High Level Transcription of the Complement Regulatory Protein CD59 Requires an Enhancer Located in Intron 1*

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CD59 is a complement regulatory protein and may also act as a signal-transducing molecule. CD59 transgenic mice have been generated using a CD59 minigene (CD59 minigene-1). Although this minigene contained a 4.6-kilobase pair 5′-flanking region from the human CD59 gene as a promoter, the expression levels of the CD59 mRNA were substantially lower than those observed in humans, suggesting that CD59 gene expression might also require other transcriptional regulatory elements such as an enhancer. To investigate the transcriptional regulation of the CD59 gene, we used three cell lines that express CD59 at different levels. We have identified DNase I-hypersensitive sites in intron 1 in HeLa cells, which express CD59 at high levels, but not in Jurkat (intermediate level) or Raji cells (low level). Furthermore, cell line-specific enhancer activity was detected in a fragment containing these DNase I-hypersensitive sites. The CD59 enhancer was mapped to between −1155 and −888 upstream of the 5′-end of exon 2. To investigate the enhancer activity in vivo, a new CD59 minigene was constructed by the addition of the enhancer fragment into CD59 minigene-1. High expressor CD59 transgenic mice were generated using the new minigene.

The human complement regulatory protein CD59 is a 20-kDa glycoprotein anchored to the membrane via glycosylphosphatidylinositol (1), which restricts human complement lysis by inhibiting assembly of the complement membrane attack complex (2–4). Human CD59 transfectants generated with a rat T cell line (5) and a Chinese hamster ovary cell line (6) were observed to develop resistance to the lytic activity of human complement. These results suggested that the transfer of the human CD59 gene together with genes encoding other complement regulatory proteins (e.g. decay-accelerating factor and membrane cofactor protein) might be used to protect xenogeneic transplants at the endothelial interface. Indeed, studies in CD59 transgenic animals have demonstrated that expression of human CD59 may offer some protection to xenografted tissues (7–9).

CD59 protein can be detected on cells of a wide range of living tissues and on cell lines (2). It has also been reported that cells undergoing apoptosis lose expression of CD59 and other complement regulatory proteins (10), suggesting that expendable cells might also exploit complement activation to assist their removal. CD59 is associated with an intracellular protein kinase, p56<sup>±±</sup> (11), suggesting some signaling role for CD59, perhaps in cell recovery from complement attack (12). How the glycosylphosphatidylinositol-anchored molecules associate with the intracellular protein kinase is unknown. Northern blot hybridization of CD59 mRNAs from many cell lines suggests that the expression of CD59 is controlled in a cell linespecific manner (see “Results”). Transcriptional regulation of the CD59 gene might be important for these functions.

One goal in the creation of CD59 transgenic animals is to ensure adequate expression in the tissue required for xenogeneic transplantation to prevent the deleterious effects of human complement. Although it may be possible to generate high expressor CD59 transgenic animals using a constitutive CD59 expression system (e.g. controlled by a strong promoter such as cytomegalovirus), the consequences of unrestricted expression of CD59 might be disadvantageous if it also functioned as a ubiquitous signal transducer.

In order to achieve a greater understanding of the mechanisms controlling CD59 gene regulation, we isolated the CD59 gene and determined its structure (13). This gene comprises a 5′-noncoding and three coding exons (13). In a previous report, we estimated (by Southern blot hybridization) that the size of intron 1 was larger than 35-kb<sup>1</sup>. We have now cloned this region and calculated it to be only 14 kb. Recently, an additional alternative 5′-noncoding exon was identified in intron 1 (14). Five different CD59 mRNAs were demonstrated as the products of alternative polyadenylation (13).

In this paper, we have demonstrated that CD59 gene expression is regulated by an enhancer located in intron 1. We have identified a DNA sequence that functions as an enhancer element in HeLa cells, which express CD59 at high level, but not in Jurkat (intermediate level) or Raji cells (low level). Two lines of transgenic mice were generated using CD59 minigenes that either contained the enhancer or did not. High levels of CD59 mRNA expression were observed only in transgenic mice generated with the CD59 minigene containing the enhancer, suggesting that this enhancer also functions in vivo.

EXPERIMENTAL PROCEDURES

Construction of CD59 Minigene and Generation of Transgenic Mice—CD59 minigene-1 and CD59 minigene-2 were constructed using CD59 genomic DNA fragments. The structure of the minigenes is indicated in

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1 The abbreviations used are: kb, kilobase pairs; TK, thymidine kinase; CAT, chloramphenicol acetyltransferase; HSs, hypersensitive sites; bp, base pairs.
Northern Blot Hybridization—Total RNAs from transgenic mice were isolated by the acid phenol extraction method (15), and total RNAs from cell lines were isolated by guanidine thiocyanate/CaCl₂ method (16). RNAs were electrophoresed on a agarose/formaldehyde gel and transferred to a nylon membrane. The RNA filter was hybridized with ³²P-labeled CD59 cDNA and β-actin cDNA as a probe.

Fig. 1. The resulting minigenes were micro-injected into fertilized mouse eggs to obtain transgenic mice. Founder mice were C57BL/6 × SJL F2 crosses. Lines were established by subsequent breeding back to C57BL/6. The copy number of the CD59 minigenes was estimated by Southern blot hybridization.

RESULTS

Expression of CD59 mRNA—To investigate the transcriptional regulation of the CD59 gene, transgenic mice were generated using a CD59 minigene (CD59 minigene-1) (Fig. 1). The human CD59 gene was detected by tail blot hybridization in seven lines of transgenic mice. However, human CD59 mRNA was detected in only four lines by Northern blot hybridization. The expression levels of human CD59 mRNA in liver, heart, and kidney from these transgenic mice were compared with that in the human cell line HeLa and are shown in Fig. 2 with the copy number of the transgene. Although this minigene contained a 4.6-kb 5’-flanking fragment as promoter, expression levels of CD59 mRNAs were extremely low. In HeLa cells, the CD59 probe hybridized to 0.7-, 1.3-, 1.9-, and 2.1-kb CD59 mRNA molecules, which differ in size from their 3’-untranslated sequence produced by alternative polyadenylation (13). In the transgenic mice, the probe only hybridized to an unusual 3.2-kb mRNA molecule (Fig. 2). This molecule may be produced by alternative splicing of transcribed products from the transgene (see “Discussion”). We have failed to generate high expression CD59 transgenic mice using CD59 minigene-1, suggesting that this minigene might lack important transcriptional regulatory elements.

We also observed by Northern blot hybridization that expression levels of CD59 mRNA were regulated in a cell line-specific manner (Fig. 3). Extremely high level expression of CD59 mRNA was observed in the fibroblast cell lines MRC5 and HF19. In addition, high level expression was detected in HeLa (epithelioid carcinoma), HepG2 (hepatocyte carcinoma), and JEG3 (colon carcinoma), Jurkat (T cell) and NALM1 (B cell) lines, and low expression in Raji (B cell) cells. To investigate whether this cell line-specific expression of CD59 mRNA was determined by promoter activity in these cells, a luciferase reporter assay was performed. The reporter plasmids were constructed using a 2-kb 5’-flanking fragment as the CD59 promoter and analyzed using cells expressing high (HeLa), intermediate (Jurkat), and
low level (Raji) CD59 mRNA. Since it is difficult to compare the promoter activities directly in different type cells, the CD59 promoter activity in these three cell lines was compared with negative control (pGL3-Basic Vector) and TK and SV40 promoter activities by luciferase assay, as shown in Table I. CD59 promoter activity was detected in all cells. In HeLa cells (which express CD59 mRNA at high level), CD59 promoter activity was 5.6-fold and 4.2-fold weaker than TK and SV40 promoter activities, respectively. On the other hand, in Jurkat cells (intermediate level), this promoter was only 2.6- and 1.5-fold weaker than TK and SV40 promoter activities, respectively. In Raji cells (low level), CD59 promoter activity was 3.6- and 2.4-fold weaker than TK and SV40 promoter activities, respectively. Based upon these results, it appears that CD59 promoter activity might not be reflected in the expression levels of CD59 mRNA. Taken together with results of CD59 expression levels in mice transgenic for the CD59 minigene-1, these results suggest that CD59 expression is regulated not only by a promoter but also by other transcriptional regulatory elements such as an enhancer.

**Cell Line-specific DNase I-hypersensitive Sites and an Enhancer**—DNase I hypersensitivity assays were performed to identify other transcriptional regulatory elements (Fig. 4A). Two DNase I-hypersensitive sites (HSs) were identified using probe E2 on EcoRI-digested DNA from HeLa nuclei but not from Jurkat and Raji nuclei (Fig. 4B). These DNase I-HSs were mapped to between 1.0- and 0.8-kb upstream of exon 2 (Fig. 4A) using probe E2 with BamHI-, EcoRI/XbaI-, BgII-, and PvuII-digested DNA (data not shown). To confirm these cell line-specific DNase I-HSs were not artifacts due to the procedure, probe E2 was washed off the filter, and the same DNA filter was hybridized with probe E1 (Fig. 4A). A cluster of DNase I-HSs was detected in all three cell lines (Fig. 4C) and mapped in the CD59 promoter region (Fig. 4A) using probe E1 with EcoRI/XhoI-digested DNA (data not shown).

DNase I-HSs have been associated with a number of functionally specific positions (e.g., promoters, enhancers, silencers, and origins of replication) (20). To investigate whether CD59 expression is controlled by the region containing the DNase I-HSs located upstream of exon 2, we analyzed the enhancer activity of a 1.4-kb SmaI fragment (1.4-kb upstream region of exon 2 plus 5 bp of exon 2 and containing the HSs) by luciferase reporter assays. The 1.4-kb fragment was introduced into the downstream region of the luciferase gene in plasmid CD59 P containing a 308-bp fragment (-291 to +17) as CD59 promoter (promoter activity of this fragment was similar to that of the 2-kb fragment observed in Table I) in both orientations (Fig. 5A). The resulting plasmids CD59 P/5 and CD59 P/13 (Fig. 5A) were transfected into HeLa (which expresses CD59 at high level), Jurkat (intermediate level), and Raji (low level) cells, and generated luciferase activities were compared with that generated using plasmid CD59 P. In HeLa cells, CD59 promoter activity was up-regulated 2-fold by insertion of the 1.4-kb fragment in both orientations. Surprisingly, this fragment suppressed 40 and 55% of CD59 promoter activities in Jurkat and Raji cells, respectively (Fig. 5B). The activities of the cell line-specific enhancer and the negative transcriptional regulatory elements were also investigated using the TK promoter in pBLCAT2 (Fig. 6). A 7-fold enhancement of TK promoter activity was detected in HeLa cells, and suppression of promoter activity was observed in Jurkat and Raji cells by insertion of the 1.4-kb fragment upstream of the TK promoter in both orientations.

**Mapping and Nucleotide Sequence of CD59 Enhancer**—To establish a more accurate position of the cell line-specific enhancer, deletion mutants of the fragment were constructed and introduced downstream of the luciferase gene in plasmid CD59 P (Fig. 7A). The nucleotide sequence of the 1.4-kb fragment and positions of the 5’- and 3’-ends of deletion mutants are shown in Fig. 8. In HeLa cells, a 2.8-fold enhancement over CD59 promoter activity was observed when an enhancer fragment with a 248-bp deletion was used (Fig. 7, P/5 d1). Disappearance of the enhancer activity was caused by a further 266-bp deletion from P/5 d1 fragment (Fig. 7, P/5 d2), suggesting that the CD59 enhancer is present in this 266-bp sequence (i.e., between -1155 and -888 upstream of the 5’-end of exon 2). Although enhancer activity was absent in P/5 d2, this mutant seemed to suppress the CD59 promoter activities in Jurkat and Raji cells, suggesting that the negative transcriptional regulatory elements may be located in the 3’-flanking region of the enhancer. It is difficult to identify the silencer activity using this result because the reduction of luciferase activity was not great. However, an increase in luciferase activity was caused by a deletion of the 3’ region from P/5 d1 fragment in HeLa cells (Fig. 7, P/5 d1, P/5 d5). Taken together, CD59 gene expression might also be regulated by negative regulatory elements.

**Binding of Nuclear Factors to the CD59 Enhancer Region**—The enhancer activity located between -1155 and -888 might be regulated by cell type-specific nuclear factors. To investigate this, a gel mobility band shift assay was performed using six 62-bp probes (probe 1, -1170 to -1109; probe 2, -1123 to -1062; probe 3, -1076 to -1105; probe 4, -1029 to -968; probe 5, -982 to -921; and probe 6, -935 to -874) with nuclear extracts from HeLa, Jurkat, and Raji cells (Fig. 9). To confirm DNA-dependent complex formation, a competition assay was also performed with a 100-fold excess of unlabeled probe (Fig. 9, lane 1). HeLa cell-specific or enriched complexes (C1 to C7) were detected with probe 1, probe 3, and probe 6 and are shown in Fig. 9 (lane 2). The enhancer activity observed here might be regulated by these nuclear factors binding to the -1170 to -1109 (probe 1), -1076 to -1105 (probe 3), and -982 to -921 (probe 5) regions. Band A was detected both with and without competitors (Fig. 9, lanes 1 and 2), suggesting that this band was an artifact of the procedure (i.e., DNA-independent). Potential transcription recognition sequences were screened using the transcription factor data base (21), and some recognition sequences are shown in Fig. 8.

**Generation of CD59 Transgenic Mice Using a CD59 Minigene Containing the Enhancer Region**—We have identified and mapped a putative CD59 enhancer. To investigate activity of this element in vivo, we created a second CD59 minigene construct (CD59 minigene-2) by adding an additional 0.85-kb fragment that includes the enhancer element (Fig. 1, CD59 minigene-1 and CD59 minigene-2). 10 lines of transgenic mice were generated, and CD59 mRNA was detected in all lines (three of them are shown in Fig. 10 with the copy number of the
CD59 gene expression was analyzed by luciferase assays. Luciferase activities generated using CD59, TK, and SV40 promoters were compared with that generated using negative control plasmid (no insert) pGL3-Basic Vector in HeLa, Jurkat, and Raji cells. Luciferase assays were repeated more than three times. To normalize transfection efficiency, the internal control plasmid pRL-TK (Promega) for transfection using the Basic, CD59, and SV40 reporter plasmids and the pRL-SV40 (Promega) for transfection using the Basic, CD59, and TK reporter plasmids were used.

**DISCUSSION**

CD59 with other complement regulatory proteins protects human cells against bystander complement lysis. Therefore, expression of CD59 could be regulated in a wide range of cells.
We have demonstrated that CD59 gene expression is regulated by an enhancer. Extremely high level expression of CD59 was observed in two lung fibroblast cell lines, MRC5 and Hf19. It is not clear why fibroblasts should express this gene so well, but as they migrate into regions of tissue damage and inflammation, they may be exposed to natural, potentially lytic, complement activity. Their high level expression of CD59 probably results from use of the enhancer element. Accordingly, we have identified DNase I-HSs in the enhancer region in MRC5 cells (data not shown).

The CD59 enhancer seems not to be functional in the lymphocyte cell lines Raji (B cell) and Jurkat (T cell). These cells might also suppress gene expression of CD59 through activity of the negative transcriptional elements in the upstream region of exon 2. Indeed, the expression of CD59 in Raji cells was extremely low. It is conceivable that some expendable cells (e.g. cancer cells, virus-infected cells, damaged cells, and apoptotic cells) down-regulate expression of the CD59 gene by turning off the enhancer and using the negative transcriptional regulatory elements and thereby allow complement-mediated cell destruction. Jones and Morgan (10) reported that CD59 and decay-accelerating factor expression on the cell surface was reduced by the apoptotic process. Perhaps signaling for apoptosis, such as the activation of caspases (22), may down-regulate CD59 gene expression by inactivation of the enhancer activity and through harnessing this negative transcriptional regulatory activity.

A 3.2-kb CD59 mRNA was detected in CD59 transgenic mice. In human cells, 0.7-, 1.3-, 1.9-, 2.1-, and 5.8-kb mRNA molecules are produced by alternative polyadenylation. This unusual 3.2-kb molecule might appear to be due to lack of splicing
between exon 1 and exon 2. An alternative 5'-untranslated exon (45-bp) was identified 5 kb downstream of exon 1 (in intron 1) (14). Our minigenes did not contain this alternative exon. Therefore, it is possible that the 3.2-kb molecules were produced by unusual alternative splicing due to the absence of the alternative exon.

We have mapped the CD59 enhancer to 2155 to 2888 upstream of exon 2. Since exon 1 of the CD59 gene is an untranslated exon, the first ATG is present in exon 2. Therefore, if this enhancer also functions as a promoter, functional CD59 mRNA may be transcribed from a site upstream of exon 2. In order to investigate the promoter activity of the enhancer, a 1.4-kb SacI fragment containing the enhancer was introduced upstream of the luciferase gene in the pGL3-Basic Vector. Promoter activity was detected in this fragment (data not shown). We have demonstrated that some mRNA might be transcribed from approximately 50 bp upstream of the 5'-end of exon 2 by primer extension assay using total MRC5 RNA. Processing of these mRNA may, however, be inefficient because the primer extension products using poly(A)^1 RNA are difficult to identify (data not shown). These results suggest that this region might function primarily as an enhancer in vivo.

A 1.4-kb nucleotide sequence located upstream of exon 2 was screened using the transcription factor data base (21). We have also demonstrated that HeLa cell-specific or enriched nuclear factors bound to the cell line-specific enhancer region (21170 to 21109, 21107 to 21015, and 21095 to 2874), and nuclear extracts from HeLa (lane 2), Jurkat (lane 3), and Raji (lane 4) cells. Binding of nuclear factors from HeLa cells was competed with 100-fold excess of unlabelled probes (lane 1). HeLa cell-specific or enriched complexes C1 to C7 are indicated by arrows. DNA-independent (an artifact) band A is also indicated by an arrow.

CD59 mRNA may be transcribed from a site upstream of exon 2. In order to investigate the promoter activity of the enhancer, a 1.4-kb SacI fragment containing the enhancer was introduced upstream of the luciferase gene in the pGL3-Basic Vector. Promoter activity was detected in this fragment (data not shown). We have demonstrated that some mRNA might be transcribed from approximately 50 bp upstream of the 5'-end of exon 2 by primer extension assay using total MRC5 RNA. Processing of these mRNA may, however, be inefficient because the primer extension products using poly(A)^1 RNA are difficult to identify (data not shown). These results suggest that this region might function primarily as an enhancer in vivo.

A 1.4-kb nucleotide sequence located upstream of exon 2 was screened using the transcription factor data base (21). We have also demonstrated that HeLa cell-specific or enriched nuclear factors bound to the cell line-specific enhancer region (−1170 to −1109, −1076 to −1015, and −935 to −874), and nuclear extracts from HeLa (lane 2), Jurkat (lane 3), and Raji (lane 4) cells. Binding of nuclear factors from HeLa cells was competed with 100-fold excess of unlabelled probes (lane 1). HeLa cell-specific or enriched complexes C1 to C7 are indicated by arrows. DNA-independent (an artifact) band A is also indicated by an arrow.

Fig. 9. Binding of nuclear factors to the CD59 enhancer. Binding of nuclear factors to the CD59 enhancer was analyzed by a gel mobility band shift assay using probe 1 (−1170 to −1109), probe 2 (−1076 to −1015), and probe 6 (−935 to −874), and nuclear extracts from HeLa (lane 2), Jurkat (lane 3), and Raji (lane 4) cells. Binding of nuclear factors from HeLa cells was competed with 100-fold excess of unlabelled probes (lane 1). HeLa cell-specific or enriched complexes C1 to C7 are indicated by arrows. DNA-independent (an artifact) band A is also indicated by an arrow.

Fig. 10. Expression of CD59 mRNA in transgenic mice generated using CD59 minigene-2. 10 μg of RNA from liver, heart, and kidney of the CD59 minigene-2 transgenic mice were hybridized with the human CD59 cDNA. To indicate amount of the loading RNA, ethidium bromide staining of the RNA gel is shown below the Northern blot. 0.7-, 1.3-, 1.9-, and 2.1-kb CD59 mRNAs produced by alternative polyadenylation are indicated. The copy number of the CD59 minigene in these transgenic mice is indicated above the line number.

CD59 mRNA may be transcribed from a site upstream of exon 2. In order to investigate the promoter activity of the enhancer, a 1.4-kb SacI fragment containing the enhancer was introduced upstream of the luciferase gene in the pGL3-Basic Vector. Promoter activity was detected in this fragment (data not shown). We have demonstrated that some mRNA might be transcribed from approximately 50 bp upstream of the 5'-end of exon 2 by primer extension assay using total MRC5 RNA. Processing of these mRNA may, however, be inefficient because the primer extension products using poly(A)^1 RNA are difficult to identify (data not shown). These results suggest that this region might function primarily as an enhancer in vivo.

A 1.4-kb nucleotide sequence located upstream of exon 2 was screened using the transcription factor data base (21). We have also demonstrated that HeLa cell-specific or enriched nuclear factors bound to the cell line-specific enhancer region (−1170 to −1109, −1076 to −1015, and −935 to −874). We have identified AP2 (−1150 to −1153 and −887 to −880), PEA3 (−1115 to

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2 M. Tone, L. E. Diamond, L. A. Walsh, Y. Tone, S. A. J. Thompson, E. M. Shanahan, J. S. Logan, and H. Waldmann, unpublished observation.
−1110 and −923 to −918), and AP1 (−1049 and −1043) recognition sequences in these areas. However, it is difficult to explain cell line-specific CD59 enhancer activity in terms of just these factors. Therefore, this enhancer activity might be regulated by further unknown transcription factors.

We have demonstrated here that gene expression of the complement regulatory protein CD59 is regulated by an enhancer. This information will be of great value in the creation of CD59 transgenic pigs to provide organs with enhanced resistance to lysis by human complement.

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