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Immunology

Regulation of the class II-associated invariant chain gene in normal and mutant B lymphocytes

(class II regulation/major histocompatibility complex/trans-acting factors)

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ABSTRACT **The invariant chain protein is intracellularly associated with class II major histocompatibility proteins. In many cases, the expression of these molecules appears to be regulated in a similar manner. Contained within the promoter of the invariant chain gene are sequences (X and Iγ1) that are similar to the X and Y box elements of class II genes, suggesting that these sequences might be involved in its regulation. DNase I footprinting reveals additional cis-acting elements (Iγ2 and Iγ3) that contain sequence similarities to NF-κB and/or H2TF1/KBF1 recognition sequences. A series of fusion constructs with the chloramphenicol acetyltransferase reporter gene were used to analyze the role of these sequences (Iγ1, Iγ2, Iγ3, and X and Y elements) in both normal and mutant B lymphocytes. These data suggest the likelihood of multiple X box proteins in B cells, which can act as both negative and positive regulatory factors.**

The invariant γ chain (Iγ) is a transmembrane glycoprotein that is physically associated with the α/β heterodimer of class II major histocompatibility complex (MHC) molecules in intracellular compartments (1–4). The function of Iγ is not completely understood. Recent evidence suggests that it is necessary for the efficient presentation of at least some antigens for which processing is required and that are restricted by class II MHC molecules (5–8).

Although the invariant chain gene is not encoded within the MHC (9, 10), the expression of Iγ and class II genes is coordinate in many circumstances. Both are expressed in the same cell types, including B lymphocytes, thymic epithelium, and activated human T lymphocytes and macrophages. Further, the expression of both molecules can be modulated by interferon γ or interleukin 4 treatment (11–14). These observations suggest that Iγ and class II gene expression might be controlled by similar regulatory elements. By contrast, other observations implicate the presence of distinct regulatory control elements. For example, in mutant cells that have lost the expression of class II molecules, the invariant chain gene continues to be expressed, albeit at reduced levels (9, 15–17).

Controlled expression of class II MHC genes is mediated in part by the conserved X and Y elements located within the promoter region. Both X and Y elements serve as recognition sequences for a number of DNA-binding proteins, and these elements are required for appropriate tissue-specific gene expression (for review, see ref. 18). The nucleotide sequence of the Iγ gene promoter contains sequences that have been reported to be similar to the X and Y elements of class II MHC genes (19, 20), suggesting that these putative elements might be responsible for the coordinate regulation of Iγ and class II proteins. However, the X and Y elements contained within the Iγ promoter are spaced further apart [44 base pairs (bp)] than are the same sequences in class II genes (16–18 bp).

To address the issue of distinct versus coordinate regulatory control of Iγ and class II genes, a DNA fragment corresponding to the promoter element of the invariant chain gene was isolated and used as a probe for *in vitro* analyses. The X conserved element and three additional cis-acting elements (Iγ1, Iγ2, and Iγ3) were found to bind nuclear proteins in extracts of Raji B lymphocytes. In no case was the previously described Y element observed to bind nuclear proteins. Rather, Iγ1 contains sequences that are recognized by Y box binding proteins that bind to MHC class II gene promoters. The Iγ2 and Iγ3 elements display striking homologies to NF-κB and H2TF1/KBF1 responsive elements, which have been shown to be important for the expression of immunoglobulin and MHC class I proteins (21–23). In addition, evidence for both positive and negative regulatory factors that bind to the X box is discussed. Finally, we argue that the class II-negative variant RJ2.2.5 lacks a positive X box binding factor and that Iγ expression in these cells is directed by the κB-like elements, which are not present in class II MHC gene promoters.

MATERIALS AND METHODS

Cell Lines. Raji is a human Epstein–Barr virus-positive Burkitt lymphoma cell line. RJ2.2.5 is a mutant of Raji that does not express class II antigens due to a defect in a trans-acting factor required for their expression (15, 24, 25).

DNA Constructs. The 5′ flanking sequences of the invariant chain gene were identified from a genomic clone, 029B, isolated from a previously reported library (26) by using the Iγ cDNA p33-1 as a probe (27). A 640-bp *Hin*cII (−512) to *Apa* I (+128) fragment was cloned into pUC18, and a series of 5′ deletion constructs were generated by using BAL-31 nuclease. The 3′ end of the DNA containing the Iγ promoter was excised with *Hin*dIII and cloned into M13 vectors for DNA sequence analysis by using the dideoxy chain-termination method. A series of truncated deletion constructs (at −343, −194, −141, −119, and −106 bp) were isolated from the double-stranded M13 plasmids by using *Bam*HI and a *Sau*3A (+44 bp) partial digest and subcloned into the promoterless chloramphenicol acetyltransferase (CAT) expression construct pOCAT (28) at the *Bam*HI site. Orientation was determined by DNA sequence analysis.

DNase I Footprinting. A 205-bp *Hin*fI fragment (−248 to −44 bp) was subcloned into pUC12 for DNase I footprinting experiments, which were carried out as described (29).

Gel Retardation Assays. Synthetic oligonucleotides were labeled with [γ-^{32}P]ATP, annealed, and purified on an 8% polyacrylamide gel. Nuclear extracts were prepared (30), and the products of the binding reaction were separated by using 4% polyacrylamide gels as described (29).

Mutagenesis. Deletion mutants −343ΔX, −343ΔIY1, −343-ΔY, and −343Δγ2 were constructed by site-directed oligonu-

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Abbreviations: Iγ, invariant γ chain; MHC, major histocompatibility complex; CAT, chloramphenicol acetyltransferase.

cleotide mutagenesis using the −343Iγ-M13 template (31). Mutations were confirmed by DNA sequence analysis and subcloned into the CAT expression plasmid as described above.

Oligonucleotides. The DNA sequences of the synthetic oligonucleotides are as follows: H2TF1/KBF1, 5′-GATCCGGCGGACTTTCCATCTCGGATC-3′ (22); NF-κB, 5′-TCGACAGAGGGGACTTTCCGAGAGGCTCGA-3′ (21, 32); mutant NF-κB, 5′-TCGACAGAATTCACTTTCCGAGAGGCTCGA-3′ (33); Iγ1, 5′-TGAGGGCCCTGGGCAGCCAATGGGATCGTGCTGGCCTTTCTACC-3′; Iγ2, 5′-GATCGGGAGCCCCCCCGCCCCACACAGATC-3′; Iγ3, 5′-GATCCCAAAGTGGGGTATTTCCAGCCTTTGTAGGATC-3′; DQβ X box, 5′-AATCTGCCCAGAGACAGATGAGGTC-3′; DQβ Y box, 5′-AGTGCTGATTGGTTCCTTTCCA-3′; DQβ Y + spacer, 5′-GTCCTTCAGCTCCAGTGCTGATTGGTTCCTTTCCAAGGGTC-3′ (29).

Transfection and CAT Assays. Suspension cells (10^7) were transfected in a 16- to 20-hr incubation with 2 μg of CsCl-purified DNA and 5 μg of Lipofectin (BRL) in 1 ml of OptiMEM (GIBCO). Cells (0.5×10^6 per ml) were maintained in complete RPMI 1640 for 48 hr. Nuclear extracts were prepared (34) and the protein concentration was determined by the Lowry method. Extracts (Raji: 10 μg, 1-hr assay; RJ2.2.5: 50 μg, 2-hr assay) were then assayed for acetylation of [^{14}C]chloramphenicol. Acetylation products were separated by thin-layer chromatography in chloroform/methanol, 95:5 and quantified by using a Betascope (Betagen).

RESULTS

Distinct Cis-Acting Sequences Within the Iγ Gene Promoter. The DNA sequence of the promoter region of the human Iγ gene is shown in Fig. 1. The putative X and Y elements and two additional elements with homology to NFκ-B sequences are indicated. A 205-bp *Hin*fI fragment (−248 to −44 bp) was end-labeled and incubated with extracts of nuclear protein isolated from Raji B lymphocytes. A typical DNase I footprinting pattern is shown in Fig. 2. Four distinct regions of protection can be seen, which include the X conserved sequence (−234 to −220 bp), a 28-nucleotide sequence (−213 to −186 bp) termed Iγ1, which is located between the X and putative Y elements, and two additional regions that contain putative NFκ-B sites, termed Iγ2 (−170 to −155 bp) and Iγ3 (−124 to −96 bp). The putative Y element (−176 to −167 bp) was not shown to bind nuclear factors, either by DNase I footprinting or by gel retardation assay (data not shown). In control experiments in which the promoter region of the DQβ gene was used as a probe, these same extracts were shown to contain proteins that bind to the Y sequence.

The assignment of the sequence at −176 to −167 bp as a Y element was based on a DNA sequence (TTGCTTGGGG) reported by O'Sullivan *et al.* (20) and matched the Y consensus sequence at 7 of 10 positions (see Fig. 3*A*). The DNA sequence presented in this report (CTGCCTGGGG) is in agreement with the sequence reported by Kudo *et al.* (19) and differs from that of O'Sullivan *et al.* at two residues. Importantly, the revised sequence (CTG*CC*TGGGG) diverges from the consensus at two residues (CTG*AT*TGGCT) that are absolutely conserved among all murine and human class II genes. Thus, the previous assignment of the Y box element at −176 to −167 bp is likely to be incorrect.

The crucial nucleotides within the Y box element are the central ATTGG: a CCAAT box in reverse; a number of CCAAT box binding proteins in addition to the well-characterized NF-Y protein can bind to this sequence (35, 36). Interestingly, this sequence is contained within the Iγ1 protected region at a distance of 14 bp from the X box element. The sequence GGGTAACCGA (−196 to −205 bp) contained within Iγ1 is a Y box in reverse and matches the complement of the Y box consensus sequence at 9 of 10 positions (see Fig. 3*A*). In order to determine whether this

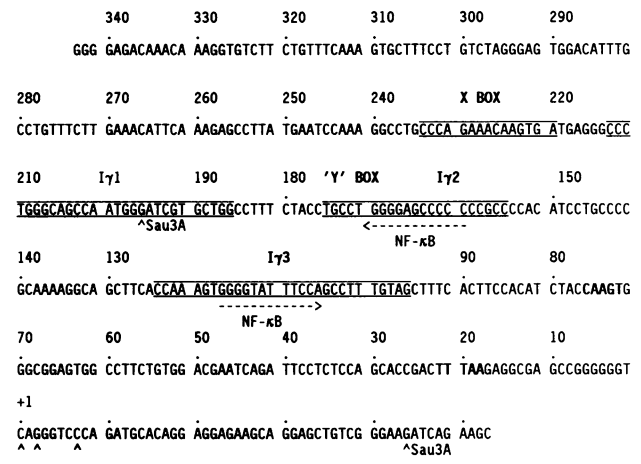

FIG. 1. Schematic representation and DNA sequence of the promoter of the human invariant chain gene. Initiation sites for the start of transcription are indicated at +1, +3, and +8 bp by carats. The TTTAA box (−22 to −18 bp), CAAGT box (−76 to −72 bp), Iγ3 protected region (−124 to −96 bp), Iγ2 protected region (−170 to −155 bp), putative Y conserved sequence (−176 to −167 bp), Iγ1 protected region (−213 to −186 bp), and X conserved sequence (−234 to −220 bp) are indicated. NF-κB recognition sequences contained within Iγ2 and Iγ3 are designated by arrows. The *Sau*3A site at +44 bp was used to fuse the promoter to the CAT gene for analysis of Iγ–CAT fusion products.

sequence would actually bind NF-Y or other CCAAT box binding proteins, a synthetic oligonucleotide 44 bp in length corresponding to the Iγ1 sequence (−219 to −176 bp) was used in a mobility shift assay (Fig. 3*B*). At least four specific DNA–protein complexes were detected with Raji B-cell nuclear extracts; the formation of all of these complexes was blocked by unlabeled Iγ1 competitor DNA. In addition, competition experiments were performed with oligonucleo-

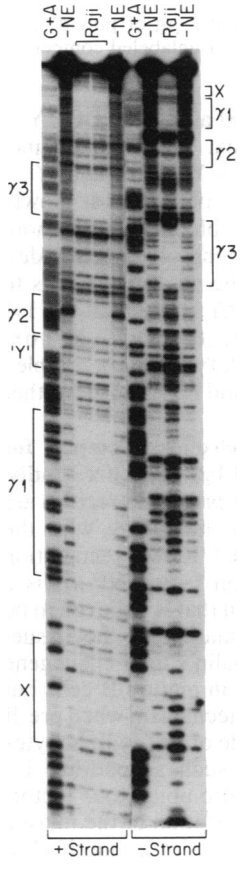

FIG. 2. DNase I footprinting analysis. A 205-bp *Hin*fI fragment (−248 to −44 bp) was end-labeled and incubated with (100 μg) or without (−NE) Raji B-cell nuclear extract prior to digestion with DNase I. The cleavage products were analyzed on a 6% polyacrylamide/urea gel along with G+A reactions. The protected regions and the putative Y element are indicated.

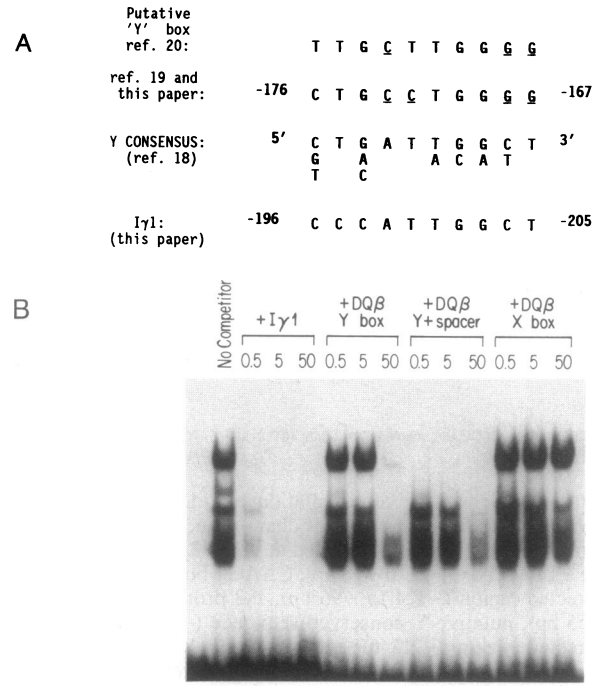

FIG. 3. (*A*) DNA sequences of the Y consensus sequence (18), the putative Y box element (−176 to −167 bp), and the Y homologous sequences contained within Iγ1 (−205 to −196 bp). Nucleotides within the putative Y sequence (−176 to −167 bp) that differ from the consensus sequence are underlined. Shown directly below the Y consensus sequence are nucleotide substitutions within the Y element that have been observed among murine and human class II genes. The sequence contained within Iγ1 matches the consensus at 9 of 10 positions. (*B*) Iγ1 contains sequences that are recognized by Y box binding proteins. The gel retardation assay was performed using a 44-bp synthetic oligonucleotide (−219 to −176 bp) containing the Iγ1 protected sequence (−213 to −186 bp) plus 6 bases 5′ and 10 bases 3′ to it. The radiolabeled Iγ1 probe was incubated with 2 μg of Raji B-cell extract (0.4 M KCl extract) and 2 μg of poly(dI-dC). Increasing amounts (0.5, 5.0, and 50 pmol) of unlabeled competitor oligonucleotide were used as indicated.

tides containing the DQβ Y box element alone, the DQβ Y + spacer element, and the DQβ X box. Although distinct differences between competition with Y alone and Y + spacer were seen (i.e., the formation of the most slowly migrating DNA–protein complex was blocked much more effectively by the Y + spacer-containing oligonucleotide), both blocked the specific binding of the nuclear proteins to the Iγ1-labeled probe, although less efficiently than the Iγ1 oligonucleotide itself. By contrast, the DQβ X box-containing oligonucleotide did not block Iγ1–protein complex formation at all. Thus, Iγ1 appears to bind NF-Y and/or other CCAAT box binding proteins.

NF-κB and H2TF1/KBF1 Oligonucleotides Compete for Binding of Nuclear Factors to Iγ2 and Iγ3 Sequence Motifs. Contained within both the Iγ2- and Iγ3-protected regions are sequence motifs that share extensive homology with the consensus for the NF-κB and H2TF1/KBF1 recognition sequences (Fig. 4*A*). The transcription factor NF-κB is a sequence-specific DNA-binding protein that is believed to be at least partially responsible for regulation of the tissue-specific expression of the immunoglobulin κ light-chain gene (21, 32). It is constitutively expressed in mature B cells but can be induced by a posttranslational mechanism when pre-B cells are treated with lipopolysaccharide or in T lymphocytes and nonlymphoid cells by mitogens such as phorbol 12-myristate 13-acetate (21, 38, 39). A more ubiquitous factor, H2TF1/KBF1, which stimulates transcription of the murine *H-2K*^{b} class I MHC gene, recognizes a similar motif (22). These proteins can be distinguished on the basis of the affinity for their cognate recognition sequence. H2TF1/KBF1 has high affinity for the *H-2K*^{b} site but low affinity for the NF-κB binding site in the κ enhancer, whereas NF-κB has relatively high affinity for both κ enhancer and *H-2K*^{b} sites (22). In addition, certain predictions can be made based on comparisons between sequences that bind NF-κB predominantly (e.g., in the mouse κB and human κB enhancer) with sequences that presumably bind both NF-κB and H2TF1/KBF1 (e.g., in the *H-2K*^{b} and β_2-microglobulin gene promoters) (37). κB sequences have additional homologies in sequences flanking the core-binding site, and at least several of these residues are conserved in the Iγ3 sequence. In addition, *H-2K*^{b} sequences contain at least three consecutive cytosine

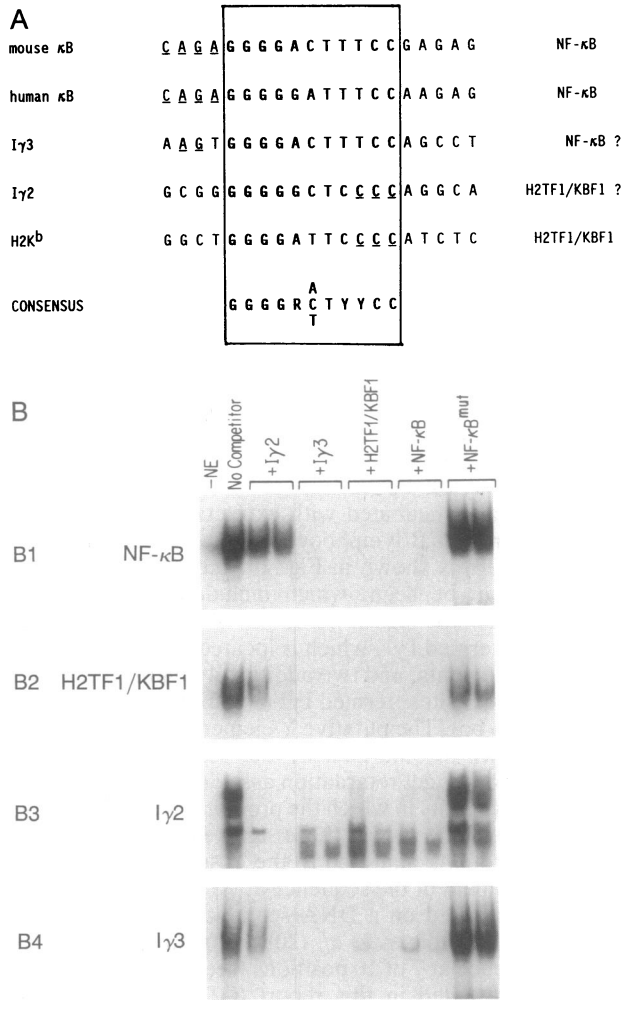

FIG. 4. (*A*) Iγ2 and Iγ3 are similar to NF-κB and/or KBF1/H2TF1 recognition sequences. Core-binding sequences and the derived consensus sequences (23) are boxed. The 5′ flanking residues (CAGA) that are conserved among κB recognition sites as well as the series of cytosine residues (>3) contained within the *H-2K*^{b} recognition sequences are underlined. The factors that preferentially bind to these sequences are indicated in the right-hand column (37). (*B*) Iγ2 and Iγ3 bind NF-κB and H2TF1/KBF1 *in vitro*. The gel retardation assay was performed with synthetic oligonucleotides containing the NF-κB recognition site from the human κB enhancer (*B1*), the H2TF1/KBF1 recognition site from the mouse *H-2K*^{b} gene (*B2*), the Iγ2 protected region (*B3*), and the Iγ3 protected region (*B4*). See *Materials and Methods* for the DNA sequence of the oligonucleotides. In each case, the radiolabeled probe was incubated with 2 μg of Raji B-cell (0.4 M KCl) nuclear extract, in the absence or presence of unlabeled competitor oligonucleotides (5.0 and 50.0 pmol, the left and right lanes within each set of competitor DNAs). −NE, No nuclear extract was added.

residues at the 3′ end of the core-binding site, whereas the sites that bind NF-κB strongly and H2TF1/KBF1 weakly have only two cytosine residues. By these criteria, Iγ2 most resembles the *H-2K*^{b} sequence, and again, the Iγ3 sequence is most similar to κB sequences.

The mobility shift assay was again used to analyze protein binding to these sequences. Oligonucleotides were synthesized that contained either an NF-κB site, the recognition sequence for H2TF1/KBF1, the Iγ2 element, the Iγ3 element, or a mutant NF-κB site. The DNA sequence of the mutant NF-κB site was altered at several guanine nucleotides that have been shown to be important contact residues by methylation interference analysis (33). Reciprocal competition experiments (Fig. 4*B*) gave the following results. Complex formation with the NF-κB (*B1*) or H2TF1/KBF1 (*B2*) radiolabeled probes was effectively blocked by Iγ3 sequences. However, Iγ2 did not block binding of protein to the NF-κB probe and only blocked complex formation with H2TF1/KBF1 probe at high molar concentrations (>100×) (Fig. 4*B2*, the fourth lane from the left). Complex formation with radiolabeled Iγ3 as a probe was poorly blocked by Iγ2. The H2TF1/KBF1 recognition sequence (Fig. 4*B4*) competed effectively at a 100-fold molar excess, whereas some specific binding to Iγ3 remained even at the higher concentration of NF-κB competitor.

A complex pattern of protein binding was seen when Iγ2 was used as a probe (Fig. 4*B3*). The formation of the most slowly migrating species, which probably corresponds to a complex with κB-like protein(s), was effectively blocked by all but the mutant NF-κB oligonucleotide. Yet there remained a more rapidly migrating complex whose formation was only blocked by Iγ2 itself. These data suggest that all of these sequences bind the same or related factors in the *in vitro* protein-binding assay. Thus, based on titration of competitor DNAs as well as sequence comparison, it would appear that Iγ3 is most like the NF-κB site, whereas Iγ2 is most similar to H2TF1/KBF1 recognition sequence.

Analysis of Iγ–CAT Fusion Constructs. A series of 5′ deletion constructs at −343, −194, −141, −119, and −106 bp were generated by using BAL-31 nuclease. Additional constructs with specific deletions of putative regulatory elements (i.e., X, Y, Iγ1, and Iγ2) were generated by site-directed oligonucleotide mutagenesis using the longest construct (−343) as a template. The deleted or mutated promoter sequences were subcloned into the promoterless CAT expression plasmid pOCAT (28) and were assayed by transient transfection into Raji and RJ2.2.5 cell lines. The activity of each truncation or deletion was normalized to the full-length construct (−343) (Fig. 5). Strikingly, the −343ΔX construct, which carries a precise deletion of the 15 nucleotides that constitutes the X conserved sequence, displayed greatly reduced activity in Raji cells (40% of the full length) but a dramatically increased (400%) activity in RJ2.2.5. Deletion of the DNA sequences contained within Iγ1 virtually abolished activity in both cell lines. By contrast, a precise deletion of the putative Y box (−175 to −171 bp) or Iγ2 (−168 to −150 bp) sequences had little or no effect in either cell line. However, with the X box already deleted, the additional deletion of Y + Iγ2 (5′ deletion to −196 versus 5′ deletion to −141 bp) had a drastic effect in RJ2.2.5 cells (0.1 versus 1.2) and a minimal effect in Raji cells.

These data suggest the likelihood of multiple X box proteins in B cells, which can act as both negative and positive regulatory factors. Analysis of Iγ–CAT constructs in the mutant B cell RJ2.2.5 suggests that these cells lack a positive-acting X box binding protein. Rather, a negative regulatory element binds in its place, thereby suppressing the level of transcription. Notably, the absolute value (% conversion) of −343ΔX in Raji (16%) is approximately equal to the absolute conversion (15%) of the same construct in RJ2.2.5 after the values have been normalized for relative transfection efficiency, amount of cell extract used in the assay, and incubation time. This observation suggests that the observed level of invariant chain mRNA present in RJ2.2.5 (≈20–30% of Raji, C.D., unpublished data; ref. 16) is likely to reflect the absence of a strong positive regulatory factor (which is present in Raji) and the presence of a negative regulatory protein, both of which can bind to the X box sequence.

DISCUSSION

Previous studies have suggested that the X and Y elements found within the promoter region of the Iγ gene might be responsible for the coordinate expression of Iγ and class II MHC proteins that has been observed in a number of cell types (20). By contrast, expression of Iγ in mutant cells (16, 17) that have lost the expression of class II molecules suggested the likelihood of distinct regulatory control elements. The results presented here provide evidence for both

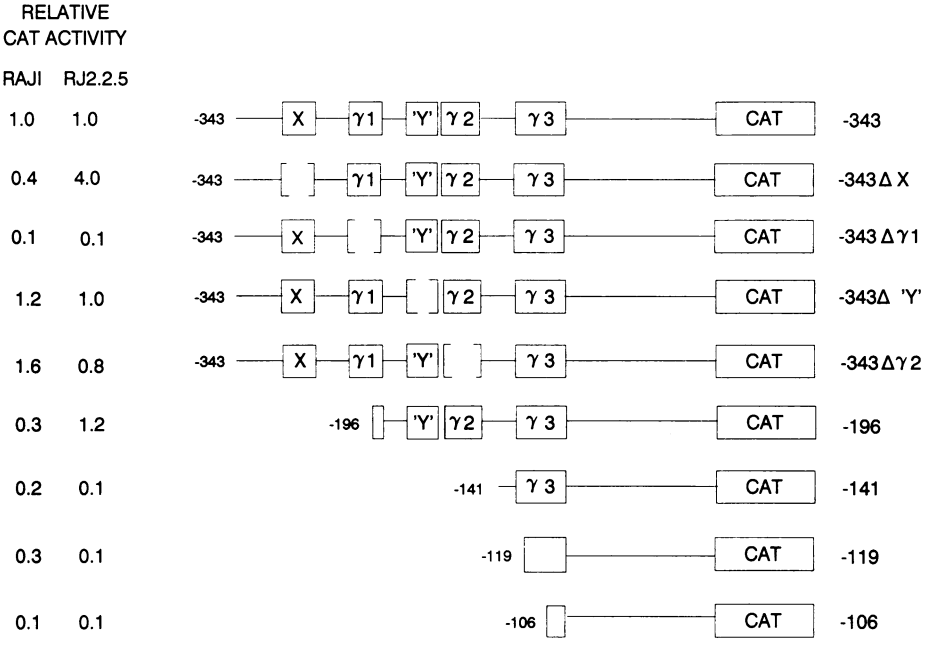

FIG. 5. Schematic representation of Iγ–CAT fusion constructs. A series of 5′ truncated deletion constructs (at −343, −196, −141, −119, and −106 bp) were fused to the CAT gene using the *Sau*3A site at +44 bp. In addition, the −343 template was used to generate precise deletion(s) of the X box (−343ΔX), the Iγ1 element (−343Δγ1), the Y box (−343-Δ'Y'), and the Iγ2 element (−343ΔIγ2) by site-directed oligonucleotide mutagenesis. The resulting constructs were assayed for the transient expression of the CAT gene after transfection into Raji and RJ2.2.5 B cells. Values for relative CAT activity are normalized to the full-length construct (−343) and represent the average of four to seven experiments. Typical values for fractional conversion of the [^{14}C]chloramphenicol to the acetylated chloramphenicol reaction products are as follows: Raji: SVCAT = 0.12, −343 = 0.41, −343ΔX = 0.16; RJ2.2.5: SVCAT = 0.05, −343 = 0.04, −343ΔX = 0.15.

distinct and shared regulatory elements contained within the promoter of the Iγ gene and provide a model for the expression patterns observed in normal and mutant B cells.

DNase I footprinting experiments defined three cis-acting elements, termed Iγ1, Iγ2, and Iγ3, all of which bind nuclear proteins present in extracts made from Raji B lymphocytes. An additional footprint is present in the region of the X consensus sequence but not within the putative Y element, which had been tentatively localized at −176 to −167 bp (20). Analysis of the DNA sequence in this region suggests that this assignment is likely to be incorrect, because of important differences within the ATTGG core element, which has been shown to be crucial to protein binding (35, 36). Moreover, this assignment would position the putative X (−234 to −220 bp) and Y conserved sequences in the Iγ promoter at a greater distance from each other (44 bp) than is seen for class II genes (16–18 bp) (for review, see ref. 18). However, Iγ1 sequences recognized by Y box binding proteins have now been localized at −205 to −196 bp, which positions the Y element 14 nucleotides away from the conserved X sequence, in accordance with the observed length of the interspace element contained within other class II MHC genes.

Both the Iγ2 and Iγ3 elements display striking homologies to NF-κB and H2TF1/KBF1 responsive elements and bind these or related factors in the mobility shift assay. These studies have been carried out with Raji B-cell nuclear extracts, in which both H2TF1/KBF1 and NF-κB proteins are expressed constitutively. Similar experiments in cells such as pre-B and/or T lymphocytes, in which κB-like protein(s) are inducible, may allow a more accurate description of the selectivity of these proteins for the Iγ2 and Iγ3 sequences.

Finally, constructs containing various subfragments from the promoter element fused to the CAT reporter gene were transiently expressed in Raji B lymphocytes and the class II-negative variant RJ2.2.5 in order to study the activity of the various cis-acting elements in these cells. Deletion of the X box conserved element from within the Iγ promoter greatly up-regulated its transcription in RJ2.2.5 while decreasing its expression in Raji. These data suggest the likelihood of multiple X box proteins in B cells, which can act as both negative and positive regulatory factors. By using gel retardation assays, DNase I footprinting, and methylation interference, a number of investigators have described a number of X box binding proteins (27, 29, 40–42). In addition, the molecular cloning of a number of distinct X box binding proteins has been reported (43, 44).

These activities may not represent distinct proteins at all but may simply reflect the presence of two forms of the same factor, modified and unmodified. One form, specifically that which functions as a positive trans-acting factor, is missing from RJ2.2.5. We have not been able to detect differences in protein binding between the Raji and RJ2.2.5 cell lines when the Iγ X box is used as a probe, either by gel retardation assay or by DNase I footprinting (data not shown).

In the case of RJ2.2.5, expression of the Iγ gene is controlled by proximal elements Iγ2 and Iγ3, thus providing an explanation for the paradox between class II expression and expression of the Iγ gene in these cells. While expression of class II MHC genes is strictly regulated by X and Y conserved elements and is dependent on positively acting trans-acting regulatory factors, transcription of the Iγ gene is more complex. Expression is enhanced by the presence of a positive-acting X box binding protein, but it is not strictly required in that other cis-acting elements contained within the proximal promoter region can suffice to mediate transcription. It may be significant that the proximal promoter elements Iγ2 and Iγ3 contain recognition sequences for DNA-binding proteins that have been shown to be important for tissue-specific expression of other molecules of the immune system.

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