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Gene Structure, Promoter Characterization, and Basis for Alternative mRNA Splicing of the Human *CD58* Gene[1]

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The 60-kDa lymphocyte function-associated Ag-3 (LFA-3/CD58), a highly glycosylated adhesion molecule that serves as ligand for the T cell-restricted glycoprotein CD2, is encoded by a gene at the human chromosome locus 1p13. We have elucidated the exon-intron organization of the entire human *CD58* gene, including ~2.5 kilobases (kb) of 5′-flanking DNA. Four overlapping genomic clones, spanning ~65 kb, contained the entire ~1-kb coding sequence of CD58 and consisted of six separate exons, which varied from 72 to 294 bp in size. At least two different CD58 mRNA precursors can be generated from the human gene as a result of alternative choice of one of the two acceptor splice sites located within exon 5. DNA sequence analysis of about 2.5 kb of 5′-flanking sequence of the *CD58* gene indicated the absence of a CAAT box. However, potential binding sites for the transcriptional activators AP-2, GATA, PU.1, and Sp-1 are present. Two consensus TATAA elements, located ~2.4 kb upstream of the transcriptional start site, have been identified. The 2.5-kb CD58 promoter sequence displayed functional activity in transient transfection assays in the hepatocellular carcinoma cell line HepG2. Comparing the response of CD58 promoter-driven luciferase plasmids to several cytokines and other agents suggests that the CD58 promoter is regulated by up-regulatory, enhancer-like and down-regulatory, silencer-like elements. Further analysis of this region should allow researchers to gain insight into the molecular mechanisms by which this gene is regulated, e.g., during inflammatory responses. *The Journal of Immunology*, 1998, 160: 2862–2871.

Human CD58 (lymphocyte function-associated Ag-3) represents an accessory molecule for costimulation of T cells via binding to its receptor CD2 (1–6). The interaction of CD2 on T lymphocytes with CD58 on the surface of endothelial cells, thymic epithelium, erythrocytes, fibroblasts, and APCs is critical for the regulation and effector function of T lymphocytes (2, 7). CD58 is expressed on the cell surface of nucleated cells in both a transmembrane and a glycosylphosphatidylinositol (GPI[3])-anchored form (8–10). The biologic significance of these variants and the signals regulating their expression are unresolved. However, marked up-regulation at sites of inflammation (11, 12) and down-regulation of CD58 in at least some tumor cells have been observed (13–20). Moreover, the existence of a soluble form of the adhesion receptor CD58 in infectious and autoimmune diseases was described (21–23). It has been postulated that CD2 and CD58 may have evolved from a common ancestor by gene duplication, which is supported by the fact that human *CD2* and *CD58* genes as well as *CD106* (VCAM-1) are located on chromosome 1p and are structurally related (24–27).

To seek evidence for these postulated evolutionary events and to better assess the role of differential expression of CD58 molecules, we report here on the cloning and characterization of the complete human *CD58* (*LFA-3*) gene. There is one copy of the *CD58* gene per human haploid genome. This gene encompasses about 65 kilobases (kb) and contains six exons. The general distribution of exons and intervening sequences reflects the structural and functional organization of the protein sequence. We suggest that the two forms of CD58 observed in humans, the transmembrane and the GPI-linked form, arise from an alternatively spliced common pre-mRNA by the choice of one of the two splice acceptor sites in exon 5. Characterization of the corresponding cDNAs has shown that the two mRNAs differ by 35 bases in the 3′ region.

Expression of CD58 mRNA has been studied in various tissues and cell lines (9, 10). To investigate the basis of regulation of *CD58* gene expression and to provide a foundation upon which to examine the relationship between T lymphocyte activation via *CD2* and *CD58* expression, we also identified and characterized the promoter region of *CD58*. In transient transfection assays, a 2.5-kb fragment comprising the 5′-flanking sequence of the *CD58* gene demonstrated functional activity when transfected into the hepatoma cell line HepG2.

Materials and Methods

Isolation and characterization of CD58 genomic clones

We have screened a human T cell genomic library in λ EMBL3 phage vector (Stratagene, Heidelberg, Germany) representing partially *Mbo*I-digested DNA. Approximately 1×10^6 clones in *Escherichia coli* P2/392 were probed with five overlapping ^{32}P-labeled ≈0.3-kb fragments of the CD58 cDNA. In addition, a human cosmid genomic library constructed in pcos2EMBL (a gift of A. Poustka, German Cancer Research Center, Heidelberg, Germany) (28) and a human chromosome 1-specific genomic cosmid library constructed in Lawrist4 (library no. 112, library name L4/FS1 from the Reference Library DataBase at the MPI for Molecular Genetics, Berlin, Germany) (29) were screened. Approximately 1×10^6 cosmid clones were separately screened with overlapping CD58 cDNA probes. Four overlapping clones were selected for further analysis: λ4-1 contains the 5′ end of the gene, ICRFc112D0797Q6 contains exons 1 and 2, λ1-2 contains exon 2, and pcosII 3-4 contains the 3′ end-harboring exons 3, 4, 5, and 6. The recombinant genomic clones were purified, and the inserts

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[1] The nucleotide sequences reported here have been submitted to EMBL/GenBank under accession nos. Y14780, Y14781, Y14782, Y14783, Y14784, and Y14785.

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[3] Abbreviations used in this paper: GPI, glycosylphosphatidylinositol; kb, kilobases.

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were characterized by restriction enzyme digestions and by Southern blot analysis with synthetic oligonucleotides based on sequences derived from the CD58 cDNA. The endonuclease digestion products were fractionated by electrophoresis on 1% agarose gels, and their size estimated by comparison with standard DNA markers (New England Biolabs GmbH, Schwalbach, Germany). Appropriate DNA fragments were isolated by gel electrophoresis and subcloned into the Bluescript vector (Stratagene) for sequencing.

DNA sequencing

Exons and the junctions between exons and intervening sequences were sequenced (30) after subcloning of appropriate fragments into pBluescriptII SK$^+$ (Stratagene). Specific oligonucleotides chosen from the coding sequence of the CD58 cDNA were used. The complete CD58 cDNA sequences have already been published (9, 10) and have been deposited in the EMBL/GenBank database (accession nos.: X06296 and Y00636).[1] DNA sequence was determined using a T7 DNA sequencing kit (Pharmacia, Freiburg, Germany) in accordance with the manufacturer's recommendation (31).

Primer-extension analysis

Primer-extension studies were performed according to published methods (32). For primer-extension studies, a 26-mer oligonucleotide primer complementary to the CD58 mRNA was synthesized. The oligonucleotide was designated 58-St2 (5′-TACGCGGTCGGCCAGAAGTAGTAGGGCTC-3′), located 64 bp upstream of the ATG of the signal peptide. The oligonucleotide was ^{32}P-labeled using T4 polynucleotide kinase, and 40 ng of labeled primer was hybridized to 5 μg of total RNA extracted from the human hepatoma cell line HepG2. The hybridization reaction was conducted at 60°C for 1 h. The annealed primer was extended with 23 U of reverse transcriptase (Promega, Mannheim, Germany) for 120 min at 42°C. The reaction mixture was digested with RNase A, phenol/chloroform extracted, and ethanol precipitated. The reaction product was separated by 7.5 M urea/8% PAGE followed by autoradiography. The primer extension product was visualized by autoradiography on x-ray films (X-OMAT, Kodak).

Construction of CD58 promoter/luciferase reporter gene plasmids

Two of the genomic clones (λ4-1 and ICRFc112D0797Q6) were shown to contain the 5′ end of the coding region and flanking DNA sequences (see *Results*). To develop *CD58* promoter/reporter gene (luciferase) chimeric constructs, a dsDNA extending from position −1 to −2551 (counting upstream from the translation initiation site) was generated by digestion of the λ4-1 clone with the *Nco*I and *Kpn*I endonucleases, "filled-in," and subcloned into the *Sma*I site of pGL-Enhancer (Promega), which represents a construct lacking a promoter but containing the entire luciferase gene, SV40 T intron, polyadenylation signal, and enhancer. Thus, starting from plasmid p58-25, we have made various constructs with nested deletions of the 5′-end sequence of the 2.5-kb upstream region. Plasmid pBluescriptII SK (Stratagene) containing the 2.5-kb upstream sequence was first cleaved at the *Sal*I site at the 5′ end of the insert followed by digestion with *Exo*III and mung bean nuclease (Stratagene) for various time intervals. The insert fragments with various deletions at the 5′ end were released from the plasmids by digestion with *Xba*I followed by blunting with Klenow and *Bam*HI digestion. These fragments were inserted into the *Sma*I/*Bgl*II sites of pGL2-Enhancer, yielding constructs p5′Δ−2370 (construct p58-24), p5′Δ−2140 (p58-21), p5′Δ−1670 (p58-17), p5′Δ−1445 (p58-14), p5′Δ−662 (p58-6), p5′Δ−536 (p58-5), p5′Δ−426 (p58-4), p5′Δ−183 (p58-2), and p5′Δ−110 (p58-1) (see Fig. 4*B*). The appropriate orientation relative to the luciferase gene in pGL2-Enhancer and endpoints of the gene inserts were verified by DNA sequencing. A construct with a nested deletion at the 3′-end sequence of the 2.5-kb upstream region was created by making use of appropriate restriction sites. To obtain plasmid p58ΔC1, a fragment extending from −[148-1] was removed by digesting vector p58-25 with *Sac*II and *Sac*I, flushing with Klenow, and religation. Plasmid p58Δ45 was constructed by deleting the region between −2265 and −1815 through digestion with *Bst*XI, flushing with Klenow, and religation. Deletion of the sequence between −1790 and −1240 from p58-25 by *Afl*II digestion and religation resulted in vector p58Δ55. To obtain plasmids p58Δ39, p58Δ164, and p58Δ211, fragments extending from −534, −1790, and −2265 to −148 were removed by digesting with *Apa*I and *Sac*II, *Afl*II and *Sac*II, and *Bst*XI and *Sac*II, respectively, flushing with Klenow, and religation (see Fig. 4*A*).

DNA transfection and transient expression

The promoter/luciferase constructs, as well as pGL-Enhancer (a negative control) and pGL2-Control (a positive control), 5 μg/28-cm^2 plate, were used in transient transfections of the human hepatoma cell line HepG2 (HB-8065; American Type Culture Collection, Rockville, MD) cultured in RPMI 1640 medium (Life Technologies, Eggenstein, Germany) supplemented with 10% FCS. Approximately 4×10^5 cells/plate were cultured 24 h before transfection. The transfections were performed in serum-free RPMI 1640 with a commercial kit (LipofectAMINE; Life Technologies) according to the manufacturer's instructions. To control for variations in transfection efficiency, plasmid pUHD16.1 (a gift of H. Bujard, ZMBH, University of Heidelberg), containing the SV40 promoter and the *Escherichia coli lacZ* gene, was cotransfected and the amount of cell extracts was adjusted according to the β-galactosidase activity. Following an overnight incubation, cells were washed and transferred to RPMI 1640/10% FCS for 24 h.

Luciferase and β-galactosidase assays

Forty hours after transfection, cells were pelleted and washed twice with PBS. The cell pellet was then resuspended in 1 ml of PBS, and one half was assayed for β-galactosidase activity and the other half was assayed for luciferase activity using the respective assay systems purchased from Promega. Luciferase activity, assessed as light output, was measured using a Berthold model Lumat 9501 luminometer (Wildbad, Germany) integrating peak luminescence 10 s after injection of assay buffer. β-Galactosidase activity was determined in the same samples (32), and the luciferase activity values were corrected for β-galactosidase activity to allow direct comparison of the CD58 promoter activity in different cell cultures transfected in parallel.

Northern analysis

To examine the expression of the *CD58* gene at the mRNA level, total RNA for Northern blot analysis was isolated (32) from untreated HepG2 cells and from cells treated with either PMA (10^{-8} M), IFN-γ (400 U/ml), or TNF-α (25 ng/ml) for 24 h. Blotted RNA was hybridized with a ^{32}P-labeled human CD58 or CD54 cDNA as described previously (33). The same filters were also hybridized with a human β-actin cDNA to verify even loading of the RNA to each lane.

FACS analysis

HepG2 cells incubated in RPMI with 10% FCS and treated with either PMA (10^{-8} M), IFN-γ (400 U/ml), or TNF-α (25 ng/ml) for 48 h were stained with AICD58 or 84H10 (CD54) mAb (Coulter-Immunotech Diagnostics, Hamburg, Germany) and goat anti-mouse Ig-FITC as a second Ab. Analysis of the HepG2 cells was conducted on FACSCalibur (Becton Dickinson, Mountain View, CA).

Results

Structure of the CD58 *gene*

Using partial and full-length CD58 cDNA probes, we screened three different human genomic libraries. From a number of positive clones, two λ-phage clones (λ4-1 and λ1-2) and two cosmid clones (pcosII 3-4 and ICRFc112D0797Q6) were further analyzed. No single clone contained the complete *CD58* gene, although the average size of the analyzed cosmid clones was about 40 kb. The restriction map of a ≈65-kb region containing the complete *CD58* gene was constructed by analysis of overlapping bacteriophage (λ) and cosmid clones (Fig. 1*A*). The genomic organization was elucidated by subcloning restriction fragments of the respective phage and cosmid clones into pBluescriptII SK$^+$ plasmids (Fig. 1*B*). Restriction maps were refined by Southern blotting with cDNA, genomic, and specific oligonucleotides probes. The sizes of subcloned fragments correspond to the size of the hybridized bands observed on Southern blots of digested human DNA probed with a CD58 cDNA clone (data not shown); this pattern is consistent with a single copy gene being present in the human (haploid) genome. Exon/intron boundaries were identified by DNA sequencing on both DNA strands using synthetic oligonucleotides chosen from the CD58 cDNA sequence. In areas where the sequences overlap, there were no nucleotide differences between the previously published human cDNA sequences (9, 10) and the genomic sequence.

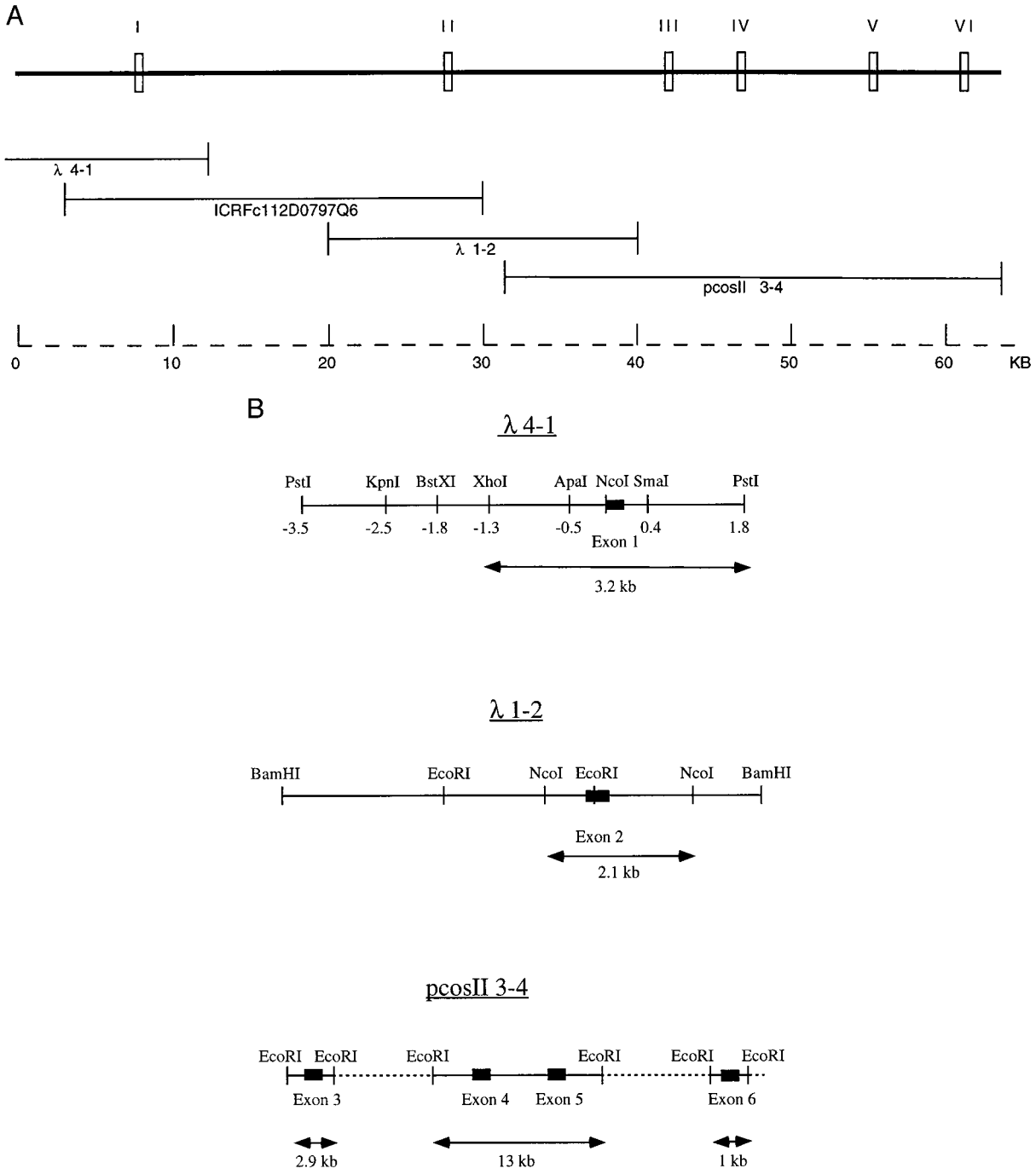

FIGURE 1. *A*, Isolation of genomic DNA clones corresponding to the human *CD58* gene. Four genomic clones (λ4-1, ICRFc112D0797Q6, λ1-2, and pcosII 3-4), varying from 20 to 40 kb in size, were isolated. The segments corresponding to the coding region of CD58 mRNA span ≈60 kb of genomic sequences. The entire coding region of the CD58 consists of six exons numbered starting from the 5′ end, drawn to scale. The individual exons are depicted by open boxes, and the introns are shown by horizontal lines. *B*, Partial restriction map of clones λ4-1, λ1-2, and pcosII 3-4. Exons are indicated by filled boxes and designated exon 1 to 6. Horizontal arrows under each map denote the fragments that were subcloned for sequencing.

This suggests that polymorphism of the *CD58* gene is, if at all, not extensive.

The *CD58* gene structure implies a correlation between exon/intron architecture and protein structure. Exon 1 encodes the 5′-untranslated region and 24 of 28 amino acid residues comprising the signal peptide (Fig. 2). Each of the two extracellular Ig-like domains is contained in a separate exon. In this respect, the *CD58* gene is similar to other members of the Ig gene superfamily, including *CD2* (34). The second exon contains 14 bp encoding the C-terminal portion of the signal peptide and 280 bp encoding the N-terminal Ig-like domain of the CD58 protein, whereas exon 3 encodes the second Ig-like 264-bp domain (Fig. 2). Exon 4 is 78 bp long and encodes a hydrophobic polypeptide sequence that is used as the transmembrane spanning domain (CD58 membrane-integrated form) or, in the GPI form, is replaced for the GPI anchor. Exon 5 is 72 bp long and contains two consensus 3′ splice acceptor sites $(T/C)_{11}N(C/T)AG$, suggesting that the upstream site is utilized to generate the mRNA encoding the GPI-linked CD58 glycoprotein, whereas the downstream site may be used for expression of the transmembrane form of CD58 (Fig. 2) Consequently,

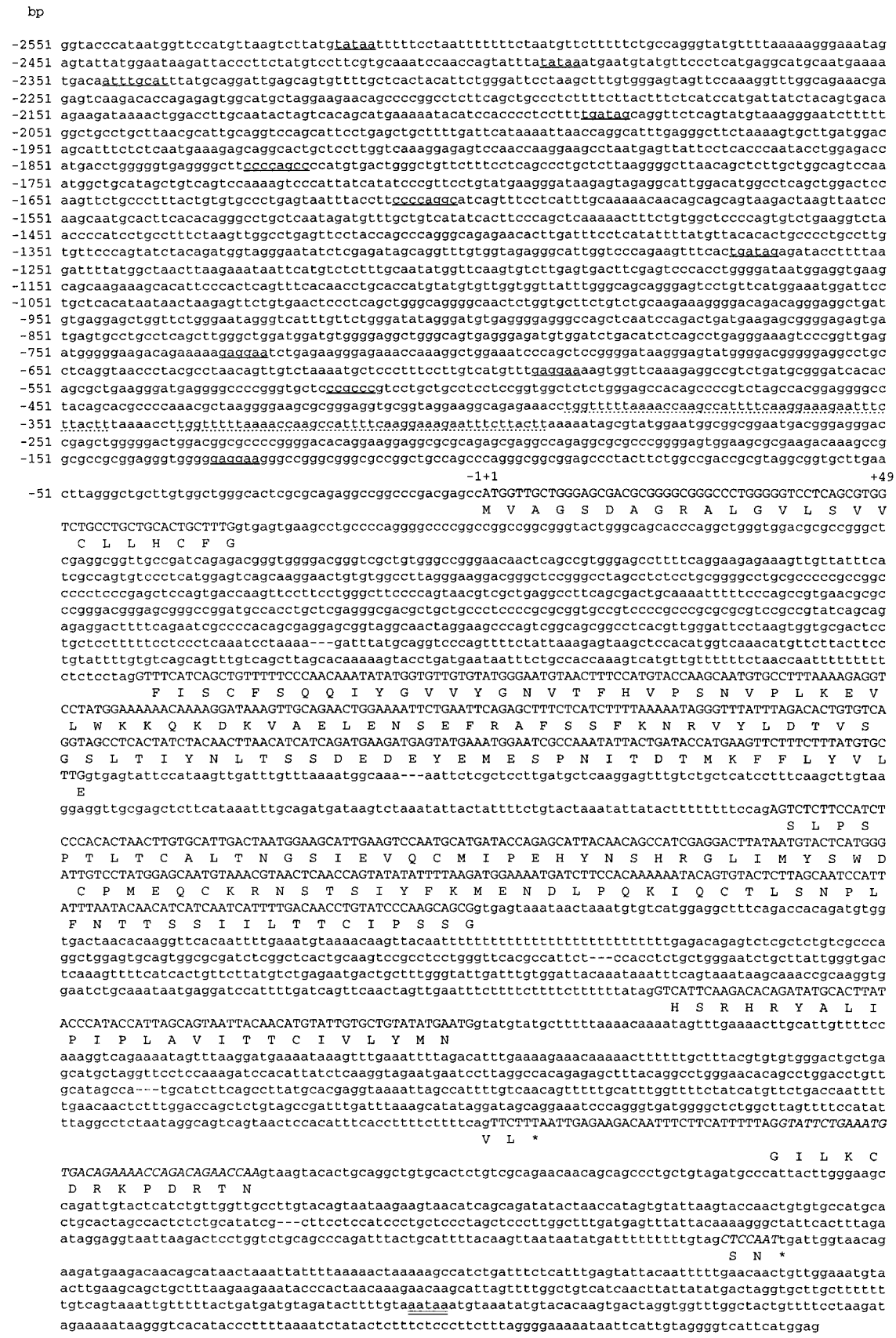

FIGURE 2. Nucleotide sequence of the *CD58* gene. The translated nucleotide sequences of CD58 exons are denoted in uppercase letters. Amino acid sequences are displayed underneath the corresponding nucleotide sequences. Putative binding sites for regulatory elements are underlined, and notes for the underlines are as follows: exon 1, TATA (tataa); exon 2, IgH, enhancer (atttgcat); exon 3, GATA (tgatag); exon 4, AP2 (ccccaggc); exon 5, PU.1 (gaggaa); exon 6, Sp-1 (ccgccc). The 45-bp direct repeats are indicated as dotted underlines. A polyadenylation consensus sequence (aataaa) is double underlined. PI = GPI-linked form; TM = transmembrane form.

Table I. *Boundaries at the exon-intron junctions, and the sizes of the exons of human* CD58 *gene*[a]

Exon	3′ Splice Acceptor Site	5′ Splice Donor Site	Exon Size (bp)
1		.. TTT G gtgagtgaag	190
2	ttttttctctcctag GT TTC	.. CTT G gtgagtattc	294
3	actttttttttccag AG TCT	.. AGC G gtgagtaaat	264
4	tttctttttttatag GT CAT	.. AAT G gtatgtatgc	78
5 (PI)	ccttttctttttcag TT CTT	.. ACC AA gtaagtaca	72
(TM)	ttcttcatttttag GT ATT	.. ACC AA gtaagtaca	37
6	attttttttttgtag C TCC	.. TTTGTAAATAAA	288

[a] The exon numbers correspond to those shown in Figure 1. Nucleotide sequences in the introns are indicated by lower case letters and those in exons by capital letters. Note the presence of a polyadenylation consensus sequence, AATAAA, at the 3′ end of exon 6 (underlined). The exon sizes were determined by direct nucleotide sequencing of the corresponding cDNAs. All exons demonstrate the presence of split codons. PI = GPI-linked form; TM = transmembrane form.

the translation termination codon for the GPI-linked form of CD58 is located in exon 5 whereas the respective stop codon of the CD58 transmembrane form is located within exon 6. In addition, exon 6 (288 bp) contains the 3′-untranslated region for both CD58 mRNA variants (Fig. 2).

Based on Southern blot analysis on human genomic DNA and mapping by in situ hybridization, CD58 is encoded by a single gene in the human genome located on chromosome 1 (35) (our unpublished observations). Thus, the two different CD58 glycoproteins (transmembrane and GPI form) are most likely generated by differential mRNA splicing, as has been demonstrated recently for CD56 (N-CAM) (36). All splice acceptor and donor sequences agree with the "GT-AG" rule (37) and confirm to the consensus proposed by Mount (38). The codons at the splice site demonstrate a characteristic pattern. With the exception of the alternatively spliced exon 5, where splice junctions occur after the second nucleotide of a codon, all other splice junctions occur after the first nucleotide (Table I).

Determination of the transcription start site

To define the start site of transcription, we performed primer extension experiments with two different primers. Poly(A)$^+$ RNA from HepG2 cells, which are reported to show a very high constitutive expression rate of CD58, was primed with an oligonucleotide extending from −90 to −65 (Fig. 2) and reverse transcribed. By using a sequence reaction primed with the same oligonucleotide as the marker, a transcription start site was localized 120 nucleotides upstream of the translation ATG start site (Fig. 3). Primer extension experiments with another primer extending from +41 to +70 were unsuccessful, possibly due to the presence of a secondary structure on the 5′ end of the mRNA. Therefore, this experiment cannot exclude the possibility that an additional transcription start site is located between the most 5′ transcriptional start site (−120) and the initiation ATG (+1).

Structure of the 5′- and 3′-flanking regions of the human CD58 *gene*

A 2.5-kb *Kpn*I-*Nco*I fragment of the genomic clone λ4-1 was found to contain a sequence representing the 5′ end of the previously characterized CD58 cDNA (Fig. 1*B*). A 500-bp segment immediately preceding the ATG start codon of CD58 (Fig. 2 and 4*A*) displays no characteristic eukaryotic promoter elements; i.e., neither a TATA nor a CAAT box could be detected. Instead, this particular region of the *CD58* gene contains several potential binding sites for transcription factors, e.g. a G · C-rich region harboring three putative Sp-1 sites (39), and three potential binding sites for PU.1 (40). Interestingly, a 45-bp direct repeat (5′-TGGTTTTT AAAACCAAGCCATTTTCAAGGAAAGAATTTCTTACTT-3′, marked by a dotted line in Fig. 2) separated by 8 bp was identified. Further upstream of the initiation ATG, two potential binding sites for the 'GATA' family of transcription activators (41, 42), and two potential binding sites for AP-2 (43) are present (Table II). Further elucidation of the 5′-flanking sequence of the *CD58* gene revealed the presence of two TATA boxes in the region >2.4 kb upstream of the ATG initiation codon. Furthermore, the sequence ATTTG CAT is found at position −2346. This sequence resembles the consensus sequence for a potential IgH enhancer, which is well conserved in all Ig gene promoters and has been implicated in promoter function (44, 45).

The 3′ end of the *CD58* gene contains a consensus motif for polyadenylation, AATAAA (46). The position is consistent with the observed size of the CD58 mRNA transcript. The predicted size of the CD58 mRNA derived from the length of the 5′-untranslated region (120 bp), the open reading frame of the transmembrane CD58 form (750 bp), as well as the size of the 3′-untranslated region (260), and poly(A) tail (≈100 bp), is 1230 bp. This is in agreement with the previously reported size of 1.3 kb for the CD58 mRNA (9).

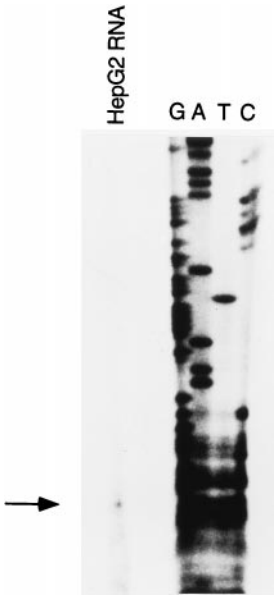

FIGURE 3. Mapping of the CD58 transcriptional start site. The transcriptional start site was determined by primer-extension analysis. Oligonucleotide 58-St2 corresponding to the 5′ end of the CD58 cDNA was labeled and hybridized to human hepatoma cell (HepG2) RNA. The product of annealing served as a template for reverse transcriptase. The extension product was run on a denaturing polyacrylamide gel (arrow). A sequence reaction, conducted with genomic CD58 DNA and the same oligonucleotide as used for the reverse transcriptase reaction, was used as size marker. Nucleotides are indicated (G, A, T, C).

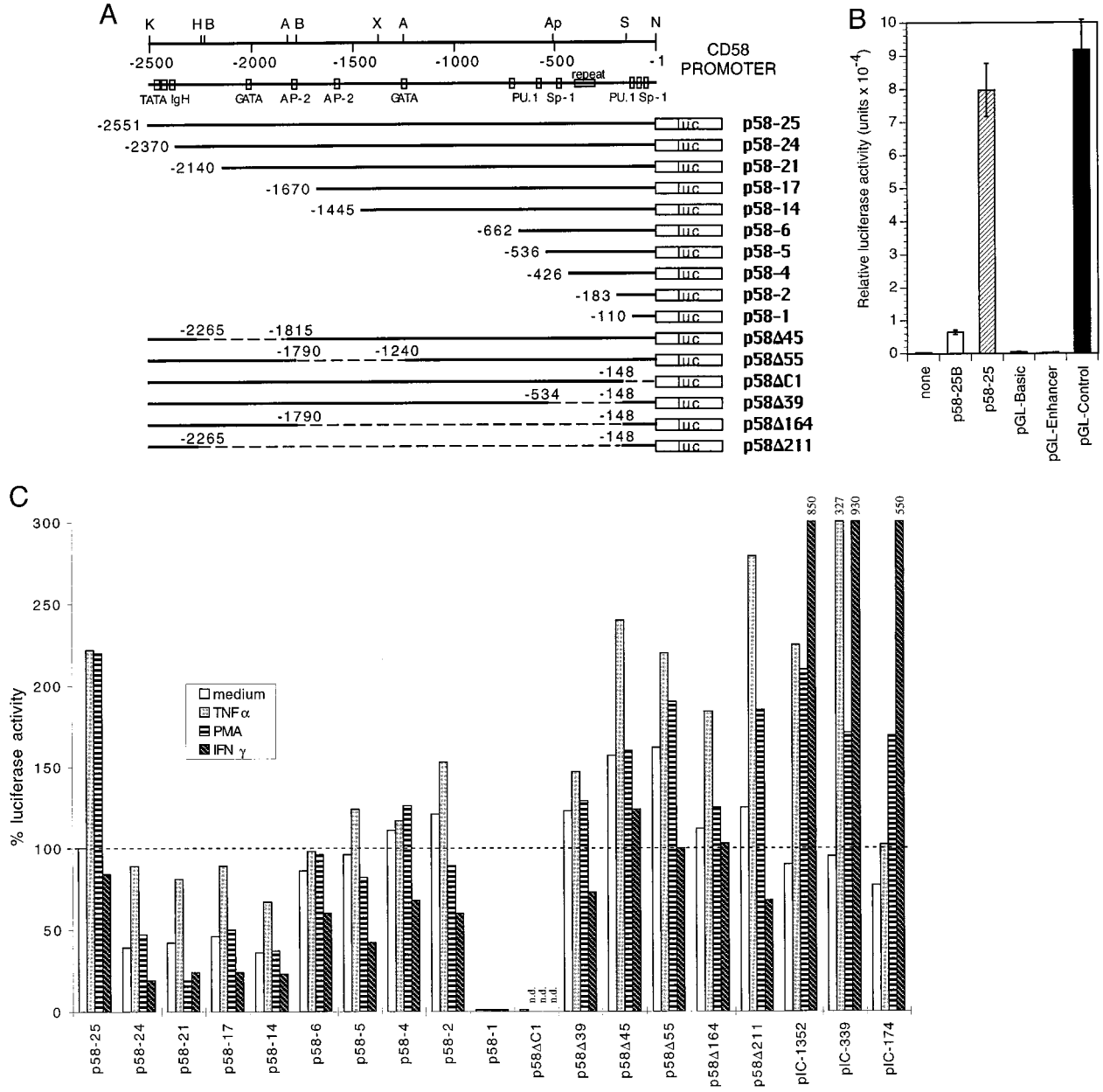

FIGURE 4. *A*, The 5′ promoter region of the human *CD58* gene. Schematic representation of the 5′ region of the *CD58* gene and of the promoter-luciferase constructs used in this study. This figure shows the restriction sites used for cloning subfragments of the promoter region upstream of a promoterless firefly luciferase cDNA in the pGL2-Enhancer vector (see *Materials and Methods*). Restriction sites are indicated as follows: A, *Afl* II; Ap, *Apa*I; B, *Bst*XI; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I; S, *Sac*II; X, *Xho*I. *B*, CD58 promoter (2.5 kb)-driven luciferase activity in HepG2 cells. The CD58 promoter was incorporated into the vector pGL-Basic (p58-25B) and pGL-Enhancer (p58-25). Each construct and the control plasmids (pGL-Basic, pGL-Enhancer, pGL-Control) were transiently transfected into HepG2 monolayers and assayed for luciferase activity. Luciferase activity is expressed relative to β-galactosidase activity derived from a cotransfected control plasmid (pUHD16.1). This value was then normalized to protein content (mg). The results shown are means of triplicate tests (±SD). *C*, Effect of deletions on the transcriptional activity of the CD58 promoter. A series of constructs containing 5′ deletion mutants (p58-1, p58-2, p58-4, p58-5, p58-6, p58-14, p58-17, p58-21, and p58-24), a 3′ deletion mutant (p58ΔC1), and internal deletions mutants (p58Δ39, p58Δ45, p58Δ55, p58Δ164, and p58Δ211) linked to the firefly luciferase gene (luc) were cloned into a multiple cloning site of the vector pGL2-Enhancer and tested for transcriptional activity after transient transfection into HepG2 cells. For control, three CD54/luciferase constructs, designated pIC-1352, pIC-339, and pIC-174, were tested. Equal aliquots of the cell extract were assayed for luciferase activity. The values are expressed as the activity of luciferase after correction for transfection efficiency by β-galactosidase activity in the same sample, and the relative luciferase activity of HepG2 cells transfected with construct p58-25 without further treatment (medium) was designated 100%. Relative activities corresponding to each construct transiently expressed in HepG2 cells untreated (medium) and induced with TNF-α, PMA, or IFN-γ are given (n.d. = not done). The data shown are representative of four experiments; SDs were less than 15%.

Table II. *Feature table of the human CD58 promoter sequence*

Name	Sequence	Position (bp)
TATA	TATAA	−2518 → −2514
		−2393 → −2389
IgH	ATTTGCAT	−2346 → −2339
AP2	CCCCAGGC	−1829 → −1822
		−1611 → −1604
GATA	TGATAG	−2088 → −2083
		−1270 → −1265
PU.1	GAGGAA	−732 → −727
		−594 → −589
		−133 → −128
Sp-1	CCGCCC	−519 → −514
		−122 → −117
		−98 → −93

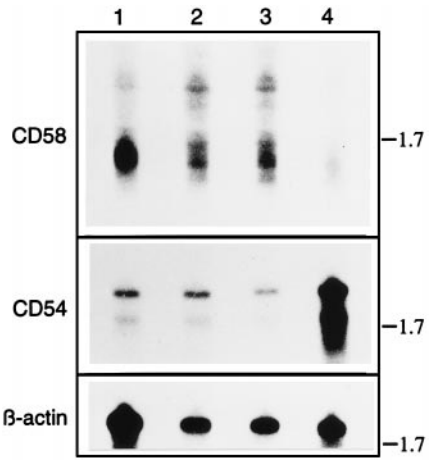

FIGURE 5. Northern blot analysis. A total of 10 μg of total RNA from HepG2 cells untreated (1) or treated with either TNF-α (2), PMA (3), or IFN-γ (4) for 24 h were subjected to electrophoresis in a 1% formaldehyde-agarose gel and transferred to nitrocellulose. The RNA blots were hybridized with ^{32}P-labeled CD58, CD54, or β-actin cDNA. The marker 1.7-kb transcript length is indicated.

Expression of CD58 *promoter/luciferase gene constructs in cultured HepG2 cells*

A DNA fragment (*Nco*I-*Kpn*I) of the λ4-1 clone containing 2.5 kb of the CD58 upstream sequence (Fig. 4*A*) had been filled in using the large Klenow fragment of *Escherichia coli* DNA polymerase I and was subcloned into the *Sma*I site of pGL2-Enhancer and pGL2-Basic (Promega) to generate plasmid p58-25 and p58-25B, respectively. The pGL2-Basic vector lacks eukaryotic promoter and enhancer sequences, whereas the pGL2-Enhancer vector contains an SV40 enhancer located downstream of the luciferase gene and the poly(A) signal. When constructs p58-25 and p58-25B were transfected, luciferase activity in HepG2 cells increased 12-fold in the presence of the SV40 enhancer region (Fig. 4*B*).

To test for the functional promoter activity of the 5′-flanking region of the *CD58* gene, several chimeric promoter/luciferase reporter gene plasmids were constructed (see *Materials and Methods*). Starting from plasmid p58-25, a series of deletion constructs were made (Fig. 4*A*) and cloned into pGL2-Enhancer. These constructs were then utilized for transient transfection of human HepG2 cells, in the presence of a cotransfected β-galactosidase expression plasmid. Previously, it has been shown that HepG2 cells express high levels of CD58 (12, 21). An assay of luciferase activity in HepG2 cells transfected with these constructs revealed clearly detectable activity, while essentially no activity was detected in cells transfected with pGL2-Enhancer as a negative control (Fig. 4*B*). After correction of the luciferase activities by the β-galactosidase activity in the same sample, the activity of construct p58-25 was chosen as the reference (activity set to 100%) and the activities of all other constructs were expressed relative to the activity of this plasmid vector (Fig. 4*C*).

To test whether the 2.5-kb upstream fragment of CD58 could confer inducibility in HepG2 cells to different cytokines or PMA, cells were transiently transfected with plasmid p58-25 followed by an incubation with these agents, and luciferase activity was measured after 16 h. Treatment with TNF-α or PMA resulted in a twofold increase in luciferase activity (Fig. 4*c*). In contrast, incubation of cells with IFN-γ leads to a slightly reduced luciferase activity. Thus, the 2.5-kb CD58 upstream region does mediate responsiveness to TNF-α and PMA, but not to IFN-γ. For positive control, the effect of cytokines and PMA on the induction of luciferase driven by CD54 upstream sequences was tested. As expected, HepG2 cells transfected with three CD54/luciferase constructs, designated pIC-1352, pIC-339, and pIC-174 (47, 48), showed about a 2-fold induction of luciferase activity upon treatment with either TNF-α or PMA, whereas IFN-γ strongly ($\approx$10-fold) increased the transcriptional activity, indicating that IFN-γ response elements (47) must be contained in these CD54 constructs that are active in this cell type (Fig. 4*C*).

To further investigate the 2.5-kb upstream region of the human *CD58* gene, a series of 5′ deletion mutants, shown in Figure 4*B*, was tested to localize putative *cis*-acting elements in this region. In the absence of cytokines or PMA, deletions of up to 1 kb (p58-24, -21, -17, and -14) resulted in a decrease of activity to about 40% of the level obtained with plasmid p58-25. However, deletion of up to 1.2 kb (p58-6, -5, -4, and -2) resulted in a stepwise increase in luciferase activity to values similar to those obtained with construct p58-25. Construct p58-1, containing 110 bp of the 5′-flanking region, abolished luciferase expression to background levels. Similarly, a 3′ deletion mutant, designated p58ΔC1, resulted in a strong decrease of luciferase activity, thus localizing an important regulatory element necessary for transcription of the *CD58* gene or, alternatively, deletion of a region close to the transcriptional start site abrogates transcriptional activity. Nevertheless, deletion analysis clearly revealed the essential role of the CD58 promoter sequence between position −183 and −110 in transcription initiation. A series of internal deletions was created (p58Δ39, Δ45, Δ55, Δ164, and Δ211) starting at position −148 and extending for increasing distances into the 5′ region (Fig. 4*B*). All deletions tested resulted in increased expression up to 162% relative to p58-25 in HepG2 cells. These observations suggests the presence of up-regulatory, enhancer-like elements within the regions −[2551-2370] and −[426-110], and the occurrence of down-regulatory, silencer-like elements within the region between −2370 and −426.

To determine whether the cytokine-induced CD58 promoter-driven luciferase activity in HepG2 cells can be correlated at the endogenous CD58 mRNA level, the effects of either TNF-α, IFN-γ, or PMA treatment on mRNA accumulation was measured. A clear signal corresponding to the 1.3-kb mRNA transcript of CD58 was detected in untreated HepG2 or cytokine-treated cell cultures. After 24 h of incubation with either TNF-α or PMA, the CD58 mRNA accumulation slightly increased, whereas treatment of HepG2 cells with IFN-γ results in decrease in CD58 mRNA (Fig. 5). This finding is in good agreement with the data obtained with our expression analysis of the *CD58* promoter/luciferase gene constructs in HepG2 cells. In addition, flow cytometry analysis of

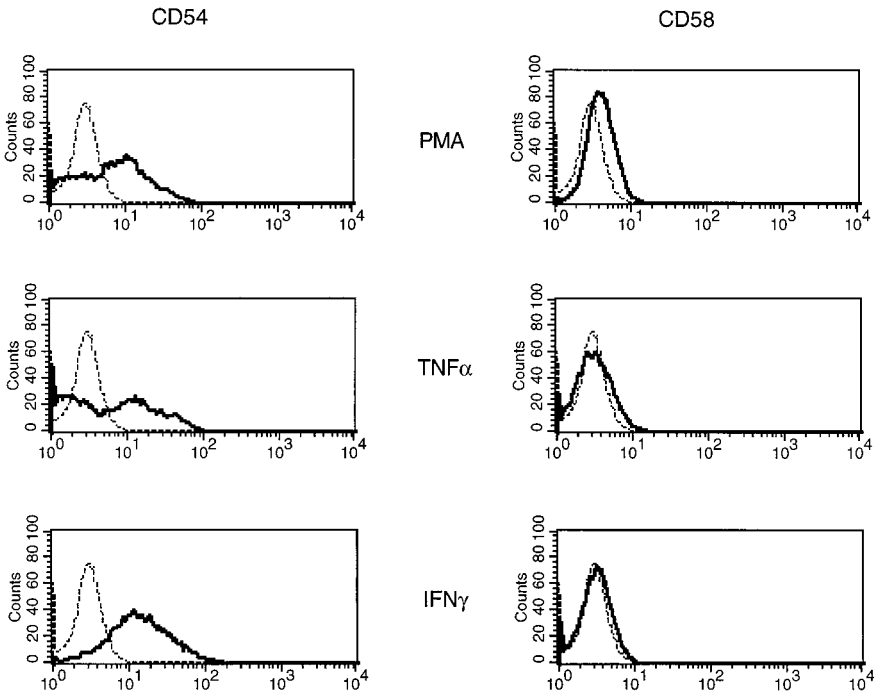

FIGURE 6. FACS profiles (relative fluorescence vs cell number) for HepG2 cells stained with AICD58 mAb (CD58) and 84H10 mAb (CD54). The cells were left untreated (dotted profile) or were treated with either TNF-α, PMA, or IFN-γ (bold profile) for 48 h.

CD58 and CD54 expression on the surface of HepG2 cells 48 h after incubation with either TNF-α, IFN-γ, or PMA showed similar responses as found in the expression assays (Fig. 6).

Discussion

Human genomic libraries were screened with a mixture of overlapping probes covering the entire CD58 cDNA, and four independent clones were isolated that together contain the complete coding sequence for *CD58* as well as 18 kb of the 5′-flanking region. Comparison of the genomic DNA sequence with the reported cDNA sequences (9, 10) revealed five introns residing within the coding region. The six exons encoding CD58 are dispersed over about 65 kbp and contain the leader sequence encoding for a signal peptide, the two Ig-like domains, and the transmembrane domain, followed by the cytoplasmic region and the polyadenylation site.

The extracellular domain of CD58 is encoded by two exons, which is in good agreement with the Ig-like structure of the protein. The exon structure is similar to that reported for other members of the Ig supergene family, including *CD2* (49, 50). In contrast, exon 5, harboring two consensus splice acceptor sites that appear to be involved in differential splicing of the CD58 mRNA, is missing from the *CD2* gene. It is therefore an attractive explanation that the GPI-linked form of human CD58 is generated from the upstream splice acceptor site, whereas mRNA encoding the transmembrane form of CD58 is derived from alternative RNA splicing to the downstream site. The 35-bp DNA segment located between the two splice acceptor sites is present only in the mRNA encoding for the GPI form of CD58 and harbors the stop codon for this glycoprotein.

Previously, we have isolated a soluble form of CD58 (sCD58) from human sera, urine, and culture supernatants of several cell lines, including HepG2 (21). The exact mechanism that causes the release of CD58 into the supernatant of these cells is as yet unknown. Many soluble forms of leukocyte adhesion molecules have been described, and only soluble P-selectin could be directly derived from a splice variant lacking a transmembrane domain (51, 52). From our limited genomic sequence data, the existence of a splice variant that could give rise to a soluble CD58 glycoprotein is not formally excluded. Nevertheless, initial experiments employing phospholipase-treated cells support the assumption that sCD58 is generated by proteolytic shedding (21, and our unpublished observations). In several pathologic situations, the levels of sCD58 in serum are elevated (21, 22). The function of this soluble form is still speculative. Shedding may be a rapid mechanism to initiate de-adhesion and subsequently inhibit T cell/target cell interaction. Alternatively, the soluble adhesion molecules might compete with their membrane-bound correlates and thereby regulate adhesion. Soluble forms also have costimulatory effects in the activation of T cells (23).

DNA sequence analysis of the 2.5-kb CD58 promoter revealed several potential binding sites for transcriptional activators that may be involved in CD58 transcription. Although the CD58 promoter lacks a TATA box close to the transcriptional start site as was observed for several other genes, including *CD2* (49), two putative TATAA sequences 2.4 kb upstream of the translational start site were identified. The functional significance of the TATAA motifs of the CD58 promoter is unclear at present. In addition, there are three putative Sp-1 binding sites located in the CD58 promoter. In a model of TATA-less transcription initiation proposed by Pugh and Tjian (39), Sp-1 interacts with a tethering factor that binds transcription factor TFIID in the absence of a TATA box, serving to anchor the transcription complex to the promoter. Other upstream activators may be important in the tissue-specific expression of CD58, and sequence analysis of the CD58 promoter indicates that the transcriptional activator PU.1 may be involved in CD58 transcription. In addition, the CD58 promoter contains three PU boxes. PU boxes bind a tissue-specific DNA binding protein, PU.1, which is a transcriptional activator in macrophages and B cells (40). Finally, two putative AP-2 binding sites are present in the CD58 promoter. AP-2 is a site-specific DNA binding protein that can activate transcription (43). Binding sites for AP-2 have also been demonstrated in *cis*-regulatory regions of several viral and cellular genes. The AP-2 sites are flanked

by the sequence motif TGATAG, which represents another potential binding site for the 'GATA' family of transcription factors (42, 53). Members of this family, GATA-1 and GATA-3, are lineage-specific hemopoietic transcription factors that have been shown to regulate erythrocyte (54) and T cell-specific (41, 42) genes, respectively.

Analysis of a series of deletion fragments of the CD58 promoter revealed that the regulatory region is composed of various *cis*-acting elements that contribute positively and negatively to the CD58 promoter activity. Apparently, sequences upstream of position −2370 may contain enhancing elements, since their removal decreased activity (>60%) of the remaining promoter (p58-24). The transcriptional activity was not changed significantly upon further deletion of the 5′-flanking region (p58-21, p58-17, p58-14, p58-6, and p58-5). It should be noted, however, that deletions to −426 (p58-4) and −183 (p58-2) retained appreciable sp. act. as observed for the CD58 promoter construct harboring 2.5 kb of upstream sequence (p58-25). Further deletion to −110 completely abolished expression of the reporter gene.

It is legitimate to suggest that silencing elements are contained in the CD58 promoter region between −2370 and −426. This assumption is supported by the fact that CD58 promoter constructs with internal deletions between −2265 and −148 (p58Δ39, p58Δ45, p59Δ55, p58Δ164, and p58Δ211) exhibit higher sp. act. compared with expression plasmid p58-25. The CD58 promoter thus exhibits a modular structure (55, 56) and the control of differential expression of CD58 might result from a combination of activity of the various regulatory elements.

Several immunohistologic and flow cytometry studies have shown that increased CD58 expression is found on cells of both hemopoietic and nonhemopoietic origin at inflammatory sites in several diseases (11, 12, 18, 57, 58). In contrast, CD58 is down-regulated on certain carcinomas, lymphocytic leukemia, and lymphoma cells (13, 14, 16, 19, 20). In acute lymphoid leukemia, the expression of CD58 was inversely correlated with the presence of a clinical tumoral syndrome, leukocytosis, and the percentage of peripheral blast cells (16, 20). Therefore, low expression of CD58 might facilitate, at least in part, the escape of tumor cells from immune surveillance. Most interestingly, culturing tumor cells with rTNF-α resulted in reinduction of CD58 expression and susceptibility to lymphocyte-mediated lysis in vitro (20). In contrast, CD58 expression in human intestinal epithelial cell lines was virtually unaffected by cytokine (IFN-γ, TNF-α, IL-1, IL-6) stimulation (59). These data indicate that CD58 expression is differentially regulated in different cell types. We have used the hepatocellular carcinoma cell line HepG2, which previously has been shown to produce high levels of CD58, to study the induction of the *CD58* gene by cytokines and by PMA in vitro (21).

Transient transfection experiments with plasmid p58-25 showed that 2.5 kb of the 5′-flanking region is sufficient to mediate responsiveness to TNF-α and PMA (about twofold increased expression) but not to IFN-γ, indicating that IFN-γ-responsive elements may not be located within this region. In contrast, the induction rate was highest with the CD54 promoter, which has been shown before to harbor IFN-γ response elements (47). With the use of the series of deletion constructs, definite regulatory sequences responsible for either PMA or TNF-α induction could not be localized within the 2.5-kb promoter region. We have observed that TNF-α and PMA treatment is almost uneffective in CD58 mRNA accumulation, suggesting that the control of CD58 expression may take place on the post-transcriptional level. Moreover, it will be interesting to investigate the mechanisms of induction of CD58 expression by different viruses, such as human T cell leukemia virus-1 or EBV, in combination with different cytokines (15, 18, 60).

Further studies on *CD58* gene expression will be required, including DNA footprinting analysis and in vitro mutagenesis of proposed regulatory regions, to analyze more precisely the location of controlling elements of the gene. In the *CD2* gene locus, the control of expression is regulated by a well-characterized 3′ enhancer together with a promoter and upstream elements (61, 62). Characterization of the additional structural elements of the *CD58* gene may lead to a better understanding of the role of CD58 in inflammatory and malignant processes.

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