

# YB-1 DNA-binding Protein Represses Interferon $\gamma$ Activation of Class II Major Histocompatibility Complex Genes

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## Summary

Interferon  $\gamma$  (IFN- $\gamma$ ) is the most potent inducer of class II major histocompatibility complex (MHC) genes. This induction is uniquely mediated by three DNA elements in the promoter region of class II MHC genes. One of these DNA elements, Y, contains an inverted CCAAT box. Previously, we have screened a  $\lambda$ gt11 library for Y-binding proteins and identified the YB-1 gene. Here we provide evidence that YB-1 can repress the IFN- $\gamma$  induction of class II MHC promoter as well as the Invariant chain (Ii) gene which also contains a Y element in its promoter. This was demonstrated by cotransfecting a YB-1 expression vector with promoter-reporter gene constructs. As an alternate approach, an efficient transient transfection system was developed which resulted in a >70% transfection efficiency. Transfection of YB-1 by this procedure resulted in the near abrogation of IFN- $\gamma$  induced HLA-DR antigen and mRNA expression. These findings show the functional suppression of class II MHC gene induction by the YB-1 protein.

Class II MHC gene products play a variety of important roles in immune regulation (reviewed in 1–3). These molecules control the acquisition of the mature T cell repertoire and serve as restriction elements for CD4<sup>+</sup> T cells. These functions place the regulation of class II MHC antigens as an important topic in immune regulation. The expression of class II MHC genes is primarily regulated at the level of transcription. In the past few years, we and others have delineated an array of *cis*-acting elements important for optimal class II gene regulation and have identified proteins that bind to these elements. The *cis*-acting regulatory elements of the DRA gene are probably the best analyzed, and include three elements (S, X, and Y) that are also found in other class II MHC promoters (4–7). The X and Y elements constitute the conserved class II box present in the upstream region of all class II MHC promoters studied to date (8, 9). These two elements are separated by a 19–21-bp spacing that is conserved in length but not in sequence. The Y element contains an inverted CCAAT element, and the X element has been functionally divided into two subregions, X1 and X2, based on the separate interactions of these subregions with the RF-X and hXBP-1 recombinant DNA-binding proteins, respectively (10, 11). The S sequence (also known as H, or W/Z which is a larger DNA sequence) is a heptamer sequence located upstream of the class II box (12–17). These

three elements are important for basal gene transcription, and thus far, they are inseparable from elements required for the IFN- $\gamma$  induction of class II MHC genes (12–19).

The YB-1 DNA-binding protein was initially identified by using radiolabeled Y element sequence to screen a  $\lambda$ gt11 expression cDNA library (20). The recombinant YB-1 protein exhibits specificity for the Y element because mutations of an inverted CCAAT sequence in the Y element can abrogate its ability to interact with YB-1. An intriguing feature is the inverse relationship between levels of YB-1 and DRA in IFN- $\gamma$ -activated cell lines. Consequently, we suggested a model wherein YB-1 negatively regulates class II MHC gene expression (20). The findings reported here provide strong evidence to support this model.

## Materials and Methods

**Plasmids.** pSFFV-YB-1 and the control pSFFV-neo expression plasmids (19) are depicted in Fig. 1 A. DRA-chloramphenicol acetyl transferase (CAT)<sup>1</sup> constructs and the control plasmid (CAT-SK+) that lacks DRA sequence have been described in detail previously

<sup>1</sup> Abbreviations used in this paper: CAT, chloramphenicol acetyl transferase; Ii, invariant chain gene;  $\beta$ -gal,  $\beta$ -galactosidase; SFFV, spleen focus forming virus.

(19). 5'Δ-152 contains 152 bp of DRA promoter linked to the CAT reporter gene. DQB2500CAT (12) contains 2400 bp of the DQB promoter linked to the CAT gene and is a kind gift from Dr. Jeremy Boss (Emory University, Atlanta, GA). 790-InCAT contains 790 bp of the invariant chain (Ii) promoter linked to the CAT gene (21). HTK-CAT (kindly provided by Dr. Judy Fridovich-Keil, Emory University) and 77HSP70CAT (provided by Dr. Albert Baldwin, University of North Carolina, Chapel Hill, NC) contain the promoters of human thymidine kinase and the heat shock protein 70, respectively, linked to the CAT gene.

**Transfection of U373-MG for CAT Assays.** The transfection of constructs containing the CAT reporter gene into U373-MG cells was performed as described previously using a gene-pulser electroporation device (Bio-Rad Laboratories, Richmond, CA). CAT analysis has been described previously (19).

**High Efficiency Transfection of U937.** High efficiency electroporation of U937 was performed as follows. U937 cells were grown in RPMI 1640 (CELL GRO) in 10% FCS to  $4 \times 10^5$  cells and transferred to fresh media 18 h before transfection.  $10^7$  cells in 400 μl RPMI 1640 were mixed with 100 μl containing 125 μg of salmon sperm DNA and 20 μg of plasmids, 10 mM Tris, 0.1 mM EDTA, isotonic HeBS (20 mM Hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM Na<sub>2</sub>HPO<sub>4</sub>, and 6 mM dextrose), and transfected in 2-mM gap cuvettes at 150 V and 600 μF with an electroporator (Model 300; BTX Inc., San Diego, CA). 5 min after transfection, cells were spun down and resuspended in 10 ml of RPMI 1640, 10% FCS, and placed in an incubator. Cells were treated with 500 U/ml recombinant human IFN-γ (Sigma Chemical Co., St. Louis, MO) 1–2 h post-transfection. Cells were harvested 15–18 h after treatment and analyzed by Northern hybridization and FACS® (Becton Dickinson & Co., Mountain View, CA).

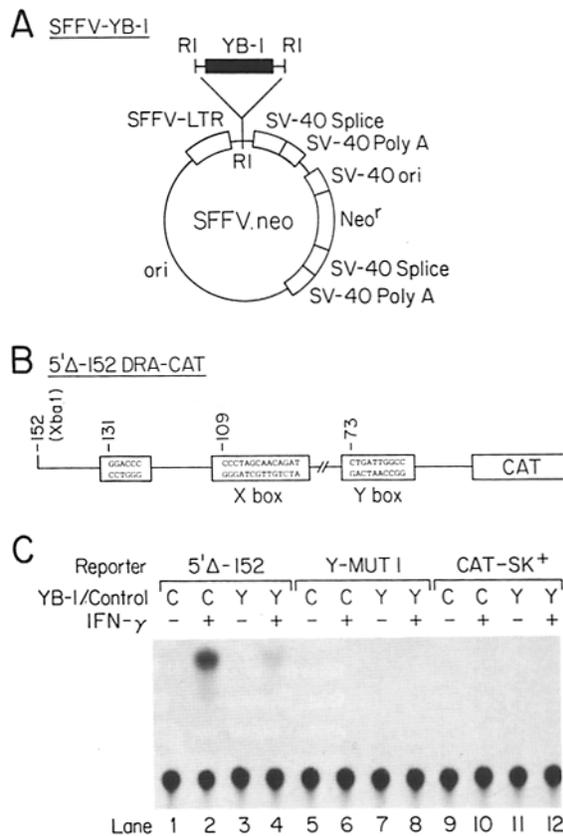
**β-Galactosidase (β-gal) Assay.** Cells transfected with pSV-βGal (Pharmacia LKB, Piscataway, NJ) were assayed for β-gal activity as follows (22). 24 h post-transfection, cells were incubated in 300 μM chloroquine for 30 min. 1.5 ml of cells were spun down and resuspended in 50 μl RPMI 1640, 10% FCS, and 600 μM chloroquine. Cells were warmed to 37°C, and an equal volume of 2 mM fluorescein di-β-galactopyranoside (Sigma Chemical Co.) in 1% dimethyl sulfoxide, 1% ethanol was added and incubated at 37°C for a minute, followed by the addition of 1 ml ice cold PBS. Cells were incubated on ice in the dark for 10 min and analyzed by FACS®.

**Northern Blot and FACS® Assays.** Cells transfected with spleen focus forming virus p(SFFV)-neo and pSFFV-YB-1 were assayed for DRB mRNA expression by Northern blot analysis and HLA-DR surface antigen expression by staining with anti-class II DR monoclonal antibody, L243, and quantitated by FACS®.

## Results

**YB-1 Suppresses Class II MHC Promoter Activity.** The effect of YB-1 protein on IFN-γ-activated DRA promoter function was assayed by introducing an expression vector containing the YB-1 cDNA into an IFN-γ-responsive, human glioblastoma line U373-MG. This line was chosen because our previous analysis and delineation of the class II MHC promoter were performed with this line. In addition, IFN-γ-induced class II MHC gene expression in this line is high (23), better permitting the detection of any suppressive effects of YB-1.

The YB-1 cDNA was subcloned into an expression vector driven by the SFFV long terminal repeat (24). The resulting plasmid is shown in Fig. 1 A. SFFV-YB-1 was cotransfected



**Figure 1.** YB-1 protein suppresses IFN-γ induction of a DRA-CAT reporter construct. (A) The construction of pSFFV-YB-1 was achieved by cloning YB-1 in an expression vector pSFFV-neo (9). (B) A depiction of the reporter construct 5'Δ-152 DRA-CAT (20). (C) The *trans*-activation of 5'Δ-152 by pSFFV-YB-1: YB-1 expression vector (designated Y) or control vector pSFFV-neo (designated C) was cotransfected with 5'Δ-152, Y-MUT1 (a mutant of 5'Δ-152 with a mutated Y box, [19]), or CAT-SK+ (the parent plasmid without the DRA sequence, [19]). Transfection was performed as described previously in the presence (+) or absence (–) of 400 U/ml IFN-γ (19).

with a DRA-CAT construct, 5'Δ-152, (Fig. 1 B) (20). As a negative control, 5'Δ-152 was also cotransfected with pSFFV-neo which lacks the YB-1 sequence. 5'Δ-152 was inducible by IFN-γ as shown previously (Fig. 1 C, lanes 1 and 2). The effect of IFN-γ is mediated through the DRA promoter sequence because removal or mutation of these sequences in the control plasmid CAT-SK+ abrogated IFN-γ induction (Fig. 1 C, lanes 5, 6, 9, and 10). Cotransfection of the control vector, pSFFV-neo, had little effect on either the basal expression or IFN-γ-induced expression of 5'Δ-152 DRA-CAT. In striking contrast, the cotransfection of pSFFV-YB-1 with 5'Δ-152 resulted in a substantial decrease of IFN-γ-induced CAT expression, although there was only a small effect on basal expression (Fig. 1 C, lanes 3 and 4). In the experiment shown, pSFFV-YB-1 reduced IFN-γ-induced CAT reporter gene expression from 21.0 to 6.8×. The average suppressive effect from four separate experiments ranges from 56 to 85% (Table 1). These experiments show that YB-1 can suppress the IFN-γ-induced activation of the DRA promoter.

**Table 1.** *YB-1 Protein Suppresses the IFN- $\gamma$  Induction of DRA, DQB and Ii Promoters*

Experiment no.	Promoter	Reporter construct	<i>trans</i> -acting construct	Percent acetylation $\pm$ interferon	Fold induction by interferon <sup>†</sup>	Percent inhibition by YB-1
1	DRA	5' $\Delta$ -152	pSFFV-YB-1	5.88/0.33	16.9X	74
			pSFFV-neo	36.30/0.55	66.0X	
2			pSFFV-YB-1	2.03/0.30	6.8X	
			pSFFV-neo	7.03/0.35	20.1X	
3	DQB	DQB2500CAT	pSFFV-YB-1	3.74/0.25	15.0X	56
			pSFFV-neo	13.00/0.38	34.2X	
4			pSFFV-YB-1	1.75/0.25	7.0X	
			pSFFV-neo	25.00/0.52	48.1X	
1	DQB	DQB2500CAT	pSFFV-YB-1	4.47/0.80	5.6X	70
			pSFFV-neo	19.00/1.00	19.0X	
2			pSFFV-YB-1	17.00/1.00	17.0X	
			pSFFV-neo	41.00/1.00	41.0X	
1	Ii	790-Ii CAT	pSFFV-YB-1	39.00/2.00	19.5X	37
			pSFFV-neo	62.00/2.00	31.0X	
2			pSFFV-YB-1	12.00/2.00	6.0X	
			pSFFV-neo	29.00/2.00	14.5X	
1	HSP70	-77HSP70CAT	pSFFV-YB-1	1.07/1.14	0.9X	18
			pSFFV-neo	1.11/1.00	1.1X	
2			pSFFV-YB-1	1.07/0.62	1.7X	
			pSFFV-neo	0.98/1.00	1.0X	
1	HTK	HTK-CAT	pSFFV-YB-1	0.84/0.87	1.0X	NI
			pSFFV-neo	0.61/1.00	0.6X	
2			pSFFV-YB-1	1.82/1.44	1.3X	
			pSFFV-neo	0.85/1.00	0.9X	

The experiments were performed as described in the legend to Fig. 1.

\* No inhibition.

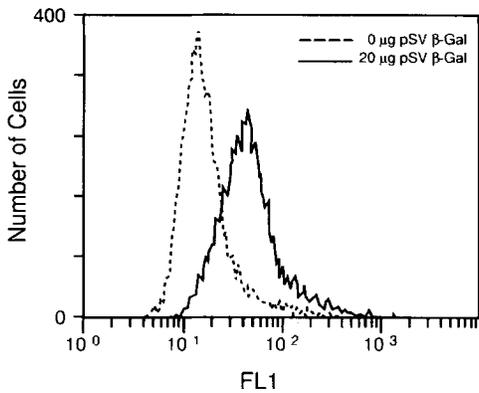
† Percent CAT-mediated acetylation in the presence of IFN- $\gamma$ /Percent acetylation with IFN- $\gamma$ .

To determine if YB-1 could suppress other class II MHC promoters, the effect of YB-1 on IFN- $\gamma$ -induced DQB promoter activity was similarly tested by cotransfecting pSFFV-YB-1 with DQB2500CAT (12). DQB2500CAT promoter contains the S, X, and Y elements that are necessary for IFN- $\gamma$  induction. As shown in Table 1, pSFFV-YB-1 suppressed the IFN- $\gamma$  induction of DQB2500CAT by an average of 65% in two experiments, but had little effect on the basal expression of this plasmid. Furthermore, the effect of YB-1 on the Ii chain promoter was also tested. The Ii chain plays an important role in the presentation of specific antigens due to its ability to target class II MHC antigens to appropriate cellular compartments (25–27). The expression of Ii is coregulated with the class II MHC genes (28, 29). Interestingly, the Ii promoter contains S, X, and Y homologues that mediate both basal and IFN- $\gamma$  inducibility (21, 30–32). pSFFV-YB-1 also suppressed the IFN- $\gamma$  inducibility of a construct (790-

InCAT) that contains the Ii chain promoter linked to a CAT reporter gene.

To ascertain if the suppressive effects of YB-1 are specific to these promoters, and not due to a general downregulation of transcription, the effects of pSFFV-YB-1 on two other unrelated promoters were tested (Table 1). This was tested as described above by cotransfecting pSFFV-YB-1 with a promoter-driven CAT construct. As shown in Table 1, neither pSFFV-neo nor pSFFV-YB-1 effected CAT expression of HTK-CAT or the -77HSP70CAT. This was true regardless of the presence of IFN- $\gamma$ .

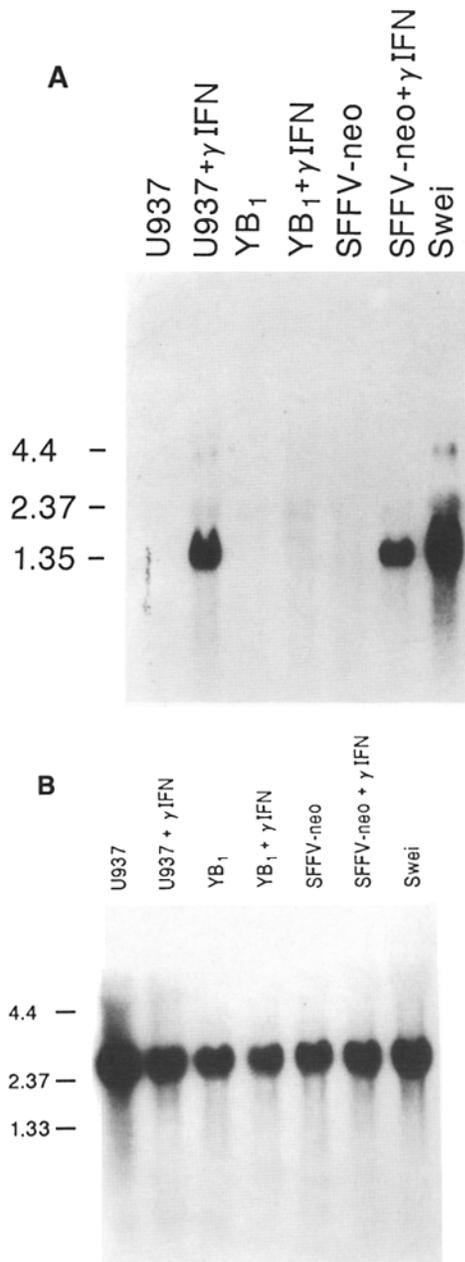
*Development of a High Efficiency Transfection System.* To address the possibility that the usage of a reporter gene system may not reflect the state of the endogenous gene, the suppressive effect of YB-1 on endogenous class II MHC gene expression was assessed. These experiments were made possible by the development of a high efficiency transfection



**Figure 2.** Transfection of U937 results in high-efficiency  $\beta$ -gal gene expression. U937 cells were transfected with pSV  $\beta$ -gal as described in Materials and Methods. The expression of  $\beta$ -gal was assayed by the conversion of fluorescein di- $\beta$ -galactopyranoside to a fluorescent enzymatic product which was measured by FACS<sup>®</sup>. (Dotted line) mock transfected cells. (Solid line) cells transfected with 20  $\mu$ g pSV  $\beta$ -gal.

system. The efficiency of this system was revealed by the percentage of cells that express the  $\beta$ -gal enzyme after transfection with a pSV $\beta$ -Gal construct.  $\beta$ -gal positive cells were scored by the conversion of a nonfluorescent substrate to a fluorescent enzyme product that was quantitated by FACS<sup>®</sup>. As shown in Fig. 2, transfection of U937 with 20  $\mu$ g of pSV  $\beta$ -gal resulted in a complete shift of the curve, and >70% of the cells expressed  $\beta$ -gal at a level significantly above background.

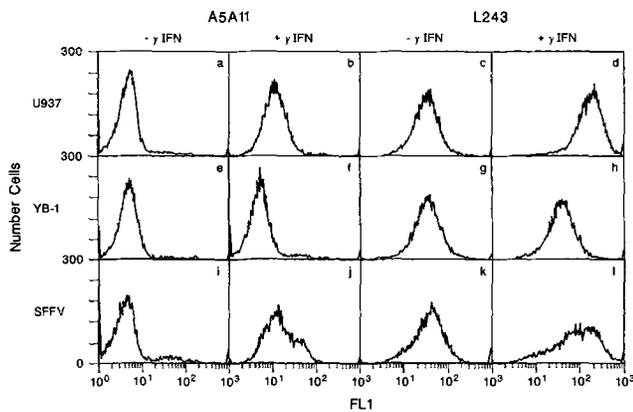
**YB-1 Suppresses IFN- $\gamma$ -induced MHC Class II mRNA and Protein Expression.** Using this protocol, the effect of YB-1 on DRB was assessed. In these experiments, pSFFV-YB-1 was transiently transfected into U937 (20), an IFN- $\gamma$ -responsive human macrophage cell line. These cells were incubated in the absence of IFN- $\gamma$ , and the RNA was isolated and assayed for DRB transcript expression by Northern hybridization analysis. In addition, surface HLA-DR antigen expression on cells from the same experiment was assayed by FACS<sup>®</sup>. As shown in Fig. 3 A, Northern hybridization shows that IFN- $\gamma$  enhanced DRB-specific mRNA in U937 as expected. In contrast, the transfection of pSFFV-YB-1 into U937 greatly diminished the level of DRB mRNA that was induced by IFN- $\gamma$ , although the negative control plasmid pSFFV-neo did not produce such an effect. The difference in DRB mRNA levels is not due to variations in the quantity of RNA in these samples, because the hybridization signals produced by an actin cDNA probe are similar among these samples (Fig. 3 B). Parallel FACS<sup>®</sup> analysis revealed that the transfection of pSFFV-YB-1 into U937 also greatly reduced IFN- $\gamma$ -induced HLA-DR surface antigen expression. Transfection with the negative control pSFFV-neo had little effect (Fig. 4). Likewise, YB-1 did not affect the staining pattern with a negative control antibody (ASA11) significantly. These results have been reproduced 19 times out of 23. Taken together, these results show that YB-1 can suppress IFN- $\gamma$ -induced endogenous class II gene expression.



**Figure 3.** YB-1 protein suppresses the levels of IFN- $\gamma$  induced DRB transcript. (A) U937 cells were mock-transfected (lanes 1 and 2), transfected with pSFFV-YB-1 (lanes 3 and 4) or with pSFFV-neo (lanes 5 and 6). Lane 7 is RNA from Swei, a B lymphoblastoid cell line. 2 h after transfection, some of the samples were treated with 500 U of IFN- $\gamma$  (+  $\gamma$ IFN). All the samples were harvested 24 h later, RNA isolated and analyzed by Northern hybridization with a DRB probe. (B) Identical samples from (A) were hybridized with a  $\gamma$ -actin probe (kindly supplied by Dr. Peter Gunning, Stanford University, Stanford, CA).

## Discussion

The ability of YB-1 to suppress IFN- $\gamma$ -induced class II MHC and Ii chain gene expression has broad implications in immune regulation. IFN- $\gamma$  represents a potentiator of the immune response via its ability to upregulate a number of genes important in immune reactivity, most notably the class



**Figure 4.** YB-1 suppresses IFN- $\gamma$ -induced DR surface antigen expression. Mock-transfected (designated U937), pSFFV-YB-1-transfected (YB-1), and pSFFV-neo-transfected (SFFV) cells from the experiment described in Fig. 3 were analyzed for surface DR antigen expression by immunofluorescence. L243 is a monomorphic anti-DR monoclonal antibody, A5A11 is an isotype-matched negative control monoclonal antibody. FL1 represents fluorescence intensity expressed as an arbitrary unit.

I and class II MHC genes (33). IFN- $\gamma$ -induced Ii gene transcription is immunologically important in light of the role Ii plays in targeting class II antigens to the appropriate endosomal compartment during antigen processing (25–27).

The demonstration that YB-1 suppresses class II MHC gene activation by IFN- $\gamma$  treatment is reminiscent of the findings with IRF-2, although several differences are also evident (34). IRF-2 is a nuclear protein with specificity for the Interferon Response Sequence (IRS also known as ICS for Interferon Consensus Sequence; reference 35, 36) present in the promoter region of class I MHC, type I IFN- $\alpha/\beta$  genes, and a number of IFN- $\alpha/\beta/\gamma$ -inducible genes. The transfection of cDNA-directed IRF-2 can reverse the effect of a positive transcriptional regulator, IRF-1 (34). Both IRF-1 and IRF-2 bind to an identical DNA sequence, thus it has been suggested that this sequence can serve as either a positive or a negative IFN-responsive *cis*-acting element depending on which nuclear factor is interacting with it. Similarly, YB-1 was identified by its ability to recognize a known positive regulatory DNA sequence, the inverted CCAAT box in the Y element of the DRA promoter (20). Homologues of YB-1 have been identified in rat, mouse, cow, and frog by using a CCAAT box-containing DNA probe (37–40). The con-

tention that YB-1 binds to the CCAAT element is further supported by footprinting and gel-shift analyses (39, 41). Functional analysis of a Y element binding protein NF-Y/YEBP by *in vitro* transcription has shown that this protein positively regulates DRA transcription via binding to the CCAAT element of the promoter (42, 43). Thus, it is possible that NF-Y/YEBP (or potentially other Y-box binding proteins) and YB-1 represent a pair of positive/negative regulators of IFN- $\gamma$ -regulated class II MHC gene expression.

Despite the similarities discussed above, the regulation of class II MHC by IFN- $\gamma$  is distinct from either IFN- $\alpha/\beta$  regulated gene expression or IFN- $\gamma$  regulated class I MHC gene expression. For example, IFN- $\gamma$ -regulated class II MHC gene expression requires three separate elements (S, X, and Y) (12–19) with stereospecific and distance constraints (44), whereas IFN- $\alpha/\beta$ -regulated gene expression as well as IFN- $\gamma$ -regulated class I MHC gene expression are primarily mediated by a singular IRS. In addition, proteins that interact with the S, X, and Y elements do not cross-react with proteins that bind to the IRS. Regulatory mutant cell lines that are defective in IFN- $\gamma$  responsiveness also confirm these previous findings (45, 46). Finally, preliminary findings in our laboratory show that YB-1 does not suppress the IFN- $\gamma$  induction of a class I MHC gene, supporting the contention that the induction of class I and class II MHC genes by IFN- $\gamma$  occurs by distinct pathways.

In addition to the CCAAT element, YB-1 also binds to several unrelated sequences as well as single-strand DNA and mRNA (47–53). There are many precedents for the ability of a DNA-binding protein to recognize unrelated sequences (54–56). A possible scenario is that the regulatory effects of YB-1 may vary with the target sequence and YB-1 could serve as either a positive or a negative regulator of gene expression depending on the target sequence (57). In addition, the function of YB-1 may not be limited to transcription, as suggested by others (49, 50) and as suggested by the study of other DNA-binding proteins.

In conclusion, the findings reported here demonstrate for the first time that the YB-1 DNA-binding protein can suppress IFN- $\gamma$ -inducible class II MHC gene expression. The suppressive effect of YB-1 on class II MHC genes is broad in that different class II MHC promoters as well as the coordinately regulated Ii chain promoter are all affected. Considering that IFN- $\gamma$  represents a potent immune modulator via its ability to upregulate class II MHC antigens, the ability of YB-1 to effectively reverse this effect places YB-1 as an important component in the circuit of immune regulation.

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