Regulation of Mouse Mammary Tumor Virus env Transcriptional Activator Initiated Mammary Tumor Virus Superantigen Transcripts in Lymphomas of SJL/J Mice: Role of Ikaros, Demethylation, and Chromatin Structural Change in the Transcriptional Activation of Mammary Tumor Virus Superantigen

Rajan M. Thomas, Kamran Haleem, Abu B. Siddique, William J. Simmons, Namita Sen, Da-Jun Zhang and Vincent K. Tsiagbe

*J Immunol* 2003; 170:218-227; doi: 10.4049/jimmunol.170.1.218

http://www.jimmunol.org/content/170/1/218

---

**References**

This article cites 56 articles, 25 of which you can access for free at:
http://www.jimmunol.org/content/170/1/218.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Regulation of Mouse Mammary Tumor Virus \textit{env} Transcriptional Activator Initiated Mammary Tumor Virus Superantigen Transcripts in Lymphomas of SJL/J Mice: Role of Ikaros, Demethylation, and Chromatin Structural Change in the Transcriptional Activation of Mammary Tumor Virus Superantigen$^{1,2}$

Rajan M. Thomas, Kamran Haleem, Abu B. Siddique, William J. Simmons, Namita Sen, Da-Jun Zhang, and Vincent K. Tsiaqbe$^3$

Mammary tumor virus (\textit{Mtv29}),$^4$ is an endogenous retrovirus integrated in the genome of the SJL/J mouse strain, and it encodes \textit{Mtv29} superantigen (vSAg29) in its 3$'$ long terminal repeat (LTR). vSAg29 is implicated in the pathogenesis of germinal center (GC)-derived B cell lymphomas, also called reticulum cell sarcoma (RCS), which spontaneously arise in $>$90% of SJL/J mice by the age of 12 mo (1, 2). In previous studies, we have shown that stimulation of CD4$^+$ V\beta16$^+$ T cells by vSAg29 is required for the growth of GC-derived lymphomas (3). The activated T cells secrete cytokines, such as IL-4 and IL-5, that promote lymphoma growth. In SJL lymphomas, the \textit{Mtv29} provirus shows the expression of a 1.8-kb vSAg29 mRNA transcript that is initiated from mouse mammary tumor virus (MMTV) \textit{env} transcriptional activator (META) region located within the \textit{env} gene (2, 4). Although META \textit{env}-initiated 1.8-kb vSAg29 transcripts are overexpressed in SJL B cell lymphomas, they are not transcribed in normal B lymphocytes or in other somatic cells. We have observed, however, that META \textit{env} allows the expression of 1.8-kb vSAg29 transcripts in Peyer's patch B cells from 6-mo-old SJL mice (4).

Actively transcribing genes typically have a relaxed chromatin structure with enhanced accessibility to transcription factors. Inactivation (silencing) of genes is frequently due to methylation (5), which causes condensation of chromatin, due to interactions with methylcytosine binding proteins (6). Condensed and heterochromatinized gene loci are resistant to digestion with \textit{DNaseI} (7). DNA methylation plays a major role in X chromosome inactivation (8) and imprinting (9, 10) and in the loss of expression of tumor suppressor and cell cycle-controlling genes in cancer cells (11–15). CpG islands in the promoter elements of many tumor
suppressor genes are unmethylated in normal cells but hypermethylated in many different types of cancer cells (16, 17).

Proviral DNA usually exists in a hypermethylated state (18, 19). This process can cause latency of viral infection (20) and is also the reason for a lack of expression of many integrated Mtv29 (21–23), even when the Mtv-LTR is used as a promoter for a transgene (24). Normal methylation patterns are frequently altered in cancer cells, and de novo demethylation of methylated retroviral elements is known to activate silenced viral genes and to play a role in carcinogenesis (25).

Ikaros is a lymphocyte-specific repressor protein, reported to be associated with silenced genes (26). It is a member of the zinc finger family of proteins and is expressed in several isoforms derived from splice variants of primary transcripts. Ikaros functions in the regulation of several genes expressed in lymphocytes. Dysregulation of Ikaros isoforms has been reported in human leukemias and lymphomas (27, 28). The Ikaros proteins, master regulators of B and T cell differentiation (29), act by associating with distinct histone deacetylase, causing chromosome remodeling and maintenance of repression in specific gene loci (26, 30). Therefore, a loss of repression may lead to a gradual de-repression of the essential genes that occur over a number of mitoses.

To the best of our knowledge, there are no reports on the regulation of META env-initiated Mtv29 transcripts in SJL GC-derived B cell lymphomas. In the present study, we have investigated the roles of Ikaros, promoter demethylation, and chromatin structural changes in the transcriptional activation of META env promoter in vSAg29-transcribing lymphoma cells. Our results suggest that the expression of META env-initiated vSAg29 transcript is associated with demethylation at a CpG site in the distal HgOl site located very close to the Ikaros binding site. The dominant negative effect produced by Ikaros isoform (Ik6) expression may contribute to chromatin remodeling in the META env region, leading to demethylation at the vSAg29 initiation region.

Materials and Methods

Reagents

The restriction endonucleases and methylation-sensitive restriction endonucleases used for the experiments were purchased from New England Biolabs (Beverly, MA). The enzyme Thul was purchased from Life Technologies (Gaithersburg, MD). Fluorescent dye SYGR green was purchased from Molecular Probes (Eugene, OR). Rabbit polyclonal C-terminal anti-Ikaros Ab (cross-reactive with mouse and human Ikaros) was a gift from Dr. S. T. Smale (Howard Hughes Medical Institute, University of California School of Medicine, Los Angeles). Custom-made oligonucleotide primers were obtained from Gene Link (Thornwood, NY). All of the reagents used for the experiments were analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO).

Mice

Female SJL/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were bred and maintained in an animal care facility of New York University School of Medicine, and all animal experiments were performed in accordance with institutional guidelines and with approval from the Institutional Animal Care and Use Committee. The in vitro lymphoma cell lines, cNJ117 and cRCS-X, were originally derived from aging SJL mice. A vSAg29-negative lymphoma cell line, cNJ101 (IgM+), unlike any of the typical GC-derived lymphomas of SJL origin, was derived from a 2-year-old SJL mouse that had received chronic treatments above (31).

Cell culture

SJL/J-derived B cell lymphoma cell lines (cRCS-X, cNJ117, and cNJ101) were grown in RCS medium (IMDM containing 10% FBS supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 8 μg/ml insulin, 1 mM ascorbic acid, 0.5 mM sodium pyruvate, and 0.05 mM 2-ME). These cell lines were cultured at standard cell culture conditions. vSAg29-responsive CD4+ Vβ16+ T cell hybridoma (1D1-E7) was derived in our laboratory (3).
methyltransferase activity in an enzyme extract from drug-treated and control cells was measured as described in Fig. 1. M13 refers to primers generated by phosphor imaging.

Drug treatment

To examine the effects of a demethylating agent, 5-azacytidine (5-Aza), which is an inhibitor of DNA methyltransferase, and a histone deacetylase inhibitor, trichostatin A (TSA), on a vAg29 nontranscribing lymphoma, cNJ101 cells (5 × 10⁶/flask) were treated with different amounts of these drugs separately and in combination. The cells were grown in RCS medium in the presence and absence of these drugs for 48–72 h. In a separate set of flasks, cNJ101 cells were treated with 20 nM TSA first and then grown in RCS medium for 24 h, followed by addition of different amounts of 5-Aza and incubated for 48 h at 37°C in a cell culture incubator. The cells were harvested at different intervals and washed three times with 1× DPBS. Total RNA was extracted from drug-treated and control cells using RNA Stat-60 (Tel-Test, Friendswood, TX), followed by chloroform extraction and precipitation with isopropanol. DNA was also extracted from the aliquots of drug-treated cells as described above.

Northern blot analysis

Northern blotting was performed to examine whether drug treatment can induce the expression of the META-initiated 1.8-kb vAg29 transcript in cNJ101 cells. Equal amounts (20 μg) of total RNA isolated from control and drug-treated cells were electrophoresed under denaturing conditions through 1.2% agarose/formaldehyde gel, and then were transferred to nylon membrane and cross-linked by UV irradiation. The LTR-open reading frame and META env probe were randomly primed with 32P-labeled LTR-open reading frame in ULTRAhyb buffer at 42°C. The membrane was prehybridized for 1 h at 42°C in ULTRAhyb buffer and hybridized overnight with random primed 32P-labeled LTR-open reading frame in ULTRAhyb buffer at 42°C. The membrane was washed as recommended by the manufacturer of the hybridization buffer. The washed filters were exposed to x-ray films. The membrane was stripped and rehybridized with 32P-labeled Meta29 META env and GAPDH probes.

Table I. List of PCR primers and oligonucleotide sequences used for experiments

<table>
<thead>
<tr>
<th>Sequence Name*</th>
<th>Sequence (5′→3′)</th>
<th>Position in Mtv29 env</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 reverse</td>
<td>GTAAAACGACGGCCAG</td>
<td>6596–6617</td>
</tr>
<tr>
<td>M13 forward</td>
<td>CAGGAAAAACGCTTGA</td>
<td>7128–7152</td>
</tr>
<tr>
<td>LL1</td>
<td>CCGACCTTTTACGAGG</td>
<td>6836–6857</td>
</tr>
<tr>
<td>RR1</td>
<td>CTTTTGGAGAAAAATTCAGC</td>
<td>7335–7358</td>
</tr>
<tr>
<td>MRPA</td>
<td>CTTTCCGCTGACGGCGGAA</td>
<td>7137–7341</td>
</tr>
<tr>
<td>MRP1</td>
<td>ACCCTGGAGGAGGGGTAATAAG</td>
<td>6596–6671</td>
</tr>
<tr>
<td>MRP2</td>
<td>TACCCCTTGGCAGAGGTGCCA</td>
<td>7355–7358</td>
</tr>
<tr>
<td>MP1</td>
<td>TTGATTTTTTTATTTGAGGAAA</td>
<td>7329–7358</td>
</tr>
<tr>
<td>MP2</td>
<td>CTTTTTTAAAAAACATTACCCCTTC</td>
<td>6873–6896</td>
</tr>
<tr>
<td>PR1</td>
<td>GATTTATTTTTTTTTTTTTTTGTTT</td>
<td>7135–7152</td>
</tr>
<tr>
<td>Aβ</td>
<td>ATTTAATATTATAATTATTTTT</td>
<td>7267–7297</td>
</tr>
<tr>
<td>Bβ</td>
<td>AAATATTGATTTGGGTTGTT</td>
<td>7267–7297</td>
</tr>
<tr>
<td>Cβ</td>
<td>AGAAGGGGTTGATAAAAGG</td>
<td>7267–7297</td>
</tr>
<tr>
<td>Ikaroς</td>
<td>forward</td>
<td>ATGGATGTCGATGAGGGTCAAG</td>
</tr>
<tr>
<td>Ikaroς</td>
<td>reverse</td>
<td>TTAGCTCAGTGGTAAAGCTGC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>forward</td>
<td>AGAACATCATCCTGATCC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>reverse</td>
<td>AGCCGTATCCTCATGCA</td>
</tr>
<tr>
<td>ODN1 (sense strand)</td>
<td>AAGGAGGCCCTTCTGAGGAGACGAGTCG</td>
<td>7267–7297</td>
</tr>
<tr>
<td>ODN2 (antisense of ODN1)</td>
<td>CAGACTGCTGCTCTTCCCCAGAAGGCGTCCTT</td>
<td>7267–7297</td>
</tr>
<tr>
<td>ODN3 (sense strand)</td>
<td>AAAGGACCCCTCCTGCGGAGAGACGAGGTGTCG</td>
<td>7267–7297</td>
</tr>
<tr>
<td>ODN4 (antisense of ODN3)</td>
<td>CAGACTGCTGCTCTTCCCCAGAAGGCGTCCTT</td>
<td>7267–7297</td>
</tr>
</tbody>
</table>

* The names used are arbitrary descriptions of positions of primers in Mtv29 env, as described in Fig. 1. M13 refers to primers specific for DNA from filamentous bacteriophage which is the plasmid containing our cloned Mtv29 env. MP1, and MP2, ODN1, 2, 3, and 4 refer to oligonucleotide positions within Mtv29 env.

* Ms-SNuPE primer.

* Mouse Ikaroς primers were designed from GenBank accession no. G0677650.

* Mouse GAPDH primers were designed from GenBank accession no. GI13624248.

Sodium bisulfite conversion and methylation-sensitive single nucleotide primer extension (Ms-SNuPE) analysis

Ms-SNuPE analysis using PCR product from sodium bisulfite-converted DNA was used to determine the degree of methylation at CpG sites located outside the methylation-sensitive enzyme sites. DNA (2 μg) was digested with EcoRI. Nspl, an enzyme that digests the Mtv8 META env region, was also used to exclude Mtv8 in the analysis. Digested samples were purified by phenol-chloroform extraction and resuspended in 20 μl of Tris EDTA buffer. Sodium bisulfite conversion of cytosine to uracil was performed as described (32), with a slight modification as described (33). To amplify bisulfite-converted DNA, PCR primers were designed as described (34). Nested primer PCR was performed to amplify the top strand (5′→3′) of the Mtv29 META env region using a first round of PCR with MP1 and MP2 primers (Table I). The thermal cycling was performed as follows: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 1 min, extension at 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR products derived from above reaction were used as template for the second round of PCR with PR1 and PR2 primers (Table I) and the PCR done for 35 cycles at the same conditions, except for annealing at 63°C for 1 min. The PCR products of expected size were gel purified using the QUII quick gel extraction kit (Qiagen, Valencia, CA) and used as a template for Ms-SNuPE. Some purified PCR products were cloned into pCR2.1 TOPO TA cloning vector (Invitrogen, Carlsbad, CA) and sequenced.

Degree of methylation at CpG sites located at positions 6897, 6962, and 7153 was analyzed by quantitative Ms-SNuPE analysis. Multiplex Ms-SNuPE analysis was performed for three CpG sites of the top strand of the Mtv29 META env region using PCR products as template that were generated by PCR with bisulfite-converted DNA. Internal primers (A, B, and C in Table I) were designed to analyze the methylation of CpG sites located at positions 6897, 6962, and 7153, respectively. During the reaction, the 3′ terminus of oligonucleotide primer is extended by incorporation of a single [32P]dTTP (representing unmethylation) or [32P]dCTP (representing methylation) by Taq polymerase (based on the methylation of the cytosine residue at CpG sites). The reaction was performed in a 25-μl mixture containing 30 ng of gel-purified template (PCR product) and incubated in a final concentration of 1× PCR buffer, 1 μM of each primer, 1 μCi of either [32P]dCTP or [32P]dTTP, and 1 U of Taq polymerase. Conditions for primer extensions were as follows: 95°C for 1 min, 50°C for 2 min, and 72°C for 1 min. Samples were then analyzed through 12% denaturing polyacrylamide gel containing 7 M urea, and incorporated radioactivity was quantified by phosphor imaging.
In vitro stimulation by vSAg29

The drug-treated and control cNJ101 cells were cocultured with vSAg29-responsive CD4+Vβ16+ (ID1-E7) T hybridoma cells (105 cell/well) in flat-bottom 96-well plates (Costar, Cambridge, MA) using RPMI 1640 medium as described (3). Plate-bound anti-TCR αβ mAb (H57-597), anti-CD3 mAb (145-2C11), or PHA (5 μg/ml) was used as positive control stimulus. The cells were harvested after 24 h of culture, and supernatants from replicate cultures were separated and stored at −20°C. IL-2 production was determined by ELISA using mouse rIL-2 standard (Minikit KM-IL-2; Endogen, Woburn, MA).

RT-PCR

RT-PCR was performed to examine the expression of mRNA transcripts of Ikaros isoforms. cDNA was synthesized from 5 μg of RNA (pretreated with RNase-free DNaseI) using Superscript II Reverse Transcriptase and oligo(dT)20-19 primers (Invitrogen). The Ikaros primers used are indicated in Table I. PCR conditions were the same as described below for semi-quantitative PCR, except for amplification with 40 cycles.

Western blot analysis

Western blotting was performed to analyze the Ikaros isoforms expressed in cytoplasmic and nuclear extracts of normal and lymphoma B cells. Cytoplasmic and nuclear extracts from different cell samples were prepared using a kit (Pierce, Rockford, IL) following manufacturer’s directions. A mixture containing protease inhibitors (Halt; Pierce) was added during sample preparation to avert protein degradation. Ikaros isoforms were detected using a polyclonal anti-Ikaros Ab (36), which was reactive with all Ikaros isoforms. Nuclear extracts (25 μg) were size fractionated on SDS-PAGE and transferred to nitrocellulose membrane by electroblotting. The membrane was blocked (5% dry milk powder and 0.1% Tween 20 in PBS) for 1 h at room temperature. The membrane was then incubated with appropriate dilution of anti-Ikaros primary Ab (in blocking buffer) in a sealed plastic bag for 1 h at room temperature. After this, the membrane was washed for 10 min three times with wash buffer (0.1% Tween 20 in 1× PBS). It was then incubated with appropriate dilution of peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h. The membrane was washed for 10 min three times with wash buffer and then developed using ECL (Amersham Pharmacia Biotech). After stripping, the membrane was also immunoblotted for the expression of CDK2 (control) using rabbit anti-mouse cyclin-dependent kinase 2 (CDK2) followed by peroxidase-conjugated anti-rabbit IgG.

Nuclei isolation and analysis of DNasel accessibility to chromatin

To determine DNasel accessibility to chromatin at a locus containing the vSAg29 initiation site, cell nuclei were isolated (37). Aliquots of isolated nuclei were incubated with 15 U of DNasel (Promega) for 3 min at 25°C, and reaction was terminated by the addition of stop buffer. DNA was extracted from control and DNasel-treated nuclei by phenol-chloroform extraction. The accessibility of DNasel to a region containing the vSAg29 initiation site in the Mtv29 META env region was determined by quantitative real-time PCR using MRP1 and MRP2 primers (Table I) following the same conditions as described previously.

EMSA

EMSA was performed to examine the ability of Ikaros to bind to an oligonucleotide representing the sequence between positions 7267 and 7297 in the META env, which contains an Ikaros binding site adjacent to the distal Hgal site. Oligonucleotide sequences (ODN 1, 2, 3, and 4) used for the EMSA are shown in Table I. The duplex oligonucleotides with or without a point mutation at the Ikaros binding site were 5’ end-labeled with [32P]ATP using T4 polynucleotide kinase. The labeled probe was purified using Chromaspin 10 columns (Clontech Laboratories, Palo Alto, CA). The probe was incubated with 15 μg of nuclear or cytoplasmic extract in a binding buffer (10 mM Tris-HCl, 1 mM DTT, 75 mM KCl, 10% glycerol, 100 μM spermine, and 1 μg of sheared salmon sperm DNA) and incubated at 25°C for 30 min in a final reaction volume of 20 μl. Ab supershift was performed with incubation of probe with nuclear and cytoplasmic extract preincubated with rabbit anti-Ikaros Ab for 30 min at 25°C. The reaction products were analyzed through 6% nondenaturing polyacrylamide gel in 1× Tris-borate-EDTA buffer for 3 h at 200 V and 4°C. The bands were detected by phosphor imaging (Bio-Rad).

Results

Methylation profile of the Mtv29 META env region

In SJL/J RCS lymphomas, 1.8-kb Mtv29 vSAg29 mRNA transcripts are initiated from the META region of the env gene, as previously characterized (4). Fig. 1 shows the schematic representation of the Mtv29 META env region. To determine the degree of methylation at the META env region in vSAg29-negative normal cells and vSAg29-positive lymphoma cells, we digested the DNA with methylation-sensitive restriction endonucleases. Methylation at AvaI, FspI, Hgal, HpaiII, and Thal sites (Fig. 1) of the Mtv29 META env region was quantified by real-time PCR using Mtv29-specific primers. The degree of methylation at these sites in both normal and lymphoma cells is shown in Fig. 2. The normal SJL liver DNA from 3-, 6-, and 9-mo-old mice exhibited hypermethylation at all analyzed sites (Fig. 2A). DNA from splenic B lymphocytes of 3-, 6-, and 9-mo-old normal SJL/J mice showed hypermethylation at the distal Hgal site. In contrast, DNA from Peyer’s patch B cells of the 6- and 9-mo-old mice showed a significant decrease in methylation at the distal Hgal site (Fig. 2B).

The semiquantitative densitometric analysis of PCR product amplified from methylation-sensitive enzyme-digested samples showed a pattern comparable to that generated for these sites using real-time PCR. Fig. 2C shows the comparison of both quantitative and semiquantitative PCR methods used for the analysis of methylation at the META env region of DNA from primary lymphomas. The results showed a significant level of demethylation at the distal Hgal site (Fig. 2C), whereas methylation at the AvaI, FspI, HpaiII, and Thal sites was not significantly different. DNA from RCS lymphoma cells showed heterogeneity in the methylation pattern in the META env region (Fig. 2D). A unique vSAg29-negative and T cell-independent SJL lymphoma cell line, cNJ101, showed hypermethylation at all of the analyzed sites in the META env region, but the vSAg29-transcribing cell lines, cNJ117 and cRCS-X, showed a significant level of demethylation at AvaI, Hgal, and HpaiII sites. Whereas cNJ117 showed hypomethylation at the FspI site, cRCS-X showed no significant change in methylation at the same site. The Thal site in the META env region showed hypermethylation in all lymphoma cell lines. Southern blot analysis was also used to examine the methylation pattern at AvaI and FspI sites of the Mtv29 META env region (Fig. 3). cNJ101,

FIGURE 1. Schematic representation of Mtv29 META env region. CpG sites (indicated by filled circles and numbered) present between position 6596 and 7358. The analyzed CpG sites within the methylation-sensitive restriction sites (AvaI, FspI, Hgal, HpaiII, and Thal) and CpG sites (outside the restriction endonuclease site) at positions 6897, 6962, and 7153 (primers A, B, and C, respectively) are also indicated. The META D enhancer element, Mtv29 transcription start site, and positions of primers are also indicated. Base numbering corresponds to the Mtv29 env sequence that we previously reported (2).
which does not transcribe the vSAg29 mRNA, exhibited more methylation at FspI and Aval sites in the META env region than did the vSAg29-transcribing SJL lymphomas (cNJ117 and cRCS-X). Liver DNA from 3-mo-old SJL mice showed hypermethylation at these sites, a result consistent with data obtained by PCR analysis.

We also examined the degree of methylation at three CpG sites (positions 6897, 6962, and 7153) by multiplex Ms-SNuPE analysis (Fig. 4). Heterogeneity in the methylation pattern was revealed, but methylation was seen at these sites in all of the analyzed samples of both liver and lymphomas, indicating that these sites may not influence vSAg29 transcription.

**Effects of 5-Aza and TSA treatments on cNJ101 cells**

We subsequently analyzed the effects of demethylating agent 5-Aza (5 μM) and histone deacetylase inhibitor TSA (20 nM) on cNJ101 cells (the atypical SJL/J-derived lymphoma cell line that neither transcribes META env-initiated 1.8-kb vSAg29 transcripts nor stimulates Vβ16+ T hybridoma cells). The changes in the degree of methylation at Aval, FspI, Hgal, HpaII, and ThaI sites induced by drug treatment were quantified by real-time PCR. The results shown in Fig. 5 demonstrate that cNJ101 cells treated with 5-Aza and TSA showed a significant level of demethylation at Aval, Hgal, and HpaII sites. The FspI site and ThaI sites did not show any significant changes in methylation. These drugs showed similar effects, irrespective of their use alone or in combination.

Next, we performed Northern blot analysis to examine whether 5-Aza and TSA treatment can induce the expression of META env-initiated 1.8-kb vSAg transcripts in cNJ101. MMTV-LTR-probed RNA from cells treated with both 5-Aza and TSA (20 nM) for 72 h showed a 1.8-kb vSAg transcript (Fig. 6A). The cells treated with 5-Aza alone also expressed 1.8-kb transcripts; however, in combination, the drugs were much more effective in
inducing the cells to express vSac29 transcripts. The cells treated with 5-Aza alone or in combination with TSA also up-regulated 2.9- and 4.1-kb env transcripts in a dose-dependent manner (Fig. 6, A and C). TSA treatment alone also caused the expression of 1.8-kb transcripts in cNJ101. The 5-Aza-induced expression of 1.8-kb vSac29 transcripts was remarkably increased after 48 h of treatment, when this drug was added to the cNJ101 cells pretreated with 20 nM TSA alone for 24 h (Fig. 6B). The 1.8-kb vSac29 transcripts in drug-treated cNJ101 cells also hybridized with a METAv region in DNA of cNJ101 cells was significantly increased after drug treatment.

To examine whether vSac29 induced by drug treatment can stimulate CD4^+Vβ16^- T cell hybridoma cells, we cocultured vSac29-responsive T hybridoma cells with cNJ101 cells pretreated with 20 nM TSA and 2.5 μM 5-Aza for 72 h and measured the IL-2 secreted by the responding hybridoma cells (Fig. 6D). Whereas the cells treated with 5-Aza and TSA (in combination) stimulated Vβ16^- T hybridoma cells, untreated cells did not. cNJ101 cells treated with 5-Aza or TSA alone did not induce any detectable levels of IL-2 production.

**FIGURE 5.** Methylation profile of Mtv29 META env region in DNA from cNJ101, after treatment with 5-Aza (5 μM) and TSA (20 nM) for 72 h. DNA extracted from drug-treated cells was digested and the degree of methylation at the indicated sites was quantified by real-time PCR. The assay was performed in triplicate. Methylation profile of untreated cNJ101 DNA is shown in Fig. 2D. The values are means ± SEM for three treatments. *, p < 0.05; **, p < 0.01; ***, p < 0.001; comparison between DNA from untreated (control) cNJ101 and drug-treated cNJ101 cells.

**FIGURE 6.** Effect of drugs (5-Aza and TSA) on a vSac29-negative lymphoma cell line (cNJ101), analyzed by Northern blot hybridization of RNA from drug-treated cNJ101 cells. A, cNJ101 cells treated with 5-Aza alone or together with TSA for 72 h at 37°C. RNA extracted from treated cells was probed with Mtv29-LTR for the expression of 1.8-kb vSac29 mRNA transcripts. The transcripts (2.9 and 4.1 kb) represent env. Expression of housekeeping gene GAPDH is shown at the bottom. B, cNJ101 cells were exposed to 20 nM TSA alone for 24 h and then to different amounts (100–5000 nM) of 5-Aza for 48 h. RNA extracted from treated cells was probed with Mtv29-LTR for the expression of 1.8-kb vSac29 transcripts. C, Mtv29 META env-initiated 1.8-kb vSac29 transcripts in drug-treated cells. The blot shown in B was stripped and then hybridized with Mtv29 META env probe (7136–7339). D, cNJ101 cells exposed to 5 μM 5-Aza and 20 nM TSA for 72 h stimulate IL-2 production in RCS-responsive CD4^+Vβ16^- T hybridoma cells. RSC stimulation is the positive control.

**DNaseI accessibility to chromatin**

Nuclei prepared from vSac29-transcribing (cRCS-X and cNJ117) and nontranscribing (cNJ101) cells, incubated with DNaseI, were examined for DNaseI accessibility to a region in META env containing the initiation site for 1.8-kb vSac29 mRNA by quantitative PCR using the DNA isolated from the DNaseI-treated nuclei. DNaseI treatment of nuclei isolated from normal SJL liver and splenic B cells, as well as from cNJ101 cells, resulted in partial digestion in the target DNA region, whereas the same site in vSac29^+ (cNJ117 and cRCS-X) nuclei showed significantly increased accessibility to DNaseI (Fig. 7). The ability of DNaseI to digest the chromatin at a locus containing the vSac29 initiation site in META env DNA of cNJ101 cells was significantly increased after drug treatment.

**Role of Ikaros**

Analysis of the META env sequence by a promoter matrix analysis program (http://bimas.dcmr.nih.gov) revealed an Ikaros binding site close to the distal Hgal site. Because the distal Hgal site showed a significant level of demethylation in lymphomas, we performed EMSA to determine whether Ikaros, present in the nuclear and cytoplasmic extracts, could bind to an oligonucleotide sequence containing the distal Hgal and Ikaros binding sites of the META env promoter. EMSA results are shown in Fig. 8. EMSA showed...
two prominent closely migrating bands (Fig. 8, see arrows). Preincubation of nuclear and cytoplasmic extracts with anti-Ikaros Ab prevented binding of Ikaros to labeled probe, as revealed by a decrease in the intensity of bound complexes. Oligonucleotide containing a point mutation at the Ikaros binding site showed a 40–60% reduction (by densitometric analysis) in the binding efficiency of bound complexes in nuclear and cytoplasmic extracts (Fig. 8, A and B). The nuclear and cytoplasmic extracts from cNJ101 cells treated with 5-Aza and TSA showed ~50% reduction in the intensity of bound complexes with probe, as compared with binding in untreated cells. Cytoplasmic extracts from all analyzed samples contained relatively large amounts of Ikaros complexes, relative to nuclear extracts (Fig. 8B).

RT-PCR and Western blotting were used to examine the expression of Ikaros isoforms in normal and lymphoma cells. Fig. 9 shows the Ikaros isoforms (Ik1, 2, 3, 4, and 6) detected in the analyzed samples at transcriptional and translational levels. Although RT-PCR results showed the message for a smaller size Ikaros isoform (Ik6) in lymphoma cells, normal B cells did not show expression of Ik6 (Fig. 9A). Western blot analysis gave similar results (Fig. 9B). Normal SJL B cells expressed larger isoforms (Ik1–4), but not Ik6. In vivo SJL lymphoma cells (RCS-X in vivo) expressed relatively more Ik6 protein than did in vitro SJL lymphoma cell lines. Cytoplasmic extracts from all cells analyzed contained Ik1, 2, 3, and 4 isoforms, but no detectable Ik6 (Fig. 9C).

Discussion

B cell lymphomas of SJL/J mice depend for their growth on help from CD4+ Vβ16+ T cells, which they stimulate by expressing an Mtv29-encoded proviral superantigen (vSAg29) (1, 4). Unlike most other strains of MMTVs, which use promoter elements in the 5′ LTR for superantigen transcription, the Mtv29 employs a promoter, META, in the env gene for the transcriptional initiation of mRNA encoding vSAg29 (2). After splicing of a 1.2-kb intronic sequence, the METAenv-initiated 1.8-kb mRNA transcripts contain a part of noncoding (92 base) sequence from the META env region and a coding sequence for vSAg29 transcribed from the 3′ LTR. In the present investigation, we examined the factors responsible for the regulation of META env-initiated superantigen (1.8-kb) transcripts in SJL/J lymphomas. In view of the lack of META env-initiated vSAg29 transcription in normal B and other somatic cells, we first examined whether DNA methylation of CpG sites in the META env region plays any role in the regulation of vSAg29 transcription. For this purpose, we examined the Mtv29 META env methylation pattern in superantigen-transcribing and nontranscribing cells. We determined the degree of methylation at CpG sites in the methylation-sensitive restriction endonuclease sites (Avai, FspI, Hgal, HpaII, and Thul) by quantitative real-time PCR and also by semiquantitative densitometric analysis of PCR products, using the enzyme-digested DNA as template in the PCR. Our results (Fig. 2, A and B) indicate hypermethylation at all sites analyzed in the META env region of DNA isolated from liver and splenic B lymphocytes of normal SJL/J mice. In primary SJL/J lymphoma cells, we detected a significant level of demethylation at the distal Hgal site, located in the vSAg29 transcription initiation region, but observed no significant change in methylation at any of the other sites examined. Analysis of methylation at CpG sites, not within methylation-sensitive enzyme sites, by Ms-SNuPE also showed more methylation at the three analyzed CpG sites in both normal and lymphoma cells. These results suggest that demethylation of the distal Hgal site may be important for vSAg29 transcription, whereas methylation at the proximal META env region may not directly influence the transcriptional activity of the META env promoter. It has been reported that EBV latency C promoter, methylated in tumor cell lines, requires demethylation at a single CpG site for transcriptional activation (38). DNA from SJL/J lymphoma cell lines (cRCS-X and cNJ117) showed demethylation at more CpG sites (Avai, FspI, Hgal, and HpaII) than did primary lymphomas.
Demethylation at the distal \( HgAI \) site is of interest because this site is located in proximity to an Ikaros binding site. Our results on EMSA indicate the ability of Ikaros to bind to an oligonucleotide probe representing this region in the META \( env \) site. The efficiency of binding was drastically reduced by introducing a point mutation at the Ikaros binding site and by pretreating nuclear and cytoplasmic extracts with an anti-Ikaros Ab. The blot was also analyzed with anti-CDK2 Ab, as a control for protein load.

FIGURE 9. Expression of Ikaros isoforms in SJL lymphomas and normal splenic B cells. vSAg29\textsuperscript{+} lymphoma (cNJ101) cells were treated with 20 nM TSA and 2.5 \( \mu \)M 5-Aza. A, RT-PCR showing the expression of mRNA transcripts of Ikaros isoforms. Expression of housekeeping gene (GAPDH) is shown at the bottom. B, Western blot analysis showing Ikaros isoforms. Nuclear proteins from control cNJ101, drug-treated cNJ101 cells, cRCS-X, RCS-X in vivo, and splenic B cells were analyzed with anti-Ikaros Ab. The blot was also analyzed with anti-CDK2 Ab, as a control for protein load. C, Western blotting of cytoplasmic extract from the above-mentioned samples (B) with anti-Ikaros Ab and anti-CDK2 (as control).

results strengthen our previous observation (4) that GC-derived B cell lymphomas arise in Peyer’s patches, as was originally suggested by Siegler and Rich (40). The Peyer’s patch B cells, which are under chronic antigenic stimulation from the surrounding environment, may accelerate the demethylation as a result of rapid proliferative responses of these cells.

Even though CpG methylation is a property of several silenced genes, the recent view is that methylation is a secondary event targeted to genes that are already made silent by other mechanisms (41). DNA methylation can lead to the stable silencing of genes as a result of interaction of DNA binding proteins, leading to repression of gene expression by promoting condensation of chromatin. Methylated sites on DNA bind 5-methyl cytosine binding protein (42), which exists in a complex with Sin3A and histone deacetylase (43). This complex decreases the level of histone acetylation, resulting in a compact chromatin structure (44). The methylation of cytosine bases of DNA is catalyzed by DNA 5-methyl cytosine transferase (45). This process is reported to be reversible by the activity of DNA demethylase (46). A correlation has been reported between the expression of DNA demethylase and demethylation in the promoter region of the c-erb B2 gene and exon I of the survivin gene in ovarian cancers (47). It is likely that DNA in rapidly proliferating cells is more susceptible to demethylation. During DNA replication, the impairment in the activity of DNA methyl transferase (DNMT1) or its access to methylated sites might contribute to a progressive decrease in methylation.

The lymphoma cell line cNJ101, which does not express META \( env \)-initiated vSAg29, showed hypermethylation and exhibited methylation patterns comparable to that observed for the META \( env \) region in liver DNA. Because the META \( env \) methylation pattern in cNJ101 was significantly different from that of vSAg29 transcribing lymphomas, this cell line was used as a suitable in vitro cell line to study the role of methylation in vSAg29 transcription. Treatment of cNJ101 cells with TSA and 5-Aza caused transcriptional activation of META \( env \) promoter and the transcriptional initiation of 1.8-kb vSAg29. The methylation profile of the drug-treated cNJ101 cells was similar to that observed for cRCS-X and cNJ117. It is interesting to note that the superantigen that was induced by drug treatment stimulated T cell hybridoma cells bearing TCR \( V\beta 16 \). These results are consistent with that of our previous findings demonstrating the ability of vSAg29 to stimulate CD4\textsuperscript{+}V\beta 16\textsuperscript{+} T cells (3).

In addition to methylation, the conformation of chromatin at the locus containing the vSAg29 initiation site appears to play a major role in the transcriptional activity of META promoter. The vSAg29-negative cells showed less accessibility of chromatin at this locus to \( DNase I \) digestion compared with a transcriptionally active locus in cNJ117 and cRCS-X cells. The cNJ101 cells treated with TSA and 5-Aza showed similar effects of increased accessibility to \( DNase I \) digestion at the vSAg29 initiation locus. It is interesting to note that TSA treatment alone was sufficient to induce the expression of 1.8-kb vSAg transcripts in cNJ101 cells. This result suggests that an open chromatin conformation can lead to simultaneous decrease in methylation at the crucial sites and activation of the promoter. Although TSA is not a demethylating agent, it enhances histone acetylation (48, 49) and chromatin remodeling (50), and these changes may be responsible for the observed decrease in methylation. TSA is known to cause selective loss of DNA methylation in \textit{Neurospora} (51). Histone deacetylation plays a role in the maintenance of viral latency, and histone acetylation at the promoter of the immediate early gene, BRLF1, of EBV allows the virus to express Rta and to activate the viral lytic cycle (52). Our results also suggest that inhibition of
histone deacetylase activity by TSA can activate the META env promoter in cNJ101 cells. Both control and drug-treated cNJ101 cells expressed Ik6, as did other Mtv29+ SJL/lymphoma cells analyzed. However, nuclear and cytoplasmic extracts from drug-treated cNJ101 cells revealed a prominent decrease in the ability of Ikaros complexes to bind to oligonucleotide probe (Fig. 8). It is not yet clear whether Ik6 has a direct role in the modulation of transcriptional activity of the META env promoter in cNJ101 cells. Demethylation at the distal Hgal site and chromatin structural changes at the vSAg29 initiation locus could be at least two major factors responsible for induction of vSAg29 META env transcripts by drug-treated cNJ101 cells.

5-Aza can cause genome-wide hypomethylation and is reported to activate and induce the expression of several silenced cellular genes (53–55). Inhibition of DNA methyltransferase by 5-aza-2'-deoxycytidine (5-Aza-CdR), treatment induces the expression of genes transcriptionally down-regulated by de novo methylation in HT29 adenocarcinoma. Indeed, microarray expression analysis of 5-Aza-CdR-treated tumor cells showed induction of STAT1, 2, and 3, which are responsive to IFN-α signaling (54). 5-Aza and 5-Aza-CdR are cytosine analogs that substitute for cytosine during DNA replication and are recognized by DNMT1 on newly synthesized DNA, with covalent trapping of DNMT, leading to its sequestration. Depletion of cellular level of DNMT1 may be responsible for drug-induced DNA hypomethylation (56). It is not clear whether 5-Aza-induced expression of the META env-initiated 1.8-kb vSAg transcript in cNJ101 cells is due to a direct effect of this drug on META env promoter or if it is an indirect effect caused by activation of transcription factors that activate the silenced genes.

Our results suggest that META env-initiated vSAg transcription in lymphoma cells is correlated with dysregulation of Ikaros isoforms, local hypomethylation at the distal Hgal site of META env promoter, and an alteration in chromatin structure at the vSAg29 initiation site. Thus, demethylation and a relaxed chromatin structure at the vSAg29 initiation site are crucial factors responsible for the transcription of vSAg9, an effect that is required for the development of SJL lymphomas.

Acknowledgments

We are most grateful to the late Dr. G. Jeanette Thorbecke for her insightful discussions and suggestions in the conduction of these studies. We are indebted to Dr. S. T. Smale (Howard Hughes Medical Institute, University of California School of Medicine, Los Angeles) for a generous gift of anti-Ikaros Ab. We thank Dr. N. M. Ponzo (University of Medicine and Dentistry of New Jersey, Newark) for cNJ101 cell line. We also thank the late Prof. G. M. Hochwald (Department of Neurology, New York University School of Medicine, New York) for proofreading the manuscript.

References