Identification and characterization of a silencer regulatory element in the 3′-flanking region of the murine CD46 gene

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The murine membrane cofactor protein (CD46) gene is expressed exclusively in testis, in contrast to human CD46, which is expressed ubiquitously. To elucidate the mechanism of differential CD46 gene expression among species, we cloned entire murine CD46 genomic DNA and possible regulatory regions were placed in the flanking region of the luciferase reporter gene. The reporter gene assay revealed a silencing activity not in the promoter, but in the 3′-flanking region of the gene and the silencer-like element was identified within a 0.2-kb region between 0.6 and 0.8 kb downstream of the stop codon. This silencer-like element was highly similar to that of the pig MHC class-I gene. The introduction of a mutation into this putative silencer element of murine CD46 resulted in an abrogation of the silencing effect. Electrophoretic mobility-shift assay indicated the presence of the binding molecule(s) for this silencer sequence in murine cell lines and tissues. A size difference of the protein–silencer-element complex was observed depending upon the solubilizers used for preparation of the nuclear extracts. A mutated silencer sequence failed to interact with the binding molecules. The level of the binding factor was lower in the testicular germ cells compared with other organs. Thus the silencer element and its binding factor may play a role in transcriptional regulation of murine CD46 gene expression. These results imply that the effects of the CD46 silencer element encompass the innate immune and reproductive systems, and in mice may determine the testicular germ-cell-dominant expression of CD46.

Key words: complement, fertilization, innate immunity, silencer, testicular germ cell.

INTRODUCTION

Complement (C)-regulatory proteins contain unique structural domains known as short consensus repeats (SCRs), form a gene cluster on chromosome 1q32, can inactivate the C effector C3b either reversibly or irreversibly, serve as virus/bacterial receptors, and are expressed at high levels on placenta and spermatozoa [1–4]. To date, six C-related SCR proteins with similar structural properties have been identified [1,5]. Due to the clustering of SCR genes on chromosome 1q32, it has been suggested that homologous recombination and unequal crossover gave rise to a variety of SCR gene members and a divergence of their functions *in vivo*. In humans, membrane cofactor protein (MCP, CD46) and decay-accelerating factor (DAF, CD55) are SCR proteins with a major role in protection of host cells from homologous C attack [6,7]. Indeed, these SCR proteins can be defined as safeguards against homologous C attack.

Human CD46 was first identified as a serine protease (Factor I) cofactor for the irreversible inactivation of homologous C3b, and can functionally complement another C-regulatory protein, decay-accelerating factor [6]. CD46 also acts as a measles virus (H protein) receptor [8,9]. All human nucleated cells and organs ubiquitously express CD46, which mediates their survival during C-mediated injury but allows systemic infection by the measles virus. The functional domains of CD46 consist of four SCRs which serve as binding sites for the ligands C3b/C4b and measles H protein [10–12].

Recently, a murine cDNA homologue of human CD46 was cloned in our laboratory [13]. The predicted mouse CD46 protein showed 45% identity in the primary structure with human CD46. Like human CD46, it consisted of four short consensus repeats, a serine/threonine-rich domain, a transmembrane domain and a short cytoplasmic tail. Strikingly, murine CD46 is expressed only in the testicular germ cells 29 days after birth [13]. Its distribution is limited to the inner acrosomal membrane of murine spermatozoa and its expression is induced by the acrosomal reaction (N. Inoue, M. Nomura, M. Okabe and T. Seya, unpublished work), as is the case with human CD46 expressed in testis [14,15]. These findings with murine CD46 support reports detailing the unique structural and functional properties of CD46 expressed on human sperm [16,17], which is distinct from the ubiquitous isoforms of human CD46 [1,2]. One possible interpretation of these findings is that sperm CD46 may act as an egg-binding protein expressed on the inner acrosomal membrane. The fact that monoclonal antibodies against human CD46 partially block binding of human sperm and hamster eggs may support this hypothesis [18,19]. Although conclusive evidence has yet to be discovered, CD46 may be involved in homologous

Abbreviations used: C, complement; CDPSE, CD46 putative silencer element; EMSA, electrophoretic mobility-shift assay; PD1 silencer, pig MHC class-I silencer; SCR, short consensus repeat; SV40, simian virus 40; TNFR2, tumour necrosis factor receptor 2; AP-2, activator protein-2; Sp-1, simian virus 40 protein-1.

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The nucleotide sequence data reported will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases under accession numbers AB014480 (5′-flanking region of murine CD46) and AB025974 (3′-flanking region of murine CD46).

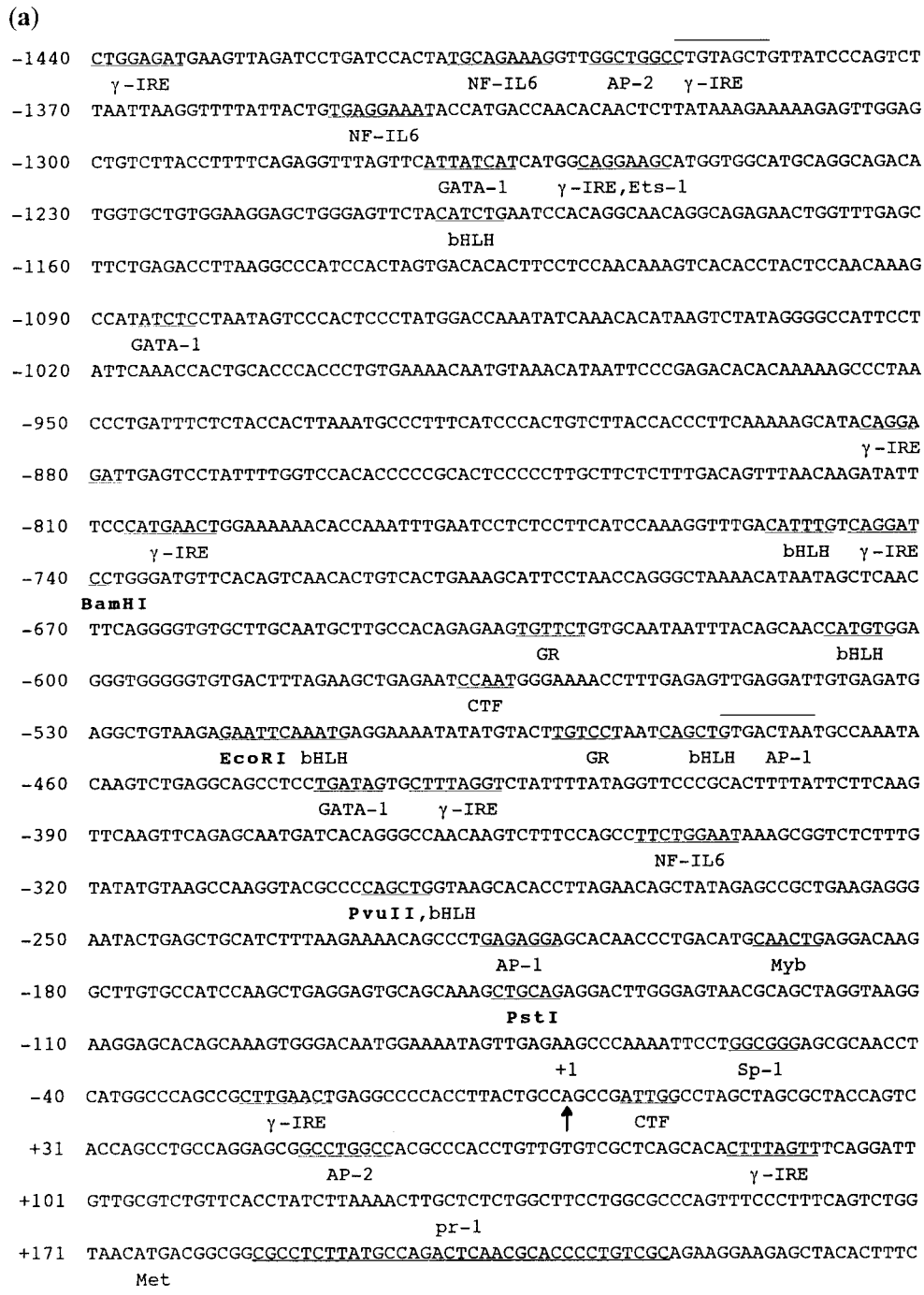

Figure 1 For legend see facing page

species recognition by functioning in C-immune effector cell responses (innate immunity) and in sperm–egg adhesion (fertilization).

The purpose of this study was to determine the mechanism through which CD46 is expressed in a testis-specific fashion in mice. The message size of the testis CD46 is 1.5 kb in both humans and mice, while that of the ubiquitously expressed form of CD46 is 4 kb in humans [2,13]. The murine equivalent of the 4-kb form is present only at very low levels in all organs tested, offering one possible explanation for the testis-specific expression of CD46. However, little is known about the transcriptional or post-transcriptional events that regulate CD46 expression in mouse cells. Our preceding study suggested that expression of CD46 is most likely to be regulated through transcription (M. Nomura and T. Seya, unpublished work). Hence, we conducted gene-regulation analysis of murine CD46 and found that a novel silencer sequence located in the 3′-untranslated region of the putative 4-kb transcript acts as a transcriptional regulator in mouse cells. Taken together, the results of our promoter analysis may in part explain the mechanism of tissue-specific expression

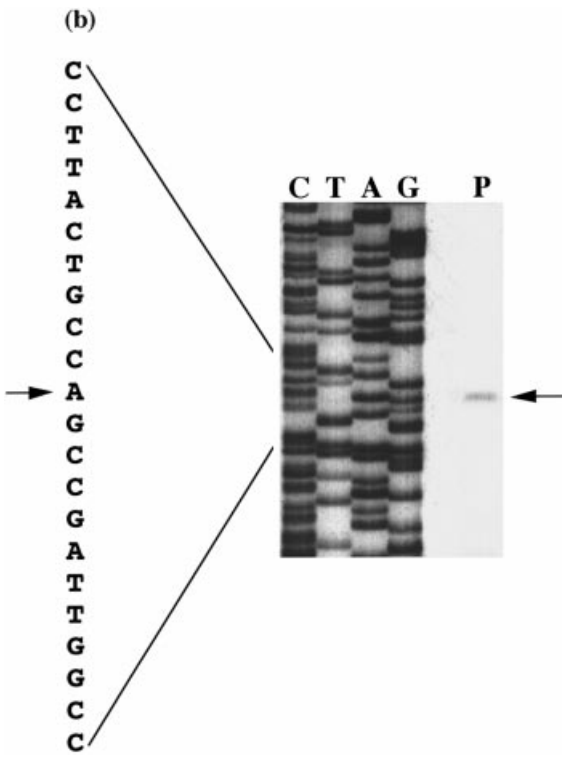

Figure 1 Sequence analysis of the 5′-flanking region of murine CD46 gene

(**a**) The nucleotide sequence of the 1.44-kb 5′-flanking region and the first exon of murine CD46 is shown. The major transcription start point is indicated by an arrow. The nucleotide corresponding to the major transcription start point is numbered as +1, which is 174 bp upstream from the start codon (Met) of murine CD46. pr-1 primer used for primer-extention analysis is underlined. The sites of restriction enzymes (*Bam*HI, *Eco*RI, *Pvu*II and *Pst*I) used for promoter analysis are underlined. Potential binding sites for regulatory factors are underlined with names of the factors listed below. γ-IRE, γ-interferon-responsive element; NF-IL6, interleukin-6-responsive nuclear factor; GATA-1, GATA-binding protein-1; Ets-1, transforming oncogene-1 of the avian erythroblastosis virus; bHLH, basic helix–loop–helix protein; GR, glucocorticoid receptor; CTF, CCAAT-box-binding transcription factor; AP-1, activation protein-1; Myb, transforming oncogene of the avian myeloblastosis virus. The nucleotide sequence data reported will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases under accession number AB014480. (**b**) Primer-extension analysis of the transcription start point(s). The lanes marked C, T, A and G show the dideoxy sequencing ladders of the 5′-upstream region which served as sequence markers, and P indicates the extended products. The deduced nucleotide sequence close to the major transcription start point is shown on the left. One major transcription start point is indicated by an arrow.

of mouse CD46. These data suggest the existence of a novel gene-regulatory pathway encompassing both the innate immune system and the reproductive system.

MATERIALS AND METHODS

Cell culture

Mouse embryonal carcinoma F9 cells [20,21], mouse fibroblast L cells and NIH 3T3 cells [22,23] were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, and the mouse hepatoma G5 cells [24] were maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum.

DNA sequencing

Murine CD46 genomic DNAs were subcloned into the pUC19, pCRII (Invitrogen), pGV-E2 and pGV-C2 (Picagene) vectors. Subcloned products were sequenced on both strands by the cycle sequencing method, using the 373A automated DNA sequencer (Perkin Elmer, Applied Biosystems Division).

Primer-extension analysis

A 35-mer oligonucleotide corresponding to the antisense sequence of the murine CD46 gene encoding signal peptide, named pr-1 (5′-GCGACAGGGGTGCGTTGAGTCTGGCATAAG-AGGCG-3′), was used for primer-extension analysis. Total RNAs of mouse testis were first extracted by Trizol (Gibco-BRL) and poly(A)$^+$ RNAs were then isolated by an mRNA purification kit (Pharmacia) and analysed as described by Triezenberg [25]. The oligonucleotide was end-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase. The labelled oligonucleotide was annealed to poly(A)$^+$ RNAs (1 μg) extracted from mouse testis. The extention reaction was carried out with SuperScript reverse transcriptase (Gibco-BRL). The extended product was analysed on a 6% sequencing gel and compared with a dideoxy sequencing ladder generated using the same primer and a genomic DNA fragment subcloned into the pUC19 plasmid.

Promoter assay

The murine CD46 promoter region (p1.6) and the human CD46 promoter region (pHum) were amplified by PCR using primers containing an *Xho*I site at the 5′ end (pr1.6-5X, 5′-GTCTCG-AGCAGATGAAGTTAGATCCTGATCC-3′; prHum-5X, 5′-GTCTCGAGCAAGAGCTTTGATCACAGCTCTGATG-3′) and a *Hin*dIII site at the 3′ end (pr1.6-3H, 5′-GTAAGC-TTCACAGACTGAAAGGGAAACTGG-3′; prHum-3H, 5′-GTAAGCTTCAGTCCAGATATGGGACGCAACAATC-3′). After digestion by *Xho*I/*Hin*dIII, the PCR products were ligated to the upstream region of the luciferase gene (*Xho*I/*Hin*dIII site of enhancer vector, pGV-E2). The PCR-amplified regions were confirmed by DNA sequencing. To generate deletion clones, the four plasmids containing fragments of the murine CD46 promoter region (pPst, pPvu, pEco, pBam) were constructed using the sites of these restriction enzymes (*Pst*I, *Pvu*II, *Eco*RI, *Bam*HI) existing within the murine CD46 promoter region. These plasmid DNAs were transiently transfected into F9 and L cells using LipofectAMINE Plus (Gibco-BRL). After 48 h cells were harvested and luciferase activity was measured according to the manufacturer's protocol (Picagene). As an internal control, we also used a double luciferase assay system, which included co-transfection with pRL-SV40 (Picagene).

Silencer assay

Three fragments of different sizes (0.6, 0.8 and 2.0 kb) of the murine CD46 3′-flanking region were amplified by PCR using primers containing a *Bam*HI site at the 5′ end (pr3′-5B, 5′-GTGGATCCCAATGTATCTGCAGCAAGATG-3′) and a *Sal*I site at the 3′ end (pr0.6-3S, 5′-GTGTCGACCCTATGA-ATACGTTCAAGTG-3′; pr0.8-3S, 5′-GTGTCGACCACCAA-GCTTTCTGCTGAGC-3′; pr2.0-3S, 5′-GTGTCGACGAGTA-GGCATTGCAGGTGG-3′). The fragments were digested by *Bam*HI/*Sal*I, and ligated to the downstream region of the luciferase gene (*Bam*HI/*Sal*I site of the enhancer vector, pGV-E2) containing CD46 promoter. The PCR-amplified regions were identified by DNA sequencing. These plasmid DNAs were transiently transfected into F9, L, NIH 3T3 and G5 cells using LipofectAMINE Plus. After transfection (48 h), we harvested cells to measure luciferase activity according to the manufacturer's protocol (Picagene). As an internal control, we used a

double luciferase assay system with co-transfection of pRL-SV40.

To examine the orientation effect, we prepared the 0.8-kb fragment of the murine CD46 3′-flanking region by PCR using primers containing a *Sal*I site at the 5′ end (pr3′-5S, 5′-GTGTCGACCAATGTATCTGCAGCAAGATG-3′) and a *Bam*HI site at the 3′ end (pr0.8-3B, 5′-GTGGATCCCACCA-AGCTTTCTGCTGAGC-3′). After digestion by *Bam*HI/*Sal*I, the fragment was ligated to the downstream region of the luciferase gene (*Bam*HI/*Sal*I site of the enhancer vector, pGV-E2) containing the CD46 promoter, which was designated as 3′-0.8R(CD). To examine the position effect, we prepared the 0.8-kb fragments of the murine CD46 3′-flanking region by PCR using forward-primer combination (pr3′-5S and pr0.8-3B) and reverse-primer combination (pr3′-5B and pr0.8-3S). After *Bam*HI/*Sal*I digestion, the fragment was ligated to the upstream region of the luciferase gene (*Bgl*II/*Xho*I site of the enhancer vector, pGV-E2) containing the CD46 promoter. The construct with the fragment made by the forward-primer set was designated 5′-0.8F(CD) and that made by the reverse primer set was designated 5′-0.8R(CD). To investigate the effect on the heterologous promoter, we used the simian virus 40 (SV40) promoter-derived luciferase control vector (pGV-C2), named 5′-0.8F(SV), 5′-0.8R(SV), 3′-0.8F(SV) and 3′-0.8R(SV) for all of the respective constructs described above.

Construction of the mutant silencer sequence

Introduction of mutations into the putative silencer sequence was performed by PCR according to the method described by Cormack [26], using the oligomers containing the mutations as primers (mut sil-5, 5′-TA**GG**AA**TT**CCCAAATTTTCAAAGA-CTTG-3′; mut sil-3, 5′-GG**AATTCC**TAGGTAGAATTAAA-CTCTG-3′). The four point mutations (shown in bold) were introduced into the silencer-binding site 2 (TATTAAAA TA-**GG**AA**TT**) of murine CD46.

Preparation of nuclear extracts from cells and tissues

Nuclear extract from L cells was prepared according to the protocol of Paludan et al. [27] using 0.6% Nonidet P-40 as a solubilizer. Whole-tissue nuclear extracts were prepared by homogenization in a Waring blender in 3 vols of 250 mM KCl/50 mM Tris/HCl (pH 7.5)/5 mM $MgCl_2$/0.5 mM PMSF/0.5 mM dithiothreitol/0.2% Triton X-100. Nuclei were pelleted by centrifugation for 5 min at 100 ***g*** at 4 °C, washed once in buffer without Triton X-100, and collected by centrifugation. In some experiments, Nonidet P-40 (final concentration, 0.6%) was added to the Triton X-100 extract. The material was then processed as described by Dignam et al. [28]. Protein concentrations were measured using the micro BCA protein assay reagent kit (Pierce), and DNA concentrations were estimated by absorption at 280/260 nm.

Electrophoretic mobility-shift assays (EMSAs)

Following a procedure described previously [29], the nuclear cell extracts (2 μg) were incubated with 1 μg of poly(dI:dC) at room temperature for 5 min in a final volume of 25 μl of binding buffer (7.5 mM Hepes/KOH, pH 7.9/0.5 mM EDTA/0.5 mM dithiothreitol/0.5 mM PMSF/50 mM KCl/10% glycerol). The double-stranded oligonucleotides used as probes were prepared as follows: 3′mut CDPSE (3′ part-mutated CD46 putative silencer element), 5′-AGAAAATCAGAGTTTAATTCTACC-TA**GG**AA**TT**-3′ and 5′-**AATTCC**TAGGTAGAATTAAACTC-TGA TTTTCT-3, 5′mut CDPSE (5′-part mutated CDPSE), 5′-AG**CTCGAG**AGAGTTTAATTCTACCTA-TTAAAA-3′ and 5′-TTTTGGTAGGTAGAATTAAACTCT**CTCGAG**CT-3′ (bold indicates the mutated sites); activator protein-2 (AP-2) probe, 5′-CGTGCTCCCCAGGCCTTGCC-3′ and 5′-GGCA-AGGCCTGGGGAGCACG-3′. The synthesized oligonucleotides were annealed, and end-labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase. A 1-μl amount containing 0.3–2 ng of the labelled DNA (20000 c.p.m.) was added to the extracts and incubated at room temperature for 30 min.

For the competitive DNA-binding assay, the extracts were preincubated at room temperature for 5 min with poly(dI:dC) plus an increasing molar excess of the competitors, and end-labelled probes were added as described above. 3′mut CDPSE was used as competitor for a negative control. DNA–protein complexes were analysed by electrophoresis on a 4% non-denaturing polyacrylamide gel. 32-mer double-stranded oligonucleotides consisting of random sequence were used as non-specific probes.

RESULTS

Analyses of the 5′-flanking region of the murine CD46 gene

Murine CD46 is selectively expressed in the testis, in contrast to human CD46 which is expressed ubiquitously [2,13]. Given the importance of promoters in the expression of a variety of testis-specific genes [30–33], we focused on the 5′-flanking region of murine CD46 to determine the regulatory mechanisms governing its expression. Primer-extension analysis with the primer pr-1, a 35-mer corresponding to a portion of the signal peptide of murine CD46 revealed a major transcription start point, at 171 bp upstream of the Met start codon (Figure 1a and 1b). We designated the nucleotide corresponding to this site as +1 of the CD46 gene (Figure 1a).

In order to determine the putative regulatory sequences of the murine CD46 gene, we subcloned and sequenced a 1.6-kb 5′-flanking DNA fragment extending from the initiation codon (Figure 1a). This region was tested first because the 5′ region of human CD46 gene (−624 to +96) has been shown to be sufficient for promoter activity [34]. Neither TATA-like nor CCAAT-like boxes were found in this region. TATA-less promoters can be divided into two classes [35]. One class are the constitutively expressed GC-rich promoters that are found mainly in housekeeping genes, and usually contain several potential binding sites for the transcription factor, Sp-1 (simian virus 40 protein-1). The other class of TATA-less promoters is not GC-rich, is regulated tissue-specifically and contains few binding sites for Sp-1. A motif search of the murine CD46 promoter region revealed the presence of several basic helix–loop–helix (bHLH) motifs and potential sites for many other regulatory factors such as Myb and activator protein-1 (AP-1); however, only one site for Sp-1 was found (Figure 1a). These features suggest that the murine CD46 promoter may belong to the latter class of TATA-less promoters, and thus is regulated tissue-specifically.

In constrast, the characteristics of the promoter sequence of human CD46, which is TATA-less, GC-rich and Sp-1-rich, suggested that the promoter of the human CD46 gene may belong to the constitutively expressed class of TATA-less promoters [34,35].

Promoter assay of the human and murine CD46 gene

To examine whether the promoter region (−147 to −1435) determines the tissue-specific expression of CD46, we performed

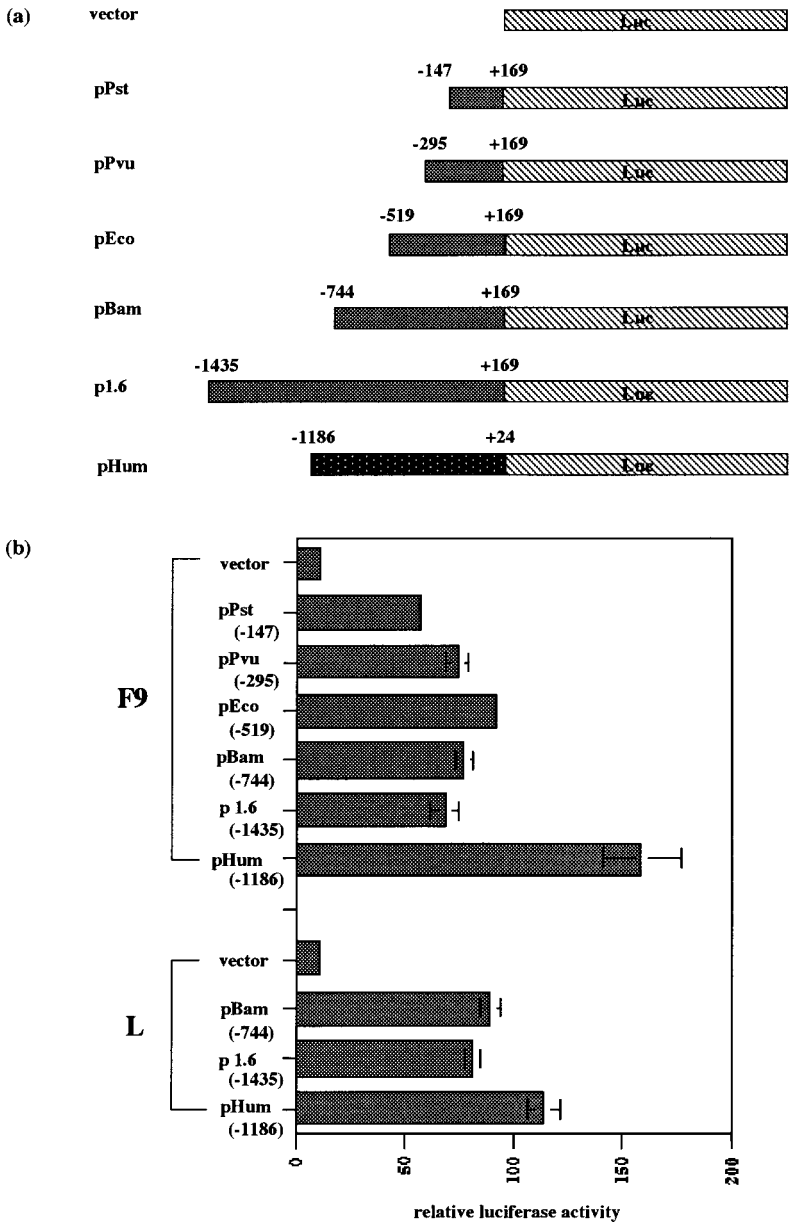

Figure 2 Promoter analyses of murine CD46 gene

(**a**) The plasmid constructs for promoter analyses are shown. Five different-sized fragments of the murine CD46 promoter region and full-length human CD46 promoter region were ligated to the luciferase enhancer vector as described in the Materials and methods section. Luc, luciferase reporter gene. (**b**) The plasmid DNAs described in (**a**) were transiently transfected into F9 and L cells. After 48 h, cells were harvested and luciferase activity was measured as described in the Materials and methods section. Relative luciferase activities are shown as means ± S.D. In three separate experiments the relative luciferase activities of the constructs were similar to those in the experiment shown.

a promoter analysis. Five different-sized fragments of the murine CD46 promoter region and the full-length human CD46 promoter region were cloned into a luciferase vector that lacked a promoter (pPst, −147 to +169; pPvu, −295 to +169; pEco, −519 to +169; pBam, −744 to +169; p1.6, −1435 to +169; and pHum, −1186 to +24; Figure 2a). The human CD46 promoter was used as a positive control because it was shown to function in mouse cells of various tissue types (results not shown). These plasmids were transfected into F9 cells (testis origin), which express a minute amount of CD46 mRNA, and L cells (fibroblast), which do not express CD46 mRNA. We hypothesized that the murine CD46 promoter constructs, unlike the human CD46 promoter construct, would not function in mouse cells other than testis, since murine CD46 expression was marginal in these cells. Unexpectedly, in both the F9 cells and L cells, comparable levels of promoter activity were detected using murine and human CD46 promoter constructs (Figure 2b), suggesting that the promoter region did not play a major role in determining the tissue-specific expression of murine CD46. Since significant promoter activity was detected in the pPst construct, the 147 bp of the 5′-flanking region may contain the main promoter activity. The highest level of luciferase activity was detected in the pEco construct, suggesting that an upstream region beyond −519 might contain some subtle inhibitory

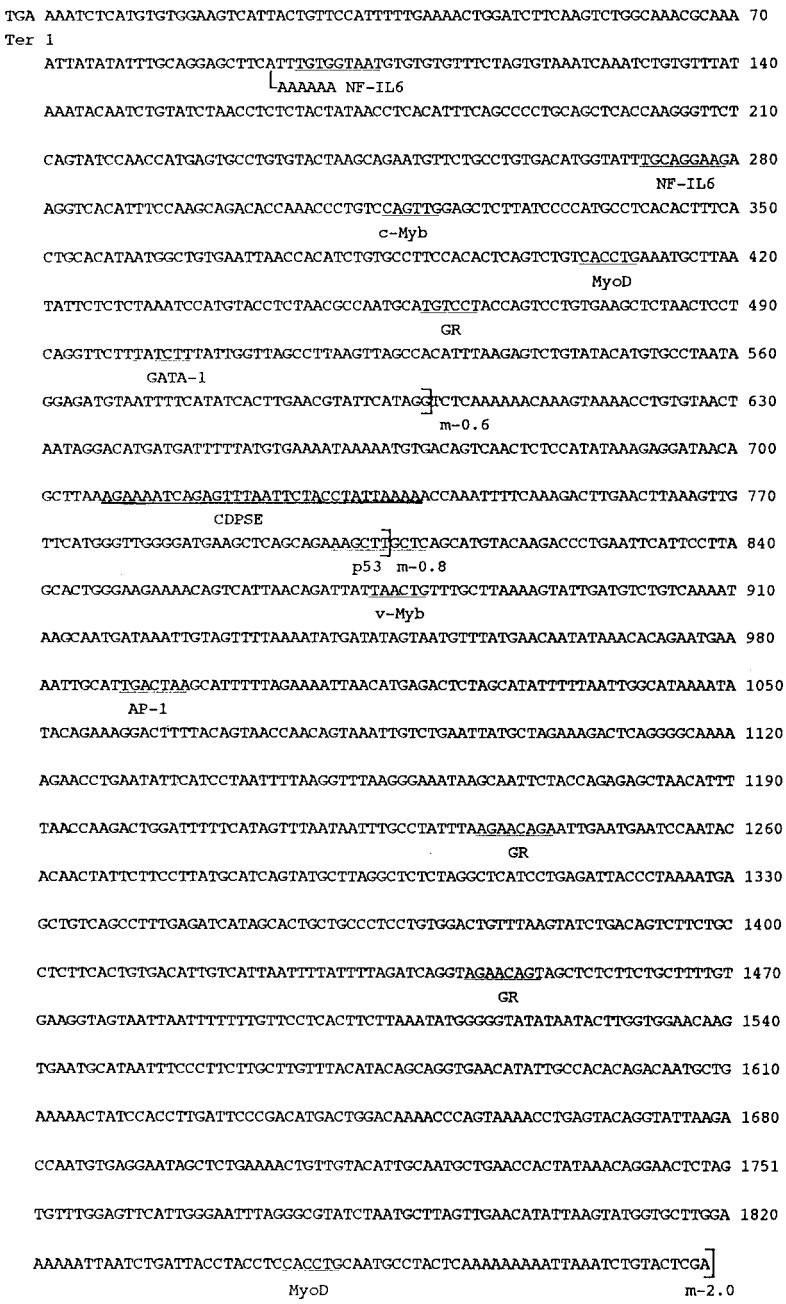

Figure 3 DNA sequence of the 3′-flanking region of the murine CD46 gene

Nucleotide sequence of the 1.88-kb 3′-flanking region (downstream of the termination codon, TGA) is shown. The end of murine CD46 mRNA is indicated by poly-As at nucleotide number 93. m-0.6, m-0.8 and m-2.0 indicate the sites of three different-sized fragments used in the experiment shown in Figure 4. Underlined sequence labelled CDPSE indicates the probe used in the EMSA shown in Figure 7. Potential binding sites for regulatory factors are underlined with names of the factors listed below the lines. NF-IL6, interleukin-6-responsive nuclear factor; v-Myb, the transforming oncogene of the avian myeloblastosis virus; c-Myb, cellular homologue of v-Myb; MyoD, myoblast-determining factor; GR, glucocorticoid receptor; GATA-1, GATA-binding protein-1; AP-1, activation protein-1. The sequence data reported here will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the accession number AB025974.

sequences similar to in humans. In the human CD46 gene, the 600-bp upstream region (-1204 to -624) beyond -624 showed the weak inhibitory effect [34].

Silencer assay of the human and murine CD46 gene

We next focused on the 3′-flanking region of murine CD46 due to the fact that in our previous study we detected an inhibitory effect in the 3′-untranslated region of human CD46 gene in mouse cell lines, but not in primate cells [36]. The 2.0 kb 3′-flanking sequence is shown in Figure 3. This region was inserted downstream of the luciferase gene in the vector containing the CD46 promoter (p1.6 shown in Figure 2a) and its effect on luciferase activity was measured in L cells. Promoter activity was decreased to one-quarter compared with the control vector (Figure 4b, m-2.0). Hence, we analysed several deletion constructs to identify the portion involved in the silencing effect. The

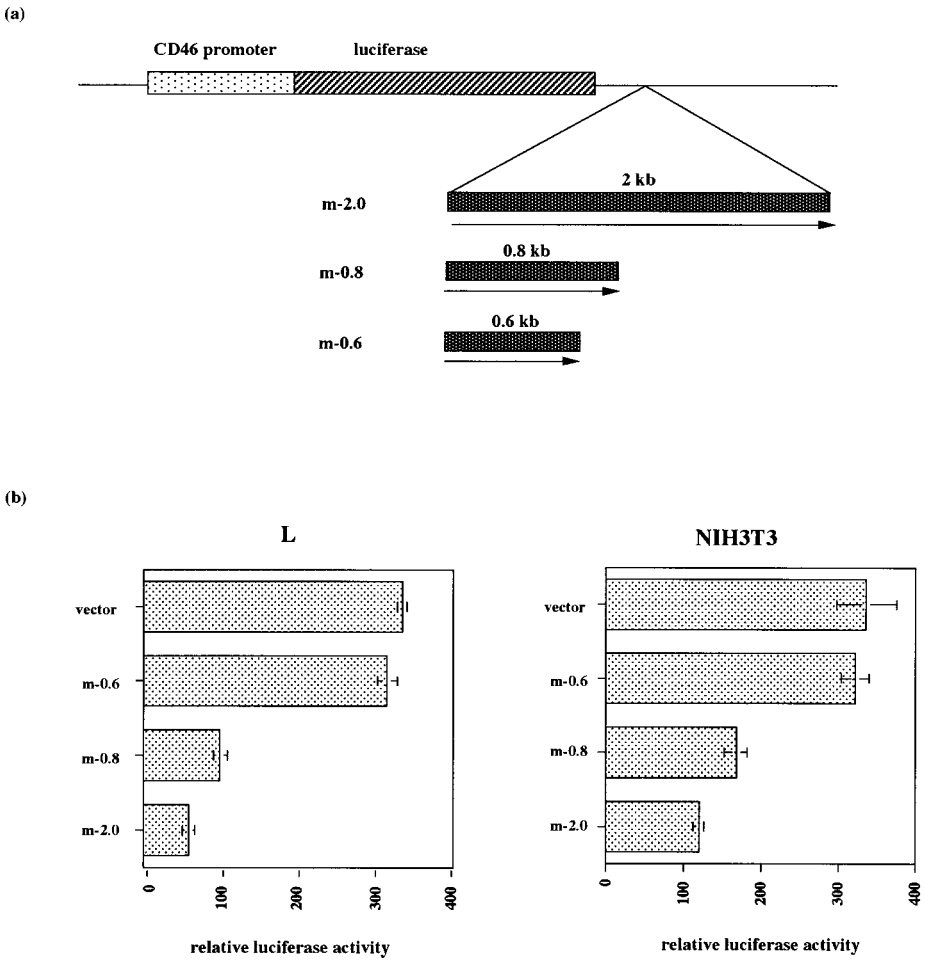

Figure 4 Effect of the 3′-flanking regions on murine CD46 promoter

(**a**) The plasmid constructs for silencer analyses are shown. Three fragments of the murine CD46 3′-flanking regions (approx. 0.6, 0.8 and 2.0 kb) were ligated to the luciferase under the control of the CD46 promoter, p1.6 shown in Figure 2(a), (m-0.6, m-0.8 and m-2.0) as described in the Materials and methods section. (**b**) Relative luciferase activities (shown as means ± S.D.) were determined similarly to Figure 2(b), using the plasmid constructs described in (**a**) and two cell lines, L and NIH 3T3, as host cells. In three separate experiments the relative luciferase activities of the constructs were similar to those shown.

representative constructs, three different-sized fragments of the murine CD46 3′-flanking region (approx. 0.6, 0.8 and 2.0 kb), were cloned into the luciferase vector containing the CD46 promoter (m-0.6, m-0.8 and m-2.0) (Figure 4a). These plasmid DNAs were transiently transfected into murine cell lines of various origins, and the effects of the murine 3′-flanking region were examined by luciferase assay. Interestingly, in mouse fibroblast L cells and NIH 3T3 cells, the 2.0- and 0.8-kb fragments of the 3′-flanking region showed significant silencing activity while the m-0.6 construct did not (Figure 4b). F9 (testis) and G5 (liver) cell lines showed similar silencing effect with these constructs (results not shown). Thus, the 3′-flanking region showed an inhibitory effect on a CD46 promoter, suggesting that the silencer element may exist in this 0.2 kb region between 0.8 and 0.6 kb (Figure 4b).

Characterization and identification of putative silencer sequence of murine CD46 gene

The 0.8-kb fragment of the 3′-flanking region of murine CD46 was inserted into the upstream or downstream regions of the luciferase reporter plasmid with authentic CD46 or heterologous SV40 promoters, in forward or reverse orientation, to study the position- and orientation-dependencies of the murine CD46 putative silencer (Figure 5a). The forward-oriented construct placed in the upstream region (5′-0.8F; Figure 5b) showed the same inhibitory effect as that in the downstream region (3′-0.8F). These constructs worked in conjunction not only with the CD46 promoter but also with the SV40 promoter. The reverse constructs (3′-0.8R and 5′-0.8R), however, showed the inhibitory effect in neither the CD46 promoter nor the SV40 promoter. Thus the 3′-flanking region of murine CD46 works as a silencer even on a heterologous promoter in a position-independent, orientation-dependent manner. In general, silencers work independent of orientation [37], but there are several reported silencer sequences that are exceptionally dependent on orientation [38,39].

The CD46 silencer most probably belongs to an orientation-sensitive silencer group. A motif search revealed the presence of a silencer-binding site 2 (TATTAAAA), which is the 3′-part of the PD1 silencer (silencer sequence of the pig MHC class-I gene) in this 0.2-kb region (Figure 3). The PD1 silencer consists of two

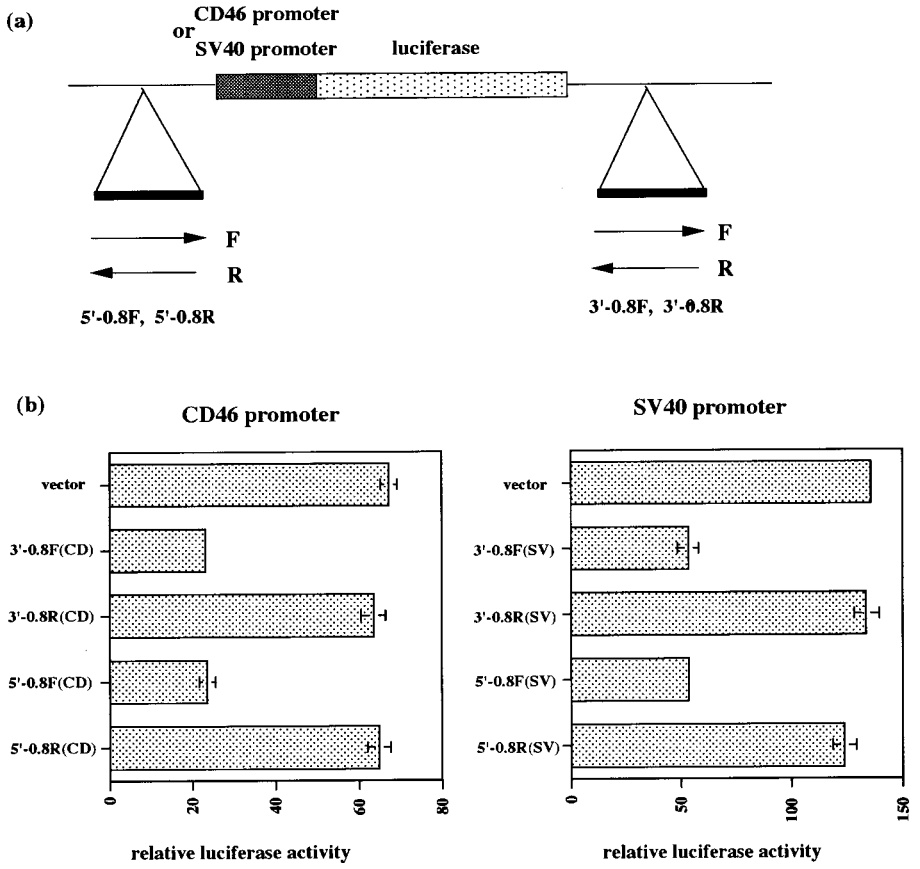

Figure 5 Position and orientation effects of murine CD46 3′-flanking region

(**a**) The plasmid constructs for silencer analyses are shown. The 0.8-kb fragment of murine CD46 3′-flanking region was inserted into the upstream or downstream regions of the luciferase vector containing the CD46 or SV40 promoters in the forward or reverse orientation as described in the Materials and methods section. F, forward; R, reverse; CD, CD46 promoter; SV, SV40 promoter. (**b**) Relative luciferase activities were determined similarly to Figure 2(b), using the plasmid constructs described in (**a**) and L cells as host cells. In three separate experiments the relative luciferase activities of the constructs were similar to those shown.

relevant sites, one 5′ and one 3′ [40]. The 3′ site is called silencer-binding site 2 and is identical to the putative silencer of murine CD46 (Figure 6a). Murine tumour necrosis factor receptor 2 (TNFR2) gene was also reported to contain a similar sequence to the PD1 silencer in its region, responsible for an inhibitory effect [41]. The similarites between the sequences of the PD1 silencer and the putative silencer of murine CD46, as well as those of the PD1 silencer and the silencer of murine TNFR2, are shown in Figure 6(a).

To determine whether this sequence was responsible for the silencing effect, we analysed a construct encoding a four-nucleotide mutation in silencer-binding site 2 of the putative CD46 silencer by the luciferase assay (Figure 6b). The silencing effect on luciferase expression was abrogated by this mutation, suggesting that this sequence is essential for the silencing effect (Figure 6c). These results imply that the 3′-flanking region of murine CD46 contains an orientation-dependent silencing region that shares the homologous sequence with the PD1 silencer.

EMSA

To search for the factors bound to the silencer sequence by EMSA, a nuclear extract of L cells, which showed a significant silencing effect, was first analysed. As shown in Figure 7(a), a binding molecule was detected using 32-mer double-stranded oligonucleotides, including CDPSE (Figure 3), and this binding was specifically decreased by the addition of unlabelled competitor probe (Figure 7a, lanes 2 and 3). The addition of the mutated repressor sequence did not affect binding, indicating that the binding was specific for CDPSE (Figure 7a, lanes 4 and 5). To exclude the possibility that the A/T-rich properties of the CDPSE (78% A/T) contribute to irrelevant binding, a random 78% A/T-containing sequence was used as a probe for EMSA, but no binding was observed using this probe (results not shown).

The binding molecule(s) was also detected in liver nuclear extracts prepared with an alternative method using Triton X-100 (Figure 7b, lane 1). As in the L cells, the binding was abrogated in the presence of unlabelled competitor probe (Figure 7b, lanes 2 and 3), and was not affected by the addition of mutated repressor sequence (Figure 7b, lane 4). During repetitive experiments, we found that the shifted bands in the Nonidet P-40 extract (L cells) did not align with those observed in the Triton X-100 extract (liver). The faster-migrating and the slower-migrating bands were detected in L cells and liver, respectively. The stability of the molecular complex binding to CDPSE may depend on the solubilizers used for the preparation of nuclear extracts. Indeed, the slower-migrating band shifted down to the

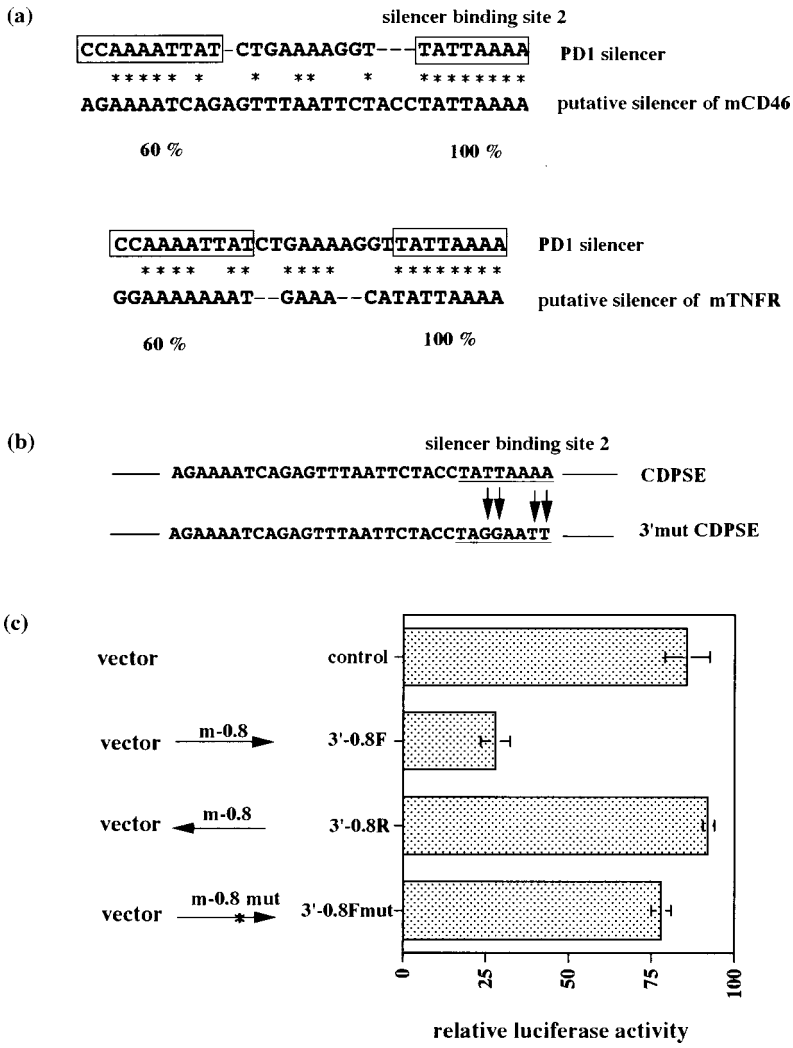

Figure 6 Mutational analysis of the putative silencer element of murine CD46

(**a**) Pairwise comparison between the PD1 or TNFR2 silencers and the putative silencer of murine CD46. (**b**) Four nucleotide mutations introduced into the silencer-binding site 2 in CDPSE are indicated by arrows, named as 3′mut CDPSE. (**c**) Relative luciferase activities were determined similarly to Figure 2(b), using the following plasmid constructs; 3′-0.8F and 3′-0.8R, in which the 0.8-kb fragment was introduced in the reverse orientation, and mutated 3′-0.8F mut, containing 3′mut CDPSE shown in (**b**). L cells are used as host cells. In three separate experiments the relative luciferase activities of the constructs were similar to those shown.

position of the faster-migrating band if the Triton X-100 nuclear extracts of tissues were treated further with Nonidet P-40 (Figure 7c). Thus in both cell lines and tissues there appear the faster-migrating and slower-migrating bands that may reflect the presence of low- and high-molecular-mass complexes, respectively.

The silencer element seems to consist of two parts, according to previous reports [40,41]. Two mutated CDPSEs (5′mut CDPSE and 3′mut CDPSE) containing nucleotide substitutions in the 5′ and 3′ parts were constructed (Figure 7d). The significant decrease in the binding of any nuclear factor to the 5′mut CDPSE (with six nucleotide substitutions) and complete failure of the binding of any nuclear factor to the 3′mut CDPSE (with four nucleotide substitutions) was observed using the same assay conditions (Figure 7d). This result reinforces the finding that murine CDPSE is involved in the silencing effect of the CD46 gene and consists of the two parts, the 3′ part (silencer-binding site 2) being more effective than the 5′ part.

The level of the binding molecules in the testis was lower than those in other organs tested to date: liver, kidney and brain (Figure 7e). Control EMSA using the AP-2-binding site as a probe showed similar binding ability in all of the tissues tested (Figure 7f). Relative depletion of the binding factor to CDPSE in the testis compared with other tissues may reflect the connection of the CD46 silencer element and its binding factor with testis-dominant expression of murine CD46.

DISCUSSION

Here, we identified a silencer element in the 3′-flanking region of the murine CD46 gene involved in transcriptional regulation. The CDPSE consisted of two parts, with the silencer-binding site 2 in the 3′ part being 100% identical to those of the PD1 and TNFR2 silencers, which control the mRNA levels of MHC class I and TNFR2, respectively. Using EMSA we also identified a silencer factor that bound specifically to the CDPSE. The level of

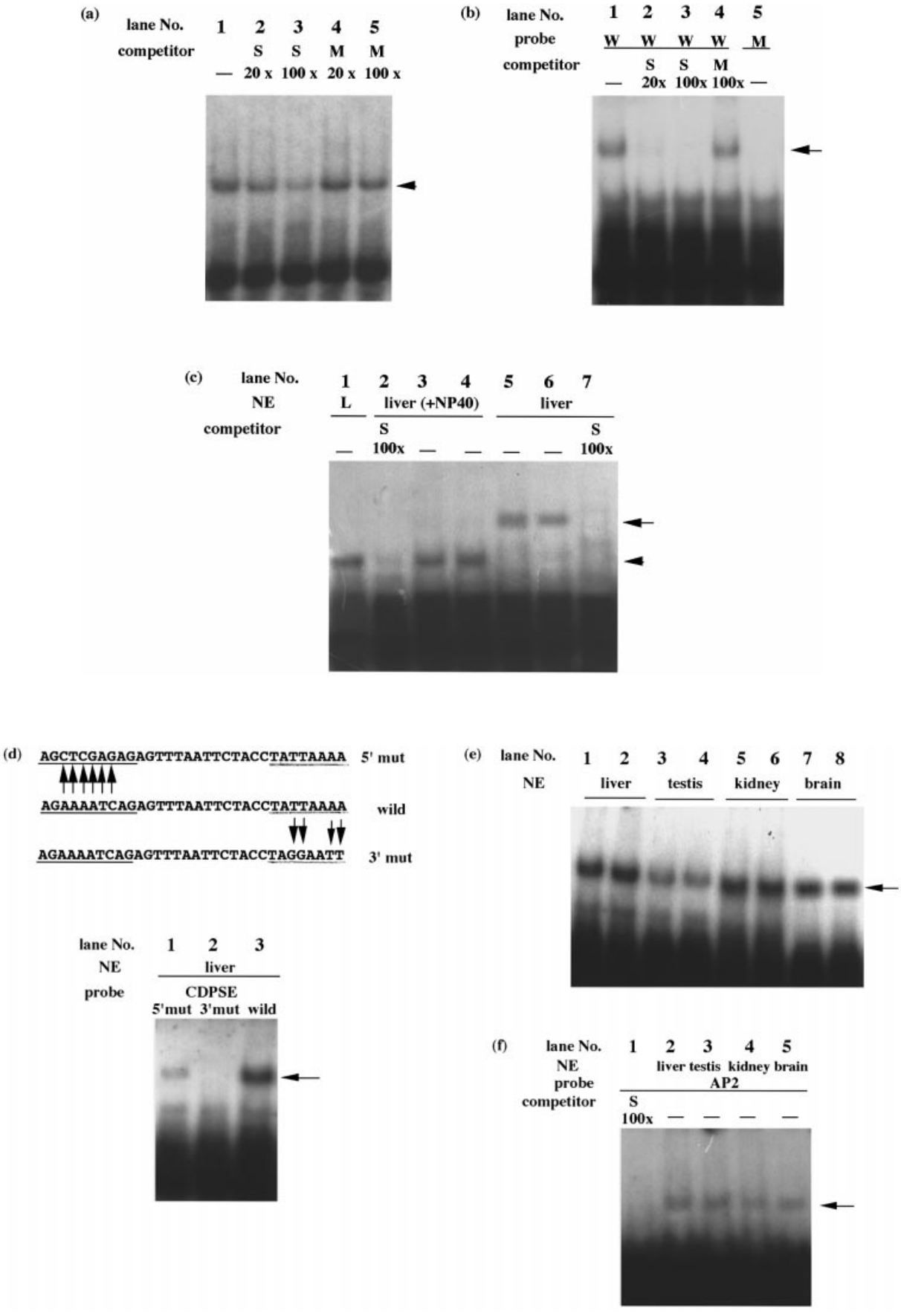

Figure 7 For legend see facing page

this silencer factor capable of binding CDPSE was higher in the liver than in the testis. In the present study, we could not identify the mechanism by which only a low level of the silencer factor was detected in the testis compared with the other organs. Protein-complex constitution, protein modification or folding sustaining the silencer activity besides the level of the silencer-binding factor may explain the tissue-specific differential binding capacity of this factor to CDPSE. If we had successfully isolated the testicular germ cells, far less of the silencer-binding factor would have been detected in the germ cells than was found in the whole testis. Our results on the transcriptional regulation of murine CD46 by CDPSE may partly contribute to the germ cell-specific expression of CD46.

The MHC class-I promoter of pig (PD1) and murine TNFR2 promoter regions contain a similar sequence to the CD46 silencer. The expression of these two genes are regulated tissue-specifically; i.e. PD1 is highly expressed in the spleen, but not in the kidney [40], and TNFR2 is expressed primarily on the myeloid and lymphoid cells [41]. Interestingly, these three molecules are also expressed on macrophages and dendritic cells, and have important roles in the innate immune system. These silencer systems may work simultaneously in these immune-competent cells under certain activating conditions.

Our hypothesis is that the core silencer-binding factors for the PD1-like silencer may be the same as or similar to other silencer-binding factors, but the functional units consisting of the core plus some accessory factors may vary according to the particular silencer. This idea is in part supported by the fact that the 5′ part of the CD46 silencer element is only 60% identical to the other silencer elements, while the 3′ part (the silencer-binding site 2) is 100% identical. Two different preparations of nuclear extract resulted in the unequal mobility shift of the bands in EMSA. The detergents in the preparative buffers may be a key to determine the gel-shift mobility, which suggest the presence of the high- and low-molecular-mass complexes in the nuclear extracts from cell lines and tissues. The presence of the high- and low-molecular-mass complexes may reflect the multimer formation of the silencer-binding protein. Further analysis is required to clarify this issue.

The silencer elements found in human interferon-γ [38] and CD8α [38], the Pc-G gene in *Drosophila* [42], and the homothallic genes HMR and HML in *Saccharomyces cerevisiae* [43] are defined as orientation-dependent silencers. Also the PD1 silencer tends to act in an orientation-dependent manner [40]. Hence, it is not surprising that the CD46 silencer described in this report acts even on the heterologous SV40 promoter position-independently, but orientation-dependently.

To date, several genes showing testis-specific expression have been studied, including histone H1t [30,31], calmodulin gene II (CaMII) [32] and lactate dehydrogenase (LDHC) [33]. Transgenic studies showed that expression of the rat H1t gene was restricted to the male germ line by as little as 141 bp of 5′-flanking sequence, and identified a G/C-rich region (C tracts) of the promoter as the inhibitory sequence. These studies suggest that inhibitory factor(s) could bind to poly-C tracts and function as potential repressor(s). Similar results were obtained with CaMII and LDHC; 294 bp of CaMII and less than 100 bp of LDHC promoter sequences were sufficient for testis-specific expression in transgenic mice. Although these three sequences do not share a consensus motif, these findings highlight the importance of the promoter region in testis-specific expression.

The homology between the promoter sequences of human and pig CD46 (Dr T. Kinoshita, personal communication), which are both expressed ubiquitously, is 62.4%, a relatively high homology compared with that of human and mouse (42.5%). Interestingly, human and pig CD46 promoter sequences are TATA-less and both contain GC-rich regions and many Sp-1 sites, which are features of the TATA-less promoters found mainly in housekeeping genes. Murine CD46 promoter sequence, however, did not contain these characteristics. These significant differences in the CD46 promoters of mouse and human/pig may allow us to divide the CD46 promoters into two categories of TATA-less promoter. One category is ubiquitous and Sp-1-rich, while the other is tissue-selective and Sp-1-poor [35]. We have not detected a significant inhibitory effect in the promoter region (-1435 to $+169$) of murine CD46 using an *in vitro* transient expression assay. Yet the possibility still remains that upstream regions beyond -1435 exert an inhibitory effect in concert with the 3′ region of CDPSE.

Recently, it was reported that the full regulation of human CD46 in transgenic mice can be generated using a YAC clone including the whole of human CD46 [44,45]. These reports suggest that a significant length of the 5′ and 3′ regions and intron sequences are necessary for the fully regulated expression of CD46. Although the transgenic approach to murine CD46 has not yet been explored, this approach with genomic remodelling will be the subject of further studies to clarify the molecular mechanism of human and murine CD46 gene expression.

The role of murine CD46 is a matter of interest. Since CD46 is not expressed ubiquitously in mice, it is unlikely that the major role of membrane CD46 is to protect host cells from autologous C attack. Even though the soluble forms of murine CD46 are produced in the liver to regulate C activation [46], CD46 in the testicular germ cells must have an alternative role unrelated to C regulation. Many groups have speculated that human CD46 is a fertilization-related protein, based on indirect evidence related to its structure and biology. These include its sperm-dominant high expression of protein [14,15], its lack of charged sugars [16,17], its limited distribution to the inner acrosomal membrane until the acrosomal reaction, when it is expressed on the surface of sperm [18], and its deficiency possibly being related to ideopathic infertility [47]. In addition, although monoclonal antibodies

Figure 7 EMSA of the putative silencer element of murine CD46

(**a**) The presence of binding molecule(s) in the nuclear extract of L cells using CDPSE as probe. EMSA was performed as described in the Materials and methods section, and autoradiographed for 3 h. The arrowhead shows the shifted band. S, specific CDPSE as competitor; M, mutated repressor sequence 3′mut CDPSE as competitor. (**b**) The binding molecule was also detected in liver nuclear extracts, but was not detected using the mutant probe 3′mut CDPSE (see Figure 6b). EMSA was performed using wild-type CDPSE (W) and 3′mut CDPSE (M) as probes and autoradiographed for 16 h. The arrow shows the shifted band. (**c**) A size difference of the protein–silencer-element complex depending upon the solubilizers for preparation of the nuclear extracts (NE). The Triton X-100 nuclear extracts of liver (lanes 5–7) were treated further with Nonidet P-40 (final concentration, 0.6%; lanes 2–4). Specific competitor (CDPSE) was added to the samples (lanes 2 and 7). Lane 1, control L-cell (L) nuclear extracts. EMSA was performed using L-cell nuclear extracts and liver nuclear extracts with or without Nonidet P-40 (NP-40) and autoradiographed for 16 h. The arrowhead and arrow show the faster- and slower-migrating bands respectively. (**d**) Binding of nuclear factor(s) to two mutated CDPSE (5′mut CDPSE and 3′mut CDPSE) probes. EMSA was performed using liver nuclear extracts and autoradiographed for 16 h. Lane 1, 5′mut CDPSE probe: lane 2, 3′mut CDPSE probe: lane 3, wild-type CDPSE probe. The arrow shows the shifted band. (**e**) Differential EMSA activity of testis and other organs. Nuclear extracts from four organs (liver, testis, kidney and brain) were analysed by EMSA using CDPSE as probe and autoradiographed for 16 h. The arrow shows the shifted band. (**f**) Control EMSA using the AP2-binding site as a probe. Nuclear extracts from liver, testis, kidney and brain tested in panel (**e**) were analysed by EMSA and autoradiographed for 48 h. In lane 1, specific competitor (CDPSE) was added to the liver sample.

against CD46 were reported to block human sperm binding to hamster eggs or to human eggs [18,19], the inhibition of binding was always incomplete. Furthermore, the epitopes recognized by monoclonal antibodies competent to block sperm binding to human and hamster eggs are wholly different [18,19]. Although the role of CD46 in fertilization has not been established definitively, we propose that the novel mechanism of CD46 gene regulation and its tissue-specific expression in the testicular germ cells supports this putative role of CD46.

The functional phenotypes of murine and human CD46 appear to be different. We surmise that CD46 is primarily engaged in species-discrimination. Rodents adopt this for species identification in fertilization, whereas primates use it for self-protection from homologous C in the immune system. The murine CD46 has only one Ser/Thr-rich (ST) domain, ST^C, similar to the human testicular isoform, whereas the ubiquitous human isoforms have combinations of the three ST domains, ST^A, ST^B and ST^C [48,49]. Thus both human and mouse testicular germ cells/spermatozoa constitutively express only the ST^C isoform of CD46 [16,17]. These results, together with our findings on testis-dominant CD46 gene regulation, suggest the main function of mouse CD46 has evolved into a germ-cell/sperm-specific one. Gene targeting of murine CD46 should settle this issue.

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