MOLECULAR AND CELLULAR BIOLOGY, Aug. 1990, p. 3906–3916
0270-7306/90/083906-11$02.00/0

Vol. 10, No. 8

# Transcriptional Control of the Invariant Chain Gene Involves Promoter and Enhancer Elements Common to and Distinct from Major Histocompatibility Complex Class II Genes

LI ZHU AND PATRICIA P. JONES*

*Department of Biological Sciences, Stanford University, Stanford, California 94305-5020*

Received 27 December 1989/Accepted 2 May 1990

**The invariant chain ($I_i$) is a glycoprotein coexpressed with the major histocompatibility complex (MHC) class II antigens. Although $I_i$ is encoded by a single gene unlinked to the MHC gene complex, $I_i$ and MHC class II appear to have similar patterns of tissue specific expression and generally are coordinately regulated by cytokines. Here we present evidence that transcription of the murine $I_i$ gene is controlled by multiple *cis*-acting elements. The 5′ regulatory region of the $I_i$ gene appears to be combined of conserved class II regulatory elements with promoter elements commonly found in other eucaryotic genes. A region containing characteristic class II promoter elements (H box, X box, and a modified Y box) serves as an upstream enhancer in the $I_i$ gene and might contribute to the coexpression of MHC class II and $I_i$ genes. A series of positive control elements, the κB element, Sp1-binding site, and CCAAT box, are present in the $I_i$ promoter and apparently serve distinct regulatory functions. The κB site in the $I_i$ gene is a cell type-specific element, contributing to expression in a B-cell line but not in a fibroblast cell line, and the Sp1 site is required by the H-X-Y′ enhancer element to stimulate promoter activity. In addition, an $I_i$ enhancer in the first intron that specifically stimulates its own promoter has been identified. Our results suggest that a sequence match between enhancers and certain promoter elements is critical.**

The major histocompatibility complex (MHC) class II molecules (also called Ia antigens in the mouse) are highly polymorphic cell surface proteins that play key functions in immune responses. A specific immune response is initiated with the recognition by T-cell receptors of processed foreign antigen peptides bound to MHC proteins (36). Multiple MHC class II genes encode the α and β chains of class II heterodimers, and they all share several common sequence motifs in their promoters. These motifs are named H (heptamer) (3, 12, 38), X, and Y boxes (42). In the last several years, much evidence has been found to support the importance of these motifs for the appropriate expression of MHC class II genes (3, 5, 12, 14, 39, 46). Some experiments also showed that these motifs can serve as enhancers in transient assay systems using the simian virus 40 (SV40) or herpes simplex virus (HSV) thymidine kinase gene (*tk*) promoter (43).

During the course of investigation of MHC class II structure, it was found that a nonpolymorphic glycoprotein called the Ia-associated invariant chain ($I_i$) is noncovalently associated with MHC class II α and β dimers of all isotypes (21). The murine invariant chain is restricted to class II proteins in the intracellular membrane compartments; little or none is found in cell surface class II proteins (44). Analysis of genes from both humans (9) and mice (40) has shown that the structure of $I_i$ bears no resemblance to that of MHC class II proteins and that the $I_i$ gene is not linked to the MHC complex (10, 35). Recent transfection experiments suggested that the $I_i$ chain may not be needed for cell surface expression of class II proteins (29, 37) but may be required for the processing or presentation of some foreign antigens in at least some cell types (41).

Two intriguing characteristics of $I_i$ make it an interesting subject for the investigation of gene regulation. First, all of the tissues that express the MHC class II proteins have been found to express $I_i$ (25); specifically, $I_i$ and MHC class II are both expressed constitutively in B cells, and both are absent from murine T cells (23). Moreover, levels of both $I_i$ and MHC class II expression can be induced by gamma interferon (11) and tumor necrosis factor (TNF) (8) in monocytes and macrophages and by interleukin-4 in pre-B cells (33). Second, despite the similarities in the expression of $I_i$ and MHC class II, some differences exist. The expression of $I_i$ has been observed in the absence of MHC class II, such as in some pre-B lymphoma, plasmacytoma, and L fibroblast cell lines (1, 24, 26). Reith et al. found that in human class $II^-$ immunodeficiency patients, none of the MHC class II chains are expressed, apparently because of the absence of an X-box-binding activity named RF-X; nevertheless, the level of $I_i$ expressed was apparently normal (34) despite the presence of an X box in its 5′ regulatory region.

The studies reported here identify *cis*-acting elements contributing to the transcriptional control of the mouse $I_i$ gene. We have found a tissue-specific promoter and multiple enhancers in the $I_i$ gene, and they appear to interact cooperatively. In $I_i$, an upstream sequence containing the H-X-Y′ motif serves as an enhancer, whereas similar motifs are required for promoter activity in the MHC class II genes. The function of the 5′ enhancer was found to be dependent on the presence of an Sp1-binding site in the promoter region. In addition, an intronic enhancer may require other elements of the $I_i$ promoter. These unique features probably are responsible for the distinct regulation of the $I_i$ gene relative to the MHC class II genes.

## MATERIALS AND METHODS

**Cells.** The cells used in this study were M12, a B-lymphoma line (22); BW5147, a T-lymphoma line (20); and WEHI-3, a myelomonocytic line (47). All three cell lines were grown in RPMI 1640 medium supplemented with 10%

* Corresponding author.

fetal calf serum (Hyclone), $5 \times 10^{-5}$ M β-mercaptoethanol, penicillin (50 U/ml), and streptomycin (50 μg/ml). The L $tk^-$ murine fibroblast cell line (16) was also used as a transfection recipient; it was grown in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum and 5% calf serum.

**Plasmids.** The starting plasmid pA10CAT-2 (abbreviated pA10-2) is a pBR322-derived chloramphenicol acetyltransferase (CAT) expression vector containing the minimum length of the SV40 early promoter. To assay enhancer activity, foreign DNA fragments were inserted either at the promoter-proximal or -distal positions, and the CAT signals derived from the new constructs were compared with that from the parental plasmid pA10-2. The SV40 promoter itself can also be removed and replaced by appropriate DNA fragments for assaying promoter activities. Plasmid pSVCAT-2 (abbreviated pSV-2) differs from pA10-2 in that it also contains the SV40 enhancer; it was used as a positive control throughout the work. Both pA10-2 and pSV-2 were obtained from J. Parnes (Stanford University, Stanford, Calif.). A *SalI/Bam*HI fragment from pA10-2, which contains the SV40 promoter and CAT-coding sequences as well as the poly(A) adenylation signal, was inserted into the same restriction sites in pUC18 to construct pUCA10-2, which contains more convenient cloning sites than pA10-2 but generally gave higher background CAT activity than pA10-2. All of the $I_i$ genomic sequences were derived from cosmid clone 10.7, generously provided by L. Hood (California Institute of Technology, Pasadena, Calif.). About 9.6 kilobase pairs (kb) of DNA from this cosmid, covering the entire $I_i$ gene and 843 base pairs (bp) of 5′-flanking region, was sequenced and published previously (48).

Plasmid $pI_i843$ was constructed by inserting a 931-bp *Bgl*II-*Nco*I fragment covering the region from −843 to +88 relative to the $I_i$ transcription initiation site into pA10-2 to replace the SV40 promoter after treating the 3′ end with S1 nuclease to make sure that the ATG start codon at +88 no longer existed. Plasmids $pI_i183$ and $pI_i660$ were made by deleting either the distal *Bgl*II-*Stu*I 660-bp fragment or the proximal *Stu*I-*Nco*I 271-bp fragment from $pI_i843$. Further deletions in the promoter-proximal region were made in clone $pI_i843$ by digestion first with *Stu*I at −183 and then with BAL 31 (2). The remaining promoter-distal sequences were removed by digestion with another restriction enzyme that cuts in the polylinker adjacent to −843. The exact break points of these deletion clones were identified by double-stranded sequencing. The deletion clones are named according to their lengths, counted from −1 to the 5′ end.

$I_i$ enhancer activities were tested with several heterologous promoters. Plasmids $pA_\alpha$CAT-209 and $pA_\alpha$CAT-58 (12) carry 209 and 58 bp, respectively of the murine $A_\alpha^d$ class II promoter region. pTE-2 is a plasmid provided by B. M. Peterlin (University of California, San Francisco) that contains the HSV *tk* promoter driving the CAT gene in the pUC18 vector. $p\beta_2$MCAT is a plasmid provided by J. Parnes that contains 594 bp of promoter sequence of the murine $\beta_2$-microglobulin gene. pdhfrCAT was created by replacing the SV40 early promoter from pUCA10-2 with mouse dihydrofolate reductase gene (*dhfr*) promoter in the 5′→3′ or 3′→5′ orientation. The *dhfr* promoter was a *Hin*dIII fragment of 280 bp extending from −3 to −283, provided by R. T. Schimke (Stanford University). A 701-bp *Stu*I fragment of the $I_i$ 5′ region containing the principal enhancer activity was inserted in both orientations into these various promoter-controlled CAT expression plasmids in the promoter-distal region.

The luciferase expression plasmid pSVALΔ5′ (13), used as the internal transfection efficiency control, was a generous gift from S. Subramani (University of California, San Diego, La Jolla, Calif.). Construction and manipulation of all plasmids were done according to standard procedures (2). All plasmids were purified by banding twice by ultracentrifugation in an ethidium bromide-CsCl gradient.

**Transfections.** DEAE-dextran-mediated DNA transfection was carried out as described by Lopta et al. (27), with minor changes. Cells ($2 \times 10^6$) were seeded 1 day before the transfection in a 10-cm-diameter tissue culture dish. Cells were washed once with serum-free medium and then covered with 3.5 ml of medium containing 10 μg of pA10-2 or other plasmid DNAs of molar equivalent and 15 (for M12 and WEHI-3 cells) or 30 (for BW5147 and L cells) μg of DEAE-dextran per ml. After 3 h of incubation, the cells were shocked in 2 ml of 5% (for L and BW5147 cells) or 10% (for M12 and WEHI-3 cells) dimethyl sulfoxide in phosphate-buffered saline for 2 min. A 2-day incubation period in complete medium followed to allow the transfected DNA to be expressed. In some experiments, luciferase was used as an internal control by cotransfecting each dish of cells with 2 μg of plasmid pSVALΔ5′.

**Enzyme assays.** CAT activity in transfected cells was measured according to the standard procedure (2). Briefly, cells were harvested from the dishes and transferred to 15-ml screw-cap tubes, washed once with phosphate-buffered saline, suspended in 100 to 200 μl of 25 mM Tris hydrochloride pH 7.5, and then lysed with three cycles of freeze-thaw. The supernatant was collected after centrifugation, and the protein concentration was measured by the Bio-Rad protein concentration quick-assay method (Bio-Rad Laboratories, Richmond, Calif.) (6). The CAT assay was carried out by mixing four-fifths of each cell extract normalized by protein concentration with $1.5 \times 10^5$ cpm of [$^{14}$C]chloramphenicol and acetyl coenzyme A at a final concentration of 1 mM in a 100-μl volume at 37°C for 1 h. The mixture was then extracted with ethyl acetate and spotted on a thin-layer silica plate (Eastman Kodak Co., Rochester, N.Y.). The acetylated products were separated by chromatography in 95:5 chloroform-methanol for 3 h, visualized by autoradiography, and quantified by scintillation counting of the cut-out spots. Luciferase activity was measured from one-fifth of each normalized cell extract by the method of deWet et al. (13), using a Moonlight 2001 luminomitor (Analytical Luminescence Laboratory, San Diego, Calif.). The final values for CAT activity in each assay were normalized by luciferase activity.

**Primer extension.** The primer extension analysis was performed by the standard procedure (2). Briefly, total RNA was extracted from $2 \times 10^7$ cells 40 h after DEAE-dextran-mediated CAT plasmid transfection. RNA (40 μg) was annealed at 30°C for 12 h to $5 \times 10^4$ cpm of $^{32}$P-labeled 18-mer oligonucleotide primer (5′-GGGATATATCAACGGT GG-3′) that was synthesized according to a CAT-coding region sequence. Thus, no endogenous $I_i$ transcript would be detected. An 80-U sample of avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was added to the annealed mixture to extend the oligonucleotide primer for 90 min at 40°C in the presence of four deoxynucleoside triphosphates and RNasin (Promega Biotec, Madison, Wis.). A brief RNase A digestion followed the incubation. The mixture was then phenol-chloroform extracted, ethanol precipitated, and resolved in a 6% sequencing gel.

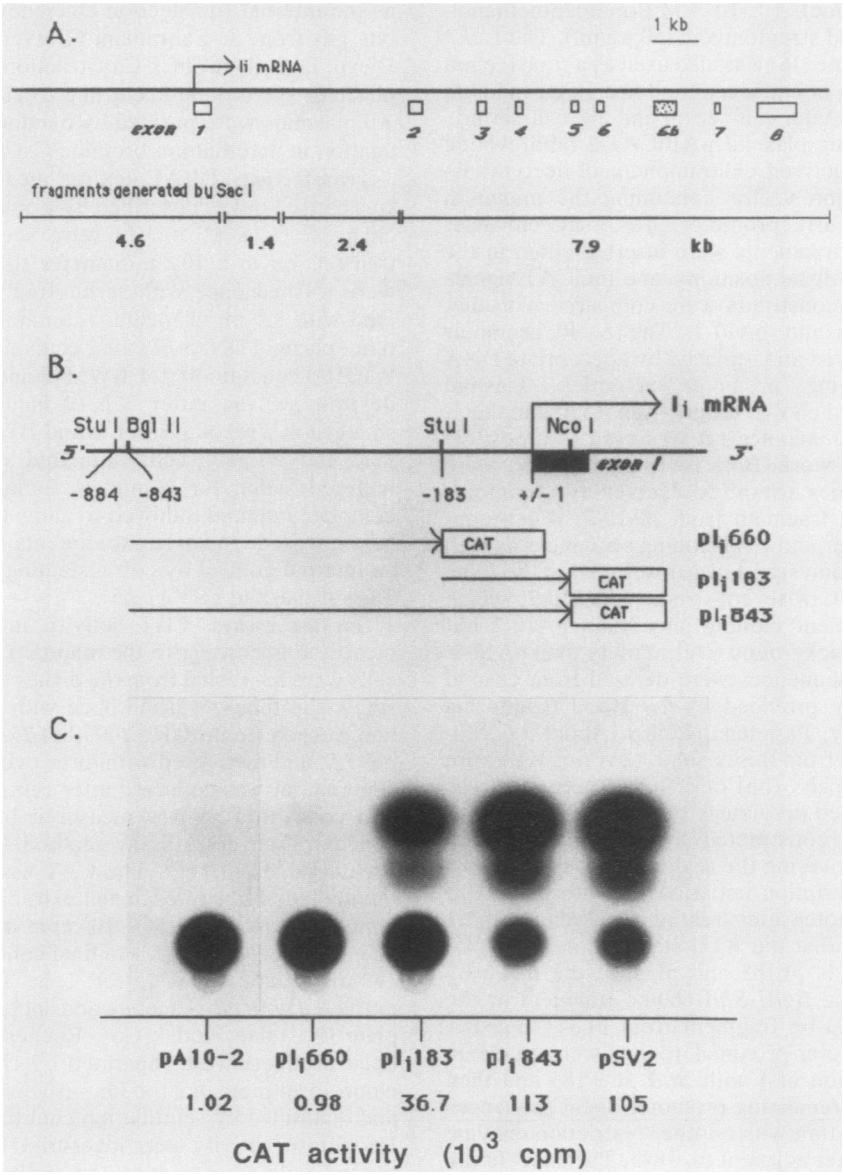


FIG. 1. $I_i$ gene organization and promoter screen. (A) Genomic organization of the $I_i$-chain gene. The arrow indicates the initiation site and the orientation of $I_i$ transcription. The open squares below the solid line represent exons. Exon 6b is differentially spliced in different $I_i$ mRNAs (40). (B) The CAT constructs for $I_i$ promoter screening. The 5′ upstream region is enlarged. Several genomic DNA fragments were connected to the CAT-coding sequence in promoterless pA10-2 with 5′→3′ orientations to generate expression clones (names are given on the right). (C) CAT assays of the different promoter constructs in M12 cells. The activity below each construct was determined by counting the $^{14}C$-acetylated chloramphenicol spots.

## RESULTS

**5′-Flanking region enhancer and promoter elements.** Schematic maps of the murine $I_i$ gene organization (48) and of constructs generated from the 5′ region of the $I_i$ gene are shown in Fig. 1A and B. To analyze sequence elements important for promoter activity of the murine $I_i$ gene, we replaced the SV40 early promoter in pA10CAT2 with a *Bgl*II-*Nco*I fragment extending from −843 to +88 ($pI_i843$) to drive the expression of the CAT reporter gene. This fragment showed strong promoter activity when tested in the M12 B-cell line. When a 660-bp fragment was deleted from the 5′ end of this fragment to −183, promoter activity of this fragment ($pI_i183$, containing 183 bp from the cap site) dropped to about one-third that of 931-bp fragment. The 660-bp fragment from the 5′ end alone did not show any promoter activity (Fig. 1B and C). These results suggested that sequences 3′ of −183 are required for $I_i$ promoter activity and that this activity can be further enhanced by sequences contained in the 660-bp fragment.

The results of the promoter screen prompted an examination of the 660-bp *Bgl*II-*Stu*I fragment for enhancer activity. Indeed, a 701-bp *Stu*I fragment including the 660-bp *Bgl*II-*Stu*I fragment showed strong enhancer activity when it was inserted either 5′ or 3′ of the SV40 early promoter (Fig. 2C). Thus, this enhancer activity increased the level of expression both of the heterologous promoter (SV40) and of its own cognate promoter ($pI_i183$). When the 701-bp *Stu*I fragment was inserted into the distal region of another SV40 early

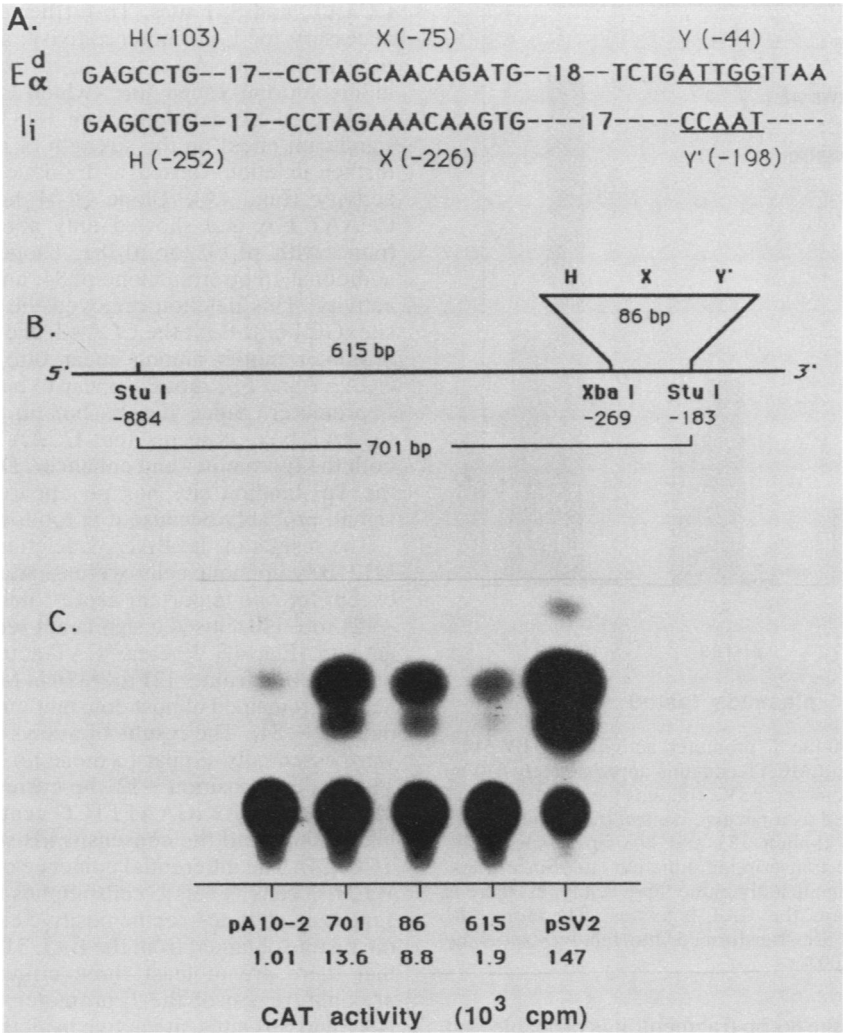


FIG. 2. Screening for the upstream enhancer core. (A) Alignment of the conserved H, X, and Y (Y') boxes of the $E_\alpha^d$ and $I_i$ genes. Numbers between the box sequences represent the numbers of nonconserved nucleotides. Numbers in parentheses indicate the distance between the center of the conserved boxes and the transcription initiation site of the respective genes. (B) The three enhancer test fragments: 701-bp *Stu*I, 615-bp *Stu*I-*Xba*I, and 86-bp *Xba*I-*Stu*I. The 86-bp *Xba*I-*Stu*I contains all three conserved boxes. (C) A representative CAT assay for transfected constructs in which the three restriction fragments were inserted into the downstream region of the SV40 early promoter in pA10-2, in the 5'→3' orientation. The values of the CAT signal for each construct are same as in Fig. 1.

promoter-containing construct, pUCA10-2 (see below), the enhancer activity was still very strong. Furthermore, this *Stu*I fragment in both orientations was capable of stimulating expression of the HSV *tk* promoter as well as the SV40 early promoter (see below). Thus, by all established criteria, this 701-bp *Stu*I fragment is a true enhancer.

The conclusion from these initial studies is that the immediate 5'-flanking region of the $I_i$ gene contains one strong promoter region and one strong enhancer region. The total activity obtained with the $I_i$-chain gene promoter and enhancer was comparable to that obtained with the positive control, pSV-2; it was much higher than that of the MHC class II $A_\alpha^d$ gene promoter, which is also constitutively expressed in M12 cells (see below) and other B-cell lines (12).

**The upstream enhancer contains the H-X-Y' boxes.** To examine the $I_i$ upstream enhancer in more detail, we took advantage of the existence of an *Xba*I site that divides the 701-bp *Stu*I fragment into 5' 615-bp and 3' 86-bp fragments (Fig. 2B). When these fragments were compared for their abilities to serve as enhancers in pA10-2, it was found that the 5' 615-bp fragment had no activity on its own, whereas the 3' 86-bp fragment showed significant activity, although the level was about half that of the intact 701-bp fragment (Fig. 2C). This result suggested that 86-bp *Xba*I-*Stu*I fragment contains the enhancer core; however, the full activity was also dependent on cooperation with some upstream sequences.

The 86-bp fragment contains sequences homologous to the H-X-Y boxes that have been found in the promoters of all human and murine MHC class II genes so far sequenced (7, 12, 44). Figure 2A compares the H-X-Y boxes from the $E_\alpha^k$ (45) and the murine $I_i$ (48) genes. The H-box and X-box sequences are highly conserved. The Y box seems to be not conserved. However, the core sequence of the Y box, AT TGG, as a reversed CCAAT box is found in the opposite orientation in the $I_i$ gene, and we call this the Y' box. The distances between the H, X, and Y' boxes in $I_i$ are conserved relative to class II genes. The human $I_i$ gene shows very similar features in the same region (31). Since this H-X-Y'

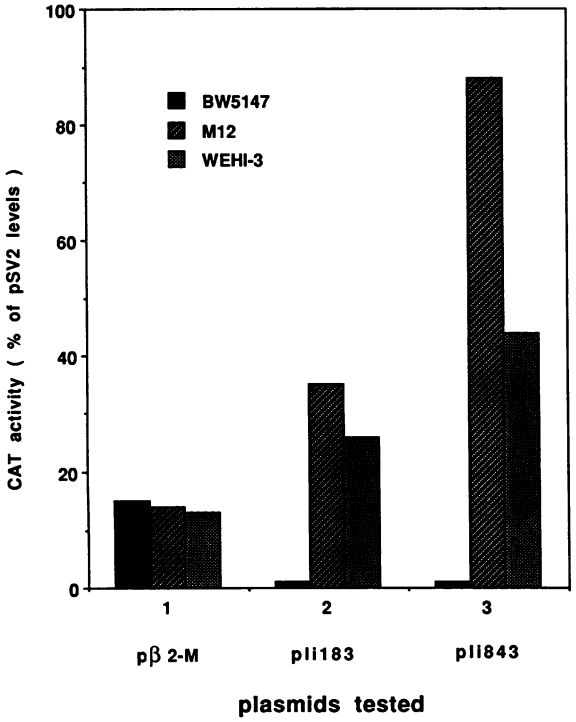


FIG. 3. Comparison of the $I_i$ promoter activities in BW5147, M12, and WEHI-3 cells. $p\beta_2$MCAT contains approximately 530 bp of the $\beta_2$-microglobulin promoter; pSV2 contains the SV40 enhancer and promoter and was used as a positive control (not shown in the plot). $pI_i183$ and $pI_i843$ contain 183 and 843 bp, respectively, upstream from the $I_i$ gene transcription initiation site. Since transfection efficiency varied significantly in the three cell types, there is no direct way to compare the CAT activities. Therefore, the measured CAT activities are transformed into relative values by setting pSV2 activity as 100%.

motif occupies most of the 86-bp fragment, it is probable that these boxes actually contain the majority of the enhancer activity observed with the whole fragment.

**The $I_i$ promoter determines tissue-specific expression.** $I_i$ expression is known to have a tissue specificity very similar to that of the MHC class II genes. To explore the basis for the tissue-specific expression of $I_i$, we tested the $I_i$-CAT constructs in three representative mouse cell lines: M12, a B-lymphoma line; BW5147, a T-lymphoma line; and WEHI-3, a monocyte line. The expression levels of $pI_i843$ and $pI_i183$ were high in M12 cells and somewhat lower in WEHI-3 cells (Fig. 3). In contrast, there was no detectable expression of either $pI_i843$ or $pI_i183$ in BW5147. Although the transfection efficiency was lower in BW5147, as revealed by the internal control of luciferase activity (data not shown) and by the positive control, pSV-2, the complete absence of CAT signals in the samples from $PI_i183$- and $pI_i843$-transfected BW5147 cells implies that the $I_i$ promoter is a B-cell- and macrophage-specific *cis*-acting element. As a positive control, a CAT construct driven by the $\beta_2$-microglobulin promoter was expressed similarly in all three cell lines. When the $I_i$ enhancer alone was tested for tissue specificity by connecting the 701-bp *Stu*I fragment to either the *tk* or *dhfr* promoter, it did not show cell type specificity because it is also active in the BW5147 T-cell line (data not shown). In conclusion, the $I_i$ promoter confers upon the $I_i$ gene its strict cell type specificity.

**Promoter region deletions reveal the importance of the κB, CCAAT, and Sp1 sites.** To further delineate the sequences necessary for $I_i$ promoter activity, a series of 5′-to-3′ deletions in the $I_i$ promoter region was generated. When assayed in the murine L-cell line (which constitutively expresses transfected $I_i$), deletions from −183 to −100 did not have significant effect on the strength of the promoter; however, further deletion caused a dramatic decrease of promoter activity (Fig. 4A). Clone $pI_i84$ has deleted a proximal CCAAT box and showed only about 20% of the activity found with $pI_i103$ or $pI_i183$. Clone $pI_i68$ has deleted an additional 16 bp from clone $pI_i84$, and it showed even lower activity. This deletion removed the conserved Sp1-binding site (GC box). Both the CCAAT and GC boxes are common promoter motifs among eucaryotic genes (30). Thus, the CCAAT and Sp1 motifs appear to be crucial elements in the $I_i$ promoter. Since the Y′ box in the $I_i$ gene is another CCAAT box, it seems that CCAAT boxes are present in both the $I_i$ promoter and enhancer. Deletions further beyond the Sp1-binding site has no apparent effect on the CAT signal, probably because it is too low already.

The results of the BAL 31 deletion constructs assayed in M12 B-lymphoma cells were similar to those for L cells except for one important aspect: deletion of sequence from −121 to −110 caused a significant reduction in the promoter activity (Fig. 4). Whereas CAT activity dropped markedly with deletion from −121 to −110 in M12 cells, in L cells CAT activity remained almost constant until the CCAAT site was deleted (−84). The results of assays in the WEHI-3 cell line were essentially similar to those for the M12 cells (data not shown). The position −121 bp corresponds to the 5′ border of a κB site: GGGGAATTTCC centered at −116, which is highly similar to the consensus κB sequence, GGGGAAAT TCC (24). The differential influence of the κB site in M12 and WEHI-3 cells versus L cells implies that the κB site in the $I_i$ gene is a cell type-specific positive element. Taken together, the results obtained from the BAL 31 deletion clones suggest that there are at least three critical *cis* elements in the proximal region of the $I_i$ promoter, two of which, the CC AAT and Sp1 sites, are active in all three cell types; the third one, the κB site, is active in only two cell types tested.

To be certain that the deletions in the 5′ upstream region did not cause any change in the site of initiation of RNA transcription, we performed primer extension analysis of the CAT transcripts obtained after transfection of five representative deletion clones in M12 cells. These clones differ from each other only in the length of the 5′ region of $I_i$ promoter sequence, with the longest being 183 bp and the shortest 68 bp from the initiation site. A single extended product with 158 nucleotides was detected in all cases (Fig. 4B). This product correlated very well with the reported initiation site for $I_i$ transcription. These results strongly indicate that the CAT assay data of the deletion clones reflect faithful transcription from the shortened $I_i$ promoter.

**The $I_i$ upstream enhancer requires the Sp1 site to be functional.** Since the 701-bp *Stu*I fragment showed high enhancer activity, we tested whether we could increase MHC class II promoter activity by inserting it into two $A_\alpha^d$ promoter-driven CAT plasmids (12), containing 58 or 209 bp of the $A_\alpha^d$ promoter sequence. The former does not contain the $A_\alpha$ H-X-Y boxes; the latter contains all three. When these constructs were transfected into the M12 B-lymphoma cells, neither showed any enhancer activity (Fig. 5). We suspected that this might be because some element(s) required for $I_i$ enhancer function is not present in the $A_\alpha^d$ promoter. Therefore, other promoters were also tested for their ability to be enhanced by the 701-bp *Stu*I fragment.

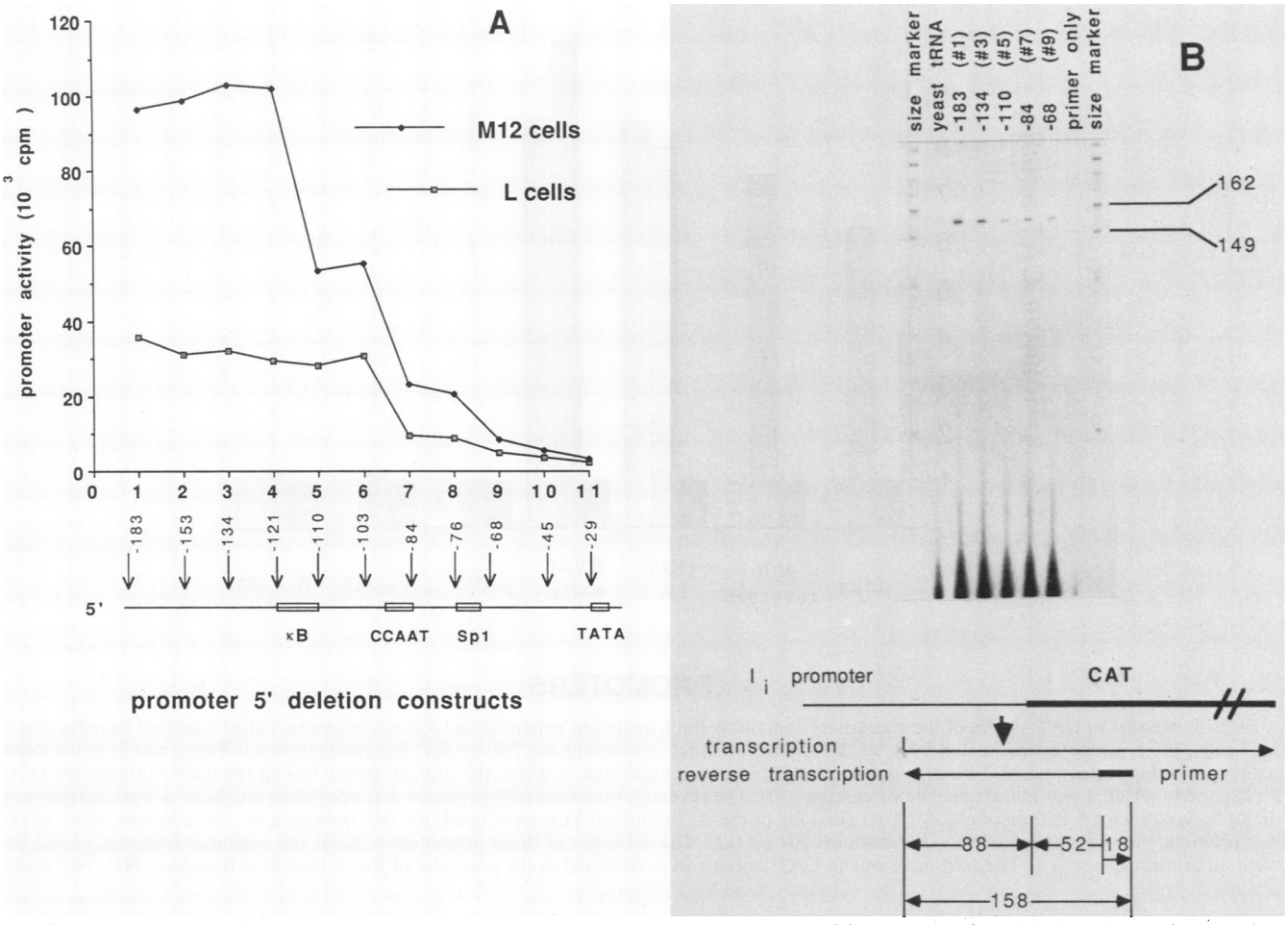


FIG. 4. (A) Analysis of promoter elements using 5′ deletion clones. Results are shown of CAT assays of the deletion clones after transient transfection of M12 cells and L cells. Numbers indicate the 5′ break point for each deletion and thus also represent the length of promoter fragment contained in each clone. Conserved regulatory sequence elements in the 183-bp fragment of the $I_i$ upstream region are indicated. (B) Primer extension analysis of the $I_i$ promoter deletion clones driving CAT expression in the transient assay. A 40-μg sample of total RNA from transfected M12 cells was annealed to a $^{32}$P-labeled 18-mer oligonucleotide corresponding to part of the CAT-coding region as shown schematically in the diagram. Products extended by avian myeloblastosis virus reverse transcriptase were resolved in a 6% sequencing gel. *Hpa*II-digested pBR322 was used as size markers after [$^{32}$P]-dCTP fill-in labeling.

Among six promoters tested, four (HSV *tk* promoter, SV40 early promoter, *dhfr* promoter, and $I_i$ promoter itself [the HSV *tk* and SV40 early promoters were tested with the *Stu*I 701-bp fragment inserted in both orientations]) were positive and two ($\beta_2$-microglobulin and $A_\alpha^d$ promoters) were negative (see Fig. 1C for the $I_i$ results and Fig. 5 for the others). The *dhfr*-CAT constructs were made in two ways: the *dhfr* promoter was oriented in the 5′→3′ direction relative to CAT sequences, as in its native orientation, or in the 3′→5′ direction, in the reversed orientation. The latter has been shown to have a weak promoter activity (18). Interestingly, both of these constructs responded to the $I_i$ enhancer.

When we compared the identifiable sequence motifs among these promoters, we immediately noticed that all of the promoters stimulated by the $I_i$ upstream enhancer contained one or more Sp1 sites (GGGCGG), whereas the unresponsive promoters lacked it. Therefore, it seemed very likely that the Sp1 site in the $I_i$ promoter is required for the upstream enhancer to be functional. This conclusion was confirmed by the behavior of some of the $I_i$ deletion mutations. The 701-bp *Stu*I fragment or the 86-bp *Xba*I-*Stu*I fragment could significantly enhance expression from $pI_i84$ or $pI_i76$, which both retain the Sp1-binding element, but not from $pI_i68$, from which the GC box has been deleted (Fig. 6). These findings strongly support the hypothesis that the upstream $I_i$ enhancer requires an Sp1 site in the promoter to fulfill its enhancer function.

**An intronic enhancer exists and may interact specifically with other promoter motif(s).** Since we have found that the $I_i$ upstream enhancer requires at least one special promoter sequence element (Sp1) to be functional, we tested the ability of various fragments of the $I_i$ gene to enhance expression. We first used the $I_i$ 5′ deletion mutation construct, $pI_i76$, as a basal promoter because it gave lower CAT activity than did the full-length promoter clone $pI_i83$, potentially allowing the identification of weak enhancers. When four *Sac*I fragments of the $I_i$ gene (Fig. 1A) were inserted into this plasmid, two (4.6 and 2.4 kb) of the four gave positive signals in M12 cells (Fig. 7). The 4.6-kb *Sac*I fragment was expected to be positive because it contains the major upstream enhancer, and $pI_i76$ does contain an intact Sp1 site. The most striking finding was that the 2.4-kb *Sac*I fragment, which is located in the first intron, showed definite enhancer activity. We have previously failed to detect

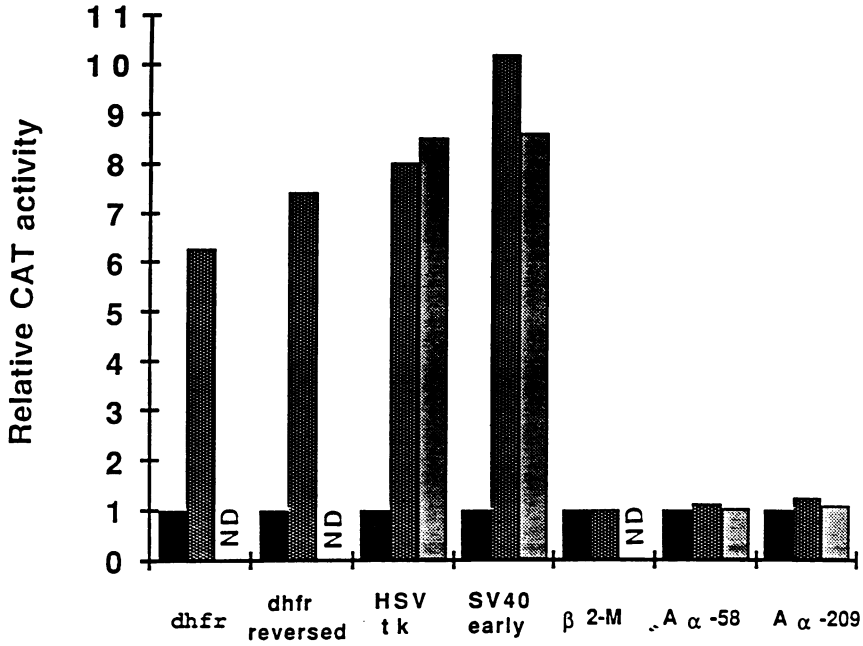


FIG. 5. Results of CAT assays of the constructs containing the $I_i$ upstream enhancer and various promoters after transient transfection of M12 cells. Each promoter was tested by itself (■) and in constructs containing the 701-bp *Stu*I fragment (shown in Fig. 1 and 2) in the distal region at random orientations. Two *dhfr* promoter plasmids were used: one in which the *dhfr* promoter was in the native orientation (5′ to 3′; ▥) and in which it was in the opposite orientation (▨). The reverse-orientation *dhfr* promoter has been shown to have a weak activity and direct an unknown short transcript (18). Two versions of the $A_\alpha^d$ promoter also were used; the one indicated as −58 contains 58 bp of the $A_\alpha^d$ promoter; the one indicated as −209 contains 209 bp (12). The activities of these promoters without the enhancer fragment varied but were all arbitrarily set at 1. The fold increases in CAT activity were obtained in the presence of the 701-bp *Stu*I fragment. ND, Not done.

enhancer activity with this fragment by using the pA10-2 vector, which has the SV40 early promoter (data not shown). However, it might be that this intron enhancer needs an element present in its own promoter but not in the SV40 early promoter as a target for its activity. This $I_i$-specific promoter element must be contained in the sequence remaining in clone $pI_i76$ and must differ from Sp1 or TATA, since both are present in the SV40 early promoter.

We further tested deletion mutation clone $pI_i68$ which, as mentioned before, is identical to $pI_i76$ except that it lacks the Sp1 site. As expected, the 4.6-kb *Sac*I fragment containing the upstream enhancer did not stimulate the 68-bp $I_i$ promoter, and the 1.4- and 7.9-kb *Sac*I fragments stimulated neither the 76-bp nor the 68-bp promoter. However, the 2.4-kb *Sac*I fragment did have strong enhancer activity with the 68-bp promoter (Fig. 7). These results indicate that whereas the upstream enhancer requires an Sp1 site to be functional, the intron enhancer does not. We are in the process of testing other deletion clones, using this strategy to identify the target element(s) in the $I_i$ promoter region for the intronic enhancer, which appears to be located in the 68 bp proximal to the initiation site.

## DISCUSSION

In this report, we have described properties of the transcriptional control of the mouse $I_i$-chain gene, with the goal of understanding the features of its expression common to and distinct from the class II genes. Four positive *cis*-acting elements in the $I_i$ upstream flanking region have been identified by functional analysis, using CAT assays after transient transfection. These are, from 5′ to 3′, the H-X-Y′ boxes centered at −210, the κB site centered at −116, the CCAAT site centered at −80, and the Sp1 site centered at −71. Among these elements, the $I_i$ H-X-Y′ region has been shown to have enhancer activity, whereas the other elements appear to be required for promoter function. Whether the κB site contributes to enhancer activity remains to be seen; it is certainly possible that it could be a lymphoid-monocyte-specific enhancer element, as has been observed for other genes (24). Our results indicate that this κB site shows positive effects in B cells and WEHI-3 cells but not in L cells. In addition, CAT assays in WEHI-3 cells with the series of promoter deletion clones showed that the sequence coinciding with the κB site is required for the TNF response (L. Zhu and P. P. Jones, unpublished data). This suggests that the κB motif might be involved in mediating the TNF response of the $I_i$ gene in monocytic cells. In fact, other studies indicate that κB sites in the class II $A_\alpha$ gene (18a) and interleukin-2 receptor gene (28) contribute to TNF inducibility. The κB site has been found to be involved in mediating both inducible and tissue-specific gene control (4, 24). Our results seem to provide an example of both κB functions in a single gene. The Sp1 site in the $I_i$ gene also seems to play dual functions. It is not only a critical promoter element but also an essential element for the activity of the upstream enhancer.

An additional enhancer was identified in the first intron of the $I_i$ gene. It appears to require promoter elements other

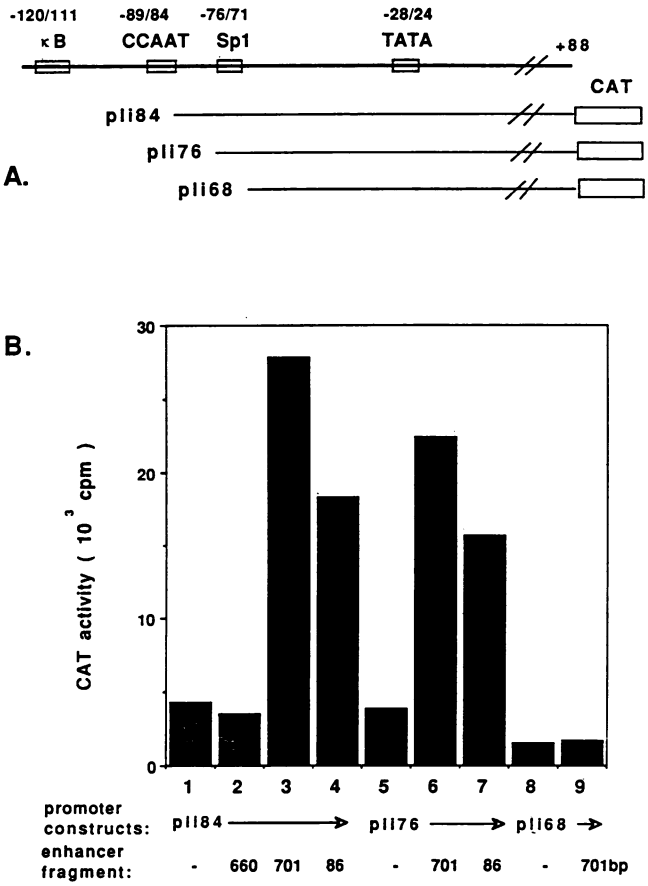


FIG. 6. Mapping the enhancer-responsive element. A representative CAT assay in M12 cells is shown for three promoter deletion clones with various insertions. The 5′ break points of three clones are indicated in panel A. Columns 1 to 4 in panel B contained samples from cells transfected with clone $pI_i84$ and its derivatives; columns 5 to 7 were obtained with $pI_i76$, and columns 8 and 9 were obtained with $pI_i68$. The inserts were one of following three fragments: 701-bp *StuI*, 86-bp *XbaI-StuI*, and 615-bp *StuI-XbaI*. Their relative positions are shown in Fig. 2B. All insertions were downstream of the CAT-coding region.

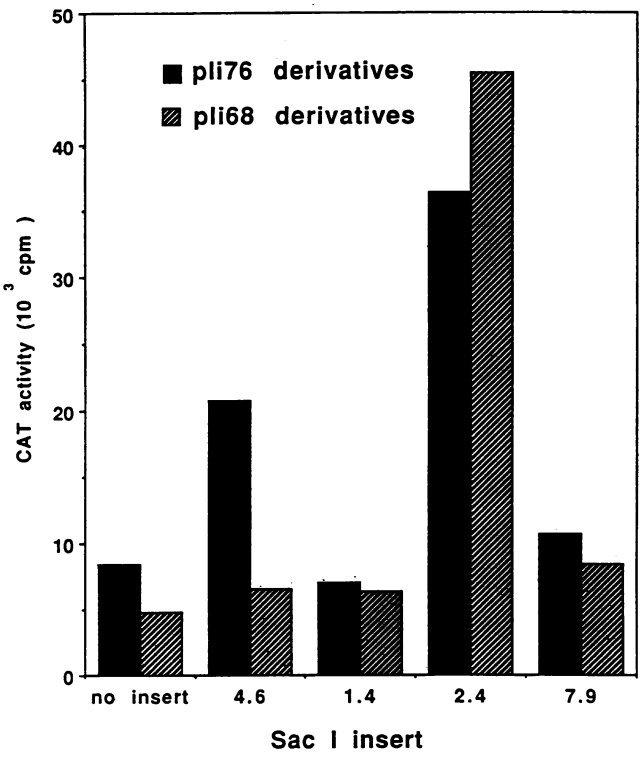


FIG. 7. $I_i$ enhancer screen. Results are presented of CAT assays of M12 cells transfected with the promoter deletion clones $pI_i76$ and $pI_i68$ with insertion of a series of genomic *SacI* fragments. The sizes and relative positions of the *SacI* fragments are indicated in Fig. 1A. Data are presented as counts per minute of $^{14}$C-acetylated chloramphenicol.

than Sp1, probably located in the 68 bp proximal to the initiation site. Thus, the $I_i$ promoter is enhanced by both the upstream and downstream enhancers via motifs including Sp1 and perhaps others in the promoter region.

Comparison of the *cis*-acting elements involved in transcriptional regulation of the $I_i$ and class II genes is summarized in Fig. 8. A set of regulatory elements shared by MHC class II and $I_i$ genes is the H-X-Y (Y′) boxes. A unique feature of the H-X-Y′ boxes in the $I_i$ gene relative to the MHC class II genes is that they are located significantly further upstream from the transcription initiation site. In MHC class II genes, the average length of the distance between the center of these three boxes and the transcription start site is around 100 bp (Fig. 8). The corresponding distance in the $I_i$ genes, however, is more than 200 bp (31). The possible biological significance of the difference in H-X-Y position between the class II genes and the $I_i$ gene was not apparent until their functions were examined.

The H-X-Y motifs of the MHC class II genes have all been shown to be critical promoter elements (12, 14, 18a, 19, 39, 42). In contrast to the results of the class II analyses, our results showed that the $I_i$ H-X-Y′ boxes are distinct from the basic promoter elements. The $I_i$ promoter minus the H-X-Y′ boxes ($pI_i183$) confers high constitutive activity in B cells and full TNF inducibility in monocytes (our unpublished results). A longer fragment containing additional upstream sequences (p843 in Fig. 1) does have stronger promoter activity, but obviously the additional sequences are not absolutely required for $I_i$ gene expression. This result immediately suggested that the $I_i$ H-X-Y′ boxes could be functioning as an enhancer that influences the expression of the $I_i$ gene through its promoter. This has been confirmed by enhancer-promoter construct experiments. Thus, the H-X-Y (Y′) boxes can be part of a promoter, as they are in the class II genes, or can be part of an enhancer, as they are in the $I_i$ gene. In both cases, they are involved in the positive control of transcription, and this probably is important for the coordinate expression of the $I_i$ and class II proteins. However, the mechanisms through which the H-X-Y (Y′) boxes act in class II and $I_i$ genes appear to differ, and this difference may explain the phenotype in the human class II$^-$ immunodeficiency disease. Since the H-X-Y′ boxes of the $I_i$ gene appear to serve as an enhancer, they do not determine $I_i$ gene expression but instead influence $I_i$ promoter activity. Therefore, the defect in the function of an X-box-binding protein (RF-X) found in class II$^-$ immunodeficiency disease patients has only a moderate effect on $I_i$ gene expression (34). On the other hand, because the H-X-Y boxes of the class II genes are critical promoter elements, the defect in RF-X function will prevent all class II genes from being expressed.

As mentioned before, we showed that the H-X-Y′ con-

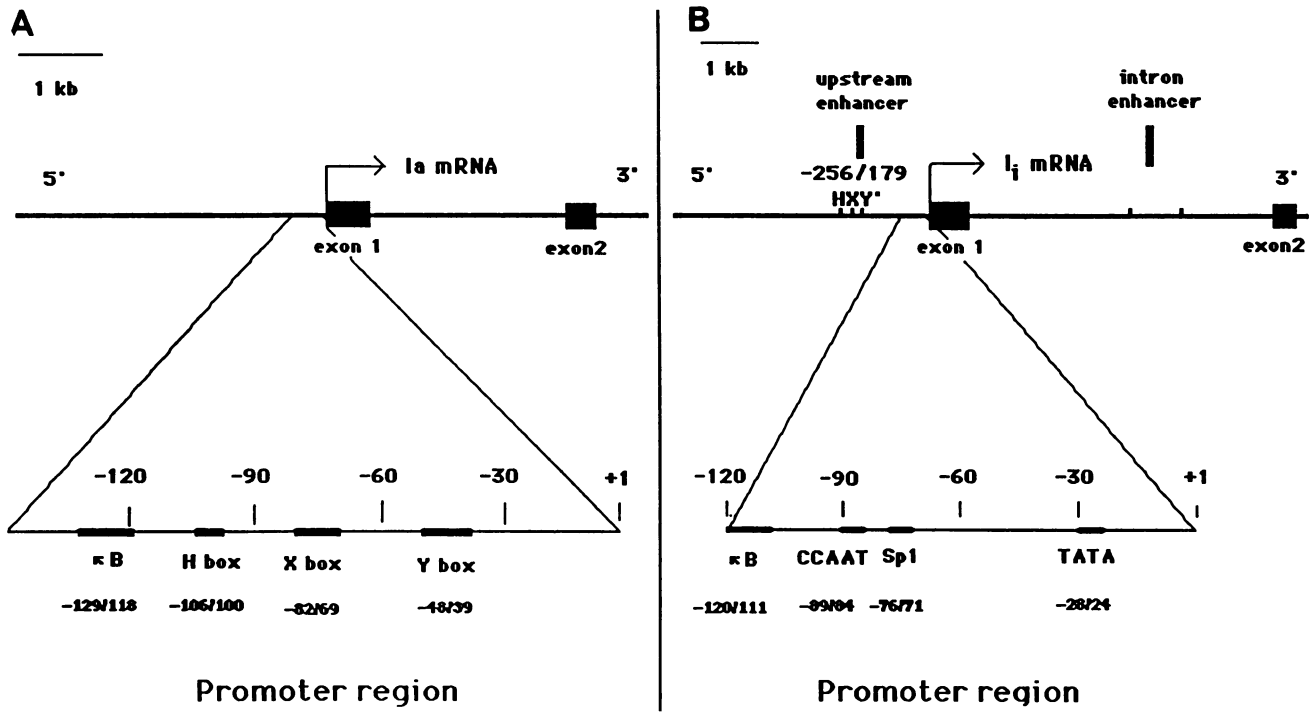


FIG. 8. Comparison of transcriptional control elements of the $I_i$ gene and class II genes. (A) The $A_\alpha^d$ promoter as a model of class II genes (12; Freund et al., submitted); (B) the murine $I_i$ gene (48). Some elements like κB may not be present in all class II genes (15). The exact positions of individual elements also vary among different class II genes.

taining $I_i$ upstream enhancer requires an Sp1 site in the promoter region to exert its activity. This finding may have implications for understanding the mechanism of how enhancers participate in gene control. It is the H-X-Y (in some cases only X-Y) boxes from the MHC class II genes that were first identified as enhancers by positive activity on the SV40 or HSV *tk* promoter (3, 43). The SV40 early promoter and the *tk* promoter have advantages such as little cell type specificity and relatively low background. However, both of them contain Sp1 sites, which is absolutely required for the $I_i$ H-X-Y′ boxes to show enhancer activity indicated by our study. Two of our assay constructs, containing the $A_\alpha^d$ or the $\beta_2$-microglobulin promoter (both of which lack an Sp1 site), failed to show the $I_i$ H-X-Y′ box enhancer activity. This led to the prediction that the Sp1 site is the key motif involved in H-X-Y′-mediated $I_i$ enhancer-promoter interaction. The 5′ deletion clones of the $I_i$ promoter provided further evidence for this prediction. Clone $pI_i76$ differs from $pI_i68$ only in the presence of the Sp1 site; however, the former is able to respond to the enhancer, whereas the latter is not. Interestingly, the two clones are equally able to respond to the intron enhancer. Our data so far are consistent with the hypothesis that Sp1 is necessary for receiving the upstream enhancer activity.

These findings suggest that many genes may have more than one enhancer, as we have described for the murine $I_i$ gene but have been unable to detect because of the particular promoter used in the enhancer screen. On the other hand, some previously identified enhancers may not function as enhancers in situ, perhaps including the enhancer function ascribed to the MHC class II gene X-Y boxes, again because of the promoter construct used. The results presented in this paper suggest that a more reliable way to screen for enhancers for a specific gene is to use either its own promoter or a combination of multiple heterologous promoters.

Our studies have opened an avenue for exploring the mechanisms of enhancer action. How enhancers operate over some distance in the DNA has remained a mystery. A current model suggests that the enhancer folds back to the proximity of the promoter and interacts with RNA polymerase II (30). In this model, there is no fundamental difference between promoter and enhancer. They all consist of basic elements recently called modules (17); each module is able to bind to a certain specific nuclear factor(s). It is believed that the constitution and organization of the modules present in promoter and enhancer(s) of a particular gene determine the features of expression of the gene (17, 30). Earlier work on immunoglobulin genes showed a synergy between cognate promoters and enhancers (19). Our studies further suggest that a precise matching between the enhancer and the promoter modules seems to be critical. We believe that more detailed analysis of the promoter-enhancer function in $I_i$ gene regulation will lead to a better understanding of enhancer-promoter recognition mechanisms.

## ACKNOWLEDGMENTS

We are greatly thankful to people who kindly provided various plasmids used in this work. We also thank R. L. Dedrick for his assistance and comments on the manuscript.

This work was supported by Public Health Service grant AI 19512 from the National Institutes of Health.

## LITERATURE CITED

1. **Accolla, R. S., G. Carra, and J. Guardiola.** 1985. Reactivation by a trans-acting factor of human major histocompatibility complex MHC class II gene expression in interspecies hybrids between an Ia-negative human B-cell variant and an Ia-positive mouse B-cell lymphoma. Proc. Natl. Acad. Sci. USA **82:** 5145–5149.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, P. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1988. Current

protocols in molecular biology. John Wiley & Sons, Inc., New York.
3. **Basta, P. V., P. A. Sherman, and J. P.-Y. Ting.** 1988. Detailed delineation of an interferon-γ-responsive element important in human HLA-DRα gene expression in a glioblastoma multiform line. Proc. Natl. Acad. Sci. USA **85**:8618–8622.
4. **Blanar, M. A., L. C. Burkly, and R. A. Flavell.** 1989. NF-κB binds within a region required for B-cell-specific expression of the major histocompatibility complex class II gene $E_\alpha^d$. Mol. Cell. Biol. **9**:844–846.
5. **Boss, J. M., and J. L. Strominger.** 1986. Regulation of a transfected human class II major histocompatibility complex gene in human fibroblasts. Proc. Natl. Acad. Sci. USA **83**: 9139–9143.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
7. **Celada, A., M. Shiga, M. Imagawa, J. Kop, and R. A. Maki.** 1988. Identification of a nuclear factor that binds to a conserved sequence of the $I\text{-}A^b$ gene. J. Immunol. **140**:3995–4002.
8. **Chang, R. J., and S. H. Lee.** 1986. Effects of interferon-γ and tumor necrosis factor-α on the expression of an MHC class II antigen on a murine macrophage cell line. J. Immunol. **137**: 2853–2856.
9. **Claesson, L., D. Larhammar, L. Rask, and P. A. Peterson.** 1983. cDNA clone for the human invariant γ chain of class II histocompatibility antigens and its implications for the protein structure. Proc. Natl. Acad. Sci. USA **80**:7395–7399.
10. **Claesson-Welsh, L., P. E. Barker, D. Larhammar, L. Rask, F. H. Ruddle, and P. A. Peterson.** 1984. The gene encoding the human class $I_i$ antigen-associated γ-chain is located on chromosome 5. Immunogenetics **20**:89–93.
11. **Collins, T., A. J. Korman, C. T. Wake, J. M. Boss, D. J. Kappes, W. Fiers, K. A. Ault, M. A. Gimbrone, J. L. Strominger, and J. S. Pober.** 1984. Immune interferon activates multiple class II major histocompatibility complex genes and the associated invariant chain gene in human endothelial cells and dermal fibroblasts. Proc. Natl. Acad. Sci. USA **81**:4917–4921.
12. **Dedrick, R. L., and P. P. Jones.** 1990. Sequence elements required for activity of a murine major histocompatibility complex class II promoter bind common and cell-type-specific nuclear factors. Mol. Cell. Biol. **10**:593–604.
13. **deWet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7**:725–737.
14. **Dorn, A., B. Durand, C. Marfing, M. Le Meur, C. Benoist, and D. Mathis.** 1987. Conserved major histocompatibility complex class II boxes—X and Y—are transcriptional control elements and specifically bind nuclear proteins. Proc. Natl. Acad. Sci. USA **84**:6249–6253.
15. **Dorn, A., H. J. Fehling, W. Koch, M. Le Meur, P. Gerlinger, C. Benoist, and D. Mathis.** 1988. B-cell control region at the 5′ end of a major histocompatibility complex class II gene: sequences and factors. Mol. Cell. Biol. **8**:3975–3987.
16. **Dubbs, D. R., and S. Kit.** 1964. Effect of halogenated pyrimidines and thymidine on growth of L-cells and a subline lacking thymidine kinase. Exp. Cell Res. **33**:19.
17. **Dynan, W. S.** 1989. Modularity in promoters and enhancers. Cell **58**:1–4.
18. **Farnham, P. J., J. Abrams, and R. T. Schimke.** 1985. Opposite strand RNAs from the 5′ flanking region of the mouse dihydrofolate reductase gene. Proc. Natl. Acad. Sci. USA **82**:3978–3982.
18a. **Freund, Y. R., R. L. Dedrick, and P. P. Jones.** 1989. Cis-acting sequences required for class II gene regulation by interferon-γ and tumor necrosis factor-α in a murine macrophage cell line. J. Exp. Med. **171**:1283–1299.
19. **Garcia, J. V., L. Bich-Thuy, J. Stafford, and C. Queen.** 1986. Synergism between immunoglobulin enhancers and promoters. Nature (London) **322**:383–385.
20. **Hammerling, G. J.** 1977. T lymphocyte tissue culture lines produced by cell hybridization. Eur. J. Immunol. **7**:743–746.
21. **Jones, P. P., D. B. Murphy, D. Hewgill, and H. O. McDevitt.** 1978. Detection of a common polypeptide chain in I-A and I-E sub-region immunoprecipitates. Immunochemistry **16**:51–60.
22. **Kim, K. J., C. Kanellopoulous-Langevin, R. M. Merwin, D. H. Sachs, and R. Asofsky.** 1979. Establishment and characterization of BALB/c lymphoma lines with B cell properties. J. Immunol. **122**:549.
23. **Koch, N., and A. W. Harris.** 1984. Differential expression of the invariant chain in mouse tumor cells: relationship to B lymphoid development. J. Immunol. **132**:12–15.
24. **Lenardo, M. J., and D. Baltimore.** 1989. NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. Cell **58**:227–229.
25. **Long, E. O.** 1985. In search of a function for the invariant chain associated with MHC class II antigens. Surv. Immunol. Res. **4**:27–34.
26. **Long, E. O., B. Mach, and R. S. Accolla.** 1984. Ia-negative B-cell variants revealed a coordinate regulation in the transcription of the HLA class II gene family. Immunogenetics **19**:349–353.
27. **Lopta, M. A., D. W. Cleveland, and B. Sollner-Webb.** 1984. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. Nucleic Acids Res. **12**:5707–5717.
28. **Lowenthal, J. W., D. W. Ballard, H. Bogerd, E. Bohnlein, and W. C. Greene.** 1989. Tumor necrosis factor-α activation of the IL-2 receptor-α gene involves the induction of κB-specific DNA binding proteins. J. Immunol. **142**:3121–3128.
29. **Miller, J., and R. N. Germain.** 1986. Efficient cell surface expression of class II MHC molecules in the absence of associated invariant chain. J. Exp. Med. **164**:1478–1489.
30. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245**:371–378.
31. **O'Sullivan, M. M., D. Larhammar, M. C. Wilson, P. A. Peterson, and V. Quaranta.** 1986. Structure of the human Ia-associated invariant (γ)-chain gene: identification of 5′ sequences shared with MHC class II genes. Proc. Acad. Natl. Sci. USA **83**:4484–4488.
32. **Paulnock-King, D., K. C. Sizer, Y. R. Freund, P. P. Jones, and J. R. Parnes.** 1985. Coordinate induction of Ia α, β, and $I_i$ mRNA in a macrophage cell line. J. Immunol. **135**:632–636.
33. **Polla, B. S., A. Poljak, J. Ohara, W. E. Paul, and L. H. Glimcher.** 1986. Regulation of class II gene expression: analysis in B cell stimulatory factor 1-inducible murine pre-B cell lines. J. Immunol. **137**:3332–3337.
34. **Reith, W., S. Satola, C. Herrero Sanchez, I. Amaldi, B. Lisowska-Gospierre, C. Griscelli, M. R. Hadam, and B. Mach.** 1988. Congenital immunodeficiency with a regulatory defect in MHC class II gene expression lacks a specific HLA-DR promoter binding protein, RF-X. Cell **53**:897–906.
35. **Richards, J. E., D. D. Prvtcheva, C. Day, F. H. Ruddle, and P. P. Jones.** 1985. Murine invariant chain gene: chromosomal assignment and segregation in recombinant inbred strains. Immunogenetics **22**:193–199.
36. **Schwartz, R. H.** 1985. T-lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. Annu. Rev. Immunol. **3**:237–261.
37. **Sekaly, R. P., C. Tonnelle, M. Strubin, B. Mach, and E. O. Long.** 1986. Cell surface expression of class II histocompatibility antigens occurs in the absence of the invariant chain. J. Exp. Med. **164**:1490–1504.
38. **Servenius, B., L. Rask, and P. A. Peterson.** 1987. Class II genes of the human major histocompatibility complex. The $DO_\beta$ gene is a divergent member of the class II β gene family. J. Biol. Chem. **262**:8759–8766.
39. **Sherman, P. A., P. V. Basta, T. L. Moore, A. M. Brown, and J. P.-Y. Ting.** 1989. Class II box consensus sequences in the HLA-DRα gene: transcriptional function and interaction with nuclear proteins. Mol. Cell. Biol. **9**:50–56.
40. **Singer, P. A., W. Lauer, Z. Dembic, W. E. Mayer, J. Lipp, N. Koch, G. Hammerling, J. Klein, and B. Dobberstain.** 1984. Structure of the murine Ia-associated invariant (Ii) chain as deduced from a cDNA clone. EMBO J. **3**:873–877.

41. **Stockinger, B., V. Pedssara, R. H. Lin, J. Habicht, M. Gvez, and N. Koch.** 1989. A role of Ia-associated invariant chains in antigen processing and presentation. Cell **56**:683–689.
42. **Sullivan, K. E., A. F. Calman, M. Nakanishi, S. Y. Tsang, Y. Wang, and B. M. Peterlin.** 1987. A model for the transcriptional regulation of MHC class II genes. Immunol. Today **8**:289–293.
43. **Sullivan, K. E., and B. M. Peterlin.** 1987. Transcriptional enhancers in the HLA-DQ subregion. Mol. Cell. Biol. **7**:3315–3319.
44. **Sung, E., and P. P. Jones.** 1981. The invariant chain of murine Ia antigens: its glycosylation, abundance and subcellular localization. Mol. Immunol. **18**:899–913.
45. **Thanos, D., G. Mavrothalassitis, and J. Papamatheakis.** 1988. Multiple regulatory regions on the 5′ side of the mouse $E_\alpha$ gene. Proc. Natl. Acad. Sci. USA **85**:3075–3079.
46. **Tsang, S. Y., M. Nakanishi, and B. M. Peterlin.** 1988. B-cell-specific and interferon-γ-inducible regulation of the HLA-$DR_\alpha$ gene. Proc. Natl. Acad. Sci. USA **85**:8598–8602.
47. **Warner, N. C., M. A. S. Moore, and D. J. Metcalf.** 1969. A transplantable myelomonocytic leukemia in BALB/c mice: cytology, karyotype and muramidase content. J. Natl. Cancer Inst. **43**:963.
48. **Zhu, L., and P. P. Jones.** 1989. Complete sequence of the murine invariant chain ($I_i$) gene. Nucleic Acid Res. **17**:447–448.