MOLECULAR AND CELLULAR BIOLOGY, Feb. 1990, p. 593–604
0270-7306/90/020593-12$02.00/0

Vol. 10, No. 2

Sequence Elements Required for Activity of a Murine Major Histocompatibility Complex Class II Promoter Bind Common and Cell-Type-Specific Nuclear Factors

RUSSELL L. DEDRICK AND PATRICIA P. JONES*

Department of Biological Sciences, Stanford University, Stanford, California 94305

Received 8 August 1989/Accepted 28 October 1989

We have examined the sequence elements and corresponding DNA-binding factors required for transient expression of the A_α^d promoter fused to the bacterial chloramphenicol acetyltransferase reporter gene in a variety of cultured cell lines. Deletion analysis demonstrated that only about 110 nucleotides of sequence 5′ of the transcription start site are required for constitutive expression in the murine B-lymphoma cell line A20 or for gamma interferon-induced expression in the murine monocytic cell line WEHI-3. Linker-scanner mutation of this region indicated that at least three sequence elements are required for promoter activity. These elements correspond to the conserved sequence elements found in other human and mouse class II genes, the X box, the Y box, and the H box. Analysis of DNA-binding activity showed that the three most predominant factors present in extracts from WEHI-3, A20, or L cells (which do not express the class II genes) are actually a family of factors that bind to a fourth sequence element, overlapping the 3′ end of the X-box sequence, that is homologous to the cyclic AMP-responsive enhancer element. A single common factor that binds to the Y box was detected in extracts from all cells tested, as has been seen with the Y-box elements of other class II genes. Another common factor was found that binds to the more conserved 5′ region of the X-box element, although A20 extracts contained a second, distinct binding activity for this region. A common binding factor for the H-box element was detected in extracts from WEHI-3 and L cells. However, this activity was absent in A20 cell extracts. Instead, two different H-box-binding activities were detected, suggesting that different components are involved in class II gene expression in B cells and macrophages. Finally, gamma interferon treatment did not significantly alter the DNA-binding activity in WEHI-3 cells for any of the sequence elements shown to be required for induced chloramphenicol acetyltransferase expression.

The class II genes of the major histocompatibility complex (MHC) encode highly polymorphic, cell surface glycoproteins (also called Ia antigens). These molecules play a central role in the immune response by forming a fundamental part of the ligand for the antigen-specific T-cell receptor. The Ia antigen–T-cell receptor interaction is required for both the development of the T-cell repertoire in the thymus (4, 25) and the presentation of antigenic peptides to helper T cells in the periphery (48). Proper function of the Ia antigens depends not only on the polymorphic nature of their structures (26) and their ability to bind the antigenic peptide but also on the regulated expression of these proteins on the surface of cells interacting with the appropriate T lymphocyte (24). Aberrant expression of Ia is thought to be involved in the development or progression of certain autoimmune disorders (10, 35, 45).

In the mouse, there are two isotypic forms of Ia, I-A and I-E. Each is a heterodimer composed of an α chain (33 to 34 kilodaltons) and a β chain (28 kilodaltons) associated noncovalently on the cell surface. The four murine class II genes are coordinately regulated (44) and display a complex pattern of expression. Expression is developmentally regulated in B lymphocytes; pre-B cells are nonexpressing, mature resting B cells constitutively express high levels of Ia, and differentiated, immunoglobulin-secreting plasma cells are nonexpressing. The level of Ia expression in mature B cells can be further augmented by interleukin-4 (41) or by the cross-linking of surface immunoglobulin (40). A second phenotype is displayed by cells such as monocytes, macrophages, and certain epithelial and endothelial cells that are normally Ia negative but can be induced to high levels of expression by the T-cell-derived lymphokine gamma interferon (IFN-γ). The IFN-γ-induced expression can be further modulated by a number of other soluble factors in both a positive (tumor necrosis factor [13, 45]) or negative (beta interferon [31], endotoxin [54], prostaglandins [52], serotonin [55], norepinephrine [21], and glucocorticoids [53, 60]) manner. Finally, most other somatic cells do not express Ia and do not respond to inducing agents.

Because of the biological importance and complexity of Ia expression, an understanding of the mechanisms controlling class II expression has been the focus of much interest (for reviews, see reference 56 and C. Benoist and D. Mathis, *in* Renkawitz, ed., *Tissue Specific Gene Expression*, in press). Initial comparisons of the sequences of human and murine class II genes revealed two highly conserved sequence elements in the region 40 to 150 nucleotides 5′ of the start site of transcription (27, 36). These elements have been called the X and Y (56) or A and B (27) boxes, and several studies have shown them to be important for both constitutive and induced expression of class II genes in human and mouse systems (8, 19, 51). More recently, sequence analysis suggested that a third conserved region, called the heptamer sequence or the H box, exists 15 to 20 nucleotides 5′ of the X box (50). This element has also been implicated in constitutive and IFN-γ-induced Ia expression (3, 8, 58). Finally, experiments with transgenic mice indicate that another, more distant enhancer region is required for developmental expression in B lymphocytes (28, 59, 61).

An understanding of the mechanisms that control the

* Corresponding author.

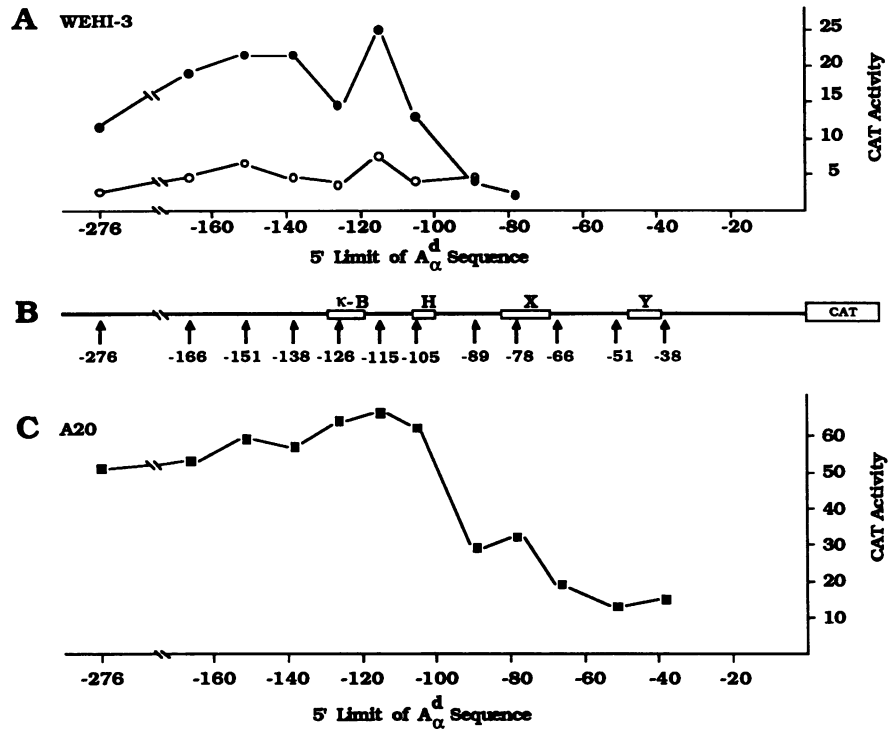


FIG. 1. Expression of A_αCAT 5' deletion mutants in WEHI-3 and A20 cells. Cells were transfected with 15 μg of each deletion mutant, and 40 μl of extract was assayed for CAT activity. (A) CAT expression in WEHI-3 cells. Transfected cells for each plasmid were divided equally into two dishes, and one dish was treated with 50 U of IFN-γ per ml for 40 h. (B) Schematic of the A_α promoter-CAT gene fusion. Arrows indicate positions of the 5' endpoints of the individual deletion mutants; the relative positions of the H, X, Y, and κB elements are shown. (C) CAT expression in A20 cells. The data shown for each graph are from one representative experiment. CAT activity is expressed as 10^3 counts per minute of chloramphenicol acetylated.

expression of the class II MHC gene family will require a detailed, comparative analysis of the components involved in regulating the expression of the various genes in cells with different phenotypes. We have investigated the *cis*-acting sequence elements required for transcription from the promoter of a murine class II gene, A_α^d, using a transient assay in various cultured cell lines that represent different phenotypes of class II expression. Our results indicate that at least three and probably four sequence elements are required for constitutive expression in a B-cell line (A20) and for IFN-γ-induced expression in a monocytic cell line (WEHI-3). These include the conserved H-, X-, and Y-box elements and a fourth element that lies between the X and Y boxes and partially overlaps the X-box element. Furthermore, the presence of DNA-binding factors for these elements in various cell types has interesting implications for the mechanisms controlling transcriptional expression of the Ia genes.

MATERIALS AND METHODS

Cell lines. Cell lines WEHI-3, A20, and M12 were maintained in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah) and 50 μM β-mercaptoethanol. The L-cell line was grown in Dulbecco Modified Eagle Medium (GIBCO) with 5% fetal bovine and 5% newborn bovine serum.

IFN-γ. Recombinant murine IFN-γ was generously supplied by Genentech, Inc. (South San Francisco, Calif.) as purified protein produced from *Escherichia coli*.

Plasmid construction. The A_α^d promoter was subcloned from plasmid pA_α^d, which contains the genomic A_α gene, including about 5 kilobase pairs of 5'-flanking DNA (generously provided by Leroy Hood, California Institute of Technology, Pasadena, Calif.), and the sequence was determined by the chain termination method (47). The A_α^d promoter was fused to the chloramphenicol acetyltransferase (CAT) reporter gene via the *Bam*HI site at position −1 of the A_α sequence by blunt-end ligation to *Hin*dIII-digested $pA10CAT_2$ (30) after filling-in with Klenow enzyme (2). An *Sst*I-*Bam*HI fragment that extended from position −276 of the A_α gene and included the simian virus 40 poly(A) site of the CAT reporter gene was isolated and blunt-end ligated into the *Eco*RI site of pUC18 (62) to construct pA_α^dCAT-276, which has the pUC18 polylinker sequences adjacent to the 5' end of the A_α promoter sequence. 5' deletion mutants were constructed by cloning a fragment of the E_β gene into the *Sma*I site of pA_α^dCAT-276 to protect the *Xba*I site of the pUC18 polylinker. This plasmid was digested with *Kpn*I and treated with BAL 31 nuclease (2). The remaining E_β DNA was removed by *Bam*HI digestion, and the plasmid ends were made blunt by filling-in with Klenow enzyme and ligated so that the intact *Xba*I site bordered the deletion endpoint. Individual plasmids were characterized by sequencing (63).

Linker-scanner (LS) mutants were constructed by first generating a set of 3' promoter deletions to join to the 5' deletions. A 346-base-pair *Sst*I fragment of the A_α promoter, spanning residues −276 to +77, was cloned into the *Sst*I site of pUC18 with the A_α coding sequences adjacent to the *Kpn*I site of pUC18, and deletions were generated with BAL 31 as described above except that the deleted ends were made

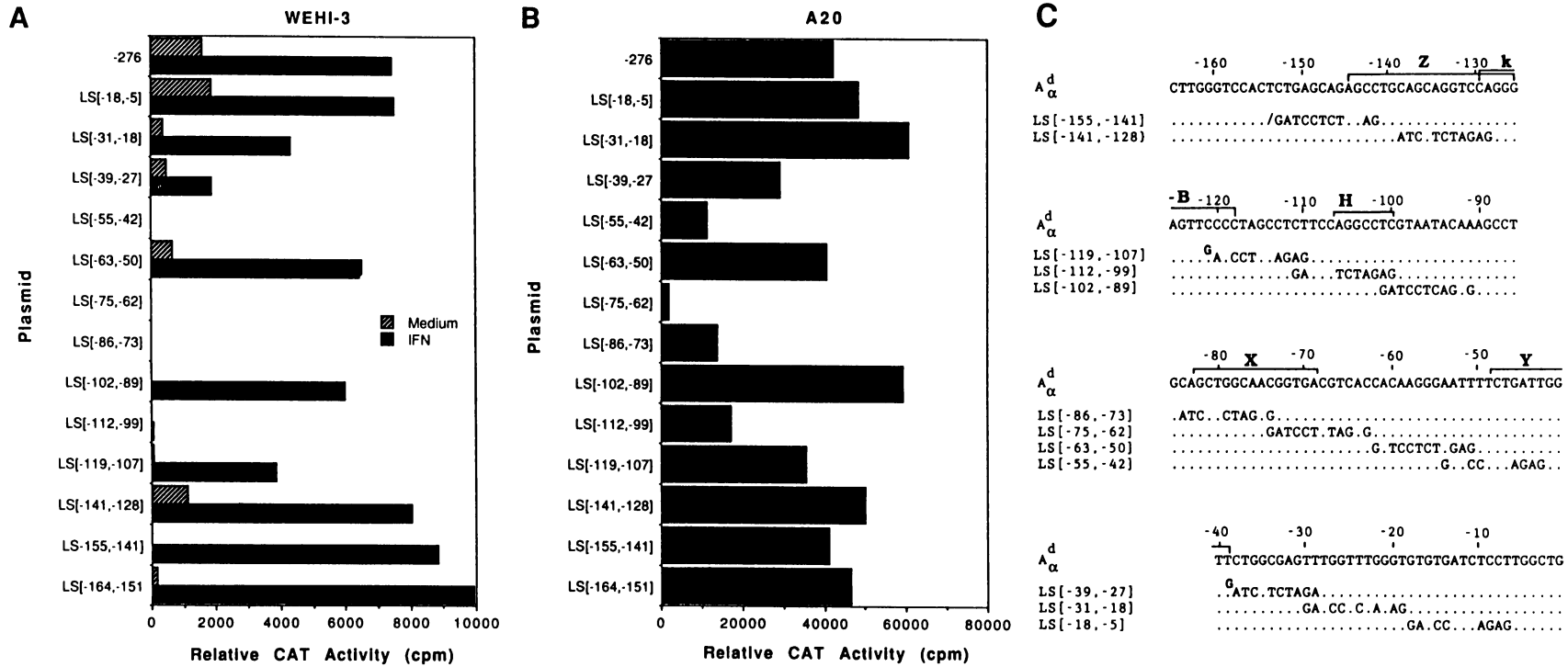


FIG. 2. Expression of A_α^dCAT LS mutants in WEHI-3 and A20 cells. Cells were cotransfected with the LS mutants and pSVALΔ5′, and relative CAT activity (expressed as counts per minute of chloramphenicol acetylated) was normalized to activity of the luciferase internal control as described in Materials and Methods. (A) CAT expression in WEHI-3 cells. Transfected cells were divided equally between two dishes, and one dish was treated with 50 U of IFN-γ per ml. In some cases (LS[−55,−42], LS[−75,−62], and LS[−86,−73]), CAT activity was less than or equal to the activity from mock-transfected cells, which has been subtracted from each sample as background. The data shown are from one representative experiment. (B) CAT expression in A20 cells. Each value is the average of results for two independently transfected samples done at the same time. (C) Sequence of the LS mutants shown in relation to the wild-type sequence. The positions of the Z-, κB-, H-, X-, and Y-box elements are outlined. The regions of wild-type sequence in the LS mutants are designated with a dot, and the altered residues are indicated. A deletion in LS[−155,−141] is indicated by a slash, and insertions in LS[−39,−27] and LS[−119,−107] are indicated by a superscript (^{G}A) where perfect register was not maintained.

blunt with mung bean nuclease before ligation. Matching 3′ and 5′ deletion mutants were digested with *Eco*RI or *Hin*dIII, respectively, and the ends were filled in with Klenow enzyme. Each plasmid was then digested with *Xba*I, and the fragment containing the 3′-deleted DNA was ligated together with the 5′-deleted vector. The resulting plasmids had the CAT gene joined to 276 nucleotides of A_α promoter DNA, with 12 base pairs substituted by the sequence GATC CTCTAGAG.

Plasmid pSVALΔ5′ (14), which contains the luciferase cDNA expressed by the simian virus 40 early promoter, was a generous gift of Suresh Subramani (University of California at San Diego, La Jolla, Calif.). All plasmids were prepared from overnight cultures by NaOH-sodium dodecyl sulfate lysis (2) and banding twice in ethidium bromide-CsCl gradients.

Transfections. Cells were transfected with 15 μg of DNA per 10-cm-diameter dish with DEAE-dextran (33), using 200 μg of DEAE-dextran per ml for L cells and 15 μg of DEAE-dextran per ml for WEHI-3 cells. A20 cells were transformed in suspension (22) in 1 ml, using 15 μg of DNA and 30 μg of DEAE-dextran per ml. When luciferase was used as an internal control, cells were cotransfected with 2 μg of pSVALΔ5′ and 13 μg of pA_α^dCAT.

Enzyme assays. CAT activity in transfected cells was measured essentially by the procedure of Gorman et al. (23), using 250,000 cpm of [^{14}C]chloramphenicol per assay, after heating the extracts 7 min at 60°C as recommended by Mercola et al. (38). Luciferase activity was measured from unheated extracts by the method of deWet et al. (14), using a Moonlight 2001 luminometer (Analytical Luminescence Laboratory, San Diego, Calif.).

To control for transfection efficiencies, CAT activities were normalized by multiplying the CAT activity of a particular test plasmid (measured as counts per minute of chloramphenicol acetylated minus background) by the ratio luciferase activity (pAaCAT-276)/luciferase activity (test plasmid). IFN-γ treatment had only a small effect on CAT expression from pSV2CAT (23) in all cell types (10 to 15% inhibition; data not shown), indicating that neither the simian virus 40 promoter nor the CAT message was greatly affected by the lymphokine. However, expression of luciferase from plasmid pSVALΔ5′ was inhibited 35 to 50% by IFN-γ treatment, particularly in the WEHI-3 cells. Because of this, CAT activities in IFN-γ-induced samples were normalized by the luciferase value of the uninduced cells from each transfection.

Nuclear extract preparation and analysis of DNA-binding factors. Nuclear extracts were prepared from logarithmically growing cells by the procedure of Dignam et al. (16). Protein concentration was measured by dye binding (9) (Bio-Rad Laboratories, Richmond, Calif.), using bovine gamma globulin as a standard. Gel mobility shift analysis was performed as described previously (2) with standard binding buffer containing 15 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.8), 12% glycerol, 75 mM KCl, 100 μg of poly(dI-dC) (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) per ml, 1 mM dithiothreitol, and 0.1 to 0.5 ng of labeled probe. Reactions were incubated for 30 min at room temperature, and complexes were separated on 4% acrylamide gels (acrylamide/bisacrylamide ratio, 80:1). Gels were dried on DE81 paper (Whatman, Inc., Hillsboro, Ore.) to prevent loss of small DNA fragments. DNA probes were end labeled with T4 kinase or Klenow enzyme as described previously (2).

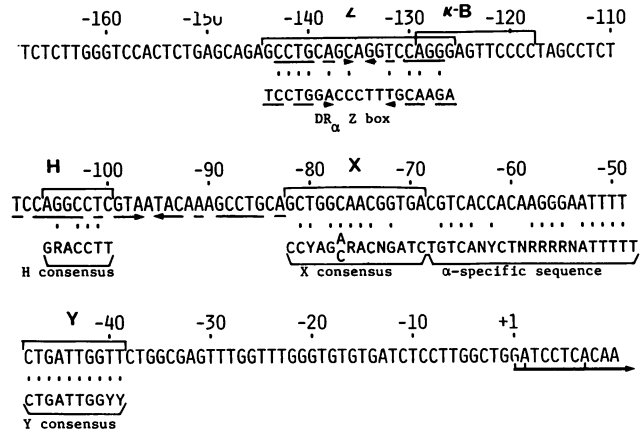


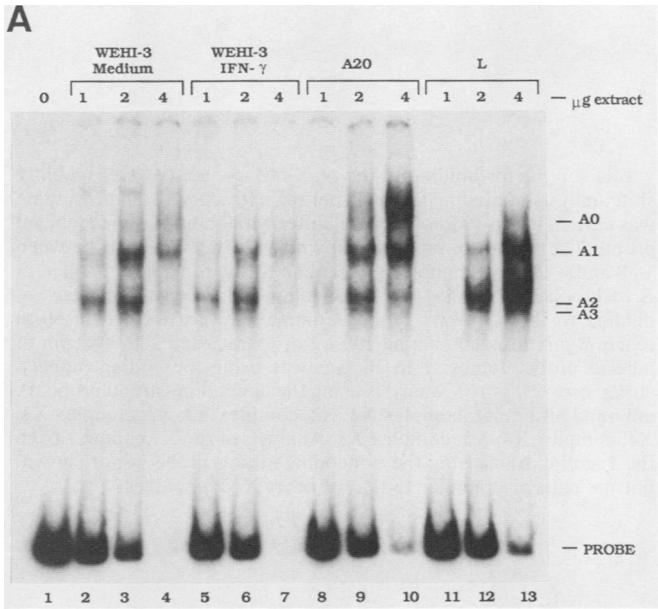
FIG. 3. Sequence of the A_α^d promoter region. The three start sites of transcription are indicated by a bold underlining arrow, with the 5′-most site designated position +1. Positions of the homologies to the Z-, κB-, H-, X-, and Y-box elements are outlined. The consensus sequences for the H box, the X box, the Y box, and the α-specific sequences are shown, as is the sequence of the DR_α Z box; a match with the A_α^d sequence is indicated by a dot; R = A or G, Y = T or C, and N = any nucleotide. Regions of dyad symmetry are underlined with convergent arrows.

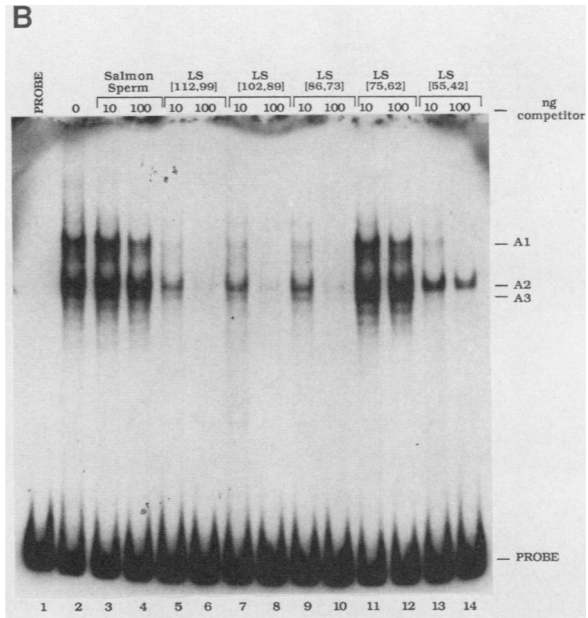
Methylation interference analysis was performed essentially as described previously (2).

RESULTS

Mutational analysis of the A_α promoter. To delineate the *cis*-acting sequence elements required for A_α promoter activity, we constructed plasmids in which the A_α coding sequences were replaced by the bacterial CAT reporter gene. These plasmids were transfected into cultured cell lines representing the three principal phenotypes of Ia expression: A20, a murine B lymphoma that constitutively expresses high levels of Ia; WEHI-3, a murine monocytic cell line that is normally Ia negative but can be induced to high levels of expression by IFN-γ; and L cells, a murine lung fibroblastic cell line that does not express Ia even after treatment with IFN-γ. Promoter activity was measured by assaying for CAT activity in cell lysates. When a plasmid containing the A_α transcription start site and 276 nucleotides of 5′-flanking sequence was transfected into these cell lines, relative CAT activity (normalized to luciferase activity expressed from pSVALΔ5′ that was cotransfected to correct for differences in transfection efficiency) accurately reflected the phenotype of Ia expression of the recipient cell. A20 cells expressed the highest level of CAT activity and were not greatly affected by IFN-γ treatment. In contrast, uninduced WEHI-3 cells expressed a modest amount of CAT activity and were induced by IFN-γ treatment to a level comparable to that seen in the A20 cells. Relative CAT activity in the L cells was low with or without IFN-γ. Including additional 5′ sequences (up to 2.5 kb) did not significantly alter the pattern of CAT expression (data not shown). Although we have not yet been able to demonstrate that transcription initiates at the expected site in our constructs because of the low transfection efficiency of the A20 and WEHI-3 cells, we have assumed that CAT expression reflects activity of the A_α promoter.

The 5′ limit of the regulatory sequences important for CAT expression from the A_α promoter was defined by





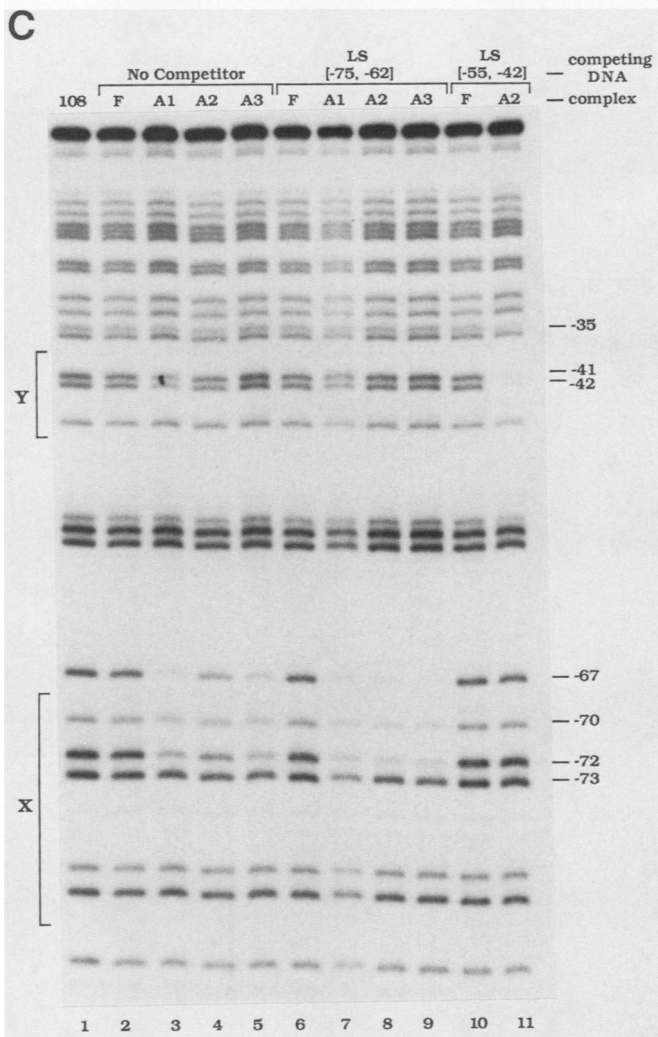


FIG. 4. Factor binding to the A_α^d promoter probe. (A) Gel mobility shift analysis of binding to a fragment of the A_α^d promoter containing sequences −137 to +3 by factors from nuclear extracts from uninduced WEHI-3, IFN-γ-treated WEHI-3, A20, or L cells. Each 10-μl reaction mixture contained about 0.5 ng of labeled probe in binding buffer with 5 mM $MgCl_2$. (B) Competition analysis for binding to the A_α-137 probe. Competing LS mutant DNAs were 525-nucleotide-long fragments that included the entire A_α^d promoter region (to −276) and about 250 nucleotides of the CAT gene. Nuclear extract from uninduced WEHI-3 cells (2 μg) was preincubated with the competitor DNA for 5 min on ice before addition of the labeled probe and the remaining components. (C) Methylation interference analysis of A_α promoter complexes formed in the presence or absence of competitor DNA, using a probe containing nucleotides −108 to +3. Binding reaction mixtures contained 12 μg of extract, 250 ng of competitor DNA, and 2 ng of labeled probe in 50 μl. $MgCl_2$ was omitted from the binding reactions to inhibit DNase activity. Lanes: 108, methylated probe: F, unbound probe; A1, complex A1; A2, complex A2; A3, complex A3.

constructing a series of 5′ deletion mutants and assessing their activity in WEHI-3 and A20 cells. In WEHI-3 cells, CAT activity was inducible by IFN-γ until sequences between −105 and −89 were deleted (Fig. 1A). The basal activity was also reduced at this point and remained low with further deletion to −38 (data not shown). In A20 cells, a similar pattern was observed: constitutive expression remained high with deletion to position −105 (Fig. 1C). Deletion to position −89 resulted in a 50 to 60% drop in CAT activity. Another drop in CAT expression was observed when deletions were extended to position −66, where it remained low.

To further define regulatory sequence elements, a series of LS clustered point mutations (37) was made in the region of the A_α promoter spanning the minimum promoter sequences defined by the deletion analysis. In WEHI-3 cells, four LS mutations resulted in a complete loss of IFN-γ-induced CAT expression (Fig. 2A). These included LS[−112,−99], LS [−86,−73], LS[−75,−62], and LS[−55,−42]. The effects of the mutations on CAT expression in uninduced WEHI-3 cells were less reproducible, although in no case did we observe an appreciable increase in basal expression.

The effect of the LS mutations on constitutive CAT

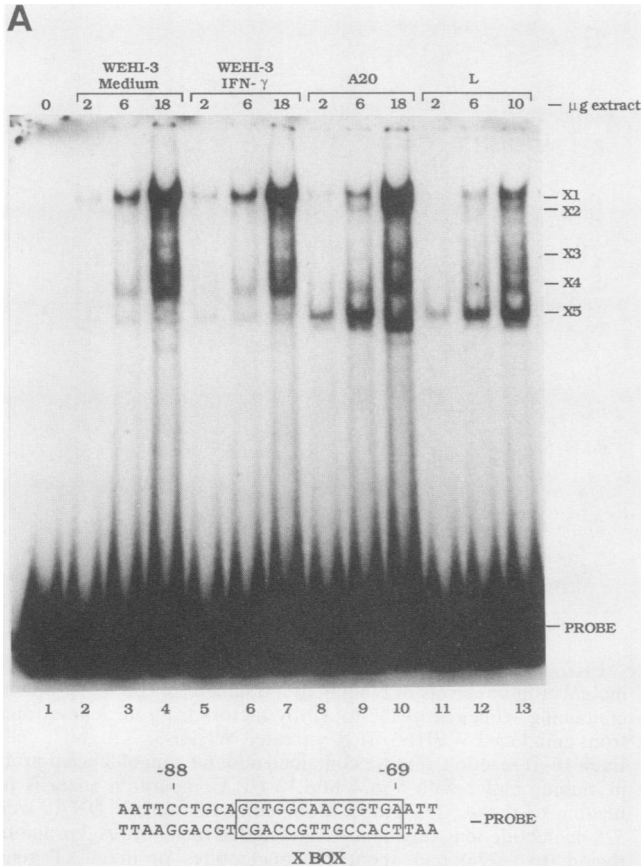
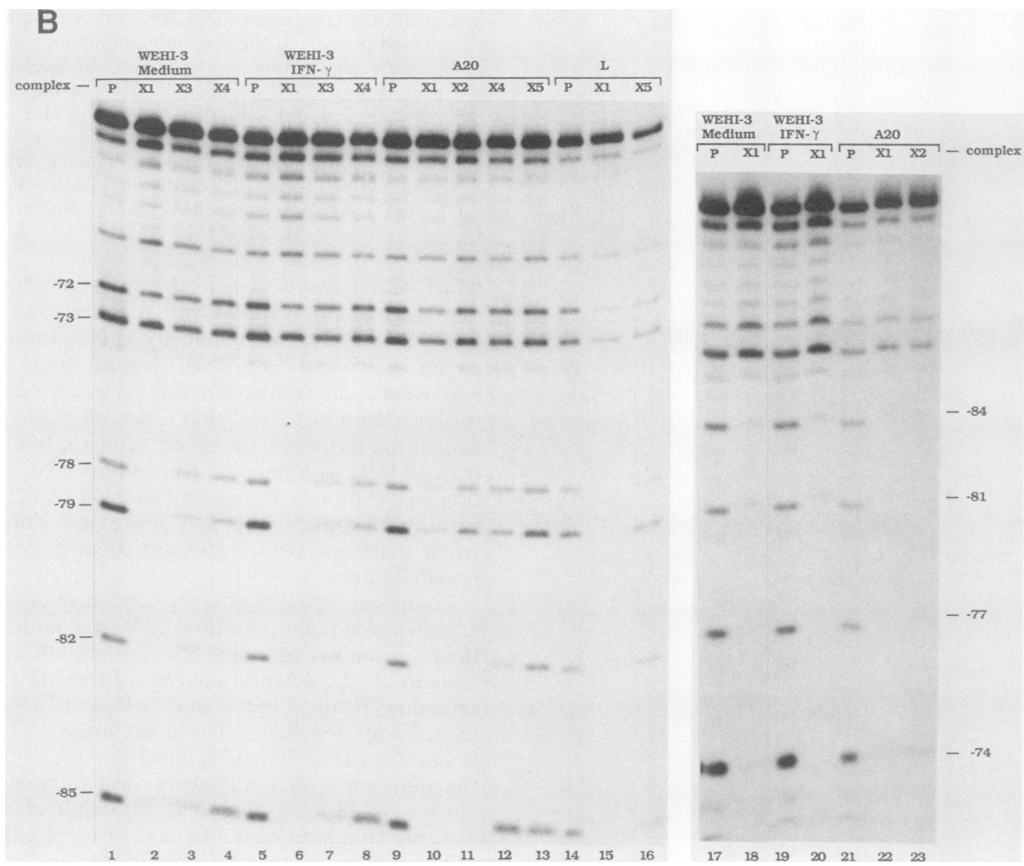


FIG. 5. Factor binding to the 5′ X-box probe. (A) Gel mobility shift analysis of binding factors from the extracts indicated. Binding was carried out in 10 μl of binding buffer with about 0.1 ng of labeled probe. The 5′ X-box probe is shown, with the sequence between −88 and −69 representing A_α^d sequences; the remainder is filled-in *Eco*RI cohesive ends. (B) Methylation interference analysis of binding to the 5′ X-box probe. Complexes were formed in 50-μl reaction mixtures containing 100 μg of extract and 200,000 cpm of labeled probe. Lanes: 1 to 16, analysis using the coding (upper)-strand probe; 17 to 23, analysis using the noncoding-strand probe; P, unbound probe; X1, complex X1; X2, complex X2; X3, complex X3; X4, complex X4; X5, complex X5. Analysis of the X1 complex from the L-cell extract using the noncoding strand probe is not shown, but the pattern is similar to that of other X1 complexes.



expression in the A20 cell line was similar to that on IFN-γ-induced expression in the WEHI-3 cells (Fig. 2C). Mutations LS[−112,−99], LS[−86,−73], and LS[−55,−42] reduced CAT expression to less than 50% of the level of the pA_α^dCAT-276 control. Mutation LS[−75,−62] reduced CAT expression about 90%.

Sequence analysis of the A_α^d promoter region. The sequence of the A_α^d promoter region is shown in Fig. 3. Three closely spaced start sites of transcription were mapped by S1 nuclease and primer extension analysis (data not shown); the numbering of the sequence is based on assignment of the 5′-most start site as residue +1. No TATA homology was found in the −30 to −20 region, and mutations in this region (LS[−31,−18] and LS[−18,−5]) did not significantly affect CAT expression in either A20 cells or IFN-γ-induced WEHI-3 cells.

The mutational analysis indicated that the sequences between about −110 and −30 are important for both IFN-γ-induced expression in WEHI-3 and constitutive expression in A20 cells. Within this region are found the conserved upstream sequences seen in all other murine and human class II genes (27, 36, 42). Residues −48 to −39 show a perfect match to the consensus sequence for the Y-box element, and mutation of this sequence (LS[−55,−42]) and closely bordering residues ([LS[−39,−27]) reduced CAT expression in both cell lines. Homology to the X-box element is found between residues −82 and −69. The fit to the consensus sequence is weakest in the 3′ end, although mutation of both the 5′ end (LS[−86,−73]) and the 3′ end (LS[−75,−62]) reduced promoter activity. The sequence between the X- and Y-box elements is of the appropriate length (20 residues) and shows strong homology to the α-specific sequence found in other class II α genes (42). A sequence similar to the H-box element (50) is found between residues −106 and −100, and mutation of this sequence affected both constitutive and IFN-γ-induced CAT expression (pA_α^dCAT-89, LS[−112,−99]). This loosely conserved sequence is found about 17 nucleotides 5′ of the X-box element in all class II promoters (50; R. Dedrick, unpublished observation) and has been included in more extended sequence elements termed the Z box (56, 58) or W box (39). However, since mutation of the surrounding sequences had a minimal effect on promoter activity in our experiments (LS[−119,−107] and LS[−102,−89]) and because there is some confusion about the identity of the W sequence (17, 39), we have used the H-box nomenclature to indicate the more limited element described by our data. The A_α^d H-box sequence is also part of an extended, discontinuous palindromic sequence that extends from positions −109 to −83. However, LS[−102,−89], which mutates a number of residues in the middle of this structure, has little effect on CAT expression, indicating that this palindrome might not be of functional significance. In addition, a repeat of the H-box sequence is found between residues −91 and −85. Unfortunately, the mutations generated for this study do not alter this region sufficiently to allow any firm conclusions as to its possible significance.

At least three additional regions of the A_α^d promoter have homology to sequence elements associated with other class II genes. Residues −144 to −126 show sequence homology and the potential palindrome formation characteristic of the Z-box element found in the human DR_α gene (56, 58). However, in our experiments neither deletion (pA_α^dCAT-138; Fig. 1) nor mutation (LS[−141,−128]; Fig. 2) of this sequence significantly altered expression from the A_α promoter. In addition, residues between −96 and −89 constitute a 6-of-8-base fit (CTTTGTAT versus GTTTGCAT) with the octamer element found in the immunoglobulin promoter elements (43). This element has also been shown to be important in DR_α promoter function (51), but mutation of this sequence did not significantly reduce CAT expression in our system (LS[−102,−89]; Fig. 2). Finally, residues −129 to −118 show a strong similarity to the κB sequence element found in the immunoglobulin kappa-chain enhancer (49) and also in the 5′-flanking region of the murine E_α gene important for appropriate developmental expression in B cells (6, 20, 59, 61). Although this element is not required for A_α promoter activity, CAT expression in the IFN-γ-induced WEHI-3 cells was reduced when the 5′ end of the homology was first deleted (pA_α^dCAT-126; Fig. 1A). Activity seemed to increase with further deletion to −115, suggesting that there could be both positive- and negative-acting elements in this region. In addition, we have recently constructed an LS mutation of this element that causes reduced expression of CAT in IFN-γ-induced WEHI-3, providing further evidence that the κB element is active in this situation (Y. Freund, personal communication).

Analysis of DNA-binding activities for the A_α^d promoter. To characterize DNA-binding factors that might bind to the sequence elements revealed by our mutational analysis, a 140-base-pair DNA fragment corresponding to residues +3 to −137 was radioactively labeled and used in a gel mobility shift assay with nuclear extracts prepared from untreated or IFN-γ-induced WEHI-3 cells, from A20 cells, or from L cells (Fig. 4A). With all extracts, three prominent complexes formed (A1, A2, and A3; compare lanes with 2 μg of extract in Fig. 4A), along with several bands of lesser intensity (seen more clearly in Fig. 4B). At higher concentrations of A20 and L-cell extract, a fourth complex appeared (A0), but this complex was easily abolished by low amounts of nonspecific competitor DNA (calf thymus; data not shown) and has not been characterized further.

The DNA sequences involved in the complex formation were characterized by a binding competition experiment in which nuclear extract from uninduced WEHI-3 cells was preincubated with an excess of unlabeled DNA prepared from the LS mutant plasmids. The labeled probe was then added, and complex formation was assessed by the gel mobility shift assay. In this experiment, the unlabeled DNA would be expected to compete for factor binding to the labeled probe unless the sequence element involved in the formation of a particular complex had been destroyed by the clustered mutations. Competitor DNA prepared from LS[−112,−99], LS[−102,−89], and LS[−86,−73] efficiently competed with the labeled probe for binding of factors responsible for all three complexes (Fig. 4B). In contrast, the LS[−75,−62] fragment failed to eliminate the formation of any of the binding complexes (A1, A2, and A3), suggesting that these complexes are formed by proteins that bind to the 3′ end of the X-box element. This was confirmed by methylation interference analysis (which detects purine residues that are involved in close contacts with the binding factor). In the presence of the LS[−75,−62] competitor DNA, methylation of G residue −67, −70, or −72 efficiently inhibited the formation of the A1, A2, and A3 complexes (Fig. 4C), indicating that they all involve contacts with these nucleotides in the 3′ end of the X-box element. Curiously, competition with the LS[−55,−42] fragment eliminated formation of A1 and A3 but apparently not A2, indicating that A2 is formed by a Y-box-binding protein. Indeed, methylation interference analysis indicated that the A2 complex that formed in the presence of the LS[−55,−42] competitor did

not involve contacts with X-box residues but instead contacted G residues −42 and −41 within the Y-box element and probably −35 (Fig. 4C). Thus, at least four complexes formed with this A_α promoter probe. One (A2Y) was formed by a factor binding to the Y-box element. The other complexes formed from factors binding to sequences in the 3′ end of the X-box element and in the region between the X and Y boxes, and one of these complexes (A2X) comigrated with the Y-box complex (A2Y). Similar results were obtained with the other extracts shown in Fig. 4A, indicating that these factors are ubiquitous and that their activity is not affected by IFN-γ treatment (see Fig. 7 for a summary of these results).

Complexes similar to those just described have been observed with the A_α^k X-box element by Boothby et al.(7), who have noted that the sequence of the 3′ region of the A_α X box is homologous to the cyclic AMP-responsive enhancer (CRE) element (L. Glimcher, personal communication). Recently, they have cloned two genes that encode factors (XBP-1 and XBP-2) that bind to this element (32).

Factor binding to the 5′ end of the X-box element. In light of the mutational data implicating the 5′ end of the X box (LS[−86,−73]) and the H box (LS[−112,−99]) as necessary for A_α promoter function, it was surprising that no complexes were observed with the A_α promoter probe that corresponded to factors bound to either of these elements. To assay for factors that might bind to the 5′ end of the X box, we synthesized a 27-base-pair oligonucleotide probe containing the X-box homology with some 5′-flanking sequence (−88 to −69) but lacking the sequences outside the 3′ end of the X box that were seen to be contacts for the factors characterized in Fig. 4. Gel mobility shift analysis with the four different nuclear extracts showed that a number of complexes formed with this probe (Fig. 5A). Binding competition between the X-box probe and unlabeled X-box probe or H-box probe indicated that only complexes X1 (all extracts) and X2 (A20 and possibly L-cell extracts) were specific for the X-box sequences (data not shown). Methylation interference analysis showed that the X1 complex involved contacts with G residues within or just 5′ of the conserved X-box sequences (Fig. 5B; see also Fig. 7), indicating that this is indeed an X-box-specific complex. The B-cell-specific complex, X2, also contacted similar residues, although the interference pattern was somewhat different than for the X1 complex. Whereas methylation of residues −84, −81, −80, −77, and −74 on the noncoding strand and residues −85 and −82 on the coding strand inhibited formation of both the X1 and X2 complexes (Fig. 5B), methylation of coding-strand residues −79 and −78 seemed to inhibit X1 formation much more than X2 formation. Methylation of residues −85, −82, −79, and −78 had a less dramatic effect on the formation of complexes X3, X4, and X5. Considering these data combined with results of the competition analysis mentioned above, we feel at this point that these were probably nonspecific complexes. Finally, the common X1-binding activity was present in L cells and was not significantly altered by IFN-γ treatment in WEHI-3 cells; therefore, its binding activity did not correlate with expression of the class II genes.

Although the previously identified binding factors for the A_α X-box element (7) have been thought to differ from those that bind to the E_α gene X box, this conclusion needs to be reevaluated, since our identification of contact residues clearly demonstrates that the A_α X-box element contains two distinct elements (see Fig. 7). Both a common factor (X1) present in all cells tested and a B-cell-specific factor

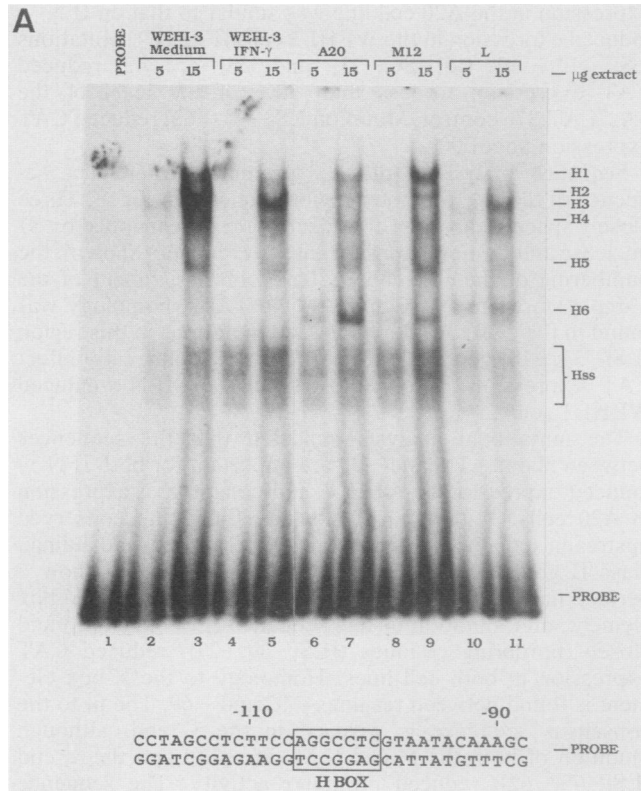


FIG. 6. Factor binding to the H-box probe. (A) Gel mobility shift analysis of H-box-binding factors in various nuclear extracts. Binding reactions were done in 10 μl of binding buffer with 0.1 ng of labeled probe. The sequence of the double-stranded oligonucleotide H-box probe is shown. (B) Methylation interference analysis of H-box-binding complexes.Lanes: 1 to 12, analysis of the noncoding (lower) strand; 13 to 23, analysis of the coding (upper) strand; P, unbound probe; H1, complex H1; H3, complex H3; H4, complex H4; H5, complex H5. The Hss complex forms from a factor(s) binding to the single-stranded form of the labeled probe (Dedrick, unpublished results).

(X2) bind to the 5′ region of the A_α X box that shows the greatest similarity to the canonical X-box sequence. Although we have not yet investigated directly the relationship between the factors that bind to this region of the A_α X box and the E_α X box, the available biochemical characteristics (gel mobility shift and methylation interference [19, 29]) indicate that they are very similar. As mentioned above, an additional family of the XBP-like factors, present in all cell types, binds to the CRE-like element found in the less conserved 3′ region of the A_α X-box element.

Factor binding to the H-box element. To analyze factors that might interact with the H-box element, a 32-base-pair oligonucleotide probe containing the A_α H box and surrounding sequences (residues −119 to −88) was synthesized. When assayed by the gel mobility shift procedure, nuclear extracts prepared from untreated and IFN-γ-induced WEHI-3 cells produced one major (H3) and one minor (H5) complex (Fig. 6A). A complex with the same mobility as that of H3 was also observed with the L-cell extract, along with other unique complexes (H4, H5, and H6). In contrast, the H3 complex was not seen with the A20 extract. Instead, several different complexes formed (H1, H2, H4, H5, and H6). An identical pattern was seen with an extract prepared from a second B-lymphoma cell line, M12, that also consti-

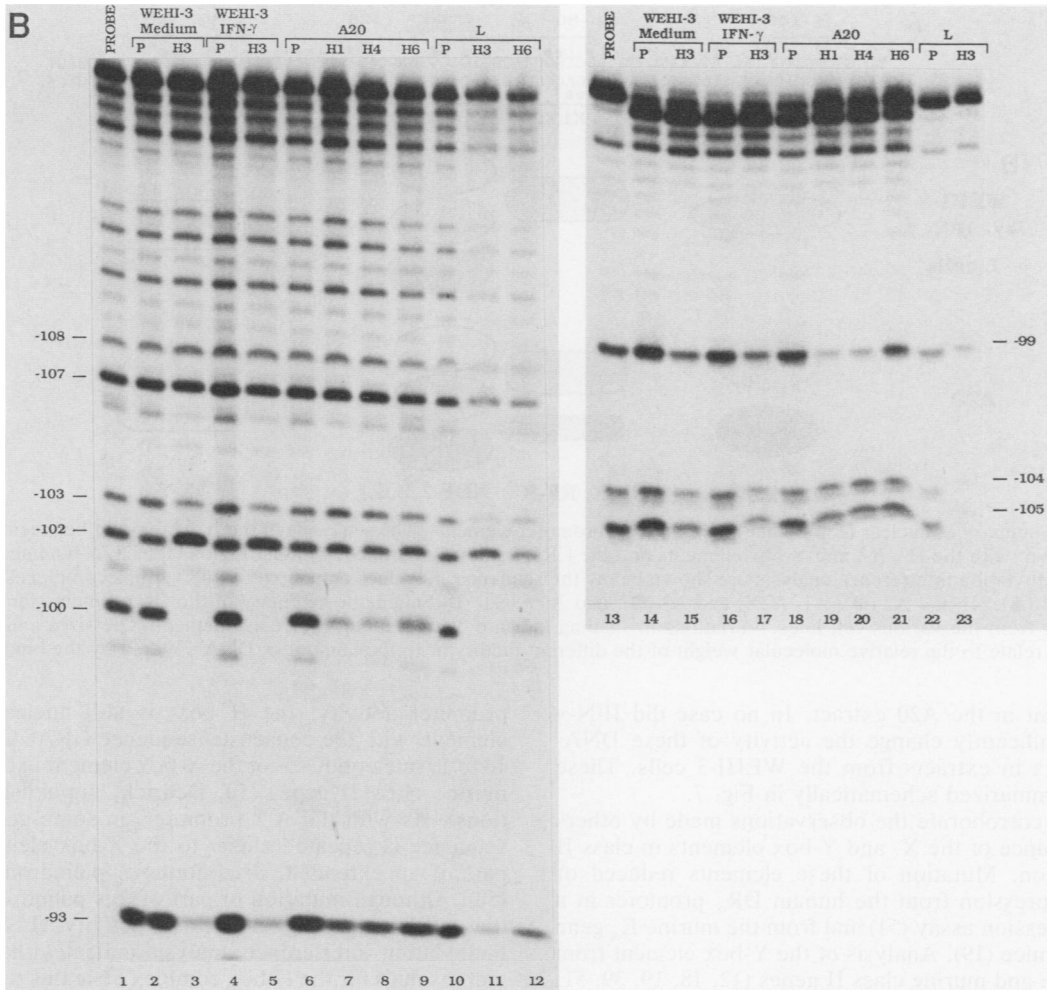


FIG. 6—*Continued.*

tutively expresses Ia. Binding competition analysis with unlabeled H-box probe, X-box probe, and a mutant H-box probe in which residues −106 to −100 had been changed to correspond to those in LS[−112,−99] indicated that only H3 from the WEHI-3 and L-cell extracts and H1 and H4 from the A20 extracts were specific for the H-box sequences (data not shown). Methylation interference analysis demonstrated that these complexes involved contacts with residues within the H-box element. Thus, the H3 complex from the WEHI-3 or L-cell extracts contacted G residues −100 and −93 on the noncoding strand and −104, −105, and −99 on the coding strand (Fig. 6B and 7). The interference patterns of the H1 and H4 complexes from the A20 extracts differed somewhat from that of H3 in that methylation of residues −105 and −104 on the coding strand and −93 on the noncoding strand did not seem to interfere with binding as greatly as for the H3 complex. However, the patterns for the H1 and H4 complexes were distinct from the patterns for the nonspecific H6 complex (Fig. 6B). These results suggest that WEHI-3 and L cells contain a common H-box-binding factor and that the activity of this factor is not significantly affected by IFN-γ treatment. However, two different H-box-binding factors seem to be present in extracts from B-cell lines that constitutively express Ia.

DISCUSSION

Expression of the class II MHC antigens is regulated in a complex manner subject to tissue-specific and developmental influences and to modulation by a number of extracellular agents. To understand the molecular mechanisms involved in the control of Ia expression, it is important to define the sequence elements and *trans*-acting factors that are involved in regulating transcription of the various class II genes in cells with different Ia-expressing phenotypes. Our analysis of A_α^d promoter activity in cultured cell lines of nonexpressing, constitutively expressing, or IFN-γ-inducible phenotypes indicates that at least three and probably four sequence elements are required for expression. These elements include the H-, X-, and Y-box homologies found in the promoters of other human and mouse class II genes. In addition, the A_α promoter contains an element homologous to the CRE. Extracts from all cells examined contain a common nuclear factor that binds to the Y-box element, a set of common factors that bind to the CRE-like element, and a common X-box-binding factor. The A20 cells and possibly the L cells have an additional X-box-binding factor. WEHI-3 and L cells contain a common factor that binds to the H-box element, but two different H-box-binding activi-

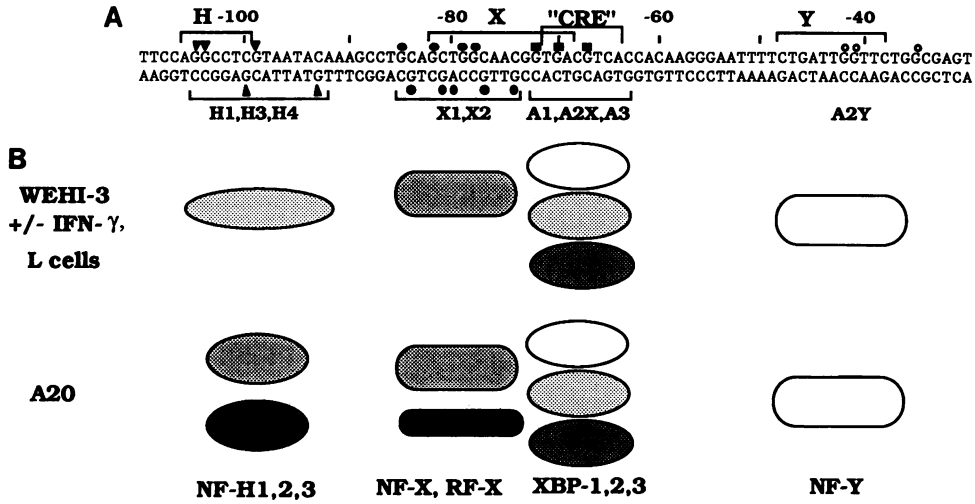


FIG. 7. (A) Summary of nuclear factors that bind to the A_α promoter elements. The sequence of the A_α^d promoter between residues −110 and −30 is shown, with the H-, X-, and Y-box elements and the CRE homology outlined. Contact regions for DNA-binding complexes, as indicated by methylation interference analysis are shown below the sequence. Residues contacted by the various complexes are indicated: H1, H2, and H4 (▲); X1 and X2 (●); A1, A2X, and A3 (■); and A2Y (○). (B) Schematic summary of the DNA-binding factors present in nuclear extracts from the various cell lines. Variations in shading indicate distinct factors for each element. The sizes and shapes of the symbols do not relate to the relative molecular weight of the different factors or to the amount of DNA covered by the binding complex.

ties are present in the A20 extract. In no case did IFN-γ treatment significantly change the activity of these DNA-binding factors in extracts from the WEHI-3 cells. These results are summarized schematically in Fig. 7.

Our results corroborate the observations made by others on the importance of the X- and Y-box elements in class II gene expression. Mutation of these elements reduced or eliminated expression from the human DR_α promoter in a transient expression assay (51) and from the murine E_α gene in transgenic mice (19). Analysis of the Y-box element from several human and murine class II genes (12, 18, 19, 39, 51; this report) indicates that the Y-box element is a positively acting element that binds a common factor that has been termed NF-Y (19). However, these results seem to be discordant with the recent observation that expression of a cloned factor that binds to the DR_α Y box is inversely correlated with expression of the DR_α gene, which might suggest that this is a negatively acting regulatory factor (15).

Whereas factors that bind to the X-box element have been more difficult to detect, DNA-binding activities have been found for the E_α (NF-X [19, 29]) and the DR_α (RF-X [11, 46]) X-box elements. In each of these cases, as with the A_α X box in our studies, methylation interference patterns indicated that contacts with purine residues were limited to the 5′ side of the element and did not extend to the three nucleotides on the 3′ end. In light of the CRE-like element that overlaps the 3′ end of the A_α X box, these results suggest that the X-box element is probably more limited than previously thought. Furthermore, the number of X-box-binding factors is still unclear. We (this report) and others (7, 11, 20) have detected multiple X-box-binding complexes in B-cell extracts. However, we observed a unique complex for the A_α X box when WEHI-3 extracts were studied. Similarly, a unique complex was observed for the DR_α X box with extracts from human B-cell lines (46). Finally, it is interesting to note that the murine X-box factor is present in the murine fibroblastic L cells that do not express the class II genes, suggesting that this factor might be involved in the expression of other genes.

The nature of the final conserved element required for A_α^d promoter activity, the H box, is still unclear. A similar element, with the consensus sequence GRACCTT, is found 16 to 19 nucleotides 5′ of the X-box element in all human and murine class II genes (50; Dedrick, unpublished observations). As with the A_α^d promoter, in some genes a similar sequence is repeated closer to the X-box element (3) or is part of an extended, discontinuous palindromic sequence (58). Although mutation of part of this palindromic element did not significantly affect A_α activity (LS[−102,−89]), methylation interference analysis indicates that some contact residues for the H-box complex lie in this region, outside the H-box consensus sequence (Fig. 7). The precise structure of this element will require a more fine-scale mutational analysis and a comparison of binding factors for H-box elements of the various class II genes. The fact that we find distinct binding factors in extracts prepared from the WEHI-3 cells compared with the A20 cells may partially explain the distinct mechanisms that are apparently involved in the regulation of class II expression in these two cell types (34).

It is interesting to find a CRE homology associated with the A_α promoter, since IFN-γ-induced class II expression in macrophages is normally thought to be inhibited by agents that elevate cyclic AMP levels (52, 54). Since the LS[−75,−62] mutant alters both the CRE-like sequence and some of the residues involved in binding to the more conserved X-box sequence, the potential role of this element in A_α expression will require a higher-resolution mutational analysis than we have done in these experiments.

Although great advances have been made in defining the components involved in the activity of the class II MHC promoters, we are still far from understanding how these factors and sequence elements interact to achieve the complex pattern of regulation characteristic of Ia expression. For instance, it is unclear how IFN-γ induces A_α expression in WEHI-3 cells. It has been suggested from mutational analysis of the DR_α (58) and the DQ_β (8) promoters that the H-box sequence binds a negative regulatory factor that is released by IFN-γ. In our analysis, mutation of the H box, as well as the X or Y box, destroys A_α promoter activity in the WEHI-3 cells, with or without IFN-γ, suggesting that these

are all positive-acting elements. In this case it is not possible to determine through which element IFN-γ is acting, since all of the mutations have essentially the same phenotype. Furthermore, it has been shown that transmission of IFN-γ responsiveness to a heterologous promoter requires both the H-box and the X-box elements (57, 58; Dedrick, unpublished observations), indicating that a single element is not sufficient. Although we have detected specific DNA-binding factors for each of the elements identified by mutation analysis, in no case was there a significant change in the activity of these factors in extracts prepared from untreated or IFN-γ-induced WEHI-3 cells. This result indicates that IFN-γ does not act by inducing a unique DNA-binding protein. Instead, it seems likely that IFN-γ acts by inducing a factor that modifies or binds to one or more of the existing factors. Though it has been suggested that de novo protein synthesis is necessary for transcriptional induction of the DR_α by IFN-γ (5), this matter remains controversial (3).

The existence of additional components required for class II MHC expression that are distinct from the factors that bind to the promoter elements has also been suggested by analysis of class II-deficient B-cell variants that have been isolated in several laboratories (1, 11). Class II gene expression can be rescued in these cells by fusion to other Ia-positive B cells, suggesting that there is a deficiency in some *trans*-acting factor; however, none of the relevant DNA-binding factors has been found to be missing (11, 29). Furthermore, the role of additional factors is indicated by the analysis of the L cells in this study. Although these cells seem to have a full complement of DNA-binding factors for the A_α promoter, the promoter remains weak in this cell line.

ACKNOWLEDGMENTS

We thank S. Sazer for technical advice, S. Subramani, L. Hood, and M. Davis for generously providing plasmid DNA, and T. Zimmer and Y. Freund for critical reading of the manuscript.

This work was supported by Public Health Service grants AI 19964 and AI 19512 from the National Institute of Allergy and Infectious Diseases. R.D. was supported by Public Health Service training grant AI 07290 from the National Institute of Allergy and Infectious Diseases and a fellowship from the Arthritis Foundation.

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