Genomic *Ly49A* Transgenes: Basis of Variegated *Ly49A* Gene Expression and Identification of a Critical Regulatory Element[1]

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Several gene families are known in which member genes are expressed in variegated patterns in differentiated cell types. Mechanisms responsible for imposition of a variegated pattern of gene expression are unknown. Members of the closely linked *Ly49* inhibitory receptor gene family are expressed in a variegated fashion by NK cells. Variegated expression of these genes results in subsets of NK cells that differ in specificity for MHC class I molecules. To address the mechanisms underlying variegation, a 30-kb genomic fragment containing a single *Ly49* gene was used to generate a panel of murine transgenic lines. The results demonstrated that, in almost all of the lines, the isolated *Ly49A* gene was expressed in a variegated pattern, remarkably similar in nearly all respects to the expression pattern of the endogenous *Ly49A* gene. Furthermore, the developmental timing of gene expression and regulation by host MHC molecules closely mirrored that of the endogenous *Ly49A* gene. Therefore, Ly49 variegation does not require competition in *cis* between different *Ly49* genes, and the sequences imposing variegation are located proximally to *Ly49* genes. Efforts to define regulatory elements of the *Ly49A* gene led to the identification of a DNase I hypersensitive site 4.5 kb upstream of the *Ly49A* gene transcription initiation site, which was shown to be essential for transgene expression. Highly related sequence elements were found upstream of other *Ly49* genes, suggesting that a similar regulatory element controls each *Ly49* gene. ***The Journal of Immunology*****, 2004, 172: 1074–1082.**

Natural killer cells can recognize and attack infected, transformed, and foreign cells. Target cell specificity is controlled by the balance of signals imparted by inhibitory and stimulatory receptors. The known inhibitory receptors are specific for MHC class I molecules, and function to prevent NK cells from attacking cells expressing high levels of MHC class I molecules (1–3). The known stimulatory receptors are specific for ligands which are often up-regulated in transformed, infected, or stressed cells (4, 5).

Murine NK cells use two families of receptors for MHC class I recognition, both of which are encoded in the *NK* gene complex on chromosome 6: the Ly49 family (6, 7) and the CD94/NKG2 family (8). The *Ly49* gene family is composed of 10 or more genes in a given strain (9–11), with the majority of the genes clustered in a region spanning ~600 kb. Ly49 receptors bind directly to class Ia D and K MHC molecules. However, different Ly49 receptors bind to distinct sets of class I MHC alleles (reviewed in Ref. 3).

Ly49 receptors are expressed in a variegated pattern, such that each receptor is expressed by 10–50% of NK cells (reviewed in Refs. 3 and 12). Receptor expression is quite stable once initiated, as suggested by experiments in which purified NK subsets are expanded in vitro or transferred in vivo (6, 13, 14). Importantly, Ly49 receptors are expressed in an overlapping fashion, such that the average NK cell expresses three to four different receptors (15). The different receptors are expressed with a considerable degree of independence, as indicated by the finding that the frequency of NK cells coexpressing a given Ly49 receptor pair or trio can be estimated by multiplying the frequencies of cells that express each of the receptors individually (the product rule) (12). This unique variegated expression pattern of the Ly49 receptors suggest that the initial choice of which receptors are to be expressed in a developing NK cell is governed by a stochastic mechanism. Combinatorial expression of receptor genes creates a large and useful NK cell receptor repertoire.

Ly49 genes exhibit allelic polymorphism, allowing one to separately monitor the expression of each allele of a given gene using RNA analysis or allele-specific mAbs. Examination of *Ly49* heterozygous mice has led to the finding that *Ly49* genes are expressed in a predominantly monoallelic fashion (13, 16–18). For example, mAbs specific for $Ly49A^{B6}$ or $Ly49A^{BALB}$ were used to demonstrate that most $Ly49A^{+}$ NK cells express only one or the other *Ly49A* allele, with approximately equal likelihood, whereas a small subpopulation of cells expresses both alleles (18). Similar results were obtained in the case of Ly49G2. In both cases, the frequencies of biallelic cells were somewhat higher than predicted by the product rule, suggesting that some cells are intrinsically more likely than others to initiate expression of the corresponding gene. Interestingly, in NK cells that coexpress Ly49A and Ly49G2, the expressed alleles may be on the same or different chromosomes, with approximately equal likelihood (17, 18). Sorted populations expressing a given allele maintain expression of that allele as the cells proliferate in culture for at least a week, suggesting that the chosen allele is activated in a stable fashion

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(16, 18). Based on these findings, we proposed that the initial choice of NK receptor genes for expression is imposed by mechanisms that act locally to stably regulate *cis*-acting control sequences associated with each *Ly49* allele, independent of neighboring *Ly49* genes or of the *Ly49* allele on the opposite chromosome (12, 13).

Monoallelic and variegated expression has been reported for other genes, including those for certain cytokines, the Pax-5 transcription factor, and olfactory receptors (19). Therefore, the mechanisms involved are of general interest. One category of models to account for the variegated expression pattern of *Ly49* genes postulates an important role for interactions or competition in *cis* between the various neighboring *Ly49* genes in a given gene cluster. For example, it might be proposed that one or a few *Ly49* genes in a given chromosomal array stably associate with master regulatory elements in or near the cluster, allowing only the chosen genes to be expressed. Another category of models would propose that each *Ly49* gene is independently regulated, with variegation arising as a consequence of limiting access of each gene to unidentified components necessary for stable activation of the gene. For example, the developmental time window allotted to assemble a complex of nuclear factors on a critical gene-activating regulatory element may be limiting, resulting in stable activation of a subset of the genes.

Because *Ly49* gene expression is likely to involve chromatin level events such as chromatin remodeling, we began our investigations of *Ly49* gene expression by examining the in vivo expression of ectopically integrated *Ly49* transgenes. The approach was used to address whether variegated *Ly49* gene expression involves interactions between different *Ly49* genes in the normal multigene array, or occurs independently for each gene. Extending the approach allowed us to identify a novel element several kilobases upstream of the promoter that is required for *Ly49A* transgene expression.

Materials and Methods

Bacterial artificial chromosome (BAC)[4] library screening and plasmid construction

A C57BL/6J (B6) BAC library (Genome Systems, St. Louis, MO) was initially screened by hybridization with Ly49A^{B6} and Ly49C^{B6} cDNAs. BAC clones containing *Ly49A*, *Ly49G2*, or *Ly49C* were confirmed by hybridization with gene-specific oligonucleotide probes and ultimately by sequencing selected exons. Two *Bam*H1-*Cla*I fragments containing Ly49A^{B6} genomic sequences were subcloned from BAC clone 101:A20 and rejoined to recreate the natural 30-kb *Bam*HI fragment containing the complete *Ly49A^{B6}* gene. To construct the Δ*HS* transgene, a 1.9-kb *Hin*dIII-*Xba*I fragment containing the hypersensitive site (HSS)-1 region was deleted from the upstream region of the 30-kb fragment. For sequencing upstream regions of *Ly49G2* and *Ly49C*, we subcloned a 7-kb *Hin*dIII fragment from BAC clone 116:M19 and a 7-kb *Bam*HI fragment from BAC clone 43:F9, respectively. The identities of the subclones were confirmed by partial exonic sequencing.

Mice

C57BL/6J (B6) and BALB.B (The Jackson Laboratory, Bar Harbor, ME) and transgenic mice were bred in the animal facility at the University of California, Berkeley. Transgenic founders were generated by injection of the purified genomic fragment into (CBA × B6)F_2 fertilized oocytes. Transgenic founders were identified by PCR using a forward primer (5′-CGGTATATGGCTGTAGGGAGG-3′) homologous to sequences near the 3′ end of the transgene, and a reverse primer (5′-GGCCGCTCTAGAAC TAGTGG-3′) homologous to the junction of the 3′ end and a short polylinker sequence included in the transgene fragment. The transgene was backcrossed between two and five times to BALB.B mice. The backcrossed mice were selected based on typing for the NK complex (NKC) as previously described (18), and for MHC by staining with anti-K^k and anti-K^b mAbs. The resulting transgenic lines were therefore *NKC$^{BALB/BALB}$* and *H-2$^{b/b}$* at the MHC locus and homozygous for most BALB.B alleles at other loci.

To generate the mice examined in Fig. 3*A*, the backcrossed *Ly49A* transgenic (TG)2 line mice were crossed with BALB/c mice, and the previously described (20) *Ly49G2* cDNA transgenic mice (on a C57BL/6 background) were crossed with BALB.B mice. Transgenic pups from each cross were intercrossed. *Ly49A* transgene$^+$*NKC$^{BALB/BALB}$* pups were selected for analysis. Among these pups were H-2$^{b/b}$ and H-2$^{b/d}$ mice, as well as *Ly49G2* transgene$^+$ and *Ly49G2* transgene$^-$ mice. Typing for the NKC and H-2 was as described (18).

Quantitative PCR

Transgene copy number was determined by the kinetics of PCR amplification with a GeneAmp 5700 sequence detection using the SYBR Green reagent kit machine (PerkinElmer Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Triplicate samples of tail DNA from transgenic mice of each line were analyzed concurrently against a standard curve of equivalent amounts of BALB.B tail DNA spiked with scaled concentrations of the transgene plasmid. Primers used were as follows: forward primer, 5′-ATCAGCACTCCTGAAAGACAAGCT-3′; reverse primer, 5′-TACACGCAAAACTGACCCTGTG-3′. Tail DNA was also amplified with γ-actin primers (5′-GCACCTAGCACGATGAAGAT TAAG-3′ and 5′-GCCACCGATCCAGACTGAGT-3′) as a control to normalize input DNA.

Antibodies

Anti-Ly49A^{B6} mAb A1 (21), anti- Ly49A^{BALB} mAb TNTA (18), and the anti-FcR mAb 2.4G2 (22) have been previously described. Abs were conjugated to FITC (Boehringer Mannheim, Mannheim, Germany) or biotin (Pierce, Rockland, IL). Anti-NK1.1-PE and DX5-PE conjugates were purchased from BD PharMingen (San Diego, CA). Anti-CD3-tricolor, anti-CD8-tricolor, streptavidin-tricolor, goat anti-mouse IgG, and mouse IgG were purchased from Caltag Laboratories (Burlingame, CA). Streptavidin-RED613 was purchased from Life Technologies (Rockville, MD).

Flow cytometry

Nylon wool nonadherent splenocytes were preincubated with 2.4G2 mAb to block FcRs. NK cell expression of the *Ly49A* transgene and endogenous Ly49A was determined by staining the cells with A1-FITC, TNTA-biotin, DX5-PE, anti-CD3-tricolor, and anti-CD8-tricolor followed by streptavidin-613. For comparable analysis of CD8$^+$ T cells, the cells were stained with anti-CD8-tricolor, anti-CD44-PE, A1-FITC, and TNTA-biotin, followed by streptavidin-613. To assess Ly49A^{Tg} expression on B cells, RBC-lysed splenocytes were preincubated with 2.4G2 mAb to block FcRs, and stained with anti-CD19-PE and A1-FITC. To assess Ly49A^{Tg} expression in peritoneal macrophages, cells were harvested by peritoneal lavage and stained with anti-MAC1-FITC and A1-biotin followed by streptavidin-PE. Flow-cytometric analysis was performed on Coulter XL-MCl flow cytometers at the University of California, Berkeley, Flow Cytometry facility. The FlowJo program (Tree Star, Ashland, OR) was used for further data analysis.

Northern blotting

Total RNA from various cells and organs was isolated with the Ultraspec RNA isolation solution (Biotecx Laboratories, Houston, TX) according to the manufacturer's instructions. A standard Northern blotting technique was used (23) with a full-length Ly49A cDNA probe. The hybridized blot was washed with 0.5× SSC/1% SDS at 65°C for 1 h. After stripping, the blot was reprobed with an actin cDNA probe.

DNase I hypersensitivity assay

For analysis of NK cells, IL-2-activated A-LAK cells depleted of CD4$^+$ and CD8$^+$ T cells were prepared essentially as previously described (24). After 7–9 days of culture, Ly49A$^-$ and Ly49A$^+$ cells were separated by staining with JR9-318-FITC and anti-FITC MACS beads and processing through an autoMACs machine (Miltenyi Biotec, Auburn, CA). The separated populations were >90% pure. Nuclei prepared as described (25) from ~5 × 10^7 Ly49A$^-$ or Ly49A$^+$ cells, were incubated with varying concentrations of DNase I (Sigma-Aldrich, St. Louis, MO) for 5 min at 37°C (25). DNase I HSS in liver chromatin was assayed as described (26). Following DNase I digestion, genomic DNA was purified, digested with a mixture of *Xba*I and *Bam*HI enzymes, and analyzed by Southern blotting

[4] Abbreviations used in this paper: BAC, bacterial artificial chromosome; HSS, hypersensitive site; NKC, NK complex; TG, transgenic; PEV, position effect variegation; LCR, locus control region; KIR, killer Ig-related receptor; TCF, T cell-specific factor.

with the probes shown in Fig. 1*A*. After hybridization, the filters were washed with 0.5× SSC/1% SDS at 65°C for 30 min.

DNA sequencing and alignments

Sequencing reactions were performed and resolved either with the Big Dye Terminator Ready Mix according to the manufacturer's instructions and an Applied Biosystems Prism 310 Sequencer (PerkinElmer Applied Biosystems), or by the Sequencing Facility at University of California, Berkeley. Sequences were aligned with CLUSTAL W (27) through VectorNTI (InforMax, Frederick, MD), and consensus binding sites were determined with the MacVector 7 program (Oxford Molecular, Oxford, U.K.).

Results

Mice harboring a genomic Ly49A^{B6} *transgene*

We subcloned a 30-kb *Bam*HI fragment containing the *Ly49A* gene from a B6-derived BAC (Fig. 1*A*). The genomic fragment contains all seven exons of the *Ly49A*^{B6} gene, ~10 kb of upstream sequence and 3 kb of downstream sequence (28). The purified 30-kb fragment was used to generate eight transgenic founders, each of which was backcrossed several times to BALB.B mice to generate transgenic lines that were homozygous for the NKC and MHC (H-2^b) of the BALB.B strain. Using this scheme, we could use flow cytometry to simultaneously discriminate cell surface expression of the transgene-encoded and endogenously encoded Ly49A receptors using the Ly49A^{B6}-specific A1 mAb and the Ly49A^{BALB}-specific TNTA mAb, respectively.

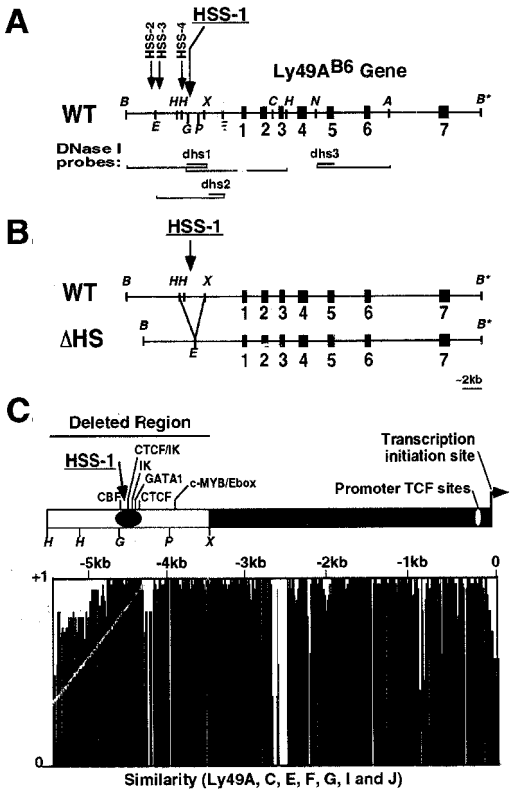

FIGURE 1. *Ly49A* transgene constructs with DNase I HSS and homology of the upstream regions of known inhibitory *Ly49* genes. *A*, Representation of the 30-kb *Ly49A*^{B6} wild-type (WT) genomic transgene construct used to generate transgenic mice. DNase I HSS associated with the transgenic and endogenous *Ly49A* loci are marked. Probes (dhs) and restriction fragments used in DNase I hypersensitive assays are indicated. A, *Hpa*I; B, *Bam*HI; B*, filled-in *Bam*HI site; C, *Cla*I; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; N, *Nhe*I; P, *Pst*I; X, *Xba*I. *B*, Representation of the deleted (Δ*HS*) transgene construct used to generate transgenic mice. *C*, Upstream sequences of *Ly49A*, *-C*, *-E*, *-F*, *-G*, *-I*, and *-J* were compared with CLUSTAL W through the VectorNTI program. Graph represents the resulting similarity plot. Location of the deleted region of the Δ*HS* transgene, restriction enzyme sites, and DNase I HSS are indicated along with selected consensus transcription factor binding sequences found in these *Ly49* genes. Also marked are previously described TCF sites near the transcription start site (44). CBF, CCAAT-binding factor; CTCF, CCCTC-binding factor; IK, Ikaros; GATA1, GATA binding protein 1; c-MYB, myeloblastosis proto-oncogene; Ebox, bHLH protein recognition motif (CANNTG).

Ly49A *transgene expression by NK cells*

Freshly isolated splenic NK cells (DX5$^+$CD3$^-$CD8$^-$ cells) from each transgenic line were examined for expression of Ly49A by multicolor flow cytometry and compared with nontransgenic littermates. In seven of the eight lines, a clear Ly49A^{B6+} population was evident, showing that the *Ly49A* transgene was expressed by NK cells in these lines (Fig. 2*A*, Tables I and II). Notably, in six of these lines (TG2, TG3, TG8, TG11, TG15, and TG16), the transgene was expressed by only a relatively small subpopulation of NK cells, representing 12.9–28.0% of the cells, and clearly distinct from the negatively staining population. The size of this subset was similar to that of the Ly49A$^+$ subset in normal B6 mice (average of 18.5% of NK cells), and larger than the fraction of NK cells in BALB.B mice that express Ly49A^{BALB} (average of 11.2%). Within a line, the size of the transgene-positive subset varied little from mouse to mouse, as shown by the relatively small SDs of the mean (Table I). In the remaining line that expressed the transgene, TG7, a majority of NK cells (average of 91.2%) expressed the transgene. It is possible that this transgene integrated in a chromosomal site that is active in NK cells. One transgenic line, TG9, failed to express the transgene. Thus, six of the eight lines exhibited a variegated transgene expression pattern very similar to that of the endogenous *Ly49A*^{B6} gene.

Importantly, the expression of the *Ly49A* transgene by NK cells was not coordinate with expression of the endogenous *Ly49A*^{BALB} gene. Instead, clear subpopulations were evident that expressed only the *Ly49A*^{B6} transgene, only the endogenous *Ly49A*^{BALB} gene, or both (Fig. 2*A*). A similar pattern with respect to expression of Ly49A^{B6} and Ly49A^{BALB} was previously observed in *Ly49A* heterozygous (B6 × BALB.B)F$_1$ mice (18). The degree of independence of expression of endogenous and transgenic Ly49A can be ascertained by comparing the degree of overlap with that predicted by the product rule. In the six lines that exhibited variegated transgene expression, the frequencies of cells that coexpressed the endogenous and transgene-encoded Ly49A receptors exceeded the predictions of the product rule by 2- to 3-fold (Table I). This comparison suggests that, although there is significant independence in the expression of these genes, the independence is incomplete, and some NK cells exhibit a higher probability than others of expressing both the transgene and the endogenous *Ly49A* gene. Strikingly, we previously observed that the frequency of NK cells in *Ly49A* heterozygous mice that coexpress two endogenous *Ly49A* alleles is also two to three times higher than predicted by the product rule (18). These data indicate a similar bias in the expression of the transgene and endogenous *Ly49A*^{B6} genes.

A comparison of the transgenic mice and their nontransgenic littermates revealed that transgene expression did not significantly alter the percentages of NK cells that express endogenous Ly49G2 and Ly49C/I receptors (data not shown). Interestingly, however, in all lines where the transgene was expressed, the percentages of NK cells expressing endogenous Ly49A (Ly49A^{BALB}) were marginally reduced compared with littermates (Table I). The reductions could be the result of competition between the transgenes and endogenous genes for nuclear factors or could arise as a consequence of a cellular education process. The differences were statistically

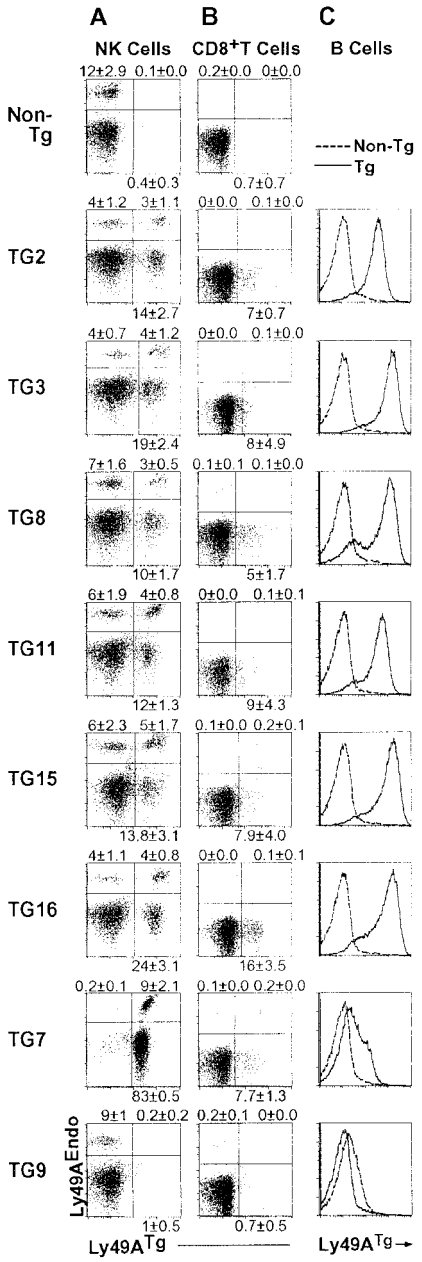

FIGURE 2. Expression of transgene-encoded and endogenous Ly49A in eight transgenic lines. *A*, Electronically gated $DX5^+CD3^-CD8^-$ splenic NK cells were analyzed with A1 (specific for the $Ly49A^{B6}$ transgene) and TNTA (specific for endogenous $Ly49A^{BALB}$) mAbs. Data represent mean percentages ± SD from three to seven animals. *B*, Electronically gated $CD8^+$ splenic T cells were stained with A1 and TNTA mAbs. Data represent mean percentages ± SD from three to six animals. *C*, $CD19^+$ gated B cells from transgenic (solid line) and nontransgenic littermates (dotted line) were stained with A1 mAb, specific for the transgene-encoded Ly49A receptor. The data are representative of several experiments.

significant in only two of the lines, however, and in all cases modest.

Previous research showed that the interaction of a Ly49 receptor with MHC during development can inhibit the expression of a second endogenous Ly49 receptor. Thus, the percentage of NK cells expressing endogenous *Ly49* genes was substantially reduced when the mice were provided with a transgene consisting of a Ly49A or Ly49G2 cDNA driven by the K^b class I promoter, which is expressed early in all developing NK cells (20, 29). A substantial reduction only occurred when the mice also expressed a cognate MHC molecule (i.e., H-2^d) for the cDNA transgene-encoded receptor. To investigate whether the genomic transgene is subject to similar regulation, we compared H-$2^{b/d}$ littermates harboring both the genomic *Ly49A* transgene and the *Ly49G2* cDNA transgene with those harboring the *Ly49A* transgene alone. The *Ly49G2* transgene caused a 50% reduction in the frequency of NK cells expressing the genomic *Ly49A* transgene (Fig. 3*A*), comparable with the reduction it caused in endogenous Ly49A expression (20). The reduction was considerably smaller in mice that did not express a cognate MHC ligand, that is, in H-$2^{b/b}$ mice. Thus, the genomic transgene, like endogenous *Ly49* genes, is regulated by interactions between other Ly49 receptors and MHC ligands that occur during development.

Table I. *Percentages of NK cells expressing transgene-encoded and endogenous* Ly49A*in eight transgenic lines*[a]

Line	$Ly49A^{Endo}$	$Ly49A^{Tg}$	$Ly49A^{Tg}$ and $Ly49A^{Endo}$	
			Observed	Predicted[b]
TG2	7.7 ± 2.2	17.0 ± 3.6	3.5 ± 1.1	1.3
NTG2[c]	12.1 ± 3.0[d]	0.5 ± 0.3		
TG3	7.8 ± 1.8	23.0 ± 2.7	4.3 ± 1.2	1.8
NTG3[c]	13.8 ± 1.6[e]	0.4 ± 0.1		
TG8	9.8 ± 2.0	12.9 ± 1.4	2.6 ± 0.5	1.3
NTG8[c]	13.0 ± 2.4	0.4 ± 0.1		
TG11	9.6 ± 2.5	16.0 ± 2.1	4.0 ± 0.8	1.5
NTG11[c]	10.5 ± 1.4	1.0 ± 0.5		
TG15	10.4 ± 3.9	18.4 ± 4.6	4.6 ± 1.7	1.9
NTG15[c]	12.2 ± 1.6	1.0 ± 0.6		
TG16	7.8 ± 1.8	28.0 ± 3.2	4.2 ± 0.8	2.2
NTG16[c]	8.7 ± 0.1	0.8 ± 0.1		
TG7	9.4 ± 2.2	92.2 ± 2.5	9.2 ± 2.1	8.7
NTG7[c]	15.1 ± 4.9	0.8 ± 0.2		
TG9	9.0 ± 1.3	0.9 ± 0.7	0.2 ± 0.2	0.1
NTG9[c]	9.5 ± 0.1	0.8 ± 0.2		

[a] Nontransgenic (NTG) and TG mice from the indicated lines were analyzed for transgenic (Tg) and endogenous (Endo) Ly49A expression as in Fig. 2.

[b] The predicted frequency of $Ly49A^{Tg}$- and $Ly49A^{Endo}$-expressing NK cells was calculated by multiplying the frequency of cells expressing the endogenous Ly49A receptor by the frequency of cells expressing the transgenic Ly49A receptor.

[c] These mice are nontransgenic; therefore, the $Ly49A^{Tg}$ column represents background staining with A1 mAb, and values for the observed and predicted columns are not shown.

[d] $p < 0.05$ vs group above by Student's *t* test.

[e] $p < 0.005$ vs group above by Student's *t* test.

Ontogeny of Ly49A *transgene expression*

Expression of most endogenous Ly49 receptors by NK cells is delayed until after birth, with the percentage of positive cells increasing progressively in the first few weeks of life (14). Examination of three lines that exhibited variegated expression in NK cells (TG2, TG3, and TG8) revealed that the transgene exhibited a strikingly similar expression pattern in ontogeny (Fig. 3*B*). NK cells from 2-day-old mice were essentially devoid of transgene-expressing cells, with the percentage increasing to the adult value over the next several weeks.

Tissue and cell type specificity of transgene expression

The endogenous *Ly49A* gene is expressed by NK cells and by a small fraction of memory $CD8^+$ T cells (30), but not by the vast majority of $CD4^+$ T cells, macrophages, B cells, or nonhemopoietic cells. Northern blot analysis of RNA from the TG2 line revealed abundant Ly49A mRNA in lymph node, lower levels in thymus, and none detectable in kidney or lung (Fig. 3*C*). These data suggested that the transgene expression is limited to lymphohemopoietic cell types. Staining analysis showed no transgene expression by macrophages (Fig. 3*D*) or by the vast majority of

Table II. *Expression of* Ly49A *transgene in different cell types*[a]

		NK Cells		T Cells		B Cells
				$CD8^+$	$CD4^+$	
Line	Copy No.	$Ly49A^{Tg+}$	MFI[b]	$Ly49A^{Tg+}$	$Ly49A^{Tg+}$	MFI[b]
TG7	1.6 ± 0.3	92.2 ± 2.5	57	7.7 ± 1.3	0.2 ± 0.0	6
TG9	2.3 ± 1.1	0.9 ± 0.7	45	0.7 ± 0.5	0.3 ± 0.2	2
TG11	4.1 ± 1.1	16.0 ± 2.1	116	9.4 ± 4.3	0.4 ± 0.1	48
TG8	9.3 ± 0.5	12.9 ± 1.4	183	4.6 ± 1.7	ND	91
TG2	13.5 ± 0.3	17.0 ± 3.6	108	7.8 ± 2.0	ND	39
TG3	18.0 ± 5.5	23.0 ± 2.7	204	8.1 ± 4.9	0.6 ± 0.0	140
TG16	22.0 ± 2.1	28.0 ± 3.2	157	15.5 ± 3.5	ND	113
TG15	51.7 ± 5.1	18.4 ± 4.6	157	7.9 ± 4.0	0.8 ± 0.2	137

[a] Transgenic lines are ordered according to copy number as determined by quantitative PCR. Percentages of transgene-positive NK cells and T cells are derived from experiments shown in Fig. 2.

[b] Mean florescence intensities (MFI) given were determined from one experiment by staining a representative of each line with a mixture of relevant mAbs.

$CD4^+$ T cells (Table II), consistent with the regulation of the endogenous *Ly49A* gene. In contrast, in all of the lines with transgene expression in NK cells, the transgene was also expressed by a fraction of $CD8^+$ T cells (Fig. 2*B*), ranging from 4.6 to 15.5% of the cells. The percentages of $CD8^+$ cells that expressed the transgene were higher than observed for expression of the endogenous $Ly49A^{B6}$ gene in B6 mice of the same age (2.5–5 mo old, 1–2% of CD8 T cells). Notably, the level of surface expression of the transgene was lower on $CD8^+$ T cells than on NK cells, a pattern also observed in the case of endogenous Ly49A expression (30). Parenthetically, BALB.B mice contain substantially fewer $Ly49A^+$ $CD8^+$ T cells than do B6 mice (Fig. 2, *B* and *C*) (C. W. McMahon and D. H. Raulet, unpublished data).

An unexpected finding was that B cells from most of the transgenic lines aberrantly expressed the transgene (Fig. 2*C*, Table II), suggesting that the transgene fragment lacks flanking sequences that repress B cell expression. Although B cell expression of the transgene is inappropriate, the pattern of transgene expression in these cells was informative, because it contrasted with the expression pattern in NK cells and $CD8^+$ T cells. In most of the transgenic lines, almost all B cells expressed the transgene (Fig. 2*C*). The exceptions were the two lines that exhibited aberrant expression patterns in NK cells. In these lines, the transgene was expressed poorly (TG7) or not at all (TG9) in B cells. The finding that, in most lines, the transgene is expressed by almost all B cells, but exhibits a variegated expression pattern in NK cells, suggests that the variegated pattern is specifically imposed in NK cells.

Interestingly, analysis of the TG2, TG3, and TG8 lines demonstrated that high-level transgene expression was already evident in most B cells in 2-day-old mice and at all other ages examined (data not shown). In contrast, NK cells in 2-day-old mice failed to express the transgene (Fig. 3*B*). These data demonstrate that developmentally delayed expression of the transgene in NK cells is a cell type-specific phenomenon.

Tissue-specific DNase I HSS near the Ly49A *gene*

To discern potential *cis*-acting regulatory elements in the *Ly49A* gene, we screened the 30-kb transgene in the TG2 line for sites that are DNase I hypersensitive in IL-2-activated NK cells. IL-2 activation did not significantly alter the percentage of NK cells that express the transgene in this or other lines examined (data not shown). DNase I hypersensitivity generally marks sites in chromatin where nuclear factors are bound. Nuclei from separated $Ly49A^+$ and $Ly49A^-$ NK cells were compared. Although the *Ly49A* probes also detected other *Ly49* genes due to high homology within this gene family, the high number of *Ly49A* transgene copies in the TG2 mice ensured that the transgene was preferentially detected and served also to enhance the sensitivity of site detection. Southern hybridization with the dhs1 probe (see Fig. 1*A* for map and probes) revealed a clear DNase I HSS, denoted HSS-1, that was localized 4.5 kb upstream of the transcription start site of the *Ly49A* gene (Figs. 1*A* and 4*A*). HSS-1 was approximately equally hypersensitive in $Ly49A^-$ and $Ly49A^+$ NK cells (Fig. 4*A*), but was absent in liver cells (*B*). Similar results were obtained with NK nuclei from the TG3, TG7, TG8, and TG11 transgenic lines (data not shown). Analysis with additional restriction enzymes (data not shown) localized HSS-1 to a position just 3′ of a *Bgl*II restriction enzyme site (Fig. 1*A*). No other bands indicative of HSS were observed in the upstream region, or in the remaining portions of the transgene that were screened (see Fig. 1*A* for the regions examined).

HSS-1 was also clearly evident in the endogenous $Ly49A^{B6}$ locus, as determined in assays using nuclei from IL-2-activated NK cells from nontransgenic (B6) mice. Again, the site was approximately equally hypersensitive in $Ly49A^+$ and $Ly49A^-$ NK cells (Fig. 4*C*), but absent in liver (*D*). The hypersensitivity of HSS-1 was also confirmed using *Eco*RI-digested DNA and the dhs2 probe (data not shown). Three weaker HSS were also detected at the endogenous locus that were absent or possibly obscured by other bands in all of the transgenic lines analyzed (Figs. 1*A* and 4). Whereas HSS-2 was prominent, HSS-3 and HSS-4 were very weak. HSS-4 was more clearly evident, although still weak, using *Eco*RI-digested DNA and the dhs2 probe (data not shown). Again, these sites were approximately similarly hypersensitive in $Ly49A^+$ and $Ly49A^-$ NK cells. Because the HSS-1 probe is predicted to cross-hybridize with other *Ly49* genes, we cannot rule out the possibility that HSS-2- to -4 arise from a *Ly49* gene other than *Ly49A*.

HSS-1 is essential for transgene expression

To address the functional significance of HSS-1, we prepared a transgene construct (ΔHS) that lacked a 1.9-kb *Hin*dIII-*Xba*I fragment containing HSS-1, but was otherwise identical with the original construct (Fig. 1*B*). Five ΔHS transgenic founders were generated, each of which was crossed back to BALB.B mice several times to generate transgenic lines. None of the ΔHS transgenic lines exhibited detectable transgene expression in NK cells (Fig. 5), $CD8^+$ T cells, or B cells (data not shown). These data demonstrate that the 1.9-kb region missing in the Δ*HS* transgene contains an element required for transgene expression.

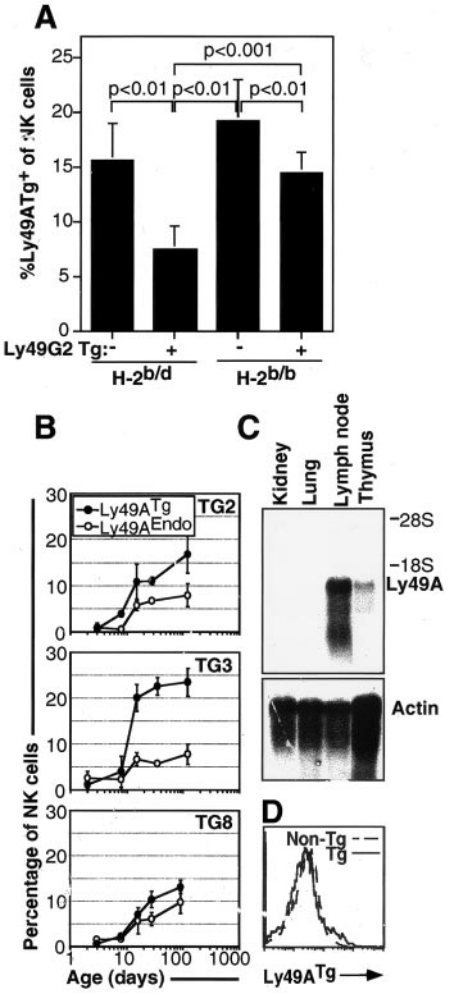

FIGURE 3. MHC regulation, ontogeny, and cell specificity of transgene expression. *A*, The percentage of splenic NK cells expressing the genomic *Ly49A* transgene is regulated by interactions of other Ly49 receptors with MHC. All mice were 11–12 wk old and harbored the genomic *Ly49A* transgene. Mice that also harbored the *Ly49G2* cDNA transgene were compared with littermates lacking the *Ly49G2* transgene. H-$2^{b/d}$ and H-$2^{b/b}$ mice were examined separately. The data represent mean percentages ± SD of three to nine mice per group. Groups were compared by Student's *t* test. *B*, The percentage of splenic NK cells expressing transgene-encoded and endogenous Ly49A is plotted vs age. Data represent mean percentages ± SD from at least three animals of each age. The expression of endogenous Ly49A is similar in nontransgenic and transgenic littermates (data not shown). *C*, Total RNA from the indicated tissues of a transgenic TG2 mouse was analyzed by Northern blotting with a Ly49A cDNA probe (*top panel*) or a control actin probe (*bottom panel*). The 18S and 28S rRNA markers are indicated next to the blot. *D*, Peritoneal macrophages were isolated from nontransgenic (dashed line) and transgenic (solid line) littermates from line TG2 and gated $Mac1^+$ cells were analyzed with A1 mAb. Similar results were obtained with the other transgenic lines (not shown).

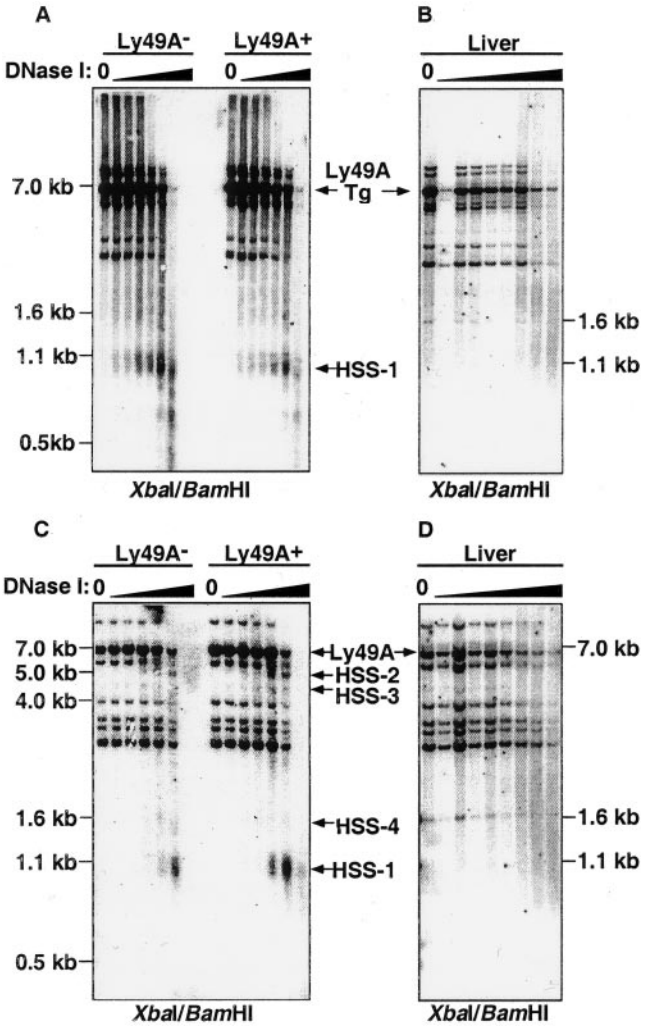

FIGURE 4. Detection of DNase I HSS in the upstream region of the *Ly49A* transgene and endogenous *Ly49A*. Nuclei from TG2 A-LAK cells (separated into $Ly49A^-$ and $Ly49A^+$ fractions) (*A*), TG2 liver cells (*B*), nontransgenic B6 A-LAK cells (separated into $Ly49A^-$ and $Ly49A^+$ fractions) (*C*), or nontransgenic B6 liver cells (*D*) were treated with increasing amounts of DNase I. *Xba*I- and *Bam*HI-digested DNA was examined by Southern blotting with the dhs1 probe (see Fig. 1*A*). Size markers and relevant bands are denoted.

Conservation of upstream regions among Ly49 *genes*

The nucleotide sequences of the upstream regions of all the inhibitory *Ly49* genes in the database were compared (Fig. 1*C*). The comparison includes *Ly49* genes that differ significantly in their protein-coding sequences. Overall, the upstream regions are highly conserved. A region of moderate homology flanks a region with a high degree of homology that includes HSS-1. The remainder of the upstream sequence extending to the transcription initiation site is also highly conserved. Thus, the upstream regions of the inhibitory *Ly49* genes are very similar across a 4.8-kb stretch, including HSS-1. Selected transcription factor consensus binding sequences located in the deleted region of the Δ*HS* transgene and conserved in all the genes are depicted in Fig. 1*C*. Although it is currently unknown whether any of these sites are functional, it is notable that several binding sequences exist in all of the genes, and that there is a clustering of conserved binding sites that coincides with HSS-1.

Discussion

Expression pattern of the Ly49A *transgene*

In several important respects, the ectopically integrated 30-kb genomic fragment containing the *Ly49A* gene was expressed much like the endogenous gene in most of the independent transgenic lines. Expression occurred in lymphocytes but not in several nonlymphoid tissues tested including macrophages, kidney, and lung. Like the endogenous gene, the transgene was expressed by a fraction of $CD8^+$ T cells (albeit a larger fraction than for the endogenous gene), but not by $CD4^+$ T cells. More importantly, in NK cells, a variegated expression pattern was observed in six of the

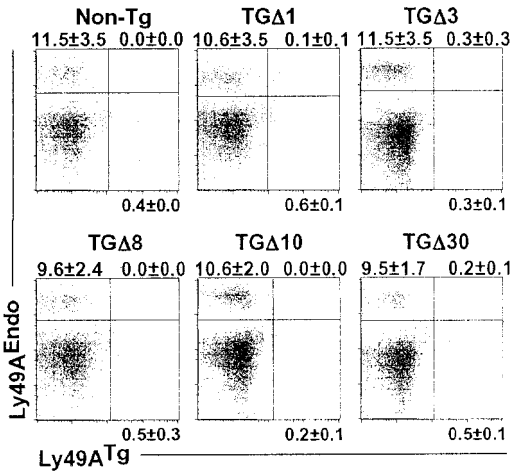

FIGURE 5. *Ly49A* Δ*HS* transgene is not expressed by NK cells. Electronically gated $DX5^+CD3^-CD8^-$ splenic NK cells were analyzed with A1 (specific for the $Ly49A^{B6}$ transgene) and TNTA (specific for endogenous $Ly49A^{BALB}$) mAbs. Data represent mean percentages ± SD from three to four animals.

eight lines that was strikingly similar to that of the endogenous *Ly49A* gene. Furthermore, the ontogeny of transgene expression by NK cells in the variegated lines was also essentially identical with that of the endogenous gene. The neonatal expression of the transgene by B cells, and not NK cells, also implies that the *Ly49A* gene is specifically inactive in developing NK cells. Finally, the percentage of NK cells expressing the transgene was regulated by interactions of other Ly49 receptors with MHC molecules, in a manner identical with the regulation of the endogenous *Ly49A* gene.

The major exception to this list of similarities is that the transgene was expressed by almost all B cells in most of the lines. One likely explanation is that the 30-kb fragment lacks a flanking element(s) that represses expression in B cells but not NK cells or $CD8^+$ T cells. Alternatively, a repressive element may reside in the transgene, but its activity may be overridden as a result of the tandem cointegration of multiple transgene copies. We observed little or no transgene expression in B cells from two lines that had low numbers of transgene copies (one to three copies), but the significance of this finding is difficult to assess given that one of these lines (TG9) exhibited no transgene expression in any cells. In conclusion, although the expression in B cells is a notable exception, the transgene in most lines was regulated in a normal fashion in other key respects, especially in NK cells. The similar ontogeny, variegation, and regulation of transgene expression by other Ly49-MHC interactions leaves little doubt that the 30-kb transgene fragment contains most of the key sequences necessary for normal *Ly49A* gene expression.

Variegation of transgene expression

An important finding of this study is that the transgene is expressed in a variegated fashion in NK cells that recapitulates the pattern of endogenous *Ly49A* gene expression in all respects examined. The frequency of $Ly49A^{Tg+}$ NK cells in most cases was comparable with the frequency of NK cells that express the endogenous *Ly49A* gene in B6 mice. Furthermore, the subset of NK cells that expresses the transgene is distinct from but overlaps with the population of NK cells that expresses the endogenous *Ly49A* gene.

Before considering the significance of this finding further, it is necessary to consider the possibility that the variegated expression pattern is due not to specific *Ly49A* regulatory elements, but rather reflects the phenomenon called transgene position effect variegation (PEV), after a similar phenomenon in *Drosophila*. It has been observed that transgenes lacking chromatin-opening regulatory regions are sometimes expressed in a variegated fashion when the corresponding endogenous gene is not (31). Inappropriate variegated expression has been attributed to integration of transgenes near regions of constitutive heterochromatin, resulting in variable creeping of heterochromatin into the transgene (32).

Several observations argue strongly that the variegated expression of the *Ly49A* genomic transgene is not due to PEV. Perhaps the most compelling was the observed expression of the transgene by all or nearly all B cells in all six of the lines that exhibited the characteristic variegated expression pattern in NK cells. The cell-type specificity of variegation argues that it is imposed by a dedicated molecular mechanism operative in NK cells. A second observation inconsistent with PEV was that the HSS-1 site was equally hypersensitive in cells that expressed the transgene and cells that did not. PEV is normally due to heterochromatinization of a gene in nonexpressing cells, which would result in a closed chromatin configuration that should be inaccessible to DNase I. A third relevant finding was that the variegated NK cell expression pattern was largely independent of transgene integration site, because it was evident in nearly all of the lines (six of seven) in which the transgene was expressed. In contrast, PEV is highly dependent on integration site (32–35). A fourth significant observation was that the percentage of NK cells expressing the transgene was quite similar in all six of the lines that exhibited variegation, and averaged close to the percentage of NK cells in B6 mice that express the endogenous $Ly49A^{B6}$ gene. In contrast, PEV of transgenes results in substantial variation in the percentages of expressing cells in different transgenic lines (35, 36). Finally, the fraction of cells expressing the transgene was regulated much like that of the endogenous gene. For example, both were similarly regulated by other Ly49-MHC interactions, and the overlap between transgene and endogenous gene expression deviated from randomness in precisely the same manner as the overlap in the expression of two endogenous *Ly49A* alleles. As previously reported, the deviation in allelic coexpression occurs even in MHC class I-deficient mice, suggesting that it is not due to the effects of an MHC-dependent cellular selection process and is more likely explained by a generally higher likelihood of initiating Ly49A expression in some NK cell progenitors than others (18). In conclusion, the characteristics of *Ly49A* transgene variegation are incompatible with PEV, and argue instead for a dedicated and regulated molecular mechanism that imposes a variegated *Ly49A* expression pattern in NK cells.

Significance of variegated transgene expression

This study allows several important conclusions to be drawn concerning *Ly49* gene variegation. First, the results indicate that the sequences required for variegation are present in the 30-kb *Ly49A* transgene fragment and that the transgenic system is suitable for analysis of *Ly49* gene variegation. This will facilitate the identification of the relevant sequences and the elucidation of the underlying mechanisms.

Second, the finding that the transgene-expressing subset is to a significant extent independent of the fraction that expresses endogenous *Ly49A*, albeit overlapping, implies that the expression of *Ly49A* is not directed to a predetermined subpopulation of NK cells. The data argue that variegation of *Ly49A* gene expression cannot be explained by the variegated expression of essential *Ly49A*-specific transcription factors in only a subset of NK cells.

Third, and perhaps most important, the results indicate that appropriate *Ly49A* gene variegation does not require interactions or

competition between different *Ly49* genes in the endogenous multigene array. This finding argues against models in which a master control element(s) in the endogenous *Ly49* array somehow selects a subset of the *Ly49* genes for expression. For example, it could be imagined that an element such as a locus control region (LCR) in the endogenous locus stably associates in *cis* with only one or a few of the 10 or so *Ly49* genes in the array, activating only these genes. Because the ectopically integrated *Ly49A* transgene exhibits a variegated expression pattern, this possibility is rendered highly unlikely. One might propose that the putative LCR resides within the 30-kb *Ly49A* transgene sequence, and selects among the different tandemly integrated *Ly49A* transgene copies as if each copy were a different *Ly49* gene in the endogenous array. However, this would result in all NK cells expressing the transgene, albeit different copies of it. Because this was not observed, the data argue against models in which the primary determinant of variegation is a competition in *cis* between neighboring *Ly49* genes for a common regulatory element. However, this does not mean that *cis* competition between genes does not play some role in the process.

Based on our findings that *Ly49A* gene variegation is largely independent of neighboring *Ly49* genes, and occurs in largely independent sets of NK cells for different *Ly49* alleles, genes, or transgenes, we favor a model in which variegation results from limitations in *trans*-acting factors that bind to *Ly49* gene regulatory elements and are necessary to stably activate gene expression. As discussed elsewhere, such factors might be limiting in the sense that only a few molecules are available in the nucleus or in a nuclear compartment in which *Ly49* genes are localized. An alternative possibility is that complete assembly of the necessary factors on the relevant regulatory sequence(s) is an inefficient process, and therefore occurs in only a fraction of developing NK cells in a limited time window allotted for this to occur (3, 16).

A recent report described mice with transgenes representing a large (160-kb) genomic segment, each containing 10 human killer Ig-related receptor (*KIR*) genes, and showed that the *KIR* genes are expressed in an appropriate variegated pattern in NK cells (37). Their conclusions are largely distinct from ours, which emphasize appropriate variegation in the absence of competing genes in *cis*, nonoverlapping expression with endogenous genes, and variegated expression with a relatively small genomic fragment.

A curious feature of our results was that neither the size nor the cell surface staining intensity of the transgene-expressing subset correlated well with transgene copy number (see Table II, which includes transgene copy numbers). Our understanding of the *Ly49* gene variegation mechanism is too limited to allow simple predictions to be made as to how transgene copy number should influence the frequency of NK cells expressing the transgene. However, the relative independence of the extent of variegation from copy number observed in this study is an interesting clue with respect to the mechanism of variegation. In terms of the level of transgene expression, copy number dependence of transgene expression is often due to the action of chromatin-opening elements such as LCRs, defined as elements that confer copy number dependence and position independence to a transgene (38). The fact that seven of eight transgenic lines exhibited substantial levels of expression might suggest that an element with chromatin-opening activity is present, but further studies will be necessary to definitively address whether the *Ly49A* gene contains a bona fide LCR.

A tissue-specific DNase I HSS, HSS-1, is essential for transgene expression

The transgenic system facilitated the identification of HSS-1 4.5 kb upstream of the transcription start site of the *Ly49A* gene. HSS-1 was DNase I hypersensitive in NK cells but not in liver cells, and was essential for transgene expression. Therefore, HSS-1 is highly likely to play an important role in regulating the *Ly49A* gene in its natural genomic location, and is a strong candidate for an element involved in the variegation of *Ly49A* gene expression.

Interestingly, HSS-1 is equally DNase I hypersensitive in $Ly49A^+$ and $Ly49A^-$ NK cells. It is known that the DNase hypersensitivity of elements involved in cell-specific regulation is not always correlated with the expression pattern (39, 40). It is possible that the repressed and expressed *HSS-1* alleles are occupied with different proteins or are differentially modified in other respects, for example, by DNA methylation or local histone modifications. It is also important to emphasize that the monoallelic expression pattern of the gene means that a significant fraction of *Ly49A* alleles in the $Ly49A^+$ population are inactive, complicating the comparison between $Ly49A^+$ cells and $Ly49A^-$ cells.

The comparison in Fig. 1*C* reveals an extremely high degree of conservation of the HSS-1 region in all inhibitory Ly49 sequences even though the coding sequences in some of the sequences exhibit as little as 56% identity in pairwise comparisons. The high degree of sequence conservation begins just 5′ to HSS1, consistent with an important role for this region in expression of all *Ly49* genes. Several transcription factor consensus binding sequences are identical in all of the inhibitory *Ly49* genes in the region deleted from the Δ*HS* transgene. Among these are possible sites for CCCTC-binding factor, which have been shown to define insulator sequences in vertebrate genes (41), and Ikaros, which mediates chromatin relocalization within the nucleus (42).

Interestingly, HSS-1 corresponds to a homologous site in the *Ly49G* gene called Pro-1, which was recently shown to exhibit promoter activity in immature NK cells in the bone marrow (43). In mature NK cells, a downstream promoter called Pro-2 was active and Pro-1 was not, leading to the suggestion that Pro-1 plays some role in initiating *Ly49* gene expression. Tests of whether the HSS-1 fragment of Ly49A exhibits promoter activity in an immature NK cell line, LNK5E6, yielded inconclusive results (data not shown). In line with expectations (43), the HSS-1 fragment did not exhibit detectable promoter or enhancer activity in the assays we performed in a mature cell line, EL-4.

A previous study implicated T cell-specific factor (TCF)-1 in regulation of the *Ly49A* gene, and suggested that the dosage of TCF might influence the percentage of cells expressing this gene (44). On this basis, it was proposed that TCF-1 might represent the limiting factor that determines *Ly49A* gene variegation. Several sites in the proximal promoter region of *Ly49A* were identified that bind TCF-1 in vitro and which might therefore be targets for TCF-1 in vivo. Our results presented in this study demonstrate that the promoter-proximal TCF-1 sites are not sufficient for transgene expression, because they are present in the Δ*HS* transgene, which is not expressed. Notably, many other potential TCF-1 sites exist in the upstream region of *Ly49A*, including one that is conserved in the deleted region of the Δ*HS* transgene in all but one (*Ly49F*) of the genes aligned here. It will be important to assess the role of the various TCF-1 sites in the promoter and the upstream region in future studies.

The results presented in this report concerning the variegation of *Ly49* genes are likely to have significance for understanding the basis of variegated expression observed in other systems, such as KIR, olfactory receptors, cytokine genes, and transcription factors such as Pax-5 (19). Although a recent report suggests that variegated expression of *KIR* genes is maintained by gene methylation, the study did not address how *KIR* gene variegation is initially established (45). Variegated gene expression may play a role not only in forming repertoires but also in directing stochastic developmental decisions. Thus, it is likely that the number of examples

of variegated gene expression will continue to grow. An understanding of the mechanisms of variegation will be essential in elucidating the regulation of this category of genes.

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