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DNA Alkylating Agents Alleviate Silencing of Class II Transactivator Gene Expression in L1210 Lymphoma Cells

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MHC class II (Ia) Ag expression is inversely correlated with tumorigenicity and directly correlated with immunogenicity in clones of the mouse L1210 lymphoma (1). Understanding the mechanisms by which class II Ag expression is regulated in L1210 lymphoma may facilitate the development of immunotherapeutic approaches for the treatment of some types of lymphoma and leukemia. This study demonstrates that in MHC class II Ag expression among clones of L1210 lymphoma is due to differences in the expression of the class II transactivator (CIITA). Analysis of stable hybrids suggests that CIITA expression is repressed by a dominant mechanism in class II-negative L1210 clones. DNA-alkylating agents such as ethyl methanesulfonate and the chemotherapeutic drug melphalan activate CIITA and class II expression in class II negative L1210 cells, and this effect appears to be restricted to transformed cell lines derived from the early stages of B cell ontogeny. Transient transfection assays demonstrated that the CIITA type III promoter is active in class II+ L1210 cells, despite the fact that the endogenous gene is not expressed, which suggests that these cells have all of the transacting factors necessary for CIITA transcription. An inverse correlation between methylation of the CIITA transcriptional regulatory region and CIITA expression was observed among L1210 clones. Furthermore, 5-azacytidine treatment activated CIITA expression in class II-negative L1210 cells. Collectively, our results suggest that 1) CIITA gene expression is repressed in class II− L1210 cells by methylation of the CIITA upstream regulatory region, and 2) treatment with DNA-alkylating agents overcomes methylation-based silencing of the CIITA gene in L1210 cells. The Journal of Immunology, 2002, 169: 3085–3093.

The expression of class II Ags of the MHC is regulated in both a developmental and tissue-specific manner. Constitutive MHC class II expression is restricted to professional APCs, which include B cells, dendritic cells, macrophages, and thymic epithelial cells (2). Studies of mouse cell lines derived from transformed B lineage cells suggests that the expression of Ia Ags varies during the course of B cell ontogeny; class II Ags are absent on pro-B cells, and are expressed on immature and mature B cells, but expression is down-regulated as B cells differentiate into plasma cells (3–9). In contrast to APC, fibroblasts and cells of endothelial and epithelial origin do not express class II Ags constitutively, but exposure to IFN-γ induces class II expression (2, 10). Finally, trophoblasts and sensory neurons do not express class II Ags, either constitutively or after exposure to cytokines (11–13).

The developmental and cell-type-specific pattern of class II gene transcription is due to differential expression of a transacting factor termed the class II transactivator (CIITA) (14–19). Expression of CIITA is constitutive in mature B cells and dendritic cells, and is activated by IFN-γ in fibroblasts and other IFN-γ-inducible cells (15–19). The down-regulation of class II expression during differentiation of B cells to plasma cells is correlated with decreased CIITA mRNA (20). Furthermore, the inability of trophoblast cells to express class II genes, even in the presence of IFN-γ, is due to the absence of CIITA (21–22). Transfection of CIITA expression vectors into plasma cells, trophoblasts, and IFN-γ-inducible cells such as HeLa results in constitutive class II gene expression (15–18, 20–22). Thus, CIITA has been called the master regulator of MHC class II transcription (14, 23).

The expression of MHC class II Ags on a series of clones of the L1210 lymphoma is inversely correlated with tumorigenicity and directly correlated with immunogenicity (1). Mice injected with class II-positive L1210 clones mounted CTL- and Ab-based immune responses, and were protected from subsequent challenge with class II-negative clones. Similar results were observed in studies of Sa1 sarcoma and K36.16 lymphoma cells transfected with class II expression vectors (24–26). Subsequent studies in Sa1 cells demonstrated that expression of the costimulatory molecules B7-1 and/or B7-2 in conjunction with class II Ags is critical for enhancement of antitumor immunity (27). These observations led to the proposal that tumors expressing class II Ags may function as APC for tumor-associated Ags, and promote enhanced antitumor immune responses.

Defining the mechanisms by which the expression of class II Ags is regulated in L1210 cells may facilitate the development of strategies for enhancing immune responses against some types of lymphomas and leukemias. In this report, we demonstrate that CIITA gene expression is silenced in class II-nonexpressing L1210 clones, and that CIITA transcription is inversely correlated with methylation of the CIITA upstream regulatory region in L1210 clones. Exposure of class II-negative L1210 cells to DNA alkylating agents (DAAs) alleviates silencing of CIITA gene expression, and this phenomenon appears to be restricted to tumor cell lines derived from the early stages of B cell ontogeny.
Materials and Methods

Cell culture

DBA/2 lymphoma L1210 and its clones (2, 3-3, 4, 5, 6, and 7-15.6) were cultured as previously described (1). The NFS lymphoma lines NFS-1, NFS-5, and NFS-7 (4) and Merwin plasma cell tumor-11 (MPC-11) plasmacytoma cells (28) were purchased from the American Type Culture Collection (Manassas, VA), and grown as suggested by the supplier. The 300-18 cells (29) were a generous gift from Dr. R. Lynch (University of Iowa, Iowa City, Iowa). All other cell lines were grown as previously described (21, 30).

Generation of stable heterokaryons

L1210 clone 3-3 cells resistant to G418 were generated by electroporation with pSV2-neo (31). G418-resistant cells were selected with 400 μg/ml G418 and cloned by limiting dilution. L1210 clone 7-15.6 cells resistant to 8-azaguanine were generated in vitro by growing the cells in gradually increasing concentrations of 8-azaguanine from 0.5 to 8 μg/ml. Cell fusions between G418-resistant 3-3 cells and 8-azaguanine-resistant 7-15.6 cells were performed with polyethylene glycol using a modified version of the procedure described by Oi and Herzenberg (32). Drug selection was initiated 24 h postfusion by adding 200–400 μg/ml G418 and 2–6 μg/ml 8-azaguanine. Three hybrid cell lines, including IAn6, were subsequently established and examined for MHC class II expression.

Treatment with DAAs and 5-azacytidine

Toxicity curves for each of the DAAs and 5-azacytidine were generated for each of the cell lines before examining the effects on gene expression. Based upon these initial studies, cells (2.5–5 × 10^6/ml) were incubated with or without 0.8–4.0 mM ethyl methanesulfonate (EMS) (Sigma-Aldrich, St. Louis, MO), 0.05–0.20 mM methyl methanesulfonate (MMS) (Sigma-Aldrich), or 0.5–2.5 μM melphalan (Sigma-Aldrich) in complete medium for 24 h at 37°C and 5% CO₂, subsequently washed three times with PBS, resuspended in the original volume of fresh complete medium, and cultured for various times thereafter. Cells were continuously treated with 0.5–1.5 μM 5-azacytidine (Sigma-Aldrich) for 1–7 days. Cells were harvested for examination of CIITA and class II expression 1–6 days after initiation of DAA treatment. Exposure of the L1210 cells to the DAAs resulted in a dramatic increase in cell diameter, from an average of 12 ± 1.8 μm before treatment to 18 ± 2.8 μm 3 days after initiation of exposure, which corresponded to an average of 3.4-fold increase in cell volume. The cell viability decreased from 91% at day 1 to an average of 62% at day 5 after EMS treatment, as measured by trypan blue exclusion.

mAbs and membrane fluorescence analysis

Affinity-purified mouse mAbs MK-D6, specific to mouse Iα (33) and 14-4-4S, specific to mouse Iγ (34) were labeled with H-lysozyme-succinimidobiotin (Sigma-Aldrich) as described by Gsedon et al. (35). Purified mouse myeloma proteins RPC-5 (IgG2α, κ) were similarly biotin-labeled and used as an isotype-matched control. mAbs that react with MHC class I Ags Kβ (31-3-4S), Dβ (34-2-12), and Lβ (30-5-7), respectively, were described previously (1). L1210 cells (1 × 10^6) were incubated with biotin-labeled MHC class II mAbs, MK-D6 or 14-4-4S (or biotin-labeled RPC-5 mAbs and membrane fluorescence analysis

Results

Hybridomas between class II-positive and class II-negative L1210 subclones do not express MHC class II Ags

The previous observation that MHC class II Ag expression varies among L1210 clones (1) can be accounted for by at least two different possibilities: 1) class II gene expression is differentially regulated in the class II-positive versus the class II-negative L1210 clones; or 2) mutations in either the class II genes or in genes encoding regulatory proteins that control class II transcription may be responsible for the lack of expression in the class II-negative clones. To distinguish between these possibilities, stable heterokaryons between class II- L1210 clone 3-3 and class II+ clone 7-15.6 were made using polyethylene glycol. L1210 hybridomas were examined for cell surface expression of class II Ags by FACS analysis using the mAbs MK-D6 and 14-4-4S, which are specific for I-A and I-E, respectively (33, 34). As previously observed, clone 7-15.6 expresses high levels of class II Ags (Fig. 1; Ref. 1), while the expression of both I-A and IE Ags was undetectable in clone 3-3. Neither I-A nor I-E Ags were expressed in clone 1A6 (Fig. 1), the stable heterokaryon between clones 7-15.6 and 3-3. Because all of the clones expressed comparable levels of the class I Ags Kα, Dα, and Lα (data not shown),
the absence of class II Ag expression on the hybridomas was not
due to a general loss of cell surface proteins. Identical results for
both class I and II expression were observed for three different
heterokaryons derived from fusion of clones 3-3 and 7-15.6 (data
not shown). If mutations were responsible for the lack of MHC
class II expression in the non-class II expressing clones, one would
expect that the stable heterokaryons would express class II genes
due to complementation by the wild-type (WT) genes from the
class II-positive clones. These studies demonstrate that the class
II− phenotype is dominant over the class II+ phenotype; and there-
fore, suggests that class II gene expression is repressed in class
II-negative L1210 clones.

Treatment of class II-negative L1210 cells with the DAA EMS
results in activation of class II expression

Glimcher et al. (38) previously used chemical mutagenesis of B
cells as a genetic means to identify putative positive regulatory
molecules that control expression of class II Ags. If class II−
L1210 clones contain a repressor factor that functions to suppress
class II Ag expression, treatment of the cells with DAAs might
mutate this factor, resulting in activation of class II gene expres-
sion. To test this hypothesis, L1210 clone 3-3 was treated for 24 h
with various concentrations of EMS, and examined for I-A
expression at several time points thereafter by FACS analysis. Inter-

FIGURE 1. Hybridomas of class II-positive and class II-negative L1210 subclones do not express MHC class II Ags. Stable heterokaryons of class II− clone 3-3 and class II+ clone 7-15.6 were made using polyethylene glycol as described in Materials and Methods, and were established in culture by drug selection. Hybridomas were examined for cell surface expression of class II Ag by FACS analysis using the mAb MK-D6, which is specific for I-A (open histogram). Isotype-matched myeloma proteins served as a negative control (filled histogram). Ab staining on the hybrids was compared with that on class II− clone 3-3 and class II+ clone 7-15.6.

FIGURE 2. Treatment of class II-negative L1210 cells with a DAA results in activation of class II Ag expression and generation of stable class II-positive clones. L1210 clone 3-3 was treated for 24 h with 4 mM EMS, washed, and resuspended in fresh medium lacking the mutagen. A, I-A Ag expression was examined by FACS analysis at various times after the initiation of EMS treatment. mAb MK-D6 was used to stain I-A Ags (open histogram) and isotype-matched myeloma proteins provided a negative control (filled histogram). B, FACS analysis of an L1210 clone derived from clone 3-3 treated with EMS. Five days after the EMS treatment, class II−-cells were subjected to four sequential rounds of purification by FACS and expansion in vitro. Stable class II− clone 54 was subsequently isolated by limiting dilution cloning.

expressed levels of class II that were intermediate between clones
3-3 and 7-15.6, and class II expression has remained stable after
>100 passages in vitro. Thus, EMS treatment of class II− L1210
cells resulted in activation of class II expression, and generation
of stable class II+ clones. Collectively, these results are consistent
with a model in which class II gene expression is repressed in the
class II− L1210 clones, and activated by EMS.

MHC class II gene expression is directly correlated with the
presence of CIITA in clones of L1210

To determine whether there is a relationship between CIITA and
the variability in the presence of class II Ags on the L1210 clones,
CIITA and class II mRNA expression were examined by RT-PCR.
Fig. 3 demonstrates that there is a direct correlation between
CIITA and class II gene expression in the L1210 clones. None of
the class II− tumorigenic L1210 clones (3-3, 4, 5, or 6) or parental
L1210 cells expressed detectable CIITA or Iα mRNA, comparable to

Stable class II-expressing variants of clone 3-3 cells were also
isolated from the EMS-treated cultures (Fig. 2B): these clones (54)
A20 B lymphoma cells. Furthermore, L1210 clone 1A6, one of the stable hybridomas derived from fusion of clones 3-3 and 7-15.6, did not express either CIITA or class II mRNA. Both CIITA and I\(\alpha\)\(\alpha\) were expressed in L1210 clone 54, the stable class II\(^{+}\) clone derived from EMS-treated clone 3-3 cells, although the levels were reduced relative to clones 2 and 7-15.6. L1210 clone 46, which was also isolated from EMS-treated clone 3-3 cells, but subsequently lost cell surface class II Ag expression, does not express either CIITA or class II message. The pattern of I\(\alpha\)\(\alpha\) mRNA expression was identical with that of CIITA and I\(\alpha\)\(\alpha\) in all of the clones (data not shown). The levels of actin mRNA were comparable in all of the clones, demonstrating that the differences in CIITA and class II gene expression were not due to inconsistent sample preparation or RNA degradation (Fig. 3A).

These results suggest that the absence of class II expression in tumorigenic clones 3-3, 4, 5, 6, 46, and 1A6 and in parental L1210 cells is due to the lack of CIITA. To test this hypothesis, class II\(^{-}\) L1210 cells were transfected with the expression vector pSVK-CIITA, which contains the CIITA gene under the control of the SV40 enhancer/promoter, or the control vector pSVK-FLAG. Transfection of L1210 cells with the control plasmid pSVK-FLAG had no effect on class II mRNA expression (Fig. 3B). However, a clear increase in the levels of I\(\alpha\)\(\alpha\) mRNA was observed in L1210 cells transfected with pSVK-CIITA (Fig. 3B). These results suggest that L1210 cells contain all of the transacting factors necessary for class II gene transcription with the exception of CIITA, and ectopic expression of CIITA activates class II mRNA transcription in parental L1210.

**DAAs activate CIITA and class II expression in class II-negative L1210 cells**

To investigate the effects of DAAs on CIITA gene expression in L1210 cells, the kinetics of CIITA and class II mRNA expression were examined at various times after exposure of clone 3-3 to EMS. Fig. 3C demonstrates that both CIITA and I\(\alpha\)\(\alpha\) mRNA are clearly detectable within 48 h after initiation of EMS treatment, and can be observed for up to 6 days. The earliest time point at which I\(\alpha\)\(\alpha\) message was detected was 48 h after the initiation of EMS treatment (Fig. 3C), while CIITA mRNA was observed within 36 h (data not shown). Identical kinetics were observed for the EMS-induced expression of CIITA and I\(\alpha\)\(\alpha\) mRNA in parental L1210 cells, L1210 clone 4, and the 3-3/7-15.6 hybridoma 1A6 (data not shown). These results demonstrate that CIITA mRNA accumulation is coincident with that of class II message in EMS-treated L1210 cells, suggesting that CIITA is responsible for the activation of class II expression by EMS.

To determine whether the EMS-mediated activation of CIITA gene expression in L1210 cells is a general response to DAAs, L1210 cells were exposed to several other DNA alkylating compounds and examined for CIITA mRNA using RT-PCR. CIITA and class II mRNA were also up-regulated in L1210 cells treated with MMS, the chemotherapeutic agent melphalan, and methanesulfonyl chloride (data not shown). The kinetics of induction were similar for all of the agents. Collectively, our studies clearly demonstrate that 1) MHC class II gene expression is differentially regulated among L1210 clones; 2) the variability in class II expression is due to differences in the synthesis of CIITA; 3) CIITA expression is silenced in L1210 clones that lack class II Ags; and 4) DAAs activate CIITA expression in class II-negative L1210 clones.

**The effects of DAAs on CIITA expression are specific to early stages of B cell differentiation**

There is little information available in the literature regarding the specific cell lineage from which L1210 lymphoma was derived.

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**FIGURE 3.** Correlation between CIITA and class II gene expression in clones of L1210 cells. A, RNA was isolated from various L1210 clones and subjected to RT-PCR using primers specific for CIITA, I\(\alpha\)\(\alpha\), and actin. RNA from A20 B lymphoma cells was used as a positive control for CIITA and I\(\alpha\)\(\alpha\) gene expression. Actin mRNA expression was included as a control for sample loading and integrity. B, Transfection of class II-negative L1210 cells with CIITA expression vectors reconstitutes MHC class II expression. L1210 cells were electroporated with the CIITA expression vector pSVK-CIITA, CIITA expression vectors reconstitutes MHC class II expression. L1210 cells were electroporated with the CIITA expression vector pSVK-CIITA or the control vector pSVK-FLAG (L1210), and analyzed for CIITA and I\(\alpha\)\(\alpha\) mRNA expression by RT-PCR. Duplicate samples are shown for each. A20 RNA was used as a positive control for CIITA and I\(\alpha\)\(\alpha\) mRNA expression. C, Kinetics of induction of CIITA and class II gene expression in EMS-treated L1210 clone 3-3 cells. L1210 cells were treated with 4 mM EMS for 24 h, washed, and incubated in fresh medium for various times. RNA was subsequently isolated at the times shown (relative to the start of EMS exposure) and subjected to RT-PCR.
However, our RT-PCR analyses demonstrated that L1210 cells express the mRNAs encoding PAX5/BSAP, Bob-1, A5, CD19, and MB-1 (S. P. Murphy and R. Holtz, unpublished observations), and FACs analysis showed that L1210 cells express the B cell surface markers CD19 and B220 (H. Fuji, unpublished observations). Furthermore, L1210 cells retain the germline configurations of the Ig H and L chain genes (H. Fuji and A. Sood, unpublished observations). Thus, L1210 lymphoma appear to be derived from cells representing an early stage of B cell development such as pro-B cells. To determine whether there are similarities in the regulation of class II expression in L1210 cells and pro-B and pre-B cells, transformed mouse cell lines representing different stages of B cell development were analyzed for the expression of both CIITA and class II mRNA by RT-PCR. NFS-70 cells, which have many of the characteristics of pro-B cells (4), do not express either CIITA or Iaα mRNA (Fig. 4). Similarly, the pre-B cell line NFS-5 (4) expresses very low to undetectable levels of both CIITA and Iα messages. In contrast, NFS-1.0 cells, which represent early B cells, express significant levels of both CIITA and class II (data not shown). Finally, the IgG2b-secreting plasmacytoma cell line MPC-11 (28) does not express either CIITA or Iaα message (Fig. 4). Thus, the levels of CIITA mRNA expression correspond closely with that of class II in mouse-transformed cell lines that represent different stages of B cell differentiation.

To investigate the effects of DAAs on CIITA and class II gene expression in pro-B, pre-B, and plasma cell lines, RNA was isolated from NFS-70 pro-B, NFS-5 pre-B, and MPC-11 plasma cells treated with EMS after 0, 1, 3, and 5 days, respectively, and analyzed by RT-PCR. EMS induced both CIITA and Iaα mRNA expression in NFS-70 pro-B cells (Fig. 4) with kinetics identical to those observed for L1210 cells. Similar results were observed with both NFS-5 pre-B and 300-18 pro-B cells (29), although the induction of CIITA expression was weaker than that observed for L1210 and NFS-70 (data not shown). Interestingly, EMS had no effect on CIITA expression in MPC-11 plasmacytoma cells (Fig. 4), or P3 × 63Ag8 and J558 plasma cells (data not shown), despite the fact that exposure of these cells to the alkylating agent resulted in 2- to 3-fold increases in cell volume, as observed for L1210 cells. These studies suggest that CIITA expression is induced by DAAs in pro- and pre-B cell lines, but not in plasma cell lines.

To further test whether the effects of EMS on CIITA expression are specific to pro-/pre-B cell lines, we extended our analysis to include a variety of other class II-negative cell lines. Among the cell types tested were NIH-3T3 fibroblasts, which express CIITA and class II in response to IFN-γ, and multiple cell lines that do not express CIITA or class II Ags either constitutively, or after exposure to IFN-γ, including mouse EL4 T cells, P19 and F9 embryonal carcinoma cells, Sa1 sarcoma cells, human JEG-3 and JAR choriocarcinoma cells, and K562 erythroleukemia cells. EMS had no effect on CIITA or class II expression in any of the additional cell lines examined (data not shown). Furthermore, neither CIITA nor class II gene expression was induced in JAR, JEG-3, F9, P19, or Sa1 cells treated sequentially with EMS and IFN-γ (data not shown). These data suggest that the effects of DAAs on CIITA expression are restricted to transformed cell lines derived from pro-B and pre-B cells.

**FIGURE 4.** DAAs activate CIITA expression in pro-B cells, but not plasma cells. NFS-70 pro-B and MPC-11 plasmacytoma cells were treated for 24 h with 4 mM EMS, washed, and cultured in fresh medium lacking the DAA for the times shown (relative to the start of EMS treatment). RNA was subsequently isolated and subjected to RT-PCR using primers specific to CIITA, Iaα, and actin.

**FIGURE 5.** CIITA mRNAs are transcribed from the B cell-specific CIITA promoter in class IIs L1210 clones and EMS-treated L1210 cells. RT-PCR analysis was performed using primers specific to the first exon of CIITA type III mRNA, or primers that correspond to sequences that are present at the 3′ end of all CIITA messages (CIITA). Type III CIITA message is clearly expressed in the class IIs L1210 clones 7-15.6 and 54, as well as EMS-treated (5 days) parental L1210 cells (WT), but not in class IIs clone 3-3, or untreated parental L1210 cells (WT).

**Type III CIITA transcripts are expressed in class IIs L1210 and NFS-70 cells**

The regulation of CIITA gene expression is controlled primarily at the level of transcription from at least three promoters that function in a cell-type specific manner (19, 39-42). The type I promoter functions in class II + dendritic cells (19), and the type III promoter is active in B cells (19, 39, 40). IFN-γ-inducible CIITA gene transcription is mediated primarily by the type IV promoter (19, 40-42), although the type III promoter is weakly activated by IFN-γ in select cell types (40, 42). To determine which CIITA promoter is functional in class IIs-expressing L1210 cells, oligonucleotide primers specific for the unique first exons of the mouse CIITA type I, III, and IV transcripts were designed, and used in RT-PCR analysis of RNA from L1210 clones 3-3, 7-15.6, 54, and EMS-treated (5 days) and untreated parental L1210 cells. The results, shown in Fig. 5, demonstrate a precise correlation between the presence of type III-specific CIITA mRNAs in L1210 clones, and RNAs detected using primers that recognize the 3′ end of all CIITA messages. As expected, A20 B cells predominantly express the type III-specific CIITA mRNA (Fig. 5). L1210 clones 7-15.6 and 54 and EMS-treated parental L1210 cells (WT + EMS) also clearly express the B cell-specific CIITA mRNA. In contrast, type III transcripts were not observed in clone 3-3 or untreated parental L1210 cells. CIITA mRNA expression was not detected in any of the L1210 samples using primers specific to the type IV CIITA transcripts, respectively (data not shown). Furthermore, very low levels of CIITA type I transcripts were observed in...
L1210 clone 7-15.6, but not in clone 54 or EMS-treated parental L1210 cells (data not shown). Therefore, CIITA gene transcription is differentially regulated at the type III promoter in L1210 clones, and the activation of CIITA gene expression by EMS occurs via the type III promoter.

**Activity of the CIITA type III promoter in class II-negative L1210 cells in transient transfection assays**

Characterization of the CIITA type III promoter by Ghosh et al. (43) revealed that the sequences between −319 and +1 (relative to the start site of transcription) are sufficient for optimal transcriptional activity in the human B lymphoma line Raji. To study CIITA type III promoter activity in L1210 cells, a DNA fragment spanning the region of the human type III promoter from −322 to +1 was generated by PCR amplification of Raji genomic DNA and cloned into the firefly luciferase vector pGL3-basic (Promega). L1210 cells were transfected with pGL3-basic and pCIITA proIII (322)luc and harvested 48 h later for measurement of luciferase activity. As a control for transfections of plasmid DNA, data are represented as relative luciferase activity from the cotransfected vector pRL-tk (Promega). As shown in Fig. 6, L1210 cells transfected with pCIITA proIII (322)luc expressed an average of 762 U of luciferase activity per microgram protein, which was a 20.3-fold increase over luciferase activity in L1210 cells transfected with pGL3-control, respectively. These results indicate that the sequences of the type III promoter from −322 to +1 are sufficient to direct transcription in L1210 cells at levels comparable to A20 B cells, and suggests that L1210 cells contain the factors necessary for B cell-specific CIITA transcription.

The CIITA upstream regulatory region is methylated in non-class II-expressing L1210 clones

The mechanism responsible for repressing CIITA expression in class II-negative L1210 clones could be epigenetic, such as methylation of the CIITA promoter, and/or insufficient acetylation of histones, or through negative regulatory elements up- or downstream of the 322-bp type III promoter. To investigate the methylation status of the CIITA upstream regulatory region, genomic DNA was isolated from parental L1210, clones 3-3 and 7-15.6, and A20 B cells, and subsequently digested with the methylation-sensitive enzymes HpaII and HhaI, or methylation-insensitive MspI. Restricted DNA was then subjected to PCR using primers that span regions of the CIITA upstream regulatory region containing the methylation-sensitive enzyme recognition sites (Fig. 7A). PCR products were only detected in these assays when either HpaII or HhaI cleaved genomic DNA from either A20 or L1210 clone 7-15.6 were subjected to PCR using primers spanning the 2.0-kb region encompassing both the type III and IV promoters. Identical results were observed when the type IV promoter region alone was examined. In contrast, PCR products were clearly detected from the DNA samples of parental L1210 and L1210 clone 3-3 cells digested with HpaII or HhaI, indicating that these enzymes did not cut. In addition, the pattern of DNA methylation of the CIITA upstream regulatory region in non-class II-expressing L1210 clones is differentially regulated at the type III promoter in L1210 clones, and the activation of CIITA gene expression by EMS occurs via the type III promoter.

**FIGURE 6.** Activity of the CIITA type III promoter in L1210 cells in reporter gene assays. L1210 cells were transfected with either pCIITA proIII (322)luc, which contains 322 bp of the CIITA type III promoter (−322 to +1 relative to the transcriptional start site) upstream of the firefly luciferase gene, or the promoterless plasmid pGL3-basic (Promega). Transfection assays were repeated four times in duplicate using two independent preparations of plasmid DNA. Data are represented as relative luciferase units per microgram of protein normalized to Renilla luciferase.

**FIGURE 7.** The CIITA upstream regulatory region is methylated in class II-negative L1210 cells. A, Schematic of the murine CIITA upstream regulatory region. The start sites of transcription for the type III and IV promoters are denoted by large filled arrows. The sites that are complementary to the primers (5′-III, 3′-III, 5′-IV, 3′-IV) used in PCR of genomic DNA are indicated on the upper schematic. The lower schematic shows the positions of the HpaI/MspI and HhaI restriction sites, respectively. B, Genomic DNA was isolated from A20, L1210 clones 3-3 and 7-15.6, and parental L1210 cells, digested with restriction enzymes, and subjected to PCR using CIITA promoter-specific primers. Lanes correspond to DNA cleaved with 1) SstI alone; 2) SstI and HhaI; 3) SstI and HpaII; and 4) SstI and MspI.
upstream regulatory region in L1210 clone 1A6, which was derived by fusion of clones 3-3 and 7-15.6, was identical with that of clone 3-3 (data not shown). PCR bands were not observed from any of the DNA samples cleaved with MspI. As a control for DNA integrity and quantity, all samples were subjected to PCR with primers spanning the type III promoter from -322 to +1 relative to the transcriptional start site, which does not contain any HpaII, HhaI, or MspI sites. Comparable levels of PCR products were observed from all of the samples, indicating that the DNA was intact. Based on these results, we conclude that the HpaII and HhaI sites immediately downstream of the transcriptional start site of the type III promoter, as well as the HhaI site in the type IV promoter, are methylated in L1210 clones 3-3 and 1A6, and parental L1210 cells, but not L1210 clone 7-15.6 or A20.

To determine whether methylation of the CIITA upstream regulatory region plays a role in repression of transcription in L1210 lymphoma, parental L1210 cells were treated with 5-azacytidine (0.5–1.5 μM) for various periods of time, and subsequently analyzed for CIITA and IAα mRNA expression by RT-PCR. CIITA mRNA expression was detected within 2 days after initiation of treatment, and remained at comparable levels for up to 7 days; IAα expression was coincident with that of CIITA (Fig. 8). Identical results were observed with 300-18 pro-B cells and L1210 clone 3-3; however, 5-azacytidine had no effect on CIITA mRNA expression in J558 plasmacytoma cells (data not shown). Lastly, the extent of methylation of the CIITA promoter region was reduced in parental L1210 cells treated with 5-azacytidine (data not shown). Collectively, our studies strongly suggest that methylation of the CIITA locus plays a central role in silencing transcription in class II-negative L1210 lymphoma clones.

Discussion
Silencing of CIITA transcription in pro-B and L1210 cells by methylation of the CIITA promoter
In this study, we demonstrate that the variation in MHC class II transcription observed among clones of L1210 lymphoma is accounted for by differential expression of CIITA. An inverse correlation between CIITA mRNA expression and methylation of the CIITA upstream regulatory region was observed among the L1210 clones, which suggests that silencing of CIITA transcription (and therefore, class II) in class II-negative L1210 clones is mediated by methylation of the CIITA type III promoter. Furthermore, DAAs activate CIITA transcription in class II-negative L1210 clones by a mechanism which is restricted to tumor lines derived from the early stages of B cell development.

Examination of MHC class II expression in transformed murine cell lines derived from different stages of B cell ontogeny suggests that class II is differentially regulated during mouse B cell development: class II Ags are absent on pro-B and pre-B cell lines, and are expressed on immature and mature B cells, but expression is down-regulated during differentiation into plasma cells (3–9). We observed methylation of the CIITA upstream regulatory region in multiple different class II-negative pro-B and pre-B cell lines (data not shown), which is consistent with our proposal that methylation of the CIITA promoter plays a central role in repressing CIITA, and therefore, class II transcription in these cells. In contrast to transformed cell lines, pre-B cells isolated from mouse bone marrow express cell surface class II Ags (36, 37). However, distinct differences in class II expression have been described in murine pro-B and pre-B cells isolated from fetal liver compared with adult bone marrow: whereas bone marrow-derived pro-B cells expressed very low levels of class II mRNA and protein, both pro-B and pre-B cells isolated from fetal liver lacked class II RNA and protein (44, 45). Thus, it is currently unclear whether the methylation-based silencing of CIITA gene transcription observed in L1210 lymphoma and other pro-B/pre-B cell lines, and the activation of CIITA by DAAs, are a reflection of a normal regulatory pathway that functions during B cell development, or whether they reflect changes that occurred during transformation or adaptation of the cells to tissue culture.

CIITA is silenced in stable heterokaryons between CIITA-positive and CIITA-negative L1210 clones (Figs. 1 and 3A), as well as stable hybrids of B cells and plasma cells (46), indicating that the repressor phenotype is dominant. However, our work suggests that the mechanism by which CIITA is silenced in L1210 lymphoma/pro-B cells is distinct from that observed in plasma cells. Recent studies by Piskurich et al. (47) and Ghosh et al. (48) suggest that CIITA transcription in plasma cells is silenced by BLIMP-1 (also known as PRD1-BF1). In contrast to plasma cells, our RT-PCR assays indicate that BLIMP-1 mRNA is not expressed in parental L1210 cells (data not shown). Moreover, exposure of J558 plasmacytoma cells to the histone deacetylase inhibitor trichostatin A activates CIITA expression (49). Conversely, trichostatin A has no effect on CIITA expression in L1210 cells (data not shown). Lastly, both DAAs and 5-azacytidine activate CIITA mRNA expression in L1210 and pro-B cell lines, but not in J558, MPC-11, or P3 × 63Ag8 plasma cells. Thus, although silencing of CIITA transcription appears to have an epigenetic basis in both L1210 lymphoma and plasma cells, the specific mechanisms differ.

Methylation of the CIITA type IV promoter also plays a role in repressing IFN-γ-inducible CIITA transcription in human chorioncarcinoma cells (50, 51) as well as other developmental tumor cell lines such as erythroleukemias and retinoblastomas (52). The CIITA type IV promoter is methylated in JAR and JEG-3 chorioncarcinoma cells, and sequential treatment with 5-azacytidine and IFN-γ resulted in low levels of CIITA mRNA expression (50, 51). In vivo footprinting indicated that the CIITA type IV promoter is unoccupied in IFN-γ-treated human JAR and JEG-3 cells (50), and chromatin immunoprecipitation assays demonstrated a lack of both factor assembly and histone acetylation after IFN-γ treatment (53), which are consistent with multiple studies demonstrating that the chromatin of methylated DNA is in a “closed” state, and effectively prevents binding of the transacting factors necessary for transcription (54). Methylation may function in a similar capacity at the CIITA type III promoter in L1210 cells, because the constitutive activity of the type III promoter in these cells in transient transfection assays suggests that L1210 cells contain the transacting factors necessary for transcriptional activation.
Collectively, our data indicate that DAAs alleviate methylation-mediated silencing of CIITA transcription in L1210 lymphoma cells. However, the precise mechanism involved is not known. The fact that a significant percentage (30–35% by day 5) of the L1210 cells exposed to EMS are activated to express class II Ags makes it unlikely that mutations are responsible for the activation of CIITA gene expression. Kinetic analysis demonstrates that CIITA mRNA is not observed until 36–48 h after initiation of treatment, suggesting that DAA-mediated activation of CIITA transcription may consist of a number of steps, including alterations of chromatin, and/or that the effects of DAA on CIITA transcription are indirect. One possible mechanism is that the DNA damage that results from exposure to DAAs directly cause changes in chromatin conformation at the CIITA locus that allow the requisite transcription factors to gain access to their respective binding sites on the CIITA type III promoter. A related possibility is that repair of DNA damage “opens” the chromatin, resulting in enhanced factor access. Alternatively, DAAs may alter the degree of CIITA promoter methylation, either directly or by affecting the expression or activity of the methyltransferase enzymes. However, this appears unlikely, because no changes in methylation of the CIITA upstream regulatory region were detected in preliminary studies in which class II-positive L1210 cells were isolated from DAA-treated cultures using class II-Ah-coated magnetic beads (data not shown).

In contrast to the possibilities outlined above, DAAs may also activate CIITA expression by more indirect mechanisms, such as by inducing the expression of cytokines, which subsequently activate CIITA synthesis. Treatment of primary human monocytes with EMS, MMS, or mitomycin C has been shown to enhance expression and secretion of IL-1α, IL-1β, and IL-6 (55). A related possibility is that alkylating agents induce differentiation of L1210 cells/pro-B cells. Several studies have demonstrated a differentiation-inducing capacity of alkylating agents on different cell types, including mouse neuroblastoma cells (56), human U-937 cells/pro-B cells. Several studies have demonstrated a differentiation-inducing capacity of alkylating agents on different cell types, including mouse neuroblastoma cells (56), human U-937 cells/pro-B cells. Several studies have demonstrated a differentiation-inducing capacity of alkylating agents on different cell types, including mouse neuroblastoma cells (56), human U-937 cells/pro-B cells. These statements are based on data from the studies cited.

Role of CIITA in DAA-induced increases in immunogenicity of tumor cells

Multiple studies have demonstrated that low-dose chemotherapeutics enhance the immunogenicity of certain types of mouse lymphoma lines, including L1210, and that following treatment these cells are efficiently cleared by the host immune system (60–62). B7-I (CD80) is expressed in MOPC315 and P815 cells exposed to low-dose melphalan, as well as in splenic lymphocytes, and expression of these costimulatory molecules plays a crucial role in enhancing tumor immunity in these systems (63–65). Similarly, DAAs may confer upon L1210 lymphoma the ability to act as APCs for their own tumor-associated Ags via up-regulation of MHC class II and costimulatory molecules. Thus, L1210 lymphoma provides a novel model system for determining the mechanisms by which chemotherapeutic agents enhance the immunogenicity of lymphomas.

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