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# Characterization of the *Ly49I* promoter

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**Abstract** Fourteen potential *Ly49* genes have been identified in the C57Bl/6 mouse strain, and cDNAs containing a complete coding region have been isolated for 10 members of this gene family. Ly49 proteins are primarily expressed in natural killer (NK) cells. Although the sequence of the *Ly49a* promoter region has been published, no study of the cell-specific activity of the promoter has been reported. A 12-kb genomic fragment of the *Ly49I* gene was isolated and characterized by DNA sequencing. Approximately 5 kb of DNA sequence upstream of the first *Ly49I* exon was determined and this region was used to perform promoter analysis using luciferase reporter plasmid constructs. A core promoter was identified that was preferentially transcribed in a Ly49-expressing cell line, EL-4. Electrophoretic mobility shift assays using oligonucleotide probes from the core *Ly49i* promoter and comparable regions from the *Ly49a* promoter demonstrated the importance of TATA-related elements in generating EL-4 and NK cell-specific DNA/protein complexes.

**Key words** Mouse · Natural killer cells · Major histocompatibility complex receptor · *Ly49* · Promoter

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## Introduction

To date, 14 members of the *Ly49* gene family have been identified in the C57Bl/6 (B6) mouse genome (Brown et al. 1997; McQueen et al. 1998) Ten of these (*Ly49a-j*) have been shown to produce mRNAs with a complete coding region, two are likely to represent transcribed pseudogenes (*Ly49k* and *n*), and the remaining two potential *Ly49* genes (*Ly49l* and *m*) have not yet been shown to produce any mRNAs in B6 mice (Brennan et al. 1994; McQueen et al. 1999; Smith et al. 1994). The *Ly49* genes are closely spaced with some intergenic regions less than 5 kb, and they appear to be arranged in a tandem fashion with identical orientation of transcriptional units. The functional role of several family members has been studied with specific monoclonal antibodies. Ly49A, C, I, and G have been shown to inhibit natural killer (NK)-cell function in response to specific major histocompatibility complex (MHC) class I ligands on target cells (Karlhofer et al. 1992; Mason et al. 1995; Yu et al. 1996), while Ly49D has been shown to activate NK-cell function (Mason et al. 1996). Inhibitory Ly49s have been shown to signal via SHP-1 (Mason et al. 1997; Nakamura et al. 1997; Olcese et al. 1996), while the activators signal through DAP-12 (Gosselin et al. 1999; Mason et al. 1998; Smith et al. 1998). There is no apparent clustering of *Ly49* genes with respect to activating/inhibitory function or gene homology.

Analysis of *Ly49* expression has demonstrated the existence of distinct subsets of NK cells that have various combinations of Ly49 molecules on their surface. The activation of *Ly49* genes during murine NK-cell development presents an interesting system for the study of selective gene expression. *Ly49a* and *C* are subject to allelic exclusion (Held et al. 1995); however, this exclusion is not complete, suggesting that the activation of *Ly49* gene expression is controlled by a probabilistic mechanism, and thus the activation of two identical family members in the same NK cell is a rare event. It has been proposed that the activation of *Ly49*

gene transcription is controlled by a stochastic process, since the proportion of NK cells that express two Ly49 proteins is roughly equivalent to the product of the proportion of NK cells expressing the individual Ly49s (Held and Kunz 1998). Single-cell RT-PCR analysis of *Ly49* expression (Kubota et al. 1999) has shown that the majority of NK cells express from one to four different Ly49s per cell and NK cells with five or more Ly49s are extremely rare, supporting the theory that the *Ly49* genes are activated in a random fashion by a limiting factor present in bone marrow.

The promoter region of *Ly49a* has been cloned, and the transcriptional start site identified (Kubo et al. 1993); however, no analysis of promoter elements required for cell-specific expression has been reported. Although Ly49 protein expression has been studied extensively, no treatments have been described that increase *Ly49* gene transcription. The level of Ly49 protein expression on NK cells has been related to the expression of the corresponding MHC class I ligand in a given mouse strain (Karlhofer et al. 1994). For example, Ly49c expression levels are lower in congenic strains that express the $H2^b$ or $H2^k$ MHC haplotypes recognized by Ly49c when compared to mice expressing the $H2^d$ haplotype (Gosselin et al. 1997). In addition, adoptive transfer experiments have shown that down-regulation of Ly49a occurs as a rapid adaptation process in mature NK cells after interaction with the H2Dd ligand in vivo (Kase et al. 1998). This suggests that Ly49 levels are not fixed but can be changed in mature NK cells if they are exposed to a different MHC class I environment. Whether or not there is any transcriptional component to this type of Ly49 regulation is unknown. The study reported here describes the promoter region of the *Ly49i* gene and the cell-specific transcriptional activity of the core promoter region.

## Materials and methods

### *Cell lines*

BFS, EL-4, P815, and NIH3T3 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

### *Isolation of mouse NK cells*

Mouse NK cells were from livers or spleens of 20- to 25-week-old interleukin (IL)-2-injected 129/J mice. Mice were injected intraperitoneally with 150,000 IU of IL-2 (Hoffman-LaRoche, Nutley, N.J.) twice daily for 3 days. On the 4th day, liver NK cells were isolated as previously described (Watanabe et al. 1997). Routinely, 75–80% of the resulting cells were $NK1.1^+$. Spleen cells were sorted for $DX5^+CD3^-$ NK cells on a MoFlo Cell Sorter (Cytomation, Fort Collins, Colo.).

### *Isolation of λ clones*

A 129 genomic library in λ Dash-2 (provided by J. Rossant, Mount Sinai Hospital, Toronto, Canada) was screened with a 158-bp fragment from the 5′ end of the mouse *Ly49I* cDNA (Stoneman et al. 1995) using conditions previously described (Anderson et al. 1993). Five clones were isolated and characterized by restriction mapping with *Eco*RI and *Pst*I followed by DNA sequencing.

### *Sequencing of Ly49I clones*

*Pst*I fragments of λ clones were subcloned into pBluescriptII KS (Stratagene, La Jolla, Calif.), amplified, and the fragment purified. The fragments were cut with either *Hae*III or *Alu*I and subcloned into M13mp18 at the *Sma*I site. M13 single-stranded DNA was purified using an M13 DNA purification system (Promega, Madison, Wis.) and sequenced with Sequenase (United States Biochemical, Cleveland, Ohio). Synthetic oligonucleotides were used to obtain sequence not covered by the M13 clones. Direct sequencing of promoter fragments containing the *Pst*I site confirmed the orientation of the *Pst*I fragments and the continuity of sequence. Sequence analysis was performed with the University of Wisconsin GCG package version 10, at the FCRDC supercomputing center.

### *Generation of Ly49I/luciferase reporter plasmids*

Several restriction enzyme fragments of the *Ly49I* promoter region were subcloned into pBluescriptII KS at the *Eco*RV site. The 3′ end of each fragment was the *Msp*I site contained within exon 1 of the *Ly49I* gene. The 5′ ends were: *Pst*I (pI-1, 258 bp), *Fnu*4HI(pI-2, 404 bp), *Dra*I (pI-3, 876 bp), and *Spe*I (pI-4, 1675 bp). The promoter fragments were then subcloned into pGL3-basic (Promega) using *Kpn*I and *Hin*dIII. All plasmid constructs were sequenced to confirm the presence and orientation of the insert.

### *Cell transfection*

EL-4, P815, or BFS cells ($4 \times 10^6$) in RPMI medium without serum were transfected with 10 μg of the individual expression constructs by electroporation using a GenePulser (Bio-Rad, Richmond, Calif.) set at 240 mV, 960 μF. NIH3T3 cells were plated at $3 \times 10^5$ cells per well in a six-well plate and transfected with lipofectamine (Life Technologies, Gaithersberg, Md.) using the manufacturer's protocol. All transfections contained 1 μg of a CMV-β-gal construct as a control for transfection efficiency.

### *Luciferase and β-galactosidase assays*

Cells were washed with phosphate-buffered saline (PBS), pelleted, and lysed by vortexing for 15 s in 100–200 μl of reporter lysis buffer (Promega). A clear lysate was obtained by centrifugation at 12,000 rpm and 2 μl of the supernatant was assayed for protein content using the BCA kit (Pierce Biochemical, Rockford, Ill.). Luciferase activity was measured from 20 μl of supernatant using the luciferase assay system (Promega) according to the manufacturer's directions. The β-galactosidase activity of 80 μl of cell lysate was assayed using the conditions of Eustice and co-workers (1991). The cell lysate was preincubated at 50 °C for 1 h and β-galactosidase activity was measured by a standard colorimetric assay using the CPRG substrate. Luciferase units were corrected for protein content and normalized for β-galactosidase activity.

### *Preparation of nuclear extracts*

Nuclear extracts were prepared as described previously (Yu et al. 1999). Briefly, cells were washed once with ice-cold PBS, once with buffer A (10 mm HEPES, pH 7.9, 10 mm KCl, 1.5 mm

$MgCl_2$, 0.5 mm dithiothreitol), then lysed in buffer A containing 0.05% NP40. Lysate was placed on ice for 10 min, then centrifuged at 4000 rpm, 4 °C for 4 min to remove cytoplasmic proteins. Nuclear proteins in the pellet were extracted in high-salt buffer (400 mm KCl, 25% glycerol, and 0.2 mm EDTA in buffer A). Insoluble material was removed by centrifugation at 15,000 rpm for 10 min. Protein concentration was measured with a Bio-Rad protein assay and samples stored at −70 °C.

*Electrophoretic mobility shift assays*

Electrophoretic mobility shift assays (EMSAs) were performed as previously described (Yu et al. 1999). Probes were as follows:

- GS-1: AGACAATGTTTTCCCCTCTCTGCAGGAGTGTGTCT
- GS-2: AGTGTGTCTTCTCTATCTTCAAATATAAATTATTCACATTTGTTTTGTCCA
- GS2-A: AGTTTTCATCTTCTCCTTCTTAAAAATATCAGTTATGGACATTGTTTTGCA
- A-PRO: TGCTTTGATGACGAGGAGGAGCATAAAATCATGAGGTTGAGTATCTC
- GS-2B: AGTGTGTCTTCTCTATCTTCA
- GS-2C: TCTTCAAATATAAATTATTCACATTTGTTTTGTC
- GS-2D: TGACATTTGTTTTGTC
- GS-3: TGTCCATCCAATACTATATGTTGTTTCAGATTGCAATAAGCA
- GS-4: TGCAATAAGCAATTTCCTCTTTTTGCTTTGTTGACA

The double-stranded oligonucleotides were end-labeled with [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol; DuPont, Boston, Mass.) using the Klenow fragment of DNA polymerase I (New England BioLabs, Beverly, Mass.). Labeled DNA probe was purified on G25 columns (Pharmacia, Piscataway, N.J.). Purified DNA probe was adjusted to 20 − 30 000 cpm/μl and stored at −20 °C until use. Five micrograms of nuclear extract was incubated with labeled probe for 30 min at room temperature. DNA-protein complexes were resolved on a nondenaturing 5% polyacrylamide gel. Cold competition experiments were performed by preincubating with unlabeled probes for 15 min on ice prior to addition of $^{32}$P-labeled DNA probe. Results were visualized by autoradiography after 1 − 4 days exposure at −70°.

## Results and discussion

*Cloning and sequencing of the 5′ region of the Ly49I gene*

A 129/J genomic library cloned in λ DASH-2 was screened with a 5′ fragment of *Ly49I* containing the region corresponding to the first and second exons of the *Ly49a* gene. Five clones were isolated and characterized by restriction mapping and sequencing with a *Ly49* exon 2 primer. Two of the clones were found to contain *Ly49I* sequences, and one of these, GenI-1, was chosen for further analysis. Figure 1A shows the restriction map of clone GenI-1, with the location of four *Ly49I* exons identified by sequencing with specific primers. The location of introns within the *Ly49I* coding region corresponded exactly to intron positions within the *Ly49A* gene (Kubo et al. 1993). Clone GenI-1 contains approximately 5 kb of DNA upstream of the 5′ end of the *Ly49I* cDNA. A 4.5-kb *Pst*I fragment was subcloned into pBluescript KS and sequenced. In addition, a 1.2-kb *Pst*I fragment containing exon 1 was subcloned and sequenced. Promoter fragments containing the *Pst*I site were isolated and sequenced, confirming the orientation and continuity of the *Pst*I fragments. The complete sequence of the 5.7-kb gene segment containing the promoter region and exon 1 has been deposited in GenBank and can be found under accession number AF173846.

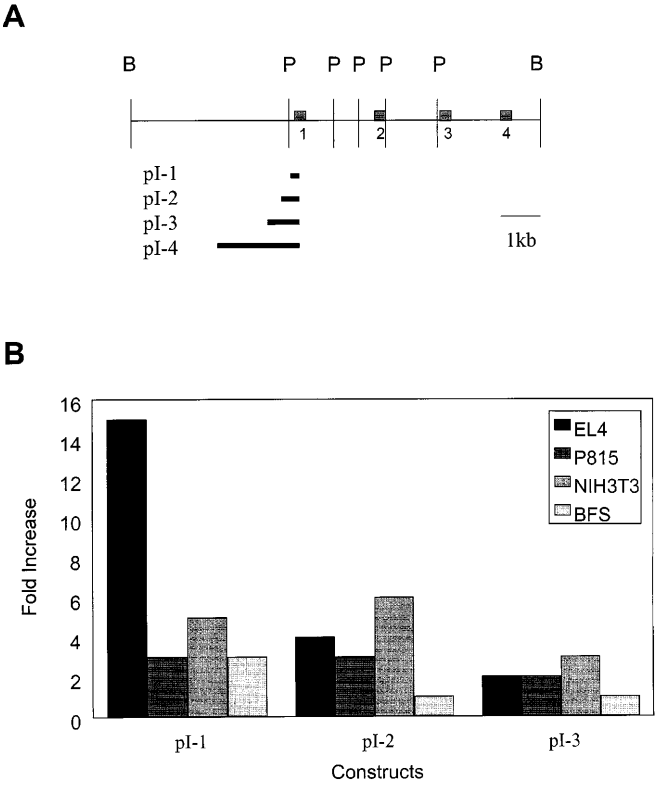


**Fig. 1A,B** *Ly49I* promoter region. **A** A restriction enzyme map of the isolated 129/J *Ly49I* genomic clone. The position of restriction enzyme sites for *Bam*HI (*B*) and *Pst*I (*P*) are shown. Exons corresponding to the *Ly49I* cDNA are indicated by the *shaded boxes*. Exon boundaries and numbering correspond to the previously reported *Ly49A* gene (Kubo et al. 1993). The position and size of restriction enzyme fragments used for luciferase reporter constructs are indicated by the *thick black lines* underneath the map. **B** Activity of *Ly49I* promoter fragments. The activity of luciferase reporter constructs transfected into different cell lines is shown relative to the activity of empty pGL3 vector. Representative data are shown, selected from five similar experiments. All cell lines were cotransfected with CMV-β-gal and values are corrected for transfection efficiency using β-galactosidase activity

*Promoter activity of Ly49I*

Four *Ly49i* promoter fragments were used to generate luciferase reporter constructs in the pGL3 vector. Figure 1A shows the regions of the *Ly49i* promoter contained in each construct. All the constructs end at an *Msp*I site that is contained within the first exon of the *Ly49i* gene. Figure 1B shows the luciferase activity of the constructs when transiently transfected into the Ly49-positive cell line EL-4, or the Ly49-negative cell

lines BFS (T cell), P815 (mastocytoma), or NIH3T3 (fibroblast). Only the core promoter construct pI-1 had significant activity in EL-4 cells and the longer promoter constructs had minimal activity in all cell lines tested, including EL-4. The core promoter contains a TATA element (TATAAA) that is not conserved in the *Ly49a* gene, and the potential TATA element reported in the *Ly49a* gene is not conserved in the *Ly49I* core promoter. These results indicate that the transcriptional start sites of these two family members have not been conserved although there is considerable homology of the region contained in construct pI-1 between *Ly49a* and *Ly49i* (71% identity). An additional *Ly49i* promoter construct containing 1675 bp upstream (pI-4) had luciferase activity similar to that of construct pI-3 (data not shown). Construct pI-2 contains 147 additional nucleotides of promoter sequence that has an inhibitory effect on the core *Ly49i* promoter. An analysis of this region for potential transcription factor-binding sites revealed the presence of a potential Oct-1 site as well as several putative homeodomain-binding sites. The lack of NK-specific enhancer elements in the upstream region was unexpected, and the apparent suppressive effect of the 147-nucleotide region flanking the core promoter in all cell types tested including the Ly49-positive EL-4 cells suggests that additional *Ly49* gene enhancer elements exist elsewhere. The unusual nature of this promoter region may reflect the global regulation of the *Ly49* gene family. Current evidence suggests that *Ly49* genes are activated one at a time in a random fashion and require the interaction of the NK cell with factors produced by the bone-marrow stroma (Dorfman and Raulet 1998). NK cells with a selected Ly49 phenotype can give rise to cells expressing additional Ly49 proteins after in vivo transfer. The exception to this rule is Ly49A-negative cells, which cannot give rise to Ly49A-positive cells after in vivo transfer. Since individual Ly49 family members are chosen for expression from the cluster of at least 14 tandemly arranged genes, we postulate the existence of an *Ly49* gene locus control region (LLCR). This locus control region would be activated when the NK cells are stimulated by bone-marrow stromal cells, and would then randomly select one of the *Ly49* genes for activation. The most likely location of an LLCR is at the 5′ end of the gene cluster in close proximity to the *Ly49a* gene. The *Ly49a* gene is the first gene of the *Ly49* cluster, so the region 5′ to it should be surveyed for the presence of *Ly49* enhancer elements. The region of the *Ly49i* promoter identified as an inhibitory element in this study may be the target region for the LLCR. Perhaps proteins bound to this region have an inhibitory effect that can only be overcome by a complex brought to the promoter by the LLCR. To fully test this hypothesis, a full promoter analysis of the *Ly49a* promoter, as well as an additional *Ly49* gene, will be required.

*EMSA analysis of the Ly49I core promoter*

EMSA analysis was used to demonstrate binding of NK cell-specific transcription factors to the core promoter. Four overlapping oligonucleotide probes covering the region contained within the core promoter were synthesized and used for EMSA analysis. Figure 2A shows the size and location of the oligonucleotides used. Figure 2B shows the results of an EMSA experiment using the oligonucleotide probes with nuclear lysate from the EL-4 or P815 cell lines. Of the four probes tested, only the GS-2 probe showed specific complexes that were present in the Ly49-positive EL-4 cell line but not the Ly49-negative P815 cell line. Three major complexes were observed, and they have been labeled C1, C2, and C3 in Fig. 2B. The GS-2 probe was subsequently subdivided into three additional probes GS-2B, GS-2C, and GS-2D as shown in Fig. 3A. Figure 3B shows the result of EMSA analysis with these probes using EL-4

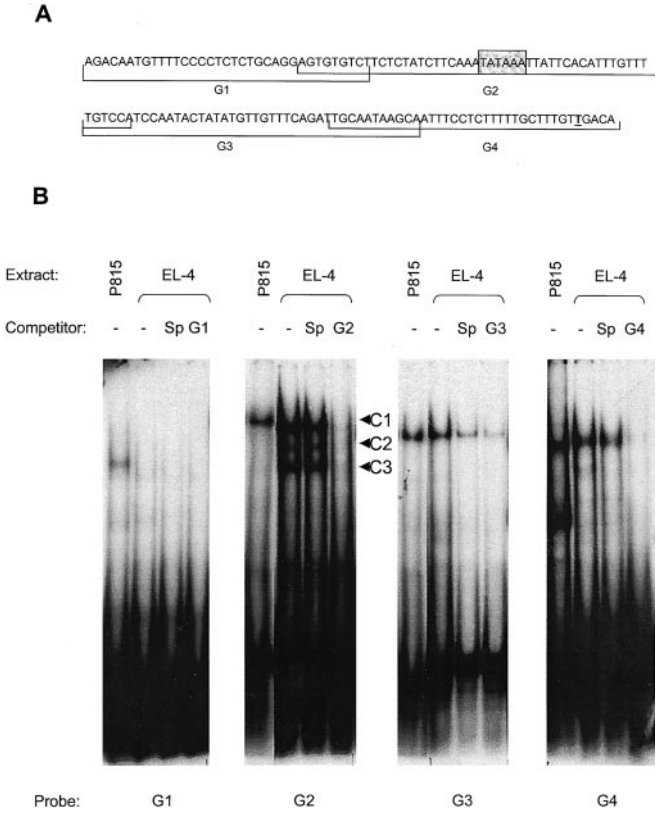


**Fig. 2A,B** Electrophoretic mobility shift assay (EMSA) analysis of the core promoter region. **A** The region corresponding to the core promoter. The regions contained within the four EMSA probes are indicated by *brackets*. The TATAAA element contained in probe GS-2 (*G2*) is indicated by the *shaded box*. The first nucleotide of the *Ly49I* cDNA is *underlined*. **B**. EMSA analysis of the four subfragments of the core promoter region. A nuclear extract of P815 cells is compared with an extract from EL-4 cells for each of the four probes. The EMSAs were competed with either a nonspecific competitor Sp-1 oligonucleotide (*Sp*), or cold probes GS-1–GS-4 (*G1–G4*). *Arrowheads* labeled *C1*, *C2*, and *C3* indicate the specific complexes observed with the GS-2 probe

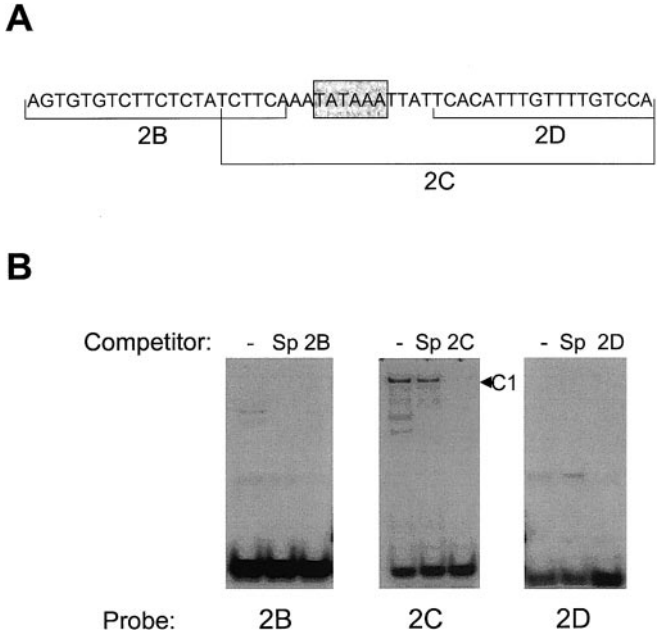


**Fig. 3A,B** Analysis of the TATA region of the *Ly49I* promoter. **A** The sequence of the GS-2 probe is shown, with the regions used to generate subfragments indicated by *brackets*. The TATAAA element is indicated by a *shaded box*. **B** EMSA analysis of the GS-2 subfragments using EL-4 nuclear extracts. Cold competitor fragments were either a nonspecific Sp-1 oligonucleotide (*Sp*) or the appropriate unlabeled probe fragment. The *arrowhead*-labeled *C1* indicates the major specific complex generated by probe 2C

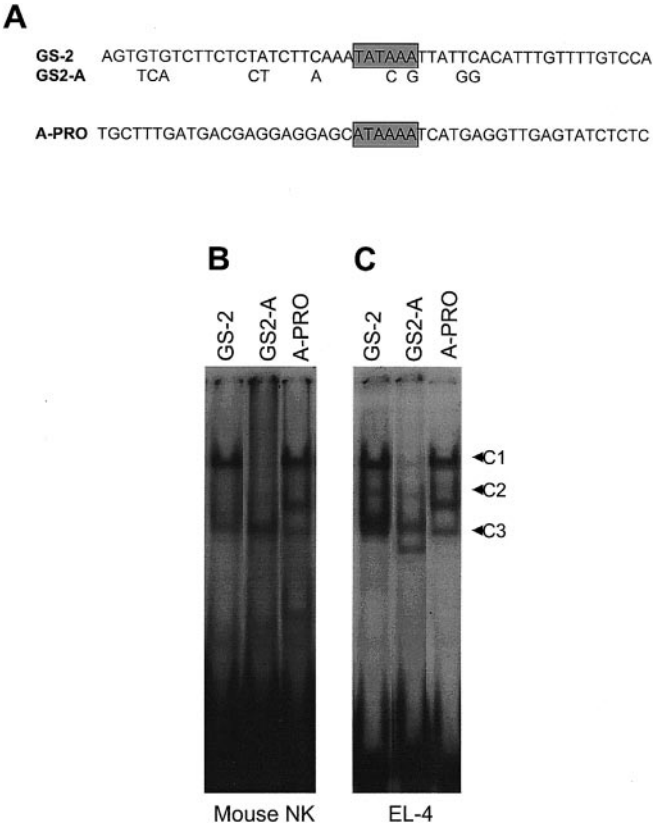


**Fig. 4A–C** Detection of NK cell-specific DNA/protein complexes in *Ly49A* and *I* core promoters. **A** Sequence of the GS-2, GS2-A, and A-PRO probes. The nucleotide sequence of the probe fragments used for EMSA analysis is shown. Only nucleotides that differ from the *Ly49I* GS-2 sequence are shown for the *Ly49A* GS2-A fragment. The A-PRO fragment represents the region containing the predicted TATA element of the *Ly49A* promoter. The *shaded boxes* indicate TATA elements of the *Ly49I* and *A* promoters. **B** Freshly isolated 129/J murine NK-cell nuclear extracts were used for EMSA analysis. **C** EL-4 nuclear extracts were used for EMSA analysis. The *labeled arrowheads* indicate the positions of the three specific GS-2 complexes observed in EL-4 nuclear extracts (as shown in Fig. 2)

nuclear lysates. Only the GS-2C probe was able to produce complexes in this experiment, indicating that the region containing the TATAAA element is involved in producing the specific complexes. Complex C1 was the only major complex observed in this experiment, suggesting that C2 and C3 depend on 5′-flanking sequence. The apparent tissue specificity of this TATAAA element is unusual; however, the recent discovery of human TATA box-binding protein (TBP)-related factors (Rabenstein et al. 1999) suggests that "basal" transcription machinery in mammals can also direct gene-specific transcription through these TBP-related factors as has been demonstrated in *Drosophila*.

### *Identification of NK cell-specific DNA-binding proteins that interact with the Ly49I and Ly49A core promoters*

To further investigate the complexes observed with the *Ly49i* core promoter fragment, additional experiments were performed using nuclear extracts from freshly isolated NK cells as well as comparable fragments of the *Ly49A* promoter. Figure 4A shows the sequence of two oligonucleotide fragments from the *Ly49a* core promoter that were analyzed. The first, GS2-A, contains the region of *Ly49a* that is homologous to the GS-2 fragment of the *Ly49i* core promoter; however, the TATA element is not conserved. Since the predicted TATA element of the *Ly49a* promoter is located 90 nucleotides downstream of the GS2-A fragment, a second probe of similar size containing the *Ly49a* TATA element was generated (A-PRO). These probes were used to perform EMSA analyses with nuclear extracts obtained from 129/J spleen/liver NK cells (Fig. 4B) as well as EL-4 nuclear extracts (Fig. 4C). As shown in Fig. 4B, complex C1 was visible in both the NK-cell and EL-4 extracts. Interestingly, this complex was not detected with the GS2-A probe that does not conserve the TATA element. In addition, when the A-PRO probe containing the predicted TATA element of *Ly49a* was used, complex C1 was present. These results suggest that C1 is the complex responsible for recognizing the TATA elements of the *Ly49* genes.

In this study we identified a core promoter element in the *Ly49i* gene that is preferentially transcribed in the EL-4 cell line that expresses Ly49 proteins. A 36-bp

region of the core promoter containing a TATAAA element was shown to form cell-specific DNA/protein complexes. Further study of the *Ly49i* core promoter will be directed towards identifying NK cell-specific DNA-binding proteins responsible for the formation of the complexes identified here.

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