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A Role for DNA Hypomethylation and Histone Acetylation in Maintaining Allele-Specific Expression of Mouse NKG2A in Developing and Mature NK Cells

Sally L. Rogers,* Arefeh Rouhi,*† Fumio Takei,*‡ and Dixie L. Mager2*†

The repertoire of receptors that is expressed by NK cells is critical for their ability to kill virally infected or transformed cells. However, the molecular mechanisms that determine whether and when NK receptor genes are transcribed during hemopoiesis remain unclear. In this study, we show that hypomethylation of a CpG-rich region in the mouse NKG2A gene is associated with transcription of NKG2A in ex vivo NK cells and NK cell lines. This observation was extended to various developmental stages of NK cells sorted from bone marrow, in which we demonstrate that the CpGs are methylated in the NKG2A-negative stages (hemopoietic stem cells, NK progenitors, and NKG2A-negative NK cells), and hypomethylated specifically in the NKG2A-positive NK cells. Furthermore, we provide evidence that DNA methylation is important in maintaining the allele-specific expression of NKG2A. Finally, we show that acetylated histones are associated with the CpG-rich region in NKG2A positive, but not negative, cell lines, and that treatment with the histone deacetylase inhibitor trichostatin A alone is sufficient to induce NKG2A expression. Treatment with the methyltransferase inhibitor 5-azacytidine only is insufficient to induce transcription, but cotreatment with both drugs resulted in a significantly greater induction, suggesting a cooperative role for DNA methylation and histone acetylation status in regulating gene expression. These results enhance our understanding of the formation and maintenance of NK receptor repertoires in developing and mature NK cells. The Journal of Immunology, 2006, 177: 414–421.

Natural killer cells play an important role in the immune response to viral infection and tumors. They are activated to lyse target cells by responding to molecules associated with infection, transformation, or cellular distress. To prevent lysis of healthy autologous cells, NK cells express inhibitory receptors that monitor levels of MHC class I on potential target cells. NKG2A is a C-type lectin-like inhibitory receptor expressed by both humans and mice as a heterodimer with CD94. The ligands for NKG2A are the nonclassical class I MHC molecules HLA-E in humans (1) or Qa-1 in mice (2), which are expressed at the cell surface with the leader peptide from classical class I MHC molecules. In this way, NKG2A provides a broad detection system for monitoring expression levels of class I MHC. In contrast to NKG2A, mouse Ly-49 inhibitory receptors specifically recognize individual allelic forms of MHC class I molecules to detect subtle changes in MHC class I on target cells (3–6). The outcome of an interaction between a potential target cell and an NK cell is a result of a fine balance of signals delivered via the activatory and inhibitory receptors. The combination of increased expression of markers of cellular distress and down-regulated expression of MHC class I on target cells, a common consequence of viral infection or transformation, will tip the balance of signals delivered to the NK cell in favor of activation and lysis of the target cell.

The NKG2A and Ly-49 receptors are C-type lectin-like receptors, and the genes are closely linked in the mouse NK complex (7). Human NK cells do not express the Ly-49 family of receptors. Instead, they express killer Ig-like receptors (KIR), a functionally homologous group of Ig-like receptors encoded in the leukocyte receptor complex on human chromosome 19 (8). Despite belonging to different structural groups, and being encoded by genes on different chromosomes, the KIR and Ly-49 receptors are strikingly similar in function and expression patterns. Both gene families are polymorphic in sequence and in gene number (9–12) and are expressed in a clonal, stochastic, and predominantly monoallelic fashion to provide a diverse repertoire of individual NK cells (13–17).

Little is known about how the repertoire of receptors is determined on developing adult NK cells. A role for epigenetic mechanisms in determining the allele- and locus-specific expression of KIR has been described in adult mature NK cells (13, 18–20). It appears that the whole KIR gene cluster is in an open chromatin conformation with acetylated histones, and expression of individual alleles is associated with hypomethylated clusters of CpG dinucleotides in the promoter region, whereas methylated promoters correlate with lack of expression. Previous work in our laboratory has shown that both DNA hypomethylation and histone acetylation correlate with expression of Ly-49A on ex vivo NK cells (21). In this study, we show for the first time a role for epigenetic mechanisms in regulating expression of NKG2A both in developing and mature adult NK cells.

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3 Abbreviations used in this paper: KIR, killer Ig-like receptor; 5azaC, 5-aza-cytidine; ChIP, chromatin immunoprecipitation; COBRA, combined bisulfite and restriction analysis; DN, double negative; DP, double positive; Hprt, hypoxanthine phosphoribosyltransferase; HSC, hemopoietic stem cell; NKP, NK progenitor; SP, single positive; TSA, trichostatin A; UTR, untranslated region.
Materials and Methods

Mouse strains

C57BL/6 (B6) and BALB/c mice were purchased from The Jackson Laboratory and bred in our animal facility. The use of animals for this study was approved by the Animal Care Committee of the University of British Columbia, and animals were maintained in accordance with the guidelines of the Canadian Council on Animal Care.

Isolation of mouse splenocytes

Spleens were homogenized, and RBC were removed from single-cell suspensions by flash lysis in ammonium chloride solution (0.8% NH₄Cl with 0.1 mM EDTA). Remaining cells were washed in DMEM supplemented with 10% FBS (DMEM-10) and incubated for 1 h in medium-filled packed nylon wool column at 37°C. Cells were slowly eluted from the column using 37°C DMEM-10. Cells were blocked with anti-FcRγII/III Ab Ab (2.4G2). Primary Abs against the following surface markers were used: NK1.1 allophycocyanin (PK136; BD Pharmingen); NKG2A/CE FITC (20d5; BD Pharmingen); NKG2A/biotin (16a11; eBioscience); Streptavidin-PE (BD Pharmingen) was used as a secondary reagent with the biotinylated Ab. All reagents were purchased from StemCell Technologies, unless otherwise stated. Flow cytometry and sorting were performed on either a FACSVantage or FACSaria (BD Biosciences).

Isolation of hematopoietic stem cells (HSC), NK progenitor cells (NKP), and NK cells from mouse bone marrow

Bone marrow cells were flushed from femurs and tibiae into PBS containing 2% FBS. Lineage-positive cells (CD5 +, TER119+, CD45R-, Ly-6G-, CD11b-, or 4^-) were depleted using StemSep mouse progenitor enrichment mixture, and HSC (line^-, Sca^-, c-Kit^-) were subsequently sorted using the biotinylated lineage Ab mixture, as described above, and anti-Sca1 PE (E13-161; BD Pharmingen), anti-c-Kit allophycocyanin (2B8; BD Pharmingen), and streptavidin FITC (BD Pharmingen) Abs. NK (NK1.1^-, CD122^-) were isolated by negative selection of NK1.1^- cells using anti-NK1.1 FITC Ab (PK136; BD Pharmingen) and EasySep mouse FITC selection kit, followed by sorting using anti-NK1.1 FITC (as described previously) and anti-CD122 PE (TM-β1; BD Pharmingen) Abs. NK cells were isolated by enrichment of NK1.1^- cells using Ab PK136 and EasySep mouse FITC selection kit, followed by sorting using anti-NK1.1 FITC and anti-NKG2A/biotin (16a11; eBioscience) Abs. Streptavidin-PE (BD Pharmingen) was used as a secondary reagent with the biotinylated NKG2A Ab.

5’ RACE

5’ RACE was performed using the FirstChoice RLM-RACE kit (Ambion), according to the manufacturer’s instructions. The primers used were as follows: forward control primer, 5’–CACAATCAATAGCTGCAGACAT-3’; reverse flanking primer, 5’–CCATGAAATAAACAATTGTTG-3’; reverse nested primer, 5’–GATATCACCCTTGGACAACT-3’.

Cell culture

Mouse lymphoid cell lines EL4, LNK, and KY-2 were grown in RPMI 1640 containing 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin, 50 μM 2-ME, and 1-glutamine. LNK and KY-2 cells were supplemented with 200 U/ml IL-2, respectively.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using a ChIP assay kit (Upstate Biotechnology), according to the manufacturer’s instructions. The following Abs were used to perform immunoprecipitations: polyclonal anti-acetyl-histone H3 (lysine-9) and polyclonal anti-acetyl-histone H4 (multiple residues) (both Upstate Biotechnology). The DNA was purified via QIAquick PCR purification (Qiagen) and resuspended in 50 μl of deionized water.

Quantitative real-time PCR

Quantitative PCR amplification of a 150-bp product from the Cpg-rich region of mouse NGK2A was performed using forward primer 5’–TGT GGCCGAGGGTAGAAATGGTC-3’ and reverse primer 5’–CCATG CAGTGATCTGTTTGTAGGC-3’ of the transcriptional start site of NKG2A (Kpasame et al., 2003). The primer sets were designed to amplify a single and specific product.

Treatment with chromatin-remodeling drugs

The EL4 cell line was cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were treated with 5a-cembranol (5a-Cb) (80 μM) and trichostatin A (TSA) for 96 h before RNA extraction. Total RNA was extracted from EL4 cells with TRIzol reagent (Invitrogen Life Technologies), per manufacturer’s instruction. RT-PCR was performed for β-actin and NGK2A (primer sequences: β-actin forward primer, 5’–GAGGCGTATGCTCTCCCCTA-3’; β-actin reverse primer, 5’–GGCGCAAGTTAGGGTTTGTCA-3’; NGK2A forward primer, 5’–CGAACGACGACAGAATG-3’; NGK2A reverse primer, 5’–ATGGCCA CAGTTACATTCAATC-3’). The primers used were as follows: initial denaturation at 95°C, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 55°C, respectively, for 30 s, 72°C for 40 or 30 s, respectively, with a final 7 min-extension at 72°C.

Bisulfite conversion

Bisulfite conversion of DNA was performed using the EZ DNA methylation kit (Zymo Research), according to the manufacturer’s protocol, with minor modifications (21). Treated DNA was cleaned and eluted to 15 μl following the manufacturer’s instructions. PCR amplification of the 461-bp Cpg-rich region identified in mouse NGK2A was amplified from converted DNA using the forward primer 5’–TTATATGTGTTAGATGTA GTAAGT-3’ and the reverse primer 5’–AACAAACAAATAACCTCTA ATTC-3’ with Platinum Taq (Invitrogen Life Technologies) under the following conditions: initial denaturation at 94°C for 8 min, followed by 40 or 45 cycles of 94°C for 90 s, 58°C for 90 s, and 72°C for 45 s. A final elongation step of 10 min was included in all reactions. PCR products were analyzed by gel electrophoresis and products purified with MiniElute (Qiagen). Three separate PCRs were performed on each sample to control for PCR bias in the subsequent analysis.

Sequencing

PCR products were pooled from individual samples and cloned using the pGEM-T Easy Kit (Promega). Sequencing was performed by McGill University and Genome Quebec Innovation Centre Sequencing Platform. Only sequences were identified that met all criteria for non-CpG cytosines, and all sequences were GC-methylated in the original sample, then the restriction enzyme site is conserved during bisulfite treatment and the PCR product will be digested. All enzymes were purchased from New England Biolabs, and digests were performed overnight following manufacturer’s instructions. Control reactions were performed each time to ensure complete digestion, and uncut reactions were run on agarose gels with digests for comparison.

Results

Identification of a cluster of CpG dinucleotides downstream of the transcriptional start site of NGK2A

Inspection of cDNA sequences obtained by 5’ RACE in GenBank revealed that mouse NGK2A appeared to have two alternative transcriptional start sites (22). These result in short 5’ UTRs of either 46 or 265 bp (23). This is conserved in the original sample, then the restriction enzyme site is conserved during bisulfite treatment and the PCR product will be digested. All enzymes were purchased from New England Biolabs, and digests were performed overnight following manufacturer’s instructions. Control reactions were performed each time to ensure complete digestion, and uncut reactions were run on agarose gels with digests for comparison.
previous report described that there is some sequence identity between human NKG2A exon 1b and the genomic region upstream of mouse NKG2A (23), suggesting that there may be an as yet unidentified splice variant of mouse NKG2A containing an extra 5' UTR exon. To investigate this possibility, we performed 5' RACE and found the same splice variants as described previously for mouse NKG2A plus a previously undescribed transcriptional start site 21 bp upstream of the translational start site (Fig. 1B). We also designed multiple primers in the region upstream of mouse NKG2A that is conserved with human exon 2, and although these primers amplified mouse genomic DNA, we were unable to generate PCR products from cDNA of multiple cell and tissue origins (data not shown). This would suggest that, at least in the samples we have tested, the major NKG2A transcriptional start sites are those identified previously. Having confirmed this fact, we identified a cluster of 10 CpG dinucleotides within a 461-bp region downstream of the transcriptional start site of mouse NKG2A (Fig. 1C). Although this region does not conform to the original definition of a CpG island (24) (49.5% G + C content instead of >50%, and observed/expected CpG ratio of 0.39 instead of >0.6), there is a definite increased density of CpG in this region (compared with a background observed/expected ratio of ~0.2 in the mouse genome) (25), and therefore we have called this region a CpG-rich region.

**Methylation status of CpG clusters correlates with expression of NKG2A in ex vivo NK cells**

We then assessed whether methylation patterns of the CpG-rich region are linked with transcriptional activity of the NKG2A gene. NK1.1+, NKG2A+/− populations were sorted from adult mouse splenocytes with >95% purity (Fig. 2A). The DNA was isolated from these cells and treated with sodium bisulfite. Sodium bisulfite converts cytosine to uracil, but does not convert 5-methylcytosine, allowing the bases to be distinguished by PCR amplification. The 461-bp CpG-rich region shown in Fig. 1C was PCR amplified from the treated DNA, and subjected to COBRA. Four enzymes were chosen for COBRA, as shown in Fig. 1C, allowing analysis of five of the CpG dinucleotides. If the CpG was originally methylated, the restriction enzyme site will be retained in the treated DNA and the PCR product will be digested. The CpG sites tested were methylated in the NKG2A− cells, and a significant proportion of the sites was unmethylated in the NKG2A+ cells (Fig. 2B). NKG2A has previously been reported to exhibit some degree of monoallelic expression (26). Presumably, the proportion of sites that are methylated in the NKG2A-positive cells represents the transcriptionally silent allele.

**DNA methylation maintains the monoallelic expression of NKG2A in ex vivo F1 hybrid NK cells**

It has been reported previously that mouse NKG2A expression is stochastic and, to some degree, monoallelic in BALB/c × B6 F1 hybrid mice (26). To investigate whether DNA methylation is responsible for maintaining the allele-specific expression of mouse NKG2A, we stained adult B6 and B6 × BALB/c F1 hybrid mouse splenocytes with an Ab that recognizes NKG2A/C/E from both strains (20d5) and an Ab that recognizes specifically the B6 allele of NKG2A/C/E from both strains (20d5) and an Ab that recognizes specifically the B6 allele of NKG2A only (16a11), as shown in Fig. 3A. There are no NKG2A/B6 single-positive (SP) cells detected, as both Abs recognize NKG2A/B6. We observed two populations in the double-positive (DP) quadrant from the F1 mice, and it has been shown previously that the population staining brightest with the 20d5 Ab most likely contains cells expressing both alleles, while the less bright population most likely represents cells expressing the B6 allele of NKG2A only (26). Only a single, presumably DP population was observed in the splenocytes from B6. Furthermore, only 0.5% of B6 cells are NKG2A/C/E positive/NKG2A/B6 negative compared with 11.5% from F1 mice, showing that NKG2C and NKG2E are expressed only at very low levels on NK1.1-positive splenocytes and are not contributing significantly to NKG2A/C/E staining. We sorted double-negative (DN), BALB/c SP, and BALB/c plus B6 DP cells to contain <5% contamination from

**FIGURE 1. Identification of CpG dinucleotides downstream of the transcription start sites for NKG2A. A. Genomic organization of the human (top) and mouse (bottom) NKG2A genes. Exons are represented as □ and numbered underneath, with narrow portions representing UTRs. Bent arrows represent identified transcriptional start sites, and the boxed region is expanded in C. Open arrow and ▶. Indicate the location of an Alu transposable element and functional GATA-3 binding site, respectively, in the human gene. Gray-filled triangle and □. Indicate the region conserved between mouse and human. B. Starred arrows indicate the alternative transcriptional start sites described for murine NKG2A from GenBank accession numbers AF106008 and AF106009. Unstarred arrow. Indicates the position of a third transcriptional start site identified by our laboratory. The coding sequence starting in exon 1 is shown in capitals. C. Cartoon showing the location of CpG dinucleotides identified around the transcriptional start of the murine NKG2A gene. Top line. Shows the location of exons (□). Lower line. CpG dinucleotides are represented as vertical lines. Letters indicate the position of restriction enzyme sites used for COBRA: M, MluI; T, TaqI; S, Sau3A1; and H, Hinfl. Arrowed line shows the region amplified for bisulfite sequencing and COBRA.**
other populations. To test the purity of our sorts, RNA was isolated, and PCRs were designed to amplify all alleles of NKG2A performed on cDNA preparations (Fig. 3B). No NKG2A was amplified from the DN population, as expected. The PCR products from the SP and DP populations were digested with MboI, a restriction enzyme that differentially digests NKG2A\textsubscript{B6} and NKG2A\textsubscript{BALB/c} (Fig. 3C). Digests from the DP cDNA contained both the NKG2A\textsubscript{B6} and NKG2A\textsubscript{BALB/c}-specific bands, whereas the digest from the SP cDNA contained only the NKG2A\textsubscript{BALB/c}-specific band, confirming that the SP cells express NKG2A mono-allelically. DNA was isolated from the cell populations and bisulfite treated. The CpG-rich region of NKG2A was PCR amplified as before, and products were sequenced and subjected to COBRA (Fig. 3D). The COBRA results show that this CpG-rich region is methylated in DN cells and unmethylated in DP cells, and sequencing confirms this result. Only sequences from unique clones are shown, as determined by either a unique pattern of CpG methylation or a unique pattern of non-CpG-unconverted cytosines. As the pattern of CpG methylation was well conserved, and the conversion rate of non-CpG cytosines was exceptionally high, the number of unique clones was low, although many identical clones were sequenced. COBRA on the SP sample suggested the presence of both methylated and unmethylated sequences, and the sequencing results confirm that CpG dinucleotides in individual clones were either all highly methylated, or all highly unmethylated. In the absence of any polymorphisms in this region, it is impossible to identify which clones come from which alleles. However, the linear pattern of methylation provides evidence that DNA methylation status of the CpG-rich region has a role in the allele-specific expression of NKG2A. A higher proportion of uncut PCR product was observed in the COBRA for the SP sample than would be suggested by the sequencing results, but this is most likely due to the fact that any heteroduplexes formed between methylated and unmethylated amplicons would be uncut (27).

**FIGURE 2.** Methylation status of CpG motifs correlates with NKG2A expression in ex vivo NK cells. A. NK1.1\textsuperscript{+}/NKG2A\textsuperscript{+} and NK1.1\textsuperscript{−}/NKG2A\textsuperscript{−} ex vivo splenocytes from B6 mice were sorted to >95% purity. B. COBRA from NKG2A\textsuperscript{−} (top panel) and NKG2A\textsuperscript{+} (bottom panel) ex vivo B6 cells. The region shown in Fig. 1C was amplified from bisulfite-treated DNA and digested with the following enzymes: U, Uncut; M, MboI; T, TaqI; S, Sau3A1; and H, HinfI. The arrow marks the position of the uncut band.

**FIGURE 3.** A role for DNA methylation in maintaining monoallelic expression of NKG2A. A. Nylon wool-passed spleen cells from B6 or B6 × BALB/c F\textsubscript{i} mice were stained with 16a11-biotin (anti-NKG2A\textsubscript{B6}), followed by NK1.1 allophycocyanin, streptavidin-PE, and 2D5-FITC (anti-NKG2B6/BALB). NK1.1\textsuperscript{−} cells were gated, then sorted for expression of NKG2A alleles. DP (NKG2A\textsubscript{B6}/NKG2B6/BALB/\textsuperscript{BALB}), SP (NKG2A\textsubscript{B6}/NKG2B6/BALB/\textsuperscript{BALB}), and DN (NKG2A\textsubscript{B6}/NKG2B6/BALB/\textsuperscript{BALB}) cells were sorted from hybrid mice, as indicated. B. NKG2A was amplified by RT-PCR from the different populations, and GAPDH was used as a positive control PCR. C. The RT-PCR products were digested with MboI, which has a different number of digestion sites in the B6 and BALB alleles of NKG2A. D. The CpG-rich region of NKG2A was amplified from bisulfite-treated DNA from the different populations. The products were cloned and sequenced, the results of which are shown on the left-hand side. A single line represents a single unique clone, and the circles represent CpG dinucleotides. ○, Indicate the CpG was methylated; □, indicate the CpG was unmethylated. COBRA results for the PCR products are shown on the right-hand side (enzymes sites were as described in previous figures). Arrows indicate the position of the uncut band.

**Hypomethylation and histone acetylation correlate with NKG2A expression in lymphoid cell lines**

Studies suggest that CpG methylation is linked to histone deacetylation, resulting in the formation of condensed, transcriptionally inactive chromatin (28). DNA was isolated from the mouse lymphoid cell lines EL4 (NKG2A negative) and KY-2 and LNK (NKG2A positive), and bisulfite treated. COBRA showed that the DNA methylation status of the CpG-rich region correlated with NKG2A expression in these cell lines (Fig. 4A). The CpG dinucleotides were predominantly methylated in the NKG2A-negative cell line EL4, and hypomethylation of CpG was associated with NKG2A expression in KY-2 and LNK cells, suggesting that DNA methylation has a role in regulating expression in cell lines as well as in ex vivo cells. We therefore used these cell lines to investigate whether acetylated histones are associated with the hypomethylated CpG sites.

Acetylation of histones H3 and H4 is an epigenetic modification associated with an open chromatin structure and transcriptionally active genes (29). The binding of acetylated histones to the NKG2A CpG-rich region in KY-2 and LNK cells compared with
EL4 cells was analyzed using ChIP. In these analyses, cross-linked chromatin was immunoprecipitated using anti-acetyl H3 (Lys9) or anti-acetyl histone H4 (Lys5, -8, -12, and -16) Abs. As a negative control, we included a precipitation reaction containing no Ab, and the input fractions before immunoprecipitation were used as positive controls. After immunoprecipitation and reversal of the cross-linking, the cell lines were analyzed using ChIP. In these analyses, cross-linked chromatin was immunoprecipitated using anti-acetyl H3 (Lys9) or anti-acetyl histone H4 (Lys5, -8, -12, and -16) Abs. Relative acetylation (calculated as described in Results) is shown for Nfm (negative control), and NKG2A (○) compared with values for Hprt (positive control, □). Values shown are means from three independent immunoprecipitations, and quantitative PCRs were performed in duplicate for each immunoprecipitation.

Exposure to chromatin-remodeling drugs induces NKG2A expression

To determine whether DNA methylation and/or association with unacetylated histones were sufficient for silencing NKG2A expression in the EL4 cell line, we investigated the role of chromatin-modifying drugs. EL4 cells were treated with the DNA methyltransferase inhibitor 5azaC, the histone deacetylase inhibitor TSA, or a combination of the two. Fig. 5 shows that no NKG2A transcripts were detected in untreated cells, or cells treated with 5azaC alone. However, treatment with TSA alone resulted in induction of NKG2A, providing evidence that histone status and chromatin structure are most likely the major controlling epigenetic influence in regulating mouse NKG2A transcription. Treatment with 5azaC and TSA together resulted in a greater induction of NKG2A transcription, providing evidence for a combinatorial effect.

DNA methylation status correlates with timing of NKG2A expression during development of NK cells in the bone marrow

NK cells develop from HSC in the bone marrow of adult mice, and NK receptors are acquired in an ordered fashion during development (31). The earliest marker of commitment to the NK lineage found on NK precursors (NKP) is CD122 (IL-2Rβ) (32), although these cells are still negative for NK1.1 and NKG2A. NK1.1 is used as a marker of functional NK cells, and expression of NKG2A begins at around the same time. Finally, expression of Ly–49 receptors occurs on adult NK cells developing in the bone marrow. Fetal and neonatal NK cells are believed to be of an immature phenotype in that they do not express Ly–49 receptors (with the exception of Ly–49E). Instead, the majority of fetal/neonatal NK cells express high levels of NKG2A, which decreases concomitant with increasing expression of Ly–49 receptors from ~6 wk after birth (31, 33, 34), suggesting some degree of coordinate regulation. To test the hypothesis that DNA methylation is important in regulating the timing of NKG2A expression during NK cell development in adult mice, we sorted the various development stages described above from the bone marrow of adult mice and determined the methylation status of the CpG-rich region of NKG2A at each stage (Fig. 6). Using a combination of sequencing and COBRA, we show that the CpG-rich region is methylated at all of the NKG2A-negative stages tested, including HSC, NKp, and NK1.1+/NKG2A− NK cells. In contrast, COBRA revealed that about half the PCR products from the NKG2A+ NK cells were unmethylated. The pattern of sequenced clones was predominately either all methylated or all unmethylated, as expected, from a monoallelic expression pattern of NKG2A, as described above. These results show that DNA hypomethylation is linked with timing of expression of NKG2A on developing adult NK cells in the bone marrow, providing evidence that epigenetic state, as opposed to lack of transcription factors, is important in blocking transcription of NKG2A throughout development.

Discussion

It is controversial whether epigenetic changes are the cause or effect of transcriptional activity, and the exact mechanisms involved remain unclear. The CpG dinucleotides we have identified

![FIGURE 5.](http://www.jimmunol.org/DownloadedFrom). Chromatin-remodeling drugs induce expression of NKG2A in the NKG2A− cell line EL4. EL4 cells were treated with either: lane 1, 8 μM 5azaC; lane 2, 100 nM TSA; lane 3, 8 μM 5azaC plus 100 nM TSA; or lane 4, untreated. Expression of NKG2A was determined by PCR for NKG2A cDNA from the samples (top panel), and the presence of cDNA in all samples was confirmed by actin PCR (bottom panel).
in NKG2A do not conform to the strict requirements of a CpG island (24), but they are definitely increased in density above the background genome. Another unusual feature of the NKG2A CpG-rich region is that it is located not in the promoter region of the gene, as is usually the case, but instead spans exon 1 and intron 1. This would suggest that the epigenetic modifications we have observed do not work in the same way as those that occur in the promoter region, in which it is believed that DNA methylation and histone acetylation result in a closed chromatin structure in the promoter that precludes binding of critical transcription factors.

Instead, we hypothesize that an intragenic closed chromatin structure is the major influence in silencing mouse NKG2A transcription, and that DNA methylation contributes to reduce transcription efficiency only if there is an open chromatin structure. Our model is that closed chromatin structure prevents transcription in cell types that do not work in the same way as those that occur in the background genome. Another unusual feature of the NKG2A CpG-rich region is that it is located not in the promoter region of the gene, as is usually the case, but instead spans exon 1 and intron 1. This would suggest that the epigenetic modifications we have observed do not work in the same way as those that occur in the promoter region, in which it is believed that DNA methylation and histone acetylation result in a closed chromatin structure in the promoter that precludes binding of critical transcription factors upstream of the transcriptional start site (35).

Our results have provided several pieces of evidence that support this hypothesis. We found that acetylated histones (indicative of an open chromatin structure) are associated with the CpG-rich region specifically in NKG2A-positive cell lines, and that inhibition of histone deacetylation by TSA is sufficient and necessary to induce NKG2A transcription in cell lines. The methyltransferase inhibitor 5azaC was unable to induce NKG2A expression, even when used at up to 20 μM (Fig. 5 and data not shown), suggesting that CpG demethylation alone is not sufficient to induce transcription. However, when used in combination with TSA, 5azaC induces a significant up-regulation of transcription compared with TSA alone, suggesting that DNA methylation may repress transcription efficiency in this region if it is in an open chromatin conformation. Furthermore, we have shown that methylation of one allele of the CpG-rich region is associated with monoallelic expression, providing further evidence that methylation may contribute to reduced NKG2A transcription.

The exact mechanisms relating epigenetic modifications and transcription of NKG2A remain unclear. A recent report has shown that intragenic DNA demethylation can alter chromatin structure and reduce RNA polymerase II elongation efficiency in mammals (36), but this was using a much denser CpG island, and only reduced transcription efficiency by 40%. However, reports have shown that methylation of a few intragenic CpG dinucleotides can reduce transcription efficiency. It has been established that efficient IL-4 transcription is strongly correlated with demethylation of non-CpG island CpG dinucleotides in a region spanning ~400 bp containing exon 1 and intron 1 of the gene (37). Whether the epigenetic status of NKG2A influences expression by reducing transcriptional elongation remains to be determined. Alternatively, methylation of the CpG-rich region of NKG2A and a closed chromatin structure may prevent transcription factors binding to an intronic enhancer element, thereby inhibiting initiation of transcription.

The association with combined epigenetic modifications and transcription of NKG2A described in this work are remarkably similar to our results for Ly-49A (21). We have shown previously that both DNA hypomethylation and histone acetylation of an intragenic region are associated with transcriptional activity of Ly-49A. The observation that both these genes appear to be regulated in the same way may help to explain why NKG2A is regulated by epigenetic mechanisms, and exhibits monoallelic expression. This type of mechanism is often found in genes that require tightly regulated, but highly flexible control, and is often associated with allelic exclusion. For example, the epigenetic regulation of the highly polymorphic, multigene Ly-49 gene family provides a mechanism that creates a diverse NK receptor repertoire in the NK cell population, allowing for highly sensitive detection of changes in specific MHC class I genes and alleles. However, NKG2A is virtually monomorphic, and the other members of the multigene family (NKG2C and E) are expressed at very low levels on adult NK cells (Fig. 3) (38). Therefore, there appears little need for NKG2A to be expressed in a stochastic and monoallelic fashion. Nevertheless, it appears that there is some degree of coordinate regulation between Ly-49A and NKG2A during early development from neonate to adult. NKG2A is expressed by the majority of fetal and neonatal NK cells in the absence of Ly-49 receptors, but its expression declines to ~50% of mature adult cells concomitant with increased expression of Ly-49 receptors (38, 39). A highly similar mechanism of transcriptional control helps to understand how this coordination may occur. It is possible that the characteristics attributed to epigenetic regulation (ordered, but stochastic and monoallelic expression) are beneficial and necessary only to the Ly-49 family. The fact that these characteristics are also exhibited by mouse NKG2A may just be a result of common epigenetic mechanisms for gene control that was acquired early in the evolution of the NKC-encoded genes, and the real benefit in terms of NKG2A transcription lies not in stochastic and monoallelic expression, but the ability to be tightly regulated with transcription of Ly-49 genes, ensuring the correct switch in expression patterns during development of NK cells. Perhaps disruption of this epigenetic switch may be responsible for the delayed

### Table 1: Summary of Epigenetic Modifications in NKG2A and Ly-49A

<table>
<thead>
<tr>
<th>Modification</th>
<th>NKG2A</th>
<th>Ly-49A</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA methylation</td>
<td>Reduced</td>
<td>Increased</td>
</tr>
<tr>
<td>Histone acetylation</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Chromatin structure</td>
<td>Closed</td>
<td>Open</td>
</tr>
<tr>
<td>Transcription efficiency</td>
<td>Reduced</td>
<td>Increased</td>
</tr>
</tbody>
</table>

### Figure 6: A role for DNA methylation in regulating expression of NKG2A during development of NK cells in the bone marrow.

The CpG-rich region of NKG2A was amplified from bisulfite-treated DNA, and histone acetylation in immature NK cells results in expression of NKG2A, which is associated with CpG demethylation. Once the region is in an open chromatin conformation, transcription occurs so that NKG2A is expressed. Our results have provided several pieces of evidence that support this hypothesis. We found that acetylated histones (indicative of an open chromatin structure) are associated with the CpG-rich region specifically in NKG2A-positive cell lines, and that inhibition of histone deacetylation by TSA is sufficient and necessary to induce NKG2A transcription in cell lines. The methyltransferase inhibitor 5azaC was unable to induce NKG2A expression, even when used at up to 20 μM.
development to a mature phenotype observed in a mouse model of stem cell transplant (40), a phenomenon that is also observed in a proportion of human recipients (41–44). It will be interesting to investigate the role of epigenetics in regulating other genes in the NK complex, as this may provide clues as to the degree and/or origins of coordinate regulation. To date, we have found that acetylated histones are associated with DNase I-hypersensitive sites in the promoter of CD94 specifically in CD94-positive, but not negative, cell lines (S. Rogers, D. Mager, and E. Lee, unpublished observations). This provides evidence that epigenetic control of transcription may have a role in coordinating transcription of functionally related genes, as CD94 forms a heterodimer with NKG2A at the cell surface.

The role of epigenetic mechanisms in regulating expression of human NKG2A remains unclear, in part due to the different gene organization at the 5′ end. As shown in Fig. 1, human NKG2A contains two extra exons encoding the 5′ UTR, resulting in a different transcriptional start site compared with mouse NKG2A. We hypothesize that the insertion of an Alu transposable element just upstream of human exon 2 (the homologue of mouse exon 1) may have disrupted the original human promoter, resulting in the gain of an alternative, upstream promoter region. Despite the apparent lack of a syntenic CpG-rich region in human NKG2A, there is evidence to suggest that epigenetic mechanisms may have a role in the regulation of human NKG2A. The Alu element is particularly CpG rich, and it has been shown that hypomethylation of this region is associated with transcription of NKG2A in NK cell lines (M. Uhrberg, personal communication).

Human KIR are the functional equivalents of the mouse Ly-49 genes and, like Ly-49, they appear to be coordinately regulated with NKG2A such that NKG2A expression decreases as KIR expression increases on developing NK cells (17, 45–47). However, in contrast to Ly-49, in which we have shown previously that both DNA methylation and acetylated histones are associated with gene silencing (21), it is believed that DNA methylation status alone is the primary mechanism determining the expression of KIR genes in adult NK cells (13, 20). This difference may arise from the different CpG distribution observed between the gene families. KIR genes contain CpG islands in their promoter regions (18, 19), in contrast to Ly-49A, which has discreet intragenic CpG dinucleotides (21). The results presented in this work demonstrate that the epigenetic modifications associated with NKG2A and Ly-49 transcription are highly similar, and we have proposed that this may be to allow coordinate regulation in the mouse. It will be interesting to determine whether both histone deacetylation and DNA methylation are associated with NKG2A gene silencing in humans, or whether human NKG2A will more closely resemble KIR genes in that DNA methylation alone may be sufficient. It is possible that the KIR and NKG2A regulatory mechanisms may be more different from each other in humans, as the genes are on separate chromosomes. Conversely, the presence of Alu elements near KIR promoters and in intron 2 of human NKG2A may provide a mechanism for coordinated epigenetic regulation, conceivably via methylation spreading.

In summary, we have demonstrated that an intragenic region of mouse NKG2A exists in strikingly different epigenetic states that are associated with transcriptional activity during development of primary adult NK cells. These results significantly enhance our understanding of mechanisms and timing involved in the formation and maintenance of NK receptor repertoires.

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Disclosures
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