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Evidence for high bi-allelic expression of activating Ly49 receptors

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ABSTRACT

Stochastic expression is a hallmark of the Ly49 family that encode the main MHC class-I-recognizing receptors of mouse natural killer (NK) cells. This highly polygenic and polymorphic family includes both activating and inhibitory receptor genes and is one of genome's fastest evolving loci. The inhibitory *Ly49* genes are expressed in a stochastic mono-allelic manner, possibly under the control of an upstream bi-directional early promoter and show mono-allelic DNA methylation patterns. To date, no studies have directly addressed the transcriptional regulation of the activating Ly49 receptors. Our study shows differences in DNA methylation pattern between activating and inhibitory genes in C57BL/6 and F1 hybrid mouse strains. We also show a bias towards bi-allelic expression of the activating receptors based on allele-specific single-cell RT–PCR in F1 hybrid NK cells for *Ly49d* and Ly49H expression in *Ly49h*$^{+/-}$ mice. Furthermore, we have identified a region of high sequence identity with possible transcriptional regulatory capacity for the activating *Ly49* genes. Our results also point to a likely difference between NK and T-cells in their ability to transcribe the activating *Ly49* genes. These studies highlight the complex regulation of this rapidly evolving gene family of central importance in mouse NK cell function.

INTRODUCTION

Natural killer (NK) cells constitute an important part of the body's defence both as centurions of innate immunity and as communicators and collaborators of the adaptive immune system. They exert their function via the interpretation of signals received from their surface receptors. Normal cells display major histocompatibility complex (MHC) class I molecules that are recognized by a number of inhibitory receptors on the surface of NK cells. The inhibitory receptors prevent activation of NK cells and the destruction of normal, MHC class I-possessing cells (1). NK cells also possess stimulatory (activating) receptors that recognize other molecules on the surface of potential target cells which are mostly pathogen-encoded or stress-induced self proteins (2–4).

NK activation and the killing of target cells therefore depend on the balance between stimulatory and inhibitory signals received from these surface receptors. In primates and some other mammals, the killer cell immunoglobulin-like (*KIR*) family of genes code for the main MHC class-I recognizing receptors (5). However, in rodents, the structurally unrelated Ly49 family provides this function. Both KIR and Ly49 families are polygenic and polymorphic among different individuals and mouse strains (6,7).

The *KIR* gene cluster of human is ~150 kb containing approximately 14 genes and pseudogenes with very high coding and regulatory sequence similarity (8) indicating that they arose through recent gene duplication events (9). There is great diversity in both gene number and sequence polymorphisms for the *KIRs* among different people (6). The *Ly49* gene cluster has also arisen from recent duplications and gene conversions of ancestral genes (10–12). Hence, there is high sequence similarity both in the coding and non-coding regions among the majority of the genes. The *Ly49* cluster includes 16 genes and pseudogenes spanning over 600 kb in the C57BL/6 (B6) mouse strain and is located in the natural killer gene complex (NKC) region of chromosome 6 (8,13). There are two functional Ly49 activating receptors, Ly49D and H, coded by the B6 genome. Ly49D binds to the MHC-class-I allele H2-D^{d} (14,15) and Ly49H binds to the m157 protein of mouse cytomegalovirus (MCMV) conferring resistance towards virus infection in the strains of mice that express Ly49H (2,3,16,17). Unlike the

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inhibitory Ly49 receptors that are expressed on T and NKT cells as well as NK cells, the stimulatory Ly49 receptors are only expressed on NK cells (18). Lack of a well-defined upstream promoter-1 (Pro-1) region, which likely acts as a stochastic switch for inhibitory receptor genes in immature NK cells (19,20) and reports of the higher co-expression of Ly49D and H and deviation from the product rule (21), suggest that the activating *Ly49* genes, despite being surrounded by inhibitory genes in the *Ly49* gene cluster, are subject to distinct regulatory mechanisms.

DNA methylation of the 5′-region of the inhibitory *Ly49* genes correlates with their expression. In the case of *Ly49a* and *Ly49c*, where stochastic mono-allelic expression has been demonstrated, we have previously shown that DNA methylation and mono-allelic receptor expression correlate (22). The existence of stochastic mono-allelic expression and/or correlation with DNA methylation is unknown for the activating receptors. Here, we have investigated the link between DNA methylation of the 5′-region of activating receptors and the maintenance of their expression. Furthermore, we show evidence for a mode of transcriptional regulation for the activating *Ly49* genes that differs from that of the inhibitory genes.

MATERIALS AND METHODS

Mice

All C57BL/6 mice were bred and maintained in the animal facility of the British Columbia Cancer Research Centre (Vancouver, British Columbia, Canada). 129SvEvTac and 129SvEvTac/C57BL6 F1 hybrids were ordered from Taconic farms. NOD/ShiLtJ and C57BL6/NOD-ShiLtJ F1 hybrids were ordered from the Jackson Laboratory (Bar Harbor, Maine, USA). All mice used in this study were more than 6-weeks old. The B6.BXD8/B6 (23) and age-matched B6 mice were 6.5-weeks old. All experiments were according to a protocol approved by the Committee on Animal Care of the University of British Columbia.

Antibodies, cell separation and flow cytometry

The monoclonal antibody (mAb) anti-FcRγ (2.4G2) (24,25) and 3D10-FITC (21) have been described before. Anti- CD3ε-PerCP-Cy5.5, anti- NK1.1-PE, anti-NK1.1-APC, anti-DX5-PE (alpha-2 integrin, CD49b), 1F8-FITC (anti-Ly49C/I/H), 5E6-biotin (anti-Ly49C/I), 4E5-FITC (anti-Ly49D), A1-biotin (anti-Ly49A^{B6}) and fluorochrome-conjugated streptavidin were purchased from BD Biosciences (Mississauga, Ontario, Canada). The anti-mouse NKp46-PE antibody was purchased from eBioscience (San Diego, CA, USA). The 12A8 purified antibody was generously provided by Dr Stephen Anderson (NCI, Frederick, MD, USA) and was conjugated to FITC via Thermo Scientific Pierce EZ-label fluorescein isothiocyanate protein labelling kit (Rockford, IL, USA) per manufacturer's protocol. Flow cytometry for cell sorting was performed on Cytopeia Influx cell sorter and Becton Dickinson FACSAria Cell Sorting System (Mississauga, Ontario, Canada). All sorted samples were >95% pure or resorted for high purity. For the single-cell sorting, NK cells were sorted into wells containing JEG-3 (human choriocarcinoma) as carrier cells. Doublet discrimination was applied to avoid more than one cell per well.

Primary cell and tissue genomic DNA extraction

Genomic DNA (gDNA) was obtained from FACS sorted cells as described before (22). gDNA was extracted from fresh B6 mouse liver using DNAzol reagent (Invitrogen) per manufacturer's instructions. Further proteinase K digestion and phenol–chloroform extraction was performed.

Sodium bisulfite conversion and PCR

Bisulfite conversion was performed as described previously (22). The conversion rate was >98%. First-round PCR amplification of *Ly49h* 5′ region was performed using *Ly49h* forward and reverse flanking bisulfite primers. Converted DNA was used as template in a 45 µl reaction volume, containing 30 pmol of each primer, 1 mM dNTPs, 3 mM $MgCl_2$ and 0.5 U *Taq* Platinum DNA polymerase (Qiagen). After initial denaturation for 7 min at 95°C, 30–40 cycles were performed, each consisting of 90 s at 95°C, 55 s at 50°C and 40 s at 72°C with a final extension of 7 min at 72°C. Two microliters of the first PCR was used for nested amplification using *Ly49h* forward and reverse nested bisulfite primers. The same amplification conditions were chosen as for first-round PCR with the exception that the annealing temperature was changed to 48 + 0.1°C/cycle. All bisulfite primer sequences are shown in Table 1.

For the *Ly49d* 5′ region of the B6 strain the following primers were used to amplify two regions for COBRA analysis of two CpG dinucleotides: first round (flanking) PCR was performed with *Ly49d* forward and reverse flanking bisulfite primers. After initial denaturation for 7 min at 95°C, 35 cycles were performed, each consisting of 90 s at 95°C, 55 s at 51°C and 50 s at 72°C with a final extension of 7 min at 72°C. Two separate nested PCRs were performed on 3 µl of the product of the flanking PCR to amplify two regions containing an upstream and a downstream (in relation to transcription start site) CpG dinucleotide. For the upstream CpG-containing fragment the *Ly49d* forward flanking and reverse nested bisulfite primers were used. After initial denaturation for 7 min at 95°C, 35 cycles were performed, each consisting of 90 s at 95°C, 55 s at 51°C and 17 s at 72°C with a final extension of 7 min at 72°C. The downstream CpG-containing fragment was amplified via the *Ly49d* forward nested and reverse flanking bisulfite primers. After initial denaturation for 7 min at 95°C, 35 cycles were performed, each consisting of 90 s at 95°C, 55 s at 53°C and 40 s at 72°C with a final extension of 7 min at 72°C. The resulting products were subjected to combined bisulfite and restriction enzyme analysis (COBRA).

For *Ly49d* and *Ly49r* 5′ region amplification from the 129SvEvTac/C57BL6 F1 hybrid cells, first round PCR was performed with the same flanking primers as *Ly49d* as their sequence is identical to *Ly49r* with the same PCR conditions as that used for *Ly49d* of B6. Nested PCR was

Table 1. Oligonucleotide sequences of primers and probes

Ly49h forward flanking bisulfite primer	5′-ATA GGG GAA TGT TAG GGT TAA AAA G-3′
Ly49h reverse flanking bisulfite primer	5′-ATT TAA CCT AAT ATA ACA CAA CCA A-3′
Ly49h forward nested bisulfite primer	5′-GGA TAT ATG TTT TGT TTT TTT TGG T-3′
Ly49h reverse nested bisulfite primer	5′-TAA CAC AAC CAA AAA AAC TCT CAA C-3′
Ly49d forward flanking bisulfite primer	5′-TAT TAA GAT GTA ATT AGT ATG ATT TAA T-3′
Ly49d reverse flanking bisulfite primer	5′-ACA ATA CAT TTA TAC ACT TCA CCT AA-3′
Ly49d reverse nested bisulfite primer	5′-CCA AAT ACT ACA AAA AAA ATA ACT ATA T-3′
Ly49d forward nested bisulfite primer	5′-AGG TAG AGT TAT AGG TAA TAA TAG T-3′
Ly49d/*r* reverse nested bisulfite primer	5′-TTC CTC TAC CTT AAT TTC TTA AC-3′
Ly49d Exon 2 forward primer	5′-CGG AAG CCT GAA AAA GCT CG-3′
Ly49d Exon 4 reverse primer	5′-TCA CAC AGT ATG TTT TGA TCC C-3′
Ly49h Exon 2 forward primer	5′-GAA CAG CCA GGT GAG ACT T-3′
Ly49h Exon 3-4 reverse primer	5′-TGT TTG TGA CAA AGT TTT TTC AGT-3′
Nkg2d Exon 2 forward primer	5′-ACT ACC AGT CAA CCT GGA GAA-3′
Nkg2d Exon 6 reverse primer	5′-GAC ATA TCC AGT TGT TAG GGC AT-3′
$Ly49d^{B6/NOD}$ forward Exon 2 primer	5′-GCT GTG AGA TTC CAT AAG TCT TC-3′
$Ly49d^{B6/NOD}$ reverse Exon 4 primer	5′-GAT GCT GCA GTT ATT GTG GTG-3′
$Ly49d^{B6}$-specific oligonucleotide probe	5′-CGG AAG CCT GAA AAA GCT CG-3′
$Ly49d^{NOD}$-specific oligonucleotide probe	5′-AGC CTC GAA AAG CTG GCC TCA-3′
Nkg2d-specific oligonucleotide probe	5′-CAA TTC GAT TCA CCC TTA ACA CAT T-3′
Ly49h Exon 2 inner reverse primer	5′-AAA GTG ACC TCC TGC TCA CT-3′
Ly49d forward EMSA probe	5′-AGA AAA GGC CCA CAT TAC CCC AAC AGG GAC ATC CAT TCC TTC TAC-3′
Ly49h forward EMSA probe	5′-AGT AAA GGC CCA CAT TAC CCC AAT TGA GGC ATC CAT TCT TTC TAC-3′
Ly49a forward EMSA probe (Plus two T-10-mers)	5′-ttttttttttt AGA AAA AGC CAA CTT TTT CCT CCA C ttttttttttt-3′
Ly49c forward EMSA probe (Plus two T-4-mers)	5′-tttt AGA AAA CGC CAA CGT TTC AGA CAA ATT TTC CCT CCA C tttt-3′

performed on 2 µl of the flanking PCR product with the forward nested *Ly49d* primer (which is identical to *Ly49r* sequence) and *Ly49d*/*r* reverse nested bisulfite primer. After initial denaturation for 7 min at 95°C, 35 cycles were performed, each consisting of 90 s at 95°C, 55 s at 50 + 0.1°C/cycle and 45 s at 72°C with a final extension of 7 min at 72°C. The PCR products were electrophoresed on 1% agarose gels and correct size bands were extracted using the MinElute gel extraction kit (Qiagen). The purified products were cloned into the T-vector using the pGEMT-vector kit (Promega). Sequencing was performed using the SP6 primer by McGill University and Genome Québec Innovation Centre sequencing facility. All clones included in the figures are unique as per criteria stated before (22).

Combined bisulfite and restriction enzyme analysis

For COBRA, gel purified fragments of *Ly49d* upstream and downstream regions were digested with $Taq^{\alpha}I$ and BmgBI restriction enzyme (NEB), respectively to distinguish between methylated (CpG) and unmethylated (TpG). Only fragments that were originally methylated in the gDNA and therefore not converted by sodium bisulfite treatment are cut. Gel purified *Ly49h* fragments were digested with $Taq^{\alpha}I$ (NEB) and BsaAI (NEB) restriction enzymes.

RNA extraction, RT–PCR and 5′ amplification of cDNA ends

cDNA was generated from total B6 and NOD/ShiLtJ spleens and FACS sorted B6 T-cell RNA per SuperScript III protocol. PCR was performed on the generated cDNAs using primers specific for *Ly49d*, *g*, *h*, *Nkg2d* and *actin*. *Ly49g* and *actin* primers have been described before (22). All RT–PCR primer sequences are shown in Table 1.

5′-Rapid amplification of cDNA end (5′-RACE) was performed on B6 spleen RNA using the FirstChoice RML-RACE kit (Ambion) per manufacturer's protocol with *Ly49h*-specific primers. For the outer PCR, the *Ly49h* Exon 3–4 reverse primer used for RT–PCR was used as the outer 5′-RACE primer in combination with the kit's outer forward primer. For the inner PCR, *Ly49h* Exon 2 inner reverse primer in combination with the kit's inner forward primer. The products were cloned into the T-vector using the pGEMT-vector kit (Promega). Sequencing was performed using the T7 primer by McGill University and Genome Québec Innovation Centre sequencing facility.

Electrophoretic mobility shift assay

Nuclear extract from FACS sorted B6 T-cells and IL-2-expanded FACS-sorted B6 NK cells were prepared as described before (26). Electrophoretic mobility shift assay (EMSA) was performed with the following double-stranded probes located downstream of the transcriptional start site within exon1 of various Ly49 genes (only the forward strand is shown in Table 1): to control for length differences among the probes, poly-T tails were added to the shorter *Ly49a* and *c* probes. The YY1 antibody (sc-281) was purchased from Santa Cruz Biotechnology and supershift experiments were performed as described before (26).

Single-cell RT–PCR

Single-cell RT–PCR and Southern blotting was performed as described before (27) with minor modifications. RNA from JEG-3, a human choriocarcinoma cell line (28), was used as carrier. *Ly49d* forward exon 2 and reverse exon 4 primers were used to specifically amplify *Ly49d* cDNA of both B6 and NOD/ShiLtJ. $Ly49d^{B6}$-specific oligo probe and $Ly49d^{NOD}$-specific oligo probe were used to

specifically detect cDNA from $Ly49d^{B6}$ or $Ly49d^{NOD}$ in Southern blot analysis. For *Nkg2d*, the same primers used for RT–PCR (previous section) were used and the products were identified after Southern blotting with an *Nkg2d*-specific oligo probe. Up to five sets of independent PCRs were performed on each single cell.

RESULTS

Lack of detectable activating *Ly49* transcripts in T-cells

Unlike the inhibitory Ly49 receptors, the activating Ly49 receptors of the B6 strain (Ly49D and H) are not expressed on the surface of T and NKT cells (29,30). This absence of surface expression on T and NKT is most likely due to the negligible amounts of the activating adaptor protein DAP12 that seems to be required for cell surface expression of the B6 activating Ly49 receptors (31,32). However, there are reports of the association of Ly49D with the activating adaptor protein CD3ζ and DAP10 (33,34). We also did not detect Ly49R (an activating receptor in 129 mice) or Ly49D and R surface expression on 129SvEvTac or 129SvS6/B6 F1 hybrid fresh *ex vivo* splenic T-cells ($DX5^-$, $CD3\varepsilon^+$) respectively by flow cytometry (data not shown). To determine if transcripts for the activating receptors exist in T-cells, fresh *ex vivo* splenic T-cells were sorted with high purity and RT–PCR with *Ly49d* and *Ly49h*-specific primers was performed on the isolated RNA (Figure 1A). Transcripts from both *Ly49d* and *Ly49h* were detected in whole spleen but very little to none in splenic T-cells, which correlates with the lack of surface expression of these receptors on T-cells. We detected similar amounts of the inhibitory *Ly49g* and *Nkg2d* cDNA in both splenic lymphocytes and sorted T-cells.

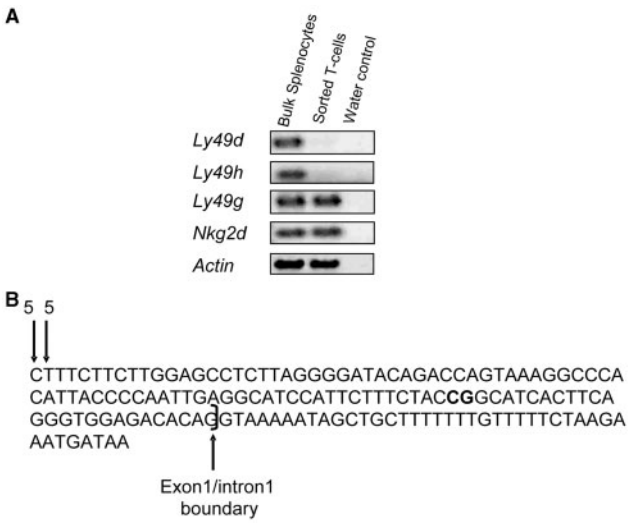

Figure 1. Transcription of activating *Ly49* genes. (**A**) RT–PCR on whole spleen and sorted splenic T-cells was performed for actin (25 cycles), the inhibitory *Ly49g* and activating *Ly49d* and *h* as well as *Nkg2d* (30 cycles) with gene-specific primers. (**B**) 5′RACE of *Ly49h* on cDNA from whole spleen. Vertical downward-pointing arrows show the transcription start sites assayed by 5′RACE. The numbers on top of the arrows show the number of sequenced clones beginning at a given nucleotide position. The exon 1/intron 1 boundary is also indicated.

5′-region DNA methylation of *Ly49d* and *Ly49h* correlates with state of expression

Ly49 genes have multiple promoters but most are transcribed from a region called Pro-2, which is thought to be the main promoter in mature NK cells (19,35). The transcriptional start site of *Ly49d* has been mapped to the Pro-2 region (35). To determine the promoter of origin for *Ly49h*, 5′RACE was performed on whole spleen RNA with gene-specific primers. The *Ly49h* transcripts detected originated from Pro-2. However, unlike other *Ly49* genes (35) we did not detect transcriptional start site variability for *Ly49h* (Figure 1B).

Based on the 5′RACE results, the region equivalent to the Pro-2 of the inhibitory *Ly49* genes is the main area of transcriptional start for *Ly49h*. We examined the DNA methylation status of the CpG dinucleotides of this region that we shall refer to as the 5′-region of *Ly49h* from here onwards. There is a cluster of six CpGs within the proximal 5′-region of *Ly49h* with three CpGs located upstream and three located downstream of the transcription start site (Figure 2A).

Sodium bisulfite sequencing revealed heavy methylation of this region in FACS-sorted Ly49H-negative ($1F8^-$) splenic NK and T-cells but not in Ly49H-positive ($1F8^+$, $5E6^-$) NK cells (Figure 2B). As with the inhibitory *Ly49a* gene (22), the CpG sites downstream of the transcriptional start site are most heavily methylated in Ly49H-negative cells. However, unlike for *Ly49a* and *Ly49c* which display a 'half-and-half' mono-allelic methylation pattern, the Pro2 region was hypo-methylated in all clones sequenced in the $Ly49H^+$ population. The bisulfite sequencing results were also confirmed by COBRA (Figure 2C).

There are very few CpG dinucleotides in the 5′-region of *Ly49d*. Hence, we only used COBRA to analyze two CpG dinucletides, one upstream and the other downstream of the transcription start site (Figure 2D). $Taq^{\alpha}I$ and BmgBI restriction endonucleases were used to assay the upstream and downstream CpGs, respectively. Ly49D expressing and non-expressing NK cells of fresh *ex vivo* spleen were FACS sorted and analyzed for DNA methylation at these two CpGs. The results are the combination of four individual PCRs per each sorted population. Ly49D negative NK cells show moderate amounts of DNA methylation at both assayed CpGs where as the Ly49D positive NK cells show almost no DNA methylation at either site (Figure 2E) reflecting a DNA methylation pattern similar to that observed for the *Ly49h* 5′-region in Ly49H positive NK cells (Figure 2B and C).

5′-region DNA methylation of *Ly49d* and *Ly49r* does not correlate with stochastic mono-allelic receptor expression in 129S6/B6 F1 hybrid

Mono-allelic gene expression has not been shown for the activating *Ly49* genes and we did not observe the bimodal

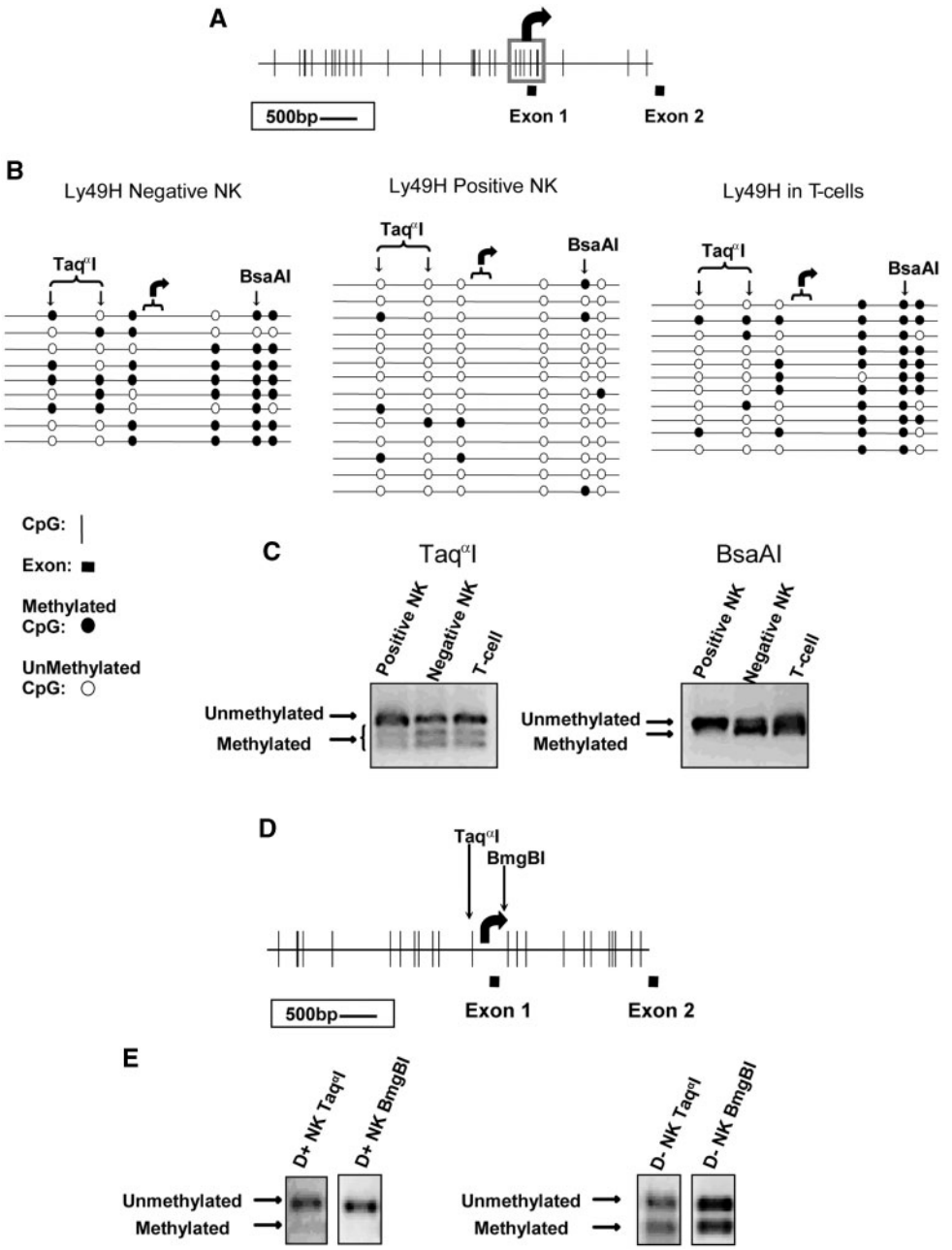

Figure 2. DNA methylation patterns of the 5′ regions of *Ly49d* and *h* in the B6 strain. (**A**) Location of all CpG dinucleotides in the 5′ region of *Ly49h* is shown. CpGs are represented by vertical lines, black boxes represent exons and the bent arrow indicates the transcriptional start and the direction of transcription. The CpGs within the boxed region (~430 bp) were assayed for methylation in primary C57BL/6 splenic NK cells via sodium bisulfite sequencing (**B**) and COBRA (**C**). For bisulfite sequencing, each line represents the sequence of an independent clone. The location of the CpG dinucleotides assayed by COBRA are indicated by arrows. Fragments that contain a CpG dinucleotide at these locations are digested by restriction endonuclease indicating methylation in the original genomic DNA. Fragments that remain uncut contain a TpG instead of a CpG, which indicates that in the original genomic DNA template this CpG was unmethylated. (**D**) CpG dinucleotide distribution in the 5′ region of *Ly49d* is shown. CpGs are represented by vertical lines, black boxes represent exons and the bent arrow indicates the transcriptional start (35) and the direction of transcription. (**E**) Two CpGs indicated with vertical arrows were assayed for methylation in primary B6 splenic NK cells by COBRA.

DNA methylation pattern typical of the mono-allelically expressed inhibitory *Ly49a* and *c* (22) for *Ly49d* and *h* in receptor-expressing NK cells. In order to verify the DNA methylation status of both alleles of an activating receptor in receptor-expressing NK cells, we investigated the DNA methylation status of *Ly49d* in the 129S6/B6 F1 hybrid. Antibody binding and specificities have been examined for the 129S6 strain Ly49 receptors allowing for sorting and analysis of specific Ly49-expressing subpoulations (36). $Ly49d^{B6}$ and $Ly49r^{129}$ are considered alleles based on the criteria presented by Makrigiannis *et al.* (36,37) such as coding region homology, intron homology and gene order. We therefore chose to assay DNA methylation of the 5′-regions of *Ly49d* and *r* in the F1 hybrid.

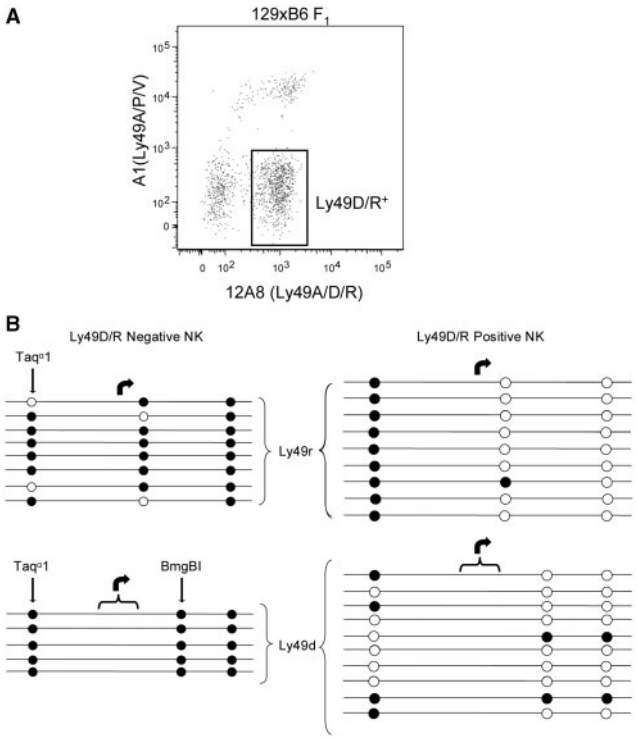

Figure 3. DNA methylation of *Ly49d* and *Ly49r* 5′-region in the F1 hybrid of 129/S6 and B6. (**A**) Ly49D/R expression profile of 129S6xC57Bl/6 F1 hybrid fresh spleen NK cells ($DX5^+$, $CD3\varepsilon^-$). The Ly49D/R-expressing ($12A8^+$, $A1^-$) population (gated) and non-expressing ($12A8^-$, $A1^-$) were sorted and analysed for DNA methylation. (**B**) Sodium bisulfite sequencing of 12A8-positive/A1-negative NK cells (Ly49D/R positive) and 12A8-negative NK cells (Ly49D/R negative). *Ly49d* and *r* differ in the position of one CpG dinucleotide but also have other polymorphisms in this region (~700 bp). All clones presented here are unique.

There are no antibodies available that bind Ly49D but not Ly49R or vice-versa, making receptor/allele-specific sorting impossible because the 4E5 and 12A8 antibodies (14) detect both receptors. However, the 4E5 antibody also detects $Ly49O^{129}$ and $Ly49V^{129}$ where as 12A8 binds to $Ly49A^{B6}$ as well (36). The percentage of NK cells expressing Ly49D (stained with 4E5) and R (stained with 12A8 antibody) in B6 and 129S6 strains respectively is 50-60% (data not shown). In order to specifically sort Ly49D and R expressing cells in 129S6/B6 F1 hybrid, splenic lymphocytes were co-stained with 12A8 (anti-Ly49D, R and A^{B6}) and A1 (anti-$Ly49A^{B6}$) antibodies. The A1 antibody also detects $Ly49P^{129}$ and V^{129} with low binding affinity (36).

Assuming *Ly49d* and *r* are allelic, we hypothesized that, based on the lack of a bimodal DNA methylation pattern observed for Ly49D and H expressing NK cells in B6, both *Ly49d* and *r* 5′-regions should be hypo-methylated. We FACS sorted 12A8-positive/A1-negative NK cells (Figure 3A). This population should include Ly49D-single positive, Ly49R single-positive and Ly49D and R double positive cells. A few polymorphisms in the 5′-region of *Ly49d* and *r*, assayed by sodium bisulfite sequencing, allow for their distinction (Figure 3B).

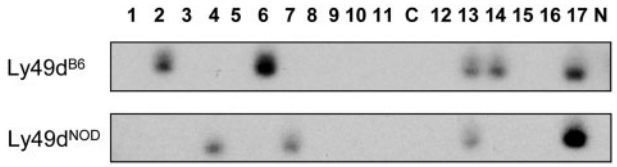

Figure 4. Detection of $Ly49d^{B6}$ and $Ly49d^{NOD}$ cDNA by single-cell RT–PCR and Southern blot. $Ly49d^{B6}$-specific and $Ly49d^{NOD}$-specific probes were hybridized to identical blots of amplified cDNA generated from single-cell RT–PCR on FACS sorted F1 hybrid splenic NK cells. This figure shows a representative experiment (one set of PCRs) where seven individual cells show one or more products. Three cells contain $Ly49d^{B6}$ only, two cells contain $Ly49d^{NOD}$ only and two cells contain both products. C indicates a lane with cDNA from JEG-3 carrier cells only (no sorted NK cell) and N indicates the no-template control lane. After five sets of independent PCRs 4 of 9 single cells in this combination of F1 cells showed bi-allelic expression for *Ly49d* (data not shown).

We also sorted 12A8 negative (Ly49D/R non-expressing) NK cells and analyzed the 5′-region of *Ly49d* and *r* for this population as well (Figure 3B). As expected, the 5′-region of *Ly49d* and *r* in the Ly49D/R-negative NK cells was hyper-methylated for all the sequenced clones. With the exception of two clones for *Ly49d* and one clone for *Ly49r*, all other clones (16 clones) are hypo-methylated in Ly49D/R-positive NK cells. The most 5′ CpG of all of the nine independent Ly49R-positive clones is methylated in Ly49D/R-positive NK cells. However, the rest of the methylation pattern does not resemble that of the Ly49D/R-negative cells. We also sequenced the *Ly49r* Pro2 region from Ly49R-postive NK cells of the 129/SvEvTac mouse (the parent of the F1 hybrid) to gauge the methylation of the most 5′ CpG. We observed the same methylation pattern as in the F1 (Supplementary Figure 1) possibly indicating that this pattern of methylation is specific to the *Ly49r* locus. The bimodal DNA methylation observed for the inhibitory *Ly49a* and *c* is not observed for *Ly49d* and *r*, rather the pattern of DNA methylation resembles that observed for *Ly49h*.

Detection of bi-allelic expression of *Ly49d*

The recent sequencing and assembly of the NOD *Ly49* cluster (7) provided an opportunity to test allelic expression of activating Ly49 receptors. While there may be some question as to the allelic relationship of $Ly49d^{B6}$ and $Ly49r^{129}$, the NOD strain has a definite *Ly49d* allele with ~98.5% identity to the B6 *Ly49d* sequence at the level of cDNA (7,38). In order to directly test the quality and quantity of allelic expression of Ly49D, we single-cell FACS sorted NK cells ($NKp46^+$, $DX5^+$, $NK1.1^+$, $CD3\varepsilon^-$) from B6/NOD F1 hybrid spleen and performed single-cell RT–PCR (27) followed by Southern blot and hybridization with allele-specific probes (see Figure 4 for example).

We assayed 88 NK cells and detected cDNA for $Ly49d^{B6}$ and/or $Ly49d^{NOD}$ in 40 cells where cDNA from both $Ly49d^{B6}$ and $Ly49d^{NOD}$ was detected in 22 of these 40 cells indicating 55% bi-allelic expression (Table 2). We never detected any products from wells that only

Table 2. Single-cell RT–PCR results

cDNA type	Ly49D^{B6}	Ly49D^{NOD}	Ly49D^{B6} and ^{NOD}	Total Ly49D$^+$	Ly49D$^-$ NKG2D$^+$	Ly49D$^+$ NKG2D$^+$	Total NKG2D$^+$	Ly49D$^+$ NKG2D$^-$	Total cells with cDNA
Number of transcribing single cells (n)	5	13	22	40	40	33	73	7	80
Percentage positive	5/40 = 12.5%	13/40 = 32.5%	22/40 = 55%		40/73 = 55%	33/73 = 45%		7/80 = 8.8%	

contained carrier cells. The single-cell RT–PCR technique will underestimate bi-allelic expression due to the fact that one allele may not be detected through inefficient cDNA generation during the RT reaction, multiple sample purification steps and random sampling error (27,39).

As a control for the efficiency of our method, we used *Nkg2d*, an activating receptor gene that is transcribed in all NK cells (40) most probably at levels similar to the *Ly49* genes, to gauge the efficiency of cell sorting, cDNA generation and detection. We only detected 73 *Nkg2d*$^+$ cells from the total cells assayed after multiple sets of independent PCRs (Table 2). *Nkg2d* transcripts went undetected in seven NK cells indicating that possibly 9% of the time the transcript of a gene and/or allele is not detected by our method. In total, we detected transcript for at least one of *Ly49d*^{B6}, *Ly49d*^{NOD} and *Nkg2d* in 80 cells from the total of 88 NK cells assayed.

Interestingly, the difference between the fraction of cells positive for *Ly49d*^{NOD} versus *Ly49d*^{B6} is nearly 3-fold. The allelic expression of Ly49G in various F1 hybrid mice using allele-specific antibodies shows that different alleles of the same gene can be expressed at different levels in various mouse strains (41). This might be due to the MHC class-I background of the mice, the Ly49 cluster gene composition as well as polymorphisms in the promoter regions.

Expression of Ly49H in a single genomic allele mouse model

Ly49H is expressed on ~50% of splenic NK cells in the B6 strain. For a completely bi-allelically transcribed gene expressed in 50% of NK cells, each allele would be expressed in all expressing cells and would contribute half of the total transcript in each cell. In a situation with only one genomic allele present, the fraction of cells expressing the gene should, however, remain the same at 50% but the amount of total transcript should be halved. For a gene expressed mono-allelically through allelic exclusion, one allele is chosen for expression and the other allele is repressed. If this gene is expressed in 50% of cells, each allele is transcribed in 25% of cells only. In contrast, for a stochastic mono-allelic expression pattern, as described for the inhibitory Ly49 receptors, every allele is equally, randomly and independently expressed (42). Based on this model, if 50% of NK cells are positive for a receptor, each allele should be expressed in 29.2% of NK cells with 8.4% of all NK cells expressing this receptor bi-allelically (see model of this situation in Figure 5A).

We attempted to distinguish between the bi-allelic versus stochastic mono-allelic model using the B6.BXD8 (B6.Ly49h$^{-/-}$) mouse, generated recently by two independent groups (23,43), which carries the B6 *Ly49* cluster with a ~26 kb deletion spanning the length of the *Ly49h* gene. Both groups have independently shown that the expression of other Ly49 receptors does not change in the B6.BXD8 mouse compared to the wild-type B6 mouse indicating that the lack of Ly49H does not affect the expression of other Ly49 receptors (23,43). Unfortunately, an inhibitory *Ly49* gene deletion mouse model does not exist for comparison. The B6.BXD8 is unique because the deleted *Ly49* genomic allele is in the same *Ly49* gene cluster as the other (non-deleted) chromosome. The variable *Ly49* clusters as well as possible differences in MHC class-I background can produce confounding effects on expression of Ly49 receptors in F1 hybrids (41).

To investigate the expression pattern of Ly49H on NK cells carrying only one genomic allele of *Ly49h* on the B6 background, we FACS analyzed peripheral blood NK cells from 6-week old *Ly49h*$^{+/-}$ heterozygous (B6.BXD8/B6) mice by staining with the 3D10 antibody. The percentage of NK cells expressing Ly49H in the heterozygous mice (*Ly49h*$^{+/-}$) was reduced to 35% from that of age-matched B6 (*Ly49h*$^{+/+}$) that have 50% Ly49H$^+$ NK cells ($P = 0.0005$) (Figure 5B). This fraction of Ly49H expressing cells in the heterozygotes is lower than expected for a completely bi-allelically expressed gene but higher than the expected 29.2% based on the stochastic mono-allelic model (Figure 5A). Thus, based on these data, the situation for *Ly49h* likely lies between these two extremes. Although we cannot predict the possible effects of the deletion of one *Ly49h* allele on the expression of the other allele, we can assume that in the wild-type B6 background both alleles should have the same probability of expression. Since Ly49H is expressed on 50% of NK cells and assuming that in the *Ly49h*$^{+/+}$ B6 mouse the second allele would also be expressed on 35% of NK cells, the bi-allelic expression of Ly49H would be ~20% (Figure 5C). This value is strikingly similar to the percentage of *Ly49d*$^{B6/NOD}$ (bi-allelic) NK cells (assuming 50% Ly49D$^+$ NK) of the B6/NOD F1 hybrid, as assayed with single-cell RT–PCR (Figure 5D).

Low sequence identity in the 5′-UTR of inhibitory and activating *Ly49* genes

The regulatory elements governing the transcription of activating *Ly49* genes have not been analyzed to date. It is unknown if inhibitory and activating genes share common transcription factors. Multiple alignment of a number of inhibitory and activating *Ly49* genes from various sequenced mouse strains (performed with Clustal W)

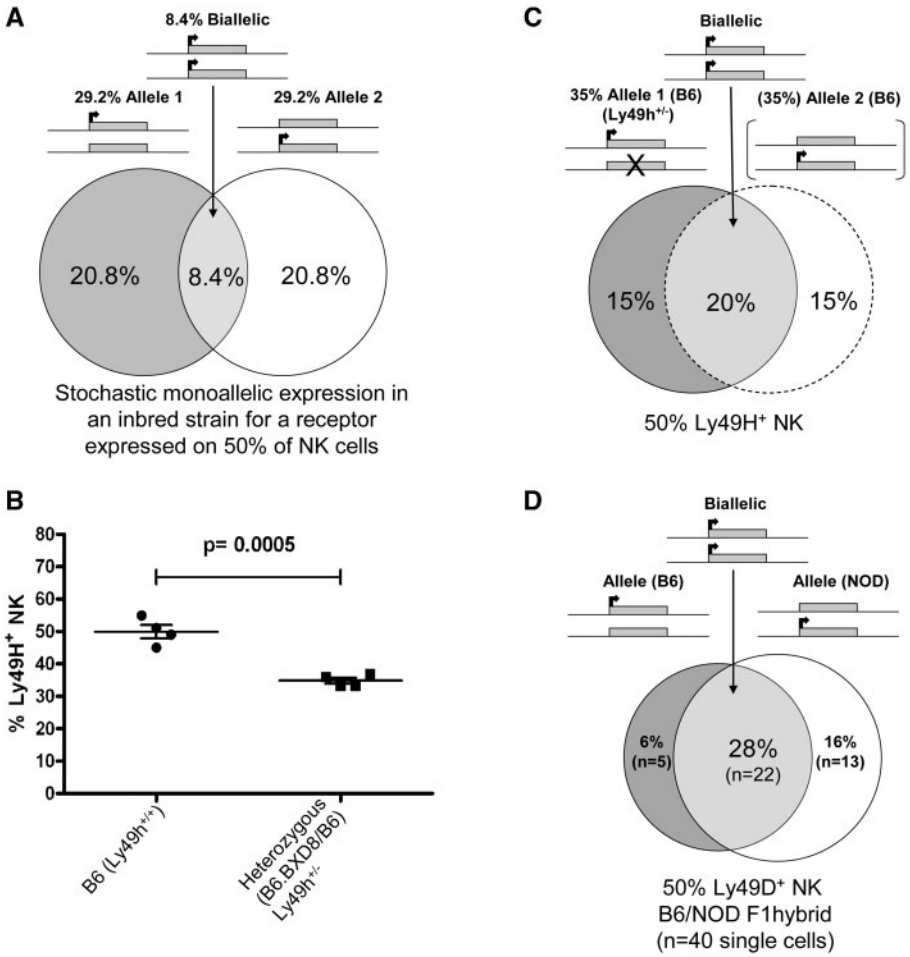

Figure 5. Expression of Ly49H in B6 *Ly49h*$^{+/-}$ NK cells and comparison to the stochastic mono-allelic expression model. (**A**) Expression of a theoretical Ly49 receptor expressed on 50% of NK cells based on the stochastic mono-allelic model in an inbred mouse strain. (**B**) The percentage of peripheral blood NK cells expressing Ly49H in B6.BXD8/B6 (*Ly49h*$^{+/-}$) mice and age-matched B6 (*Ly49h*$^{+/+}$) were determined by FACS. *P*-value was calculated according to the two-tailed unpaired *t*-test. (**C**) Allelic expression of Ly49H in a theoretical B6 mouse based on the expression of this receptor in B6.BXD8/B6 (*Ly49h*$^{+/-}$) mice. The dashed circle and brackets indicated predicted expression patterns. (**D**) Allelic expression of Ly49D on NK cells based on the single-cell RT–PCR results (Table 2) in B6/NOD F1 hybrid. Each chromosome is shown with a line, the alleles are shown with rectangle, arrows indicate transcription and cross shows the deleted allele.

shows a region in exon 1 that is present for the activating *Ly49* genes but is either absent or different for the inhibitory ones (Figure 6A). This region contains a number of potential binding sites for the transcription factor Yin Yang-1 (YY1) in the activating but not the inhibitory genes.

Since more than half of the assayed CpG dinucleotides of the 5′-region of *Ly49* genes fall downstream of the transcriptional start site, it is possible that differential methylation of this region might affect transcription of the *Ly49* genes. Furthermore, investigation of the possible differential regulatory role of this region might explain some of the differences between inhibitory and activating receptor expression. We therefore probed the possible existence of activating gene specific transcription factor binding sites by gel-shift assay with nuclear extracts from FACS sorted IL-2 cultured NK cells and T-cells (Figure 6B and Supplementary Figure 2).

Fragments spanning the region of interest from *Ly49a*, *c*, *d* and *h* were used as probe. In both NK and T-cells a differential pattern of protein binding was observed between the activating and inhibitory *Ly49* probes. A large band (indicated by a bracket in Figure 6B and Supplementary Figure 2), likely consisting of multiple protein complexes, was most prominent for the activating genes. In addition, a protein complex only bound to the activating *Ly49* probes (indicated by arrow in Figure 6B and Supplementary Figure 2) disappeared (supershifted) in the presence of YY1 antibody.

DISCUSSION

Here, we have analyzed the DNA methylation of the 5′-region of B6 activating NK receptor genes, *Ly49d* and *h*. We have shown that DNA methylation of this region correlates with expression patterns of these receptors as with the inhibitory Ly49A, Ly49C (22) and NKG2A (44). Furthermore, as in the case of *Ly49a*, the CpG dinucleotides downstream of the transcriptional start site of *Ly49h* seem to have higher levels of methylation compared

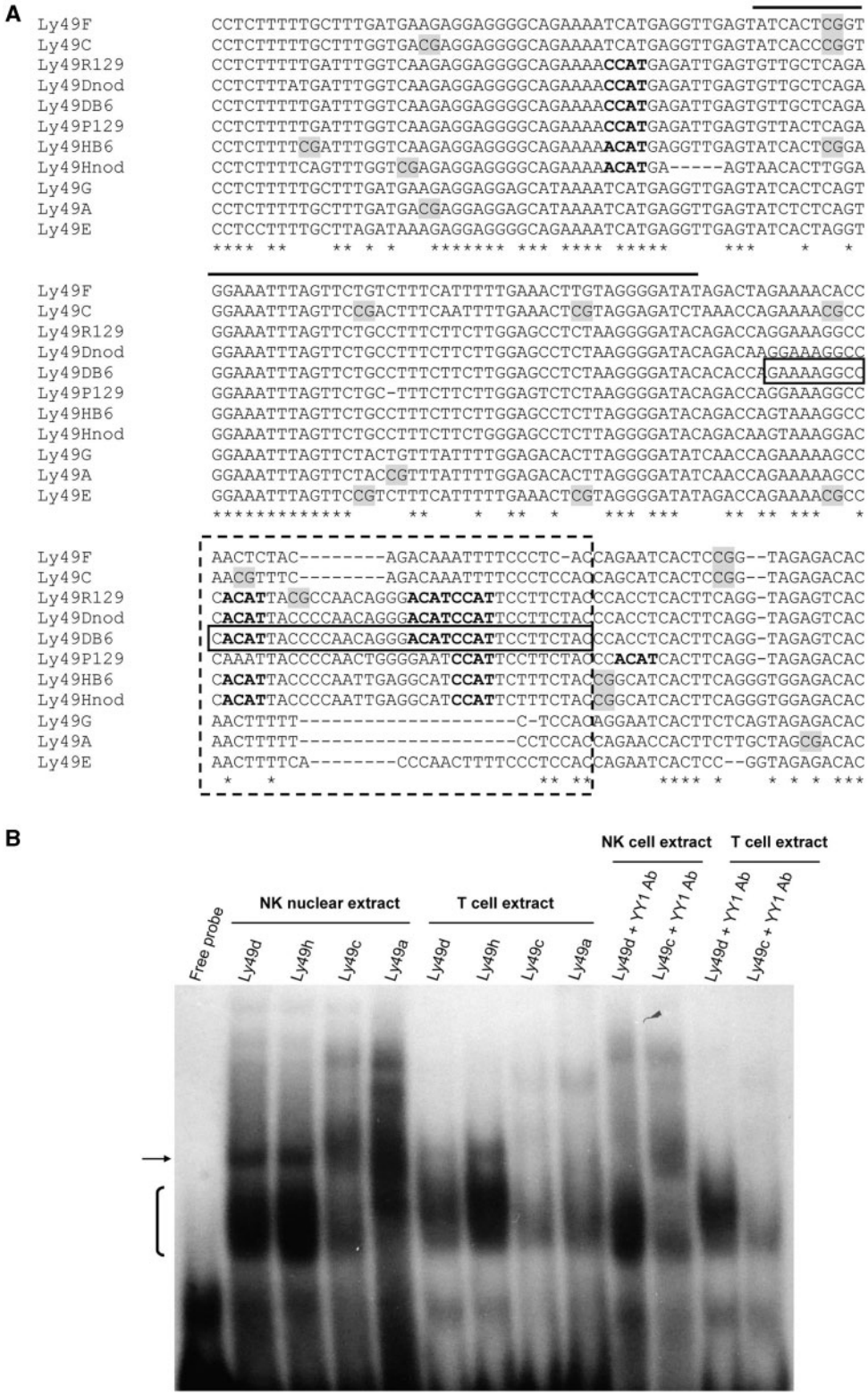

Figure 6. Identification of a region of high homology among activating *Ly49* genes. (**A**) Alignment of 5′ regions of inhibitory and activating *Ly49* genes. A region, located in exon 1, with high homology among activating receptors only, is boxed (dashed line). The region of transcriptional start site is indicated by the solid black line above the alignment. Promoter region sequences of *Ly49d* and *h* of NOD, *Ly49p* and *r* of 129/S6 as well as *Ly49a, c, d, e, f, g* and *h* of the B6 strain are shown. *Ly49a*, *c*, *e*, *f* and *g* are inhibitory genes and all other sequences are from activating genes. CpG dinucleotides are also highlighted. The sequence used as probe in the supershift experiment is indicated for *Ly49d* with box (solid line). Potential YY1 binding sites (CCAT and ACAT) are shown in bold font. (**B**) Double-stranded probes harbouring the region of interest in exon 1 for the activating *Ly49d* and *h* of the B6 strain and the corresponding region for the inhibitory *Ly49a* and *Ly49c* were used in gel-shift experiments with nuclear extract from FACS-sorted T-cells and IL-2 cultured NK cells. A YY1 antibody was used for supershift assays. The bracket indicates the protein complexes bound most significantly by the activating *Ly49d* and *h* probes but not the inhibitory *Ly49a* and *c* in both NK and T-cells. The arrow indicates another protein complex specific to the activating *Ly49* probes that disappears (supershifts) in the presence of anti-YY1 in both NK and T-cells.

to those located upstream in Ly49H-negative cells. This find again raises the possibility of the existence of a proximal downstream regulatory element for the *Ly49* genes whose function might be affected by DNA methylation. Indeed for the KIR genes, a region spanning the transcriptional start site and the 5′-UTR provides the core promoter activity in T-cells but not NK cells (45). This region also binds different protein complexes in NK and T-cells as shown with gel shift experiments (45). The multiple alignment of the 5′-region of inhibitory and activating *Ly49* genes revealed a region of high homology in exon 1 among the activating genes that is not present in the inhibitory *Ly49* sequences (Figure 6A). Further investigation of this region, for the activating *Ly49d* and *h* versus the corresponding region for the inhibitory *Ly49a* and *c*, in gel shift experiments, showed different patterns of protein complex binding between inhibitory and activating gene regions (Figure 6B). YY1 was also confirmed as a candidate transcription factor binding to this region. YY1 is known to have both transcriptional activating and inhibiting properties (46). In addition, the tested region showed a different protein-binding pattern with nuclear extract from NK and T-cells (Figure 6B). These results combined with the lack of detectable activating *Ly49* transcripts in T-cells support a varied mode of transcriptional regulation for the two types of *Ly49* genes. Further analysis of this region is needed to confirm the extent of its role in the transcriptional regulation of the activating *Ly49* genes.

In contrast to the inhibitory receptors, the DNA methylation pattern of the 5′-region in the Ly49D and H expressing NK populations does not follow the bimodal (half-and-half) methylation pattern. The half-and-half pattern of DNA methylation correlates with the mono-allelic expression of Ly49A, C and NKG2A as was subsequently shown using F1 hybrids for *Ly49a* and *Nkg2a* (22,44). If DNA methylation does not correlate with stochastic mono-allelic expression; either stochastic gene expression is maintained at the level of histone modifications only or this pattern is possibly an indication of high, if not complete, bi-allelic expression and even the lack of stochastic expression of the activating receptors.

We have previously shown that the transcription of some *Ly49* genes and *Nkg2a* correlates with histone acetylation levels (22,44,47). *Ly49g* transcription in the EL4 cell line is activated mostly in response to histone deacetylase inhibitors but is mostly unaffected by DNA methyltransferase inhibitors. Histone acetylation levels of the Pro-2 region as assayed via chromatin immunoprecipitation (ChIP) also correlate with state of expression in EL4-derived subclones (47). It is possible that the stochastic mono-alleleic expression of Ly49G (42) is controlled at the level of histones only. Based on these results, it is also possible that the maintenance of the activating receptor expression patterns is through differential histone modifications.

However, an alternative hypothesis to explain the lack of a half-and-half DNA methylation pattern could be that the expression of the activating receptors is predominantly bi-allelic. Based on this hypothesis, in the Ly49D/R expressing NK cells of the 129/B6 F1 hybrid, the 5′-regions of both *Ly49d* and *r* should always be hypomethylated to reflect absolute bi-allelic expression. Our results show that most *Ly49d* and *r* clones are hypo-methylated (16/19) in the receptor-expressing NK cells indicating a strong deviation away from the mono-allelic DNA methylation pattern observed for the inhibitory genes (Figure 3B). If *Ly49d* and *r* are not alleles but are independent loci, based on the product rule, assuming no bias for co-expression given 50% surface expression of each receptor by NK cells, we would expect 25% of all NK cells to be co-expressing the two receptors. Since the total of NK cells expressing one or both of Ly49D and R would be 75% (assuming independent loci), we would expect that 1/3 of all clones sequenced for each gene (when sorting for D and/or R expressing NK cells) to show hyper-methylation patterns similar to the negative population. Our data indicates a much lower methylation frequency than would be expected with stochastic mono-allelic expression and hence we can conclude that neither *Ly49d* nor *r* show bimodal DNA methylation patterns. Our experiment does not prove or disprove the allelic relationship of *Ly49d* and *r*.

The bias towards bi-allelic expression of the activating Ly49 receptors is further supported by the results of the single-cell *Ly49d* allele-specific RT–PCR performed on NK cells of the B6/NOD F1 hybrid (Table 2) as well as the higher than expected expression of Ly49H from a single genomic allele (Figure 5). We detected bi-allelic transcription of *Ly49d* in 55% (22/40) of *Ly49d*-transcribing NK cells. If Ly49D is expressed on 50% of NK cells, the percentage of NK cells expressing this receptor bi-allelically would be ~28% (Figure 5D). The single-cell RT–PCR method tends to be somewhat inefficient in detecting bi-allelic gene expression. The transcription factor, Pax-5, was statistically deemed to be bi-allelically expressed by this method even though only close to 65% of the cells analysed showed bi-allelic expression (39). Transcripts for our control gene *Nkg2d*, that is expressed by all NK cells (40), went undetected in ~9% (7/80) of NK cells (Table 2), indicating a likely underestimation of the bi-allelic expression percentage calculated for *Ly49d*. However, based on the few hyper-methylated clones detected from the Ly49D/R-expressing NK cells as well as the detection of only one *Ly49d* allele in a few single cells (from the B6/NOD F1 hybrid) even after five sets of single-cell RT–PCR, we believe that a real albeit very small population of NK cells express Ly49D mono-allelically.

It is not inconceivable that the activating receptors are controlled and expressed differently from the inhibitory receptors. The lack of a well-defined Pro-1 element and its transcripts (19,20) in addition to deviation of co-expression percentages from the product rule (21) suggests a different mode of transcriptional regulation from that of the inhibitory genes. Also, in the *Ly49h* genomic transgenic mouse, Ly49H expression was restricted to NK cells as is the case with the endogenous receptor (48). This might be due to the lack of the adapter protein DAP12 in T and B cells. However, in mice carrying only an *Ly49d* cDNA transgene (driven by H-2K^b promoter and IgH enhancer) (32) some surface expression of

Ly49D was observed on T cells (49) indicating possible pairing of Ly49D with other adaptor proteins in these cells. The ability of T-cells to express some Ly49D on their surface in the absence of DAP12 in this transgenic mouse (49) supports the notion that the endogenous *Ly49d* promoter might be inactive in T-cells due to lack of necessary transcription factors and/or the presence of repressive epigenetic marks at regulatory sequences. Our results support both possibilities. The *Ly49h* 5′ region is hypermethylated in T-cells (Figure 2B) indicating a possible epigenetic hindrance to transcription of this gene in these cells. The lack of detectable *Ly49d* and *h* transcripts in T-cells (Figure 1A) also supports this notion. Furthermore, the 5′-UTRs of both *Ly49d* and *h* show different protein complex binding pattern compared to that of the inhibitory *Ly49* genes (Figure 6B).

In contrast to the endogenous receptor, in the *Ly49a* genomic transgenic mouse, expression of Ly49A was seen on the majority of splenic B-cells for all transgenic lines regardless of copy number of the transgene (50). The 30Kb *Ly49a* genomic transgene contained the Pro-1 region and it was subsequently shown that the deletion of this region in the same transgene abrogated the expression of Ly49A in NK, T and B cells (50). Mice carrying a 79kb *Ly49h* genomic transgene spanning the complete length of the gene displayed Ly49H expression patterns similar to the B6 *Ly49h* gene indicating that the transcriptional regulatory sequences necessary for the 'wild-type' expression of this gene are contained within the 79 kb region (48). *Ly49h* transcript and percentage of NK cells expressing Ly49H correlated in the transgenic mice and increased according to the transgene copy number, but only up to a certain threshold (48). This phenomenon indicates a possible control mechanism for an upper limit of expression likely at the level of transcription and/or at the level of NK selection.

The activating *Ly49* genes evolve faster than their inhibitory family members (51). This is evident from the large variation in the number and sequence of the activating *Ly49* genes and the high number of activating psuedogenes in nearly all the sequenced mouse strains (7,11). It is also possible that the regulatory sequences of the activating receptors evolved differently from that of the inhibitory receptors in order to allow a tighter control in NK cells and as a side effect of this evolution, mouse T-cells lost the ability to express these receptors. Deviation from stochastic expression might also allow more control on the number of activating receptors on the surface of NK cells because mono-allelic expression might lead to a lower number of receptors on the cell surface compared to bi-alleleic expression (41). Hence, in a non-stochastic system, the expression level of these receptors is homogeneous among different NK cells. In conclusion, our results suggest that distinct modes of transcriptional regulation govern the expression of the activating and inhibitory *Ly49* genes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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