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Histone deacetylase inhibitor Trichostatin A reduces anti-DNA autoantibody production and represses IgH gene transcription

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Abstract

Systemic lupus erythematosus is characterized by the presence of autoantibodies and hypergammaglobulinemia. To investigate the role of histone deacetylases (HDACs) in the production of autoantibody and immunoglobulin, we examined the effect of Trichostatin A (TSA), a specific inhibitor of HDACs, on anti-DNA autoantibody production and IgH gene transcription. Our results showed that inhibition of HDAC activity by TSA markedly reduced anti-DNA autoantibody production by T347 cells either by inducing apoptosis or in an apoptosis-independent manner, suggesting that TSA might be useful for treating certain autoimmune diseases. Moreover, we found that TSA strongly inhibited germline and post-switch immunoglobulin transcripts in T347 cells and in primary splenic B cells of MRL-lpr mice. Reporter gene analysis demonstrated that both Eμ and 3′-IgH enhancer activities were repressed significantly by TSA-mediated HDAC inhibition. Furthermore, we observed that HDAC1 was recruited to the 3′-IgH enhancer hs1,2 as determined by chromatin immunoprecipitation assays. Over-expression of HDAC1 increased the activity of IgH enhancers, especially 3′-IgH enhancers. These findings implicate HDAC in the IgH gene transcription via activation of 3′-IgH enhancers.

Keywords: Autoantibody; IgH gene transcription; Trichostatin A; Histone acetylation; Histone deacetylases; IgH promoter; IgH intronic enhancer (Eμ); 3′-IgH enhancers

In the nucleus, 146 bp of DNA are wrapped around the histone octamer to form a nucleosome core. Histone modifications such as acetylation and methylation play an important role in the activation or silencing of gene transcription [1]. Histone acetylation is regulated by HDACs and histone acetyltransferases (HATs). Mammalian HDACs are grouped into three classes, according to homology with their yeast counterparts. Of these three classes, class I HDAC encompasses HDAC1–3, 8, and 11, while class II HDAC contains HDAC4–7, 9, and 10 [2]. HATs activate genes by altering chromatin configuration and facilitating transcription factor access, while HDACs have the opposite effect, mediating transcriptional repression [3]. TSA is a potent and specific inhibitor of histone deacetylase that acts by binding directly to its catalytic site. Both class I and class II HDACs are sensitive to inhibition by TSA [4]. Furthermore, TSA has been shown to modulate transcriptional activity, thereby affecting a broad variety of cellular processes, such as cell cycle, differentiation, and apoptosis [5,6]. Histone acetylation is

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emerging as a central theme in the regulation of gene expression in several diseases [7,8]. Accumulated evidence indicates that histone acetylation plays an important role in the development of cancer, and TSA has potent anti-tumor activity in vitro and in vivo [9,10].

The formation of a functional immunoglobulin heavy chain (IgH) gene involves class switch recombination (CSR) and somatic hypermutation (SHM) in secondary lymphoid organs following rearrangement of V, D, and J gene segments in bone marrow. Histone acetylation has been proposed to regulate VDJ recombination by modifying the accessibility of the target locus to recombination activating gene (RAG) proteins in an enhancer-dependent manner [11]. Recently, histone acetylation of the IgH switch region and variable region has been associated with CSR and SHM, respectively [12,13]. These results largely advance our understanding of the molecular mechanism of antibody gene assembly. However, the role of HDACs in IgH gene transcription remains unclear. The development of anti-DNA autoantibody results in systemic autoimmunity and is a hallmark of mouse and human lupus. It is not known whether HDACs play a role in the production of anti-DNA antibody. Therefore, we investigated the effect of HDACs on autoantibody production and IgH gene transcription. Inhibition of HDAC activity by TSA markedly reduced anti-DNA autoantibody production in T347 cells in a dose-dependent manner. Such reduction was associated with TSA-induced apoptosis. Meanwhile, TSA also significantly reduced autoantibody production in a T347 cell clone that is insensitive to TSA-induced apoptosis. These results suggest a potential application of TSA for treating autoimmune diseases. Moreover, we found that TSA represses germline or post-switch transcription of *γ2a* in T347 cells and *γ1*, *γ2a*, and *μ* in primary splenic B cells of MRL-lpr mice. Reporter gene analysis showed that the activities of the IgH intronic enhancer (Eμ) and 3′-IgH enhancers (hs1,2, hs3A, hs3B, and hs4) were significantly decreased by TSA treatment. Transient expression of HDAC1 increased the activity of IgH enhancers, especially 3′-IgH enhancers. Furthermore, chromatin immunoprecipitation experiments showed that HDAC1 is recruited to the hs1,2 locus of 3′-IgH enhancers. These results indicate a role for HDAC1 in 3′-IgH enhancer activation and implicate HDAC in the regulation of IgH gene transcription.

Materials and methods

Chemical agents. TSA (Sigma–Aldrich) was dissolved in dimethyl sulfoxide (DMSO), and stored at −80 °C until use.

Cell line, mice, and cell culture. The T347 cell line (provided by Dr. Davidson A, Albert Einstein College of Medicine) is a hybridoma that produces IgG2a anti-dsDNA autoantibodies. MRL-lpr mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions. Cells were cultured in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin, and incubated at 37 °C with 5% CO_2.

Autoantibody detection. IgG2a anti-dsDNA autoantibody was measured by ELISA using plates pre-coated with salmon sperm dsDNA (Sigma) at a concentration of 100 μg/ml in PBS. Plates were then blocked with 3% FCS in PBS for 1 h at 37 °C and incubated with supernatant at a 1/2000 dilution for 2 h at room temperature. Plates were washed three times with PBS–0.05% Tween 20, followed by incubation with HRP-conjugated goat anti-mouse IgG2a (Southern Biotechnology Associates) diluted 1/2000 in PBS containing 3% FCS for 1 h at 37 °C. The ELISA was developed by adding the HRP substrate (Southern Biotechnology Associates), and the OD values were measured at 405 nm using an ELISA Reader.

Apoptosis assay. Apoptosis was assessed by flow cytometry. Briefly, 1×10^6 cells were treated with TSA at indicated concentrations for 12 h. Staining of cells with Annexin V-Cy5 (BD), which binds to cell surface phosphatidylserine, was performed according to the manufacturer's instructions and analyzed using a FACScalibur flow cytometer (Becton–Dickinson).

Immunoglobulin transcript detection. Total cellular RNA was extracted from 2×10^6 cells with TRIzol (Gibco). One microgram of RNA was reverse transcribed using the Promega First-Strand cDNA Synthesis Kit. The thermal cycler protocol used was 95 °C for 2 min, 35 cycles of 95 °C for 30 s plus 60 °C for 40 s and 72 °C 1 min, and a final extension step of 8 min at 72 °C. Amplification of germline (*γ1*, *γ2a*, and *μ*) or post-switch transcripts (*γ1*, *γ2a*) was performed as described [14]. The following primers were used:

p21: 5′-CGGTGGAACTTTGACTTCGT-3′,
5′-CACAGAGTGAGGGCTAAGGC-3′;
β-actin: 5′-CGTGAAAAGATGACCCAGATCA-3′,
5′-TGGTACGACCAGAGGCATACAG-3′.

Transient transfection and luciferase assay. T347 cells (5×10^6) were electroporated with either the V_H promoter (V_H luc) alone, the V_H promoter in combination with the intronic enhancer Eμ(V_H luc Eμ), or the V_H promoter combined with a group of four IgH enhancer elements (V_H luc 3A 1,2 3B 4) (provided by Dr. R.G. Roeder, Rockefeller University) [15]. After 24 h, TSA was added to the transfected cells for 6 h. To study the role of HDAC1 in IgH promoter and enhancer activity, the pBJ-HDAC1 expression plasmid (provided by Dr. S.L. Schreiber, Harvard university) was co-transfected with either V_H luc, or V_H luc Eμ or V_H luc 3A 1, 2 3B 4. After 30 h, cells were lysed and luciferase activity was measured using the Promega dual-luciferase assay system.

Chromatin immunoprecipitation assay. Primary splenic B (1×10^7) cells were used for each chromatin immunoprecipitation (ChIP) assay. Formaldehyde was added at a final concentration of 1% to the cells to cross-link protein and DNA, and cells were incubated for 10 min at room temperature. Glycine was then added to a final concentration of 0.125 M to stop the reaction. After removal of the medium, cells were washed with 1 ml ice-cold phosphate-buffered saline. Cells were resuspended in 200 μl SDS lysis buffer containing a cocktail of protease inhibitors (Sigma) and incubated for 10 min on ice. The cell lysates were sonicated (five times for 10 s each) to break up the chromatin into 200–1500 bp fragments, and debris was removed by centrifugation for 10 min at 13,000 rpm at 4 °C. Supernatant fractions were transferred to a new Eppendorf tube and diluted 10-fold in ChIP dilution buffer (Upstate Biotechnology) containing protease inhibitors and 1/20 of each chromatin solution was kept as input. To reduce nonspecific background, the rest of the chromatin solutions were pre-cleared with 60 μl of salmon sperm DNA–protein A–agarose (Upstate Biotechnology) for 1 h at 4 °C with agitation. Five micrograms of rabbit anti-HDAC1 Ab (Cell Signaling Technology) or rabbit IgG (Dingquo Biotechnology) was added to the chromatin solution, and the samples

were incubated overnight at 4 °C with rotation. Immunocomplexes were precipitated using 60 μl salmon sperm DNA–protein A–agarose for 2 h at 4 °C with rotation. The pellets were washed sequentially with low salt wash buffer, high salt wash buffer, LiCl wash buffer, and TE buffer (twice) (Upstate Biotechnology). Bound DNA–protein complexes were eluted using 250 μl of 1% SDS in 0.1 M $NaHCO_3$. Twenty microliters of 5 M NaCl was added to the eluates and inputs, and protein–DNA was reverse cross-linked at 65 °C for 4 h. After addition of 10 μl of 0.5 M EDTA, 20 μl of 1 M Tris–HCl (pH 6.5), and 2 μl of a 10 mg/ml solution of proteinase K, samples were incubated for 1 h at 45 °C and extracted with phenol–chloroform. DNA was precipitated using ethanol and 20 μg glycogen as carrier. Primers for PCR amplification of the hs1,2 and hs4 enhancer DNA were ATT TTC CTT CGG TTT AGG GTG G (forward)/GGG AGT CAC TGA TGC TAT TTC (reverse) and AGA ACA GGA ACC ACA GAG CAG AGG (forward)/GGT CAT TGA AAC TCA TCC ATA GCC (reverse), respectively. The hs1,2 downstream region from 1705 to 1929 (GenBank No. X96607) was amplified as a negative control with the primer pair TCG CAG GCA ATG AAG AAT AA and ACA GGG CTT GAT GTT GGT GA. PCR was performed for 35 cycles (95 °C for 45 s, 56 °C for 30 s, and 72 °C for 45 s), and the products were run on 2% agarose gels.

Results

TSA reduces anti-dsDNA autoantibody production

In order to investigate the role of HDACs in autoantibody production, we treated T347 cells with various concentrations of TSA. We found that TSA markedly reduced IgG2a anti-dsDNA secretion by T347 cells at 12 and 24 h culture (Fig. 1A). Addition of TSA resulted in a dose-dependent decrease in anti-dsDNA autoantibody. TSA (100 ng/ml) treatment caused a 2.7- or 3.6-fold decrease of the autoantibody production after 12 or 24 h treatment, respectively (Fig. 1A). To study the mechanism of decreased autoantibody production by TSA, T347 cells were incubated with TSA and analyzed by flow cytometry following staining for Annexin V. As shown in Fig. 1B, TSA induced apoptosis of T347 cells in a dose-dependent manner. TSA at 50 ng/ml caused 94% of T347 cells to undergo apoptosis after 12 h of treatment. To study the role of TSA further in autoantibody production, we selected a T347 cell clone that is insensitive to TSA-induced apoptosis (Fig. 2A). TSA also decreased anti-dsDNA antibody production in this cell line (Fig. 2B). These results indicate that TSA inhibits autoantibody production either by inducing apoptosis or via an apoptosis-independent pathway.

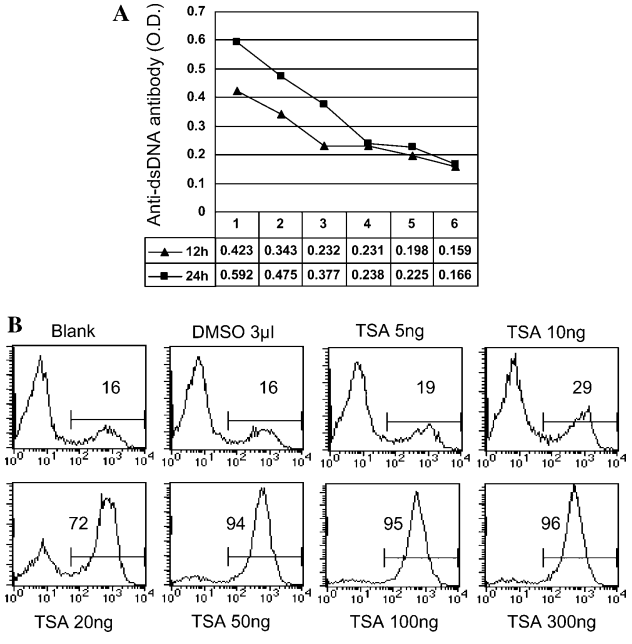

Fig. 1. TSA decreases anti-dsDNA autoantibody production by inducing apoptosis in T347 cells. (A) T347 cells (5×10^5/ml) were cultured in absence or presence of TSA (1, DMSO 0.6 μl; 2, TSA 5 ng; 3, TSA 10 ng; 4, TSA 20 ng; 5, TSA 50 ng; 6, TSA 100 ng). Supernatants were collected at 12 or 24 h for detection of autoantibody by ELISA. (B) T347 cells (1×10^6) were cultured with or without TSA as indicated for 12 h. Apoptotic cells were stained by Annexin V-Cy5 and analyzed on flow cytometer. Percentage of apoptotic cells is given in histogram.

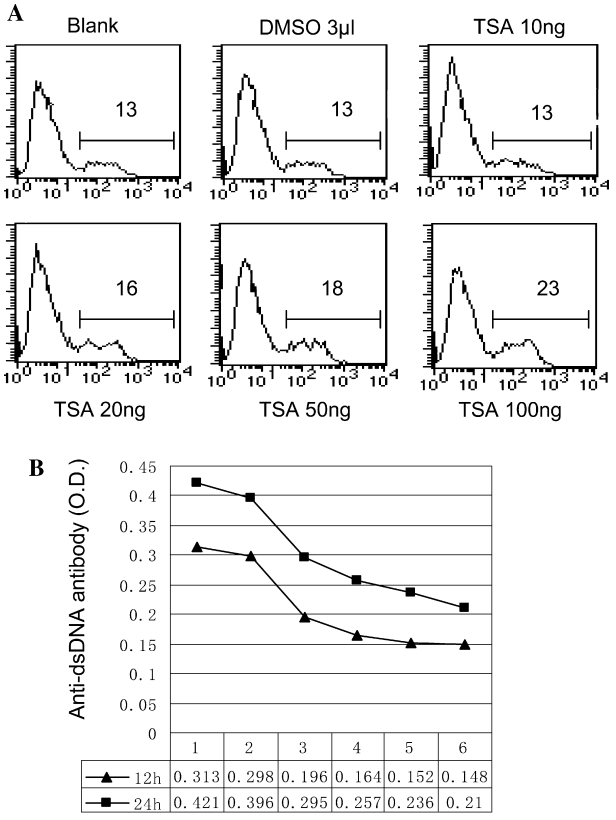

Fig. 2. TSA decreases anti-dsDNA production in a T347 cell clone that is insensitive to TSA-induced apoptosis. (A) T347 cells (1×10^6) were incubated with or without TSA as indicated for 12 h. Apoptotic cells were analyzed by flow cytometry via staining with Annexin V-Cy5. Percentage of apoptotic cells is shown in histogram. (B) T347 cells (1×10^6/ml) were treated with or without TSA: 1, DMSO 0.6 μl; 2, TSA 5 ng; 3, TSA 10 ng; 4, TSA 20 ng; 5, TSA 50 ng; 6, TSA 100 ng. Supernatants were collected at 12 or 24 h for detection of autoantibody by ELISA.

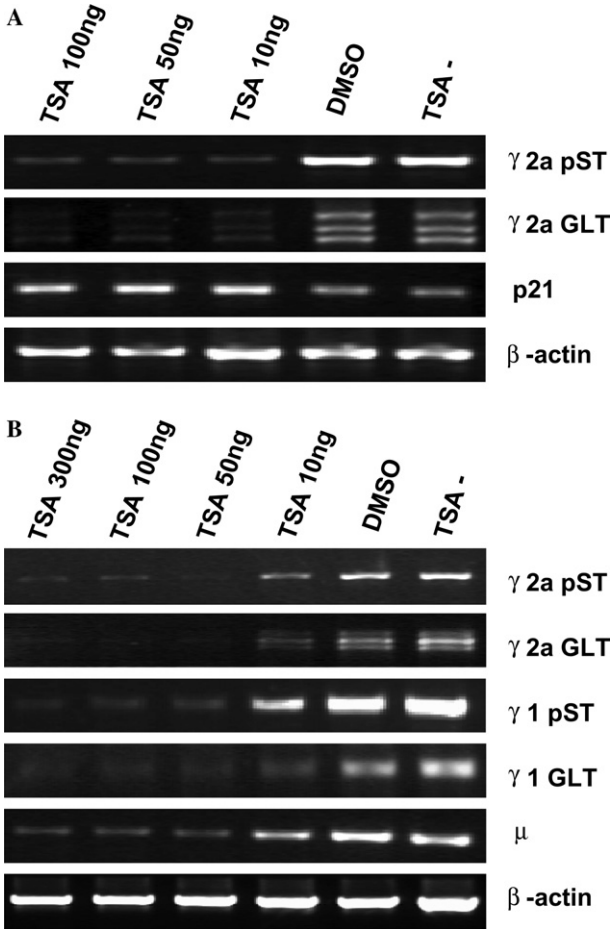

Fig. 3. TSA inhibits the production of immunoglobulin transcripts. Cells (1×10^6) from T347 cell line (A) or splenic cells of MRL-lpr mice (B) were treated with or without TSA as indicated for 6 h. Germline or switched transcripts of *γ2a*, *γ1* or *μ* and p21 mRNA were detected by RT-PCR. β-Actin was used as control.

TSA inhibits IgH transcripts in T347 cells and primary splenic B cells

In order to determine whether inhibition of HDAC activity affects immunoglobulin gene transcription, we measured the expression of *γ2a* transcript in T347 cells treated with TSA. As shown in Fig. 3A, TSA dramatically inhibited the germline and switched *γ2a* transcripts after 6 h of treatment. Although p21 gene expression was up-regulated at that time, apoptosis of the cells was not induced (data not shown). To further define the effect of TSA on IgH transcripts, we treated primary splenic B cells from MRL-lpr mice with TSA. Our data showed marked inhibition of both germline *γ1*, *γ2a*, *μ*, and post-switch *γ1*, *γ2a* transcription (Fig. 3B). Interestingly, even low-dose TSA (e.g., 10 ng) effectively reduced the amount of these transcripts in T347 cells and in primary B cells (Figs. 3A and B). These results indicate that inhibition of HDAC activity can repress IgH gene transcription.

TSA decreases and HDAC1 increases the activities of IgH enhancers

IgH transcription is tightly regulated by IgH promoters and enhancers. Therefore, we investigated the effect of TSA on the activity of IgH regulatory elements. T347 cells were transfected with either the V_H promoter alone, the V_H promoter in combination with the intronic enhancer Eμ, or the V_H promoter combined with the group of 3′-IgH enhancer elements (3A 1,2 3B 4). The addition of TSA slightly inhibited IgH promoter activity (Fig. 4). However, TSA markedly repressed the activity of the intronic and 3′-IgH enhancers. Treatment with 20 or 100 ng/ml TSA resulted in a 2.1- or 3.2-fold decrease

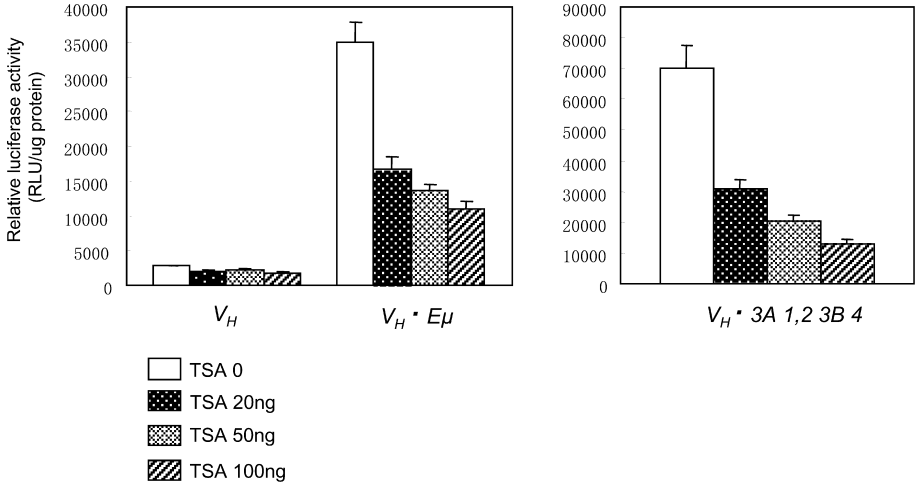

Fig. 4. TSA represses the activities of Ig heavy chain enhancers. T347 cells (5×10^6) were transfected with V_H luc, V_H luc Eμ or V_H luc 3A 1,2 3B 4. After 24 h transfection, TSA was added as indicated. Luciferase activity was measured after 6 h treatment of TSA using Promega dual-luciferase assay kit. Results are means of three independent experiments ± SE.

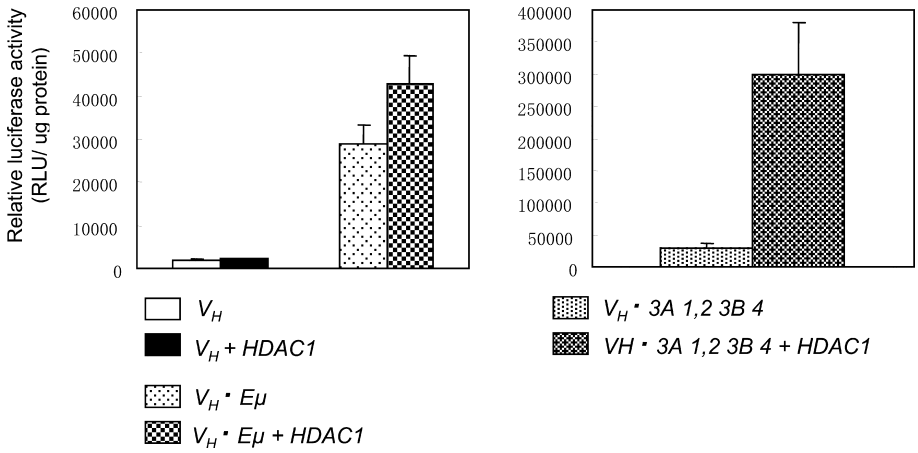

Fig. 5. HDAC1 increases the activities of Ig heavy chain enhancers. HDAC1 expression plasmid was co-transfected with either V_H luc, V_H luc Eμ, or V_H luc 3A 1,2 3B 4 as indicated. After 24 h, luciferase activity was measured. The mean ± SE of three replicates are shown.

in Eμ and a 2.3- or 5.4-fold in 3A 1, 2 3B 4 activity, respectively.

Next, we examined the role of HDAC1 in the activity of IgH promoter and enhancers. The HDAC1 expression plasmid was co-transfected with V_H luc, V_H luc Eμ or V_H luc 3A 1,2 3B 4 in T347 cells. As shown in Fig. 5, transient expression of HDAC1 had no apparent effect on the IgH promoter. However, HDAC1 expression significantly increased the activity of the Eμ enhancer. Strikingly, over-expression of HDAC1 led to a 9.9-fold increase in 3′-IgH enhancer activity. These results indicated that inhibition of HDAC activity by TSA represses the intronic and 3′-IgH enhancers, while HDAC1 promotes the activity of IgH enhancers, especially 3′-IgH enhancers.

HDAC1 is recruited to 3′-IgH enhancer hs1,2

Since HDAC1 over-expression strongly activates the 3′-IgH enhancers, we hypothesized that HDAC1 might be recruited to the loci. Therefore, we performed chromatin immunoprecipitation assays to investigate whether HDAC1 is associated with 3′-IgH enhancers. After cross-linking of chromatin and protein in primary splenic B cells, chromatin was co-precipitated with anti-HDAC1 antibody. Of the four 3′-IgH enhancers, hs1,2 and hs4 appear to have the strongest enhancer activity [15]. Therefore, we performed PCR using primers that amplify the enhancer region of hs1,2 and hs4. The results of the ChIP assays showed that endogenous HDAC1 is bound to the hs1,2 enhancer, but not the hs1,2 downstream region (1705–1929) (Fig. 6). We did not detect any band corresponding to hs4 in chromatin immunoprecipitates using several primer pairs in three independent ChIP experiments (Fig. 6 and data not shown). It would be interesting to evaluate further whether HDAC1 is selectively recruited to some of 3′-IgH enhancers.

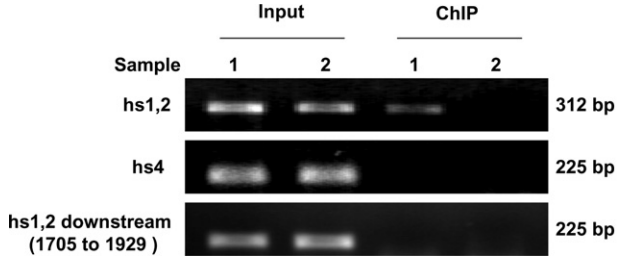

Fig. 6. HDAC1 is recruited to the hs1,2 locus of 3′-IgH enhancers. Cross-linked chromatin prepared from purified splenic B cells was precipitated with rabbit anti-HDAC1 antibody (sample 1) or rabbit IgG (sample 2). PCRs were carried out using specific primers to the hs1,2, hs4 or hs1,2 downstream region. Representative results from three independent experiments are shown.

Discussion

In this study, we used the T347 cell line, which produces anti-DNA autoantibody, to investigate the effect of HDACs on autoantibody production. We observed that TSA markedly reduced the production of IgG2a anti-dsDNA autoantibody in a dose-dependent manner by inhibiting the activity of class I and II HDACs. Similar to the effect of TSA on many tumor cell lines [16,17], TSA treatment induced apoptosis of the hybridoma lines T347and J70 (the latter not shown). Thus, apoptosis of T347 cells induced by TSA could be responsible for the decreased production of anti-DNA autoantibody observed. Based on the observation that primary B cells are not sensitive to TSA-induced apoptosis, we selected a T347 cell clone that was insensitive to TSA-induced apoptosis and found that TSA treatment significantly decreased anti-DNA antibody production in this cell line. These results provide useful information for application of TSA in treating certain autoimmune diseases.

Histone acetylation has been associated with VDJ recombination, CSR and SHM [11–13]. However, the role of HDACs in IgH gene transcription is unclear. In

this study, we found that inhibition of HDACs by TSA markedly inhibited germline or post-switch transcription of *γ1*, *γ2a*, or *μ*. These results indicated that normal transcription of *γ1*, *γ2a*, and *μ* is at least partially dependent on HDAC activity. Consistent with our results, Lee et al. [18] reported that TSA and butyrate treatment lead to decreased surface IgM expression in the mature B lymphoma cell line L10A. IgH gene transcription is controlled by two distinct classes of cis-acting elements, promoters located 5′ of the individual variable (V_H) gene segments and enhancer elements located either in the intron or 3′ end of the Ig HC locus [19]. In order to define the role of HDAC on the IgH regulatory elements, we performed reporter gene analysis to study the effect of TSA on V_H promoter and IgH enhancer activity. The results showed that inhibition of HDACs by TSA markedly inhibited the activities of Eμ and 3′-IgH enhancers in T347 cells. In accordance with these results, transient expression of HDAC1 increased the activity of Eμ, especially 3′-IgH enhancers. Moreover, ChIP assays showed that endogenous HDAC1 is bound to the hs1,2 enhancer. These findings indicated that HDAC1 is recruited to the 3′-IgH enhancer and may function as an activator for the enhancer(s). Collectively, the present study provides evidence that HDAC is involved in the regulation of IgH gene transcription via activation of 3′-IgH enhancers.

HDAC has been linked extensively with gene transcription inhibition [20]. However, we observed that HDAC1 stimulates the IgH enhancer activity and therefore activates IgH transcription. Consistent with our finding, it was reported that HDAC augments nitric oxide synthase (iNOS) and NF-κB gene promoter activity, and TSA inhibits basal or induced mouse mammary tumor virus (MMTV) promoter activity [21,22]. The data from our study and those of others suggest that there may be a different function of HDAC in gene transcription, with regard to individual genes and their promoters and/or enhancers.

Acknowledgments

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