MOLECULAR AND CELLULAR BIOLOGY, Oct. 2001, p. 6495–6506 Vol. 21, No. 19
0270-7306/01/$04.00+0 DOI: 10.1128/MCB.21.19.6495–6506.2001

Histone Deacetylase Activity Represses Gamma Interferon-Inducible HLA-DR Gene Expression following the Establishment of a DNase I-Hypersensitive Chromatin Conformation

AARON OSBORNE,[1,2] HONGQUAN ZHANG,[3] WEN-MING YANG,[4] EDWARD SETO,[2,4,5] AND GEORGE BLANCK[1,2,3,6]*

Department of Biochemistry and Molecular Biology,[1] Department of Medical Microbiology,[5] and Department of Pathology and Laboratory Medicine,[3] College of Medicine, Institute for Biomolecular Science,[2] and Immunology[6] and Molecular Oncology[4] Programs, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, Florida 33612

Received 26 April 2001/Returned for modification 31 May 2001/Accepted 27 June 2001

Expression of the retinoblastoma tumor suppressor protein (Rb) is required for gamma interferon (IFN-γ)-inducible major histocompatibility complex class II gene expression and transcriptionally productive HLA-DRA promoter occupancy in several human tumor cell lines. Treatment of these Rb-defective tumor cell lines with histone deacetylase (HDAC) inhibitors rescued IFN-γ-inducible HLA-DRA and -DRB mRNA and cell surface protein expression, demonstrating repression of these genes by endogenous cellular HDAC activity. Additionally, Rb-defective, transcriptionally incompetent tumor cells retained the HLA-DRA promoter DNase I-hypersensitive site. Thus, HDAC-mediated repression of the HLA-DRA promoter occurs following the establishment of an apparent nucleosome-free promoter region and before transcriptionally productive occupancy of the promoter by the required transactivators. Repression of HLA-DRA promoter activation by HDAC activity likely involves a YY1 binding element located in the first exon of the HLA-DRA gene. Chromatin immunoprecipitation experiments localized YY1 to the HLA-DRA gene in Rb-defective tumor cells. Additionally, mutation of the YY1 binding site prevented repression of the promoter by HDAC1 and partially prevented activation of the promoter by trichostatin A. Mutation of the octamer element also significantly reduced the ability of HDAC1 to confer repression of inducible HLA-DRA promoter activation. Treatment of Rb-defective tumor cells with HDAC inhibitors greatly reduced the DNA binding activity of Oct-1, a repressor of inducible HLA-DRA promoter activation. These findings represent the first evidence that HDAC activity can repress IFN-γ-inducible HLA class II gene expression and also demonstrate that HDAC activity can contribute to promoter repression following the establishment of a DNase I-hypersensitive chromatin conformation.

Major histocompatibility complex (MHC) class II molecules are heterodimeric cell surface glycoproteins comprised of both a heavy (alpha) chain and a light (beta) chain. MHC class II molecules (HLA-DR, -DP, and -DQ in humans) bind and display peptide antigens for recognition by $CD4^+$ T lymphocytes. Recognition of the MHC class II heterodimer-antigen complex by the T-cell receptor and the accessory protein CD4 of T lymphocytes leads to the generation of an immune response. MHC class II molecules play an important role in antitumor immunity (1–4, 11, 29, 42–44, 49). Specifically, transfection of tumor cells with syngeneic murine MHC class II genes immunizes mice against MHC class II-negative parental tumor cells (2). Vaccination of mice using this protocol also leads to eradication of an MHC class II-negative, basement membrane-invasive tumor (4). Also, tumor-specific antigens capable of eliciting HLA class II-restricted activation of tumor-infiltrating T lymphocytes have been identified (46, 57, 58).

MHC class II expression is constitutively activated during development in professional antigen-presenting cells, such as B cells, dendritic cells, and macrophages; it is inducible by cytokines, most importantly, gamma interferon (IFN-γ), in nearly all other types of cells. MHC class II expression is regulated primarily at the level of transcription through promoter elements that are conserved among the MHC class II genes and the genes encoding accessory molecules such as the invariant chain, the MHC class II chaperone. The elements are, from 5′ to 3′, S box, X1 box, X2 box, Y box, and TATA box. The transactivators RFX, X2BP (CREB), and NF-Y are required factors for MHC class II gene activation and bind the X1, X2, and Y boxes, respectively. Cooperative interactions between transactivators bound to the X and Y elements have been demonstrated to be essential for the establishment of promoter occupancy and the transcription of class II genes (60). In particular, binding of the Y box factor, NF-Y, has been demonstrated to be required for occupancy of the other promoter elements and for IFN-γ-inducible MHC class II gene expression (60). In addition to the promoter binding factors, the class II transactivator (CIITA) is a required coactivator that functions by interaction with and stabilization of the transcription factors previously assembled on MHC class II promoters (20, 24, 38, 53, 59, 68).

It has been shown that the retinoblastoma tumor suppressor protein (Rb) is also required for IFN-γ-inducible MHC class II gene expression (34, 35, 41, 67). Several Rb-defective human tumor cell lines exhibit a loss of IFN-γ-inducible MHC class II gene expression that is rescued by the reexpression of func-

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, University of South Florida College of Medicine, MDC Box 7, 12901 Bruce B. Downs Blvd., Tampa, FL 33612. Phone: (813) 974-9585. Fax: (813) 974-7280. E-mail: gblanck@hsc.usf.edu.

tional Rb (34, 35, 41). Rb-defective tumor cell lines exhibit significantly reduced or complete loss of promoter occupancy at all of the known transactivator binding sites within the HLA-DRA promoter, as detected by in vivo footprinting (41). The expression of exogenous Rb results in increased occupancy at these promoter elements, and this effect of Rb is independent of IFN-γ-mediated transcriptional activation (41). Thus, Rb apparently relieves a block to efficient, transcriptionally productive transcription factor assembly at the HLA-DRA promoter. There is also significantly reduced or absent promoter occupancy in cells from patients with bare lymphocyte syndrome (BLS), where RFX is defective or missing (26–28). In BLS cells, the HLA-DRA promoter DNase I-hypersensitive site is absent (17), indicating a close association of nucleosomes with promoter DNA.

In this report, we demonstrate that the HLA-DRA promoter retains the DNase I-hypersensitive site in non-IFN-γ-inducible, Rb-defective tumor cells. This observation separates the formation of the hypersensitive site and presumably a nucleosome-free promoter region from the transcriptional competency of the promoter. The distinction between the lack of the DNase I-hypersensitive site and the lack of transcriptionally productive transactivator binding establishes two stable levels of repression of the HLA-DRA promoter in situ. While histone deacetylase (HDAC) activity is generally accepted as mediating repression by nucleosomes, its role in other levels of promoter repression is unknown. We were interested in determining whether HDAC activity could be involved in maintaining repression following the partial derepression of chromatin that is represented by the establishment of the promoter DNase I-hypersensitive site. Here we show that the lack of HLA-DR expression in Rb-defective tumor cells was due to the repression of HLA-DRA and -DRB promoter activation by HDAC activity. We also show that the repression of HLA-DRA promoter activation by HDAC activity is facilitated by YY1 and octamer elements. The approach of mapping HDAC function to distinct states of chromatin has the potential of identifying specific target proteins that mediate stable, intermediate states of promoter repression.

MATERIALS AND METHODS

Cell cultures. 1A4 and 12-27 are subclones of the Rb-defective bladder carcinoma cell line 5637 (ATCC HTB9), while 66.1A3 and 68.2A5 are subclones of the parental Rb-defective non-small-cell lung carcinoma cell line H2009. 12-27 and 68.2A5 are transformed with both an Rb expression vector and a G418 resistance gene. 1A4 and 66.1A3 are transformed with only a G418 resistance gene. 5637, 1A4, 12-27, H2009, 66.1A3, and 68.2A5 cells were all grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U of penicillin-streptomycin/ml, 3 mM L-glutamine, and 1 mM sodium pyruvate.

DNase I-hypersensitive site assay. Approximately 5×10^7 cells were harvested, rinsed three times with ice-cold phosphate-buffered saline (PBS), resuspended in lysis buffer A (10 mM Tris [pH 8.0], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40 [NP-40]), and incubated for 5 min on ice. The nuclei were pelleted by centrifugation at $1,400 \times g$ for 5 min at 4°C and resuspended in 700 μl of cold buffer B (10 mM Tris [pH 8.0], 10 mM NaCl, 3 mM $MgCl_2$). Samples of resuspended nuclei corresponding to 5×10^6 cells were digested with various amounts of DNase I (20, 10, 5, and 2.5 μg/ml) for 10 min at 37°C. The digestion reactions were stopped by adding 20 μl of 200 mM EDTA followed by the addition of 200 μl of buffer C (10 mM Tris [pH 8.0], 140 mM NaCl, 10 mM EDTA, 1.2% sodium dodecyl sulfate [SDS]). The samples were then digested with 0.2 mg of proteinase K/ml overnight at 37°C. Genomic DNA was isolated by repeated phenol-chloroform extractions followed by ethanol precipitation. Approximately 30 μg of purified genomic DNA isolated from each sample of DNase I-treated nuclei was digested with *Pst*I, phenol-chloroform extracted, and ethanol precipitated. The samples were then loaded on 0.8% agarose gels and electrophoresed at 80 V for 6 h. The gels were soaked in 0.5 N NaOH–1.5 M NaCl for 30 to 45 min, neutralized for 60 min, and transferred to nitrocellulose membranes by the Southern blot procedure (51). The membranes were dried at 80°C for 2 h. The membranes were prehybridized for 3 h at 42°C in a solution containing 50% deionized formamide, 33 mM sodium phosphate monobasic, 12 mM sodium phosphate dibasic, 5× Denhardt's solution (0.1% Ficoll, 0.01% bovine serum albumin, 0.01% polyvinylpyrrolidone), 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.2% SDS, and 30 μg of sonicated salmon sperm DNA/ml. The prehybridization solution was then replaced with a hybridization solution of the same composition but containing a denatured probe labeled with [α-^{32}P]dCTP by the nick translation procedure (51). Hybridization was allowed to continue for 16 h at 42°C. The membranes were washed twice at 55°C, for 40 min each wash, in a solution containing 2× SSC and 0.1% SDS. The membranes were then autoradiographed for 4 days at −70°C with an intensifying screen.

RT-PCR. Total cytoplasmic RNA was prepared by the NP-40 lysis method as previously described (6). When needed, each sample was treated with sodium butyrate and trichostatin A (TSA) for 72 h and 400 U of IFN-γ/ml for 48 h prior to harvesting of total cytoplasmic RNA. Five micrograms of total cytoplasmic RNA from each sample was primed using random hexameric primers (Gibco-BRL) and reverse transcribed with Superscript II reverse transcriptase (Gibco-BRL) according to the manufacturer's instructions. PCR was performed for each sample with a 50-μl reaction mixture containing 5 μl of reverse transcription (RT) reaction product; 5 μl of 10× PCR assay buffer B (Fisher Biotech); 1.5 mM $MgCl_2$; 5% dimethyl sulfoxide; 10 pmol of each DRA-, DRB-, or γ-actin-specific primer (13, 45); 0.2 mM each deoxynucleoside triphosphate; and 1 U of *Taq* polymerase (Fisher Biotech). Each sample was incubated successively at 95°C for 30 s, 53.5°C for 30 s, and 72°C for 45 s, for a total of 30 (DRA and DRB) or 12 (γ-actin) cycles, followed by a final extension at 72°C for 5 min. PCR products were visualized on a 1.4% agarose gel containing ethidium bromide.

Flow cytometry. 5637 cells were plated at 10^6 cells per plate in 100-mm tissue culture plates and, where appropriate, treated with 1 mM sodium butyrate for 72 h prior to harvesting. Additionally, when needed, the cells were treated with 400 U of IFN-γ/ml 48 h prior to harvesting. To harvest the cells, cell monolayers were rinsed three times with cold PBS and scraped with a rubber policeman. Aliquots of 2×10^5 cells were resuspended in 500 μl of PHA buffer (in cold PBS containing 1% human serum and 0.2% sodium azide) and placed on ice to block Fc receptors. For the sample cells only, 100 μl of tissue culture supernatant containing anti-DR monoclonal antibody DA6.147 (19) was added and incubated for 30 min at 4°C. The cells were collected by centrifugation, washed once with PHA, collected again by centrifugation, and resuspended in 500 μl of PHA. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G antibody was added to both control and sample cells and incubated for 30 min at 4°C. The cells were collected by centrifugation, washed once with PHA, collected again by centrifugation, and resuspended in 500 μl of cold PBS. Four hundred microliters of each sample was analyzed with a FACStar (Becton-Dickinson).

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were carried out as follows. Chromatin from 10^7 Rb-defective bladder carcinoma cells (5637) was cross-linked by the addition directly to the tissue culture medium of formaldehyde to a final concentration of 1% and rocking at room temperature for 10 min. The cross-linking reactions were quenched by the addition directly to the tissue culture medium of glycine to a final concentration of 0.125 M and rocking at room temperature for an additional 10 min. The medium was removed by aspiration, and the cells were rinsed three times with 4°C PBS. The cells were lysed by the addition of 500 μl of cell lysis buffer (20 mM HEPES [pH 7.9], 1 mM EDTA, 0.2% Igepal, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, complete protease inhibitor cocktail [Boehringer Mannheim] to a 1× final concentration) per 150-mm plate, and the nuclei were collected by scraping the plates with a disposable cell scraper. The nuclei were pelleted by centrifugation at $8,400 \times g$ and 4°C for 15 s in a microcentrifuge. The pelleted nuclei were resuspended in 1 ml of nuclear lysis buffer (50 mM Tris-HCl [pH 8.1], 10 mM EDTA, 1% SDS, complete protease inhibitor cocktail to a 1× final concentration), rapidly frozen in a dry ice-ethanol bath, and stored at −70°C until required. The cross-linked chromatin was sheared to an average size of 400 to 600 bp by sonication. The exact sonication conditions required to shear the chromatin to this size were empirically determined during each experiment.

One hundred microliters of sheared chromatin was diluted with 855 μl of 89 mM NaCl, 20 μl of 10-mg/ml sonicated salmon sperm DNA, and 20 μl of 10-mg/ml total RNA from baker's yeast, and 5 μl of anti-YY1 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) was added to each sample. Additionally,

a no-antibody control reaction as well as an irrelevant antibody (anti-EGFR Ab [Upstate Biotechnology]) control reaction were set up as well. The samples were placed at 4°C on a rotating stand overnight. Forty microliters of a 50% slurry of protein G-agarose beads was then added to each sample and incubated at 4°C for 2 h on a rotating stand. The immunoprecipitates were washed two times at room temperature for 15 min each wash with buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 150 mM NaCl), buffer 2 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 500 mM NaCl), buffer 3 (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.1]), and buffer 4 (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA). The samples were eluted by the addition of 150 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) to the pelleted beads. The elution was repeated, and the eluate was combined with the previous eluate. The cross-links were reversed by the addition of 20 μl of 5 M NaCl and incubation at 65°C overnight. The samples were proteinase K digested, phenol-chloroform extracted, chloroform extracted, ethanol precipitated, and resuspended in 30 μl of water.

PCRs for ChIP assays. PCRs were performed with a solution containing 5.0 μl of 10× *Taq* buffer (Gibco-BRL), 2 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (dATP, dTTP, dGTP, and dCTP), 2.5 μl of dimethyl sulfoxide, 50 pmol of each gene-specific primer (see below), 2.5 U of *Taq* polymerase (Gibco-BRL), and 3.0 μl of each ChIP reaction mixture (see above) as a template in a final reaction volume of 50 μl. The HLA-DRA promoter-specific primers span the YY1 binding element (upstream +264 primer, 5′-GAAGTCAGATTGGG GTTAA-3′; and downstream −110 primer, 5′-CAGCTATGATGAAAAATCC T-3′). The β-actin promoter-specific primers were used to control for the amplification of a region of DNA not expected to interact with YY1 (upstream primer, 5′-TGCCTAGGTCACCCACTAACG-3′; and downstream primer, 5′-C TGGAGCTGCCTGCTTTTG-3′). The samples were incubated in an MJ Research thermocycler as follows: 95°C for 5 min; 35 cycles (HLA-DRA) or 30 cycles (β-actin) of 95°C for 30 s, 50°C (HLA-DRA) or 48°C (β-actin) for 30 s, and 72°C for 30 s; 72°C for 10 min; and 4°C until required. The amplification products were electrophoresed on 2.2% agarose gels in 1× Tris-acetate-EDTA running buffer at 120 V for approximately 1 h. The DNA was visualized by staining with ethidium bromide and UV transillumination.

Plasmid construction. The promoter-luciferase constructs, pDRA, pDRA-YY1mut, pDRA-Octmut, and pDRA-Oct/YY1mut, were constructed as follows. The pDRAlucWT and pDRAlucOctmut constructs (65) were digested with *Sac*I and *Hin*dIII, resulting in linear molecules lacking all DRA sequences downstream of position +32 in both constructs. A double-stranded DNA fragment containing DRA gene sequences from positions +32 to +76, including the wild-type YY1 binding site (underlined), was generated by annealing two oligonucleotides of the following sequences: sense, 5′-CTACTGACTCCCAAAAGA GCGCCCAAGAAGAAAATGGCCATAAGTCTAGA-3′; and antisense, 5′-A GCTTCTAGACTTATGGCCATTTTCTTCTTGGGCGCTCTTTTGGGAGT CAGTAGAGCT-3′. The annealed oligonucleotide was then ligated to the linearized pDRAlucWT and pDRAlucOctmut constructs to yield the pDRA and pDRA-Octmut constructs, respectively. To create the pDRA-YY1mut and pDRA-Oct/YY1mut constructs, two complementary oligonucleotides that were identical to the ones described above were annealed, except that they encoded a GCC→ATT substitution mutation (underlined) in the YY1 binding site (sense, 5′-AAATGGCCAT-3′→5′-AAATGATTAT-3′). The resulting double-stranded mutant oligonucleotide was then ligated to the linearized pDRAlucWT and pDRAlucOctmut constructs to generate the pDRA-YY1mut and pDRAOct/YY1mut constructs, respectively. The HLA-DRA promoter inserts for all of the reporter constructs generated in this report were verified by DNA sequencing.

The pCMV-HDAC1 expression vector was generated by ligation of the full-length HDAC1 cDNA obtained by digestion of the pGEX2T-HDAC1 (human HDAC1) vector (63) with *Eco*RI and *Bam*HI into pcDNA3 (Invitrogen, Carlsbad, Calif.) linearized with these same enzymes. The pCMV-YY1 expression vector was generated by ligation of full-length YY1 cDNA to the *Eco*RI site of the pcDNA1/amp vector and has been described previously (62). The pCEP4F-YY1(1-396) expression vector was generated by ligation of YY1 cDNA encoding codons 1 to 396 (*Nco*I-*Hin*cII digestion of YY1 cDNA) to an adapter oligonucleotide containing *Hin*dIII and *Bam*HI recognition sites. The ligation product was then digested with *Hin*dIII and *Bam*HI and ligated to the pCEP4F vector (63) linearized by digestion with the same enzymes.

Transient transfections. 5637 and 12-27 cells were plated at 5×10^4 cells per well in 24-well tissue culture plates 24 h prior to transfection. Various amounts of plasmid DNA were transfected in accordance with manufacturer instructions using the cationic lipid reagent TransIT-LT1 (PanVera, Madison, Wis.) at a ratio of 2 μl of TransIT-LT1 to 1 μg of DNA transfected. All cells were treated with IFN-γ at a final concentration of 400 U/ml. Cell lysates were prepared in accordance with manufacturer instructions using 100 μl of passive lysis buffer (Promega, Madison, Wis.) 24 h after IFN-γ treatment. Luciferase assays were performed with a Turner Designs TD-20/20 Luminometer using 20 μl of cell lysate and 100 μl of luciferase assay buffer (Promega). All transfection data represent the mean and standard error of the mean for at least three replicate samples analyzed on the same day under identical treatment conditions.

EMSA. Electrophoretic mobility shift assays (EMSA) were carried out as follows. Crude nuclear extracts were prepared as previously described (65). The total protein concentration was determined for each sample using bicinchoninic assay reagents (Pierce, Rockford, Ill.). The −62/−37 HLA-DRA octamer element probe and competitor oligonucleotides have been previously described (65). The HLA-DRA Y box probe has also been previously described (65). The +32/+76 HLA-DRA wild-type YY1 probe, as well as the wild-type and mutant YY1 competitor oligonucleotides, were the same oligonucleotides as those used to generate the wild-type and mutant reporter constructs in this report. The wild-type Y box and YY1 probes were labeled with [α-^{32}P]dCTP by the Klenow filling-in reaction (51). Antibody supershift reactions were performed by adding 1 μl of anti-Oct-1, anti-NF-Y (A subunit) (Rockland Immunochemicals, Gilbertsville, Pa.), or anti-YY1 antibody to each reaction. EMSA were performed by the method of Yu et al. (64), except that all binding reactions were performed at room temperature for 30 min. All EMSA reactions were separated by electrophoresis on either 7% (Oct-1) or 8% (NF-Y and YY1) polyacrylamide gels for approximately 3 h at 12 V/cm in 0.25× Tris-borate-EDTA running buffer.

RESULTS

Rb-defective tumor cells retain the DNase I-hypersensitive site at the HLA-DRA promoter. The HLA-DRA promoter possesses a prominent DNase I-hypersensitive site (Fig. 1A) in cells that are transcriptionally competent (17). However, RFX-defective, transcriptionally incompetent cells derived from patients with BLS do not possess the HLA-DRA promoter hypersensitive site (17). Furthermore, the HLA-DRA promoter in these BLS-derived cell lines exhibits a "bare promoter phenotype," in which strong or transcriptionally productive occupancy of the required transcription factor binding sites (X1, X2, and Y boxes) cannot be detected by in vivo genomic footprinting (26, 27, 28). It has been demonstrated by in vivo footprinting that the HLA-DRA promoter exhibits a loss of transactivator binding in Rb-defective tumor cells (41) that is similar to the bare promoter phenotype observed in RFX-defective BLS-derived cell lines (26, 27, 28). Thus, it is possible that the lack of transcriptionally productive factor binding to the HLA-DRA promoter in Rb-defective cells is due to condensation of promoter chromatin, as indicated by a loss of the promoter hypersensitive site.

To determine whether the block to transcriptionally productive promoter occupancy, detected by in vivo footprinting analysis, in Rb-defective tumor cell lines (41) is related to inaccessibility of the HLA-DRA promoter chromatin, we performed DNase I-hypersensitive site assays of subclones of Rb-defective non-small-cell lung carcinoma and bladder carcinoma cell lines, H2009 and 5637, respectively. The HLA-DRA promoter DNase I-hypersensitive site (Fig. 1A) (17) was present in both the Rb-defective H2009 subclone, 66.1A3 (Fig. 1B), and the Rb-defective 5637 subclone, 1A4 (Fig. 1C). These observations indicate that some stable decondensation of HLA-DRA promoter chromatin, represented by the promoter DNase I-hypersensitive site, occurs in cells that lack Rb expression. Furthermore, when considered along with the previously observed lack of transcriptionally productive promoter occupancy in these Rb-defective cells (41), these observations serve to separate the formation of the HLA-DRA promoter hypersensitive site from transcriptionally productive occupancy of the pro-

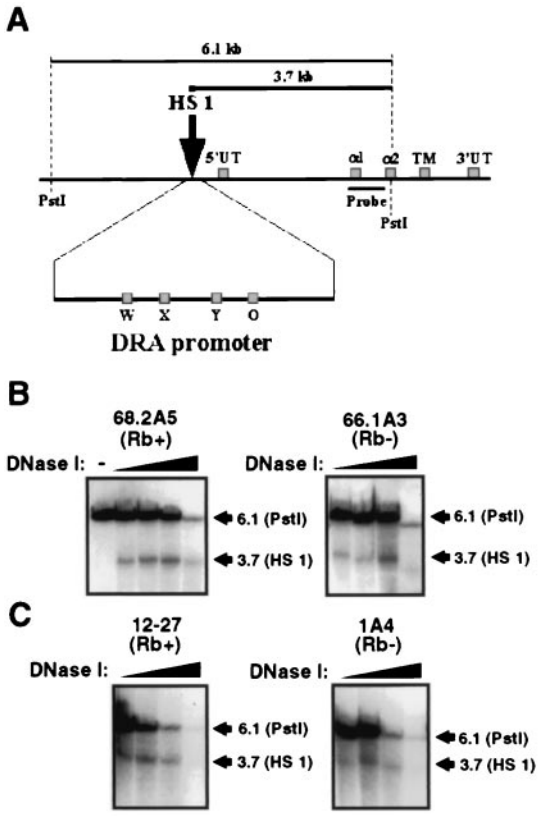

FIG. 1. Loss of Rb does not result in loss of DNase I-hypersensitive sites in the HLA-DRA promoter. (A) Schematic diagram of HLA-DRA promoter region and coding sequences. The previously described (17) major site of DNase I hypersensitivity in the HLA-DRA promoter is indicated as HS 1. W, W (S/Z) box; X, X1 and X2 boxes; Y, Y box; O, octamer element; α1, α2, and TM, regions of gene coding for α1, α2, and transmembrane domains of DRα, respectively. Digestion of genomic DNA with restriction endonuclease *Pst*I generates an approximately 6.1-kb fragment encompassing the HLA-DRA promoter and most of the coding sequences. If the promoter chromatin is in a generally accessible conformation, further digestion of the genomic DNA with DNase I results in the generation of an approximately 3.7-kb fragment. The 3.7-kb cleavage fragment generated by digestion with *Pst*I and DNase I is detected by hybridization with a radiolabeled DNA probe. (B) Rb-reconstituted (68.2A5) and Rb-defective (66.1A3) subclones of non-small-cell lung carcinoma cell line H2009 were analyzed for their sensitivity to cleavage by DNase I within the HLA-DRA promoter. HS 1 is detected in both Rb-reconstituted and Rb-defective cells and is indicated by an arrow labeled as 3.7 (HS 1). The parental *Pst*I fragment that was not digested with DNase I is indicated by an arrow labeled as 6.1 (PstI). (C) Rb-reconstituted (12-27) and Rb-defective (1A4) subclones of bladder carcinoma cell line 5637 were analyzed exactly as described in panel B for their sensitivity to DNase I cleavage within the HLA-DRA promoter.

moter by required transactivator proteins and thus the transcriptional competency of the promoter.

HDAC inhibitors rescue IFN-γ-inducible HLA-DRA and HLA-DRB mRNA expression in Rb-defective tumor lines. Inhibition of HDAC activity activates transcription and causes a change in endonuclease sensitivity at several promoters (7, 16, 39, 56). Despite the fact that the DNase I-hypersensitive site was present in the Rb-defective cells, we wished to determine whether HDAC inhibitors could restore HLA-DRA and -DRB mRNA expression in these cells. Rb-defective subclone 1A4, from the Rb-defective bladder carcinoma cell line 5637, was treated with IFN-γ and various amounts of the HDAC inhibitor sodium butyrate or TSA. HLA-DRA expression and HLA-DRB expression were then examined by RT-PCR (Fig. 2A). As reported previously (41), IFN-γ was unable to induce HLA-DRA or -DRB mRNA expression in the Rb-defective 1A4 cells (Fig. 2A, lane 2), while IFN-γ treatment of the Rb-reconstituted 12-27 cells resulted in increased expression of both HLA-DRA and -DRB mRNAs (Fig. 2A, lane 4). Treatment of 1A4 cells with sodium butyrate alone also did not result in a detectable increase in HLA-DRA mRNA expression (Fig. 2A, lane 5). However, treatment of 1A4 cells with IFN-γ and so-

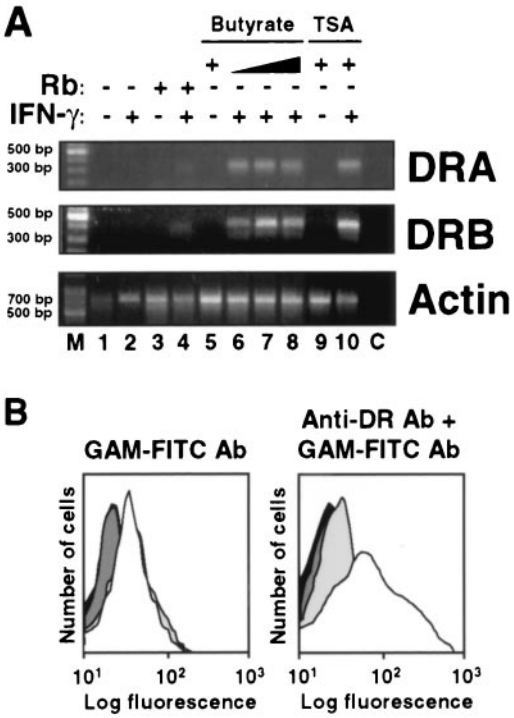

FIG. 2. HDAC inhibitors rescue IFN-γ-inducible HLA-DR gene and cell surface expression in Rb-defective bladder carcinoma cells. (A) HDAC inhibitors rescue IFN-γ-inducible HLA-DRA and -DRB mRNA expression in Rb-defective bladder carcinoma cells. RT-PCR was performed using total cytoplasmic RNA from Rb-defective (1A4; lanes 1, 2, and 5 to 10) and Rb-transformed (12-27; lanes 3 and 4) bladder carcinoma cells. Lane M, molecular size marker with 100-bp increments; lane C, negative control for amplification, as no cytoplasmic RNA was added to the RT reaction used as a template for this amplification reaction. Samples were treated with 50 nM TSA or various amounts of sodium butyrate (0.5, 0.25, 0.5, and 1.0 mM for lanes 5 to 8, respectively). Additionally, samples were treated with 400 U of IFN-γ/ml. PCR was performed for 30 cycles for DRA and DRB and 12 cycles for γ-actin to ensure that the reactions were still in the linear range of amplification. The expected band sizes of 325 bp (DRA), 402 bp (DRB), and 730 bp (γ-actin) were obtained for each set of primers. (B) Sodium butyrate rescues IFN-γ-inducible cell surface expression of the HLA-DR heterodimer in Rb-defective bladder carcinoma cells. Rb-defective (5637) bladder carcinoma cells were treated with 400 U of IFN-γ/ml and 1 mM sodium butyrate, and HLA-DR heterodimer expression was detected using primary mouse anti-DR monoclonal antibody (Ab) DA6.147, followed by staining with FITC-conjugated secondary goat anti-mouse (GAM) immunoglobulin G antibody. The plots are as follows: black, untreated; dark gray, IFN-γ; light gray, butyrate; and white, IFN-γ and butyrate.

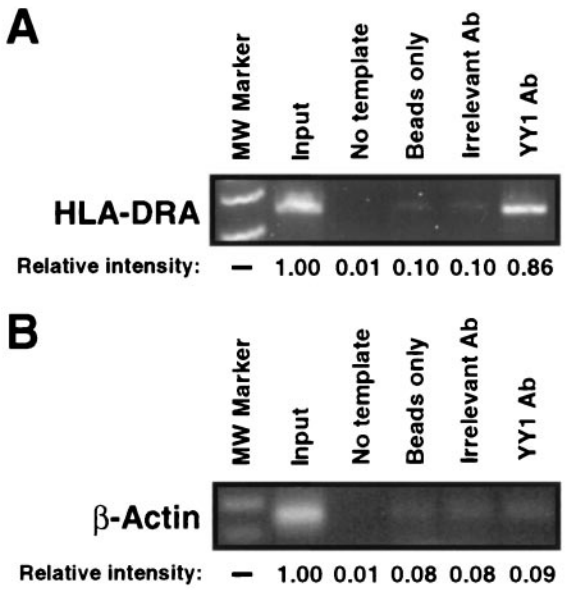

FIG. 3. YY1 is present in vivo at the HLA-DRA promoter in Rb-defective 5637 cells. ChIP assays were performed using formaldehyde cross-linked chromatin isolated from 5637 cells. The chromatin was sheared by sonication to an average size of approximately 400 to 600 bp and was immunoprecipitated using only protein G beads, protein G beads and an irrelevant antibody (Ab), or protein G beads and anti-YY1 antibody. Following immunoprecipitation, HLA-DRA promoter fragments were amplified by 35 cycles of PCR using primers spanning the putative HLA-DRA YY1 binding site (A), and β-actin promoter fragments were amplified by 30 cycles of PCR using primers specific for the β-actin promoter (B). The β-actin promoter does not possess a consensus YY1 binding element and is not believed to be regulated by YY1. Input, amplification using 1% the total chromatin introduced into each ChIP reaction as a template; No template, controls in which water was substituted for ChIP samples during PCR amplification of each region of DNA. The relative intensity of each band was determined by densitometry. MW, molecular weight markers.

dium butyrate rescued HLA-DRA mRNA expression across a range of sodium butyrate concentrations (Fig. 2A, lanes 6 to 8). The combination of TSA and IFN-γ treatments of 1A4 cells also resulted in the rescue of IFN-γ-inducible HLA-DRA and -DRB mRNAs in these cells (Fig. 2A, lane 10). The rescue of IFN-γ-inducible HLA-DRA mRNA expression in these cells was verified by an RNase protection assay (data not shown). These data demonstrate that HDAC activity represses IFN-γ-inducible HLA-DR gene expression in Rb-defective tumor cell lines.

Sodium butyrate rescues IFN-γ-inducible cell surface expression of the HLA-DR heterodimer. We next determined whether sodium butyrate treatment could rescue IFN-γ-inducible cell surface HLA-DR expression in Rb-defective 5637 cells, as detected by flow cytometry (Fig. 2B). Treatment with a combination of IFN-γ and sodium butyrate resulted in an increase in mean fluorescence, indicating an increase in cell surface HLA-DR protein expression (Fig. 2B, white plot). This increase in surface DR heterodimer expression is comparable to that observed following IFN-γ treatment of Rb-reconstituted breast carcinoma cells (34, 52), as well as normal human fibroblasts and other nonprofessional antigen-presenting cells (data not shown).

YY1 represses IFN-γ-inducible HLA-DRA promoter activation through specific interactions with the +62/+72 binding element. Although HDACs are not known to possess intrinsic DNA binding activity, they can be directed to the promoters of genes through interactions with sequence-specific transcription factors. One such factor, YY1, has been demonstrated to physically associate with three members of the human HDAC family, HDAC1, HDAC2, and HDAC3 (63). The HLA-DRA gene possesses a consensus YY1 binding element that is located in the first exon from positions +62 to +72 relative to the start of transcription and that specifically interacts with YY1 (21). Thus, we wished to determine whether YY1 can repress inducible HLA-DRA promoter activation through the +62/+72 YY1 binding element and whether this element is also required for HDAC-mediated repression of inducible HLA-DRA promoter activation.

To first determine whether YY1 was capable of interacting with the HLA-DRA promoter region in vivo in Rb-defective tumor cells, we performed ChIP assays. Chromatin was prepared from Rb-defective bladder carcinoma (5637) cells and sonicated to shear the DNA to an average size of 400 to 600 bp, and an anti-YY1 antibody was used to immunoprecipitate YY1 and associated genomic DNA fragments. PCR was performed using primers spanning the HLA-DRA YY1 binding element (Fig. 3A). Strong amplification of HLA-DRA promoter DNA was obtained only when the YY1 antibody was included in the ChIP reaction, indicating that YY1 is present in vivo at the HLA-DRA promoter in Rb-defective tumor cells.

To determine whether YY1 binding to the +62/+72 element is capable of repressing IFN-γ-inducible HLA-DRA expression, the pDRA and pDRA-YY1mut luciferase reporter constructs (Fig. 4) were transiently transfected in 12-27 cells with the YY1 expression vector, pCMV-YY1. Previous reports have indicated that high-level expression of YY1, as well as other sequence-specific factors, can result in nonspecific repression of transcription (5, 14, 25, 31, 32, 47, 48). To address this problem, the YY1 expression vector, pCMV-YY1, was

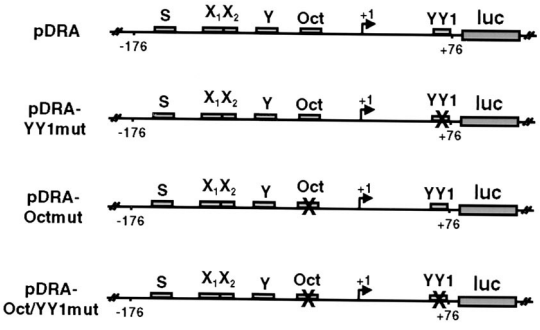

FIG. 4. Schematic diagrams of the reporter constructs used in this study. pDRA represents the region, from −176 to +80 of the HLA-DRA promoter, controlling the expression of a luciferase reporter gene. The mutant constructs, pDRA-Octmut and pDRA-YY1mut, are identical to pDRA except for a four-nucleotide substitution mutation (underlined) in the octamer element (5′-ATTTGCAT-3′→5′-CCAT GGAT-3′) and a three-nucleotide substitution mutation (underlined) in the YY1 element (5′-AAATGGCCAT-3′→5′-AAATGATTAT-3′), respectively. The double-mutant construct, pDRA-Oct/YY1mut, contains both the four-nucleotide octamer element mutation and the three-nucleotide YY1 element mutation.

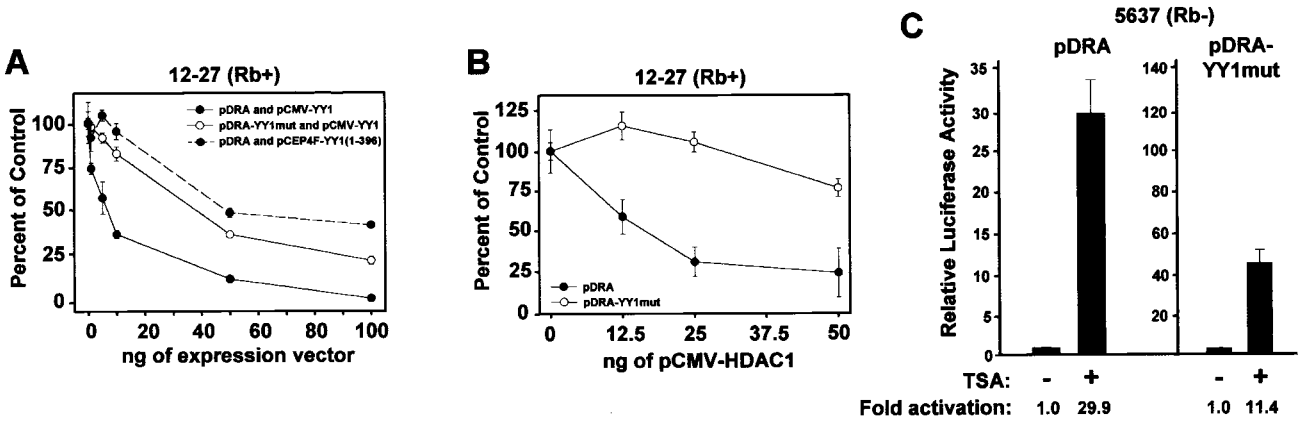

FIG. 5. YY1 overexpression and HDAC activity repress HLA-DRA promoter activation through the +62/+72 YY1 binding element. (A) YY1 requires DNA binding activity for repression of inducible HLA-DRA promoter activation. Different amounts (1, 5, 10, 50, and 100 ng) of expression vector encoding either full-length YY1 protein (pCMV-YY1) (solid line) or a C-terminally truncated mutant YY1 protein which is not capable of binding to DNA [pCEP4F-YY1(1-396)] (broken line) were cotransfected along with 25 ng of promoter-luciferase construct pDRA (closed circles) or pDRA-YY1mut (open circles) into 12-27 cells. The cells were then treated with 400 U of IFN-γ/ml for 24 h prior to assays for luciferase activity. The total amount of transfected DNA in each experiment was normalized by the addition of the empty expression vector pcDNA1/amp or pCEP4F, as appropriate. The data for each reporter construct in the presence of the YY1 expression vector are presented as the percent activation of the same reporter construct in the presence of the appropriate control expression vector (defined as 100%). Specific binding of endogenous and in vitro-translated YY1 to the +62/+72 element and elimination of YY1 binding activity by the GCC→ATT mutation in the pDRA-YY1mut construct or by truncation of the YY1 protein were confirmed by EMSA (see Fig. 7C). (B) HDAC1-mediated repression of inducible HLA-DRA promoter activation requires an intact YY1 binding site. Different amounts (12.5, 25, and 50 ng) of expression vector pCMV-HDAC1 encoding HDAC1 were cotransfected along with 25 ng of construct pDRA (closed circles) or pDRA-YY1mut (open circles) into 12-27 cells. The cells were then treated with 400 U of IFN-γ/ml for 24 h prior to assays for luciferase activity. The total amount of cotransfected DNA was normalized by the addition of empty expression vector pcDNA3. The data for each reporter construct in the presence of the HDAC1 expression vector are presented as the percent activation of the same reporter construct in the presence of control expression vector pcDNA3 (defined as 100%). (C) Activation of the HLA-DRA promoter by TSA requires an intact YY1 binding site. 5637 cells were transfected with 25 ng of promoter-luciferase construct pDRA or pDRA-YY1mut and treated with 400 U of IFN-γ/ml and, where indicated, 100 nM TSA for 24 h prior to assays for luciferase activity.

transfected within a 100-fold range of amounts, along with a constant amount of either wild-type pDRA or pDRA-YY1mut promoter-luciferase construct, into 12-27 cells. The cells were treated with IFN-γ for 24 h following transfection. Overexpression of YY1 in these cells was indeed capable of repressing IFN-γ-inducible activation of the pDRA construct, and this repressive activity was dose dependent (Fig. 5A). The pDRA-YY1mut construct was also repressed by cotransfected pCMV-YY1, consistent with previous reports of nonspecific (DNA-independent) repressive activity when YY1 is expressed at high levels. However, the pDRA-YY1mut construct was not repressed by YY1 overexpression to the same degree as the pDRA construct for any of the amounts of YY1 expression vector analyzed. Maximal derepression due to mutation of the YY1 binding site was observed at low levels of cotransfected pCMV-YY1 (Fig. 5A).

We next cotransfected 12-27 cells with a constant amount of the pDRA promoter-luciferase construct and various amounts of the pCEP4F-YY1(1-396) expression vector. This expression vector encodes a C-terminally truncated YY1 mutant protein that spans amino acids 1 to 396 and that is incapable of binding to DNA (data not shown). The cells were then treated with IFN-γ for 24 h following transfection. Overexpression of the YY1 protein spanning amino acids 1 to 396 did not repress pDRA activation to the same extent as overexpression of full-length YY1 protein for any of the amounts of expression vector analyzed (Fig. 5A). The above experiments demonstrate that YY1 is capable of repressing IFN-γ-inducible HLA-DRA promoter activation through specific interactions with the +62/+72 YY1 binding element.

HDAC activity represses IFN-γ-inducible HLA-DRA promoter activation through the +62/+72 YY1 binding element. To determine whether HDACs are capable of repressing HLA-DRA promoter activation through the +62/+72 YY1 binding element, various amounts of an HDAC1 expression vector and a constant amount of either the pDRA or the pDRA-YY1mut promoter-luciferase construct were cotransfected in 12-27 cells. The cells were then treated with IFN-γ for 24 h following transfection. HDAC1 overexpression resulted in a pronounced, dose-dependent reduction in IFN-γ-inducible DRA promoter activation in the presence of an intact YY1 binding site (Fig. 5B). However, mutation of the YY1 binding site abolished repression due to overexpression of this HDAC (Fig. 5B).

To further examine whether the repression of HLA-DRA promoter activation by HDAC activity was dependent on the +62/+72 YY1 binding element, 5637 cells were transfected with either the pDRA or the pDRA-YY1mut promoter-luciferase construct and treated with either IFN-γ alone or both IFN-γ and TSA for 24 h following transfection. The wild-type pDRA promoter-luciferase construct was activated 29.9-fold by TSA and IFN-γ relative to activation by IFN-γ alone (Fig. 5C). However, the ability of TSA to activate the HLA-DRA promoter was significantly reduced by mutation of the YY1 binding site in the pDRA-YY1mut promoter-luciferase construct (Fig. 5C). Taken together, these results indicate that HDAC activity is capable of repressing IFN-γ-inducible acti-

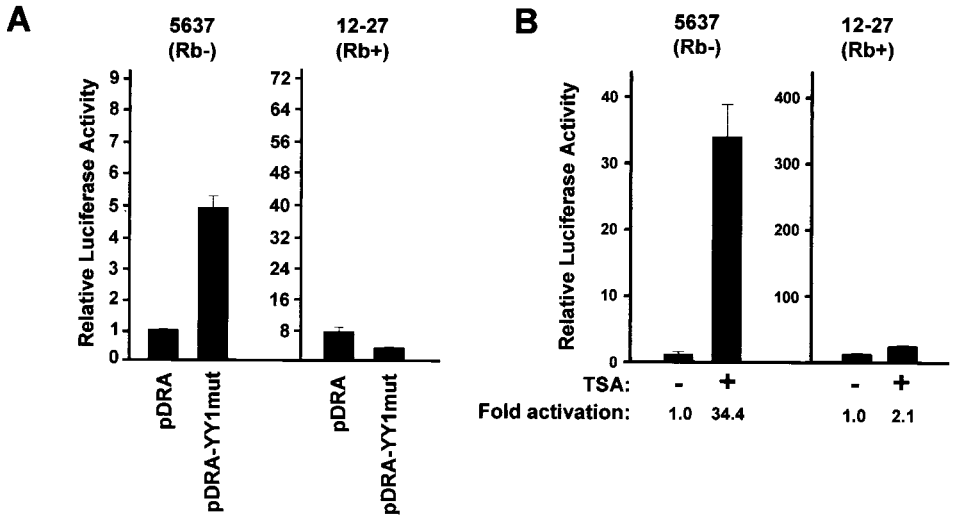

FIG. 6. Repression mediated by the +62/+72 YY1 binding element and HDAC activity is relieved by Rb expression. (A) Rb expression prevents repression of the HLA-DRA promoter mediated by the YY1 binding element. Twenty-five nanograms of promoter-luciferase construct pDRA or pDRA-YY1mut was transfected into Rb-defective (5637) or Rb-transformed (12-27) bladder carcinoma cells. The cells were then treated with 400 U of IFN-γ/ml for 24 h prior to assays for luciferase activity. (B) Rb expression prevents activation of the HLA-DRA promoter by TSA. Twenty-five nanograms of promoter-luciferase construct pDRA was transfected into either Rb-defective (5637) or Rb-transformed (12-27) bladder carcinoma cells treated with 400 U of IFN-γ/ml and, where indicated, 100 nM TSA for 24 h prior to assays for luciferase activity.

vation of the HLA-DRA promoter through the +62/+72 YY1 binding element. However, as mutation of the +62/+72 YY1 binding element does not completely prevent TSA from activating the promoter, it is possible that other HDAC activities also contribute to the repression of IFN-γ-inducible promoter activation in these cells.

The +62/+72 consensus YY1 binding element and HDAC activity confer repression of IFN-γ-inducible HLA-DRA promoter activation that is relieved by Rb expression. To further examine the role of the +62/+72 consensus YY1 binding element in regulating IFN-γ-inducible HLA-DRA expression and to determine whether Rb expression regulates HLA-DRA inducibility through this element, the pDRA and pDRA-YY1mut promoter-luciferase reporter constructs (Fig. 4) were transiently transfected into Rb-defective bladder carcinoma cell line 5637 and its Rb-reconstituted subclone, 12-27 (Fig. 6A). The cells were then treated with IFN-γ for 24 h following transfection. In the Rb-defective 5637 cells, pDRA-YY1mut was derepressed by 5.1-fold relative to the wild-type pDRA promoter-luciferase construct following IFN-γ induction (Fig. 6A). In the Rb-reconstituted 12-27 cells, the pDRA-YY1mut construct was 53% less active in response to IFN-γ than the wild-type pDRA construct (Fig. 6A). These results indicate that the +62/+72 consensus YY1 binding element is capable of repressing IFN-γ-inducible HLA-DRA promoter activation and that Rb expression eliminates this site-dependent repression.

To determine whether Rb expression affects HDAC-mediated repression of IFN-γ-inducible HLA-DRA promoter activation, we transfected Rb-defective (5637) and Rb-reconstituted (12-27) cells with the pDRA promoter-luciferase construct and treated the cells with either IFN-γ alone or both IFN-γ and TSA. Treatment of the Rb-defective (5637) cells with TSA resulted in pronounced HLA-DRA promoter activation (Fig. 6B). However, TSA treatment of the Rb-reconstituted (12-27) cells did not yield significant activation of the promoter (Fig. 6B). These results indicate that HDAC-mediated repression of HLA-DRA promoter activation is reduced in Rb-reconstituted cells.

HDAC inhibitors disrupt Oct-1 binding to the octamer element. Oct-1 interacts with the HLA-DRA octamer element with a high efficiency and represses IFN-γ-inducible HLA-DRA promoter activation in Rb-defective tumor cell lines (65). Thus, we wished to determine whether HDAC inhibitor treatment of 5637 cells could disrupt Oct-1 binding. We performed an EMSA using nuclear protein extracts from Rb-defective (5637) and Rb-reconstituted (12-27) bladder carcinoma cells to examine Oct-1 binding to a −62/−37 HLA-DRA promoter probe encompassing the HLA-DRA Oct-1 binding site. As previously reported (65), untreated 5637 cells demonstrated high levels of Oct-1 binding to the probe (Fig. 7A, lane 2) and 12-27 cells exhibited markedly reduced Oct-1 binding activity (Fig. 7A, lane 1). Extracts from sodium butyrate- and TSA-treated cells also exhibited markedly reduced levels of Oct-1 binding to the probe (Fig. 7A, lanes 3 and 4). The reduction in binding activity seen for Oct-1 was specific, as two other proteins, NF-Y and YY1, were not affected in their ability to bind DNA following treatment of the cells with HDAC inhibitors (Fig. 7B and C, compare lanes 1, 2, and 3). The upper band in Fig. 7A represents Oct-1, as it is supershifted by the addition of both an internal POU linker domain-specific Oct-1 antibody (lane 9) and a C-terminus-specific Oct-1 antibody (lane 8). Furthermore, the upper band comigrates with in vitro-translated Oct-1 protein using the same probe (Fig. 7A, lane 10) and cannot be competed by an oligonucleotide containing a mutation in the octamer element (Fig. 7A, lane 7). However, the lower band in Fig. 7A is a protein that has a binding specificity for the probe different from that of Oct-1, as it is efficiently competed by both wild-type and mutant octamer element competitor oligonucleotides (lanes 6 and 7). Furthermore, it is possible that this protein represents

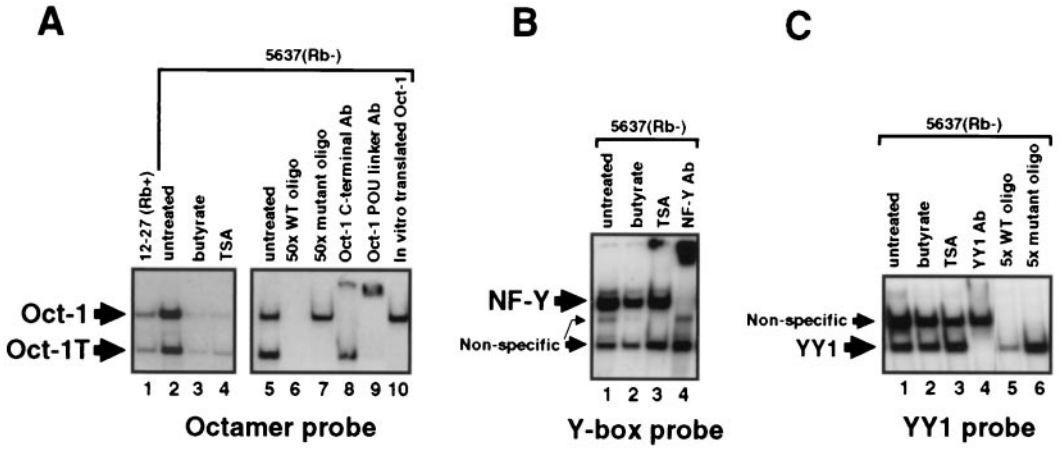

FIG. 7. Oct-1 facilitates repression by HDAC. (A) Oct-1 binding to the HLA-DRA octamer element is disrupted by HDAC inhibitors. EMSA were performed using nuclear protein extracts from Rb-defective bladder carcinoma cell line 5637 and its Rb-reconstituted subclone, 12-27. Each lane represents 3 μg of total nuclear protein and 25 fmol of labeled −62/−37 HLA-DRA octamer element probe. Lanes 1 and 2, extracts from 12-27 and 5637 cells, respectively; lanes 3 and 4, extracts from 5637 cells treated with 1 mM sodium butyrate and 200 nM TSA, respectively, for 72 h prior to the isolation of nuclear proteins. The upper band in the autoradiograph represents the Oct-1 protein-DNA complex, as it is supershifted by both anti-Oct-1 antibodies (Ab) (lanes 8 and 9), comigrates with in vitro-translated Oct-1 added to the binding reaction (lane 10), and is competed only by a wild-type (WT) octamer element oligonucleotide (oligo) (lane 6). The band labeled Oct-1T may represent a truncated isoform of Oct-1, as it is supershifted only by an antibody specific for the Oct-1 POU linker domain (lane 9) and not by an antibody specific for the C terminus of Oct-1 (lane 8). Apparently, the DNA binding specificity of this protein is different from that of Oct-1 as well, as it is competed by both wild-type and mutant octamer element oligonucleotides (lanes 6 and 7). (B and C) Binding of NF-Y to the HLA-DRA Y box and YY1 to the +62/+72 HLA-DRA YY1 binding element is not affected by treatment of cells with HDAC inhibitors. EMSA were performed exactly as described above, except that the HLA-DRA octamer element probe was replaced with either a Y box (B) or a YY1 binding site (C) probe.

a truncated form of Oct-1, as it not supershifted by an antibody specific for the C terminus of Oct-1 (Fig. 7A, lane 8). However, it is supershifted by an antibody specific for the POU linker domain of Oct-1 (Fig. 7A, lane 9).

Repression due to HDAC1 overexpression is dependent on intact YY1 and Oct-1 binding sites. To determine whether the octamer element was capable of repressing IFN-γ-inducible HLA-DRA promoter activation, Rb-defective bladder carcinoma cells (5637) were transfected with either the pDRA or the pDRA-Octmut construct (Fig. 4). The cells were then treated with IFN-γ for 24 h following transfection. Consistent with previous observations (65), mutation of the octamer element in Rb-defective cells resulted in a significant increase in promoter activation following IFN-γ treatment (Fig. 8A). However, consistent with the observed decrease in Oct-1 binding activity in Rb-transformed cells in EMSA (Fig. 7A) (65),

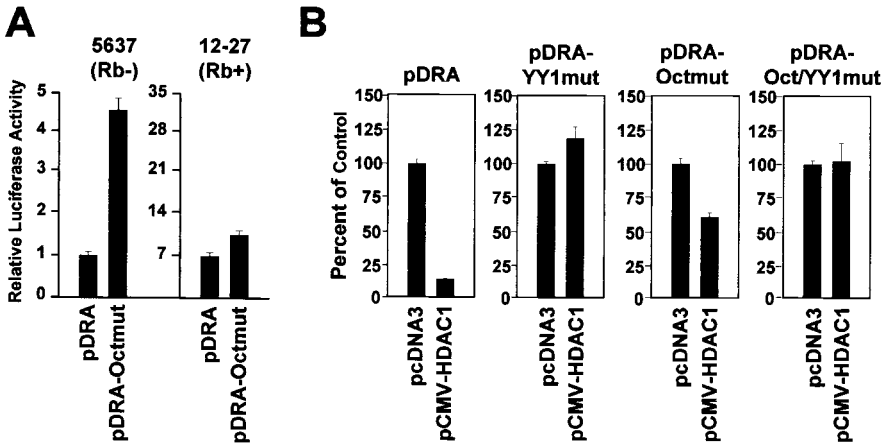

FIG. 8. The octamer element represses IFN-γ-inducible HLA-DRA promoter activation and is required for efficient HDAC1-mediated repression of the promoter. (A) The octamer element represses IFN-γ-inducible HLA-DRA promoter activation in Rb-defective cells.Twenty-five nanograms of construct pDRA or pDRA-Octmut was transfected into Rb-defective (5637) and Rb-transformed (12-27) cells treated with 400 U of IFN-γ/ml for 24 h prior to assays for luciferase activity. (B) The octamer element participates in HDAC1-mediated repression of inducible HLA-DRA promoter activation. Twenty-five nanograms of promoter-luciferase construct pDRA, pDRA-YY1mut, pDRA-Octmut, or pDRA-Oct/YY1mut was cotransfected along with 25 ng of expression vector pCMV-HDAC1 or the corresponding empty expression vector, pcDNA3, into 12-27 cells. The cells were then treated with 400 U of IFN-γ/ml for 24 h prior to assays for luciferase activity. The luciferase activity of each reporter construct in the presence of the control vector, pcDNA3, is shown as 100% in each graph, while the luciferase activity of each reporter in the presence of expression vector pCMV-HDAC1 is shown as a percentage relative to the activity obtained with the control vector.

mutation of the octamer element in Rb-transformed cells was not able to significantly activate the promoter following IFN-γ treatment (Fig. 8A).

To determine whether the octamer element can facilitate HDAC-mediated repression of HLA-DRA promoter activation, we transiently cotransfected 12-27 cells with an HDAC1 expression vector and the pDRA, pDRA-YY1mut, pDRA-Octmut, or pDRA-Oct/YY1mut construct (Fig. 4). The cells were then treated with IFN-γ for 24 h following transfection. As demonstrated in Fig. 5B, the repression of HLA-DRA promoter activation mediated by HDAC1 overexpression was dependent on an intact YY1 binding site, as mutation of this site entirely relieved HDAC1-mediated repression (Fig. 8B). Mutation of the Oct-1 binding site also relieved HDAC1-mediated repression (Fig. 8B). However, the extent of this relief was not as great as that observed following mutation of the YY1 binding site. In sum, the effect of HDAC inhibitors on Oct-1 DNA binding (Fig. 7) and the cotransfection experiment just described (Fig. 8B) indicate that Oct-1 is a candidate HDAC target and may mediate the repressive effects of HDAC activity at the HLA-DRA promoter. The finding that the pDRA-Octmut construct was only partially protected from HDAC1-mediated repression may indicate that additional proteins serve to facilitate HDAC-mediated repression of HLA-DRA promoter activation.

DISCUSSION

HDAC activity represses IFN-γ-inducible HLA-DR gene expression following the establishment of a DNase I-hypersensitive chromatin conformation. We have demonstrated that HDAC activity can repress inducible HLA-DRA promoter activation when Rb expression is lost in human tumor cells. Treatment of Rb-defective tumor cells with HDAC inhibitors rescues high-level IFN-γ-inducible HLA-DR gene expression. Thus, a net decrease in the acetylation state of HLA-DRA and -DRB promoter chromatin, possibly including nonhistone proteins such as Oct-1, likely contributes to the substantially reduced HLA-DRA promoter occupancy and lack of IFN-γ-inducible HLA-DR gene expression in Rb-defective tumor cells. Additionally, we have shown that the HLA-DRA promoter DNase I-hypersensitive site is present in these transcriptionally incompetent, Rb-defective tumor cells prior to treatment with HDAC inhibitors. Thus, the effect of HDAC inhibitors on HLA-DRA promoter activation is occurring within the context of a generally accessible chromatin environment (i.e., an apparently nucleosome-free, or topologically altered, promoter region). Several reports have indicated that nonhistone chromosomal proteins (i.e., transcription factors and components of the RNA polymerase II holoenzyme) are substrates for acetylation (8, 18, 22, 23, 37, 66), which may in turn affect their abilities to facilitate transcriptional activation. Also, both Rb transformation and HDAC inhibitors may serve to further increase histone acetylation at the HLA-DRA promoter, which would serve to further increase the general accessibility of the promoter region to sequence-specific transactivators and to DNase I. Regardless of whether the target of HDAC activity is histone protein or nonhistone protein, these inhibitors facilitate the transition from a stable state of chromatin in vivo that is accessible to DNase I but that does not permit transcriptionally productive occupancy of the promoter by transactivators to a new state whereby promoter occupancy capable of supporting transcription is permissible.

YY1-tethered HDAC activity regulates HLA-DRA promoter activation. The specificity of HDAC activity for certain promoters is believed to be due to the interaction of HDACs with sequence-specific factors bound to the promoter regions of HDAC-regulated genes (55). One such factor, YY1, is a member of the GL1-Krüppel zinc finger family of proteins and interacts with the class I deacetylases (HDAC1, HDAC2, and HDAC3) (63). Indeed, YY1 can repress promoter activation by interactions with HDAC1 (12) and HDAC2 (62). Several of the MHC class II genes possess a YY1 binding element downstream from the start of transcription (21). In the HLA-DRA gene, the consensus YY1 binding element is located in the first exon from +62 to +72 relative to the start of transcription. This element is capable of interacting with YY1 in constitutively expressing (21) and IFN-γ-inducible cell lines (Fig. 3 and Fig. 7C). Interaction of YY1 with the +62/+72 YY1 binding element in bladder carcinoma cells efficiently repressed inducible HLA-DRA promoter activation (Fig. 5A and 6A). Furthermore, TSA stimulated high-level promoter inducibility (Fig. 2A, 5C, and 6B), and overexpression of HDAC1 repressed inducible HLA-DRA promoter activation (Fig. 5B and 8B), but only when the YY1 binding element was intact (Fig. 5B, 5C, and 8B). These data support the ideas that YY1 serves to tether HDAC activity in the vicinity of the HLA-DRA promoter and that the YY1-HDAC1 complex contributes to the maintenance of postnucleosome repression of IFN-γ-inducible HLA-DRA promoter activation in Rb-defective cells (see also discussion of states of HLA-DRA promoter chromatin below).

Rb expression prevents YY1- and HDAC-mediated repression of HLA-DRA promoter activation. The mechanism by which Rb reconstitution prevents HDAC-mediated repression of HLA-DRA promoter inducibility is not yet fully understood. One possible explanation is that Rb may interact with the YY1 interaction domain of HDAC1, thereby preventing simultaneous association of Rb and YY1 with HDAC1. Rb and HDAC1 can associate in vivo, and the domain of HDAC1 that interacts with Rb has been identified (9, 15, 36, 40). However, the domain of HDAC1 that interacts with YY1 is not known. Sequestration of HDAC1 from YY1 by interaction with Rb may then allow for the previously described interaction of YY1 with coactivator proteins such as p300 and CREB binding protein (30). This scenario could account for the observation that, in Rb-transformed cells, mutation of the YY1 binding site results in a reduction in IFN-γ-inducible promoter activation (Fig. 6A). Another possibility is that Rb expression disrupts the binding of a target protein that serves to mediate repression of the promoter by HDAC activity. If the interaction of this protein with the HLA-DRA promoter were to be disrupted by Rb expression, then HDAC could theoretically remain tethered to the promoter and yet not facilitate the repression of IFN-γ-inducible promoter activation. Interestingly, reconstitution of Rb-defective tumor cells with functional Rb results in hyperphosphorylation of Oct-1 and disruption of Oct-1 interaction with the HLA-DRA octamer element (65).

Oct-1 binding and repression of HLA-DRA promoter inducibility. Oct-1 can be posttranslationally modified by phosphor-

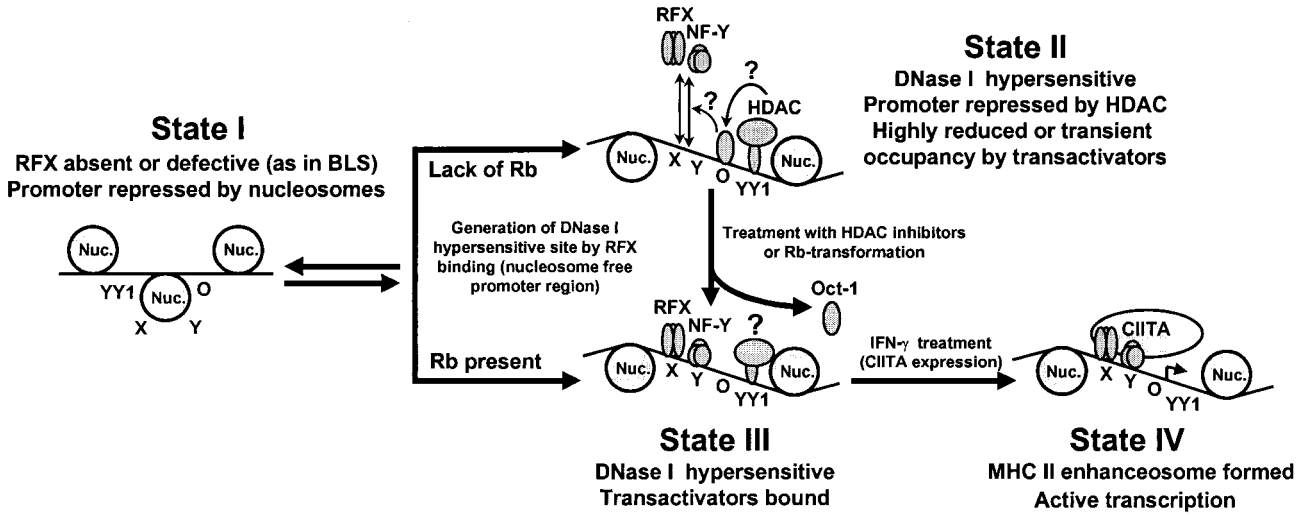

FIG. 9. Proposed model for HLA-DRA promoter chromatin transitioning through four states of chromatin and culminating in transcriptional activation by CIITA. The HLA-DRA promoter, in the absence of RFX, lacks the hypersensitive site and is repressed by association with nucleosomes (Nuc.) (state I). In the presence of RFX, the HLA-DRA promoter is DNase I hypersensitive (presumably because of a nucleosome-free or topologically altered region at the promoter) and is bound by the required transactivator proteins, such as NF-Y (state III). However, if Rb expression is lost, as in several human tumors, the HLA-DRA promoter retains the DNase I-hypersensitive site yet does not exhibit transcriptionally productive binding by the required transactivator proteins (state II). This situation may be due to prevention of individual factors from accessing their cognate binding sites or transient association of these factors with their binding sites. RFX may establish the hypersensitive site in these Rb-defective cells and then dissociate from the promoter because of its low affinity for nonnucleosomal DNA in the absence of NF-Y. The hypersensitive site is then presumably maintained by other sequence-specific repressor proteins associated with the promoter in these cells, because significant interaction of neither RFX nor NF-Y with the promoter can be detected in Rb-defective cells. HDAC activity tethered to the YY1 element may repress promoter activation by targeting, either directly or indirectly, Oct-1 or by targeting histones that continue to repress the promoter following the formation of the hypersensitive site. Treatment of Rb-defective tumor cells with HDAC inhibitors or reconstitution of these cells with functional Rb leads to decreased Oct-1 DNA binding activity (and possibly subtle alterations in promoter topology) and to complete derepression of the promoter (state III). Treatment of these cells with IFN-γ leads to CIITA expression and subsequent stabilization of transactivator binding at the HLA-DRA promoter by the formation of the MHC class II enhanceosome (state IV). This situation then leads to active transcription from the HLA-DRA promoter.

ylation, and this modification has been shown to reduce the binding of Oct-1 to DNA (54). Oct-1 is hypophosphorylated and binds the HLA-DRA octamer element in Rb-defective tumor cell lines (65), consistent with several reports that Oct-1 represses inducible HLA-DRA promoter activity (50, 65) (Fig. 8A). The data presented here indicate that HDAC inhibitors rescue IFN-γ-inducible HLA-DRA promoter activation by disrupting Oct-1 binding activity in Rb-defective tumor cells, although it is not known whether Oct-1 is a direct or an indirect target of HDAC activity. Furthermore, the mechanism of Oct-1 repression of the HLA-DRA promoter is not known. In other promoters, Oct-1 can prevent the activation of transcription by binding to an octamer element that overlaps the binding site for a required activator protein, presumably blocking binding of the activator to the promoter (for example, the interleukin-8 promoter) (61, 65). However, the HLA-DRA octamer element does not overlap any site known to be required for IFN-γ-inducible activation of the HLA-DRA promoter.

We have recently identified a multiprotein complex that interacts very efficiently with the HLA-DRA promoter termed DRAN (HLA-DRA negative). DRAN is specifically present in extracts from Rb-defective, non-IFN-γ-inducible tumor cells (A. Osborne et al., submitted for publication). DRAN is not detectable in Rb transformants of Rb-defective cells and is not detectable in Rb-defective cells treated with HDAC inhibitors (A. Osborne and G. Blanck, unpublished observations). In fact, the presence of DRAN binding activity correlates perfectly with the loss of IFN-γ-inducible HLA-DRA gene expression in every cell line examined to date, including the bladder and non-small-cell lung carcinoma cell lines examined in this study (Osborne et al., submitted). The DRAN complex requires an intact octamer element for formation and contains Oct-1 (Osborne et al., submitted). Furthermore, preliminary evidence suggests that the DRAN complex overlaps the Y box and prevents the binding of NF-Y to the HLA-DRA promoter (Osborne and Blanck, unpublished). Thus, DRAN may mediate the repressive effect of Oct-1. Alternatively, binding of Oct-1 to the HLA-DRA promoter may effect a DNA conformation that inhibits efficient cooccupancy of the promoter by NF-Y (H. Zhang and G. Blanck, unpublished observations). Finally, we have not ruled out the possibility that Oct-1 binds an HDAC that functions independently of YY1-tethered HDAC to repress the HLA-DRA promoter through an unknown deacetylation target.

Activation of the HLA-DRA promoter involves four distinct states of chromatin. Formation of hypersensitive sites in the HLA-DRA promoter region requires interaction of RFX with the X box (17). When RFX is mutated, as in BLS, there are no DNase I-hypersensitive sites at the HLA-DRA promoter (Fig. 9, stage I). As would be expected, RFX mutant B-cell lines exhibit a lack of occupancy at all of the known transactivator binding elements within the HLA-DRA promoter, as determined by in vivo genomic footprinting (26, 27, 28). RFX does not efficiently interact with naked DNA but does efficiently

interact with nucleosome-associated DNA (10). Thus, it is likely that RFX binding to an HLA-DRA promoter–nucleosome complex leads to the formation of a nucleosome-free region, as detected by DNase I-hypersensitive sites, at the HLA-DRA promoter. NF-Y also stabilizes RFX binding to DNA (10, 33). Interestingly, the NF-Y(B) and NF-Y(C) subunits consist of histone fold motifs that may torsionally constrain the promoter DNA to resemble nucleosome-associated DNA. This could explain why RFX interacts efficiently with nucleosomes and with NF-Y-bound DNA. Mutation of the Y box in HLA-DRA promoter constructs stably transfected into B cells results in a loss of occupancy at the X box and at all other activator binding sites, as detected by in vivo footprinting (60), consistent with RFX being unable to efficiently interact with naked DNA.

High-level, transcriptionally productive occupancy of activator binding sites in vivo, however, cannot be solely dependent on the generation of a DNase I-hypersensitive promoter region that forms following RFX interaction with the X box in nucleosome-associated DNA. Rather, the establishment of promoter occupancy by activator proteins is also dependent on the affinity and activity of repressors, such as Oct-1, YY1, or HDACs, at the HLA-DRA promoter. In Rb-defective cells, the lack of transcriptionally productive promoter occupancy, as well as the presence of a DNase I-hypersensitive site in the promoter region, may be explained by increased association of these repressors with the HLA-DRA promoter. The repressor proteins either directly or indirectly prevent or substantially reduce the interactions of transactivators, such as NF-Y, with the promoter DNA while also maintaining the presence of the promoter hypersensitive site (Fig. 9, stage II). The inhibition or reduction of NF-Y binding would then account for the lack of occupancy at all of the other known activator binding sites. The repression of HLA-DRA promoter activation following the formation of the hypersensitive site is maintained by YY1, Oct-1, and HDAC. This conclusion is based on the fact that the factors required for the formation of the hypersensitive site are already present in the Rb-defective, transcriptionally incompetent cells used to assess the repressive roles of these proteins. In sum, HDAC activity mediated by YY1 and the repressive functions of Oct-1 can be mapped to a stable state of chromatin that exists in vivo and that follows the formation of the hypersensitive site. This state of repression, as far as is known, immediately precedes high-level, transcriptionally productive occupancy of the HLA-DRA promoter by required transactivators, such as NF-Y (Fig. 9, stage II). The expression of Rb in tumor cells, however, prevents interactions of repressor proteins, such as Oct-1, with the HLA-DRA promoter, thus favoring the binding of RFX, NF-Y, and X2BP (CREB) to the promoter (Fig. 9, stage III). Treatment of Rb-defective tumor cells with HDAC inhibitors also prevents interactions of repressor proteins, i.e., possible HDAC targets, such as Oct-1, with the promoter and facilitates increased interactions of activator proteins with the promoter (Fig. 9, stage III). Thus, Rb expression and HDAC inhibitors serve to preset the HLA-DRA promoter by favoring transcriptionally productive interactions of the required transactivators with the promoter such that the promoter can respond to activation by CIITA (Fig. 9, stage III).

ACKNOWLEDGMENTS

We thank Guy Beresford for extremely helpful suggestions regarding the chromatin immunoprecipitation experiments. We also thank Donna Eason and Hongkang Xi for helpful discussions and other members of our laboratory for support work pertaining to the preparation of the manuscript. We also thank Jodi Kroger, Mary Beth Colter, and the H. Lee Moffitt Cancer Center and Research Institute Flow Cytometry and Molecular Biology Core Facilities.

This work was supported by American Cancer Society grant CIM 184-01 as well as National Institutes of Health grant R01-CA81497 awarded to G. Blanck.

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