THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 2, Issue of January 10, pp. 948–955, 2003

Printed in U.S.A.

Sp1 Transactivation of the *TCL1* Oncogene*

Received for publication, July 17, 2002, and in revised form, November 1, 2002
Published, JBC Papers in Press, November 5, 2002, DOI 10.1074/jbc.M207166200

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Cis-regions and trans-factors controlling *TCL1* oncogene expression are not known. We identified the functional *TCL1* promoter by mapping four transcriptional start sites 24–30 bp downstream of a TATA box. A 424-bp fragment upstream of the major start site showed robust promoter activity comparable with SV40 in both *TCL1* expressing and non-expressing cell lines. Additional constructs spanning 10 kb upstream and 20 kb downstream of the start site showed only modest increases in reporter activity indicating that *TCL1* expression is primarily controlled by the promoter. Ten putative Sp1-binding sites were identified within 300 bp of the start site, and three of these specifically bound Sp1. A dose-dependent transactivation of the *TCL1* promoter with Sp1 addition in Sp1-negative *Drosophila* SL2 cells was observed, and mutation of the three identified Sp1-binding sites significantly repressed reporter gene expression in 293T cells, confirming a key role for Sp1 in activating the *TCL1* promoter *in vivo*. In *TCL1* silent cell lines, CpG DNA methylation was rarely observed at functional Sp1 sites, and methylation of a previously reported *Not*I restriction site was associated with dense CpG methylation rather than endogenous *TCL1* gene silencing. Together, these results indicate that Sp1 mediates transactivation of the *TCL1* core promoter and that *TCL1* gene silencing is not dependent on mechanisms involving Sp1 and *Not*I site methylation.

The T-cell leukemia-1 (*TCL1*) oncogene is expressed mainly but not exclusively at specific stages of lymphocyte development in humans. In normal T-lineage cells, *TCL1* expression is restricted to CD3/CD4/CD8 triple-negative immature thymocytes (1). Interestingly, mature peripheral T-cell expansions and clonal malignancies may aberrantly express *TCL1* due to characteristic chromosomal translocations and inversions at 14q32.1 (reviewed in Ref. 2). These chromosomal rearrangements reposition T-cell receptor α/δ- or β-chain control sequences next to the *TCL1* coding region leading to T-cell-specific dysregulation. A tumorigenic role for this aberrant expression has been confirmed from transgenic studies in which *TCL1* dysregulation targeted to T-cells mainly in the thymus cause mice to exclusively develop mature peripheral T-cell malignancies (3).

In normal B-lineage cells, *TCL1* is expressed from early pro-B bone marrow precursors through mature peripheral B-cell stages of development (1, 4, 5). Terminally differentiated B-cells, such as non-proliferating memory or plasma cells, lack *TCL1* expression. *TCL1* gene silencing with terminal B-cell maturation has been shown to correlate with the conversion of *TCL1*-positive B-cells to plasmacytoid cells by growth on CD40L-expressing fibroblasts supplemented with interleukin-4 and interleukin-10 (5). Interestingly, recent studies (4–7) have also linked aberrant *TCL1* expression to specific classes of mature B-cell lymphoma. Approximately 30% of diffuse large B-cell lymphomas (DLBCL)[1] and about 75% of AIDS-related DLBCL abnormally express *TCL1* (4, 5). Evidence that this dysregulation promotes B-cell malignancies has been obtained from transgenic mouse studies in which *TCL1* is aberrantly expressed only in B-cells or in both T- and B-cells (8, 13). In this situation, dysregulation strongly favors the development of peripheral B-cell tumors *versus* T-cell malignancies. The mechanism for induction of both T- and B-cell malignancies is thought to rely on inappropriately strong co-activation of the serine-threonine kinase AKT by excessive TCL1 oncoprotein levels (9–14).

In contrast to known chromosomal rearrangements that cause aberrant expression in mature T-cell tumors, little is known about the mechanism(s) regulating *TCL1* expression during development or supporting its aberrant expression in mature B-cell malignancies. The *TCL1* promoter and other potential regulatory regions have not been characterized, and there have been no reports of 14q32.1 rearrangements with associated dysregulation of *TCL1* for B-cell malignancies, including *TCL1*-expressing Burkitt lymphomas (BL), B-chronic lymphocytic leukemias (β-CLL), or DLBCL (15). Initial studies by Yuille *et al.* (15) reported a correlation between the methylation status of two CpG sites within a single *Not*I site in the putative *TCL1* promoter and *TCL1* expression levels. Also, treatment of *TCL1* non-expressing Jurkat and CEM T-cells with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-AC) was reported to activate *TCL1* expression, suggesting a potential role for epigenetic modifications in *TCL1* gene silencing (15). While providing important first clues, further analyses are needed to improve our understanding of the mech-

* This work was supported by the Lymphoma Research Foundation, Amgen/U.C. BioStar Project S98-35, and National Institutes of Health Grants T32CA009120, T32CA009056, CA74929, CA90571, CA85841, GM07185, and GM40185. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: DLBCL, diffuse large B-cell lymphoma; BL, Burkitt lymphoma; 5-AC, 5-aza-2′-deoxycytidine; EMSA, electrophoretic mobility shift assay; PEL, primary effusion lymphoma; GC, germinal center; EMSA, electrophoretic mobility shift assay; EBV, Epstein-Barr virus.

anisms regulating *TCL1* in expressing and silent cell types during development and in cancer. Here we provide the first detailed functional characterization of the human *TCL1* promoter, and we demonstrate a key role for Sp1 in activating *TCL1* gene transcription from the core promoter.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—EBV-immortalized fetal cord blood lymphocyte line 75714 was created, and cell lines were obtained and grown as described (5) except human myeloma AF10 which was provided as a kind gift from M. Kuehl (NCI, National Institutes of Health, Bethesda). 293T fibroblasts, Raji BL, and UC 729-6 B-lymphoblastoid cells were purchased from ATCC. SL2 *Drosophila* cells (a kind gift from L. Zipursky, UCLA, Los Angeles) were grown in Schneider's *Drosophila* media (Invitrogen) supplemented with 10% fetal bovine serum at room temperature.

S1 Nuclease Protection—Total cellular RNA was isolated from Ramos, 2F7, and KS-1 cells (Qiagen). A complementary oligonucleotide was manufactured that overlapped the presumed transcriptional start site: 5′-ACTCGGCCATGGCGTCCTCGGGCCGCCTAAGAAGCAAGAGCCAGAGCCTCTCAAGGCCGCTCGCTGGTCCCTGGGATGTG-3′. A single-stranded oligo-probe was 5′ end-labeled with [γ-^{32}P]ATP and purified by G-50 Sephadex spin column chromatography. A G + A ladder was created using the Maxam-Gilbert sequencing method (16). In brief, 500,000 cpm of radiolabeled probe was incubated with 5 μg of salmon sperm DNA and 1 M piperidine/formate at 37 °C for 20 min. The reaction was frozen on dry ice and dried to completion in a Speedvac concentrator. 20 μl of deionized water was added, and the reaction was frozen and re-dried as before. 100 μl of 1 M piperidine was added, and the reaction was incubated at 90 °C for 20 min and dried. 100 μl of deionized water was added, and the reaction was dried. The reaction was resuspended in loading dye and boiled for 3 min before gel loading. S1 nuclease protection was performed as described (16), with some modifications. In brief, 500,000 cpm of radiolabeled probe was hybridized with 50 μg of total RNA at 30 °C overnight. 450 units of S1 nuclease (Protégé) was added, and probe digestion was carried out at 30 °C for 2 h. After ethanol precipitation, the reaction was resuspended in loading dye, boiled for 3 min, and loaded on an 8% acrylamide, 8 M urea denaturing gel. Gels were run at 1400 V for 2.5 h at room temperature in 1× TBE, dried, and exposed to film.

TCL1 Promoter-Reporter Gene Constructs—The sequence of the human *TCL1* genomic locus at 14q32.1 (GenBank™ HTG data base entry accession number AL139020.1) was used to engineer PCR cloning primers. The reverse primer began at the ATG translation start site (5′-ATGGCGTCCTCGGGCCGCCTAAGAAGCAAG-3′) and was paired with the following forward primers: (−191) 5′-ACGTAGCGCCTGCGCGGGACCCTCA-3′; (−350) 5′-AGAAAGGGCCAAGGTCACCCCGGTGCCTCT-3′; (−424) 5′-GTCGACTGTGAGTTCCCAGCAGAG-3′; (−543) 5′-AAGGCACAGGCTGGTGGAGATCCAGGGAAC-3′; (−760) 5′-GGTGGAAGGAGGGATTTCTTTTTAAG-3′; and (−943) 5′-TGATGTTTGAACCAGGCTGGAGCTGG-3′. PCR-amplified products from 200 ng of BL41 (and TC32 Ewing sarcoma) genomic DNA were isolated on a 1% agarose gel, cloned into the pCR2.1-TOPO vector (Invitrogen), digested with *Eco*RI, subcloned into the *Eco*RI site of the pGL3-basic firefly luciferase expression plasmid (Promega) and cycle sequenced. Reporter gene point mutants were generated with the QuikChange Kit (Stratagene) and targeted CCGCCC to CCGAAC changes made in specific Sp1 GC box-binding motifs using techniques provided by the manufacturer.

Reporter Gene Assays—10^7 mammalian cells were co-transfected with 10 μg of each test construct and 1 μg of the *Renilla* transfection control plasmid pRL-*SV40* (Promega) by electroporation (250 V, 1180 microfarads, low resistance, 4 °C in ice-cold supplemented RPMI 1640 plus 5% fetal calf serum; Invitrogen Cell-Porator). Cells were harvested at 48 h, and *Renilla*-normalized firefly luciferase luminescence was measured using the Dual Luciferase Assay System (Promega). SL2 *Drosophila* cells were co-transfected in 6-well plates using LipofectAMINE (Invitrogen) per the manufacturer's instructions. 3 μg of pGL3-basic, p*SV40*-luciferase or p424*TCL1*-luciferase constructs were used along with varying amounts of the control plasmid pP_{ac} and 0, 100, or 300 ng of the *Drosophila* Sp1 expression plasmid pP_{ac}Sp1 (generously provided by R. Tjian, University of California, Berkeley), keeping the total amount of DNA constant at 3.3 μg (17). Cells were harvested at 48 h, and protein was normalized using the Bio-Rad Protein Assay (Bio-Rad). Firefly luciferase luminescence was measured using the Luciferase Assay System (Promega).

EMSA Analysis—Preparation of crude nuclear extract from BCBL-1 and Ramos cells was as described (18). Electrophoretic mobility shift assay (EMSA) was performed as described previously (19). Briefly, EMSA probes and cold competitors were complementary double-stranded DNA oligonucleotides containing each of the 10 identified Sp1 consensus sites within the *TCL1* core promoter region (see Fig. 1). The probe sequences are as follows: Sp1(consensus), 5′-ATTCGATCGGGGCGGGGCGAGC-3′; Sp1(A), 5′-CAGCAGCAGAGGGCGGCGGTCGGTG-3′; Sp1(B), 5′-CCTCGAGGAAGGCGCGGGCCAGCTG-3′; Sp1(C), 5′-CGGGACGTAGCGCCTGCGCGGGACC-3′; Sp1(D), 5′-CCCACAAACCCCCGCCCCATCCTGCC-3′; Sp1(E), 5′-GCCTTACGCCCCGCCCCAAGGTCGT-3′; Sp1(F), 5′-ACCCGGGGTCCCGCCCCAAGACCGT-3′; Sp1(G), 5′-CCGTCCTCCCGCCCCGCCGCTTGGT-3′; Sp1(H), 5′-GCCGCTTGGTGGCGCCCGCATGCTG-3′; Sp1(I), 5′-CTTGCTTCTTAGGCGGCCCGAGGAC-3′; and Sp1(J), 5′-CTTAGGCGGCCCGAGGACGCCATGG-3′. Mutant Sp1 probes were also created by changing the GC box-binding sequence from CCGCCC to CCGAAC to create Mut-Sp1(D), Mut-Sp1(E), Mut-Sp1(F), and Mut-Sp1(G) (20). Probes were end-labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase (New England Biolabs). 10 μg of BCBL-1 or Ramos nuclear extract was incubated with 20 mM HEPES, pH 7.9, 0.2 mM EDTA, 50 mM KCl, 1 mM dithiothreitol, 5% glycerol, 2 μg of poly(dI-dC), and 10 μg of bovine serum albumin in a 20-μl reaction volume. Radiolabeled probe (20,000 cpm per reaction) was added, and samples were incubated for 20 min at 25 °C. DNA-protein complexes were resolved by electrophoresis for 2 h at 150 V in a 4% non-denaturing polyacrylamide gel and subjected to autoradiography. Competition assays were performed with a 500-fold molar excess of unlabeled probe. Antibody blocking assays were performed by adding 2 μg of either Sp1 antibody (clone 1C6, Santa Cruz Biotechnology) or control normal mouse IgG (Santa Cruz Biotechnology) to the reaction 10 min prior to incubation with probes.

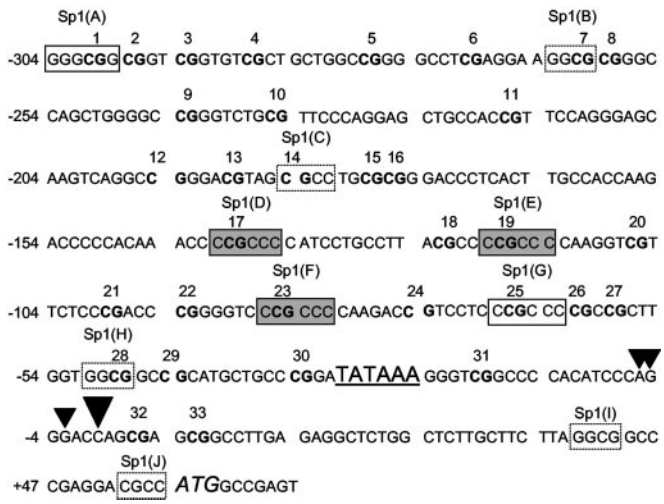

FIG. 1. **The *TCL1* promoter.** The sequence from −304 to +66 nucleotides relative to the major transcription start site (*large arrowhead*) is shown. Three minor start sites are depicted with *small arrowheads.* The TATA box is *underlined* and *enlarged* whereas the translation start site is *enlarged* and shown in *italics.* Consensus Sp1-binding sites (CCGCCC; *A, D, E, F,* and *G*) are *boxed* with a *solid line,* and those *shaded* in *gray* functionally bind Sp1 by EMSA (see Fig. 5). Potential Sp1-binding sites (*B, C, H, I,* and *J*), based on a conserved CCGC core sequence, are *boxed* with *dashed lines* (40). CpG dinucleotides that were surveyed for methylation (see Fig. 8) are indicated by *boldface type* and numbered *1–33*.

Western Blot Analysis—10 μg per lane of SL2 cell extract or 15 μg per lane of cell extracts from a variety of human *TCL1*-expressing and silent lymphocyte cell lines was resolved on a 10% denaturing polyacrylamide gel followed by transfer to nitrocellulose membranes (Micron Separations). Western blotting was performed as described previously (5) with the following modifications. The membrane was incubated with Sp1 antiserum (1:2000 clone 1C6, Santa Cruz Biotechnology), washed, and incubated with horseradish peroxidase-conjugated mouse antiserum (Cell Signaling Technology) and exposed to film following development with ECL+Plus (Amersham Biosciences).

Genomic Bisulfite Sequencing—Sodium bisulfite treatment of DNA has been described (21, 22). 2 μg of genomic DNA was restriction-digested with *Hin*dII and *Nco*I to isolate the basal *TCL1* promoter, ethanol-precipitated, resuspended in 40 μl of water, heated to 97 °C for 5 min, and placed on ice. 2 μl of 6.3 M NaOH was added, and the mixture was incubated at 39 °C for 30 min. 416 μl of fresh sodium bisulfite

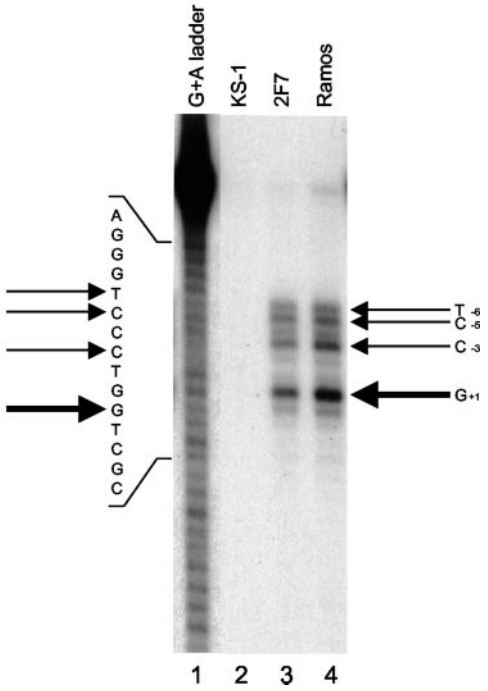

FIG. 2. ***TCL1* transcription start sites determined by S1 nuclease protection assay.** A single-stranded end-labeled probe complementary to the presumed start site(s) of *TCL1* transcription was used. The probe was incubated with 50 μg per sample of total cellular RNA from KS-1 (*TCL1*-silent), 2F7, and Ramos (*TCL1*-expressing) B-cell lines (5). The protected products were resolved on an 8% acrylamide, 8 M urea denaturing gel. *Lane 1,* G + A ladder; *lane 2,* KS-1 RNA; *lane 3,* 2F7 RNA; and *lane 4,* Ramos RNA. The major transcription start site is indicated with a *heavy arrow,* and the minor start sites are indicated with *smaller arrows* (see also Fig. 1). The sequence of the complementary oligonucleotide is indicated on the *left.*

solution (40.5 g of sodium bisulfite dissolved in 80 ml of water, pH adjusted to 5.1, add 3.3 ml of 20 mM hydroquinone; adjust final volume to 100 ml with water) was added, and the mixture was incubated at 55 °C for 15 h, with a heat shock to 95 °C every 3 h. Sodium bisulfite-treated DNA was desalted with the Wizard DNA Clean-up System (Promega); 6.3 M NaOH was added to a final concentration of 0.3 M followed by incubation at 37 °C for 15 min. 10 M ammonium acetate, pH 7.0, was added to a final concentration of 3 M, followed by ethanol precipitation, pellet drying, and resuspension in 100 μl of TE. 2 μl of sodium bisulfite-treated DNA was PCR-amplified for 47 cycles using multiple sense-strand primer pair combinations for *TCL1* followed by 30 cycles of nested PCR amplification. Primers used to amplify sodium bisulfite-treated DNA include 5′-GGGGGGGTTTTTTAGAAGAAGAA-AGGG-3′ and 5′-CCAAAACCTCTCAAAACCACTC-3′. Nested primers used were either 5′-GGTGTTTTTTTAGTAGTAGTAGAGGG-3′ or 5′-G-TTTTAGGGAGTAAGTTAGGTTGGG-3′. PCR products were cloned into the pCR2.1-TOPO vector and transformed into DH5α bacteria, and individual clones were sequenced.

RESULTS

TCL1 Transcriptional Start Site Determination—A transcription initiation site has been suggested at 41 bp downstream of a putative TATA box based upon the initial reverse transcriptase-PCR cloning of the *TCL1* cDNA (1, 15). However, the *TCL1* start site has not been experimentally determined, and this position would be inconsistent with known start sites from TATA box-dependent promoters, which are generally 25–30 nucleotides downstream of the first T in the TATA sequence (reviewed in Refs. 23 and 24). Therefore, we used S1 nuclease protection analysis to identify the transcription start site(s) of *TCL1* in order to help locate the major promoter for further investigation (Fig. 2). In *TCL1* expressing 2F7 and Ramos BL cells, the major site of transcription initiation is a cytosine located 30 nucleotides downstream of the TATA box. There are also three identical minor sites of transcription initiation in these two cell lines. These sites include an adenine at 24 nucleotides, a guanine at 25 nucleotides, and a guanine at 27 nucleotides downstream of the TATA box (summarized in Fig. 1). These sites are consistent with transcription initiation sites in TATA box-dependent promoters. In addition, S1 nuclease protection products were not seen in KS-1 primary effusion lymphoma (PEL) cells, consistent with a lack of *TCL1* transcription in this line (5), and no products were observed with a probe incubated with S1 nuclease in the absence of input RNA (data not shown).

TCL1 Promoter Identification—To date, regions suspected to contain *TCL1* promoter activity have not been analyzed. We sought to define the *TCL1* promoter and began a search just upstream of the TATA box and transcription initiation sites. A genomic fragment was cloned beginning at the ATG translation start site and extending to minus 424 bp from the major transcription start site using the originally reported upstream sequence as a guide for primer design (1). Sequencing of a BL41-derived fragment (and one derived from TC32 Ewing sarcoma cell genomic DNA, data not shown) revealed that the originally reported 5′ sequence contains an ~60-bp duplication that is not found in clones generated here or in recent data released from GenBank™ (data base entry GI 624960) (1). This fragment was inserted into pGL3-basic, creating p424*TCL1*-luciferase. Previously it was shown that EBV infection has no effect on endogenous *TCL1* expression levels and that EBV-immortalized peripheral blood cells, along with BL lines, express abundant *TCL1* (5). Reporter activity in three of these highly expressing B-cell lines (75714, BL41, and 2F7) was 15–35-fold elevated over an empty vector construct, pGL3-basic, that lacks known promoter activity (Fig. 3*A*). PEL lines silence B-cell-specific gene transcription, including *TCL1*, and are derived from terminal stages of B-cell differentiation that normally lack *TCL1* expression (25–27). In four silent PEL lines (BCBL-1, BC-1, BC-3, and KS-1), the *TCL1* reporter gene was expressed 15–45-fold over pGL3-basic. In addition, a 12-fold activation was seen in Jurkat T-cells, which also do not express the endogenous *TCL1* gene. These results indicate that the −424 *TCL1* gene fragment contains strong promoter activity and lacks functional silencing elements in transient transfection assays in lymphocytes. Furthermore, the level of activity was roughly equivalent to the activity seen with a robust *SV40* promoter positive-control construct in all lines examined.

We next performed an extended search for further 5′ sequences affecting *TCL1* promoter activity. Overlapping fragments from 191- to 943-bp upstream of the transcription start site were cloned into pGL3-basic and tested for promoter activity. All of the engineered constructs demonstrated robust expression in *TCL1*-negative BC-3 PEL and 293T fibroblast cells (Fig. 3*B*). In BC-3 PEL cells, reporter gene activity was essentially equivalent for constructs containing 350–943 bp of 5′ sequence and ranged from 17- to 25-fold stronger than pGL3-basic. This indicates that cis-elements required for activity in transient assays are localized within 350 bp of the transcription start site and that no additional strong enhancer or silencer motifs are within 1 kb upstream. Reporter gene expression in 293T cells was 15–35-fold higher compared with levels seen in BC-3 PEL cells using a similar range of reporter constructs (Fig. 3*B*). In fact, a 191-bp 5′ fragment showed reporter activity equivalent to that seen with a 424-bp fragment, suggesting that all essential core promoter components are present within the first 200 bp 5′ of the transcription start site. In sum, these findings suggest the *TCL1* core promoter region functions in a tissue-nonspecific manner. Similar findings have been reported from the analyses of many other core promoters from tissue-specific genes (28–35).

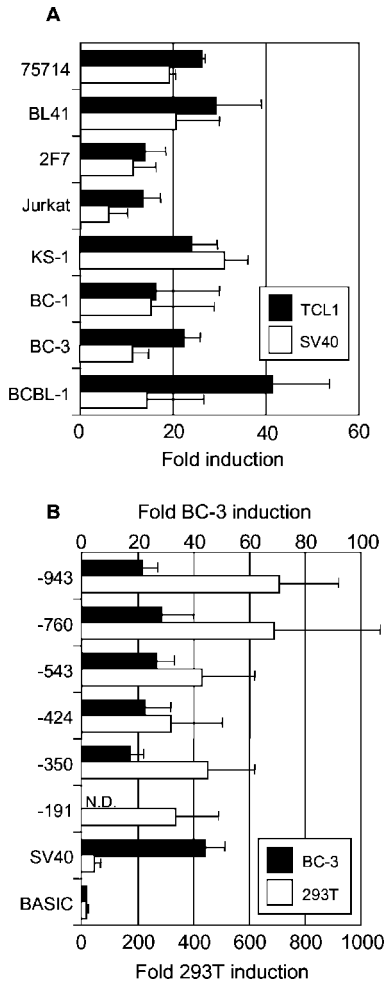

FIG. 3. ***TCL1* promoter activity in *TCL1*-expressing and non-expressing cell types.** *A*, *TCL1*-expressing (75714, BL41, and 2F7) and silent (Jurkat, KS-1, BC-1, BC-3, and BCBL-1) lines were transiently transfected with either p424*TCL1*-luciferase or pGL3*SV40*-luciferase expression and pGL3-basic (control) constructs and assayed for luciferase activity. *White bars* display *SV40* reporter activity, and *black bars* show *TCL1* reporter activity in comparison to pGL3-basic activity, which was set at 1. Each transfection was normalized to a co-transfected *Renilla*-luciferase vector (pRL*SV40*-luciferase) to control for transfection efficiency. *Error bars* denote S.D. from three separate experiments using independently isolated reporter gene DNA. *B*, DNA fragments from 191 to 943 bp upstream of the major transcription start site do not significantly affect *TCL1* promoter activity in *TCL1*-negative 293T fibroblast and BC-3 PEL cells. *White bars* display *TCL1* reporter activity in 293T fibroblasts, and *black bars* display reporter activity in BC-3 PEL cells in comparison to pGL3-basic activity, which was set at 1. Each transfection was normalized to co-transfected pRL*SV40*-luciferase to control for transfection efficiency. *Error bars* denote S.D. from at least four separate experiments using independently isolated reporter gene DNA. Scales for fold induction are shown *above* and *below* the bar diagram for each cell line.

Sp1 Binding in the TCL1 Core Promoter—The 350-bp core promoter of *TCL1* exhibited robust activity that was not significantly affected by upstream (to 10 kb) or downstream (to 20 kb) elements in expressing *versus* non-expressing or lymphoid *versus* non-lymphoid cell types.[2] This indicates that critical regions responsible for expression in transient transfections are present in the core promoter and that the factor(s) driving this expression are broadly expressed in distinct cell types. MatInspector (36) analysis of this 350-bp promoter sequence revealed 5 consensus GC box Sp1 factor-binding motifs

[2] S. W. French, C. S. Malone, R. R. Shen, M. Renard, S. E. Henson, M. D. Miner, R. Wall, and M. A. Teitell, unpublished results.

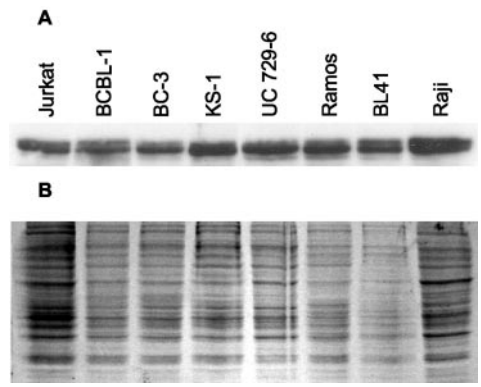

FIG. 4. **Equivalent Sp1 protein levels in *TCL1*-expressing and silent cell lines.** *A*, Western blot using Sp1 antiserum on cell lysates from *TCL1*-expressing (UC 729-6, Ramos, BL41, and Raji) and *TCL1*-silent (Jurkat, BCBL-1, BC-3, and KS-1) B- and T-cell lines. *B*, Coomassie Brilliant Blue-stained gel indicating equal total protein loading for each cell lysate examined.

(CCGCCC) that are labeled *A, D, E, F,* and *G* in Fig. 1 (1, 37–39). A previous transcription factor-binding site study showed that the core sequence CGCC was sufficient, in multiple sequence contexts, to facilitate Sp1 binding in EMSA (40). Therefore, sites with this core motif are labeled *B, C, H, I,* and *J* in Fig. 1, bringing to 10 the total number of putative Sp1-binding sites within the *TCL1* core promoter region. Sp1 is a ubiquitous transcription factor (reviewed in Refs. 41 and 42), and its known transactivating function is consistent with a role in driving *TCL1* promoter activity in all the cell types examined regardless of endogenous *TCL1* expression (Fig. 3, *A* and *B*). Supporting this postulate, Western blot analysis shows that the level of Sp1 protein in *TCL1*-expressing and silent lymphocyte lines is equivalent, which also indicates that changes in the level of Sp1 are not responsible for the tissue-specific expression of *TCL1* (Fig. 4).

Double-stranded DNA oligomers of these 10 potential Sp1 sites were tested for binding function by EMSA with nuclear extracts from *TCL1*-silent BCBL-1 PEL cells and *TCL1*-expressing Ramos cells (Fig. 5 and data not shown). Three sites, Sp1(D) Sp1(E), and Sp1(F), formed a complex resulting in a band with decreased gel mobility. This complex appears to be specific, as the band was competed away with unlabeled consensus cold Sp1 and self-oligomers. It was not competed away with a nonspecific oligomer that binds another transcription factor (LEF) (43–46). Additionally, Sp1-specific antibody significantly blocked complex formation by preincubation with nuclear extracts, whereas nonspecific IgG antibody had no effect on complex formation (Fig. 5, *D* and *E*). Furthermore, Mut-Sp1(D), Mut-Sp1(E), Mut-Sp1(F), and Mut-Sp1(G) oligomers not only failed to generate a reduced mobility band shift but they were also ineffective at inhibiting complex formation with wild-type Sp1(D), Sp1(E), and SP1(F) probes (Fig. 5 and data not shown). Taken together, the data demonstrate that Sp1 interacts with Sp1(D), Sp1(E), and Sp1(F) sites centrally located within the first 150 bp of the *TCL1* core promoter.

Sp1 Transactivates the TCL1 Core Promoter—To confirm that Sp1 can functionally transactivate the *TCL1* promoter, co-transfection assays were performed in Sp1-negative *Drosophila* SL2 cells where the effect of exogenous Sp1 expression can be tested on Sp1-dependent promoters (17). Promoter-less control (pGL3-basic), Sp1-dependent pGL3*SV40*-luciferase, or p424*TCL1*-luciferase expressing constructs were transfected into *Drosophila* cells with varying amounts of human Sp1 generated by a fly-specific Sp1 expression vector (pP_{ac}Sp1; Fig. 6*A*). The level of reporter gene expression detected for each construct in the absence of co-expression of exogenous Sp1 was

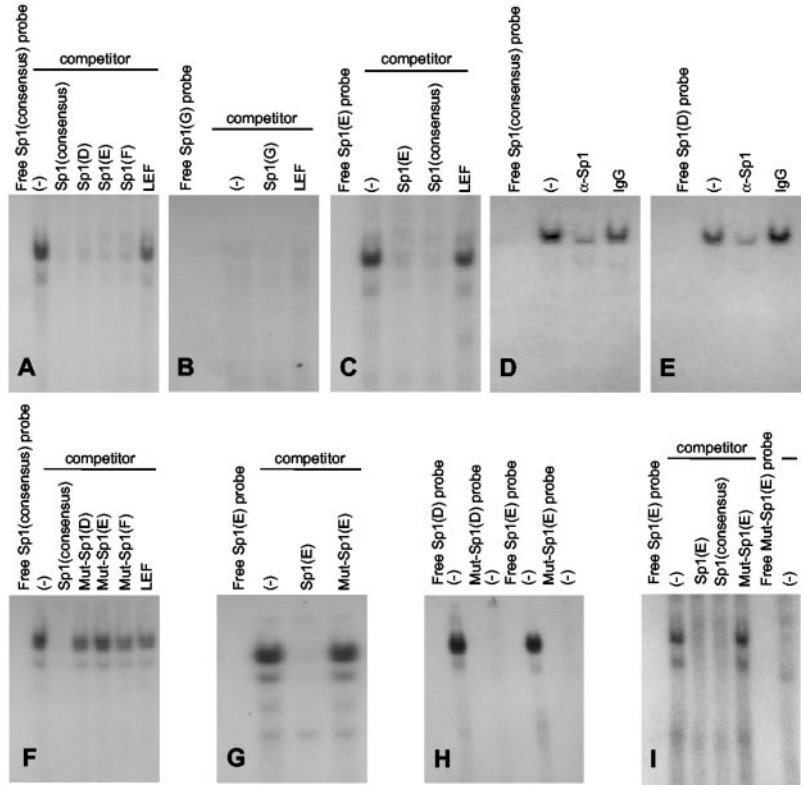

FIG. 5. **Sp1 binding to 3 of 10 Sp1 sites within the *TCL1* promoter.** EMSA was performed using BCBL-1 (*A–E* and *G–I*) and Ramos (*F*) nuclear extracts. Radiolabeled or competitor probes Sp1(A) through Sp1(J) and mutant probes Mut Sp1(D) through Mut Sp1(G), corresponding to the boxed Sp1 elements depicted in Fig. 1, along with an Sp1(consensus) sequence probe were investigated. *A–C*, nonspecific (LEF) and distinct Sp1 cold competitors were used to demonstrate binding specificity. Identical results to those presented in *C* were also obtained with Sp1(D)- and Sp1(F)-labeled probes (data not shown). *D* and *E*, EMSA was performed with radiolabeled Sp1 oligomers and pretreatment of nuclear extracts with Sp1 antiserum or control IgG. Identical results to those presented in *E* were also obtained with Sp1(E)- and Sp1(F)-labeled probes (data not shown). *F, G,* and *I,* Mut-Sp1(D), Mut-Sp1(E), and Mut-Sp1(F) probes cannot compete with radiolabeled Sp1(consensus) or Sp1(E) probes for Sp1 binding. Identical results were obtained with radiolabeled Sp1(D) and Sp1(F) probes (data not shown). *H* and *I,* Mut-Sp1(D), Mut-Sp1(E), and Mut-Sp1(F) (data not shown) oligomers do not bind Sp1. Overall, Sp1 consensus and Sp1(D), Sp1(E), and Sp1(F) sequences within the *TCL1* core promoter specifically bind Sp1 from *TCL1* silent (BCBL-1) and expressing (Ramos) nuclear extracts.

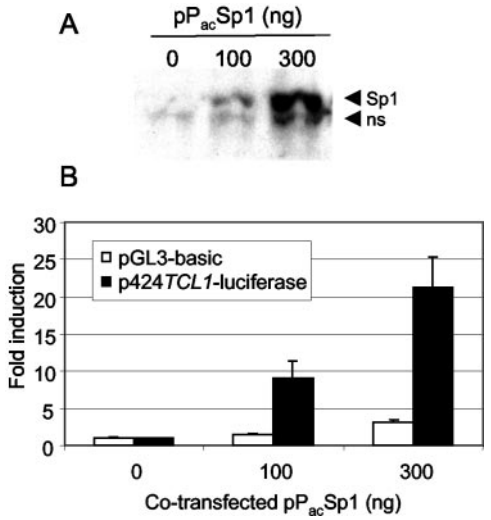

FIG. 6. **Sp1-induced expression of the *TCL1* core promoter in *Drosophila* SL2 cells.** SL2 cells were transfected with promoter-less pGL3-basic, Sp1-dependent p*SV40*-luciferase, or p424*TCL1*-luciferase expression constructs and varying amounts of the pP$_{ac}$Sp1 Sp1 expression vector. *A*, Western analysis of Sp1 level from a representative experiment. The amount of co-transfected pP$_{ac}$Sp1 vector is indicated at the *top*. *Arrowheads* at the *right* indicate the Sp1 specific immunoreactive band and a nonspecific (*ns*), lower molecular weight band. Equal protein was loaded in each lane, followed by confirmation with Coomassie Brilliant Blue gel staining prior to transfer (data not shown). *B*, dose-dependent induction of *TCL1* expression by Sp1. Amount of co-transfected pP$_{ac}$Sp1 is indicated at the *bottom*. The Sp1-dependent p*SV40* luciferase-positive control construct exhibited a 150–180-fold induction with Sp1 addition under identical conditions (data not shown).

assigned the arbitrary value of 1. The *SV40* promoter was induced 150–180-fold (data not shown), whereas the *TCL1* promoter was induced 9–21-fold with exogenous Sp1 expression compared with the induction without Sp1 expression (Fig. 6*B*). Western analysis confirmed dose-dependent expression of exogenous Sp1 with increasing amounts of pP$_{ac}$Sp1 in transfected SL2 cells. In addition, reporter constructs containing single, double, or triple mutations engineered into the Sp1(D), Sp1(E), and Sp1(F) sites, along with a control mutation engineered into the non-binding Sp1(G) site, were tested for activity in 293T fibroblast cells (Fig. 7). 293T cells are the optimal cell line for analyzing the effects of Sp1-binding site mutations, because the robust expression of *TCL1* reporter constructs in 293T cells (Fig. 3*B*) would require a strong inhibitory effect from Sp1-binding site mutants to significantly reduce expression. In this context, the occurrence of a statistically significant inhibitory effect would strongly support a powerful role for Sp1 in regulating *TCL1* promoter activity. Mutations of the Sp1D, -E, and -F-binding sites resulted in markedly decreased expression of the *TCL1* reporter construct (Fig. 7). The amount of expression was reduced in all single site mutants relative to the unmutated reporter construct and further decreased substantially in double and triple mutant constructs ($p \leq .01$). Importantly, mutation of the Sp1(G)-binding site, which failed to bind Sp1 by EMSA analysis (Fig. 5*B*), had no effect on *TCL1* reporter construct expression ($p > 0.05$). Together, the data from EMSA analysis, SL2 *Drosophila* cell studies, and mutant reporter construct investigations in 293T cells strongly indicate that Sp1 transactivating factors and cis-binding sites Sp1(D), Sp1(E), and Sp1(F) play a dominant role in regulating *TCL1* core promoter activity.

CpG Methylation Is Not Correlated with TCL1 Silencing—The region from +10 to −300 bp of the *TCL1* core promoter contains 33 CpG sites and corresponds to a classical "CpG island" (Fig. 1) (47). Based on differential sensitivity at a single *Not*I restriction enzyme site in this core promoter, Yuille *et al.* (15) previously concluded that *TCL1* silencing was mediated by CpG methylation. Their finding that *TCL1* expression was activated in silenced cells by 5-AC treatment was advanced as further support for this conclusion, although this reactivation experiment does not distinguish between direct and indirect effects resulting from 5-AC treatment. We used genomic bisul-

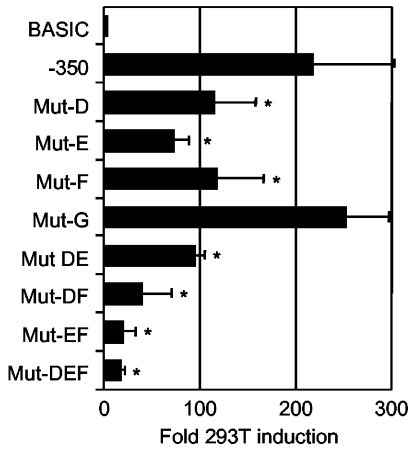

FIG. 7. **Mutation of three Sp1-binding sites inhibits *TCL1* reporter gene expression.** 293T cells were transfected with promoterless pGL3-basic (BASIC), p350*TCL1*-luciferase (−350), or mutant p350*TCL1*-luciferase expression constructs containing mutations in Sp1 protein-binding sites. Single mutant (Mut-D, Mut-E, Mut-F, and Mut-G), double mutant (Mut-DE, Mut-DF, and Mut-EF), and triple mutant (Mut-DEF) reporter constructs were tested. *Black bars* show *TCL1* reporter activity in comparison to pGL3-basic activity, which was set at 1. Each transfection was normalized to co-transfected pRL*SV40*-luciferase to control for transfection efficiency. *Error bars* denote S.D. from at least six separate experiments using independently isolated reporter gene DNA. *Asterisks* indicate statistically significant differences from unmutated p350*TCL1*-luciferase results using a two-sided Student's *t* test ($p \leq .01$). Identical results were obtained using similarly mutated and unmutated p191*TCL1*-luciferase and p424*TCL1*-luciferase expression constructs (data not shown).

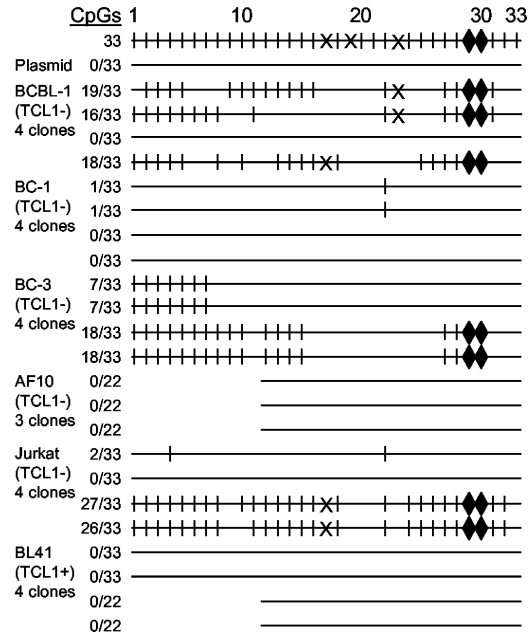

FIG. 8. **CpG DNA methylation of the *TCL1* core promoter in expressing and silent cell types.** Five *TCL1* silent (TCL1−) and one *TCL1*-expressing (TCL1+) cell lines were examined for CpG methylation by genomic bisulfite sequencing. Three to four clones were determined with genomic bisulfite sequencing from each cell line for a total of 23 clones. Clones from each cell line were determined on distinct days to reduce the possibility for crossover contamination, and each assessment included a control plasmid analysis, which always showed complete bisulfite-mediated conversion of unmethylated cytosines. The CpG positions analyzed are numbered 1–33 and correspond to the *boldface type* positions in Fig. 1. Cell lines and the number of clones examined are listed on the *left*. The span of individual sequenced clones is depicted by the extent of a *horizontal line* on the *right*. A *vertical slash* indicates a methylated CpG at that position. "*X* " corresponds to a methylated CpG position contained within Sp1(D), Sp1(E), or Sp1(F) sites within the *TCL1* core promoter. A ♦ corresponds to methylated CpG positions within a previously reported *Not*I restriction enzyme site (15).

fite sequencing to determine definitively the methylation status of every cytosine in the *TCL1* core promoter of expressing and non-expressing cell types (Fig. 8). As expected, no CpG methylation was detected at any of the 33 potential sites in core promoter clones derived from *TCL1*-expressing BL line BL41 (Fig. 8). The core promoter clones from five tested cell lines in which *TCL1* is silenced showed no, minimal (1 or 2 sites), moderate (5–10 sites), or dense (>50% of sites) CpG methylation. Both unmethylated and CpG-methylated promoter clones were seen in 2 of 5 *TCL1* silent cell lines (BCBL-1 and Jurkat), consistent with either one unmethylated allele in all cells or a mixture of cells containing both methylated and unmethylated *TCL1* alleles. Non-expressing BC-3 cells contained both moderately and heavily CpG-methylated clones, with the moderately CpG-methylated positions all clustering within 50-bp at the 5′ end of the promoter. All of the core promoter clones from the two remaining *TCL1* silent cell lines tested, BC-1 and AF10, were either totally unmethylated or contained only minimal (1–2) methylated CpG core promoter sites, indicating that neither allele is methylated in these cells. Because 4 of 5 *TCL1* negative cell lines analyzed with bisulfite sequencing contained unmethylated core promoter clones, CpG methylation is not correlated with or directly responsible for *TCL1* gene silencing. Methylation of two CpG sites contained within the previously analyzed *Not*I site was only detected in those promoter clones with the highest degree of CpG methylation, such as in clones with ≥ half of the 33 potential sites methylated in BCBL-1, BC-3, and Jurkat cell lines. This indicates that *Not*I methylation is only consistently associated with the most extensive CpG methylation and is not a marker for silencing the *TCL1* core promoter in all negative cell types. Significantly, the three confirmed functional Sp1 motifs identified in our studies were not methylated in most *TCL1* promoter clones. CpG methylation was detected in only 1 of 3 Sp1 sites, and this pattern was only seen in the most densely methylated promoter clones analyzed from BCBL-1 and Jurkat cell lines. It has been shown that CpG methylation within Sp1 sequence motifs does not affect Sp1 factor binding (48–54). In fact, Sp1 sites may be important for maintaining CpG-rich regions in an unmethylated state. Our finding with the *TCL1* oncogene is consistent with these combined reports in which methylation of functional Sp1 sites does not play a role in silencing genes. Overall, our results exclude direct *TCL1* core promoter methylation as the mechanism responsible for *TCL1* gene silencing in the cell lines examined here.

DISCUSSION

In this study we have identified a 424-bp fragment that confers robust *TCL1* reporter activity in many *TCL1*-expressing and silent cell types, indicating that we have localized the *TCL1* core promoter. This core promoter directs one major and three minor sites of *TCL1* transcription initiation. These mapped sites are longer by 11–17 nucleotides than the start site suggested by Yuille *et al.* (15), based upon the cloning of the *TCL1* cDNA (1). This discrepancy may result from an incomplete or partially degraded 5′ end of the original library-based *TCL1* cDNA. We think it unlikely to have occurred from rare intact shorter transcripts based on our sensitive S1 nuclease mapping procedure performed with freshly isolated RNA. This result strongly suggests that *TCL1* promoter activity depends on classic TATA-binding proteins and the TATA box (reviewed in Refs. 55 and 56).

We searched extensively both upstream and downstream for cis-acting elements that could affect transcription from the

TCL1 promoter in *TCL1* silent cell types. We were unable to demonstrate any significant effects on core promoter activity by sequences ~10-kb 5′ and 20-kb 3′ of the promoter in transient transfections. This suggests that regulation of expression is mediated through the core promoter rather than through upstream or downstream cis-acting elements. The *TCL1* core promoter contains 10 putative Sp1-binding sites. We confirmed that Sp1 interacts with three of these sites by cold Sp1 oligomer competition assays and by blocking with Sp1 antibody. Exogenously introduced Sp1 in *Drosophila* SL2 cells induced significant reporter gene expression indicating that Sp1 functions *in vivo* to regulate transcription from the *TCL1* core promoter. Furthermore, mutation of these three Sp1 sites, but not a site that did not bind Sp1 using EMSA, substantially inhibited reporter gene expression in 293T cells, reiterating the importance of these specific Sp1 interaction sites in regulating the *TCL1* core promoter.

Sp1 was previously shown to activate transcription from a spectrum of housekeeping, tissue-specific, and cell cycle-related gene promoters (reviewed in Ref. 57). Although Sp1 is ubiquitous and is expressed at equivalent levels in *TCL1*-expressing and silent lymphoid cell types, its function is regulated in several different ways that could explain its involvement with modulation of a tissue-specific promoter such as *TCL1*. Levels of Sp1 have been shown to be cell cycle-dependent and high in the G_1 phase of the cell cycle, whereas levels are significantly lower in other cycle stages. Sp1 levels may be reduced and thereby contribute to decreased *TCL1* expression in terminally differentiated, non-dividing B-lineage cells in which *TCL1* expression is extinguished (5). This is in contrast to the cell lines tested here that are all in cycle and demonstrate abundant Sp1 protein. In these cell lines Sp1 does not confer tissue specificity to a transiently transfected *TCL1* reporter construct.

Transcriptional control via Sp1 is also regulated by means other than absolute protein levels. Sp1 has been shown to be glycosylated and phosphorylated which may alter its activity in specific cell types (58, 59). Another level of regulation is through binding site competition with other specific transcription factors, including additional members of the Sp1 protein family. For example, promoters with multiple Sp1-binding sites as seen in the *TCL1* core promoter were repressed by interactions with Sp3 (60, 61). However, Sp3 is also ubiquitous so its detailed role in tissue-specific gene regulation is not clear.

Sp1 induced *SV40*-mediated transcription by a significantly larger amount than it did *TCL1*-mediated expression in *Drosophila* SL2 cells, yet both promoters showed comparable levels of expression in mammalian cells (Figs. 3 and 5). This implies that, in addition to Sp1, other transcription factors are likely involved in the regulation of the *TCL1* promoter. Interaction of tissue-specific factors with Sp1 has been shown to regulate transcription from tissue-specific promoters through their specific transcription factor-binding sites. Interaction of Puralpha and Sp1 results in enhanced binding of Puralpha to its binding site and increased transcription from the myelin basic protein promoter (62). Also MEF-2 and Sp1 synergistically activate transcription of myoglobin and muscle creatine kinase together through their individual specific binding sites (63). Further analysis for putative tissue-specific transcription factor binding sites will be necessary to characterize completely the *TCL1* promoter.

In other cases, tissue-specific promoter activity was mediated by tissue-specific factors that regulated the interaction of Sp1 with its cognate binding site in the absence of the tissue-specific factor binding to the promoter. For example, peroxisome proliferator-activated receptor-γ blocks expression of thromboxane by binding Sp1 and preventing its binding to an Sp1 site within the thromboxane promoter (64). Also the transcription factors retinoic acid receptor and retinoid X receptor interact with Sp1 to increase its binding to a GC box allowing increased transcription of urokinase plasminogen activator (65). Therefore, *TCL1* promoter activity may be modulated through blocking or enhancing Sp1 interactions with the core promoter Sp1-binding sites via intervention by yet unidentified tissue-specific factors.

Of particular interest is the recent finding that the POZ domain of BCL-6, a B-cell-specific transcriptional repressor, binds directly to Sp1 and blocks its DNA binding activity and subsequent ability to transactivate transcription (66). BCL-6 is expressed in germinal center (GC) B-cells but not by post-GC B-cells (67, 68). Interestingly, *TCL1* expression begins in early B-cell development in the bone marrow, markedly decreases in the GC, and disappears in post-GC B-cells (4, 5). It is possible that the transient expression of BCL-6 in GCs has a role in directing *TCL1* repression through an interaction between the BCL-6 POZ domain and Sp1. This could tip the balance toward binding of Sp3 and Sp4 transcription factors in the *TCL1* core promoter, as has been shown to mediate repression of the ADH5/FDH promoter (69). Also, the POZ domain of BCL-6 interacts with SMRT/N-CoR, mSin3a, B-CoR, and histone deacetylase transcription-inhibitory factors, leading to increased gene repression (70–72). In addition to potentially blocking Sp1 binding and augmenting the binding of inhibitory Sp1 family member proteins, the recruitment of these repressing co-factors may facilitate epigenetic modifications of chromatin structure (*e.g.* histone deacetylation and DNA methylation) involved in gene silencing. Suggesting against a role for BCL-6 in *TCL1* gene silencing, however, is the observation of high level BCL-6 and TCL1 co-expression in multiple DLBCLs (5). If BCL-6 normally represses *TCL1* expression, this co-expression argues that the repressive mechanism may be broken in lymphoid cancers.

An association between CpG promoter methylation and *TCL1* gene silencing was not found in studies presented here. Also, the association between gene silencing and methylation of two CpG sites within a single *Not*I site in the core promoter as reported previously (15) was not confirmed. Instead, an association between alleles with high level CpG methylation and *Not*I site methylation was demonstrated. Rather than acting as a marker for gene silencing, *Not*I methylation appears to indicate dense CpG methylation of the core *TCL1* promoter. Because each of the five *TCL1*-negative cell types examined here contained unmethylated or minimally methylated DNA clones, *TCL1* silencing does not appear to be due to promoter methylation. Combined with the results presented here, the prior finding that Jurkat and CEM T-cell lines treated with 5-AC-activated *TCL1* gene expression suggests that inhibition of DNA methyltransferase activity likely affected DNA methylation outside of the *TCL1* core promoter region (15). In fact, the lack of consistent high level CpG methylation in the promoter does not exclude additional mechanisms of epigenetic regulation in the control of *TCL1* gene expression. The identification of tissue- and development-specific factors that may interfere with Sp1 binding, such as BCL-6, along with the resolution of potential epigenetic mechanisms will both be necessary to understand the regulation of *TCL1* expression in lymphocyte development and malignancy.

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