Repression of Class II Major Histocompatibility Complex Genes by Cyclic AMP Is Mediated by Conserved Promoter Elements

By Lionel B. Ivashkiv, and Laurie H. Glimcher

From the Department of Cancer Biology, Harvard School of Public Health, Boston, Massachusetts 02115; and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115

Summary

The induction of cell surface expression of class II major histocompatibility complex (MHC) antigens by interleukin (IL)-4 and interferon (IFN)-γ is inhibited by elevation of intracellular cyclic (c)AMP, which is caused by immunomodulatory agents such as E series prostaglandins (PGEs). To investigate the mechanism of this downregulation, we have analyzed the consequences of elevating intracellular cAMP on cell surface expression, mRNA levels, and promoter activity of the murine Aα and Eβ class II MHC genes. Elevation of cAMP resulted in a coordinate repression of both basal and inducible Aα and Eβ expression. 151 and 192 base pairs of Aα and Eβ promoter sequence, respectively, were sufficient for conferring repression by cAMP on a reporter gene. A mutational analysis of the Aα promoter revealed that cAMP downregulation is mediated by the conserved S and X1 DNA elements, which are also necessary for induction by cytokines. Downregulation by cAMP was not dependent on an intact X2 site, which is identical in sequence to the CRE element which mediates the positive regulation of several genes by cAMP. These results identify the DNA elements which mediate repression of class II MHC genes by cAMP and show that the same DNA sequences can mediate both positive and negative regulation of class II MHC expression.

Activation of lymphocytes and macrophages is important in the pathogenesis of autoimmune disease. These activated cells are found at inflammatory sites and synthesize numerous effector molecules, such as cytokines, proteases, and prostaglandins (1). An important molecular event in the activation of lymphocytes and macrophages is the induction of class II MHC antigens. Class II MHC antigens are cell surface heterodimeric proteins which present antigens to CD4 positive T lymphocytes and thus play a central role in the regulation of immune responses (2). Class II is usually expressed, at low levels, only on antigen presenting cells such as B lymphocytes or macrophages, and is induced by activating stimuli. Aberrant expression of class II can be induced on many cell types and is characteristic of several autoimmune processes. For example, class II antigens are expressed on synoviocytes in rheumatoid arthritis and on renal tubule cells in autoimmune MRL/*lpr* mice (3, 4).

Class II MHC expression is usually coordinately regulated, primarily at the level of transcription of class II MHC genes (reviewed in references 5 and 6). Most work has focused on understanding basal class II gene transcription and induction by activating signals. Class II genes are activated by many stimuli, including crosslinking of sIg, IL-4, and LPS in B lym-

phocytes and IFN-γ, TNF-α, and granulocyte macrophage-CSF in macrophages. IFN-γ is the most potent inducer of aberrant class II gene expression. Work from many laboratories has shown that conserved DNA sequences, termed S (also known as W or H), X1, X2, and Y boxes or elements, are present in all class II gene promoters which have been sequenced and are important for the basal expression and induction of class II genes. Several genes encoding DNA binding proteins which bind to the X1, X2, and Y elements have been cloned, but the mechanism of induction of class II genes is not known (5, 6).

Physiologic mechanisms for the resolution of inflammation are not well understood, but likely include deactivation and the prevention of activation of lymphocytes and macrophages. An important component of deactivation would be downregulation or prevention of induction of class II expression. Indeed, agents which suppress immune and inflammatory responses, such as TGF-β, glucocorticoids, and E series prostaglandins (PGEs)[1] (which act through elevation of in-

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[1] *Abbreviations used in this paper:* CRE, cAMP response element; PGE, prostaglandin E; PK-A, protein kinase A.

tracellular cAMP), downregulate class II MHC expression (5, 6). The mechanisms involved and the DNA elements which mediate negative regulation of class II are not known. PGEs and cAMP have been shown to prevent induction of cell surface expression of class II by IL-4 and IFN-γ (7–9). In addition, cAMP has numerous suppressive effects on immune and inflammatory processes, including inhibition of T lymphocyte proliferation (10), inhibition of IL-2, IFN-γ, and TNF-α expression (11–13), and inhibition of expression of the protease stromelysin (14). This suggests that agents which increase intracellular cAMP may be useful as therapeutic agents for autoimmune diseases, and, indeed, subcutaneous administration of PGEs resulted in increased survival in autoimmune MRL/*lpr* and NZB mice (15).

Significant progress has been made over the last several years in understanding the positive regulation of genes by cAMP. For several well studied genes, the stimulation of transcription by cAMP is mediated by a consensus DNA sequence TGACGTCA, termed the cAMP response element (CRE). Transcription factor CRE-binding protein (CREB), which binds to the CRE, is phosphorylated after elevation of intracellular cAMP and activates transcription of cAMP inducible genes (16). The repression of several genes by cAMP has been described, but the DNA sequences and transcription factors which mediate this effect have not been characterized (11–14, 17). In this study, we investigated the coordinate repression of basal and IFN-γ–induced expression of the murine Aα and Eβ class II MHC genes by cAMP. We found that the proximal promoter sequences of both the Aα and Eβ genes were sufficient to mediate cAMP repression. Mutational analysis of the Aα promoter revealed that the conserved S and X1 DNA elements which mediate basal expression and induction of class II genes by cytokines also mediate repression of these genes by cAMP. Our data suggest that cAMP downregulates class II gene expression by inhibiting or altering positive transcription factors which bind to class II promoters and open a new avenue for investigating positive and negative regulation of class II genes.

Materials and Methods

Cell Lines. Cell lines were grown in RPMI 1640 medium supplemented with 8% FCS, 100 μg/ml penicillin and streptomycin, and 10^{-4} 2-ME. M12.4.1 cells are derived from a mature B cell lymphoma and P388D1 is a macrophage cell line, as previously described (18). Both are of the d haplotype.

Flow Cytometric Analysis. Flow cytometric analysis was performed using a Becton Dickinson FACS analyzer (Becton Dickinson and Co., Mountain View, CA). The following murine mAbs were used: K24-199 (anti-I-Aα), MK-D6 (anti-I-Aβ), 14-4-4 (anti-I-Eα), 34-1-4 (anti-I-Eβ), 34-2-12 (anti-H-2D), and 28-14-8 (anti-H-2L). The rat mAb directed against murine CD18, JB379, was the gift of Dr. T. Springer (Harvard University). Cells were washed with HBSS and incubated with 30 μl of a previously titrated mAb for 20 min on ice. Controls were performed using no primary antibody or an isotype matched mAb directed against a noncrossreacting haplotype of I-A or H-2 proteins. Cells were then washed twice with FACS media (HBSS supplemented with 3% FCS and 0.1% Na azide) and incubated with fluorescein conjugated

goat F(ab)$_2$ fragment anti–mouse or anti–rat immunoglobulin (Cappel). After two washes with FACS media, cells were analyzed on the FACS analyzer.

Preparation of Cellular RNA and Northern Hybridization Analysis. Total cellular RNA was isolated after disruption of cells in 4.4 M guanidium isothiocyanate and extraction with acid-phenol exactly as described (19). 20 μg of RNA were fractionated on 1.2% formaldehyde agarose gels and transferred to Nytran membranes (Schleicher and Schuell, Keene, NH). The membranes were then prehybridized, hybridized, and washed as recommended by the manufacturer. Hybridization was with nick translated cDNA probes: I-Aα 1.3-kb Hind III fragment (18), I-Eβ 0.5-kb Pst I fragment (20), and A50 0.9-kb Pst I fragment (21).

Plasmids and Mutagenesis. Standard recombinant technology was used (22). PCR was used to amplify promoter sequences corresponding to base pairs −151 to +48 of the Aα^k promoter and −192 to +44 of the Eβ^d promoter from subcloned genomic sequences (20, 23). Nucleotide positions in the Aα promoter are numbered according to reference 24. The PCR primers introduced unique Hind III and Xba I restriction endonuclease sites which allowed subcloning of these promoter fragments into vector Basic CAT (Promega), which contains the CAT gene but no promoter or enhancer sequences. The nucleotide sequence of both strands of each insert was confirmed using the dideoxynucleotide method with Sequenase, as recommended by the manufacturer (U.S. Biochemical).

Deletion mutations of the Aα promoter were obtained by using PCR to amplify appropriate sequences. In each case, an Xba I site was introduced at the same 3' end and a Hind III site was introduced at the appropriate 5' end. Substitution mutations of the Aα promoter were obtained by using PCR to amplify two DNA fragments which when cut with Xho I and ligated, reconstitute the Aα promoter sequences −151 to +48 with the appropriate base substitutions and with Hind III and Xba I ends. The DNA fragments generated by PCR were subcloned into vector Basic-CAT and the nucleotide sequence of both strands of each insert was confirmed. The sequences of PCR primers used are available on request. The plasmid RSV-CAT was the gift of Dr. P. Mellon (Salk Institute).

The expression plasmid CMVCα expressing the catalytic subunit of protein kinase A driven by the CMV promoter was the gift of Dr. M. Uhler (University of Michigan Medical Center). The control expression plasmid CMVC$\alpha\Delta$ was constructed by deleting 400 bp of catalytic subunit coding sequence by cutting with Bgl II, filling in the recessed termini with the Klenow fragment of DNA polymerase I, and religating.

Transfections. M12.4.1 and P388D1 cells were transfected using the DEAE-dextran method as previously described (25). All samples were done in duplicate and tested in at least three independent experiments. Pilot experiments showed that normalization for transfection efficiency using cotransfected plasmid pCH110 encoding β-galactosidase was equivalent to normalizing for protein content of extracts. Subsequent transfections were normalized for protein content using a commercially available kit (Biorad). Each sample was transfected with a total of 12.5 μg of DNA in a 2:1 molar ratio of expression plasmid: reporter plasmid. 8-Br cAMP (Sigma Chemical Co., St. Louis, MO) was added to appropriate samples at a final concentration of 1 mM 2 h post transfection. IFN-γ (Amgen) was added to a final concentration of 10 U/ml 24 h after transfection. M12.4.1 cells were harvested 48 h after transfection and P388D1 cells harvested 72 h after transfection, and CAT activity was assayed and quantified using thin layer chromatography and liquid scintillation counting as described (25).
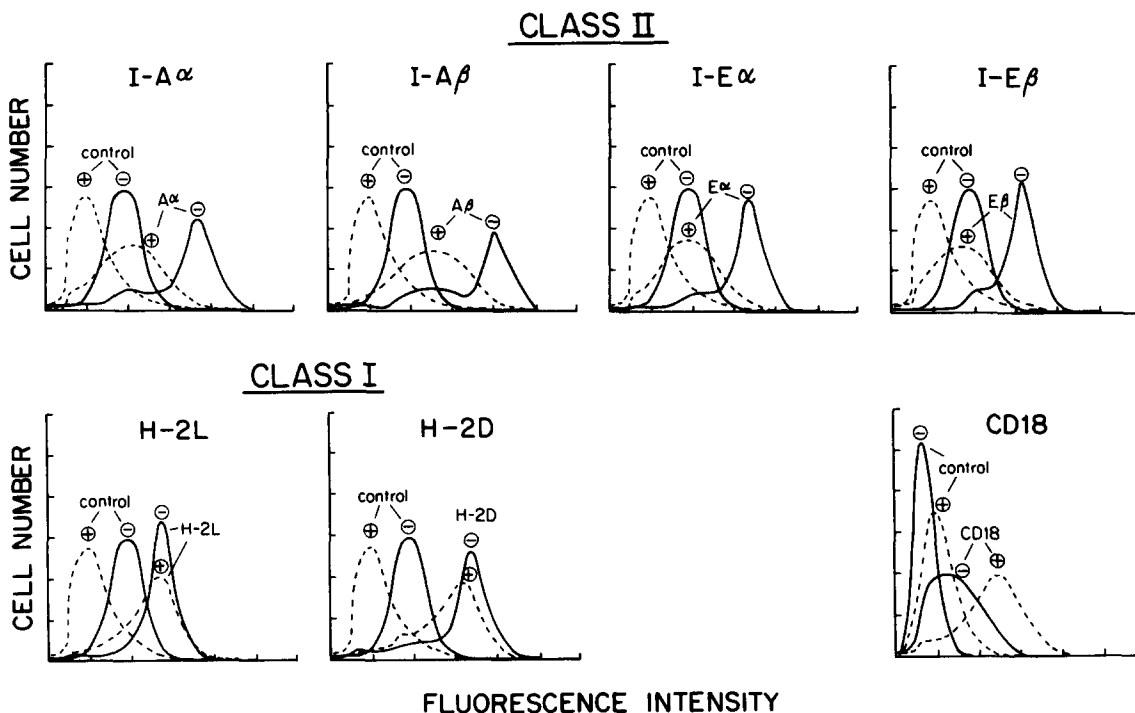
Figure 1. Flow cytometric analysis of M12.4.1 cells cultured with 8-Br cAMP. M12.4.1 B lymphoma cells (d haplotype) were analyzed for cell surface expression of class II MHC (I-A) and class I MHC (H-2) molecules using murine mAbs, and for CD18 expression using a rat mAb (as detailed in Materials and Methods). Fluorescein conjugated goat F(ab)$_2$ fragments anti-mouse or anti-rat immunoglobulin were used as secondary reagents. In control samples no primary antibody or an irrelevant primary antibody of the same isotype were used. Identical results were obtained and only one set of control histograms is shown. M12.4.1 cells were analyzed after growth for 7 d in media alone (_____) or with 1 mM 8-Br cAMP (____).

Results

Increased Intracellular cAMP Decreases Cell Surface Expression of Class II Major Histocompatibility Complex Proteins. Previous results demonstrated that cAMP prevented induction of cell surface expression of class II proteins by IL-4 and IFN-γ (7–9). In order to simplify the analysis of this repressive effect, we wished to determine if cAMP also decreased constitutive class II cell surface expression. The class II MHC positive M12.4.1 B cell line was cultured in the presence of 1 mM 8-Br cAMP (a cAMP analog which is not a substrate for phosphodiesterase) and surface expression of the four "classical" class II MHC proteins (Aα, Aβ, Eα, Eβ) was analyzed (Fig. 1). Surface expression of all four class II proteins was significantly reduced by treatment with 8-Br cAMP. The low level of background staining also decreased upon treatment with 8-Br cAMP, but to a lesser extent than class II specific staining. Thus, a significant number of treated cells were class II negative (the dotted curves overlap significantly) while untreated cells were mostly class II bright (the peaks of the solid curves are nonoverlapping). The low level of background staining was seen only when a murine (and not rat; see Fig. 1, CD18 panel) secondary FITC antibody was used, and most likely reflects the direct binding of murine secondary antibody to surface IgG (L. Ivashkiv, unpublished data). However, because of the change in background, we wished to further explore the specificity of the downregulation of cell surface class II molecules, and therefore determined the M12.4.1 cell surface expression of class I and CD18 proteins after 8-Br

cAMP treatment (Fig. 1). Unlike class II, cell surface expression of class I H-2L and H-2D did not decrease any more than background after 8-Br cAMP treatment, and cell surface expression of CD18 (detected with a rat mAb) was actually slightly induced. Therefore, treatment of M12.4.1 cells with 8-Br cAMP results in a specific and coordinate downregulation of cell surface class II molecules.

Decreased Cell Surface Expression of Class II Is Associated with a Decrease in mRNA. We determined the effect of 8-Br cAMP on class II MHC Aα and Eβ mRNA levels using Northern blot hybridization (Fig. 2). Aα and Eβ mRNA levels were significantly diminished after 8-Br cAMP treatment, but the mRNA levels for the A50 control housekeeping gene did not change significantly. Aα and Eβ mRNA levels increased after the media was changed and the 8-Br cAMP was removed (Fig. 2, lane *3*). Thus, the cAMP induced decrease in surface expression of class II correlates with a decrease in levels of class II mRNA.

Proximal Promoter Sequences of the Aα and Eβ genes Are Sufficient for Repression by cAMP and Protein Kinase A. To determine the effect of cAMP on class II gene promoter activity, we fused promoter sequences containing 151 bp of 5' flanking Aα^k DNA (23, 24) and 192 bp of 5' flanking Eβ^d DNA (20) to the bacterial CAT reporter gene in the vector Basic-CAT (Fig. 3 *A*). These constructs are similar to those used to study the cytokine inducibility of these genes (5, 6, 24) and transcription has been shown to initiate accurately
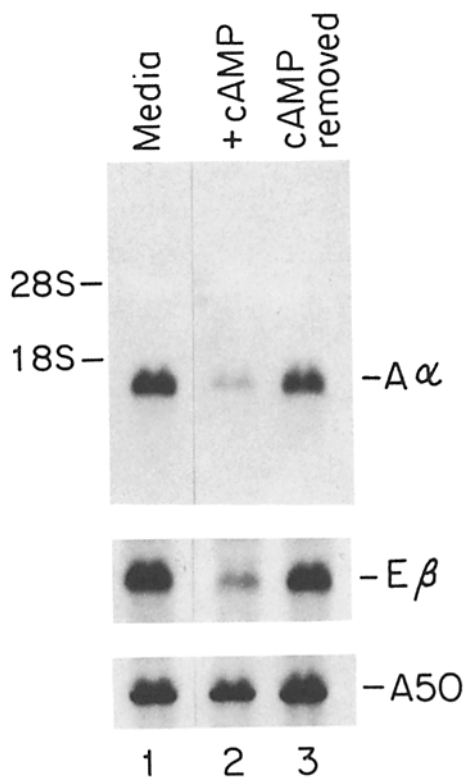
Figure 2. Northern blot analysis of RNA from M12.4.1 cells cultured with cAMP. RNA was isolated from M12.4.1 cells after 7 d of treatment with 1 mM 8-Br cAMP and 2 d after 8-Br cAMP was removed. 20 μg of RNA were fractionated on 1.2% formaldehyde agarose gels, blotted, and the same membrane sequentially hybridized with radiolabeled cDNA probes encoding Aα, Eβ, or the housekeeping gene A50.

(M. Z. Whitley, unpublished data). Promoter activity was measured by transfecting the Aα151-CAT and Eβ192-CAT constructs into M12.4.1 cells and assaying CAT activity. The constructs containing Aα and Eβ promoter sequences resulted in dramatically higher CAT activity than the Basic-CAT vector in M12.4.1 cells (Fig. 3 *B*, lanes *1*, *3*, and *5*) and this increased activity was cell type specific and correlated with endogenous class II MHC gene expression (not shown). Incubation of transfected M12.4.1 cells with 8-Br cAMP resulted in a 72.9% and 82.3% decrease in the activity of the Aα151-CAT and Eβ192-CAT constructs, respectively, but not of the RSV-CAT control (Fig. 3 *B* shows a representative experiment; % repression are those calculated based on duplicate samples tested in at least three independent experiments).

cAMP acts by binding to the regulatory subunit of protein kinase A (PK-A) and causing the release of free catalytic subunit. A common experimental approach for mimicking elevations in intracellular cAMP is to overexpress free PK-A catalytic subunit. Fig. 3 *C* shows the results of cotransfections of M12.4.1 cells with reporter constructs and an expression plasmid encoding PK-A catalytic subunit. Overexpression of the catalytic subunit (+PK-A) resulted in a >85% reduction of Aα151-CAT and Eβ192-CAT activity, but not of the RSV-CAT control. (Control lanes [−PK-A] were cotransfected with the same expression plasmid encoding a mutated catalytic subunit). These experiments show that the
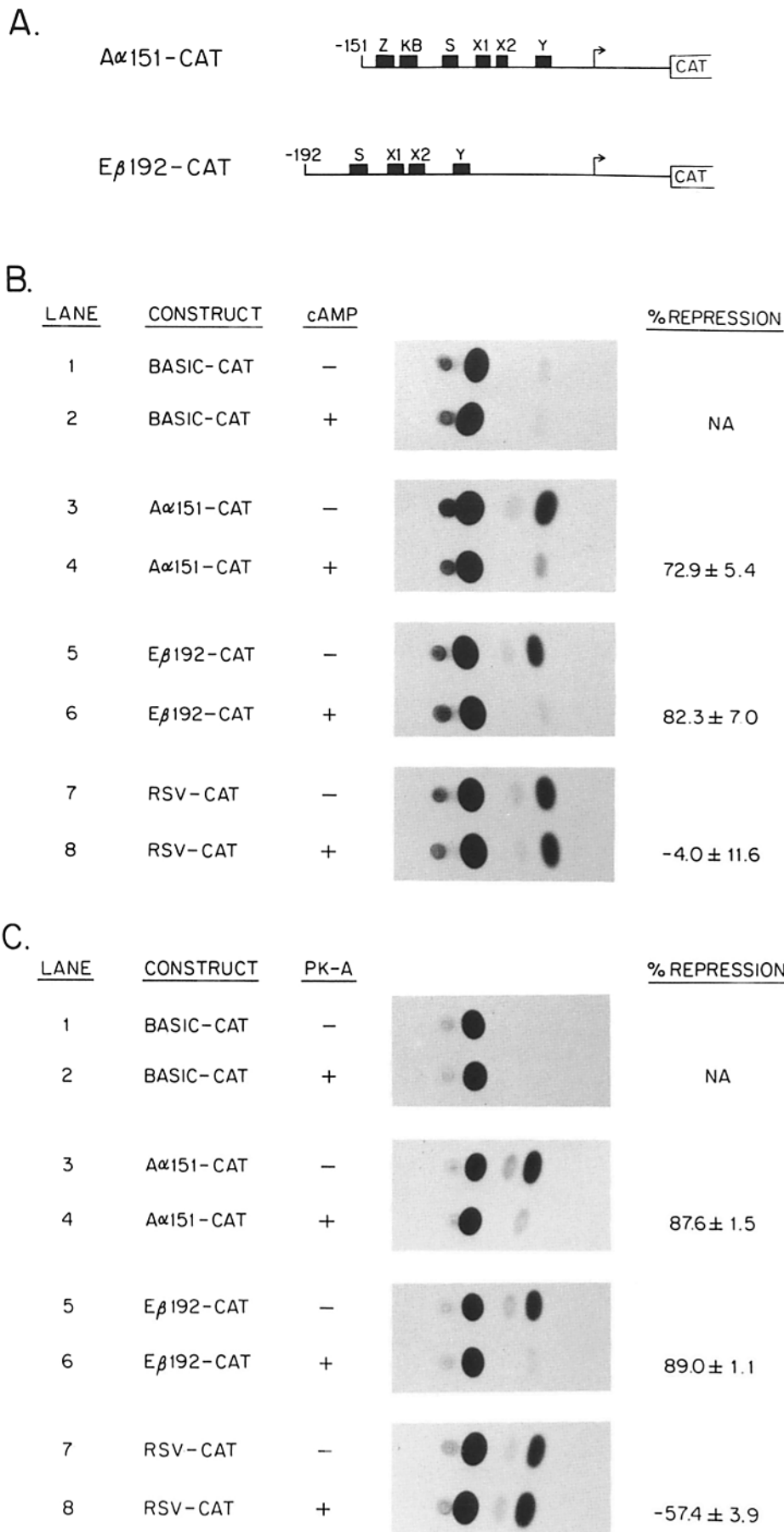
Aα151-CAT and Eβ192-CAT reporter genes accurately reflect the regulation of endogenous class II genes and that the proximal promoter sequences of the Aα and Eβ genes are sufficient to confer repression by cAMP onto a reporter gene.

We also tested the ability of protein kinase A to repress the IFN-γ–induced activity of Aα151-CAT and Eβ192-CAT in the macrophage-like P388D1 cell line (Fig. 4). CAT activity in cells transfected with Aα151-CAT or Eβ192-CAT was induced 22.7-fold and 26.0-fold, respectively, by IFN-γ, consistent with data from other laboratories (5, 6). Activity of the plasmid Basic-CAT, which does not contain any class II promoter sequences, was not affected by IFN-γ (Fig. 4, lanes *1* and *2*). Cotransfection with PK-A resulted in a 60% inhibition of IFN-γ–mediated induction of Aα151-CAT and a 78% inhibition of the induction of Eβ192-CAT activity. Thus the class II proximal promoter sequences mediate cAMP repression of both basal and inducible class II expression, and this effect is evident in two different cell lines.

Mutagenesis of Aα Promoter and Analysis of the Effect of Deletion and Substitution Mutations on Basal Aα Promoter Activity. We wished to identify the DNA sequences or transcription elements within a class II gene promoter which mediate repression by cAMP. We used PCR to generate panels of deletion and substitution mutations in the Aα promoter. The details of construction are described in Materials and Methods. Fig. 5 shows the wild type Aα promoter and the mutations which were created. The S (also termed W or H; reference 24), X1, X2, and Y elements are present in all class II gene promoters and their function in basal and inducible promoter activity has been well documented (5, 6). In addition, the Aα promoter contains the Z motif and a putative NF-κB binding site whose role in class II expression is not well characterized (24). Mutations were constructed such as to sequentially delete one or two known functional elements. Substitution mutations were introduced into the S, X1, X2, and Y elements; an additional construct contains substitution mutations in both S and X1 elements (Fig. 5).

M12.4.1 cells were transfected with wild type and mutant constructs and CAT activity was assayed. Fig. 6 shows the effect of the mutations on CAT activity. These results agree in general with the analysis of the Aα^d promoter by Dedrick and Jones (24) and provide new information about the effects of mutation of only the X2 element and double mutations of both S and X1. Small differences from the Dedrick and Jones data (24) are most likely secondary to differences in exact constructs and mutations created, but may be secondary to using a different cell line or to haplotype specific differences in promoter activity (26).

The Conserved S and X1 Promoter Elements Mediate Repression of Aα Promoter Activity by cAMP. The various Aα promoter deletion and substitution mutants were assayed for their ability to be repressed by cAMP. M12.4.1 cells were cotransfected with promoter constructs and expression plasmids encoding wild type or mutant PK-A and CAT activity was assayed. The results have been normalized to the amount of repression seen with the wild type plasmid Aα151-CAT in each experiment. This was done to facilitate averaging of results from several experiments, since the percent repression of the

A.

Aα151-CAT

-151   Z   KB   S   X1 X2   Y   CAT

Eβ192-CAT

-192   S   X1 X2   Y   CAT

B.

LANE	CONSTRUCT	cAMP		% REPRESSION
1	BASIC-CAT	−		
2	BASIC-CAT	+		NA
3	Aα151-CAT	−		
4	Aα151-CAT	+		72.9 ± 5.4
5	Eβ192-CAT	−		
6	Eβ192-CAT	+		82.3 ± 7.0
7	RSV-CAT	−		
8	RSV-CAT	+		−4.0 ± 11.6

C.

LANE	CONSTRUCT	PK-A		% REPRESSION
1	BASIC-CAT	−		
2	BASIC-CAT	+		NA
3	Aα151-CAT	−		
4	Aα151-CAT	+		87.6 ± 1.5
5	Eβ192-CAT	−		
6	Eβ192-CAT	+		89.0 ± 1.1
7	RSV-CAT	−		
8	RSV-CAT	+		−57.4 ± 3.9

Figure 3. cAMP and protein kinase A can repress the activity of reporter plasmids containing class II MHC gene promoters. (*A*) The structure of the Aα151-CAT and Eβ192-CAT reporter containing class II Aα and Eβ promoter sequences subcloned into vector Basic-CAT. The S (also termed W or H; references 5 and 24), X1, X2, and Y elements are conserved sequences found in all class II MHC promoters. (*B*) M12.4.1 B lymphoma cells were transfected with reporter constructs and appropriate samples were treated with 1 mM 8-Br cAMP beginning 2 h after infection. Cells were harvested 48 h post transfection and CAT activity assayed by thin layer chromatography and liquid scintillation counting. (*C*) M12.4.1 cells were cotransfected with reporter constructs and a twofold molar excess of an expression plasmid encoding wild type (+PK-A) or mutated (−PK-A) protein kinase A catalytic subunit. *B* and *C* show representative experiments. The values for % repression ± SE were calculated using data from duplicate samples from at least three independent experiments. NA = no significant repression of the low level CAT activity generated by Basic-CAT was detected.

LANE	CONSTRUCT	IFN-γ	PK-A		FOLD INDUCTION	% REPRESSION (MEAN) (RANGE)
1	BASIC-CAT	−	−			
2	BASIC-CAT	+	−		NA	
3	BASIC-CAT	+	+		NA	NA
4	Aα151-CAT	−	−			
5	Aα151-CAT	+	−		22.7±2.8	
6	Aα151-CAT	+	+		9.0±2.3	60.4 (45.2–80.1)
7	Eβ192-CAT	−	−			
8	Eβ192-CAT	+	−		26.0±6.2	
9	Eβ192-CAT	+	+		5.8±0.8	77.7 (69.6–86.4)

Figure 4. Protein kinase A inhibits the induction of class II MHC Aα and Eβ promoter activity by IFN-γ. The P388D1 macrophage cell line was cotransfected with reporter constructs and a two-fold molar excess of an expression plasmid encoding wild type (+PK-A) or mutated (−PK-A) protein kinase A catalytic subunit. rIFN-γ (Amgen) was added to a final concentration of 10 U/ml 24 h post transfection and cells were harvested 72 h post transfection and CAT activity assayed. A representative experiment is shown; values for fold reduction ± SE and % repression were calculated using data from duplicate samples from at least three independent experiments. NA = No significant induction of low level CAT activity generated by Basic-CAT was detected.

A.

B.
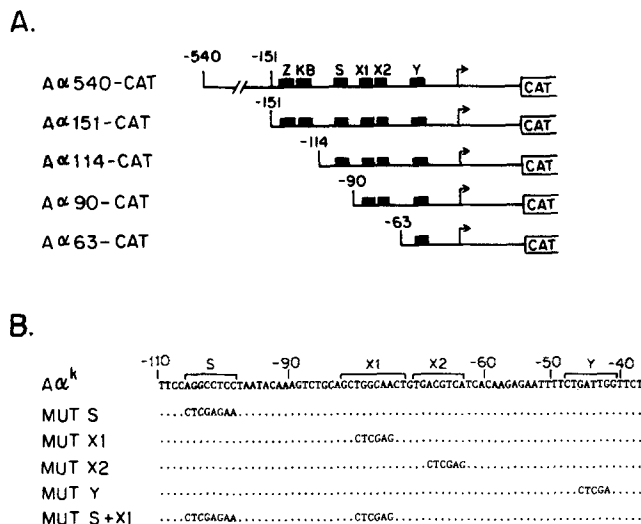
Figure 5. Mutagenesis of the class II MHC Aα promoter. (A) Structure of constructs containing progressive deletions of 5' flanking Aα sequence. The S (also termed W or H; reference 24), X1, X2, and Y elements are conserved sequences found in all class II MHC gene promoters. (B) Substitution mutations in the Aα promoter. Aα 5' flanking sequence from −110 to −38 and specific nucleotide substitutions introduced into the S, X1, X2, and Y elements are shown. Substitution mutations were made in the context of the Aα151-CAT construct. All constructs were verified by sequencing of both strands.

Aα151-CAT construct varied slightly from one experiment to the next (range 84.6–89.3%).

Fig. 7 shows the analysis of the deletion mutations. A construct which contains 114 bp of 5' flanking sequence was fully repressed by PK-A, but deletions of the S element (compare Aα114-CAT to Aα90-CAT) and X elements (compare Aα90-CAT to Aα63-CAT) progressively diminished the ability of PK-A to repress promoter activity. Three lines of reasoning suggest that this effect is not merely secondary to the lower basal activity of the deleted constructs: (a) deletion of the Z and κB elements significantly lowered basal activity but did not affect repression. (b) There was no common baseline level to which all constructs are repressed; instead, the level of CAT activity under +PK-A conditions varied according to construct (a combination of data in Figs. 6 and 7, not shown). (c) When upstream sequences were deleted, the Aα63 construct, which had the lowest basal activity, was actually induced by PK-A. Thus, these results suggest that the conserved S and X promoter elements mediated repression by PK-A. To confirm this, we tested the panel of substitution mutations (Fig. 8). Mutations in the S and X1 elements, but not the X2 element (which matches a consensus CRE), decreased repression by PK-A to 59.2% and 37.4%, respectively, of the repression seen with the wild type promoter. The Y element mutation decreased promoter activity to a low level

A.

B.

A⍺151-CAT
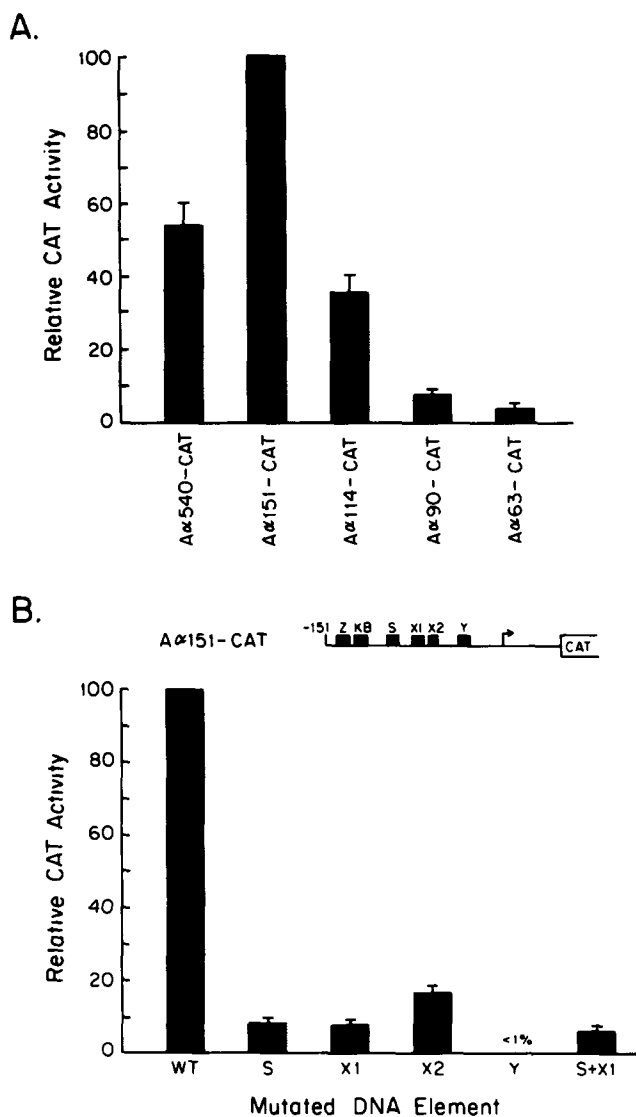
Figure 6. Effect of deletion and substitution mutations on basal expression of A⍺-CAT plasmids in M12.4.1 B lymphoma cells. M12.4.1 cells were transfected with reporter plasmids and CAT activity assayed. Each plasmid was assayed in duplicate in at least three independent transfections. The data is shown as mean CAT activity ± SE relative to the A⍺151-CAT reference construct (100% activity). Absolute % conversion with A⍺151-CAT was in the range of 30–50%, and in some experiments more extract from cells transfected with mutant constructs was used to increase the absolute % conversion. (A) Relative CAT activity generated by A⍺ promoter deletion mutations. (B) Relative CAT activity generated by substitution mutations in A⍺151-CAT.

such that any repression could not be accurately assayed (see Fig. 6). A double mutation, in both the S and X1 elements, completely eliminated repression by PK-A, even though it did not depress basal activity significantly below that seen with either the single S or X1 mutations. These results show that the S and X1 promoter elements together mediate the repression of promoter activity by PK-A.

Discussion

Intracellular cAMP levels in lymphocytes and macrophages are elevated by several immunomodulatory stimuli, such as

Elements deleted.   none   none   Z,KB   Z,KB,S   Z,KB,S,X1,X2
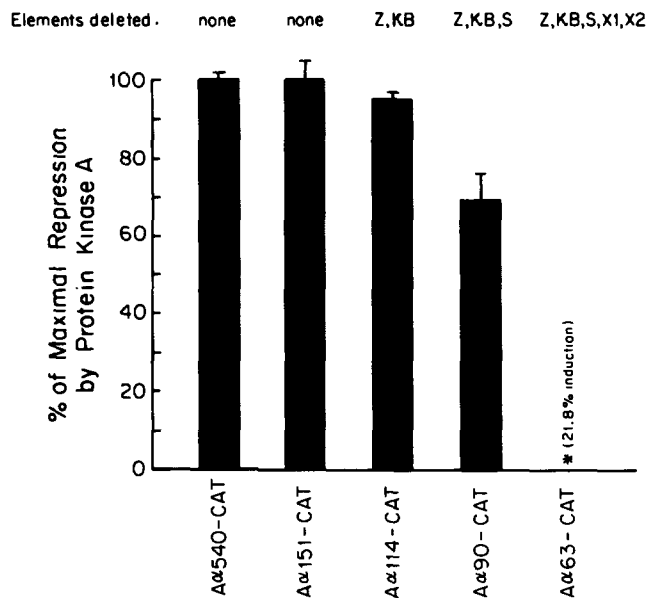
Figure 7. The effect of progressive 5' deletions on the ability of protein kinase A to repress the A⍺ class II MHC gene promoter. M12.4.1 B lymphoma cells were cotransfected with reporter constructs and a two-fold molar excess of an expression plasmid encoding wild type or mutated protein kinase A catalytic subunit and CAT activity was assayed. The absolute % conversion with A⍺151-CAT was in the range of 30–50%, and in most experiments, more extract from cells transfected with mutant constructs was used to increase the absolute % conversion. The data is shown as the mean % repression ± SE of each construct relative to the repression of the reference construct A⍺151-CAT (100%). The absolute repression of A⍺151-CAT was 87.6 ± 1.5%. Each construct was tested in duplicate in at least three independent experiments. * The A⍺63-CAT construct was not repressed by PK-A, but instead CAT activity was induced by 21.8 ± 6.3%.

PGEs, crosslinking of Fc receptors, and crosslinking of cell surface class II antigens (7–9, 27, 28). cAMP mediated signaling has numerous suppressive effects on immune and inflammatory processes, including inhibition of T lymphocyte proliferation, inhibition of induction of class II expression by IL-4 and IFN-γ, and inhibition of the expression of cytokines such as IL-2, IFN-γ, and TNF-α (7–14, 27, 28). An understanding of the mechanism by which cAMP suppresses class II and cytokine expression would be useful in developing therapeutic approaches to suppressing inflammation. In this study, we undertook an initial analysis of the mechanism by which cAMP represses class II MHC expression. We chose to study class II because the cAMP mediated inhibition of induction of cell surface expression of class II has been well described, including experiments done with whole animals, and because of the importance of class II in regulating immune responses and its aberrant expression in autoimmune disease (1, 2, 7–9). In addition, class II MHC gene promoters contain a conserved X2 site, which is identical or similar to the CRE, and thus it was possible that this element might mediate the regulation of class II by cAMP.

Our results show that cAMP downregulates basal as well as inducible cell surface expression of class II antigens. This downregulation is coordinate for the four "classical" murine

Aα151-CAT   -151 Z KB S X1 X2 Y   [CAT]
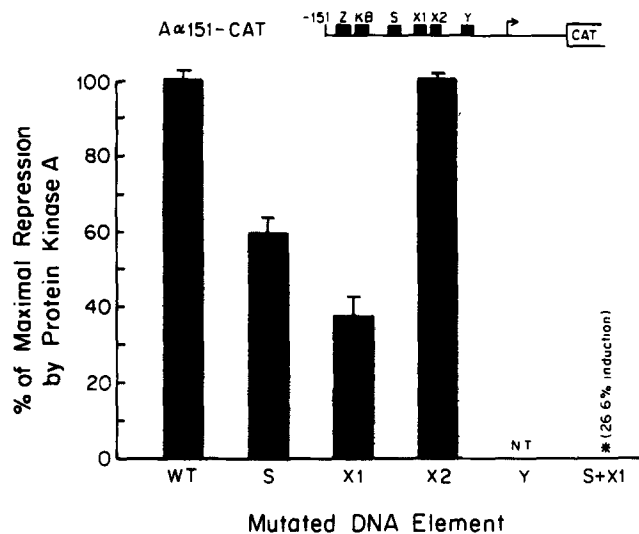
Figure 8. The S and X1 elements mediate the repression of the Aα promoter by protein kinase A. M12.4.1 B lymphoma cells were cotransfected with reporter constructs and a two-fold molar excess of an expression plasmid encoding wild-type or mutated protein kinase A catalytic subunit and CAT activity was assayed. The absolute % conversion with Aα151-CAT was in the range of 30–50%, and in most experiments, more extract from cells transfected with mutant constructs was used to increase the absolute % conversion. The data is shown as the mean % repression +/− SE of each construct relative to the repression of the reference construct Aα151-CAT (100% repression). The absolute repression of Aα151-CAT was 87.6 ± 1.5%. Each construct was tested in duplicate in at least three independent experiments. NT = The basal CAT activity generated by the construct with a mutated Y box was too low to allow a determination of repression. * The construct with both S and X1 elements mutated was not repressed by PK-A, but instead CAT activity was induced by 26.6 ± 5.2%.

class II proteins (Figs. 1 and 2), and is also seen with the human DRα gene (L. Ivashkiv and H.-C. Liou, unpublished data). The repression of class II expression correlates with a decrease in class II mRNA levels. Our observation that 151 and 192 bp of Aα and Eβ promoter sequence, respectively, can confer repression by cAMP on a reporter gene suggests that repression by cAMP occurs, at least in part, at the level of transcription. This is supported by a mutational analysis of the Aα promoter.

The mutational analysis of the Aα promoter utilized both progressive 5′ deletions and substitution mutations. The substitution mutations were designed such as to introduce discrete 5–8 bp changes into known transcription elements which have been defined in many laboratories, including our own, using functional, protein-binding, and in vivo footprinting approaches (5, 6, 24, 29). The effects of mutations on basal Aα promoter activity are in general agreement with the data of Dedrick and Jones (24), with small differences most likely explained by the differences in exact site and sequence of introduced mutations. Our data extend previous observations by demonstrating that an X2 site mutation which leaves the X1 element intact diminishes basal promoter activity by 83% and by showing that effects of simultaneous mutations in the S and X1 elements diminish basal promoter activity only slightly more than single S or X1 element mutations.

Analysis using the deletion mutants showed that 114 bp of upstream Aα sequence were sufficient for full repression by PK-A. Deletion of the S and X elements resulted in a progressive diminution of repression by PK-A, to the point where a construct which contains only an intact Y box was slightly inducible. Analysis using substitution mutations showed that individual mutations in the S and X1 elements partially decreased repression by PK-A (by 40.8% and 62.6%; Fig. 8). A construct containing mutations in both S and X1 elements did not repress at all, but instead was slightly induced by PK-A. Therefore the S and X1 elements, which are found in tandem in all class II MHC promoters sequenced so far, mediate the repression of the Aα promoter by cAMP/PK-A. This is reminiscent of the cytokine induction of class II genes, for which three elements, the S, X1, and Y boxes, are required. Our Y box mutation resulted in such low promoter activity that its effect on repression by PK-A could not be tested. However, constructs which contained intact Y boxes (Aα63-CAT, Fig. 7; S+X1 MUT, Fig. 8) were not repressed at all by PK-A, suggesting that the Y box cannot act independently to mediate repression by PK-A.

The X region of class II MHC promoters has only recently been subdivided into adjacent X1 and X2 sites. The X1 site binds proteins such as RF-X and NF-X (30, 31), whereas the X2 site is related to the CRE, and binds transcription factors of the CREB/ATF family such as mXBP and hXBP (32, 33). Our data demonstrate that the X1 and X2 sites differ functionally in their ability to mediate responses to cAMP, and thus support the subdivision of the X box into two adjacent sites. A surprising result was that regulation of the Aα gene by cAMP was not mediated by the X2 site, a perfect CRE, but instead was mediated by the S and X1 elements. cAMP effects are not necessarily mediated by CREs, as an AP-2 site can confer cAMP inducibility on a gene (34), and CREs do not always function in cAMP induction, but can serve as basal transcription elements (35) or can mediate responses to elevations in intracellular calcium (36).

Our data suggest that the repression of the Aα promoter by cAMP is mediated by proteins which bind to the S (24) and X1 elements (such as RF-X, NF-X) or proteins which do not directly contact DNA but interact with S and X1 binding proteins. Our initial analysis of the effect of cAMP on nuclear protein binding to the Aα S and X1 sites has been hampered by the low affinity of this interaction compared to the high affinity binding to the X2 site (reference 24; L. Ivashkiv, unpublished data). Experiments utilizing in vivo footprinting (29) should provide additional information about the effects of cAMP on binding of proteins to the Aα promoter.

In conclusion, our study identifies two tandem DNA sequences, the S and X1 sites, which mediate the repression of the Aα class II MHC gene by cAMP/protein kinase A. To the best of our knowledge, this is the first identification of DNA elements which can mediate repression by cAMP. The promoters of other genes which are repressed by cAMP, such as stromelysin, IL-2, and TNF-α do not contain any obvious homologies to the S and X1 sequences. This suggests that cAMP acts upon a factor which does not directly

contact DNA, but instead interacts with different proteins of variable DNA binding specificity. This is reminiscent of the induction of class II genes by cytokines such as IFN-γ. Indeed, the same S and X1 DNA elements can mediate both positive regulation by IFN-γ and TNF-α (5, 6, 37) and negative regulation by cAMP. Therefore, a better understanding of the mechanism of negative regulation by cAMP may also yield insight into positive regulation by cytokines.

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