Involvement of Sp1 and Microsatellite Repressor Sequences in the Transcriptional Control of the Human *CD30* Gene

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CD30, as a member of the tumor necrosis factor (TNF) receptor family, is expressed on the surface of activated lymphoid cells. CD30 overexpression is a characteristic of lymphoproliferative diseases such as Hodgkin's/non-Hodgkin's lymphomas, embryonal carcinoma, and a number of Th2-associated diseases. The *CD30* gene has been mapped to a region of the murine genome that is involved in susceptibility to systemic lupus erythematosus. Functionally, CD30 may play a role in the deletion of autoreactive T cells. We were interested in determining the molecular nature of CD30 overexpression. Sequence comparison has revealed significant identity between the TATA-less human and murine *CD30* promoters; they share a number of common consensus binding motifs. Transfection assays identified three regions of transcriptional importance; the region between position -1.2 kb and -336 bp, containing a CCAT microsatellite sequence, a conserved Sp1 site at positions -43 to -38, and a downstream promoter element (DPE) at positions +24 to +29. EMSA and DNase I footprinting showed specific DNA-protein interactions of the *CD30* promoter with the Sp1 site and the CCAT repeat region. The DPE element was shown to be essential for start site selection. We conclude that the conserved Sp1 site at -43 to -38 is associated with maximum reporter gene activity, the DPE element is required for start site selection, and the CCAT tetranucleotide repeats act to repress transcription. We also have shown that the microsatellite is multiallelic, when we screened a random healthy population. Further studies are required to determine whether microsatellite instability in the repressor predisposes susceptible individuals to CD30 overexpression. (*Am J Pathol* 2000, 156:1723-1731)

CD30 is a member of the tumor necrosis factor (TNF) receptor family that is expressed on the surface of activated lymphoid cells, particularly activated peripheral blood B and T cells, and a small population of cells in the

perifollicular area of hyperplastic lymph nodes and tonsils.¹⁻⁶ The cellular distribution of CD30 suggests that, like other family members, CD30 is involved in the regulation of immune processes. The exact role CD30 plays is still under investigation. However, signaling through CD30 has been shown to produce both proliferative and apoptotic effects, depending on the cell type expressing the protein.⁷⁻¹¹ Cross-linking of CD30 molecules results in an influx of intracellular Ca^{2+} and induction of NF κ B activity consistent with the positive effect of CD30 on proliferation.^{2,9,12-14} CD30 has been implicated in the control of differentiation and/or selection events within the thymus. CD30-deficient mice have been found to contain elevated numbers of thymocytes and exhibit defects in negative selection, suggesting a role for CD30 in the deletion of autoreactive T cells.¹⁵ These findings have been confirmed in transgenic mice overexpressing CD30, in which overexpression enhances the deletion of CD4⁺/CD8⁺ thymocytes and so may act as a costimulatory molecule in thymic negative selection.¹⁶

CD30 was originally identified as a surface marker on Hodgkin's and Reed-Sternberg (H-RS)³ cells, the neoplastic component of Hodgkin's disease.^{17,18} Diagnosis of Hodgkin's disease is based on the presence of a small population of these H-RS cells surrounded by a cellular infiltrate composed of normal immunoreactive lymphocytes, neutrophils, eosinophils, histiocytes, plasma, and stromal cells.^{10,19} The exact cellular origin of these H-RS cells remains to be determined, although all of these cell types have been implicated.^{20,21} Overexpression of CD30 is characteristic of Hodgkin's disease, where the interaction of CD30 with its ligand CD153 appears to be involved in the regulation of cell-cell interactions, particularly between the H-RS cells and the surrounding lymphoid cells.²² Hence it has been suggested that loss of regulatory control may lead to the observed overexpression of CD30 and contribute to the progression of the disease state. In the later stages of Hodgkin's disease, the membrane form of CD30 is proteolytically cleaved,

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and high serum levels of the soluble form of CD30 can be detected, making it a good clinical marker for disease progression.^{23,24} The soluble form of CD30 may function by competing with surface CD30 for the binding of CD153 (CD30 ligand), thus preventing signaling through the membrane form of CD30, blocking normal intracellular signaling processes, as seen with TNF.²⁵ Interestingly, CD30 overexpression is also a characteristic of other lymphoproliferative disorders, such as non-Hodgkin's lymphoma, as well as embryonal carcinoma and a number of Th2-associated diseases.^{26–32} Recently it has been shown that CD30 signaling limits the proliferative potential of autoreactive CD8 effector T cells and protects against autoimmune diabetes.³³ It is possible that the observed alterations in the normal expression of CD30 may result in the progression of these CD30-positive diseases through autocrine or paracrine signaling mechanisms. The mechanisms involved in up-regulation of CD30 expression have yet to be investigated.

As a first step toward identifying factors involved in regulating CD30 expression we have recently described the isolation and characterization of the 5' region of the human and murine *CD30* genes.³⁴ Sequence comparison revealed significant identity between the mouse and human *CD30* promoters. Both genes belong to an expanding group that lack consensus TATA and CAAT boxes, an increasingly common feature among members of the TNF receptor family.^{35–38} The human and murine *CD30* promoters share a number of common consensus transcription factor binding motifs that may be involved in the regulation of gene expression. In particular, our previous study revealed the presence of conserved Sp1 and initiator elements,³⁴ both of which have been implicated in positioning transcriptional machinery in genes that lack a TATA box.^{39–42}

In this report we present the results of functional studies carried out to investigate these conserved regions in the human *CD30* promoter. We have identified a number of functionally important regulatory regions, including an Sp1 element in the minimal promoter and a downstream promoter element (DPE) within the initiator, which is required for start site selection. In addition, the discovery of a polymorphic, upstream tetranucleotide repeat that binds proteins acting to repress transcriptional activity of the *CD30* promoter, may implicate this region in the dysregulation of CD30 expression in neoplastic cells.

Materials and Methods

Cell Culture

Cultured cell lines were obtained from the American Type Culture Collection (Bethesda, MD). All cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

DNA Transfection and RNase Protection Assays

Jurkat T cells were transfected by electroporation, using a BioRad (Hercules, CA) Gene Pulser at 240 V, 960 µF. Approximately 4×10^6 cells in a final volume of 400 µl were cotransfected with 10 µg of each test construct and 300 ng of control plasmid pRL-TK. Cells were cultured for 32 hours after transfection in 5 ml complete media before preparation of cellular extracts. Cells were harvested, washed twice in phosphate-buffered saline, and lysed in 300 µl of passive lysis buffer (Promega Corp., Madison, WI). Promoter activity of constructs and controls was measured using the Dual Luciferase Assay kit (Promega), following the manufacturer's instructions.

Total RNA was isolated from Jurkat T cells transfected with either the –336 *CD30*/luciferase construct or the DPE downstream mutant, using the RNasol B method (Geneworks, Adelaide, Australia). A fragment of the *CD30* promoter from –83 to +167 plus 153 bp (bp) of the luciferase gene derived from the WT *CD30*/luciferase construct was subcloned into pGEM-3ZF+ (Promega), linearized with an enzyme distal to the promoter, and used as a template for the T7 MAXI Script In Vitro Transcription Kit (Ambion, Austin, TX) to generate an antisense riboprobe. RNase protection assays were performed on 50 µg total RNA, using the Hybspeed RPA kit (Ambion). Protected fragments were analyzed on a 6% denaturing polyacrylamide/urea gel. Gels were dried and exposed to X-ray film.

CD30 Reporter Constructs

A 3.7-kb *CD30* DNA fragment spanning the region from approximately –3500 to +199 relative to the major transcription start site was generated by polymerase chain reaction (PCR) from a plasmid containing the human *CD30* promoter. This fragment was directionally cloned, via *SacI* and *HindIII* sites in the primers, into the polylinker of pGL3-Basic (Promega) to generate the wild-type *CD30* reporter construct. The Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce *KpnI* or *SacI* restriction sites at different positions within the wild-type construct to generate a series of mutant constructs. Mutated plasmids were subsequently digested with *SacI* or *KpnI* and religated to generate deletion constructs. The microsatellite region of the *CD30* promoter (~400 bp) was isolated from the *CD30* promoter by digestion with *KpnI* and cloned upstream from the SV40 promoter in the plasmid pGL3-Promoter (Promega) to generate a heterologous promoter construct. All DNA used in transfections was prepared using the Qiafilter plasmid maxi kit (Qiagen GmbH, Hilden, Germany), and all constructs were checked by sequencing.

Nuclear Extracts, DNase I Footprint Analysis, and Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared according to the method of Li et al.⁴³ For DNase I footprinting, a fragment of the *CD30* gene spanning the region from –311 to +152 bp

was generated by PCR and labeled with [γ - 32 P]ATP, using T4 DNA polymerase (Promega). Approximately 10,000 cpm of the labeled DNA fragment was incubated with 25 μ g nuclear extract or 5 μ g BSA and 1 μ g poly(dI-dC) for 20 minutes on ice. Samples were treated with increasing units of DNase I (0.02–0.04 U with BSA and 2–10 U with nuclear extract) at room temperature for 2 minutes. Samples were analyzed using a 6% denaturing polyacrylamide/urea gel. For comparison a G/A ladder of the same end-labeled DNA fragment was generated by Maxam-Gilbert chemistry.

Wild-type and mutant double-stranded oligonucleotides representing –57 to –22 of the *CD30* promoter or the microsatellite region [(CCAT)₅CACCTTATGCAT(CCCT)₂] were synthesized for electrophoretic mobility shift assay (EMSA) with a single 5' G overhang to facilitate end-labeling with [α - 32 P]dCTP. Nuclear proteins were preincubated on ice with 1 μ g poly(dI-dC) in a binding buffer (4% Ficoll, 20 mmol/L HEPES (pH 7.9), 1 mmol/L EDTA, 1 mmol/L DTT, 50 mmol/L KCl, 0.8 μ g ssDNA) to give a final reaction volume of 20 μ l. For competition assays a 50-fold molar excess of specific or nonspecific competitor, as indicated, was incubated with nuclear extract for 10 minutes on ice before the addition of 32 P-labeled oligonucleotide. Nuclear proteins were then incubated with 32 P-labeled oligonucleotide (0.32 pmol) for 30 minutes on ice. For supershift assays anti-Sp1 or anti-Sp3 antibody was added after 30 minutes and incubated on ice for 1 hour. Products were analyzed by electrophoresis through a 6% polyacrylamide gel at 150 V.

Microsatellite Typing

DNA was obtained by mouthwash from seven randomly chosen individuals essentially as described, but with the use of 10% Chelex (Biorad) instead of EDTA.⁴⁴ An aliquot (1/20th of the total) of DNA was used in a 50-ml PCR reaction with 25 pmol each of the microsatellite flanking primers 30MSF (5'-ACCCATTTACCCACTCACCTGC-3') and 30MSR (5'-CAACTGGCCTAGGGAGACTGC-3'). The reactions contained 2.0 mmol/L MgCl₂, 1 $\times$ PCR buffer (Promega, Madison, WI), 2 mmol/L dNTPs, and 1 U *Taq* polymerase (Promega). Reactions were treated as follows: 94°C for 5 minutes; 29 cycles of 94°C for 30s, 60°C for 30s, 72°C for 1 minute; and 72°C for 10 minutes. PCR products were analyzed on 6% nondenaturing polyacrylamide gel electrophoresis (PAGE).

Results

Identification of Functional Promoter Elements Upstream of the Human *CD30* Gene

To identify DNA elements that control the regulation of *CD30* transcription, we subcloned the 5' flanking region of the human *CD30* gene from –3.5 kb to +199 bp into the luciferase expression vector pGL3-Basic. Restriction sites were introduced into the core recognition sequences contained in the full-length *CD30*/luciferase construct (–3.5 kb) by site-directed mutagenesis to generate

Figure 1. Analysis of the promoter region of the human *CD30* gene. **A:** Expression of *CD30* 5' promoter deletion constructs in transiently transfected Jurkat cells. Constructs with different extents of *CD30* promoter driving the firefly luciferase gene were cotransfected with pRL-TK and incubated for 32 hours before cells were assayed for luciferase activity. The normalized relative transcriptional activity is expressed relative to the full-length –3.5-kb construct. Error bars are standard errors from duplicate experiments. Possible transcription factor binding elements defined by searching the TRANSFAC database are represented on the full-length construct. Numbers (in bp) represent the position of the 5' deletion end points in relation to the major transcription initiation site, shown as an arrow. **B:** Mutations were introduced into the full-length –3.5-kb *CD30*/luciferase construct at the five possible transcription factor binding elements and then transfected and assayed as in **A**. The normalized relative transcriptional activity is expressed relative to the full-length –3.5-kb construct. Error bars are standard errors from duplicate experiments.

mutant/deletion derivatives for transient transfection assays in Jurkat T cells. Potential transcription factor binding sites chosen for mutation included two Sp1 sites, two ETS sites, and an MZF site. The region of the *CD30* promoter containing the Sp1 site (–43 to –38) has been previously shown to be highly conserved between human and murine *CD30* promoter sequences. Preliminary transfection results also indicate that the region between –90 and –25 is important in driving transcription.³⁴

Analysis of transient transfection results showed that the full-length –3.5-kb construct was able to direct low-level expression of the luciferase reporter in Jurkat T cells as illustrated in Figure 1. Deletion of DNA sequences between –3.5 kb to –1.2 kb had no effect on promoter activity. However, deletion of the sequence between –1.2 kb and –336 resulted in a 3.5-fold increase in promoter activity (Figure 1A). This result suggests that the region contained strong repressor activity. Further sequential deletion of sequences between –336 and –90 had little effect on promoter activity. Deletion of the region between –90 and –40 had a dramatic effect on promoter activity and resulted in a decrease in activity to

Figure 2. Interaction of Jurkat cell nuclear proteins with the core *CD30* promoter. **A:** *In vitro* DNase I footprints of the region -311 to $+152$ of the *CD30* gene. The probe was incubated with BSA or nuclear extracts in the presence of increasing amounts of DNase I, represented by triangles. Three regions of protection were identified at -159 to -149 , -142 to -128 , and -44 to -28 . The -44 to -28 footprint region contains the consensus Sp1 site shown to be important for *CD30* promoter activity by mutational analysis. A Maxam and Gilbert G+A sequencing ladder (G/A) of the same region serves as a size marker. **B:** EMSA using a double-stranded probe representing -57 to -22 of the *CD30* promoter. Major complexes are indicated (a–f). **Lanes 1–7:** Probe plus Jurkat nuclear extracts (NE); **lane 8:** free probe. **Lane 1:** Probe alone. **Lane 2:** Probe plus 50-fold molar excess of unlabeled probe (self). **Lane 3:** Probe plus 50-fold molar excess of double-stranded consensus (GC) oligonucleotide specific for Sp1. **Lane 4:** Probe plus 50-fold molar excess of double-stranded consensus (GT) oligonucleotide. **Lane 5:** 50-fold molar excess of nonspecific GC-rich competitor (NI). **Lane 6:** Probe plus nuclear extract incubated with Sp1 antibody. **Lane 7:** Probe plus nuclear extract incubated with Sp3 antibody.

the level of the full-length construct and confirmed the presence of strong positive regulatory elements in the immediate upstream region containing the Sp1 site.

A Consensus Sp1 Binding Site at Position -43 to -38 Is Essential for Minimal *CD30* Promoter Activity

Results generated from transfection of mutant constructs in Jurkat T cells (Figure 1B) indicated that mutations within the upstream ETS sites (-238 to -233 and -158 to -153) had little effect on expression. However, mutation of the highly conserved Sp1 site at position -43 to -38 had a profound effect on transcription (Figure 1B) and completely abolished activity to the level seen with the luciferase vector alone (data not shown), suggesting that proteins binding within this region are essential for *CD30* expression. This result is consistent with data generated from analysis of the 5' *CD30* deletion series, where deletion of this potential Sp1 site essentially abolishes high-level transcription.

To define DNA-protein interactions in the region surrounding the conserved Sp1 site, we performed *in vitro* DNase I footprint assays, using a fragment from -311 to $+152$ of the *CD30* promoter as a probe. Three major regions of protection were identified in the presence of Jurkat T-cell nuclear extract. These regions encompassed nucleotides -159 to -149 , -142 to -128 , and -44 to -28 (Figure 2A). The region spanning -44 to -28 contains the conserved Sp1 site, which appears to be

essential for high-level expression of the -3.5 -kb *CD30*/luciferase construct. The two upstream regions (-164 to -148 and -144 to -131) both contain sites mutated in the transfection experiments; however, the mutations did not appear to alter basal transcription levels (Figure 1B).

A double-stranded oligonucleotide probe, representing the protected region from -44 to -28 of the *CD30* promoter, was used in EMSA to analyze the interaction of this region with Jurkat T-cell nuclear proteins. Six major complexes (a–f) were formed between the probe and nuclear proteins (Figure 2B, lane 1). All of these complexes could be competed away by using a 50-fold molar excess of unlabeled probe (Figure 2B, lane 2). Complexes a and b could be completely competed away by using a 50-fold molar excess of consensus Sp1 GC and GT box sequences, suggesting that complexes a and b recognized Sp1-binding sequences (Figure 2B, lanes 3 and 4). An unrelated GC-rich oligonucleotide sequence (NI) was able to compete for complex d and f binding but was less efficient at competing away complexes a–c (Figure 2B, lane 5). To determine whether members of the Sp family of transcription factors were involved in formation of complexes a and b, the DNA protein complexes, formed during the binding reaction, were incubated in the presence of either anti-Sp1 or anti-Sp3 antibodies. Results indicated that complexes a and b were supershifted with the Sp1 antibody, suggesting that Sp1 formed part of these complexes (Figure 2B, lane 6). No shift was seen with the Sp3 antibody (lane 7), indicating that the complexes a and b were antigenically related to Sp1 specifically.

To further characterize the nature of complexes c–e, mutated versions of the EMSA oligonucleotide were designed and used to identify the relative binding position of the observed complexes. Three mutated oligonucleotides were generated (M1, M2, M3) and used as probes or competitors in EMSAs with Jurkat T-cell extracts (Figure 3). As predicted, mutation of the consensus Sp1 binding site in M2 affected binding of complexes a and b, shown in the supershift assays to contain Sp1 (Figure 3, lanes 3 and 6). M2 also partially effected binding of complex e. The M1 mutation affected binding of complexes c and d and to some extent the binding of e compared to wild type (Figure 3, lanes 2 and 5). However, the M3 mutation completely abolished binding of complex e (Figure 3, lanes 4 and 7). These results suggest that complexes c and d are binding to the region upstream from the Sp1 consensus site, whereas complex e binds to the downstream region, including all or part of the ATTCAA sequence, which was mutated in the M3 oligonucleotide.

The Microsatellite-Containing Region of the *CD30* Promoter Acts to Strongly Repress Transcription

The transfection results (see Figure 1) identified a strong repressor element in the region between -1.2 kb and -336 bp of the *CD30* promoter; transcriptional activity increased by 3.5-fold when this region was deleted. This

Figure 3. Definition of binding sites within the -57 to -22 region of the *CD30* promoter. **A:** Double-stranded oligonucleotide EMSA probe used with regions mutated by introduction of a *SacI* site, indicated by the boxed regions (M1, M2, M3). **B:** EMSA comparing wild-type probe with M1, M2, and M3 mutated probes. **Lanes 1–7:** The probe indicated plus Jurkat cell nuclear extract. **Lanes 1–4:** The wild-type (WT) or mutated probe indicated. (Note that multiple preparations of the M1 probe showed aberrant mobility, which presumably was due to secondary structure as a result of introduction of the mutation.) **Lane 5–7:** Wild-type probe plus 50-fold molar excess of mutant oligonucleotide as indicated. **Lanes 8–11:** Labeled probe only.

segment of the *CD30* promoter was sequenced and shown to contain ~400 bp of a complex microsatellite of the type [(CCAT)_{2–12}CCACTTATGCAT]_n, although some of the repeat elements were imperfect (Figure 4). Comparison of the human sequence with the equivalent murine sequence indicated that the microsatellite was evolutionarily conserved and arose before the rodent/primate divergence (Figure 4). However, the mouse microsatellite sequence was very degenerate compared with the human sequence, although the CCAT repeat was evident throughout the sequence. To investigate the role of the microsatellite in the observed repression, a construct (Δ MS) in which ~400 bp of human microsatellite sequence was spontaneously deleted in *Escherichia coli*, was compared in transfection assays with the -1.2 kb and -336 constructs (Figure 5). Sequencing of the Δ MS construct showed that it lacked most of the microsatellite sequence but retained four copies of the CCAT repeat. The Δ MS deletion resulted in a 1.6-fold increase in transcription compared with the -1.2-kb construct. The Δ MS deletion did not completely relieve repression as compared to the -336 deletion construct (3.8-fold over the -1.2-kb construct), indicating that the (CCAT)₄ sequence alone was capable of repressing transcription from the *CD30* promoter. Although we have not tested the

Figure 4. Comparison of the human and murine microsatellite sequences within the *CD30* promoter. **A:** Schematic representation of the relationship between the human and murine microsatellite region of the *CD30* promoter. The 400-bp imperfect tetranucleotide repeat found in the human *CD30* promoter is longer and less degenerate than the 150-bp murine equivalent. The microsatellites are represented by the filled boxes. **B:** Comparison of the 3' part of the murine (GenBank accession AF069504) and human (GenBank accession no. AF065475) microsatellite sequences from -401 to -581 of the human *CD30* promoter. Regions of identity are shown (*). Sequences were aligned using Clustal V.

function of the murine microsatellite sequences, we would predict that they also are able to repress the transcription of murine *CD30*. Although the mouse sequence is degenerate, five contiguous copies of CCAT are present at least twice (see Figure 4), and we have shown that four copies are sufficient to repress *CD30* transcription.

The function of the microsatellite sequence was also tested in a heterologous system by cloning the entire

Figure 5. The *CD30* promoter microsatellite can act to repress transcription. Luciferase reporter constructs were used to transfect Jurkat cells and were assayed for luciferase activity 32 hours later. Relative activities with respect to the full-length -3.5-kb construct are shown. Error bars are standard errors from duplicate experiments. Δ MS, deletion of microsatellite except (CCAT)₄; SV, pGL3 promoter containing the SV40 minimal promoter; SV-MS, pGL3 promoter plasmid with 400-bp microsatellite inserted upstream from the SV40 promoter.

Figure 8. Mutations in the downstream promoter element effect the position of transcription initiation. **A:** Transient transfection of Jurkat cells with *CD30*/luciferase downstream mutants. Mutations were introduced into the full-length ~ 3.5 -kb *CD30*/luciferase construct at the downstream AP1-like and DPE sites. Constructs were transfected into Jurkat T cells and assayed for luciferase activity 32 hours later. The transfection-normalized relative luciferase activity is shown. Error bars are standard errors from duplicate experiments. **B:** RPA analysis of *CD30*/luciferase mRNA expression from Jurkat cells transfected with either the -336 construct (lane 2) or the DPE mutant construct (lane 1). Molecular size standards (W) were *HinfI*-digested ϕ X174.

downstream promoter element (DPE) (+20 to +26) sites directly downstream from the major transcription initiation site (TIS).⁴⁵ Transient transfection analysis of the AP1 mutant in Jurkat cells indicated that the site was not important in regulating the expression of CD30 (Figure 8A). However, mutation within the DPE element resulted in a twofold increase in reporter gene activity. To determine the effect of the DPE mutation on transcription and/or TIS selection, mRNA expression from the mutated *CD30*/luciferase DPE construct was compared with the equivalent wild-type construct, -336 . Mutation of the DPE element resulted in both a shortened transcript compared with the -336 construct and a possible decrease in the amount of transcript produced (Figure 8B). These results suggest that mutation of the DPE element results in altered start site selection to produce a transcript truncated by ~ 70 bp (TSS at +70) compared to the wild-type *CD30* gene. Comparison of the luciferase assay results and those obtained from the RNase protection assay suggests that although the amount of transcript decreases, posttranscriptional events may serve to increase expression at the protein level. For instance, the shortened transcript may exhibit a decreased turnover rate or, alternatively, may differentially regulate interaction with the translational machinery.

Discussion

CD30, as a member of the TNF receptor family, is of interest because of its potential role in mediating proliferative and/or apoptotic events in initiation or promulgation of an immune response. Our interest lies in understanding the control of CD30 expression, particularly as

its overexpression provides a diagnostic marker for a range of non-Hodgkin's lymphomas. In the current study, we have identified three regulatory regions that are important in transcriptional control: a complex microsatellite region, an Sp1 site immediately upstream of the transcription start site, and a DPE downstream from the CD30 TIS. Jurkat T cells were used as a model *in vitro* system for studying CD30 expression, as they constitutively express CD30 at both the mRNA and protein level (data not shown).

Our results demonstrated that the Sp1 binding site at position -44 to -28 is a critical element required for initiating transcription of the *CD30* gene and forms a DNA-protein complex with the Sp1 protein. Sp1 sites immediately upstream of the TIS have been shown, in other genes, to act as an anchor for the basal transcription machinery in the absence of a TATA box.^{39–42,46} Sp1 appears to recruit TATA binding proteins such as TBP, which are normally involved in the formation of transcription initiation complexes around the TATA consensus sequence. Hence Sp1 appears to act as a surrogate TATA motif in the *CD30* gene, as in other TATA-less genes.⁴⁰ The downstream element, or DPE, located at +30 has recently been described as the downstream counterpart to the TATA box.⁴⁵ Positioning of the DPE with respect to the initiator element has been found to be critical in directing both the accuracy and level of transcription,⁴⁵ and these functions have been confirmed in the case of the *CD30* promoter. The presence of these two functional sites in the *CD30* gene provides additional evidence regarding the importance of these elements in transcriptional regulation from TATA-less promoters. As the lack of a canonical TATA box appears to be a common feature in the promoters of both the TNF ligand^{47,48} and receptor families,^{35–38} it is possible that transcription of other members of the family is regulated in a similar manner and requires further investigation.

Sp1 is known to be up-regulated by viral activation, so it is possible that viral infection of lymphoid cells may up-regulate Sp1, leading to the overexpression of CD30, as seen in Hodgkin's disease. Epstein-Barr virus (EBV) is frequently found in CD30-positive lymphomas and is known for its ability to up-regulate CD30 in EBV-positive B-lymphoma cell lines.³ A number of CD30-related receptors, such as CD40 and Fas, are also up-regulated in Hodgkin's disease.¹⁰ Therefore, alterations in a common mechanism involving Sp1 may explain why the H-RS cells of Hodgkin's disease display unbalanced cytokine production and deregulated expression of surface antigens.

Reporter gene transcriptional assays identified a repressor between positions -1.2 kb and -336 bp. Sequencing of this region revealed the presence of a 400-bp tetranucleotide microsatellite. This variable repeat region was found to repress luciferase gene expression when cloned upstream from the SV40 promoter in pGL3 promoter. Deletion of almost the entire variable region (excluding four copies of the CCAT repeat) relieved transcriptional repression by twofold compared with complete removal of all CCAT repeats, which relieved repression up to 3.8-fold. EMSA established that at least four nuclear proteins are able to interact with the

tetranucleotide sequences. Increasing evidence in the literature suggests that simple repeats can exhibit regulatory activity. For instance, a tetranucleotide repeat located in the first intron of the tyrosine hydroxylase gene has been shown to act as a transcriptional enhancer *in vitro*.⁴⁹ A number of dinucleotide repeats have also been shown to cause up-regulation or down-regulation of basal transcription when cloned upstream from a minimal promoter.^{50,51} The extent of these regulatory effects has been shown to be dependent on the length of the repeating element.⁵¹ Our microsatellite typing data suggest that allelic differences in the length of the CD30 upstream microsatellite may differentially regulate CD30 expression *in vivo*.

The involvement of a microsatellite sequence in the control of CD30 expression may have significant implications for the observed overexpression in lymphoma and leukemia. The generation of the spontaneous *CD30* microsatellite deletion in *E. coli* and the degree of polymorphism seen in a small sample of randomly chosen individuals suggest that the *CD30* microsatellite has the potential to be unstable. Microsatellite instability results, at least partly, from a defect in DNA mismatch repair and has been identified as a common molecular mechanism in the development of cancer. Microsatellite instability has been identified in adult T-cell leukemia, childhood T-cell acute lymphoblastic leukemia, Hodgkin's disease, and some B-cell non-Hodgkin's lymphomas.⁵²⁻⁵⁷ Whether the degenerate microsatellite within the *CD30* promoter is specifically affected by microsatellite instability *in vivo* remains to be investigated. However, as the microsatellite in *CD30* is a strong repressor, it is possible that deletion of the *CD30* microsatellite may lead to relief of transcriptional repression, resulting in the overexpression of CD30 in Hodgkin's and non-Hodgkin's lymphomas. Further studies are required to screen for polymorphism in the CD30 microsatellite repressor in different cell lines derived from CD30-positive tumors, as well as the germline of patients from whom the cell lines are derived.

The CD30 locus occurs within a genomic region that has been shown to confer susceptibility to Lupus nephritis in (NZW × NZB) F1 mice.^{58,59} The observed regulatory effects of the CD30 microsatellite may have significant implications for Lupus in humans. Promoter polymorphisms in other genes, such as TNF,⁶⁰ have been implicated in susceptibility to autoimmunity. Considering the high degree of polymorphism in our small sampling of healthy individuals, it is a priority to screen patients to determine whether carriage of particular alleles of the CD30 microsatellite are associated with susceptibility to systemic lupus erythematosus (SLE). Determination of the degree of repression from the disease associated alleles may provide a molecular explanation for disease susceptibility.

This work provides a first step in identifying the mechanisms involved in CD30 gene expression by identifying three key regulatory elements. Further work will be required to elucidate the exact roles that the Sp1 and CCAT microsatellite regions play in the altered cytokine and

receptor expression associated with CD30⁺ disease states.

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References

- Andreesen R, Osterholz J, Lohr GW, Bross KJ: A Hodgkin cell-specific antigen is expressed on a subset of auto- and alloactivated T (helper) lymphoblasts. *Blood* 1984, 63:1299-1302
- Ellis TM, Simms PE, Slivnick DJ, Jack HM, Fisher RI: CD30 is a signal-transducing molecule that defines a subset of human activated CD45RO⁺ T cells. *J Immunol* 1993, 151:2380-2389
- Froese P, Lemke H, Gerdes J, Havsteen B, Schwarting R, Hansen H, Stein H: Biochemical characterization and biosynthesis of the Ki-1 antigen in Hodgkin-derived and virus-transformed human B and T lymphoid cell lines. *J Immunol* 1987, 139:2081-2087
- Hecht TT, Longo DL, Cossman J, Bolen JB, Hsu SM, Israel M, Fischer RI: Production and characterization of a monoclonal antibody that binds Reed-Sternberg cells. *J Immunol* 1985, 134:4231-4236
- Schwarting R, Gerdes J, Durkop H, Falini B, Pileri S, Stein H: BER-H2: a new anti-Ki-1 (CD30) monoclonal antibody directed at a formol-resistant epitope. *Blood* 1989, 74:1678-1689
- Stein H, Mason DY, Gerdes J, O'Connor N, Wainscoat J, Pallesen G, Gatter K, Falini B, Delsol G, Lemke H: The expression of the Hodgkin's disease associated antigen Ki-1 in reactive and neoplastic lymphoid tissue: evidence that Reed-Sternberg cells and histiocytic malignancies are derived from activated lymphoid cells. *Blood* 1985, 66:848-858
- Bowen MA, Olsen KJ, Cheng L, Avila D, Podack ER: Functional effects of CD30 on a large granular lymphoma cell line, YT. Inhibition of cytotoxicity, regulation of CD28 and IL-2R, and induction of homotypic aggregation. *J Immunol* 1993, 151:5896-5906
- Bowen MA, Lee RK, Miragliotta G, Nam SY, Podack ER: Structure and expression of murine CD30 and its role in cytokine production. *J Immunol* 1996, 156:442-449
- Lee SY, Kandala G, Liou ML, Liou HC, Choi Y: CD30/TNF receptor-associated factor interaction: NF-kappa B activation and binding specificity. *Proc Natl Acad Sci USA* 1996, 93:9699-9703
- Gruss HJ, Boiani N, Williams DE, Armitage RJ, Smith CA, Goodwin RG: Pleiotropic effects of the CD30 ligand on CD30-expressing cells and lymphoma cell lines. *Blood* 1994, 83:2045-2056
- Smith CA, Gruss HJ, Davis T, Anderson D, Farrah T, Baker E, Sutherland GR, Brannan CI, Copeland NG, Jenkins NA: CD30 antigen, a marker for Hodgkin's lymphoma, is a receptor whose ligand defines an emerging family of cytokines with homology to TNF. *Cell* 1993, 73:1349-1360
- Ansieau S, Scheffrahn I, Mosialos G, Brand H, Duyster J, Kaye K, Harada J, Dougall B, Hubinger G, Kieff E, Herrmann F, Leutz A, Gruss HJ: Tumor necrosis factor receptor-associated factor (TRAF)-1, TRAF-2, and TRAF-3 interact *in vivo* with the CD30 cytoplasmic domain; TRAF-2 mediates CD30-induced nuclear factor kappa B activation. *Proc Natl Acad Sci USA* 1996, 93:14053-14058
- Duckett CS, Gedrich RW, Gilfillan MC, Thompson CB: Induction of nuclear factor kappaB by the CD30 receptor is mediated by TRAF1 and TRAF2. *Mol Cell Biol* 1997, 17:1535-1542
- Tsitsikov EN, Wright DA, Geha RS: CD30 induction of human immunodeficiency virus gene transcription is mediated by TRAF2. *Proc Natl Acad Sci USA* 1997, 94:1390-1395
- Amakawa R, Hakem A, Kundig TM, Matsuyama T, Simard JJ, Timms E, Wakeham A, Mittrucker HW, Griesser H, Takimoto H, Schmitts R, Shahinian A, Ohashi P, Penninger JM, Mak TW: Impaired negative selection of T cells in Hodgkin's disease antigen CD30-deficient mice. *Cell* 1996, 84:551-562
- Chiarle R, Podda A, Prolla G, Podack ER, Thorbecke GJ, Inghirami G: CD30 overexpression enhances negative selection in the thymus and

- mediates programmed cell death via a Bcl-2-sensitive pathway. *J Immunol* 1999, 163:194–205
17. Schwab U, Stein H, Gerdes J, Lemke H, Kirchner H, Schaadt M, Diehl V: Production of a monoclonal antibody specific for Hodgkin and Sternberg-Reed cells of Hodgkin's disease and a subset of normal lymphoid cells. *Nature* 1982, 299:65–67
18. Stein H, Gerdes J, Schwab U, Lemke H, Mason DY, Ziegler A, Schienle W, Diehl V: Identification of Hodgkin and Sternberg-Reed cells as a unique cell type derived from a newly-detected small-cell population. *Int J Cancer* 1982, 30:445–459
19. de Bruin PC, Gruss HJ, van der Valk P, Willemze R, Meijer CJ: CD30 expression in normal and neoplastic lymphoid tissue: biological aspects and clinical implications. *Leukemia* 1995, 9:1620–1627
20. Drexler HG, Amlot PL, Minowada J: Hodgkin's disease-derived cell lines—conflicting clues for the origin of Hodgkin's disease? *Leukemia* 1987, 1:629–637
21. Hsu SM, Hsu PL: The nature of Reed-Sternberg cells: phenotype, genotype, and other properties. *Crit Rev Oncog* 1994, 5:213–245
22. Gruss HJ, Pinto A, Duyster J, Poppema S, Herrmann F: Hodgkin's disease: a tumor with disturbed immunological pathways. *Immunol Today* 1997, 18:156–163
23. Hansen HP, Kisseleva T, Kobarg J, Horn-Lohrens O, Havsteen B, Lemke H: A zinc metalloproteinase is responsible for the release of CD30 on human tumor cell lines. *Int J Cancer* 1995, 63:750–756
24. Nawrocki JF, Kirsten ES, Fisher RI: Biochemical and structural properties of a Hodgkin's disease-related membrane protein. *J Immunol* 1988, 141:672–680
25. Seckinger P, Isaaz S, Dayer JM: A human inhibitor of tumor necrosis factor alpha. *J Exp Med* 1988, 167:1511–1516
26. Bengtsson A, Holm L, Back O, Fransson J, Scheynius A: Elevated serum levels of soluble CD30 in patients with atopic dermatitis (AD). *Clin Exp Immunol* 1997, 109:533–537
27. Biswas P, Smith CA, Goletti D, Hardy EC, Jackson RW, Fauci AS: Cross-linking of CD30 induces HIV expression in chronically infected T cells. *Immunity* 1995, 2:587–596
28. Chilosi M, Facchetti F, Notarangelo LD, Romagnani S, Del Prete G, Almerigogna F, De Carli M, Pizzolo G: CD30 cell expression and abnormal soluble CD30 serum accumulation in Omenn's syndrome: evidence for a T helper 2-mediated condition. *Eur J Immunol* 1996, 26:329–334
29. Del Prete G, Maggi E, Pizzolo G, Romagnani S: CD30, Th2 cytokines and HIV infection: a complex and fascinating link. *Immunol Today* 1995, 16:76–80
30. Latza U, Foss HD, Durkop H, Eitelbach F, Dieckmann KP, Loy V, Unger M, Pizzolo G, Stein H: CD30 antigen in embryonal carcinoma and embryogenesis and release of the soluble molecule. *Am J Pathol* 1995, 146:463–471
31. Nadali G, Vinante F, Stein H, Todeschini G, Tecchio C, Morosato L, Chilosi M, Menestrina F, Kinney MC, Greer JP: Serum levels of the soluble form of CD30 molecule as a tumor marker in CD30+ anaplastic large-cell lymphoma. *J Clin Oncol* 1995, 13:1355–360
32. Romagnani S, Annunziato F, Manetti R, Almerigogna F, Biagiotti R, Giudizi MG, Ravina A, Gianno V, Tomasevic L, Maggi E: Role for CD30 in HIV expression. *Immunol Lett* 1996, 51:83–88
33. Kurts C, Carbone FR, Krummel MF, Koch KM, Miller JF, Heath WR: Signalling through CD30 protects against autoimmune diabetes mediated by CD8 T cells. *Nature* 1999, 398:341–344
34. Croager EJ, Muir TM, Abraham LJ: Analysis of the human and mouse promoter region of the non-Hodgkin's lymphoma-associated CD30 gene. *J Interferon Cytokine Res* 1998, 18:915–920
35. Cheng J, Liu C, Koopman WJ, Mountz JD: Characterization of human Fas gene. Exon/intron organization and promoter region. *J Immunol* 1995, 154:1239–1245
36. Kemper O, Wallach D: Cloning and partial characterization of the promoter for the human p55 tumor necrosis factor (TNF) receptor. *Gene* 1993, 134:209–216
37. Santee SM, Owen-Schaub LB: Human tumor necrosis factor receptor p75/80 (CD120b) gene structure and promoter characterization. *J Biol Chem* 1996, 271:21151–21159
38. Yoo J, Stone RT, Kappes SM, Toldo SS, Fries R, Beattie CW: Genomic organization and chromosomal mapping of the bovine Fas/APO-1 gene. *DNA Cell Biol* 1996, 15:377–385
39. Carcamo J, Buckbinder L, Reinberg D: The initiator directs the assembly of a transcription factor IID-dependent transcription complex. *Proc Natl Acad Sci USA* 1991, 88:8052–8056
40. Smale ST, Schmidt MC, Berk AJ, Baltimore D: Transcriptional activation by Sp1 as directed through TATA or initiator: specific requirement for mammalian transcription factor IID. *Proc Natl Acad Sci USA* 1990, 87:4509–4513
41. Smale ST, Baltimore D: The "initiator" as a transcription control element. *Cell* 1989, 57:103–113
42. Zenzie-Gregory B, Khachi A, Garraway IP, Smale ST: Mechanism of initiator-mediated transcription: evidence for a functional interaction between the TATA-binding protein and DNA in the absence of a specific recognition sequence. *Mol Cell Biol* 1993, 13:3841–3849
43. Li YC, Ross J, Scheppler JA, Franza BR Jr: An in vitro transcription analysis of early responses of the human immunodeficiency virus type 1 long terminal repeat to different transcriptional activators. *Mol Cell Biol* 1991, 11:1883–1893
44. Lench N, Stanier P, Williamson R: Simple non-invasive method to obtain DNA for gene analysis. *Lancet* 1988, 1:1356–1358
45. Burke TW, Kadonaga JT: The downstream core promoter element, DPE, is conserved from *Drosophila* to humans and is recognized by TAFII60 of *Drosophila*. *Genes Dev* 1997, 11:3020–3031
46. Smale ST: Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes. *Biochim Biophys Acta* 1997, 1351:73–88
47. Croager EJ, Abraham LJ: Characterisation of the human CD30 ligand gene structure. *Biochim Biophys Acta* 1997, 1353:231–235
48. Shimadzu M, Nunoi H, Terasaki H, Ninomiya R, Iwata M, Kanegasaka S, Matsuda I: Structural organization of the gene for CD40 ligand: molecular analysis for diagnosis of X-linked hyper-IgM syndrome. *Biochim Biophys Acta* 1995, 1260:67–72
49. Meloni R, Albanese V, Ravassard P, Treilhou F, Mallet J: A tetranucleotide polymorphic microsatellite, located in the first intron of the tyrosine hydroxylase gene, acts as a transcription regulatory element in vitro. *Hum Mol Genet* 1998, 7:423–428
50. Amirhaeri S, Wohlrab F, Wells RD: Differential effects of simple repeating DNA sequences on gene expression from the SV40 early promoter. *J Biol Chem* 1995, 270:3313–3319
51. Gogos JA, Karayiorgou M: Sequence-specific and length-dependent interaction of C2H2 zinc fingers and (TA)_n microsatellites. *Hum Genet* 1996, 98:616–619
52. Baccichet A, Benachenhou N, Couture F, Leclerc JM, Sinnett D: Microsatellite instability in childhood T cell acute lymphoblastic leukemia. *Leukemia* 1997, 11:797–802
53. Christensen M, Jensen MA, Wolf H, Orntoft TF: Pronounced microsatellite instability in transitional cell carcinomas from young patients with bladder cancer. *Int J Cancer* 1998, 79:396–401
54. Gamberi B, Gaidano G, Parsa N, Carbone A, Roncella S, Knowles DM, Louie DC, Shibata D: Microsatellite instability is rare in B-cell non-Hodgkin's lymphomas. *Blood* 1997, 89:975–979
55. Hatta Y, Yamada Y, Tomonaga M, Miyoshi I, Said JW, Koeffler HP: Microsatellite instability in adult T-cell leukaemia. *Br J Haematol* 1998, 101:341–344
56. Mark Z, Toren A, Amariglio N, Schiby G, Brok-Simoni F, Rechavi G: Instability of dinucleotide repeats in Hodgkin's disease. *Am J Hematol* 1998, 57:148–152
57. Tanosaki S, Inokuchi K, Shimada T, Dan K: Relation between microsatellite instability and N-ras mutation and duration of disease free survival in patients with acute leukemia. *Cancer* 1998, 83:475–481
58. Drake CG, Babcock SK, Palmer E, Kotzin BL: Genetic analysis of the NZB contribution to lupus-like autoimmune disease in (NZB × NZW)F1 mice. *Proc Natl Acad Sci USA* 1994, 91:4062–4066
59. Kono DH, Burlingame RW, Owens DG, Kuramochi A, Balderas RS, Balomenos D, Theofilopoulos AN: Lupus susceptibility loci in New Zealand mice. *Proc Natl Acad Sci USA* 1994, 91:10168–10172
60. Abraham LJ, Kroeger KM: Impact of the –308 TNF promoter polymorphism on the transcriptional regulation of the TNF gene: relevance to disease. *J Leukoc Biol* 1999, 66:562–566