elements (PREs and CRES, respectively) (see Figs. 3 and 5) are indicated by solid underlining. The transcriptional start site is shown by a bent arrow.

of the λ–DAF junction to 18 bp downstream of the transcriptional start site) was ligated to CAT sequence in pSVO·CAT (10), and CAT activity generated in COS cells was compared with that using pSVO·CAT and pRSV·CAT (10), respectively. As shown in Table 1, the −815 to −67 DAF5T 5′ flanking sequence functioned 2.5% as efficiently as the RSV long terminal repeat. After nuclease S1 analyses establishing that the DAF·CAT construct was utilizing DAF's −82 transcriptional start site (Fig. 4*C*), the regions influencing promoter activity were delimited by preparing deletion constructs retaining −355, −286, −242, and −159 to −67 sequence. As shown in Fig. 5, deletion of sequence from −815 to −355 enhanced CAT expression ≈4-fold. In contrast, further deletion from −355 to −242 and from −242 to −159 incrementally reduced promoter activity back to 11% and 5%, respectively, of basal (−815) activity.

To establish whether the DAF 5′ sequence is responsive to PMA and/or cAMP, graded doses of PMA or 8-bromo-cAMP were added to cultures 8 hr prior to harvesting cells transfected with the −815 to −67 sequence. The modulators dose-dependently increased CAT expression levels 2- and 10-fold (Table 1), respectively.

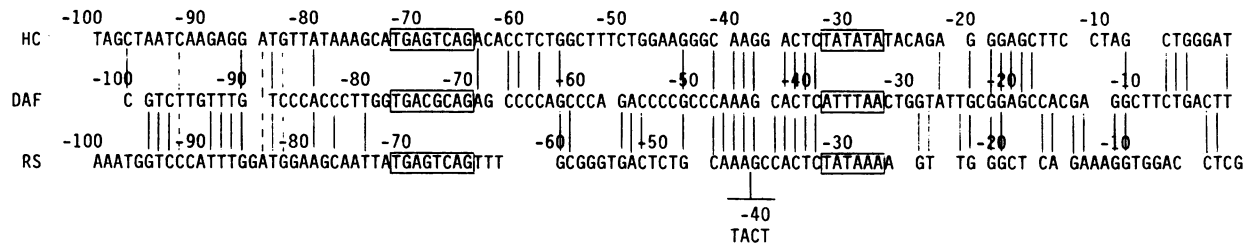

FIG. 3. The positional relationship of DAF's proximal CRE with corresponding elements in the collagenase (HC) (15) and rat stromelysin (RS) (16) gene promoters. Multiple sequence homologies are indicated. Transcriptional start sites are designated +1.

DISCUSSION

A genomic clone (DAF5) encompassing the 5′ end of the DAF coding region was identified, and ≈3.5 kb of its sequence beginning 815 bp upstream of its AUG codon was analyzed. The 5′ flanking sequence was found to contain an (A)TTTAA subsequence upstream of the major transcriptional start site identified at −82/−81. It was noted to exhibit features of atypical 5′ regulatory regions characteristic of housekeeping genes and to contain sequences homologous to PREs and CREs. The 5′ end of the coding region was found to exhibit exon–intron organization conforming to that of other SCR-containing proteins.

The positional relationship of the (A)TTTAA sequence in the DAF gene 5′ regulatory region to the DAF transcriptional start site and the alignment of a nearby sequence (ACTC) with corresponding sequences flanking the TATA boxes in the human collagenase (15) and rat stromelysin (16) gene promoters argue that this sequence represents a TATA variant. The presence, however, in the DAF gene 5′ flanking region of up to 76% G+C content, at least one typical and other potential Sp-1 binding sites, and two repeats conforming to the motif TCCTCC parallels findings for previously described promoters lacking TATA and CAAT consensuses. Among these are the hydroxymethylglutaryl-CoA reductase (18) and epidermal growth factor receptor (19) gene promoters. The former exhibits 65% G+C content and contains three GC boxes at positions −66, −152, and −221. The latter is up to 88% G+C-rich, contains five GC boxes, and exhibits four repeats conforming to the TCCTCC motif in the region −481 to −16. The TCCTCC sequence elements exhibit nuclear protein binding and removal of these sequence elements abolishes promoter activity. The effects of DAF promoter deletions on CAT expression levels observed in COS cell transfections in the present study (Fig. 5) argue that the TCCTCC elements in the DAF gene promoter may serve an analogous role. Exonuclease III protection studies and DNase I footprinting will be required to verify this point and to define the function, if any, of sequences exhibiting the related motifs TCCC and TCCCC present adjacently and at positions −417 to −413.

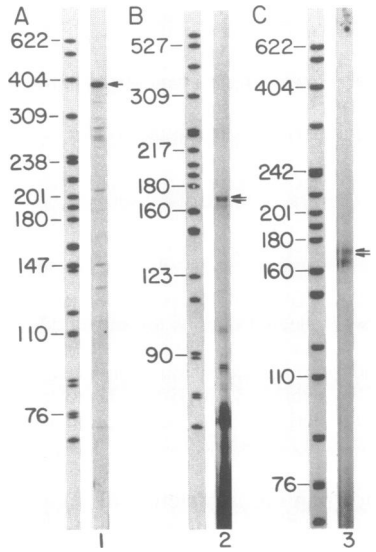

FIG. 4. Identification of the DAF gene transcriptional start site. (*A*) Reverse transcription of HeLa poly(A)$^+$ RNA primed with oligonucleotide P-4.6 is shown in lane 1. The major extension product is designated by the solid arrow. (*B*) Fragments remaining after nuclease S1 digestion of a 444-bp 5′-end-labeled *Xba* I/*Nae* I fragment of DAF5T hybridized to HeLa poly(A)$^+$ RNA are shown in lane 2. (*C*) Fragments remaining after nuclease S1 digestion of a 440-bp 5′-end-labeled *Xba* I/*Pvu* II fragment of DAF-CAT DNA annealed to DAF-CAT poly(A)$^+$ RNA are shown in lane 3.

Primer-extension analyses yielded major and minor products corresponding to transcription initiated at positions −82 and −234 (Fig. 4*A*). Nuclease S1 mapping gave protected fragments (Fig. 4*B*) corresponding to transcription initiated at −82/−81. The significance of the doublet is unclear but similar findings have been reported for other promoters (20). The −82-bp start site position overlaps precisely with the 5′ end of DF13 cDNA establishing that the composite DF13:2 sequence corresponds to a full-length transcript. Its size corresponds to one of the endogenous DAF mRNA species detected in several cell types (4, 5).

The identification of inhibitory sequence in the DAF gene promoter parallels findings for other genes including the rat insulin (21), rat growth hormone (22), and chicken lysozyme (23) genes. Although the elements in the DAF gene operative in the inhibitory effect have not yet been localized, the observation that further deletion of a downstream −285 to −266 sequence abolished the increase in DAF transcription afforded by removal of the −815 to −355 region argues that the former region (containing the downstream TCCTCC homology) is important for the augmentation. The physiological function of negative regulatory sequence in DAF's promoter remains to be determined. One possibility is that it could maintain the markedly divergent levels of DAF expression found in different cell types (2, 24). Transfections of −815 to −355 DAF sequence ligated to other promoters in different cell types will be necessary to establish whether this

Table 1. DAF promoter enhancement

Plasmid	Relative activity
SVO-CAT	0.5
RSV-CAT	40
DAF-CAT	1.0
PMA	
100 nM	1.9
10 nM	1.7
1 nM	0.8
cAMP	
1.0 mM	9.8
0.5 mM	6.5
0.1 mM	2.3

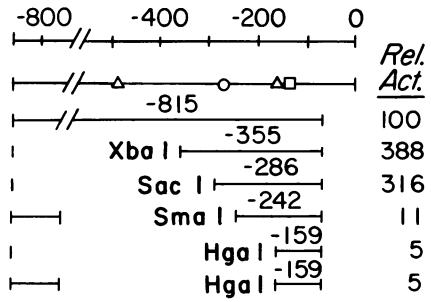

FIG. 5. Effects of sequential deletions on DAF promoter activity. CAT activity generated by −355, −286, −242, or −159 DAF-CAT/SVO constructs (without or with 34 bp of λ sequence) was compared with that generated by using −815 DAF-CAT/SVO. The positions of the GC box (□), PRE and CRE elements (△), and TCCTCC homology (○) are indicated.

sequence shares characteristics of other silencers and to determine the cell specificity of its effect.

The enhancing effects of PMA and of cAMP analogs on gene transcription are not characteristically transmitted via selective interactions of unique Ap-1 and CRE binding (CREB) proteins with PREs and CREs, but multiple nuclear proteins may participate and their interactions may involve one or both elements (14). The two sequences (TGAGTCCA and TGACGCAG) at positions −311 to −304 and −159 to −152 within DAF's 5′ flanking region resemble the PRE and CRE consensuses of the simian virus 40 promoter (25) and the α chain of human glycoprotein hormones (26) but differ in that they contain a G in place of a C and an extra C and G in place of a T. Three additional partially homologous oligonucleotides are tightly spaced in the regions −236 to −229, −220 to −213, and −208 to −201. Although our findings that DAF's 5′ flanking sequence is PMA and cAMP responsive *in vivo* do not establish that responsiveness is mediated through the above elements, recently performed gel-shift assays (27) have demonstrated that both of the −159 to −152 and −311 to −304 sequences bind HeLa cell nuclear protein(s) *in vitro*, and that the binding of the latter is inhibitable by the α chain CRE of human glycoprotein hormones. Surface DAF levels on HL60 cells (2) and on umbilical vascular endothelium (28) are augmented 3- to 4-fold by PMA and, in the latter cell type, the increase is protein kinase C dependent.

Individual SCRs in SCR-based proteins show three different patterns of exon–intron organization (reviewed in ref. 6). In 150-kDa factor H and 550-kDa C4 binding protein, composed of 20 SCRs and of 8 SCRs per 70-kDa monomer, respectively, each SCR is encoded by a separate exon. In contrast, in 160- to 240-kDa CR1 composed of 30 SCRs, and in 150-kDa CR2, composed alternatively of 15 or 16 SCRs, SCR 9 and SCRs 4, 8, and 12, respectively, are encoded by two exons. Moreover, SCRs 1 and 2, 5 and 6, 9 and 10, and 13 and 14 in CR2 derive from common exons. Our finding that the first two SCRs of DAF conform to the first pattern is in accordance with recent intron–exon junction analyses by others (29) that in DAF SCRs 1, 2, and 4 are encoded by single exons, while SCR 3 is encoded by two exons.

High-level DAF expression can protect melanoma cells from complement-mediated cytotoxicity (24) and diminished DAF expression can render lymphocytes of AIDS patients sensitive to complement-mediated injury (30). Up-regulation of DAF may play a role in protection of vascular endothelium from autologous complement-mediated injury during immune reactions (28) and this process may be involved in atherogenesis (31). In ocular tissues, DAF expression provides virtually all of the complement regulatory activity (3) and in the kidney it protects glomerular epithelial cells (32, 33). These findings argue that changes in surface DAF levels on cells may participate in disease pathogenesis not only in paroxysmal nocturnal hemoglobinuria but also in other disorders.

The authors thank B. Finke for data analyses and manuscript preparation; M. Tykocinski and B. Howard for reagents; and W. Dirksen, B. Hampson, D. Hamernik, and J. Nilson for helpful discussion. This investigation was supported by grants from the American Heart Association (89075) and the National Institutes of Health (AI23598 and P01DK38181). M.E.M. is an Established Investigator of the American Heart Association.

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