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Different and Divergent Regulation of the KIR2DL4 and KIR3DL1 Promoters

C. Andrew Stewart, Jeroen van Bergen, and John Trowsdale

The killer Ig-like receptors (KIR) are a family of highly related MHC class I receptors that show extreme genetic polymorphism both within the human population and between closely related primate species, suggestive of rapid evolutionary diversification. Most KIR are expressed in a variegated fashion by the NK population, giving rise to an NK repertoire of specificities for MHC class I. We compared the promoter for KIR3DL1, which exhibits variegated gene expression, with that for KIR2DL4, which is expressed by all NK cell clones. Maximum transcriptional activity of each was encoded within ~270 bp upstream of the translation initiation codon. The KIR2DL4 promoter drove reporter gene expression only in NK cells, while the KIR3DL1 promoter was active in a range of cell types, suggesting that the latter requires other regulatory elements for physiological expression. In NK cells, reporter gene expression driven by the KIR2DL4 promoter was greater than that driven by the KIR3DL1 promoter. DNase I footprinting revealed that transcription factor binding sites differ between the two promoters. The data indicate that while the promoters of these two KIR genes share 67% nucleotide identity, they have evolved distinct properties consistent with different roles in regulating the generation of NK repertoire. The Journal of Immunology, 2003, 170: 6073-6081.

natural killer cells were initially described as cells capable of direct cytolysis of target cells in the absence of prior stimulation (1). A critical early observation was the correlation of sensitivity to lysis and the absence of MHC class I molecules on the target cell (2). The discovery of NK cell inhibitory receptors specific for MHC class I molecules provided a molecular explanation for this phenomenon. These molecules fall into two functionally distinct groups. The complexes formed between HLA-E (human) or Qa-1 (mouse) and leader peptides from classical class I molecules provide recognition structures for the heterodimeric CD94/NKG2 family members (3, 4). In contrast to this indirect recognition of multiple MHC class I products, the killer Ig-like receptors (KIR; human) or Ly49 (mouse) families directly recognize subsets of MHC class I allele products (5, 6). In addition to the inhibitory forms, all these families comprise some activating counterparts. Some members of the human leukocyte Ig-like receptors (LIILR; previously ILT/LIR/MIR) family have also been shown to bind HLA-class I molecules. Of these, NK cells express the inhibitory LIILR1 receptor (7, 8).

The KIR family is of particular interest because individual members bind specific subgroups of HLA allele products (9–11). They permit the NK population to detect changes in the HLA expression pattern, a property crucial to the NK cell’s proposed role in immunosurveillance. Tumor cells and cells infected with certain viruses can down-regulate specific groups of HLA class I molecules (12–14). Either of these forms of HLA down-regulation would impair T cell responses, providing a rationale for inhibitory NK receptors specific for small groups of HLA class I allotypes.

The KIR genes have a unique and fascinating expression pattern. Most KIR loci are expressed on different NK clones in a stochastic manner, so that every NK cell expresses only a subset of the available KIR genes (9, 15–17). The expression of a KIR is determined at the transcription level and is stable in both NK clones and NK populations (9, 15, 16, 18–20). The expression of individual KIR molecules is largely independent of the expression of other KIR molecules, in line with the statistical test for independence termed the product rule (16). The independence of expression results in a population of NK cells with a diverse repertoire of receptors, resulting in an NK pool with a range of specificities with regard to the loss of HLA class I allotypes (21). Thus, a diverse NK repertoire is achieved through diversity of receptor expression rather than the combinatorial receptors characteristic of TCR and Ig in the adaptive immune system.

CD8 memory T cells, some CD4 T cells (22), and a population of γδ T cells also express KIR with a similar expression pattern as that observed on NK cells. Diversification of the KIR repertoire occurs following clonal expansion of naive T cells, suggesting that populations of expanded T cell clones are diverse in their capacity to be stimulated, according to their KIR repertoire (23, 24).

Study of the genetics of the KIR cluster has raised some interesting observations pertaining to the regulation of KIR expression. The KIR genes are tightly packed into a region of human chromosome 19q13.4 with approximately 2 kb between most genes. The KIR genes are highly related to each other and are arranged in a continuous head-to-tail pattern. The number of KIR loci can differ between haplotypes, although some framework loci appear to be present in most, if not all. These framework loci are KIR3DL3 at the centromeric end of the cluster, KIR3DL2 at the telomeric end, and KIR2DL4 in the middle of the cluster. Very few stretches of sequence in the cluster do not have homology with other parts of the cluster. Between the start of the KIR3DL3 gene and the end of the KIR3DL2 gene, the only major unique stretch of sequence is...
a 14-kb region upstream of KIR2DL4. Virtually all of this unique stretch is composed of genomic repetitive sequence elements (25). All KIR that have variegated expression on the NK cell population have upstream regions with >90% nucleotide identity (26). This, along with the similarity between genes, hints that the regulation of each variegated KIR is probably through the same mechanism. The NK cells of individuals heterozygous for the KIR3DL1 gene include subpopulations that have mono-allelic expression, distinguished by the DX9 binding phenotype (19, 27) or allele-specific PCR (28). While the reported coexpression of the KIR3DL1 alleles is higher than expected (27), this suggests that variegated expression of KIR genes is not due to different regulatory mechanisms. On the other hand, the two KIR genes showing divergence in expression pattern (KIR3DL3 and KIR2DL4) belong to those framework loci with upstream sequences present in unique or divergent regions of the KIR cluster. KIR3DL3 (otherwise known as KIR3DL7 or KIRC1) does not appear to be expressed by the circulating NK cell pool in many donors (29). Most reports indicate that all NK clones and KIR-positive T cell clones express KIR2DL4 transcripts (16, 23, 24, 30, 31). The upstream regions of both these KIR genes diverge significantly from those of KIR with variegated expression (26).

We therefore hypothesized that the promoter function of variegated KIR genes would differ from those of KIR2DL4 and KIR3DL1, so we decided to identify and characterize the promoters of a variegated KIR (KIR3DL1) and KIR2DL4. While the KIR2DL4 promoter is NK cell restricted and drives high expression in NK cell lines, the KIR3DL1 promoter drives lower expression, but is more promiscuous between cell types. Using DNase I footprinting and 5′RACE we show that despite the 67% nucleotide identity between these two promoters, most of the cis-acting elements that could be involved in their regulation are distinct, and they probably use different mechanisms of core promoter regulation. The lack of these core promoter elements in the equivalent upstream region of KIR3DL1 potentially explains its low or absent physiological expression. These results have relevance to regulatory control of the NK repertoire.

Materials and Methods

Cells

All cell lines, with the exception of NK92.30.1G, were maintained in RPMI 1640 (Invitrogen, Paisley, U.K.) supplemented with 10% heat-inactivated FBS (Harlan, Crawley Down, U.K.), 100 U/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine at 37°C in 5% CO2. NKL was maintained with the additional supplement of 100 U/ml recombinant human IL-2 (Proleukin; Chiron U.K., Harefield, U.K.), and NK3.3 medium was maintained with the additional supplement of 100 U/ml recombinant human IL-2 (Proleukin; Chiron U.K., Harefield, U.K.) and 100 U/ml streptomycin, 100 mg/ml of penicillin G, 15% Lymphocult T (Biotest, Solihull, U.K.) and a total of 15% FBS (Harlan, Crawley Down, U.K.), 100 U/ml streptomycin, 100 U/ml penicillin G, 1% activated FBS (Harlan, Crawley Down, U.K.), 100 U/ml streptomycin, 100 mg/ml of penicillin G, and 20% FBS (Gibco/Life Technologies, Paisley, U.K.). All cell lines, with the exception of NK92.30.1G, were maintained in activated FBS (Harlan, Crawley Down, U.K.), 100 U/ml streptomycin, 100 mg/ml of penicillin G, 1% activated FBS (Harlan, Crawley Down, U.K.), 100 U/ml streptomycin, 100 mg/ml of penicillin G, and 20% FBS (Gibco/Life Technologies, Paisley, U.K.).

KIR RT-PCR

RT-PCR for KIR was performed according to the method described by Uhrberg and colleagues (33) with the following modifications: actin was amplified in separate PCR reactions, and reactions were performed on a Peltier thermal cycler (PTK-225; MJ Research, Waltham, MA) using Platinum Taq polymerize (Invitrogen, San Diego, CA).

Reporter gene assays

Wild-type KIR2DL4 and KIR3DL1 promoters were cloned into the pGL3-Basic (Promega, Southampton, U.K.) reporter vector using PCR-facilitated cloning from PI artificial chromosomes (1060p11 and 7B8E, respectively) (25). The promoter sequence used for KIR3DL1 in this study is that linked to allotype KIR3DL1*00101 (found in accession no. AC011501), which binds well to KIR3DL1-specific Abs (27). KIR2DL4 full-length promoter PCR was performed with forward primer 5′-CGCTGCGGATCCACAAATAACCCCTTCCTCACA-3′ and the reverse primer 5′-CAATTCGCGGATCCACAAATAACCCCTTCCTCACA-3′ and the reverse primer 5′-CAATTCGCGGATCCACAAATAACCCCTTCCTCACA-3′, resulting in a fragment of the KIR2DL4 promoter spanning from base –6 to base –1947 relative to the translation initiation codon. The product was digested with HindIII/BamHI and cloned into HindIII/BglII-digested pGL3-Basic for sense promoter orientation, or BamHI/XmaI, and cloned into BglII/XmaI digested pGL3-Basic for inverse promoter orientation. KIR3DL1 full-length promoter PCR was performed with forward primer 5′-CGCTGCGGATCCACAAATAACCCCTTCCTCACA-3′ and reverse primer 5′-TCCCCCCCGGCATGTGATGCTGCGGATCCACAAATAACCCCTTCCTCACA-3′, resulting in a product including the ATG initiation codon to base –2068. Forward orientation of this promoter was achieved by cloning through Neo/BamHI digestion, and reverse orientation was achieved by XmaI/BamHI digestion. For the deletion series of each promoter, PCR amplification was performed from the full-length promoter using the above-mentioned reverse primers and forward primers with the linker 5′-CGCTGCGGATCCACAAATAACCCCTTCCTCACA-3′ connected to –20 bases of the promoter starting at the position detailed in Fig. 1, B and D.

The cloning was performed as described above, and all clones were fully sequenced. We initially performed the deletion analysis with a ~98 bp clone of the promoter from KIR3DL1 and a ~108 bp clone from KIR2DL4. These clones resulted in low expression from the KIR3DL1 promoter and relatively high expression from the KIR2DL4 promoter. We therefore also included the reciprocal clones in the experiments.

All vectors were prepared using maxi and mini purification protocols (Qiagen, Crawley, U.K.), then subjected to spectrophotometry and gel electrophoresis and assayed quantity and quality. All data in Fig. 2 used two independent creations of each vector, and other replicates were performed with independent plasmid preparations to reduce the effect of variation between preparations.

For all transient transfections, an equal mass of pRL-TK (Promega, Madison, WI) was cotransfected with the pGL3 test vector to serve as an internal control. Transfection of suspension cells was performed with 4 × 105 cells in 200 μl of RPMI and 5 μg of vector DNA by electroporation in a 4-mm gap cuvette at 260 V and 500 μF. Transfected cells were returned to prewarmed complete medium and incubated for 44–60 h depending on the experiment. Transfection of 293T cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Following incubation, the cells were harvested and assayed using the Dual luciferase assay (Promega) with a Sirius FB121 luminometer (Berthold Detection Systems, Pforzheim, Germany) and settings of a 3-s delay and a 10-s integration time for each reading. All measurements were normalized using a reading from the Renilla luciferase control provided by pRL-TK cotransfection. In all cases parallel assays were performed using the pGL3-Basic promoterless negative control (Basic-luc = pGL3-Basic), and the pGL3-promoter SV40 promoter-positive control (Promoter-luc = pGL3-promoter; Promega). Luciferase activity (light units) = test pGL3 activity × (10⁰/Renilla pRL-TK activity) (Fig. 1). Relative luciferase activity = light units from test pGL3 activity (light units)pGL3-Basic activity (light units) (Fig. 2). Statistical analysis for replicate measurement groups was performed using Student’s t test.

Nuclear extracts

Nuclear extracts were prepared according to the method of Zhang and Lichtenheld (34), except that 0.5 mM PMSF and Complete EDTA-free protease inhibitor cocktail (Roche, Lewes, U.K.) were used to counteract protease inhibitor cocktail (Roche, Lewes, U.K.) were used to counteract protease activity in the required buffers. All extracts were assayed for quantity using the Bio-Rad protein assay (Amersham Pharmacia Biotech, Little Chalfont, U.K.), and were assessed for quality using EMSA with an NFκB probe and Coomasie Blue SDS-PAGE.

In vitro DNase I footprinting

Probes for DNase I footprinting were created by PCR with primers used to create the 2DL4-luc 262 and 3DL1-luc 267 promoter constructs. These are: KIR2DL4 promoter: forward, 5′-CGCTGCGGATCCACAAATAACCCCTTCCTCACA-3′ and reverse, 5′-CAATTCGCGGATCCACAAATAACCCCTTCCTCACA-3′; and KIR3DL1 promoter: forward, 5′-CGCTGCGGATCCACAAATAACCCCTTCCTCACA-3′ and reverse, 5′-TCCCCCCCGGCATGTGATGCTGCGGATCCACAAATAACCCCTTCCTCACA-3′. To ensure that no PCR errors were introduced in abundance, PCR reactions were split before amplification and reamplified before probe labeling. Five picomoles of each PCR product was labeled using T4 polynucleotide kinase and [γ-32P]ATP (sp. act. 5000 Ci/mmol; Amersham Pharmacia Biotech, Little Chalfont, U.K.) by incubation at 37°C. Probes were then purified using Qia-gen columns and digested with the following enzymes to generate probes labeled at a single end: KIR2DL4 forward, BamHI; KIR2DL4 reverse, HindIII; KIR3DL1 forward, BamHI; and KIR3DL1 reverse, Neoi. Following digestion, probes were phenol/chloroform-purified and ethanol-precipitated. Approximately 2–3 fmol of labeled probe was used for each DNase I footprinting reaction.
Identification of potential transcription factors

Potential transcription factor binding sites were identified using the MatInspector program (36), and the TFSEARCH program (Yutaka Akiyama, http://www.rwcp.or.jp/papia/) using the TRANSFAC database (37).

Results

Expression of different KIR by overlapping fractions of the NK cell population results in a repertoire of NK cells with different KIR profiles. Namely, if not all, KIR-positive cells express the KIR2DL1 gene prompted us to compare its regulation with that of a stochastically expressed KIR, namely KIR3DL1.

KIR2DL4 and KIR3DL1 promoters are active in NK cells and are confined to −262 and −267 bp relative to the translation initiation codon, respectively.

We selected the NK-like cell line YT-Indy to study the promoter regulation of KIR2DL4 and the stochastically expressed KIR3DL1, as it is readily transfected and expresses both the KIR3DL1 and KIR2DL4 genes endogenously (Fig. 1A). The full intragenic region between the KIR3DL1 translation initiation codon and the upstream KIR2DL4 gene was cloned into the pGL3 reporter vector to constructs used in C and E, respectively. Constructs were created by PCR from P1 artificial chromosomes (PAC) DNA and cloning into the pGL3 luciferase reporter vector as described in Materials and Methods. The numbers by each of the clones describe the most 5′ position of KIR promoter DNA relative to the translation initiation codon included in the clone. C and E, Dual luciferase assay of the KIR promoter constructs in YT-Indy NK cells. Expression from the KIR2DL4 −262 bp construct (2DL4-luc 262) and the KIR3DL1 −267 bp construct (3DL1-luc 267) was statistically significant compared with the empty (Basic-luc) luciferase vector (p < 0.02, by Student’s t test). Promoter-luc (Promoter-luc) contains the SV40 promoter and served as a positive control. All readings were normalized to the internal control pRL-TK Renilla luciferase activity as described in Materials and Methods, and error bars represent the SE.
examine the expression of luciferase from the KIR3DL1 promoter. A comparable fragment was cloned from upstream of KIR2DL4. Both the KIR promoter fragments drove expression of luciferase in an orientation-dependent fashion in YT-Indy cells (Fig. 1, C and E).

Progressive 5' deletion clones of the two reporter constructs showed maximal expression to be conferred within -262 bp for KIR2DL4 and -267 bp for KIR3DL1 of the translation initiation codons. The reduced luciferase activities from assays with longer promoter constructs were consistent with the lower molar ratios between the test luciferase constructs and the normalizing pRL-TK vector, suggesting that no enhancers or silencers are present upstream of the -262 bp (KIR2DL4 promoter) and -267 bp (KIR3DL1 promoter) positions. All deletions further into the KIR promoters resulted in significant diminution of reporter gene activity, consistent with multiple regulatory regions within the promoters. The -108 bp KIR2DL4 promoter clone drove ~60% of the luciferase activity of the full-length promoter, indicating that it comprised a major functional component. In contrast, the corresponding clone of KIR3DL1 conferred ~30% of the full promoter activity, suggesting that the upstream -262 to -108 region is integral to efficient activity of this promoter. Further deletion from -108 to -98 bp reduced the reporter activity of both by a further 50%, implying that in each promoter, these 10-bp regions encompass functional regulatory elements. The data confined minimal promoter activity of the KIR2DL4 and KIR3DL1 genes to within, respectively, -267 and -262 bp of their translation initiation codons and demonstrated the presence of multiple regulatory regions within each promoter.

**Tissue specificity of promoter activity**

We next tested the capacity of the minimal KIR2DL4 and KIR3DL1 promoters to drive transcription in a variety of cell lines to determine the tissue distribution of the required trans-acting factors. Jurkat T cells, K562 erythroleukemia cells, and 293T embryonic kidney cells were tested along with YT-Indy (Fig. 2). Expression from the KIR2DL4 promoter was tightly restricted to YT-Indy. Promoter activities in Jurkat, K562, or 293T cells were equivalent to, if not lower than, the Basic-luc promoterless negative control. These data show that the combination of transcription factors necessary for KIR2DL4 promoter activity is only present in the NK cell line. They indicate that the promoter of KIR2DL4 is critical in determining the physiological expression pattern of the KIR2DL4 gene.

Conversely, the promoter of KIR3DL1 directed statistically significant expression of the luciferase reporter gene in all cell types tested. The expression from the KIR3DL1 promoter in Jurkat was approximately half that of the SV40 positive control, suggesting a physiologically relevant promoter activity. K562 showed a similar level of activity from the KIR3DL1 promoter. On the other hand, KIR3DL1 promoter activity in 293T cells was very low, suggesting that this may be due to basal promoter activity. The promiscuous activity of the KIR3DL1 promoter suggests that the trans-acting factors required for its activity are widely distributed. Given that KIR3DL1 is only expressed in cell populations that also express KIR2DL4 (16, 23, 30, 31), these data implicate mechanisms other than proximal promoter regulation in restricting the expression of the KIR3DL1 gene.

In addition to tissue restriction, a further difference between the KIR2DL4 and KIR3DL1 promoters was in the level of reporter gene expression they gave. The KIR3DL1 promoter was less efficient at driving luciferase expression than the KIR2DL4 promoter in YT-Indy cells, resulting in approximately half the luciferase activity (Fig. 2). Combined, these data revealed large differences in the regulatory properties of the KIR2DL4 and KIR3DL1 promoters.

**Transcription factor binding sites differ between the two promoters**

Above, we demonstrated marked differences in tissue specificity and functional activities of the KIR promoters. To explore the molecular basis of this differential control of the KIR2DL4 and KIR3DL1 promoters, we determined the promoter elements bound by transcription factors. We used DNase I footprinting with the full-length minimal promoter probes and nuclear extracts from YT-Indy cells (Fig. 3, A and B). These analyses revealed that the factor-bound sites are largely divergent between the two promoters.
(Fig. 3C). The sequence identity in the minimal promoter region is 67%. However, only a minority of sites bound by transcription factors were coordinate on the two promoters. One of these coordinate transcription factor-binding regions was identified as site A in the promoters of both KIR2DL4 and KIR3DL1. The sequences at these sites are highly similar, but no specific binding was observed when probes of these sequences in isolation were used in EMSAs (data not shown). This is possibly due to weak or cooperative binding of transcription factors to these sites that would be disrupted during the EMSA procedure. Transcription factors with a high score potential to bind KIR2DL4 site A include AREB-6 and SMAD-3, and AREB-6 has a potential binding site around KIR3DL1 site A. The sequence similarities of the KIR2DL4 and KIR3DL1 A sites make them good candidates as sequences binding a common KIR transcription factor. The other conserved binding region is that of KIR2DL4 site E and the gene-proximal part of KIR3DL1 site D. The sequence at these sites corresponds to a potential RUNX/AML transcription factor-binding site. The functional activity of these sites is supported, as the −108 to −96 deletions of each KIR promoter bisected these sites and reduced the activity of each promoter (Fig. 1, C and E).

The remainder of the sites identified by DNase I footprinting are disparate between the KIR3DL1 and KIR2DL4 promoters. Site B of KIR2DL4 includes strong candidate sequences for binding Nkx, AP-1, TCF-2, MYC-MAX, and GATA-3. A lower scoring sequence is present for IFN regulatory factor 2 (IRF-2) binding. The strongest candidates for binding KIR2DL4 site C are members of the AP-1 family. KIR2DL4 site D corresponds to a CREB/ATF or an AP-1 binding site, and KIR2DL4 site F is a potential binding site for RUNX family members, AP-1, estrogen receptor, and ROR-α-1.

In the KIR3DL1 promoter, site B is a potential binding site for TCF-2, AP-1, and the estrogen receptor. Potential factors binding to KIR3DL1 site C include STAT, Ets family members, and YY-1. The gene distal part of KIR3DL1 site D is a potential binding sequence for CREB and the estrogen receptor, and the KIR3DL1 site E is a binding sequence for E2F, NF-1, and SP-1, although it may be directly involved in core promoter activity as a TF IIIB recognition element (see below).

KIR2DL4 site G and KIR3DL1 site F are coordinate with the transcription initiation sites

Another marked difference between the two KIR promoters was observed in the positions of the final DNase I-protected sites. These sites, KIR2DL4 site G, and KIR3DL1 site F, corresponded with the 5′ ends of cDNAs generated by a 5′RACE analysis (Fig. 4). This coincidence of the transcription initiation sites with the transcription factor-binding sites suggests that they are generated by components of the transcription initiation apparatus. In support of this role, neither KIR2DL4 site G nor KIR3DL1 site F in isolation bound YT-Indy nuclear extracts with sequence specificity in EMSA (data not shown).

The 5′ cDNA ends of the KIR transcripts generated by 5′RACE were heterogeneous, but tightly clustered just upstream of the translation initiation site. Heterogeneous transcription start sites are often present in promoters without TATA boxes (38). A potential TATA box (AAATAAC) is present at positions −65 to −59 of the KIR3DL1 promoter. Its sequence differs substantially from the canonical TATAAA, although certain divergent sequences can act as TATA boxes (39). A sequence (GGGCGGCC) corresponding to a TF IIIB recognition element (BRE) is present immediately upstream of this TATA box within KIR3DL1 site E. These elements serve to increase the affinity of TF IIIB and therefore may confer functionality to the KIR3DL1 TATA box (40). No consensus TATA box was observed in the KIR2DL4 promoter, but a number of potential initiator (Inr) sequences are present around the start site. Inr sequences have the loose consensus Py Py A +1 N T/A Py Py (41). The data further exemplify the striking differences between the KIR2DL4 and KIR3DL1 promoters in the transcription factor binding patterns and the potential core promoter regulation.

Discussion

The KIR gene cluster uses variaged gene expression to generate an NK cell repertoire with the capacity to detect aberrations in the expression patterns of HLA class I molecules. To investigate the mechanisms of KIR regulation, we performed the first characterization of their promoters.

Several properties of the KIR2DL4 and KIR3DL1 promoters are disparate. First, the progressive deletion of the promoters revealed different regions to be major components of regulation. Second, the KIR2DL4 promoter displayed a tissue restriction not seen with the KIR3DL1 promoter. Third, the KIR2DL4 promoter consistently drove higher levels of reporter gene expression than the KIR3DL1 promoter. Fourth, most of the transcription factor-binding sites differed on the two promoters. Fifth, transcription initiation occurred at different positions within the KIR2DL4 and KIR3DL1 promoters, consistent with different mechanisms of core promoter function. While these KIR promoters share 67% nucleotide identity, there are large qualitative differences between them, consistent with evolutionary divergence driven by distinct regulatory requirements.

Certain footprinted sites within the KIR2DL4 and KIR3DL1 promoters correspond to binding sites for factors implicated in NK cell differentiation and gene regulation. All the footprinted sites in isolation, as short oligonucleotide constructs, with the exception of KIR2DL4 sites A and G and KIR3DL1 sites A and F, showed sequence-specific binding to YT-Indy nuclear extracts in EMSA (data not shown). The data revealed a selection of cis elements with the potential to confer the functional differences between the promoters. Of the potential factors involved in KIR promoter induction, IRF-2, a binding sequence for which is present within site B of the KIR2DL4 promoter, is of interest due to its importance in NK development. The number of NK cells in IRF-2-deficient mice is severely reduced, with those NK cells present having an immature phenotype (42). Likewise, Ets-1 is a critical factor in the development of murine NK cells (43), and potential Ets-1 binding sites are present in both KIR2DL4 site F and KIR3DL1 site C.

The presence of sites with the potential to bind CREB and AP-1 within the KIR2DL4 promoter raises the possibility that cellular activation is involved in the expression of KIR2DL4. KIR-expressing cells of both the CD8 T cell and NK cell lineages have properties consistent with cellular activation. In particular, KIR+ CD8 T cells are believed to be a subset of memory T cells (44, 45). Furthermore, the ability of KIR+ T cells to survive in culture may be enhanced compared with that of KIR− cells (46). NK cells show higher levels of cell surface phosphorylation than naive T cells and are more comparable with activated T cell populations (47). A precedent for the regulation of gene expression by AP-1 family members in NK cells is found in the promoter regulation of the 2B4 gene, an activating receptor expressed by NK cells and T cells capable of non-MHC-restricted cytotoxicity (48).

KIR2DL4 site E and the gene-proximal part of KIR3DL1 site D correspond to potential RUNX/AML transcription factor binding sites. Additionally, the lower expression of the −98 bp promoter constructs compared with the −108 bp promoter constructs suggest that these RUNX binding sites are functional, as deletion to −98 bp abrogates them. Vilches and colleagues (49) observed that nonexpressed variants of KIR2DL5 have upstream regions that
lack a consensus RUNX1 (AML1) site at this position. Therefore, the binding of RUNX factors could be crucial in the regulation of all KIR genes.

The mechanisms of core promoter function within the KIR2DL4 and KIR3DL1 promoters are likely to be different. We observed divergence in the position of the transcription start sites and in the potential core promoter elements. The KIR3DL1 promoter has a potential TATA box with a weak similarity to the consensus that is 30 bp distance from a major transcription start site. The presence of a consensus sequence for a BRE just upstream of this TATA box may serve to enhance its function. Within the KIR2DL4 promoter there is no TATA-like sequence. Some potential Inr sequences exist around the transcription start sites, but all differ slightly from consensus. Our data indicate that the mechanism employed for core promoter function on the KIR2DL4 promoter is different from that of KIR3DL1.

Based on these data, the observed low or nonexistent expression of KIR3DL3 (29) is possibly due to a lack of core promoter elements. In the upstream region of this gene, the sequence between −40 bp and the ATG is identical with that of KIR3DL1. However, in the equivalent region to KIR3DL1 site E there is distinct KIR3DL3 sequence that lacks the TATA box and BRE found in the KIR3DL1 promoter (26). 5′RACE of both KIR2DL4 and KIR3DL1 resulted in a series of clones with heterogeneous transcription start positions. Heterogeneous start sites are usually characteristic of TATA-less promoters (38), and have been observed for other NK-expressed MHC class I receptors. Transcription start sites for members of the Ly49, CD94, and NKG2 families have been mapped either by 5′RACE, S1 nuclease protection, or primer extension. Most members of these families have staggered or heterogeneous transcription start sites (50–52). Therefore, it is plausible that all the NK receptor

**FIGURE 3.** Identification of cis sequences within the KIR promoters by DNase I footprinting. A and B, DNase I footprinting of KIR2DL4 (A) and KIR3DL1 (B) promoters. The regions of DNA that gave full expression of the KIR2DL4 (262 bp) and KIR3DL1 (267 bp) promoters (Fig. 1, C and E) were subjected to DNase I footprinting with nuclear extracts from the YT-Indy NK cell line. The quantities of nuclear extract used for footprinting reactions were 50 μg (+), 100 μg (++) and 150 μg (+++). Regions protected from DNase I digestion compared with the naked probe are boxed. Footprinting reactions were run against a Maxam Gilbert sequencing reaction for G and A nucleotides, and the sequences corresponding to the footprints are indicated. Letters alongside the sites indicate the nomenclature, e.g., KIR2DL4 site A. C, Alignment of the minimal promoter sequences of KIR2DL4 and KIR3DL1 showing the locations of the footprints formed using YT-Indy nuclear extracts and potential transcription factor binding sites. Vertical lines indicate regions of sequence identity between the two KIR promoters. Horizontal lines above the upper strand show protected regions using the forward probe, and lines below the lower strand show those formed using the reverse probe. Bold sequence indicates the consensus sequences from these footprints. The translation initiation codon is also in bold. Site nomenclature is indicated above the bold consensus footprinted regions. Boxes show stretches of KIR promoter sequence with the potential to bind the transcription factors indicated.

**FIGURE 3.** (continues)
families for class I molecules have staggered transcription start sites.

Our study suggests that the KIR2DL4 promoter activity is restricted in expression to NK cells. We previously observed that among a panel of immortalized T cell lines, variegated KIR transcripts could be detected, but no transcripts of KIR2DL4 were amplified. In contrast, all tested NK cell lines expressed KIR2DL4 transcripts (data not shown). These data suggested that the KIR2DL4 gene is subject to an NK-specific regulation not present for other variegated KIR. The expression profiles of the KIR2DL4 and KIR3DL1 promoters detailed in this manuscript suggest that a major component of NK-restricted KIR2DL4 expression is due to its promoter, through activation by NK-specific transcription factor complexes. Whether the KIR2DL4 promoter is accessible to this regulation during NK development or development of KIR− T cells remains to be ascertained, but the tissue specificity observed suggests that an accessible KIR2DL4 promoter alone could function to determine a physiological expression pattern. On the other hand, the capacity of the KIR3DL1 promoter to drive gene expression in cells other than NK cells indicates the requirement for other regulatory elements to determine in vivo expression. This control could be due to global gene regulatory mechanisms. The idea of global transcriptional regulation of the KIR gene cluster is supported by the observations of Santourlidis and colleagues (53). They observed that demethylating treatment of NK cells resulted in the de novo expression of various variegated KIR. In contrast, demethylating treatment of Jurkat T cells resulted in dramatic de novo expression of KIR3DL2 only (53). It is noteworthy that KIR3DL2 is one of the framework loci positioned at the telomeric end of the KIR gene cluster (25) and therefore may not be as stringently regulated by global mechanisms of gene repression. One possibility is that derepression of the KIR gene cluster may be the consequence of activation of the closely linked KIR2DL4 gene. A plausible model is that either activation of the KIR2DL4 promoter or expression of the KIR2DL4 gene is a prerequisite for the expression of other variegated KIR. A temporal expression sequence of MHC class I receptors has been observed for the Ly49 genes in the mouse and has been proposed for members of the human leukocyte receptor cluster containing the LILR and KIR genes (54–57).

Comparison of the two KIR promoters used in this study suggests a mechanism for the generation of variegated KIR gene expression. The reporter gene expression driven by the KIR3DL1 promoter is approximately half that driven by the KIR2DL4 promoter in YT-Indy. This could be explained either by different rates of transcription from the two promoters or by differences in the

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**FIGURE 3.** (continued)
number of template promoters with active transcription factor complexes (58). Differences in the fraction of occupied promoters could influence the expression of KIR during the time window of repertoire development. This system would provide a mechanism for stochastic expression of the KIR. Subsequent stabilization of the KIR expression pattern has been shown to correlate with the absence of promoter DNA methylation (28, 53). With relevance to the variegated expression pattern of class I receptors, it is interesting that the role of TCF-1 in acquisition of Ly49A gene expression in vivo appears to be to determine the frequency of active Ly49A gene templates rather than the transcription rate of the active templates. Moreover, two TCF-1 binding sites exist within the proximal promoter and are important in conferring expression from this promoter in reporter gene assays (59). While there are other likely effects of TCF-1 in the development of the Ly49 repertoire (60), these data suggest that the binding of the TCF-1 transcription factor to the proximal promoter may be determining the frequency of active promoters rather than the rate of transcription from them.

The KIR promoters of those genes expressed in a variegated fashion by NK cells are 90% identical in nucleotide sequence. Comparison of the footprinted sites within the KIR3DL1 promoter and the other KIR promoters suggests that many of these sites have the capacity to bind the same transcription factors in all the promoters. The incubation of NK cell lines with 5-aza-2-deoxycytidine, an inhibitor of DNA methylation, results in the cell population inducing the expression of a large number of KIR genes (28, 53). This evidence indicates that the transcription factors necessary for KIR gene expression are fully functional in the untreated NK cell lines, but the promoters themselves are nonpermissive to expression. To elucidate whether different transcription factors are involved in the expression of different variegated KIR, we have also performed the reporter gene assays with a minimal KIR3DL2 promoter (data not shown). This promoter has one of the most divergent sequences from KIR3DL1 among the panel of variegated KIR. The KIR3DL2 promoter induced only slightly less reporter gene expression than the KIR3DL1 promoter when tested in either YT-Indy or Jurkat (data not shown).

This study has demonstrated that the mechanisms of regulation are divergent within the tightly linked KIR gene cluster. These differences are consistent with the physiological expression patterns of the KIR and divergence from a typical KIR promoter sequence. All the variegated KIR genes are likely to use similar mechanisms of regulation due to their highly related promoters. The findings resonate with genomic features of the region. The KIR cluster on chromosome 19q13.4 appears to be evolving rapidly, and the numbers of loci within it vary extensively between haplotypes (25, 61, 62). The similarities of the promoters and their regulation may ensure that the control of any novel KIR combination is readily facilitated. Significantly, the KIR2DL4 gene is well conserved in primates (61, 63, 64) and has qualitatively different regulation from the other KIR. The large differences demonstrated between the promoter of KIR2DL4 and those of variegated KIR may provide an explanation for the distinct phenotypes of these genes. Knowledge of the mechanisms involved in generating the NK repertoire is central in understanding the biology of NK cells and in the potential to manipulate these critical cells of the immune system.

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References
