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Differential Transcription Factor Use by the *KIR2DL4* Promoter Under Constitutive and IL-2/15-Treated Conditions

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Abstract

KIR2DL4 is unique among human *KIR* genes in expression, cellular localization, structure, and function, yet the transcription factors required for its expression have not been identified. Using mutagenesis, electrophoretic mobility shift assay, and co-transfection assays, we identified two redundant Runx binding sites in the *2DL4* promoter as essential for constitutive *2DL4* transcription, with contributions by a CRE site and initiator elements. IL-2-and IL-15-stimulated human NK cell lines increased *2DL4* promoter activity, which required functional Runx, CRE, and Ets sites. Chromatin immunoprecipitation experiments show that Runx3 and Ets1 bind the *2DL4* promoter in situ. *2DL4* promoter activity had similar transcription factor requirements in T cells. Runx, CRE, and Ets binding motifs are present in *2DL4* promoters from across primate species, but other postulated transcription factor binding sites are not preserved. Differences between *2DL4* and clonally-restricted *KIR* promoters suggest a model that explains the unique *2DL4* expression pattern in human NK cells.

Keywords

Human; Natural Killer Cells; Cell Activation and Differentiation; Gene Regulation; Transcription Factors

INTRODUCTION

NK lymphocytes use MHC class I-specific clonally-restricted KIR (crKIR) receptors to distinguish healthy and aberrant cells in immunity to intracellular pathogens and in cancer surveillance (1). Inhibitory crKIR have cytoplasmic tails that contain ITIM motifs, which recruit SHP-1 and SHP-2. These proteins dephosphorylate Vav1 and disrupt actin polymerization needed for signaling from a variety of activating receptors (2). Activating crKIR are missing the ITIM motif because they have shortened cytoplasmic tails, but have a transmembrane region that contain a basic residue that recruits ITAM-containing proteins, which generate activating signals (1). In contrast to crKIR, the function of KIR2DL4 is less clear. However, *2DL4* is likely very important because of all KIR, 2DL4 is the only *KIR* gene with orthologs present in all hominid species and in old world monkeys, such as the rhesus monkey (3).

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Compared with crKIR, 2DL4 has unusual expression, cellular localization, structure, and function. Although both *crKIR* and *2DL4* expression is controlled by DNA methylation, *crKIR* expression is allele-specific while *2DL4* is bi-allelically expressed by all NK cells (4–9). crKIR expression is largely absent from immature $CD56^{bright}$ NK cells and is found on more mature $CD56^{dim}$ NK cells. By comparison, KIR2DL4 is expressed by $CD56^{bright}$ NK cells and expression declines with further NK cell maturation (10–12). Unlike most crKIR, 2DL4 is poorly expressed on the cell surface, but is strongly expressed in early endosomes (13). KIR2DL4 has both a cytoplasmic ITIM motif and a transmembrane arginine that associates with the activating effector molecule, FcεR1γ (14). Despite these dual activating and inhibitory structural features, 2DL4 usually transduces activating signals (10, 15). Distinct from activating crKIR, 2DL4 ligation weakly stimulates cytotoxicity (10, 16). Instead, 2DL4 ligation stimulates NK cells to produce a unique repertoire of inflammatory and angiogenic cytokines and chemokines, including IFN-γ, IL-6, IL-8, and IL-23 (13). The classical MHC class I (HLA-A, B, C) ligands for crKIR are widely expressed. In contrast, 2DL4 recognizes HLA-G, a non-classical MHC class I protein that is expressed by invasive fetal trophoblast cells, but few other cells (13, 17–19).

NK cells dominate the uterine leukocyte population during the early stages of pregnancy, where they may be involved in uterine tissue remodeling (20). Fetal trophoblast cells invade the uterine mucosa where they encounter maternal NK cells. These uterine NK cells secrete inflammatory and angiogenic factors that encourage trophoblast cell growth, differentiation, and migration, and spiral artery remodeling, all of which are important functions for successful pregnancy (20). Recent data suggests that 2DL4 may play a role in this process. Ligation of decidual NK cell 2DL4 with mAb or HLA-G induced production of IL-1β, IL-6, TNF-α, and IL-8 inflammatory cytokines and chemokine, which have been hypothesized to promote uterine remodeling (19). KIR2DL4 expression is not absolutely required for successful pregnancy, perhaps because other leukocyte receptors fill redundant functions (19).

In addition to sense transcription from the proximal promoter, *KIR* transcription may be regulated by more complex mechanisms. Several *KIR* genes are transcribed from distal promoters that are upstream of the more proximal promoters, including a promoter ~ 10 kb from the *2DL4* translation start site (21). Furthermore, both *crKIR* and *2DL4* proximal promoters are transcribed in both sense and antisense directions (22). However, the importance of these transcripts in *KIR* gene expression regulation has not been firmly established. Focusing on the proximal promoter, we reported that sense transcription of the *3DL1 crKIR* gene depends on five different transcription factors, each of which make a small contribution to full promoter activity (23). The *2DL4* promoter has about 60% sequence identity to the cr*KIR* promoters, including homologous transcription factor binding sites, suggesting both shared and distinct transcriptional control mechanisms (24). A previous report suggested that Runx factors inhibit *2DL4* expression (25), but stimulatory transcription factors have not been identified. Given the unique role of 2DL4 in NK cell biology, we performed a systematic, unbiased study of the *2DL4* promoter. We demonstrate that Runx transcription factors are absolutely required to stimulate *2DL4* expression. Our data also provide a plausible mechanism for differential *2DL4* and *crKIR* expression in NK cell development.

MATERIALS and METHODS

Cells and constructs

Human YT-HY cells (hereafter referred to as YT) were cultured as described (23). Human NKL cells were grown in suspension in RPMI 1640 (Invitrogen, Lonza) + 10% FBS (Hyclone) + 2 mM glutamine (Invitrogen) + 1 mM pyruvate (Invitrogen) + 200 U/mL IL-2

(NIH). Mouse LNK cells were grown as monolayers in RPMI 1640 + 10% FBS + 50 μM 2-mercaptoethanol (Invitrogen), nonessential amino acids (Invitrogen), + 20 mM HEPES (Invitrogen) + 200 U/mL IL-2. Human Jurkat, Hut-78, and CEM-T4 cells were grown in RPMI 1640 + 10% FBS. Human NK 92.26.5 cells were grown as previously described (26). Primary NK cells were enriched from peripheral blood by incubating them with antibody complexes bispecific for erythrocyte glycophorin A and leukocyte CD3, CD4, CD19, CD36, and CD66b (RosetteSep™; StemCell Technologies Inc.). Erythrocyte-leukocyte rosettes were removed by density gradient centrifugation through Lymphoprep™ (Axis-Shield); flow cytometry indicated that > 90% of enriched cells were NK cells. The *2DL4* promoter pGL3 reporter plasmid has been described (24) and includes bases −7 to −269 bp upstream of the *2DL4* ATG translational start site. We performed PCR-based mutagenesis on this plasmid essentially as described, including substitution of 24 consecutive 10 bp segments beginning 10 bp upstream of the ATG site (23). Putative transcription factor binding sites were identified using the TESS computer algorithm (http://www.cbil.upenn.edu/cgi-bin/tess/tess). Human Ets1, Ets2 expression constructs and the pSG5 parent vector were generous gifts of Dennis Watson (Medical University of South Carolina, Charleston, SC, USA). Human Runx1, Runx2, Runx3, and the mouse CBF beta expressing constructs in the pEF-Bos vector were generous gifts of Yoshiaki Ito (National University of Singapore). Surface 2DL4 was detected in flow cytometry with a PE-labeled mAb (347005 PE, Biolegend).

EMSA

YT cell nuclear extracts were prepared and EMSA was carried out as described (23). The Ab used for supershift studies were from Santa Cruz Biotechnology, CREB (186X), ATF-1 (270X), and Runx1 (8584X), or from Active Motif, Runx 2 (AML-3, 39302), Runx3 (AML-2, 39301).

Transient transfection

YT and HeLa cells were transfected as described (23). Other cells were transfected by electroporation using plasmid “midipreps” (Bio-Rad), including LNK (3×10^6 trypsinized cells, 5.0 μg luciferase plasmid). All other electroporation conditions included 20.0 μg luciferase plasmid with the indicated number of cells: NKL (20×10^6); Jurkat, Hut-78 and CEM-T4 (7.5×10^6). All electroporations included 100 ng CMV-Renilla or SV40-Renilla control plasmids and were conducted in a Bio-Rad Gene Pulser II (250 V, 600 μF) with a 2 mmgap cuvette (USA Scientific) in 400 μl conditioned media (including serum), with the exception of CEM-T4 cells, which were transfected in fresh RPMI 1640 without serum. After electroporation, cells were incubated in 50% fresh/50% conditioned media. Cell lysates were prepared ~40 h after electroporation and luciferase and Renilla activity was measured as described (23). Where indicated, at 24 and 12 h before harvest, IL-2 (40 and 20 U/ml, respectively) or IL-15 (10 ng/ml each time) was added to YT transfectants. Luciferase activity was divided by Renilla activity and this value was normalized to the value for the wild type promoter vector.

ChIP

Chromatin isolation and transcription factor immunoprecipitation was carried out using the EZ ChIP Protocol (Millipore) with modifications. 80×10^6 NK 92.26.5 cells [or $15 – 20 \times 10^6$ primary NK cells—values within brackets below refer to primary NK cells] were resuspended in 40 [10] mL NK-92 media (26) and treated with 1% formaldehyde (freshly prepared as an 18.5% stock solution from paraformaldehyde (Fisher) using the instructions provided in the kit) for 10 min at room temperature; the reaction was terminated by incubating on ice for 5 min after adjustment to 0.125 M glycine, 1 × EDTA-free Roche complete protease inhibitor cocktail (PIC), and 0.4% IPA saturated with PMSF (Sigma). All

subsequent steps were carried out at 4 °C: Cells were washed twice with 40 [10] mL of PBS containing 0.5 [1.0] × PIC and 0.4 % IPA/PMSF. The cell pellet was next resuspended in 1.6 [1.0] mL of Swelling Buffer (100 mM Tris, 10 mM KCl, 15 mM $MgCl_2$, 0.5% NP-40, pH 8.0) containing 1 × PIC and 0.4% IPA/PMSF. After 10 min, cells were sheared with 5 strokes from a glass Dounce homogenizer, centrifuged at 2500 × g for 5 min, and the supernatant was removed. The cell pellet was then resuspended in 600 [200] μl of Lysis Buffer (EZ-ChIP kit) with 1 × PIC and 0.4% IPA/PMSF, and 200 [50] μl of glass beads (106 microns, Sigma-Aldrich) was added. Chromatin was fragmented to an average size of 500 bp by sonicating (Branson 450 Sonifier) for 15 pulses (10 s each at 30% of the microtip limit with 1 min breaks), and then centrifuged at 14,000 × g for 1 h. 600 [200] μl supernatant was resuspended to a final volume of 6.0 [2.0] mL in ChIP Dilution Buffer (EZ-ChIP kit) with 1 × PIC and 0.4% IPA/PMSF. 600 [150] μl of Protein A agarose/salmon sperm DNA solution (Millipore) was added and the mixture was gently rocked for 2.5 h. The resin was removed by centrifugation and the chromatin was split into eight 720 μl [two 950 μl] aliquots, with extra chromatin saved as Input. 3.0 [2.0] μg of Ab were added to individual aliquots, including mouse anti-Runx3 (clone 5G4, D235-3 and clone 6E9, D234-3, from MBL), and several Ab from Santa Cruz Biotech: mouse anti-FLAG (G-8, 166384), rabbit anti-Ets1 (C-20, 350X), rabbit anti-FLAG (D-8, 807), and normal rabbit IgG (2027). After overnight incubation with rotation, 9.0 [6.0] μg of rabbit anti-mouse H + L chain secondary Ab (Millipore, m-06-371) was added to the aliquots with mouse Ab, and incubated with rotation for 1.5 h. The samples were centrifuged at 13,000 × g for 30 min to remove non-specific precipitates, and 60 [45] μl of protein A agarose was added to the supernatants and incubated with rotation for 1 h. The samples were centrifuged for 15 min at 6000 × g to pellet the resin, and the resin was washed once with 1 mL of buffers of increasing ionic strength, for 5 min: Low Salt Immune Complex Buffer, High Salt Immune Complex Buffer, LiCl Immune complex Buffer (EZ ChIP kit), and then 2 washes of TE Buffer. Chromatin bound to the resin was eluted with two 100 μl washes of room temperature elution buffer (0.1 M sodium bicarbonate + 1.0% SDS, pH 9.0). Crosslinks were released overnight at 65 °C. Protein was digested as described in the protocol and DNA was purified using QIAquick Spin (Qiagen) columns. Quantitative PCR was carried out using 1× SYBR Green JumpStart Taq Ready mix (Sigma) or SensiFAST SYBR Hi-Rox Kit (Bioline) on an ABI 7000 qPCR system or a Bio-Rad CFX96 Real-Time System. The primer sequences used were: *2DL4* promoter, sense: AATACATCAAATTTCCTCATGTGA, antisense: TCTGCTGCCAGGACGCAGTGA at 200 nM final concentration; *2DL4* Intron 4, sense: GTCACAGGTGAGGAAAGCCAAT, antisense: CCATGCTGCATCTTCTATCCA (200 nM), *KIR3DL1*F, GTGAAGGACGC GAGGTGTCAATTCTAGTGA<u>C</u>AG and *KIR3DL1*R, ACCTCTAGGC CCATATCTTTACCTCCA<u>A</u>GT (250 nM, underline bases indicate mismatches designed to improve specificity). For the *2DL4* promoter and intron 4, an initial denaturation step at 94 °C for 2 min, was followed by cycles of 15 s at 94 °C, 1 min at 63 °C, 1 min at 72 °C. For the *3DL1* promoter, the program was 95 °C for 2 min, and then cycles of 10 s at 95 °C, 10 s at 63 °C, 40 s at 72 °C The quantity of DNA in the samples was calculated from a 4 or 5 point standard curve generated from dilution from the Input sample; this standard curve typically indicated a ~ 100% amplification efficiency with $r^2 > 0.95$. In each case, agarose gels indicated a single product of the expected size.

Statistical testing

F-statistics indicated that luciferase and ChIP data showed equivalent variances after logarithmic transformation, allowing parametric testing. Data shown in Figures 2, 3A, 4A, 7, and 8 were tested using paired, two-tailed t tests. Data shown in figure 6 were tested using unpaired, two-tailed t tests. P values are listed in the figures and figure legends. All error bars represent SEM.

RESULTS

Systematic dissection of the 2DL4 promoter reveals few important cis-acting elements

KIR3DL1, a model *crKIR* gene, contains five promoter elements that are required for optimal transcription in NK cells: CRE, Ets, Runx, and Sp1 sites and an overlapping STAT/Ets/YY1 site (Fig. 1). Each of these elements made a relatively weak contribution to *3DL1* promoter activity, but mutation of all five sites abrogated *3DL1* promoter activity (23). Of these elements, only the Ets and Runx sites are also present at homologous *2DL4* locations (Fig. 1). Juxtaposed to the Ets site in the *2DL4* promoter is a second Runx site that is not found in *crKIR* promoters. Although the *2DL4* promoter does not contain the CRE site that is found in *crKIR* promoters, an inverted CRE site is present nearby (Fig. 1). This suggested that *2DL4* and *crKIR* promoters are activated by both shared and distinct transcription factors, as predicted by DNase I footprinting (24).

Because there has been limited testing of the transcription factors that drive *2DL4* expression, we directly tested the *2DL4* promoter. Using the unbiased linker scanning mutagenesis method, we replaced twenty-four 10-bp segments with a sequence that does not contain a known transcription factor binding motif (27). In contrast to deletions, substitutions do not affect promoter activity via phasing changes of transcription factor binding sites on the DNA helix (28, 29). We tested mutants in the YT NK cell line, which expresses endogenous 2DL4 (Fig. 2A and (30)) and in which expression of the *2DL4* promoter reporter is more than 20-fold greater than the reporter plasmid without insert (pGL3-Basic, Fig. 2B). Substitution of Segments 7, 10 and 12 increased promoter activity (Fig. 2B). The increased promoter activity could be due to removal of repressive elements or creation of new activating sites at the junction of the linker and the *2DL4* sequence (data not shown). In contrast, substitutions of Segments 1, 3 and 13 each significantly decreased *2DL4* promoter activity (about 50% for Segments 1 and 13). Because activating factors have not yet been identified for the 2DL4 promoter, we focused our investigation on the regions where substitution reduced promoter activity.

A CRE element is required for optimal 2DL4 promoter activity

Because *2DL4* Segment 13 contained a predicted CRE site (Fig. 1), we targeted this site with two point mutations. Both CRE mutations substantially decreased *2DL4* promoter activity (Fig. 3A). To further test the importance of the CRE site, we performed EMSA. YT NK cell nuclear factors formed several bands with the *2DL4* CRE probe and were specifically competed by unlabeled self oligonucleotide, but not by self oligonucleotide that contained a mutated CRE site (Fig. 3B). Furthermore, the nuclear complexes were supershifted by Ab to CRE-binding transcription factors, CREB and ATF-1. Thus, mutagenesis and EMSA studies indicate that optimal *2DL4* transcription, like *crKIR* transcription, requires a CRE site. Because *2DL4* and *crKIR* expression is controlled by DNA methylation (4–9), we tested CREB and ATF binding to a methylated 2DL4 probe. As expected from other gene studies (31), CREB and ATF-containing nuclear complexes poorly bound to the methylated 2DL4 probe. This result suggests that DNA methylation inhibits *2DL4* transcription, in part, by inhibiting CREB and ATF binding.

2DL4 core promoter elements

Substitution of Segment 1 significantly inhibited *2DL4* promoter activity. Because the TESS transcription factor binding program did not predict any high probability transcription factor binding sites in this region (32) and because *2DL4* transcription start sites are upstream of Segment 1 (24, 33), it was possible that Segment 1 contained elements of the core *2DL4* promoter. Core promoters can be classified into distinct types, depending on the sequence recognized by the general transcription factor TFIID of the RNA polymerase II holoenzyme

complex, including TATA and Initiator (Inr) sequences. Most (8 of 9) *crKIR* promoters contain a TFIIB recognition element (BRE) and an adjacent A-rich sequence (AAATAAC) that has been postulated to act as a variant TATA element ((24), Fig. 1, and data not shown). The *2DL4* promoter does not contain the BRE motif or the A-rich sequence (Fig. 1). Instead, *2DL4* transcription often initiates at or near potential Inr sequences in Segments 2–4 (Table III), with putative initiating adenine residues at −28, −32, −37, and −42 relative to the ATG translation start site (24, 33). The weak effect of substitutions in Segments 2, 3, and 4 is consistent with multiple Inr sites, each contributing to *2DL4* activity (Fig. 2). To test this hypothesis, we made point mutations in the putative Inr elements. *2DL4* promoter activity was decreased by 47% by the InrA mutation, which replaced initiator adenines of the four putative Inr sites (Table III). Mutation of adenine in a consensus Inr sequence may not reduce promoter function because alternative sites can substitute for mutated Inr sites (34, 35), and so we mutated additional bp surrounding the initiating adenines (InrB). This mutation decreased *2DL4* promoter activity by 60% (Table III). Statistically significant and substantial reduction, but not elimination, of *2DL4* promoter activity by the InrA and InrB mutations is consistent with an important role for Inr elements, but suggest involvement of other initiating sites. Indeed, *2DL4* transcription often initiates from sites distinct from the Inr elements (24, 33). We conclude that *2DL4* has functional Inr elements that contribute to promoter activity.

To investigate the hypothesis that *crKIR* promoters contain a functional TATA box, we purified recombinant human TBP and tested whether it would shift oligonucleotides containing either the *2DL4* or *3DL1* core promoter sequence, as visualized by EMSA. Although purified human TBP shifted the well-characterized adenovirus major late promoter TBP positive control (AdMLP, Table I), TBP did not shift a TBP negative control probe (36) or either of the KIR probes tested (Supplemental Fig. S1A). Addition of human TFIIB to the incubation also failed to result in a shift (Supplemental Fig. S1A). Although the *2DL4* and *crKIR* core promoters are distinct, our results do not support the hypothesis that *crKIR* promoters contain a functional TATA box.

We considered whether the Segment 1 substitution decreased *2DL4* promoter activity by eliminating other putative elements of the core promoter, such as elements with sequence homology to a Motif 10 Element and a Downstream Core Element (35). However, mutagenesis studies did not show evidence for the function of these sites (Supplemental Fig. S1B) and the role of Segment 1 in *2DL4* gene expression remains to be defined.

Runx/Ets interaction in the 2DL4 promoter

3DL1 promoter activity was affected by mutations in the Ets and Runx sites (23, 37). Both of these sites are preserved at homologous locations in the *2DL4* promoter (Fig. 1), so we expected that they would be required for optimal *2DL4* transcription. Surprisingly, *2DL4* promoter activity was not decreased by substitutions of Segment 5, which contained the Ets site, or of Segment 9, which contained the distal Runx site, homologous to the *3DL1* site (Fig. 2). We then further investigated the importance of the Ets and the two *2DL4* Runx sites. A 2-bp substitution targeting the Ets site that had diminished *3DL1* promoter activity by more than 60% did not decrease *2DL4* promoter activity (Fig.4A). Similarly, three mutations of the distal Runx site that had diminished *3DL1* promoter activity by 35–45% produced little or no decrease in *2DL4* promoter activity. We also tested the Segment 5 proximal Runx site that did not have a homologous site in *crKIR* promoters. A 2-bp substitution that replaced a GG dinucleotide present in nearly all Runx sites and a second 1-bp Runx substitution did not significantly reduce promoter activity (Fig. 4A and data not shown). Therefore, with the exception of the CRE site, none of the putative transcription factor binding sites shared with *crKIR* promoters appeared to be critical for *2DL4* promoter activity. We hypothesized that the Ets site interacts with the juxtaposed proximal Runx site,

and that mutation of both sites in the same construct maybe required to reveal an interaction. Combination of the Ets mutation and the proximal Runx mutation reduced *2DL4* promoter activity by 40% (Fig. 4A). The synergy between the mutations suggests that Runx and Ets family proteins interact at the compound proximal Runx/Ets site.

Given the ability of the Ets site to synergize with the proximal Runx site, we wished to further investigate whether Ets family members were involved. We had previously shown that YT NK nuclear complexes containing Ets family members, GABP and Elf1, bound the 3DL1 Ets site in EMSA (23). Although YT nuclear proteins bound the Ets probe and were competed by mutant, but not Ets-mutant competitor, neither GABP nor Elf1 binding was shown by Ab supershift (results not shown). This suggested that other Ets family members contribute to *2DL4* transcription. To investigate possible roles for Ets1 or Ets2, which are poorly detected in EMSA, we performed transactivation studies in HeLa cells, which are deficient in several lymphoid specific Ets factors (38, 39). We co-transfected HeLa cells with plasmids encoding either Ets1 or Ets2 together with the wild-type or Ets-site mutated *2DL4* promoter reporter plasmids. Both Ets1 and Ets2 increased transcription from the intact *2DL4* promoter relative to the Ets-mutated promoter (Fig. 4B). Ets1 and Ets2 transactivation of *2DL4* was at least as great as that of *3DL1* (data not shown). This indicated that the *2DL4* Ets site is functional and responsive to both Ets1 and Ets2, although it does not rule out a potential role for other Ets family members.

Runx transcription factors are critical for 2DL4 promoter activity

We tested possible redundancy of the two Runx sites in the *2DL4* promoter. Combined mutation of both proximal and distal Runx sites reduced luciferase activity nearly to that of the pGL3-Basic empty vector (Fig. 4A). These results suggest that Runx transcription factors, acting at either of the two promoter sites, are absolutely required for *2DL4* transcription in NK cells. To further explore a role for the two Runx sites, we tested the ability of YT NK cell nuclear proteins to bind to proximal and distal Runx probes, comparing 2DL4 probes to probes from homologous regions of the *3DL1* promoter (Fig. 5A). Several YT NK cell nuclear protein complexes bound the 2DL4 and 3DL1 distal Runx probes in EMSA (Fig. 5B) and were competed by unlabeled self oligonucleotides, but not by Runx-mutated oligonucleotides. Ab specific for Runx2 and Runx3, but not Runx1, supershifted both 2DL4 and 3DL1 (Fig. 5B). These results indicate that YT NK cell Runx2 and Runx3 proteins bind the homologous distal Runx sites in the *2DL4* and *3DL1* promoters, regardless of non-identical flanking sequences. Runx2 and Runx3 also bound the 2DL4 proximal Runx probe (Fig. 5C). As expected, Runx proteins did not bind a homologous 3DL1 probe that did not contain a Runx motif (Fig. 5C). Therefore, the *2DL4* promoter differs from *crKIR* promoters in having two functional Runx sites. To test which Runx transcription factors can activate the *2DL4* promoter, we investigated *2DL4* promoter activity in HeLa cells, which do not express endogenous Runx factors (40). We co-transfected intact and Runx-mutated *2DL4* promoter plasmids with expression plasmids for Runx1, Runx2, or Runx3, together with CBFβ, the Runx heterodimer partner protein (41). All three Runx factors greatly stimulated transcription from the *2DL4* promoter, and Runx transactivation was substantially lower with Runx-mutated *2DL4* promoters (Fig. 5D). These results show that Runx factors act directly on the *2DL4* promoter. Interestingly, mutation of either the proximal or distal Runx site significantly decreased Runx transactivation, although mutation of both sites was even more destructive. These results reinforce mutation and EMSA findings, showing that Runx transcription factors are critical for *2DL4* promoter activity.

DNase I protection experiments (24) and our own TESS search had suggested additional *2DL4* promoter cis-acting sites: CP2, myogenic differentiation antigen 1, AP-1, interferon regulatory factor 2, and GATA-3 sites located in Segments 4, 4, 16, 21, and 21, respectively.

We found that mutation of these specific sites, produced little or no reduction in *2DL4* promoter activity (Supplemental Fig. S1C).

Distinct transcription factors act on the 2DL4 promoter in IL-2/15-treated lymphocytes

NK cells require IL-15 signals for maturation, proliferation and survival. Moreover, 2DL4 expression is relatively high in immature CD56bright NK cells that have recently developed from $CD122^+$ NK precursor cells under the influence of IL-2 and IL-15 in the lymph node and the bone marrow, respectively, and NK cell 2DL4 levels increase with cytokine stimulation (10–12, 42). To test whether the *2DL4* promoter uses distinct transcription factors in the presence of cytokines, we used YT NK cells that respond to both IL-2 and IL-15 ((43) and data not shown). The *2DL4* promoter was 1.55-fold more active with IL-2 stimulation (data not shown). Comparing wild type and mutated *KIR* promoters under the same cytokine condition, the 24 *3DL1* segment substitutions showed little or no relative change in promoter activity in the presence vs. the absence of IL-2 (Supplemental Fig. S1D). For the *2DL4* promoter, 22 of 24 segment substitutions showed little or no IL-2 effect, including Segment 9 (distal Runx) and Segment 13 (CRE). However, substitutions of Segment 5 (Ets) and Segment 6 (proximal Runx) significantly reduced relative transcription only when tested in IL-2-treated YT cells (Fig. 6A).

To confirm that the compound Ets and proximal Runx site is important in the context of cytokine treatment, we tested point mutations in either IL-2- or IL-15-treated YT cells. Consistent with the Segment 5 substitution data, the Ets mutation strongly reduced *2DL4* promoter activity only in the presence of IL-2 or IL-15 treatment (Fig. 6B). This indicates that Ets transcription factors are much more important for *2DL4* transcription in the presence of IL-2/15 than constitutive *2DL4* transcription in YT NK cells. Mutation of the proximal Runx site also reduced *2DL4* promoter response to IL-15 (Fig. 6B). As before, the combination of the Ets mutation and the proximal Runx mutation reduced constitutive *2DL4* promoter activity, which was relatively more reduced in the presence of IL-2 or IL-15. As a control, we tested three Segment 9 distal Runx mutations under the same conditions. The relative response was similar in the presence or absence of cytokine stimulation (Fig. 6B). Therefore, although the distal and proximal Runx sites have identical sequence motifs, mutations directed at the distal Runx site did not prevent the *2DL4* promoter from responding to IL-2 or IL-15.

Use of 2DL4 promoter elements in T and NK cell lines

Because *2DL4* is expressed by both mature and immature NK cells and by a subset of T cells, we tested several NK and T cell lines. Mouse LNK cells are a model of immature NK cells and human NKL cells are models of mature cytotoxic NK cells (44, 45). Both NK cell lines require cytokine for growth. Hut-78 is a model of KIR-expressing $CD4^+$ T cells. All cell lines, with the exception of Hut-78, were tested while being treated with IL-2 (Fig. 7). YT, LNK, NKL, and Hut-78 cells required the CRE site for optimal *2DL4* promoter activity, showing that CRE-binding elements contribute to *2DL4* transcription under all conditions tested. Similar to IL-2-treated YT cells, but distinct from untreated YT cells, the other NK and T cell lines required the Ets site and the proximal Runx site for optimal *2DL4* promoter activity (Fig. 7). Therefore, the compound proximal Runx/Ets site plays a unique and nonredundant role in cytokine-stimulated NK cells and in T cells. To further investigate a role for the distal Runx site, we mutated both Runx sites (Fig. 7). Combined mutation of both sites virtually eliminated 2DL4 promoter activity in all cell lines tested, showing that at least one *2DL4* promoter Runx site is required by NK and T cells. Similar results were seen in untreated CEM-T4 and Jurkat T cell lines (data not shown). We conclude that Runx transcription factors are absolutely required for *2DL4* transcription in both NK and T cell lines, in both resting and cytokine-stimulated conditions.

Runx3 and Ets1 bind to the 2DL4 promoter in situ

We carried out ChIP using Ab specific for Runx1 and Runx3, because these two isoforms are well expressed while Runx2 is poorly expressed in mature NK cells (46–48). First, we tested the IL-2-dependent NK92.26.5 cell line that expresses both *2DL4* and *3DL1*. Runx3 associated with the *2DL4* promoter, as shown with two anti-Runx3 mAb (5G4 and 6E9), but not with anti-Runx1 Ab (Fig. 8A and data not shown). Ets1 also associated with the *2DL4* promoter in IL-2-treated NK92.26.5 cells (Figure 8B). Runx3 and Ets1 association was significant in comparison with a control genomic location (*2DL4* intron 4, Fig. 8). We did not observe an association of Runx3 and Ets1 with the *3DL1* promoter in 3DL1-expressing NK92.26.5 cells (data not shown). We also tested whether Runx3 bound to the endogenous 2DL4 promoter in primary NK cells. To preserve chromatin structure, NK cells were purified from peripheral blood by buoyant density negative selection and fixed immediately after isolation. We found that Runx3 associated with the *2DL4* promoter, and that association was significant in comparison with a control genomic location (*2DL4* intron 4, Fig. 8C).

DISCUSSION

Alignment of the *2DL4* and *3DL1* promoters (Fig. 1) suggests that while the Ets and distal Runx sites are conserved in human *KIR* promoters, many potential *2DL4* sites (proximal Runx, CRE, AP-1) are not found at homologous positions in *crKIR* promoters. We identified potential *cis*-elements and corresponding *trans* factors required for optimal *2DL4* promoter activity in a systematic and unbiased fashion using linker scanning mutagenesis. Tentatively identified factors were confirmed in point mutation, EMSA, co-transfection, and ChIP experiments. For optimal *2DL4* promoter activity, a Runx family member is absolutely required at one of two redundant Runx sites, the CRE site and Inr sites are important, and a compound proximal Runx/Ets site is important only in certain contexts, such as T cell and cytokine-treated NK cell lines. The roles of the *2DL4* Runx, CRE, and Ets sites are supported by an examination of orthologous *2DL4* promoter sequences in chimpanzee, orangutan, and the more distantly related rhesus monkey (Fig. 9). The distal Runx site and the Ets site are perfectly conserved in these primate *2DL4* promoters. Although a CRE site is not present in a homologous position in the rhesus *2DL4* promoter, a CRE site appears in the same position and having the same sequence as human *crKIR* promoters, which was shown to be functional (23). The rhesus *2DL4* promoter does not contain a proximal Runx site and we predict that Runx proteins stimulate *2DL4* transcription through the distal Runx site in rhesus monkey NK cells. Based on DNase I footprinting, GATA-3, LEF-1/TCF-2, IRF-2, and AP-1 sites were hypothesized to regulate *2DL4* transcription (24). With the exception of AP-1, these transcription factor consensus motifs are not well matched in the human *2DL4* promoter and diverge even more from consensus in the rhesus monkey *2DL4* promoter (Figure 9 and data not shown). This observation is consistent with our mutagenesis studies showing that these sites are not required for *2DL4* promoter activity (Supplemental Figure S1C). Thus, primate *2DL4* promoter alignment supports our conclusion that *2DL4* transcription requires Runx protein binding, along with a partial dependence on Ets and CRE sites. In addition, *KIR2DL5* alleles that do not have a functional distal Runx promoter site are not expressed by NK cells (6). Hence, the distal Runx site is required for in vivo *crKIR* expression, possibly through an epigenetic mechanism (6).

The three Runx homologs (Runx1, 2, 3) act as activators or repressors, depending on the context in which they are bound. Runx proteins dimerize with CBFβ to achieve a high affinity DNA interaction and functional activity (41, 49, 50). Runx factors likely are required for NK cell development because reduction of CBFβ activity to 15% of WT levels in mouse fetal liver cells almost completely eliminated the production of immature and mature NK cells (47, 49). Furthermore, transgenic expression of a dominant negative Runx

protein reduced mature NK cell numbers and reduced expression of proteins required for NK function (46, 49). During mouse and human NK cell development, Runx1 levels remain relatively constant while Runx3 levels increase steadily, so that Runx3 becomes the dominant Runx family member expressed in mature NK cells (46–49). Additionally, mature human NK cells express Runx3 from a distal promoter that is associated with robust protein translation (48). Despite the importance of Runx factors in NK cell development, very few NK cell genes are known to be controlled by Runx factors. Our report provides evidence that Runx3 binds to the *KIR2DL4* promoter in human primary NK cells and activates its transcription.

Trompeter et al. reported that point mutation of the distal Runx site increased promoter activity 2-fold in NK3.3 cells and concluded that Runx *represses 2DL4* transcription (25). Based on multiple lines of evidence, we found that Runx proteins *activate 2DL4* transcription in several different settings. It is not clear why our findings differ because we tested the same mutation (dRx3) as reported by Trompeter et al. It should be noted that Trompeter et al. achieved *2DL4* promoter activity that was no more than 3-fold greater than that of the pGL3 Basic empty vector. In contrast, *2DL4* promoter activity was 10–35-fold above that of pGL3 Basic vector in the IL-2-treated NK cell lines that we tested. We conclude that Runx proteins are required for *2DL4* transcription.

KIR2DL4 gene expression differs significantly from that of *3DL1* and other *crKIR* genes. *2DL4* is expressed earlier in development and may be a prerequisite for *crKIR* expression in NK cells and CD8 T cells. Although both *2DL4* and *crKIR* transcription are controlled by promoter DNA methylation, *2DL4* is bi-allelically expressed in all NK cells, but *crKIR* have variegate expression that is typically mono-allelic (4–9). Our analysis of the *2DL4* promoter shows important differences with *crKIR* promoters and may explain how expression is differentially regulated. Despite overall sequence conservation of human *KIR* promoters, Runx elements are absolutely required for *2DL4* promoter activity, whereas Runx transcription factors make only a small contribution to *3DL1* promoter activity (23). This may be related to the observation that *crKIR* promoters, but not the *2DL4* promoter, contains an active Sp1 site and an overlapping STAT/Ets/YY1 site. Alternatively, the strong importance of Runx for the 2DL4 promoter may be related to the fact that *2DL4*, unlike *crKIR* promoters, contains redundant Runx sites. Given that the redundant Runx sites are critical elements required for *2DL4* promoter activity, we propose that Runx3 is a major driver of *2DL4* transcription in mature NK cells. In a second contrast with *crKIR*, *2DL4* promoter activity is increased by IL-2 and IL-15 cytokine treatment, an increase that depends upon the *2DL4* compound proximal Runx/Ets site that is not found in *crKIR* promoters. We propose that the compound site serves multiple roles. In mature NK cells, cytokine-stimulated Ets1 binding increases Runx3 driven expression in an additive manner (our findings). The site also has the potential to promote Runx1/Ets1 synergy (50). Therefore, the compound Runx/Ets site may be especially important for initiating *2DL4* transcription in pre-NK cells or early NK cells, when Runx1 is relatively more abundant (46, 47, 49). In parallel with the *TCR*α enhancer and the *TCR V*β 8.1 promoter (51–53), Runx1/Ets1 protein-protein contacts and synergistic DNA binding, in cooperation with CREB or ATF, may allow assembly of a three-dimensional multiprotein-DNA complex at the *2DL4* promoter that is not possible at *crKIR* promoters during early NK cell development, because the *crKIR* promoters lack a compound Ets-Runx site. In contrast to these *TCR* elements, however, there is much less cooperativity between CRE, Runx, and Ets factors at the *2DL4* promoter. Mutation of *TCR*α enhancer CRE, Runx, or Ets site alone nearly abrogated enhancer activity in transfected T cells (51), but mutation of *2DL4* CRE, Runx, or Ets site alone had much smaller effects in transfected NK cells. Therefore, although the *TCR*α enhancer, the *TCR*β promoter, and the *2DL4* promoter share many cis-acting sites, their three-dimensional structures likely differ.

Based on our results that define the requirements for steady state and cytokine-induced transcription, we propose a model for initiation and maintenance of *2DL4* transcription. IL-15 and IL-2 signaling in bone marrow and lymph node, which are required for maturation of pre-NK cells into CD56^{bright} NK cells, increase Ets1 levels and phosphorylate CREB (54, 55). We speculate that Runx1, which is expressed in both pre-NK cells and NK cells (46, 47, 49), forms complexes with Ets1 and CREB at the *2DL4* promoter via protein-protein interactions (50, 51, 56), compensating for CREB's poor affinity for methylated DNA (our findings and (31)). Transcription factor binding is known to block DNMT1 binding, which passively prevents methylation of replicating DNA in dividing cells and we propose a similar mechanism for *2DL4* (57). In our model, these transcription factors recruit CBP/p300, which acetylates and further stabilizes Runx (58–61). CBP/p300 also is known to acetylate histones, open chromatin, and induce transcription (62). Given that CD56^{bright} NK cells express high affinity IL-2 receptors and are sensitive to low IL-2 concentrations (63), we propose that Ets1 and Runx protein cooperation at the *2DL4* promoter plays a role in cytokine-stimulated immature NK cells, and, in part, explains why CD56^{bright} NK cells express relatively high levels of 2DL4 (10–12). In mature CD56^{dim} NK cells that are less sensitive to IL-2 signaling, we propose that *2DL4* transcription is maintained at a relatively lower level, largely under the influence of Runx3 and possibly a CRE-binding transcription factor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ATF-1	Activating Transcription Factor-1
BRE	TFIIB Recognition Element
CBFβ	Core Binding Factor β
ChIP	chromatin immunoprecipitation
CRE	cyclic AMP response element
crKIR	clonally-restricted KIR
Inr	initiator element
IPA	isopropyl alcohol
KIR	killer cell Ig-like receptor
PIC	protease inhibitor cocktail
TBP	TATA-binding protein

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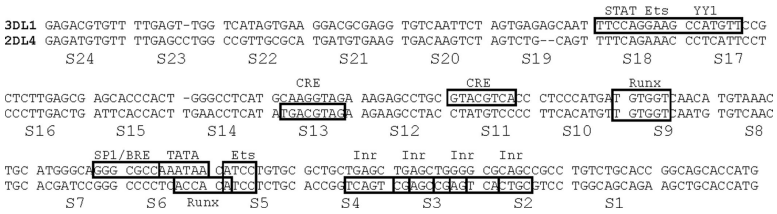

Figure 1.

3DL1 and *2DL4* promoters share overlapping but distinct sets of potential *cis*-acting elements. Shown are aligned *3DL1* (top) and *2DL4* (bottom) promoter sequences. Twenty-four contiguous *2DL4* 10-bp segments (denoted S1–S24) were replaced with the linker sequence, GCAGATCCGC. Putative *cis*-acting elements are denoted by boxes. The sequences end at the ATG translational start site.

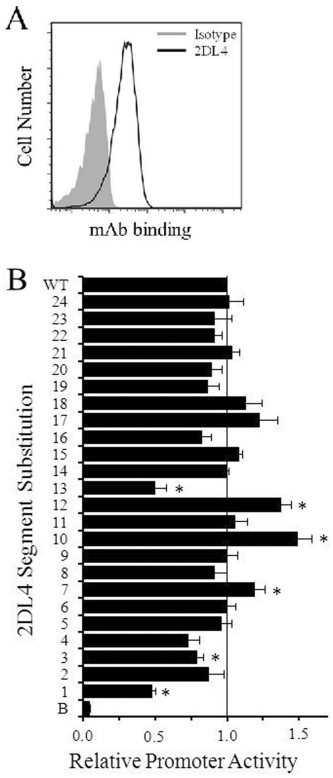

Figure 2.

A. YT cells express high levels of 2DL4. YT cells were treated either with a PE-labeled mAb specific for 2DL4 (solid line) or with an isotype control (shaded) and surface staining was detected by flow cytometry. B. Linker-scanning mutagenesis identifies few *2DL4* promoter *cis*-acting sites. YT cells were transfected with the indicated *2DL4* substitution, numbered as in Fig. 1. WT, wild type. B, background level produced by the empty pGL3-basic vector. Values represent averages from tests of 3–8 different plasmid preparations (each measured in duplicate). Asterisks indicate significant differences from WT ($p < 0.05$).

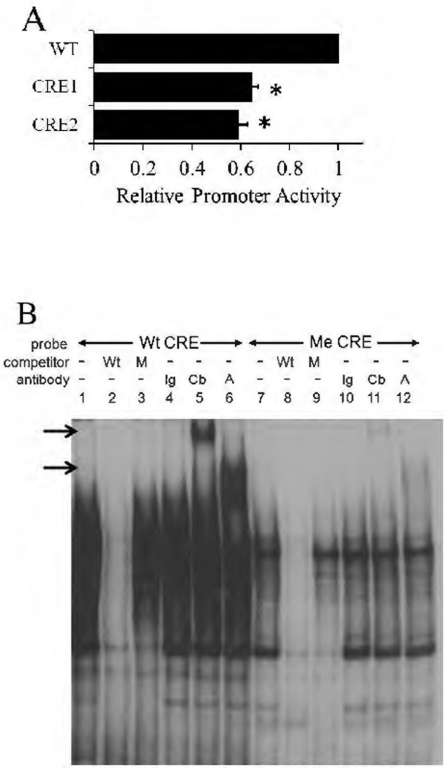

Figure 3.

An unmethylated CRE site contributes to *2DL4* promoter activity. A. *2DL4* promoter activity is reduced by mutations affecting the CRE site. Shown is *2DL4* promoter activity without (WT) and with CRE site point mutations (CRE1 and CRE2, see Table II). Promoter activity was measured as described in Fig. 2, and asterisks indicate significant differences from WT ($p < 0.01$). B. CpG methylation diminishes *2DL4* promoter DNA binding to CREB and ATF-1 in EMSA. YT nuclear extract was incubated with an unmethylated (Wt CRE, lanes 1–6) or a CpG methylated (Me CRE, lanes 7–12) probe encompassing the CRE site, either alone (lanes 1, 7) or with 150-fold excess of self-competitor (Wt, lanes 2, 8) or competitor with a mutated CRE site (M (CREm2, Table I), lanes 3, 9). Alternatively, nuclear extracts were pre-incubated with non-specific rabbit IgG (Ig, lanes 4, 10), or with Ab to CREB (Cb, lanes 5, 11) or to ATF-1 (A, lanes 6, 12) as indicated. The arrows indicate supershifted bands. The experiment shown is representative of three independent experiments with similar results.

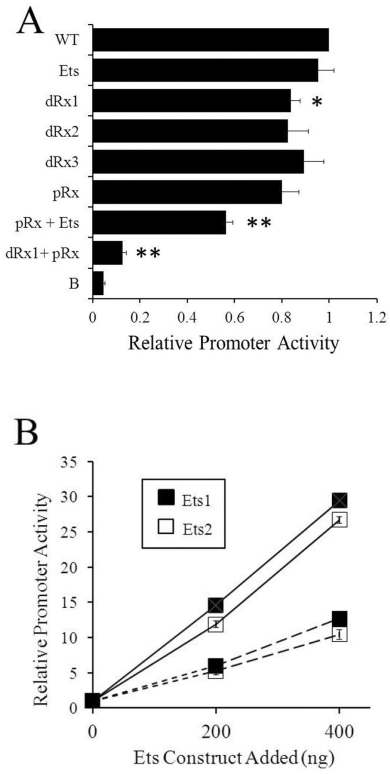

Figure 4.

2DL4 promoter activity depends on redundant activating sites. A. The activities of single site promoter mutations in Ets, distal Runx (dRx1, dRx2, dRx3), and proximal Runx (pRx) sites (described in Table II), and combinations of these mutations are shown, along with empty vector pGL3 Basic background activity (B). Promoter activity was measured as described in Fig. 2. Asterisks indicate significant differences from WT (*, $p < 0.005$; ** $p < 0.0005$). B. The *2DL4* Ets site is functional. HeLa cells were transfected with 1 ng of the control SV-40 Renilla luciferase construct, either 0, 200, or 400 ng Ets1 (solid square) or Ets2 (open square) expression plasmids and 1.5 ug *2DL4* promoter reporter that was either wild type (solid line) or mutated at the Ets site (dashed line). Empty pSG5 plasmid DNA was added as needed to equalize the total amount of transfected DNA. Each point represents the average of five tests; each test had a separate reporter plasmid preparation and one of three different Ets expression plasmid preparations. Shown is one representative experiment of three with similar results. Error bars (not always visible) show within-experiment SEM.

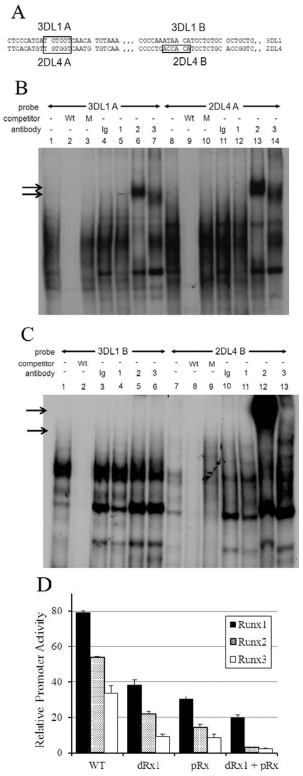

Figure 5.

Runx transcription factor family members bind the *2DL4* promoter at two functional sites. A. Shown are sequences surrounding the *2DL4* distal (left) and proximal (right) Runx sites and the aligned *3DL1* promoter sequences. Boxes denote Runx motifs and the sequences shown denote EMSA probes. B. Runx2 and Runx3 bind to *2DL4* and *3DL1* distal Runx sites. YT nuclear extract was incubated with either 3DL1 (3DL1 A, lanes 1–7) or 2DL4 (2DL4 A, lanes 8–14) probes encompassing the distal Runx site either alone (lanes 1, 8) or in the presence of 150-fold excess of self-competitor (Wt, lanes 2, 9) or competitor with a mutated Runx site (M, lanes 3 and 10 (mut 3DL1 A, mut 2DL4 A, Table I)). Alternatively, probe was added to nuclear extracts that had been pre-incubated with non-specific rabbit IgG (Ig, lanes 4, 11), or with Ab to Runx1 (1, lanes 5, 12), Runx2 (2, lanes 6, 13) or Runx3 (3, lanes 7, 14) as indicated. Arrows indicate supershifted bands. C. Runx2 and Runx3 bind to the *2DL4* proximal Runx site but not to the *3DL1* aligned region. YT nuclear extract was incubated with either a 3DL1 (3DL1 B, lanes 1–6) or 2DL4 (2DL4 B, lanes 7–13) probe encompassing the proximal Runx site (2DL4) or the aligned 3DL1 sequence alone (lanes 1, 7) or in the presence of 150-fold excess of self-competitor (Wt, lanes 2, 8) or a competitor with a mutated proximal Runx site (M (mut 2DL4 B, Table I) lane 9). Alternatively, nuclear extracts were pre-incubated with IgG (Ig, lanes 3, 10), or with Ab to Runx1 (1, lanes 4, 11), Runx2 (2, lanes 5, 12) or Runx3 (3, lanes 6, 13) as indicated. For B. and C., the experiments shown are representative of three independent experiments with similar results. D. Both proximal and distal Runx sites are functional. HeLa cells were transfected with 1 ng of a control SV-40 Renilla luciferase construct, 500 ng CBFβ expression plasmid, 500 ng Runx1, Runx2, or Runx3 expression plasmids as indicated, and 1.5 ug *2DL4* promoter reporter that was either wild type (WT), mutated at the proximal Runx site (pRx), at the distal Runx site (dRx1), or at both Runx sites (pRx + dRx1, mutations described in Table II). Each group represents the average of five tests; each test had a separate reporter plasmid preparation and one of three different Runx1, Runx2 or Runx3 expression plasmid preparations. Error bars

denote within-experiment SEM. Shown is one representative experiment of two with similar results.

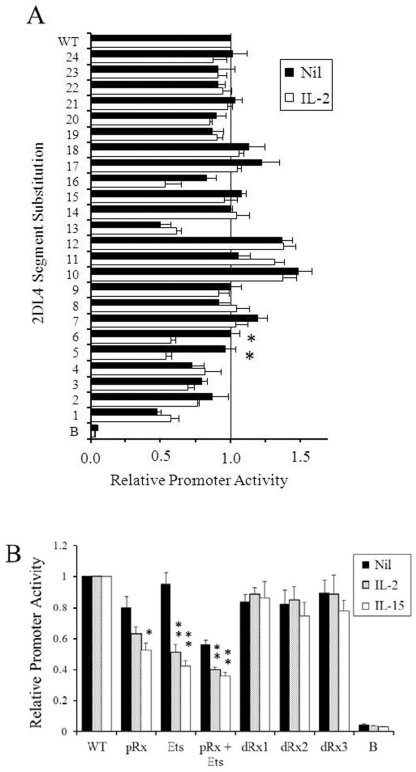

Figure 6.

Distinct *2DL4* promoter transcription factor requirements in constitutive and cytokine-treated cells. A. IL-2 treatment activates Segment 5 and 6 sites. YT cells transfected with *2DL4* segment substitution mutant reporter plasmids were either IL-2 treated or not treated (Nil), as described in Methods. Promoter activity was measured as described in Fig. 2. Asterisks indicate significant differences between the indicated groups (*, $p < 0.0005$). WT and B are described in Fig. B. IL-2 and IL-15 specifically activate the compound Ets/Runx site. YT cells transfected with *2DL4* promoter reporter plasmids with mutations in either the proximal Runx (pRx), Ets (Ets), proximal Runx and Ets site (pRx + Ets), or in the distal Runx site (dRx1, dRx2, dRx3, mutations described in Table II) were treated with IL-2, IL-15, or not treated (Nil) as described in Methods. Promoter activity was measured as described in Fig. 2. Asterisks indicate significant differences between the indicated groups (*, $p < 0.05$; ** $p < 0.0005$). WT and B are described in Fig. 2.

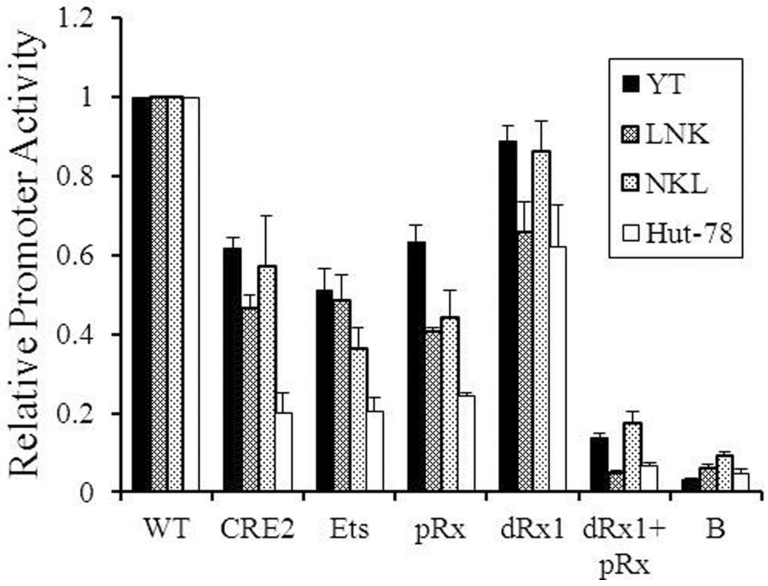

Figure 7.

CRE, Ets, and Runx sites are required for full *2DL4* promoter activity in NK and T cells. YT (black), LNK (dark gray), NKL (light gray), and Hut-78 (white) cell lines were transfected with *2DL4* reporter plasmids with mutations to either the CRE (CRE2), Ets (Ets), proximal Runx (pRx), distal Runx (dRx1), or combined distal and proximal Runx mutations (dRx1 + pRx, mutations described in Table II), and were cultured with IL-2 (with the exception of Hut-78) as described in Methods. Promoter activity was measured as described in Fig. 2. With the exception of CRE2 and dRx1 for NKL, all mutations had significant declines in activity compared to wild type ($p < 0.05$). WT and B are described in Fig. 2.

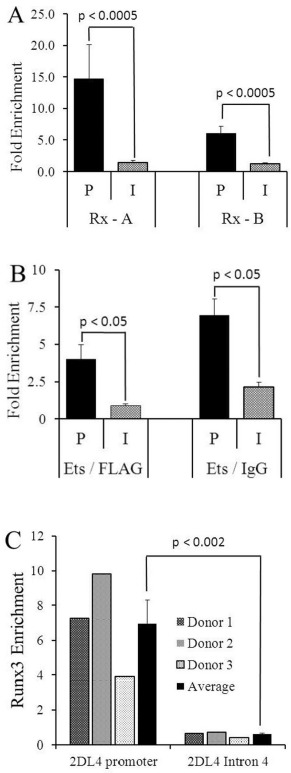

Figure 8.

Runx3 and Ets1 specifically bind to the endogenous *2DL4* promoter. Cross-linked chromatin was purified from IL-2-cultured NK92.26.5 (A, B) or freshly-isolated primary NK (C) cells and immunoprecipitated with Ab to Runx3, Ets1, or with negative control Ab. DNA from immunoprecipitates was purified and qPCR amplified using primers specific either to the *2DL4* promoter (P) or *2DL4* intron 4 (I), as indicated. A. ChIP was performed with anti-Runx3 mAb 5G4 (Rx-A) and 6E9 (Rx-B), and negative control mAb (anti-FLAG). Values represent the average enrichment of DNA bound to specific Ab (Runx/FLAG) from tests of five different chromatin preparations. B. ChIP was performed with anti-Ets1 Ab and negative control Ab, either rabbit anti-FLAG or normal rabbit IgG. Values represent the average enrichment of DNA bound to Ab (Ets/FLAG or Ets/IgG) from tests of at least three different chromatin preparations. C. ChIP was performed with anti-Runx3 mAb 5G4 and a negative control mAb (anti-FLAG). Values represent the enrichment of DNA bound to specific Ab (Runx 5G4/FLAG) using chromatin preparations from three different human donors. Averages and p values are shown.

Figure 9.

CRE, Ets, and distal Runx promoter sites are present in *2DL4* primate orthologs. Chimpanzee (C), orangutan (O), and rhesus monkey (R) promoter sequences were aligned to the human (H) sequence (−225 to +3) using CLUSTALW2. Putative *cis*-acting elements are denoted by boxes. Chimpanzee, orangutan, and rhesus monkey sequences had 98%, 95%, and 69% identity, respectively, to the human sequence.

Table I

2DL4 EMSA Probes and Competitors

Reagent	Oligonucleotide Sequence
Wt CRE	GGA CCT CAT ATG ACG TAG AAG AAG CC
Me CRE	GGA CCT CAT ATG A**C**G TAG AAG AAG CC
CRE m2	GGA CCT CAT ATA ATG TAG AAG AAG CC
3DL1 A	GGC TCC CAT GAT GTG GTC AAC ATG TAA
Mut 3DL1 A	GGC TCC CAT GAA CTA GTC AAC ATG TAA
2DL4 A	GGT TCA CAT GTT GTG GTC AAT GTG TCA A
Mut 2DL4 A	GGT TCA CAT GTA CTT GTC AAT GTG TCA A
3DL1 B	GGC GCC AAA TAA CAT CCT GTG CGC TGC TG
2DL4 B	GGC CCC TCA CCA CAT CCT CTG CAC CGG TC
Mut 2DL4 B	GGC CCC TCA CTA CAT CCT CTG CAC CGG TC
AdmLP	GGC TGA AGG GGG GCT ATA AAA GGG GGT GGG GG
TBP negative control	GGG CTG CGC CGG CTG TCA CGC CAG GCT GCG CC
3DL1 core	GGG GGC AGG GCG CCA AAT AAC ATC CTG TGC GC
2DL4 core	GGG ATC CGG GCC CCT CAC CAC ATC CTC TGC AC
2DL4 BTE	GGG ATC CGG GCG CCA AAT AAC ATC CTC TGC AC

Underling denotes mutated bases, bold lettering denotes 5-methylcytosine.

Table II

KIR2DL4 mutations

Site	Motif[a]	KIR Sequence[b]	Mutated Sequence[c]
Segments 1–24			GCAGATCCGC
CRE1	TGACGTCA	TGACGTAG	TAACTTAG; −137, −134
CRE2	TGACGTCA	TGACGTAG	TAATGTAG; −137, −135
MTE/DPE	CSARCSSAAC/RGWYVT	CTGGCAGCAGAAGC	CTGGTAGGAGTAGC; −16, −13 −10
DCE1	AGC (segment 1 of DCE)	AGC	TTT; −15 to −13
DCE2	AGC (segment 1 of DCE)	AGC	ACC; −14
Ets	CMGGAWGY[d]	ACATCCTC	ACATAATC; −56, −55
dR×1	TGTGGT	TGTGGT	ACTTGT; −100, −99, −97
dR×2	TGTGGT	TGTGGT	TGTTGT; −97
dR×3	TGTGGT	TGTGGT	TGTAGT; −97
pRx	TGTGGT	ACCACA	ATAACA; −62, −61
pRx + Ets	CCACWTCCT[d,e]	CCACATCCT	TAACATATT; −62, −61, −56, −55
CP2	CNRG(N)$_{5–6}$ CNRS	CCGGTCAGTCGAGCCGAG	CCGATCATTCGAGCCGAG; −45, −41
MyoD	CANNTG	CAGTCG	TAGTCT; −43, −38
AP1-A	TGASTCA	TGATTCA	GGCTTCA; −161, −159
AP1-B	TGASTCA	TGACTGATTCA	TGTCGGCTGCA, −163, −161, −159, −157
IRF-2	AAANYGAAA	GAAGTGACAA	GAGGTGTCAA; −211, −207
GATA-3	WGATAR	TGATGT	TAACGT; −218, −216

Footnotes:

[a] W = A, T; M = A, C; N = A, C, G, T; R = A, G; V =A, C, G; Y = C, T; S = C, G.

[b] Underlining denotes mutation target

[c] Underlining denotes new base at the position indicated

[d] Reference (56)

[e] Motif on antisense strand

Table III

Inr Mutations Decrease *2DL4* Promoter Activity

Construct	Sequence	Activity (SEM)
WT	tcAgtcGagccgagtcActGc	1.0
InrA	tcGgtcgGgccgGgtcGctgc	0.53 (0.045)*
InrB	tcGgCGgTgccgagtcGcGgc	0.40 (0.021)*

Shown are *2DL4* promoter sequences −44 to −24 with initiating adenine residues of putative Inr sites (YYANWYY motif) underlined and major transcription start sites identified by Radeloff et al (33) are shown in capital letters in the wild type (WT) sequence. The InrA and InrB substitutions are indicated by capital letters. The relative promoter activity represents averages from tests of at least 10 different plasmid preparations (with SEM).

*Significantly different from WT, $p < 1 \times 10^{-5}$.