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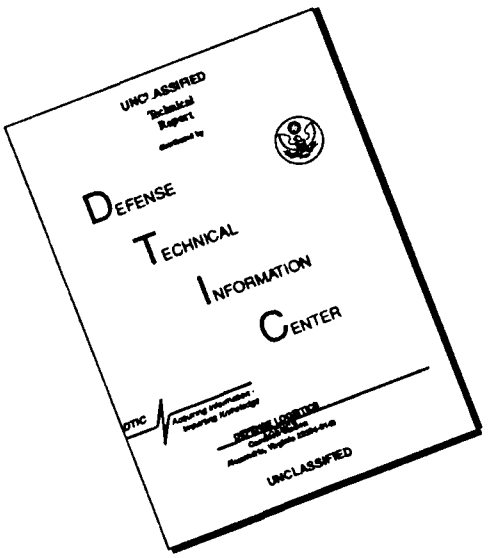

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THE GENOMIC ORGANIZATION OF THE CD28 GENE

Implications for the Regulation of CD28 mRNA Expression and Heterogeneity[1]

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CD28 is a 90-kDa homodimeric glycoprotein present on the surface of a large subset of T cells that appears to play an important role in the modulation of T cell activation. Although a number of physiologic effects associated with CD28 stimulation have been defined, relatively less is known about the structure and expression of the CD28 gene itself. We now show that CD28 is expressed in both Th cells and plasma cells as a series of four distinct CD28 mRNA species: 1.3-, 1.5-, 3.5-, and 3.7-kb transcripts. The steady state expression of all four transcripts in CD28[+] T cells was stimulated by PMA, suggesting that they might share a common phorbol-sensitive promoter. Consistent with this hypothesis, CD28 was found to be encoded by a single copy gene organized into four exons, each exon defining a functional domain of the predicted protein. All CD28 transcripts appear to initiate within a 61-bp palindrome. Generation of the four CD28 mRNA species from the CD28 gene involves two distinct posttranscriptional events. The longer pair of transcripts (3.5/3.7 kb) is generated by the use of an alternate nonconsensus polyadenylation signal. This results in the addition of 2167 bp beyond the first polyadenylation site utilized by the shorter (1.3/1.5 kb) pair of transcripts. The size difference between the 3.7- and 3.5-kb messages and between the 1.5- and 1.3-kb messages is generated by an internal splicing event that deletes 252 bp within exon 2, which encodes the extracellular domain. This deletion would result in the loss of 84 amino acids, including 4 of 5 extracellular cysteine residues. Although this deletion would result in significant disruption of CD28 secondary structure, it would not be expected to interfere with the ability of the resultant protein to be expressed on the cell surface. These findings suggest that variant isotypes of CD28 may be expressed on the cell surface with potentially different physiologic roles.

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The mechanism and regulation of T cell activation is one of the most intensely investigated paradigms of modern immunology. It is now clear that the TCR/CD3 complex plays a central role in the specific recognition of Ag and subsequent initiation of cellular activation (1, 2). Additionally, a number of accessory molecules present on the surface of T cells (3, 4) are able to function as physiologically important mediators of the immune response. The CD28 Ag (previously named Tp44), a member of the Ig gene superfamily, appears to have such an accessory function. CD28 is expressed on approximately 95% of $CD4^+$ and 50% of $CD8^+$ mature T cells (5) as an 88- to 90-kDa homodimer consisting of two identical disulfide cross-linked 44-kDa glycopeptides (6–9). The CD28 Ag was initially defined by the mAb 9.3 (6). Stimulation of CD28 in resting T cells by the binding of mAb 9.3 alone has no effect on T cell proliferation nor activation in most (8–13), but not all (14, 15), reports. However, additional cross-linking of CD28 causes an increase in intracellular calcium (16) and, in the presence of IL-6 (or monocytes), results in IL-2 responsiveness through CD25 (IL-2R) expression (10). Furthermore, in T cell preparations purified free of accessory cells, CD28 stimulation provides a co-mitogenic signal that synergizes with submaximal doses of a variety of other stimuli (anti-CD3-mAb (8, 13, 17–21), PHA (7, 8, 21), PMA alone (9, 12, 15, 20) or PMA plus ionomycin (19), Con A (8, 21), CD2 (11), or IL-2 (18)) and results in a marked enhancement of both proliferation and IL-2R expression. CD28-mediated effects are relatively resistant to the inhibitory effects of the immunosuppressant cyclosporine A, which is in sharp contrast to responses mediated by CD3 or PMA/ionomycin stimulation alone (12, 13, 19). More recently it has been shown that the expression and secretion of multiple lymphokines/cytokines (IL-2, TNF-α, lymphotoxin, IFN-γ, and granulocyte-macrophage CSF) in normal T lymphocytes are also markedly enhanced after CD28 stimulation (13) and that this enhancement is mediated primarily through the stabilization of the mRNA of these lymphokines (22). In contrast to these activational effects, binding of mAb 9.3 to CD28 results in the inhibition of proliferation to both alloantigen (in naive T cells (18)) and soluble antigen in Ag-specific (*Mycobacterium leprae*) class II MHC-restricted T cell clones (14). This inhibition may be dependent on the degree of cross-linking of CD28 (18). A physiologic role for this dual effector response of CD28 has not yet been determined.

Despite the accumulating evidence of the varied phys-

iologic effects of CD28, less is known regarding the regulation of CD28 expression and the role expression may play in the generation of these effects. Expression of CD28 at the cell surface (14, 21, 23) is enhanced by PMA, PHA, and CD3 stimulation, signals that are also comitogenic for CD28. At the mRNA level, the CD28 gene transcribes four RNA species; a smaller pair consisting of a 1.5-kb transcript and a 1.3-kb transcript, and a larger pair consisting of a 3.5- and a 3.7-kb transcript (24). cDNA cloning by Seed and Aruffo (24) reveals that the 1.5-kb transcript encodes the surface-expressed 44-kD form of CD28 recognized by the CD28 specific mAb 9.3. The remaining transcripts have not been characterized and the origin and significance of this mRNA heterogeneity is not known. We now show that all four mRNA species are inducible to varying degrees during T cell activation. In addition, all four mRNA species are expressed in a B cell line representative of plasma cells but not in less mature B cell lines. In order to characterize the origins of CD28 mRNA heterogeneity, we have cloned the genomic CD28 gene and find it to be a single copy gene organized into four exons, each corresponding to a functional domain of the predicted protein. The larger 3.5- and 3.7-kb mRNA species arise from the use of an alternate, nonconsensus polyadenylation signal located 2167 base pairs (bp) downstream from the first signal. This would account for the size difference seen between the larger (3.7/3.5 kb) and the smaller (1.5/1.3 kb) mRNA species. In addition, evidence of an internal splicing event using an unusual splice donor site was found. This internal splicing event can result in the deletion of 252 bp in the coding region, and would account for the size difference seen between the 3.7 and 3.5 mRNA species and between the 1.5- and 1.3-kb mRNA species. This deletion would translate into the loss of 84 amino acids in the extracellular domain, including four of the five cysteine residues. Nonetheless, this predicted protein is still capable of being expressed as a cell surface molecule. These data suggest that alternate products of the CD28 gene may be expressed as cell surface molecules.

MATERIALS AND METHODS

Cells. CD28$^+$ T cells were purified from normal peripheral T cells and incubated with PMA as previously described (19). The EBV-transformed B cell lines LCL-JRS (kind gift of Dr. B. Carreño) and JRSA 1001, the plasma cell line RPMI 8226 and the T cell line CEM were grown in RPMI 1640 with 10% FCS, 2 mM L-glutamine, and penicillin/streptomycin. When indicated, LCL-JRS cells were incubated with PMA (5 ng/ml) and ionomycin (0.8 μg/ml) as previously described (19). CEM cells were incubated with PHA (10 μg/ml) at 1 × 10^6 cells/ml and harvested at 24 h. Tonsillar B cells were harvested from normal tonsil and positively selected with CD20.

DNA probes. Unless otherwise indicated, the ^{32}P-labeled DNA probes used in these experiments were all the result of hexanucleotide priming (25) of CD28-specific inserts (100 ng) isolated from low melting point agarose after either digestion of the plasmid in which they were propagated with the appropriate restriction endonuclease or generation by PCR[3] (see below). The full length 1.5-kb CD28 cDNA probe (24) was the generous gift of Drs. B. Seed and A. Aruffo (Massachusetts General Hospital). Digestion of this CD28 cDNA with restriction endonuclease *Xho*I yields an 893-bp 5′ probe and a 621 bp 3′ probe. Digestion with *Rsa*I yields a 195-bp probe that contains the 5′ end of the CD28 cDNA. Generation of specific DNA probes from genomic DNA fragments using site-specific oligonucleotide primers and PCR is described below.

Northern blot analysis. Cells were harvested from culture by resuspension and centrifugation, and total cellular RNA was extracted with guanidinium isothiocyanate (26). RNA samples were equalized for ribosomal RNA, and the equalization confirmed by ethidium bromide staining of equal amounts of RNA samples separated on a nondenaturing 1% agarose gel (27). These equalized RNA samples (5 to 10 μg) were separated on 1% agarose-formaldehyde gels, transferred to nitrocellulose, baked at 80°C under vacuum for 2 h and prehybridized (12). A 1.5-kb CD28 cDNA probe was radiolabeled by nick translation and subsequently hybridized to the membrane (12), after which the membranes were exposed to x-ray film at −70°C.

Southern blot analysis. High m.w. DNA from human PBL was prepared as previously described (28). The 10-μg samples of DNA were digested with the appropriate restriction endonuclease(s), separated on 0.8% agarose gels, transferred to nitrocellulose and prehybridized (28). The membranes were subsequently hybridized to either a [α-^{32}P]-labeled 5′ *Xho*I fragment or 3′ *Xho*I fragment of CD28 cDNA (28). High stringency wash conditions were 0.1 × SSC and 0.1% SDS at 56°C. Low stringency wash conditions were 2 × SSC and 0.1% SDS at 50°C. Membranes were subsequently exposed to x-ray film at −70°C.

Isolation and characterization of genomic clones. Approximately 1 × 10^6 recombinant phage from two separate human genomic libraries, an *Mbo*I partial digest of HPB-ALL DNA cloned into EMBL3 (generous gift of Dr. J. Leiden, University of Michigan, Ann Arbor, MI) and a *Sau*3A partial digest of normal PBL DNA cloned into λFix (Stratagene, San Diego, CA) (generous gift of Dr. J. Lowe, University of Michigan, Ann Arbor, MI) were screened with the ^{32}P-labeled CD28-specific DNA probes described above. Positively hybridizing clones were purified to homogeneity through sequential dilution of positively hybridizing plaques. Each clone was then structurally characterized by restriction endonuclease mapping, Southern blot analysis, and DNA sequencing of putative exons.

Isolation and characterization of cDNA clones. Approximately 1 × 10^6 recombinant phage from two separate human cDNA libraries, HPB-MLT/λgt11 (29) and Jurkat T cell (PMA + ionomycin stimulated, poly A primed)/λgt11 (both generous gifts of Dr. J. Leiden) were screened with ^{32}P-labeled CD28 DNA probes described above. Positively hybridizing clones were purified and analyzed as described for genomic clones.

DNA sequencing. Appropriate restriction endonuclease fragments were subcloned directly from low melt agarose into pGEM3Z (Promega Biotec, Madison, WI) (30). DNA sequencing reactions were performed on double-stranded plasmid template DNA using a Sequenase kit according to suppliers' protocol (U.S. Biochemical Corp., Cleveland, OH) and separated on 50 cm wedged (0.2 to 0.5 mm) 8% polyacrylamide gels using a buffer gradient. Oligonucleotide primers used were specific for the SP6 or T7 promoter sites of pGEM3Z or were 20mer primers specific for sites within the CD28 gene. All sequences were confirmed by sequencing both strands. Sequence data analysis were performed using the DNASTAR software package (DNASTAR, Madison, WI).

PCR. PCR (31) was used to generate specific cDNA fragments from RNA templates (RNA PCR) for further analysis and to generate specific DNA fragments from DNA templates (DNA PCR) for use as radiolabeled probes. All reactions were done in a 50-μl volume. Oligonucleotide primers were synthesized on an Applied Biosystems Model 380B DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). Certain primers had the addition of *Eco*RI or *Hind*III restriction enzyme sites to their 5′ end to allow for directional cloning into pGEM3Z.

RNA PCR. RNA was extracted from PHA-stimulated CEM cells with guanidinium isothiocyanate (26). First strand cDNA synthesis was performed by mixing 1 μg of RNA with 3 μg of oligo d(T) primer, 1 μl 25 mM dNTP (Pharmacia Fine Chemicals, Piscataway, NJ), 50 mM Tris (pH 8.3), 50 mM KCl, 8 mM $MgCl_2$, 10 mM dithiothreitol, 26 U avian myeloblastosis virus reverse transcriptase (Seikagaku Inc., St. Petersburg, FL) and incubating at 42°C for 1 h. To enhance specificity and sensitivity, two sequential PCRs using internally nested 3′ primers were done. Ten μl of the first strand cDNA synthesis mixture was added to 200 ng each of primers 1811 (5′) and 1407 (3′), 7.5 μl of 1 mM dNTP, 40 mM KCl, 0.01% gelatin, and 2.5 U Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). PCR was performed in a Perkin-Elmer Cetus thermal cycler for 10 cycles (consisting of 94°C for 1.5 min and 72°C for 4 min) followed by a 72°C 10-min final extension step. Five μl of this reaction mixture was then added to 1 μg each of primer 1811 (5′) and the internally nested 3′ primer 1091, 10 μl 1 mM dNTP, 5 μl of 10× Taq polymerase buffer (100 mM Tris (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin, 20 mM dithiothreitol) and 2.5 U Taq polymerase followed by 20 thermal cycles (consisting of 94°C for 45 s, 72°C for 1.5 min) and a 72°C 10-min final extension step. PCR products were subsequently separated on 1% agarose gels, transferred to nitrocellulose, and hybridized to a radiolabeled 1.5-kb CD28 cDNA probe as described

[3] Abbreviations used in this paper: PCR, polymerase chain reaction; dNTP, deoxynucleotide triphosphates.

above. Positively hybridizing products were digested with *Eco*RI and *Hin*dIII, cloned into pGEM3Z and subsequently sequenced.

DNA PCR. To generate specific DNA fragments not found in the original cDNA clone for use as radiolabeled probes, PCR of cloned genomic CD28 fragment templates with site-specific synthetic oligonucleotide primers was used. A total of 200 ng of the appropriate genomic fragment was added to 1 μg each of site-specific oligonucleotide primers, 5 μl 10× Taq polymerase buffer, 10 μl 10 mM dNTP, 2.5 U Taq polymerase followed by 30 thermal cycles (94°C for 1.5 min, 55°C for 1 min and 72°C for 2 min) and a 72°C 10-min final extension step. PCR products were then separated from unused primers on a 1% low melting agarose gel followed by Elutip-D column purification (Schleicher & Schuell, Keene, NH).

Primer extension. Primer extension analysis was performed as described by Ausubel et al. (32). A 30mer oligonucleotide primer corresponding to the reverse complement of base pairs (AGGGGCTGGAACCCTAGCCCATCGTCAGGACAAAGAT) of the 5′ sequence of exon 1 was 5′ end-labeled with [γ^{32}P]ATP and T4 polynucleotide kinase. The 5×10^4 cpm of labeled oligonucleotide was then hybridized overnight with 50 μg of CEM cellular RNA at 30°C in 80% formamide, 40 mM PIPES (pH 6.7), 400 mM NaCl, and 1 mM EDTA. Primer extension was then performed by adding 64 U avian myeloblastosis virus reverse transcriptase (Seikagaku) and incubating at 42°C for 90 min. The reaction mix was then treated with RNAase A (30 μg/ml) for 30 min at 37°C, phenol/chloroform extracted, and ethanol precipitated. The reaction products were denatured and separated on a 6% acrylamide/urea gel run in parallel with 5′ end-labeled *Hin*fI digested ΦX174 DNA size markers.

S1 nuclease protection analysis. S1 analysis was performed by a modification of the method described by Ausubel et al. (32) by digesting a 1.7-kb genomic fragment containing the CD28 first exon and 5′ flanking sequence with the restriction endonuclease *Ava*I, producing an 850-bp fragment spanning the transcriptional start site predicted by primer extension. After separation and recovery on a low melting point agarose gel, the fragment was purified on Elutip-D columns. The fragment was then 5′ end-labeled with [γ^{32}P]ATP by T4 polynucleotide kinase. As only the antisense strand of this fragment can function as a probe in an S1 assay, the ^{32}P-label of the sense strand was removed by digesting an *Eco*RI site very close to the 5′ end of this strand. The products were separated on a low melting point agarose gel and the appropriately labeled fragment was recovered and purified through Elutip-D columns. A total of 30 μg of Jurkat RNA (kind gift of Dr. T. Lindsten, University of Michigan, Ann Arbor, MI) was hybridized to 1.5×10^4 cpm of labeled probe in 30 μl of 80% formamide, 40 mM PIPES (pH 6.7), 1 mM EDTA, 400 mM NaCl at 50°C for 12 h. S1 nuclease (Boehringer Mannheim, Indianapolis, IN) digestion was performed at a final concentration of 1000 U/ml in 300 μl of 0.28 M NaCl, 50 mM sodium acetate, 4.5 mM $ZnCl_2$ at 37°C for 90 min. The reaction products were separated on a 6% acrylamide/urea gel.

RESULTS

1.5-kb CD28 cDNA probe recognizes 4 distinct RNA species. CD28 mRNA expression was examined by Northern blot analysis of T and B cell RNA after a variety of different stimuli. A representative Northern blot of resting peripheral T cells stimulated with PMA is shown in Fig. 1*A*. By 12 h poststimulation there has been a marked increase in the larger mRNA species (3.7 and 3.5 kb) with less increase in the smaller mRNA species. These increases were even more pronounced at 36 h. These results are in agreement with previous studies that the expression of cell surface CD28 is induced after T cell activation (14, 21, 23). These data would also suggest that the increased surface protein expression is the result of increased steady state mRNA levels.

In contrast to T cells, B cells (LCL-JRS cells, JRSA 1001, and tonsillar B cells in Fig. 1*B*) do not express detectable levels of CD28. Attempts to stimulate CD28 mRNA expression with phorbol ester and/or ionomycin were unsuccessful. However, significant levels of CD28 mRNA can be detected in the myeloma cell line RPMI 8226 (Fig. 1 *B*), confirming earlier reports that anti-CD28 mAb reacts with plasma cell lines (33). Furthermore, there is expression of all four of the RNA species observed

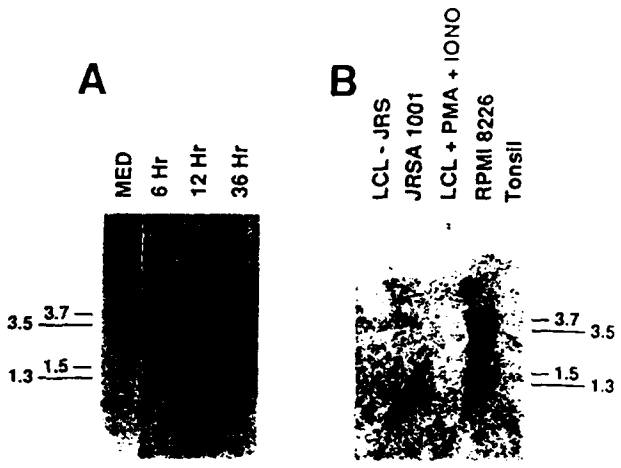

Figure 1. Expression of CD28 mRNA. *A*, Northern blot analysis of total cellular RNA of normal peripheral blood T cells after PMA stimulation, hybridized to a CD28 cDNA probe. Resting T cells are represented in the media alone (*MED*) lane. Time refers to hours poststimulation. mRNA sizes in kb are indicated. *B*, Northern blot analysis of RNA from the B cell line LCL-JRS and JRSA 1001 (unstimulated), LCL-JRS stimulated with PMA + ionomycine (IONO), the plasma cell line RPMI 8226 and normal tonsillar B cells hybridized to a CD28 cDNA probe. mRNA sizes in kb are indicated.

in T cells. Thus, as with activated T cells, this plasma cell line expresses appreciable levels of four distinct CD28 mRNA transcripts.

CD28 is not member of closely homologous gene family. Southern blot analysis of human PBL DNA hybridized to 5′ and 3′ [^{32}P]-labeled CD28 cDNA fragments was performed to gain insight into the genomic organization of CD28 and the possible existence of related genes (Fig. 2 *A* and *B*). Hybridization under high stringency and low stringency conditions yielded identical results. The failure of low stringency hybridization to demonstrate additional fragments suggests that CD28 is not a closely homologous member of a multigene family. Furthermore, given the location of the single *Eco*RI site 3′ of the *Xho*I site in the CD28 cDNA, the five unique bands seen on the *Eco*RI digests of this Southern blot analysis would predict that at least four exons are transcribed to create the 1.5-kb CD28 transcript if CD28 is a single copy gene.

CD28 has four exons organized by functional domains. The genomic organization of CD28 (Fig. 3) was established through the recovery of CD28-specific genomic phage clones from two independent human leukocyte libraries probed with CD28 cDNA fragments. A total of 51 independent clones was recovered from an HPB-ALL/EMBL3 library. Subsequent restriction endonuclease and DNA sequencing analysis revealed that these clones collectively spanned a region encompassing part of the first intron through part of the fourth exon (as verified by subsequent cDNA cloning). Three representative clones (clones 652/2, 653/2, and 787) are shown. However, no clones containing the first exon/5′ end or the 3′ end of the gene were initially recovered. A second human peripheral leukocyte/λFix library was screened with CD28 5′ or 3′ end-specific probes and 34 independent clones were recovered. One representative clone (1100/1) containing the complete fourth exon (as verified by subsequent cDNA cloning) is shown in Figure 3. Three representative clones (1053/1, 1047/1, and 1057/1) containing the first exon/5′ end are also shown. The overlap

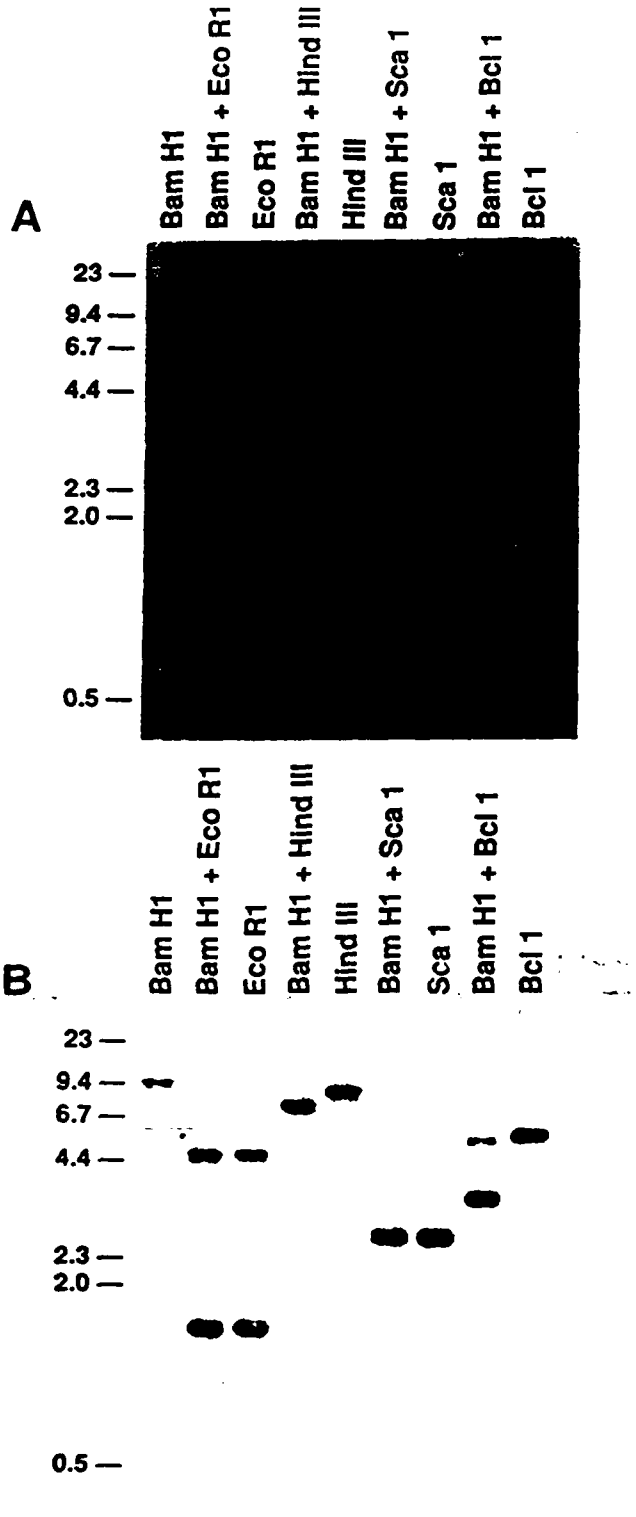

Figure 2. *A*, Southern blot analysis of human PBL DNA digested with the restriction endonucleases *BamHI*, *EcoRI*, *HindIII*, *ScaI*, *BclI* (alone and in combination). The probe was made by digesting CD28 cDNA (1.5 kb) with *XhoI* to yield an 893-bp 5′ fragment and a 621-bp 3′ fragment. The radiolabeled 5′ fragment was hybridized to this filter. *B*, Southern blot analysis of human PBL DNA hybridized to the radiolabelled 3′ fragment of CD28 cDNA. Hybridization under high and low stringency yielded identical results. The size in kb is shown to the left of each blot.

of clones 1057/1 and 653/2 was verified by sequence analysis. A large 22-kb first intron separates exon 1 from exon 2.

The start of transcription was mapped by S1 and primer extension analysis to a region of 61 bp indicated in Figure 4*A*. A large palindromic structure in this region prevented the identification of the exact start of transcription. The region 5′ of the start of transcription contains no consensus promoter elements but does contain an AP-1-like element (GTGACAAA instead of GTGACTAA) at position −39, which may be the basis of CD28 responsiveness to PMA. A human Alu family interspersed repetitive element is found approximately 170 bp 5′ of the start of transcription.

Exons were identified by hybridization to specific cDNA probes and subsequently analyzed by DNA sequencing (Fig. 4 *A* to *D*). The CD28 gene is composed of four exons spanning 36 kb of genomic DNA. Each splice junction conforms to consensus splice donor-acceptor sequences as reported by Mount (34) although the splice donor site of exon 3 is somewhat atypical (GG | GT vs consensus AG | GT). Each exon encodes a functional domain of the CD28 protein as originally defined by Aruffo and Seed (24). Exon 1 encodes the 5′ untranslated region and leader peptide, exon 2 contains the majority of the extracellular surface domain, exon 3 encodes the remainder of the surface domain and the transmembrane region, and exon 4 contains the intracytoplasmic and 3′ untranslated region. A second Alu family repeat is found in the 3′ untranslated region, 77 bp downstream of the first polyadenylation signal. The long $C(A)_N$ repeated sequence found 3′ of this Alu repeat is characteristic of many Alu repeats (35, 36).

Difference between the 1.5/1.3-kb mRNA pair and the 3.7/3.5-kb pair is due to alternate polyadenylation signal utilization. cDNA cloning of the 1.5 kb transcript by Seed and Aruffo (24) predicts that a consensus polyadenylation signal sequence (AATAAA) 739 bp 3′ from the end of translation is used. To investigate the possibility that the larger CD28 mRNA transcripts are the result of additional transcription 3′ of this signal, two independent T cell cDNA libraries (HPB-MLT and Jurkat) were screened with DNA probes specific for genomic sequences 3′ of the second Alu repeat. Positive clones were subsequently isolated and sequenced. The longest clone obtained, 1100/2.3, extended 2167 bp beyond the first polyadenylation signal and ended in a poly(A) tail. Surprisingly, sequence 5′ to the second polyadenylation site in both the cDNA and corresponding genomic DNA does not contain the highly conserved consensus polyadenylation signal AATAAA. A previously described variant AATTAA (37) is found 127 bp 5′ of the polyadenylation site but this distance is significantly greater than the usual spacing of 10 to 30 nucleotides (38) and makes use of this hexamer unlikely. However, within the constraints of spacing and sequence composition (hexamer with an invariant T at position 3 and very highly conserved As at positions 4, 5, 6), a candidate hexamer of GATAAA is found 15 bp from the polyadenylation site. Data bank searches suggest that this sequence has not been previously described as a polyadenylation signal, although a similar hexamer, CATAAA, is used in approximately 1% of the vertebrate mRNA analyzed (37).

Thus, the approximately 2.2-kb difference seen be-

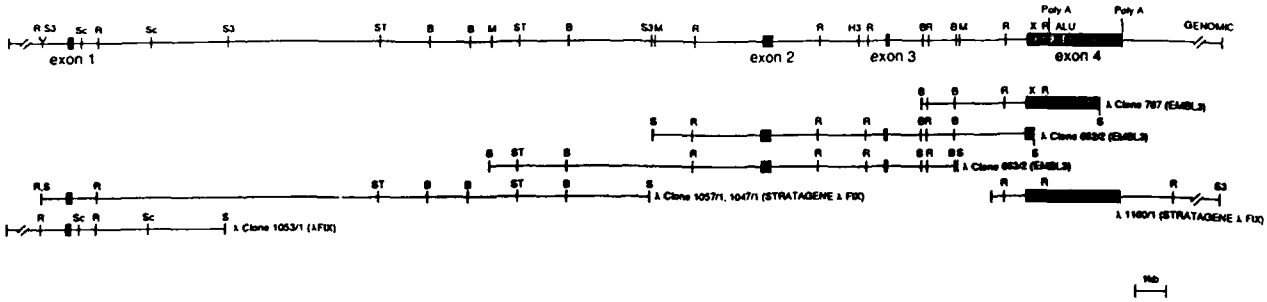

Figure 3. The genomic organization of CD28. A schematic representation and partial restriction endonuclease map is shown at the top. *Eco*RI (*R*), *Sau*3A (*S3*), *Sca*I (*Sc*), *Stu*I (*ST*), *Bam*HI (*B*), *Mbo*I (*M*), *Hin*dIII (*H3*), *Xho*I (*X*), *Sal*I (*S*) sites are shown. The six CD28 genomic clones (shown below) were obtained by screening two independent human leukocyte genomic libraries (*Mbo*I partial digest and *Sau*3A partial digest) with a radiolabeled CD28 cDNA (1.5 kb) probe under high stringency conditions (clones 1057/1 and 1047/1 are identical). The particular type of λ-vector is indicated in parentheses.

tween the two major species of CD28 mRNA can be explained by the additional transcription of 2167 bp in the 3′ untranslated region when the downstream nonconsensus polyadenylation signal is used. As noted, these sites differ in their putative poly(A) signal sequences. In addition, the sequence 3′ of the downstream poly(A) site is T (or U) rich, a motif that has been implicated as important for optimal 3′ end formation (39). In contrast, the sequence 3′ of the upstream poly(A) site is relatively devoid of such T- or GT-rich sequence. Furthermore, this upstream site is in close proximity (approximately 77 bp) to a human Alu family interspersed repeat, which is predicted to have significant secondary/stem-loop structure. It is possible that the presence of the Alu repeat accounts for the inefficient use of the proximal polyadenylation signal.

A 252-bp difference between mRNA transcripts is due to internal deletion. Alternate polyadenylation signal use explains the larger (2.2 kb) difference seen between the pairs of CD28 mRNA but not the smaller (approximately 200 bp) difference seen within each pair. One possible explanation is that this difference represents alternative splicing of an exon (such as the transmembrane domain encoding exon 3), thus generating different protein isotypes. To assess whether there exist subpopulations of CD28 mRNA that differ in their coding regions, a strategy using the PCR on total cellular T cell RNA was used (Fig. 5*A*; *Materials and Methods*). Briefly, the translated region was amplified through the use of site-specific flanking oligonucleotide primers and PCR, and the products were characterized by size separation and hybridization to a ^{32}P labeled CD28 cDNA probe. In Figure 5*A*, the 5′ primer corresponds to the first 30 bp of exon 2. Both 3′ primers were upstream of the first poly(A) signal in the 3′ untranslated region and were nested to enhance specificity. If a subpopulation of CD28 mRNA contains a deletion in this amplified region, one would anticipate two differently sized PCR products. In fact, we detected two such products that differ by approximately 250 bp (Fig. 5*A*). This difference would account for the size differences seen between the two pairs of RNA transcripts on Northern blot analysis. The larger 670-bp PCR product is the size predicted by the distance between the sense and antisense primers in the absence of an intervening deletion. The smaller 418-bp product therefore represents a 252-bp deletion between these primers present in a subpopulation of CD28 mRNA. The relative signal intensity of the smaller versus larger product is not dramatically different, demonstrating that the "deleted" mRNA constitute a significant fraction of total CD28 mRNA.

To better characterize these products, both were subcloned and sequenced. One 670-bp clone and three independent 418-bp clones were analyzed. A comparison is shown in Figure 5*B*. As predicted, the 670-bp product represents the entire cDNA sequence flanked by the oligonucleotide primers. However, the 418-bp product is the result of a 252-bp deletion within exon 2 that encodes the extracellular domain. If translated, this deletion would result in the loss of 84 amino acids. More importantly, three of the five cysteine residues in the extracytoplasmic domain would be completely lost with the conversion of the fourth at the splice junction to a tyrosine. Such a deletion would result in a major alteration in the secondary structure of the putative "receptor" portion of the CD28 molecule. This deletion appears to result from an internal splicing event that uses an uncommon splice donor signal sequence. The 3′ acceptor sequence TCCTCCTCCTTACCTAG is in good agreement with the consensus splice acceptor sequence $(T/C)_{11}N(C/T)AG$ previously described (34, 40). However, the 5′ splice donor sequence CT | GCAAGT differs significantly from the consensus splice donor sequence AG | GT(A/G)AGT (34, 40). The dinucleotide GT found just 3′ of the splice site in the consensus sequence is virtually invariant (41) with only rare cases (42–46) of the dinucleotide GC being used as in this case. It is also clear from the relative signal intensities on both the Northern blots and from PCR products that this site is used relatively efficiently.

DISCUSSION

Our studies indicate that CD28 is encoded by a single copy gene that has a complex pattern of expression on both a cellular and molecular level. On a cellular level, the activation of resting T cells by PMA results in a significant increase in the expression of four different CD28 mRNA species (3.7, 3.5, 1.5, and 1.3 kb), particularly for the larger (3.7/3.5 kb) species. Unlike T cells, resting B cells neither express CD28 nor can be induced to express CD28 by protein kinase C activation. However, in a cell line representative of the terminally differentiated stage of B cells (i.e., the secretory plasma cell), there is significant expression of CD28 mRNA. This finding is in agreement with the work of Kozbor et al. (33) in which resting B cells induced to differentiate into plasma

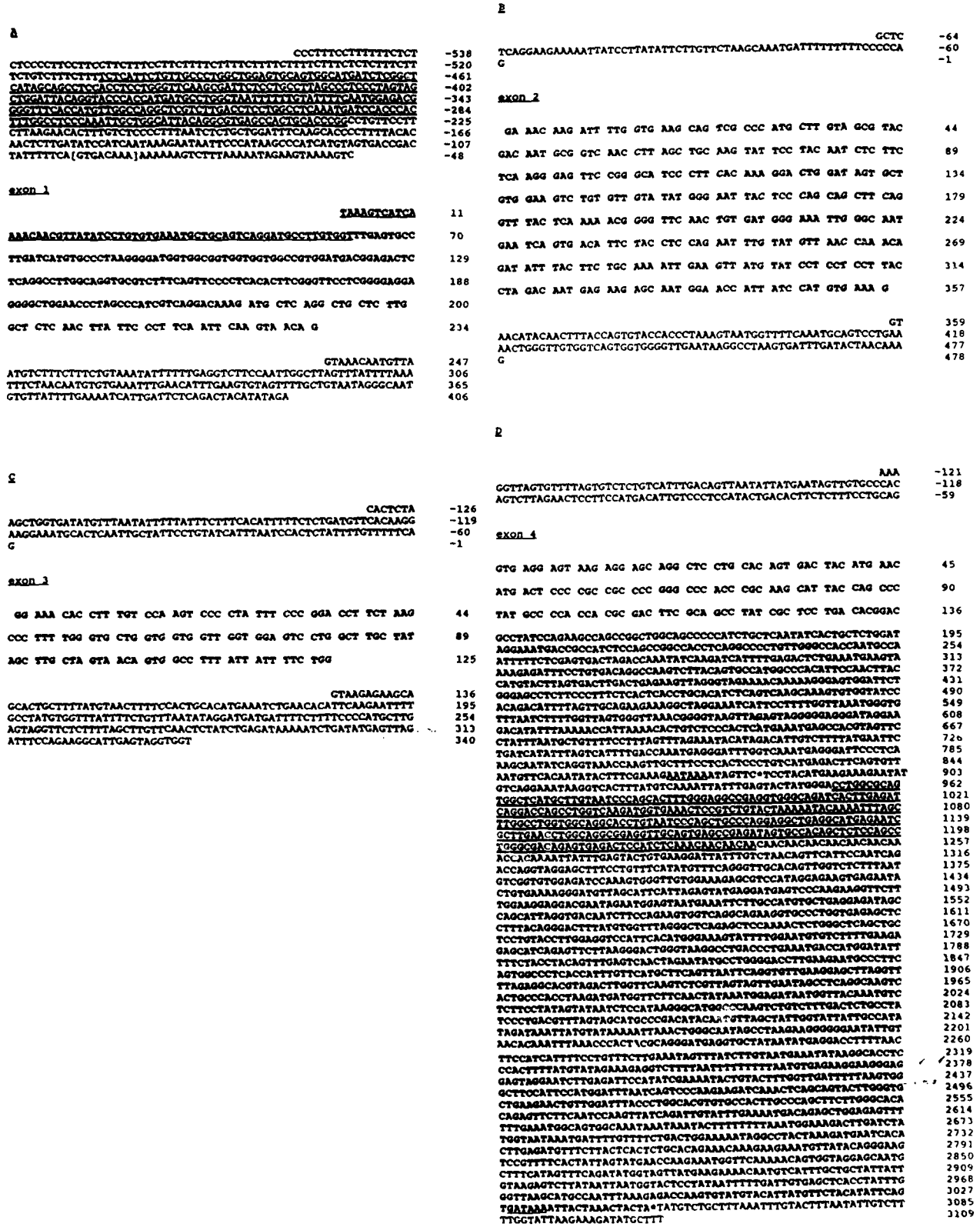

Figure 4. Exon sequence (bold type) and 5′ and 3′ flanking sequences for exon 1 (*A*), exon 2 (*B*), exon 3 (*C*), and exon 4 (*D*). Translated sequences are grouped in the proper reading frame. In *A*, the AP-1-like element is bracketed and the region of transcription initiation is underlined. Alu repetitive elements are *double underlined* below the corresponding sequence. In *D*, the polyadenylation signals are underlined. The polyadenylation sites are indicated by an *asterisk*. Distance in bp from the start of each exon is indicated to the *right* of each *figure*.

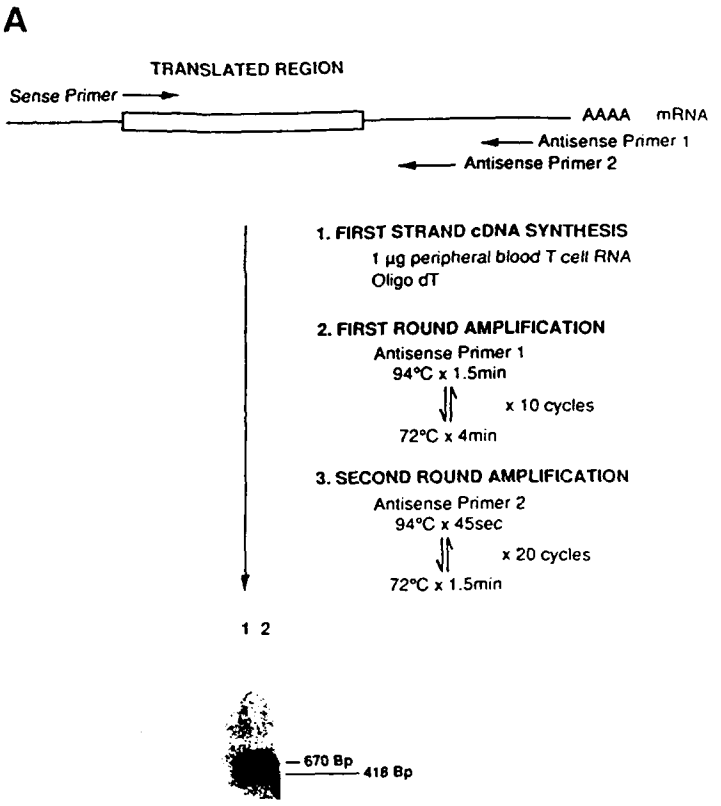

Figure 5. ***A***, The approximately 250-bp difference between mRNA transcripts is due to an internal deletion. To characterize the difference between the 1.3/1.5-kb and 3.5/3.7-kb transcripts, the PCR was used. Using primers flanking the translated region (antisense primers were nested to enhance specificity), PCR was performed on T cell RNA as indicated. The reaction products were run on an agarose gel, transferred to nitrocellulose and hybridized to radiolabelled CD28 cDNA (1.5 kb). *Lanes 1* and *2* represent two independent reactions. Size in bp is indicated to the right. ***B***, The PCR products shown in *A* were subsequently subcloned and sequenced; comparison is shown here. The larger 670-bp product is referred to as "undeleted" and the 418-bp product as "deleted." Corresponding exons are noted above the sequence and the base pairs are numbered from the start of their respective exon. The cryptic splice donor-acceptor signals are double underlined. The predicted peptide sequence is also shown. Cysteine residues are marked with *asterisks*.

B

```
                EXON 2
                |       60v        70v        80v        90v       100v
UNDELETED       GCGGTCAACCTTAGCTGCAAGTATTCCTACAATCTCTTCTCAAGGGAGTTC
                          CysLysTyrSerTyrAsnLeuPheSerArgGluPhe
                AlaValAsnLeuSer *
                          TYr  '  !
DELETED         GCGGTCAACCTTAGCT----------------------------------

                      110v       120v       130v       140v       150v
UNDELETED       CGGGCATCCCTTCACAAAGGACTGGATAGTGCTGTGGAAGTCTGTGTTGTA
                ArgAlaSerLeuHisLysGlyLeuAspSerAlaValGluValCysValVal
                                                              *
DELETED         --------------------------------------------------

                     160v       170v       180v       190v
UNDELETED       TATGGGAATTACTCCCAGCAGCTTCAGGTTTACTCAAAAACGGGGTTC
                TyrGlyAsnTyrSerGlnGlnLeuGlnValTyrSerLysThrGlyPhe
DELETED         ------------------------------------------------

                v      210v       220v       230v       240v
UNDELETED       AACTGTGATGGGAAATTGGGCAATGAATCAGTGACATTCTACCTCCAG
                AsnCysAspGlyLysLeuGlyAsnGluSerValThrPheTyrLeuGln
                 *
DELETED         ------------------------------------------------

                                                        ' '
                  v      260v       270v       280v       290v
UNDELETED       AATTTGTATGTTAACCAAACAGATATTTACTTCTGCAAAATTGAAGTTATG
                AsnLeuTyrValAsnGlnThrAspIleTyrPheCysLysIleGluValMet
                                                    *
DELETED         --------------------------------------------------

                  v      310v       320v       330v       340v
UNDELETED       TATCCTCCTCCTTACCTAGACAATGAGAAGAGCAATGGAACCATTATCCAT
                TyrProProProTyrLeuAsp
                                  AsnGluLysSerAsnGlyThrIleIleHis
                                  ..
DELETED         ------------------ACAATGAGAAGAGCAATGGAACCATTATCCAT
```

cells through surface Ig signaling expressed high levels of surface CD28. Although the role for CD28 in B cells has not yet been defined, its expression in the secretory cell of the B cell lineage suggests a similar role as in lymphokine-producing Th cells.

On a molecular level, the generation of the four different CD28 mRNA species appears to involve posttranscriptional mechanisms. The expression of the larger CD28 mRNA transcripts in T cells arises from the utilization of an alternative, nonconsensus polyadenylation signal. This results in the addition of 2167 bp to the 3′ untranslated region. This region, aside from the Alu repeat, has no significant homology to other sequences in the GenBank database. The incorporation of the Alu family repetitive element into the longer transcripts may afford an additional point of posttranscriptional control. Alu family repeats are extremely abundant in the human haploid genome (300,000 or more copies) and represent up to 25% of heterogenous nuclear RNA (35). Highly conserved Alu-like elements are an integral part of 7SL RNA (47), which in turn is a component of the signal recognition particle, a cytoplasmic riboprotein that functions to target nascent secretory and membrane proteins to the rough endoplasmic reticulum (48). Interestingly, the 3′ Alu repeat found in the CD28 gene has significant homology to the Alu element found in 7SL RNA. It has been proposed that Alu elements incorporated into transcripts and transported into the cytoplasm may have similar function as 7SL RNA (49). In view of the reported role of CD28 in the regulation of lymphokine secretion, it is tempting to speculate that the Alu repeat found in the 3′ untranslated region plays a regulatory role in both this process and CD28 surface expression. Other authors have proposed mechanisms of gene regulation involving repetitive elements and RNA-RNA duplexes (50).

In addition to alternate polyadenylation, a second process involving an internal deletion of a portion of exon 2 is involved in the generation of CD28 mRNAs. This 252-bp deletion, seen as the small difference between transcripts (i.e., 1.3 vs 1.5 kb and 3.5 vs 3.7 kb) on Northern blots, would result in the loss of 62% (84 amino acids) of the extracellular domain and in the complete disruption of the predicted Ig-like secondary structure due to the loss of four of five cysteine residues. However, this deletion does not disrupt any elements likely to be necessary for the surface expression of the putative protein. It is therefore possible that this mRNA deletion results in the surface expression of a previously undescribed form of CD28. A similar model using alternatively spliced exons to generate variant isotypes has been described for another lymphocyte-assocated cell surface protein, CD45 (51).

The mechanism resulting in this deletion appears to involve an internal splicing event involving an uncommon 5′ splice donor site, CT | GCAAGT. This mechanism functions in the presence of normal exon splicing, unlike the cryptic splice site utilization seen in such models as β-globin (52, 53), which require the inactivation of normally used splice sites to become manifest. This would suggest that the internal deletion seen in the case of the CD28 gene is not simply the result of aberrant splicing.

At present, there is no direct evidence that a "deleted" form of CD28 exists or has a physiologic role. Indirectly, evidence exists for a soluble 45-kDa protein released into the supernatant by PHA-activated T cells that appears by peptide analysis to be CD28 but is not recognized by mAb 9.3 (7). It seems less likely, however, that the "deleted" CD28 transcripts encode for this soluble protein because the soluble form was reported to have m.w. equivalent to the parent peptide. Additional indirect evidence suggestive of alternative forms of the CD28 Ag is the previously noted dual effector function of CD28, which may be mediated through different forms of the receptor or receptor complex. Further investigation will be required to determine the existence and role, if any, this hypothetical protein may play.

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