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A Synthetic DAF (CD55) Gene Based on Optimal Codon Usage for Transgenic Animals[1]

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The human DAF (CD55) gene was chosen as a representative molecule in a xenotransplantation study. The gene was synthesized in order to adapt its codons to those which are more frequent in mammals, especially pigs, and the expression levels were then examined in Chinese hamster ovarian (CHO) cells, swine endothelial cell (SEC) and transgenic mice. A significant increase in protein production with no detectable mRNA elevation was observed in the transfectants of synthetic DAF (sDAF), compared with the wild-type DAF (wtDAF) and delta-SCR1 wild-type DAF (Δ1wtDAF). Consistent with the *in vitro* data, the expression of DAF in mice that carry sDAF was higher than Δ1wtDAF in many organs, especially the pancreas. The sDAF showed a high level of expression in SEC and transgenic mice, suggesting that it will be useful in the development of transgenic pigs with high levels of expression.

Key words: codon usage, DAF, transgenic animal.

The growing numerical gap between the number of patients who would potentially benefit from allotransplantation and the number of available human donor organs has led to a revived interest in xenotransplantation (*1*). A number of trials are currently underway, the aim of which is to develop transgenic pigs that express human complement regulatory proteins (CRPs), such as MCP (CD46), DAF (CD55), and CD59 (*2–5*). Sufficient progress has been made, so that the inhibition of hyperacute rejection (HR) via the expression of CRPs on the graft has been successful (*6, 7*). As a result, the issue of xenografts has become a subject of current interest relative to acute vascular rejection (AVR) (*8*).

The origin of AVR is currently a controversial topic, but it is thought to be initiated by xeno-reactive antibodies and the subsequent activation of graft endothelium, the activation of small amounts of complement, and the incompatibility of the coagulation system between host and graft. Therefore, there is little apparent difference between HR and AVR in terms of their origins. Half-completed complement regulation, via the expression of DAF alone or DAF + CD59, simply introduces a new situation.

The purpose of this study is to investigate the issue of whether changes in the DAF coding sequence, based on codon-optimization, lead to higher levels of expression in mammalian cells. If this strategy is feasible, the effect of codon-optimization would be involved, not only in other CRPs, but also in any other molecules that would be expected to be present at high levels, such as HLA-G, -E, and anticoagulants (*9*).

MATERIALS AND METHODS

Cell Cultures—Chinese hamster ovarian (CHO) cells were obtained from the American Type Culture Collection (Bethesda, MD) and cultured in Ham's F12 medium containing 10% fetal bovine serum, L-glutamine, and kanamycin/amphotericin. The swine endothelial cell (SEC) line, MYP30, was cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, as well as L-glutamine and penicillin/streptomycin (Gibco Labs, Grand Island, NY) (*10*). The cultures were maintained in a 5% CO_2/95% air atmosphere at 37°C.

Construction of the New DAF Gene—The strategy for the construction of a completely modified DAF gene was based on the overlap extension PCR method using long oligonucleotides as starting materials (*11*). The codon usage for expression in mammals was adopted from a previous report (*12*). The actual codons used here for each amino acid are summarized in Table I. The sequences of the delta-short consensus repeat (SCR)1 synthetic DAF (sDAF) gene were verified using an ABI 310 autosequencer (Perkin-Elmer).

The cDNAs of wild-type (wtDAF), delta-SCR1 wild-type DAF (Δ1wtDAF), and sDAF with a Kozak sequence were subcloned into the pCXN site (*13*); a β-actin promoter and a cytomegalovirus enhancer.

Expression of cDNAs—The cDNAs of pCXN-DAF (50 μg)

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Abbreviations: CHO, Chinese hamster ovarian; sDAF, the synthetic DAF; wtDAF, the wild-type DAF; SCR, short consensus repeat; Δ1wtDAF, the delta-SCR1 wild-type DAF.

were introduced into CHO cells and MYP-30 by lipid-mediated DNA transfection with lipofectamine (GIBCO/BRL). Transfected cells were maintained in the complete medium up to day 3. The expression of plasmids was confirmed by flow cytometry, Western blotting and Northern blotting, as described below.

Stable transfectants with wtDAF, Δ1wtDAF, and sDAF were also established. Each clone was checked for the expression level of DAF molecules by flow cytometry, and for complement regulatory function by LDH assay, as described below.

Flow Cytometry—The transfectants with pCXN-DAF were treated with anti-CD55 mAb, 1C6 (Wako, Tokyo) at 4°C for 1 h, washed and then incubated with 2 μl of fluorescein-conjugated rabbit anti-mouse IgG (ICN/Cappel) as a second antibody for 1 h at 4°C. The stained cells were counted with a FACS Calibur flow cytometer (Becton Dickinson).

Western Blotting—Cell lysates of transfectant and naive cells were quantified by the BCA method (Pierce), and 20 μg or 50 μg aliquots of the obtained proteins in CHO cell, or SEC lysates, respectively, were subjected to 10% SDS/PAGE under non-reducing conditions by the method of Laemmli (*14*), and then transferred electrophoretically onto a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked with 5% skim milk in Tris-buffered saline/0.05% Tween 20 (TBST) for 1 h at 25°C, and then incubated in 1% bovine serum albumin (BSA)/0.5% skim milk/TBST with anti-DAF antibody, 1C6, for 1 h at 25°C. After washing, the blots were incubated with a horseradish peroxidase–conjugated secondary antibody, and the signal was developed using an ECL detection system (Amersham).

Northern Blotting—Total RNA was isolated from transfectant and naive cells with TORIZOL (Biotec Laboratories, Houston, TX), and separated by electrophoresis (5 μg/lane for CHO cells and 15 μg/lane for SEC). The probe used for hybridization was the PCR product generated from a portion of the 3'UTR (167 bp) of pCXN using the primers; 5'-ACTCCTCAGGTGCAGGCTGC-3' and 5'-GCAATGAAAATAAATTTCCT TTATTAG-3', since this probe should hybridize equally well with each mRNA. The probes were then labeled by the ECL detection system (Amersham). Hybridization signals were evaluated by FAST SCAN (Molecular Dynamics) (*15*).

LDH Assay—The LDH assay was performed according to a previously described method, using a Kyokuto "MTX LDH" kit. The stable SEC transfectants were plated at 2 × 10^4 cells per well in a 96-well tray, 1 day prior to assay. Fifteen hours later, the plates were incubated with 20 or 40% NHS diluted in Dulbecco's modified Eagle's medium for 2

TABLE I. **Codon usage in DAF genes (actual number of codons).**[a]

codon	AA	w*	s	codon	AA	w*	s	codon	AA	w*	s	codon	AA	w*	s
TTT	Phe	6	-	TCT	Ser	9	-	TAT	Tyr	8	-	TGT	Cys	7	-
TTC	Phe	3	9	TCC	Ser	5	1	TAC	Tyr	2	10	TGC	Cys	7	14
TTA	Leu	3	-	TCA	Ser	6	-	TAA	Stop	-	-	TGA	Stop	-	-
TTG	Leu	5	-	TCG	Ser	1	-	TAG	Stop	1	1	TGG	Trp	4	4
CTT	Leu	4	-	CCT	Pro	6	-	CAT	His	2	-	CGT	Arg	4	-
CTC	Leu	3	1	CCC	Pro	3	30	CAC	His	3	5	CGC	Arg	-	15
CTA	Leu	6	-	CCA	Pro	17	-	CAA	Gln	3	-	CGA	Arg	1	-
CTG	Leu	9	29	CCG	Pro	4	-	CAG	Gln	8	11	CGG	Arg	3	1
ATT	Ile	7	-	ACT	Thr	11	-	ATT	Asn	12	-	AGT	Ser	5	-
ATC	Ile	2	12	ACC	Thr	13	41	AAC	Asn	1	13	AGC	Ser	3	28
ATA	Ile	3	-	ACA	Thr	13	-	AAA	Lys	12	-	AGA	Arg	5	-
ATG	Met	3	3	ACG	Thr	4	-	AAG	Lys	3	15	AGG	Arg	2	-
GTT	Val	4	-	GCT	Ala	1	-	GAT	Asp	2	-	GGT	Gly	8	-
GTC	Val	6	-	GCC	Ala	1	11	GAC	Asp	3	5	GGC	Gly	5	26
GTA	Val	4	-	GCA	Ala	6	-	GAA	Glu	10	1	GGA	Gly	9	1
GTG	Val	6	20	GCG	Ala	3	-	GAG	Glu	6	15	GGG	Gly	5	-

[a]The actual codons used for each amino acid in sDAF are the most prevalent ones in mammals, according to a previous report (*12*). In particular, CTC, TCC, GAA, GGA-codons were used for Leu, Ser, Glu, Gly, respectively, in one place in sDAF for the sake of convenience in the construction. A.A., amino acid; w*, delta-SCR1 wild-type DAF; s, synthetic DAF.

GAATTCGCCA cc ATG ACC GTG GCC CGC CCC AGC GTG CCC GCC GCC CTG CCC CTG CTG
GGC GAG CTG CCCCGC CTG CTG CTG CTG GTG CTG CTG TGC CTG CCC GCC GTG TGG
GGC CGC AGC TGC GAG GTG CCC ACC CGC CTG AAC AGC GCC AGC CTG AAG CAG CCC
TAC ATC ACC CAG AAC TAC TTC CCC GTG GGC ACC GTG GTG GAG TAC GAG TGC CGC
CCC GGC TACCGC CGC GAG CCC AGC CTG AGC CCC AAG CTG ACC TGC CTG CAG AAC
CTG AAG TGG AGC ACC GCC GTG GAG TTC TGC AAG AAG AAG AGC TGC CCC AAC CCC
GGC GAG ATC CGC AAC GGC CAG ATC GAC GTG CCC GGC GGC ATC CTG TTC GGC GCC
ACC ATC AGC TTC AGC TGC AAC ACC GGC TAC AAG CTG TTC GGC AGC ACC AGC AGC
TTC TGC CTG ATC AGC GGC AGC AGC GTG CAG TGG AGC GAC CCC CTG CCC GAG TGC
CGC GAG ATC TAC TGC CCC GCC CCCCCC CAG ATC GAC AAC GGC ATC ATC CAG GGC
GAG CGC GAC CAC TAC GGC TAC CGC CAG AGC GTG ACC TAC GCC TGC AAC AAG GGC
TTC ACC ATG ATC GGC GAG CAC AGC ATC TAC TGC ACC GTG AAC AAC GAC GAG GGC
GAG TGG AGC GGC CCC CCC CCC GAG TGC CGC GGC AAG AGC CTG ACC AGC AAG GTG
CCC CCC ACC GTG CAG AAG CCC ACC ACC GTG AAC GTG CCC ACC ACC GAG GTG AGC
CCC ACC AGC CAG AAG ACC ACC ACC AAG ACC ACC ACC CCC AAC GCC CAG GCC ACC
CGC AGC ACC CCC GTG AGC CGC ACC ACC AAG CAC TTC CAC GAG ACC ACC CCC AAC
AAG GGC AGC GGC ACC ACC AGC GGC ACC ACC CGC CTG CTG AGC GGC CAC ACC TGC
TTC ACC CTG ACC GGC CTG CTG GGC ACC CTG GTG ACC ATG GGC CTG CTG ACC TAG

Fig. 1. **Characteristics of the synthetic DAF gene.** The nucleotide sequence of sDAF, including a Kozak sequence, is shown. The synthetic DAF gene lacks SCR1.

hr at 37°C, and the released LDH was then determined. The spontaneous release of LDH activity from target cells was less than 5% of the maximal release of LDH activity, as determined by sonication (*16*).

Transgenic Mice—B6C3F1 female mice were induced to superovulate and then crossed with B6C3F1 males. Microinjection and embryo transfer were performed by standard methods, thus generating transgenic mice expressing DAF. Mice carrying these pCXN-DAF plasmids were crossed with B6 to obtain offspring. That the mice carry the DAF plasmid was verified by PCR and Southern blotting. The primers used for the detection of the DAF gene were the 5′ primer ACCTTCTCTATCACCAAAACT and the 3′ primer TCTCCTTCATCA TTATTCACA. The primers used for synthetic DAF were also targeted for a portion of the 3′UTR of pCXN (*17*). The conditions were as follows: 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 60 s.

Southern blotting was carried out using these PCR probes. Hybridization signals were also evaluated by FAST SCAN.

Histological Detection of DAF Expression—Various organs were excised from transgenic mice and a portion of each was fixed in 4% paraformaldehyde/Dulbecco's PBS (D-PBS) for 30 min. The fixed cells were incubated with blocking solution (2% BSA/D-PBS) for 1 h and then reacted with anti-DAF mAb 2H6 (a gift from Dr. T. Kinoshita, Osaka University, Osaka) (*17*), which was conjugated with FITC in our laboratory at a 1:1,000 dilution for 1 h. The slides were viewed with a Zeiss Axioplan 2 universal microscope.

Statistics—Data are presented as the mean ± SEM. The student's *t* test was used to ascertain the significance of differences within groups. Differences were considered statistically significant when $p<0.05$.

RESULTS

Construction of the Synthetic DAF Gene—The coding region of synthetic DAF without SCR1 (sDAF) was divided into seven segments in order to permit the synthesis of the gene using oligonucleotides that could be easily constructed (Fig. 1). The actual codons used here for each amino acid were the most prevalent ones in mammals. As a result of these experiments, 73% of the codons in the coding se-

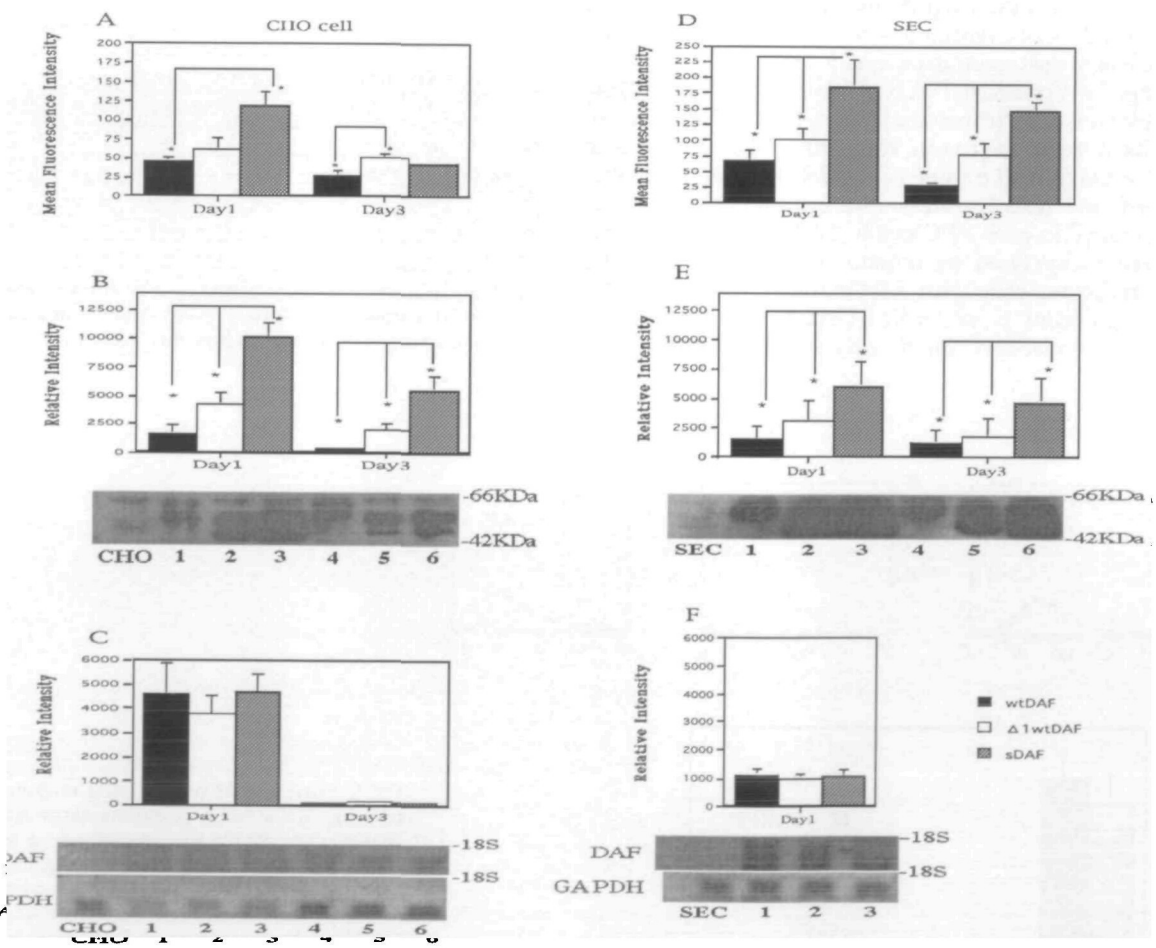

Fig. 2. **Expression of the DAF protein and mRNA in CHO cells and SEC temporarily transfected with three types of DAF gene.** Mean shift values DAF expression on the cell surface (A, D), Western blot of the DAF protein in cell lysates (B, E), and Northern blot of DAF mRNA (C, F) in CHO cells (A, B, and C) and SEC (D, E, and F) on days 1 and 3 after transfection are shown. [CHO and SEC, naive control; 1, wtDAF on day 1; 2, Δ1wtDAF on day 1; 3, sDAF on day 1; 4, wtDAF on day 3; 5, Δ1wtDAF on day 3; 6, sDAF on day 3.] A quantitative analysis of blotting bands in 6–7 independent experiments is shown in the upper panels. Each value is expressed as the mean ± SEM. *Significantly different ($p < 0.05$).

quence were replaced by more suitable codons in mammals, especially in swine cells (Table I).

In Vitro Analysis—pCXN-wtDAF, pCXN-Δ1wtDAF, and pCXN-sDAF were transfected into CHO cells and SEC. The levels of DAF expression of the transfectants with sDAF were determined by flow cytometry, and compared with those of wtDAF and Δ1wtDAF at day 1 and 3. The mean shift values for each transfectant are shown in Fig. 2, A and D. Only 1.9–2.0 times significant differences were detected between sDAF and Δ1wtDAF in SEC. In addition, differences between wtDAF and Δ1wtDAF were not detected in CHO cells on day 1.

However, Western blotting measurements of the protein levels of cell extracts indicated that the sDAF gene provided CHO cells with 11.9–17.4 or 3.3–2.9 times higher levels, and SEC with 4.0–4.1 or 1.9–2.6 times higher levels than did the wtDAF or Δ1wtDAF, respectively (Fig. 2, B and E).

DAF mRNA accumulation in CHO cells and SECs containing pCXN-DAF was next examined by Northern blot analysis. Total RNA, 5 μg from CHO cells and 25 μg from SECs, was loaded in each lane. The data showed that the mRNA levels of the sDAF gene were nearly the same as those of wtDAF and Δ1wtDAF (Fig. 2, C and F).

The data show that a significant increase in protein level was produced by the synthetic gene, indicating that the observed effect is post-transcriptional.

Function of Transfected DAF Molecules on Stable SEC Transfectants—SEC transfected with each DAF and control SEC cells were treated with 20 or 40% NHS, a source of natural antibody and complement. Amelioration of complement-mediated lysis by these transfectant molecules was then examined in each SEC line by LDH assay. Naive SEC cells were easily lysed by human complement. However, SEC transfectant cells with wtDAF, Δ1wtDAF and sDAF showed significant resistance to lysis by human complement. These molecules significantly suppressed SEC lysis by human complement with almost the same efficacy (Fig. 3).

Tissue Expression of DAF Molecules in Transgenic Mice—The in vivo expression levels of DAF were compared for pCXN-Δ1wtDAF and pCXN-sDAF.

Six independent lines of both constructs of transgenic mice were obtained, as assessed by PCR and Southern blotting.

The efficiency of DAF expression was also compared using these transgenic mice. Histochemical analysis of several types of organs from F1 hemizygous transgenic mice with DAF was performed. Tissue expression levels in transgenic mouse lines generated with both constructs are indicated in Table II. The results show that the level of DAF expression is considerably greater in the sDAF transgenic mice than in Δ1wtDAF transgenic mice, especially in the pancreas (Fig. 4).

A naive control mouse was also analyzed in exactly the same manner as for the transgenic mice. Specimens of naive organs are indicated (−), or sometimes (±). In addition, in a previous study, three transgenic mouse lines were developed with pCX-wtDAF. However, all sections of organs revealed only a weak expression for DAF, (−)–(±) (data not

TABLE II. **Tissue DAF expression in transgenic mice.**

	Δ1wtDAF						sDAF					
	#1	#8	#11	#14	#33	#39	#4	#5	#13	#30	#44	#57
Heart	±	±	±	-	-	+	+	-	-	±	+	+
Lung	±	±~+	±	-	±	±	+	+	±	-	±	-
Liver	-	±	±	++	±	±	+~++	±	+	-	+	±
Pancreas	±	-	-	-	-	-	++	+	+	+	±	±
Kidney	±	-	±	±	+	±~+	+	±	±~+	±	±	±

Grading scale: (−) = not stained, (±) = stained equivocally or weakly; (+) = stained moderately; (++) = stained intensely. Hemizygotes of each lines (F1) were used in this study.

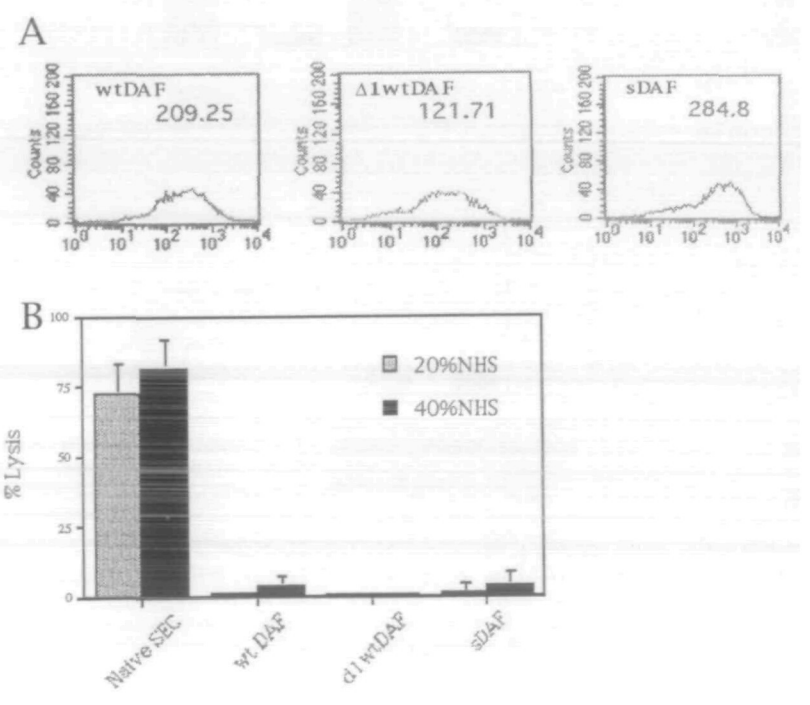

Fig. 3. **Function of transfected molecules in protecting SECs from complement-mediated cytotoxicity.** (A) Stable SEC transfectants with wtDAF, Δ1wtDAF, and sDAF genes were established, and the expression of each DAF molecule was verified by flow cytometry. (B) Control SEC and transfectants expressing equivalent amounts of DAF were tested in the LDH assay using human serum as a source of natural antibody and complement. Quantitative analysis of the data from 8 independent experiments is shown. Each value is expressed as the mean ± SEM. There are no significant differences among the transfectants.

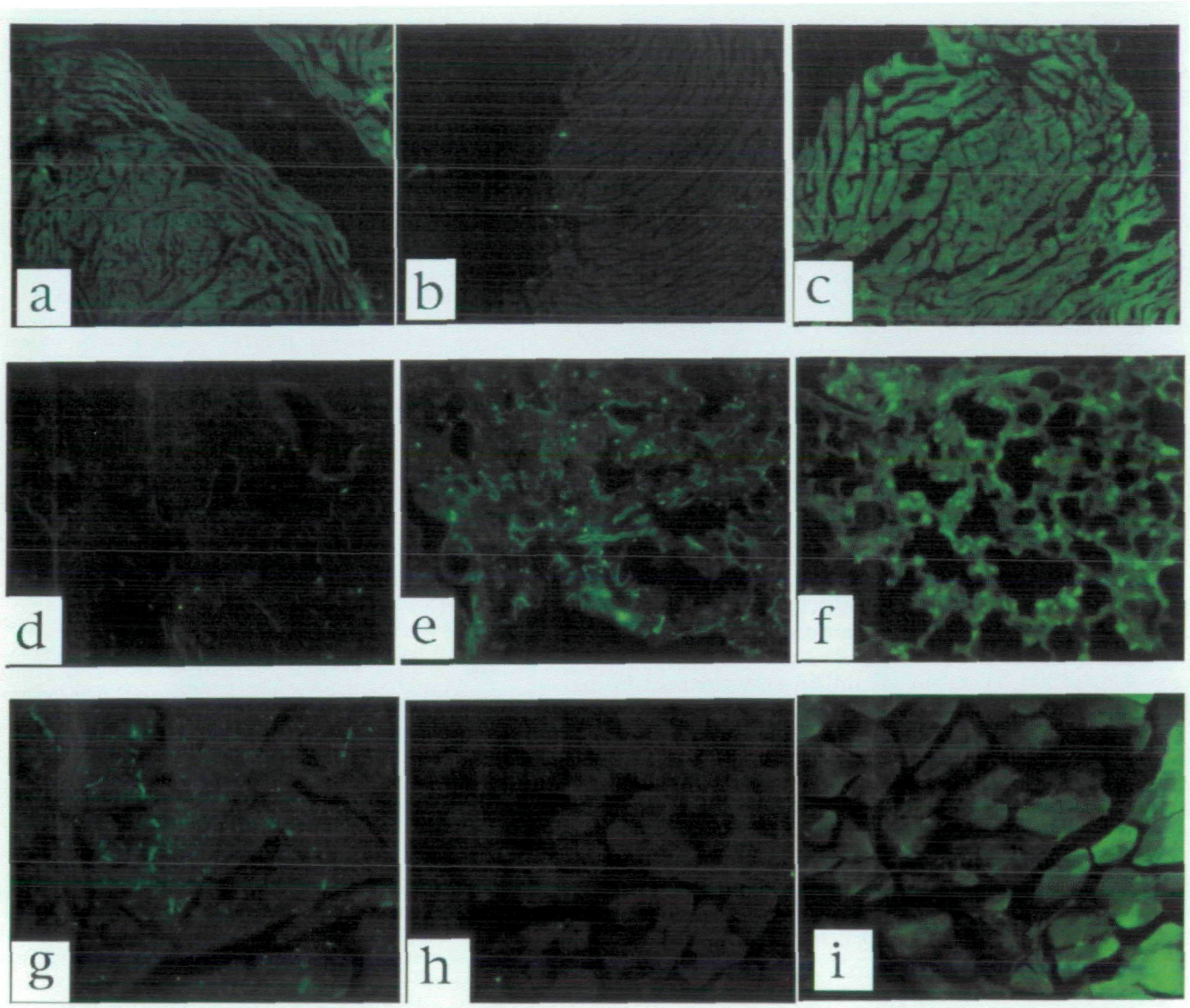

Fig. 4. **Immunostaining of tissue sections from transgenic mice.** Stainings of heart (a, b, and c) (×50), lung (d, e, and f) (×200) and pancreas (g, h, and i) (×200) are shown. a, Δ1wtDAF, #8, (±); b, Δ1wtDAF, #14, (−); c, sDAF, #44, (+); d, Δ1wtDAF, #14, (-); e, Δ1wtDAF, #11, (±); f, sDAF, #4, (+); g, naive control, (±); h, Δ1wtDAF, #14, (−); i, sDAF, #4, (++). Grading scale: (−) = not stained; (±) = stained equivocally or weakly; (+) = stained moderately; (++) = stained intensely. Representative fields of each section examined are shown.

shown).

Copy Number of Transgenes and the Expression of DAF—The copy number of transgenes in hemizygous offspring (F1) of both transgenic mouse lines was examined by Southern hybridization. The level of expression of DAF was not correlated with the copy numbers of the transgene. For example, the highest copy number was found in mouse #30 with sDAF (data not shown).

DISCUSSION

A strategy for achieving a high-level of DAF expression on a xeno-cell membrane was examined in this study. All substituted codons in the DAF gene were converted to codons that are more frequently utilized by mammals, especially pig (as defined using the Genbank sequences available in 1990) (*12*).

This strategy has usually been adapted to the expression of molecules in species more remote from mammals, such as the GFP (jellyfish *Aequorea victorio*) gene in plants or mammals (*18–20*) and the Cre recombinase (bacteriophage P1) gene in mammals (*15*). However, the pressing need for a high level of expression of CRPs in transgenic pigs, and the fact that the DAF gene has many low frequency codons in mammals, stimulated the initiation of these experiment (*21*).

In *in vitro* studies, a significant increase in protein levels produced by the synthetic gene was observed using 1C6 mAb, which binds to the SCR3 of the DAF molecule (*22*). In addition, the location of the epitope for the 2H6 mAb was also proved to be at a site other than the SCR1 of the DAF molecule. While the protein production of sDAF might be 4.0–11.9 times higher than that of wtDAF, the level of expression on the cell surface was only 2.6-2.7 fold higher.

The possibility exists, however, that considerably more DAF molecules actually exist in the caveola and that these remained undetected (*23*). However, it is much more likely that the produced molecules were distributed beneath the cell surface, such as in the Golgi apparatus, vesicules (*24*), and caveola.

A Northern blot analysis was also carried out, in order to determine of whether the high expression detected by Western blot is produced by the synthetic sequence only in the translational stage. Earlier studies, including ours, showed that the codon-optimized gene correlates with the upregulated mRNA, either through direct effects on mRNA stability determinants, or indirectly via an interplay between mRNA translation and degradation (*15, 25*), while other studies have concluded that the effect of high expression is not due to transcriptional synthesis or stability (*26, 27*). In this study, Northern blot analysis showed that the transcripts that encode sDAF are nearly the same as the transcripts which encode wtDAF and Δ1wtDAF, indicating that the effect is translational in nature.

In addition, the choice of an expression system to achieve a high level of production of a recombinant protein is dependent on a variety of factors, including promoters, enhancers, transcriptional or translational terminators, and mRNA stability. In our case, the same promoter and poly A tail were used, and a cloning site for the cDNA exists on exon 2 in pCXN. Therefore, all mRNAs are destined to decay *via* the same pathway.

We are planning to introduce a delta-SCR1 type DAF into a transgenic pig, because the removal of SCR1 has no effect on DAF function, as demonstrated in this study. The active site of DAF comprises a positively charged surface area on SCR2 and SCR3 (including $KKK^{125-127}$) and the nearby exposed hydrophobic residues ($L^{147}F^{148}$) on SCR3. SCR4 is also related in terms of its alternative pathway regulatory activity (*28, 29*). On the contrary, SCR1 functions as a receptor for viruses, such as coxsackievirus A21 (*30*) and B3 (*31, 32*), echovirus 7 (*33, 34*), and an *Escherichia coli* receptor (*35*).

The specific reason Δ1wtDAF provides much better DAF expression than wtDAF in SEC and CHO cells is currently unknown. A down regulating effect in exon 2 of DAF (SCR1) at the post-transcriptional stage is consistent with this observation (*36–38*). Further studies will be required to clarify the precise mechanism.

Finally, histochemical analysis of a section from F1 transgenic mice showed that DAF expression is much more evident in mice with sDAF than Δ1wtDAF. Regarding mouse #14 of the Δ1wtDAF line, DAF expression in the liver appears to arise from a chromosomal position effect. The difference between these two constructs in transgenic mice seems to be closer to that of the cell surface expression of DAF *in vitro*. In addition, the pCX promoter and synthetic DAF produced high expression of the DAF molecule, especially in pancreas. These data suggest that this system is suitable for xenotransplantation of pancreas or pancreatic islets, which is thought to be the nearest to actual clinical therapy (*39*). Unfortunately, the protein levels of DAF in each organ could not be investigated in detail because of the specificity of the two antibodies used in the *in vivo* Western blotting experiments.

In conclusion, a synthetic DAF gene with a codon usage optimized to the mammalian system represents a critical factor in the development of transgenic animals.

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