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Regulation of a transfected human class II major histocompatibility complex gene in human fibroblasts

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ABSTRACT **To investigate the cis-acting DNA elements that are involved in regulation of class II major histocompatibility complex genes, including γ-interferon (γ-IFN) induction, 5′ flanking DNA deletions of a DQβ "minigene" were analyzed in stable transfected cell lines. At least four elements 5′ to the gene were found to be involved in DQβ regulation. Deletion of sequences from −2500 to −159 base pairs (bp) resulted in increased transcription, suggesting that negative regulatory elements resided in the deleted region. These clones were all capable of responding to γ-IFN. Further deletion of sequences from −159 to −128 bp resulted in constitutive high level transcription and the inability of these constructions to respond to γ-IFN. A deletion to −107 bp resulted in a decrease in the basal level of expression that was restored by removal of the 5′ DNA sequence to −82 bp, suggesting the presence of a second negative element. Finally, deletion to −64 bp caused a marked decrease in expression, suggesting the loss of an element necessary for high levels of transcription. The γ-IFN control and the transcription control elements contain the conserved upstream sequences found in all class II genes, suggesting a role for these sequences.**

Class II major histocompatibility complex (MHC) molecules in humans are heterodimers encoded in the HLA-D region. These molecules are normally expressed on the surfaces of mature B cells, activated T cells, and macrophages and participate in the immune response by presenting foreign antigen to helper T cells. Normal expression of class II genes is essential for an individual to mount a correct immune response. For example, individuals lacking expression of class II molecules on their lymphocytes exhibit a severe combined immunodeficiency, called the bare lymphocyte syndrome (1). In lymphocytes at least three families of MHC class II genes are coordinately regulated—i.e., the α and the β chains of DR, DQ, and DP (reviewed in ref. 2). Cell-surface expression of class II molecules may be induced in many cell types (e.g., endothelial cells and dermal fibroblasts) that are normally class II negative by incubation with γ-interferon (γ-IFN). Such treated cells are now capable of presenting foreign antigen to helper T cells, thereby participating in the activation of the immune response (3). The induction of MHC class II genes by γ-IFN is due to an increase in mRNA levels. In addition, γ-IFN causes increased levels of the mRNA encoding the invariant chain, a protein associated with the biosynthesis of class II molecules. The gene encoding the invariant chain is not located on the same chromosome as the class II genes, indicating that γ-IFN is able to induce other genes relevant to class II gene expression (4, 5). In addition to γ-IFN, other lymphokines and cellular factors have been shown to stimulate or repress class II MHC gene expression, such as interleukin 4 (BSF-1) (6) and prostaglandins (7), respectively.

DNA sequences upstream of their respective structural genes are often important for the control of transcription. Enhancer elements dramatically increase the levels of transcription (8), whereas negative elements act to silence or reduce gene expression (9). In addition, some elements control transcription of their respective genes under specific environmental conditions (10). Comparison of the DNA sequences 5′ to all known murine and human class II genes shows a short but striking homology in a region that is otherwise divergent (11–13). These conserved upstream sequences (CUS) are located between −66 and −114 base pairs (bp) 5′ to the mRNA cap site in the DQβ gene (14). The homology is greatest in two conserved elements at the ends of this region (−114 to −100 bp and −80 to −67 bp) [upstream nucleotides indicated in this paper are counted from the most 5′ of the transcription initiation sites of DQ2β (14)]. In addition, the sequences between these elements (−99 to −81 bp) are class II α-chain or β-chain specific (13). The extreme conservation of this DNA suggests that these CUS are involved in some aspect of class II gene expression.

To investigate the cis-acting sequences that control class II gene expression, a set of 5′ promoter deletions in a modified DQβ gene was transfected into human fibroblasts. Stable cell lines were selected, cloned, and assayed for their ability to transcribe the transfected genes and respond to γ-IFN.

MATERIALS AND METHODS

Plasmid Constructions. To simplify subsequent plasmid manipulations, a DQ2β "minigene" referred to as MX was constructed (Fig. 1). The 5′ flanking sequences, a marked exon 1 (described below), intron 1, part of exon 2 (*Sst* I site), and 2 kilobases of 3′ flanking sequences (*Eco*RI to *Sal* I) were from the genomic clone λ-42 (14). Exons 2, 3, 4, and part of 5 (*Sst* I to *Eco*RI) are composed of cDNA sequences from pII-β-1 (15). To distinguish between the mRNA synthesized from the transfected gene and that of the endogenous DQβ mRNA, a *Xho* I synthetic linker was placed in frame into exon 1 by ligation into the "filled in" ends of a *Bam*HI site. This created a 12-bp or 4 amino acid insertion into the signal sequence of DQβ. DQβ gene constructions MX2500, MX590, and MX159 have ≈2500 bp (bordered at its 5′ end by a *Hin*dIII site), 590 bp (*Xba* I site), and 159 bp (*Aat* II site) of 5′ flanking sequences, respectively. All of the constructs were inserted into pUC vectors (16). MX159 and its subclones are bordered by the *Aat* II site in the pUC vector at their 5′ ends instead of the polylinker.

A blunt-ended *Aat* II and *Pvu* II restriction fragment containing sequences +195 to −159 bp (including the marked exon) was subcloned in the anti-sense orientation into pSP65 (17) and used to generate anti-sense transcripts for RNA protection analysis. This clone, pSPMX4c was also used to

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Abbreviations: MHC, major histocompatibility complex; IFN, interferon; bp, base pair(s); CUS, conserved upstream sequence(s).

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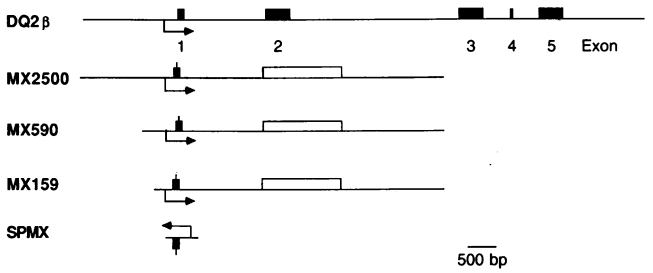

FIG. 1. Schematic representation of the DQβ gene (14). Darkened boxes represent genomic exons. DQβ constructions MX2500, MX590, and MX159 have genomic exons 2, 3, 4, and part of 5 replaced by cDNA sequences (open boxes) from the plasmid pII-β1 (15). Exon I in the MX constructs contains an in-frame *Xho* I linker that allows discrimination from the DQ2β gene. MX2500, MX590, and MX159 have approximately 2500 bp, 590 bp, and 159 bp of 5′ flanking DNA, respectively. All constructs are in pUC vectors. An anti-sense construct SPMX contains sequences +195 to −159 bp, including the altered first exon subcloned into pSP65. Transcription initiation is indicated by an arrow.

generate a series of BAL-31 deletions. pSPMX4c DNA was linearized by restriction digestion in the polylinker of pSPMX4c and digested with BAL-31 as recommended by the vendor (Bethesda Research Laboratories). The ends were repaired by T4 DNA polymerase and ligated to *Cla* I linkers. The resulting *Cla* I/*Xho* I restriction fragments (containing the deletions) were subcloned into MX159c (a clone containing a *Cla* I synthetic linker at −159 bp). The end points of four of these deletions were determined by dideoxy sequencing (18).

Cell Culture and DNA Transfection. Human cell lines M1XP (19), a xeroderma pigmentosa fibroblast, and 143b (20), an osteosarcoma fibroblast, were cultured on 100-mm plates in Dulbecco's modified Eagle's medium (DME medium) (GIBCO) and 10% fetal bovine serum with added penicillin/streptomycin. Six hours prior to transfection, cells were incubated in DME medium (GIBCO) containing colcemid (0.2 μg/ml; Sigma). Twenty micrograms of plasmid DNAs was cotransfected with 1–2 μg of pSV2neo DNA (confers resistance to the antibiotic G418) into human fibroblasts by means of electroporation as described (21). Stable cell lines were selected in 0.5 mg of G418 (GIBCO) per ml. Single colonies were cloned using cloning cylinders or pooled. Cells were treated with γ-IFN (kindly provided by Biogen, Cambridge, MA) by culturing them in the presence of 400 units/ml for 12–72 hr.

RNA Isolation and Analysis. RNA was prepared from 2–5 × 10^7 cells using the Nonidet P-40 lysis method (22). ^{32}P-labeled (uniformly) anti-sense transcripts were prepared *in vitro* as described (17). Actin anti-sense probes (23) used to standardize some experiments were labeled at a lower specific activity by incorporating 50 μM UTP in the transcription reaction. RNA protection analysis was carried out on 10 μg of total cellular RNA as described (24).

RESULTS

Transfection of the DQβ MX Constructions into Human M1XP or 143b Fibroblast Cell Lines. The functional activities of the 5′ flanking regions of the three constructions MX2500, MX590, and MX159 (2500, 590, and 159 bp of 5′ flanking DNA) were assayed in stable cell lines. The gene constructions were cotransfected with pSV2neo as a selectable marker for stable transformation into two human fibroblast cell lines, M1XP and 143b. Stable colonies were either cloned from single colonies or pooled when there were >100 colonies for a single transfection experiment (transfection efficiency: 10^{-5} to 10^{-6}). DNA dot blot hybridizations, carried out to determine the relative copy number in some of the isolated clones, showed a wide range in copy number of the transfected DNA, from about 1 copy in addition to the endogenous DQβ gene in clone MX159-5 to ≈30 copies in clone MX590-7 (data not shown). Cell lines that contained extra copies of the transfected DQβ DNA were analyzed further.

Expression of the DQ2β Minigene (MX) Constructs in Stable Cell Lines. The basal levels of expression of the transfected MX DNAs in the cloned lines were assayed by RNA protection analysis (17, 24) using a uniformly labeled anti-sense RNA probe corresponding to nucleotides +195 to −159 bp containing the marked exons (see *Materials and Methods*). In these experiments a protected fragment of 176 bases represents the MX construct mRNA, whereas protected fragments of 89 and 86 bases represent endogenous DQ2β mRNA. Endogenous DQ2β mRNA was not detected in these clones. A comparison of the basal levels of transcription from several individual clones containing the constructions MX2500, MX590, and MX159 (Fig. 2) showed that the shorter the 5′ flanking sequences in the DNA construction the higher the level of expression of MX mRNA. This is most typically demonstrated by a comparison of clones MX159-5 and MX2500-1. In addition, the increases in expression appeared to be stepwise—i.e., a comparison of all of the constructs indicated that the MX2500 clones had the lowest basal level of transcription; MX590 clones had a higher level and MX159 clones had the highest level in the approximate ratio of 1:3:10, respectively. These data suggested that there are several negative control regions in the flanking sequences that were sequentially removed by the various deletions. Pools of transfected cells containing MX2500, MX590, and MX159 also demonstrated increasing levels of transcription of MX RNA in the order listed.

To exclude the possibility that the plasmid vector interfered with the expression of the MX2500 clones, thereby reducing the level of MX mRNA, the vector was placed in the opposite orientation with respect to the MX2500 construct. A comparison of the two vector orientations in clones MX2500-54 and MX2500-1 showed approximately equal levels of MX mRNA (Fig. 2), suggesting that vector sequences in these constructions did not greatly affect transcription.

Induction of MXDQβ mRNA in the Presence of γ-IFN. The transfected cells were incubated with γ-IFN for 12–24 hr. This incubation time proved to be optimal for the expression of the transfected genes. Increased incubation periods with γ-IFN resulted in a decrease from maximal expression. RNA protection analysis of pools of MX159 and MX590 clones in the 143b cell line are shown in Fig. 3. Data obtained from other pools and individual clones in the M1XP and 143b cell lines are summarized in Table 1. Increased levels of MX mRNA in response to γ-IFN were observed in all three constructs (MX2500, MX590, and MX159) in most of the transfected clones (29 of 35 clones). Since constructs with as little as 159 bp of upstream sequence responded strongly to γ-IFN, these results demonstrated that the γ-IFN control region was contained within the MX159 construct that contains the CUS of class II genes. Again note that the basal level of expression (i.e., in the absence of γ-IFN) is much higher in MX159 than in MX590 and, in fact, the basal level in MX159 is much higher than the γ-IFN-induced level in MX590. Since the level of transcript in MX159 in the presence of γ-IFN is far higher than that seen in normal B cells, it may be referred to as "superinduced."

Deletion Analysis of the CUS of Class II Genes. To analyze the sequences required for γ-IFN induction in more detail, a set of BAL-31 deletions was constructed from the 5′ *Aat* II site (−159 bp) of MX159. The four deletions obtained (MX128, MX107, MX82, and MX64 having 128, 107, 82, and

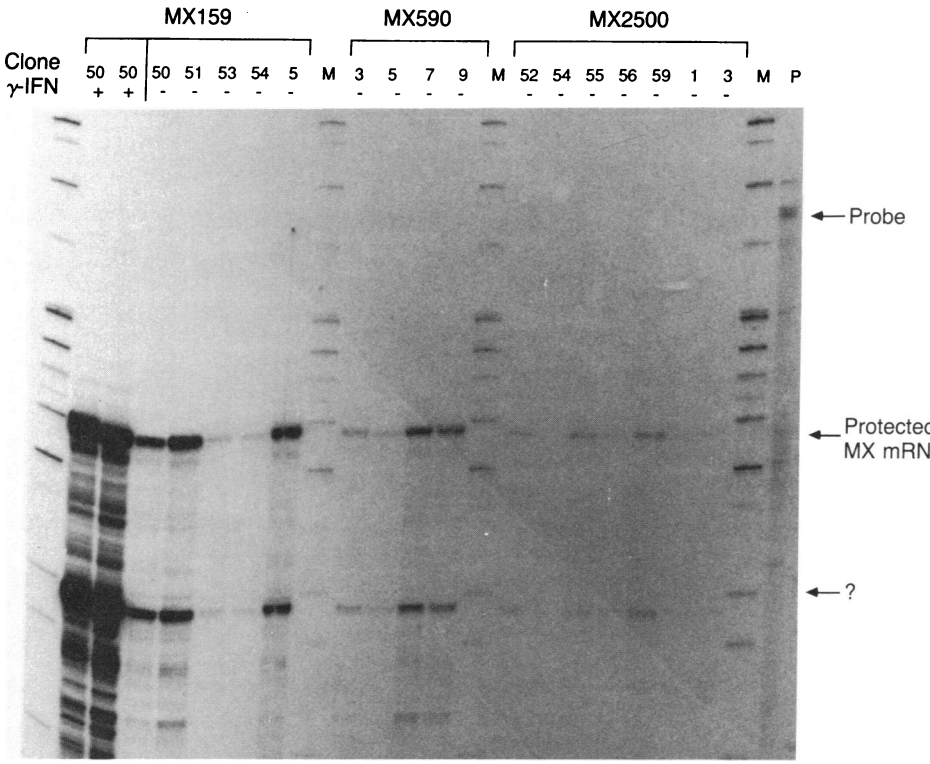

FIG. 2. RNase protection of MX159, MX590, and MX2500 transcripts. RNA isolated from G418-stable M1XP cell clones transfected with MX2500, MX-590, and MX159 DNAs was hybridized to ^{32}P-labeled anti-sense SPMX RNA. Hybrids were treated with RNase A and T1 and analyzed on sequencing gels. Clone MX159-50 was treated with 400 units of γ-IFN for 12 hr prior to RNA isolation. P, untreated anti-sense probe; M, ^{32}P-labeled *Hpa* II digest of pBR322; ?, extraneous band correlating with MX mRNA expression levels. Endogenous DQβ is not seen in these gels.

64 bp of 5′ flanking sequence, respectively) span the CUS region (Fig. 4). The constructs were transfected into the M1XP cell line and stable colonies were selected. Pools were collected and analyzed for the expression of MX mRNA before and after induction with γ-IFN (Fig. 5). Analysis of individual clones gave identical results.

All four constructs are capable of expressing the MX mRNA, indicating that the largest deletion to −64 bp still contains enough DNA to promote transcription with proper initiation although at a very low level; this clone includes intact CCAAT and TATA sequences (Fig. 4). The basal level of transcription for MX128 is higher than the parent clone MX159, suggesting again the presence of a negative regulatory element upstream of −128 bp. This element may be the same or an additional element to the one(s) suggested above. The additional deletion in the MX107 clones resulted in a decreased level of transcription, indicating the removal of a positive control element, presumably the first upstream element of the CUS (−114 to −100 bp). The lowered level of transcription in MX107 and increased levels in MX82 suggest that a negative control element was removed by the MX82 deletion. This region contains the β-chain-specific sequences (13). MX64 clones, however, showed markedly decreased levels of MX mRNA. The CUS (−80 to −67 bp), which is intact in MX82 and deleted in MX64, may function as a positive element and therefore be necessary for high expression of the DQβ gene.

None of the BAL-31 gene constructions made from MX159 responded to γ-IFN treatment (Fig. 5). [Compare MX levels and actin levels, used as an internal control (see *Materials and Methods*).] The MX128 construction contained the entire

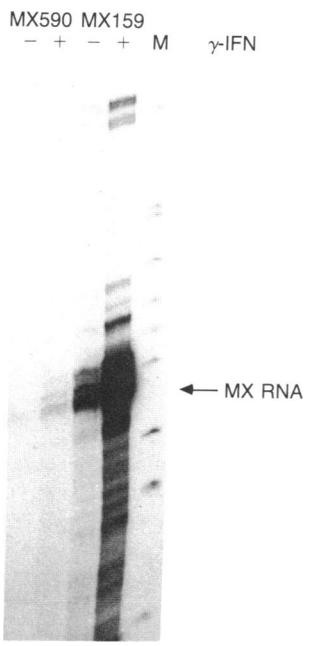

FIG. 3. Analysis of RNA from 143b cells transfected with MX159 and MX590 DNAs. RNA was isolated from pools of 143b stable cell lines (>100 individual clones) before and after a 12-hr incubation with γ-IFN and analyzed by RNA protection as described above. M, as in Fig. 2.

Table 1. Analysis of cloned cell lines

Recombinant	Cell	Neo^R	DNA$^+$	RNA$^+$	IND$^+$
MX159	M1XP	18	12/18	10/13	8/10
	143b	7	6/7	5/6	4/5
	Total	25	18/25	15/19	12/15
Pool	M1XP	2	ND	+	+
	143	1	ND	+	+
MX590	M1XP	15	8/15	5/8	5/5
	143	18	6/12	4/6	3/4
	Total	33	14/27	9/14	8/9
Pool	143	1	+	+	+
	M1XP	2	+	+	+
MX2500	M1XP	21	12/21	9/12	7/9
	143b	4	3/4	3/3	2/3
	Total	25	15/25	12/15	9/12
Pool	M1XP	2	+	+	+

ND, not determined; Neo^R, number of clones analyzed resistant to G418; DNA$^+$, number of Neo^R clones positive for MX DNA; RNA$^+$, number of DNA$^+$ clones expressing MX RNA; IND$^+$, number of RNA$^+$ clones inducible by γ-IFN. The number of pools analyzed is shown, with a "+" sign indicating a positive result for the category.

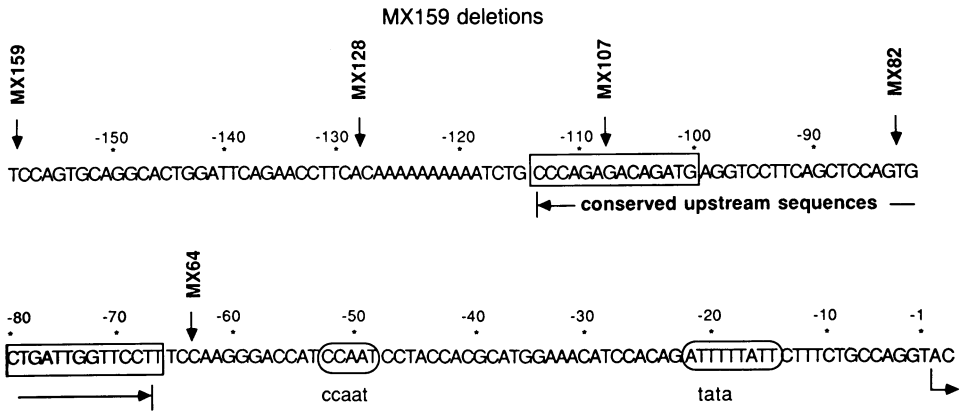

FIG. 4. DNA sequence of DQ2β from −159 to +3 bp (14). (Numbering is from the 5′ most site of transcription initiation.) End points of MX clones created by digestion with BAL-31 are shown. Open boxes denote the two highly conserved ends of the CUS element. Open ovals highlight the CCAAT and TATA elements. The arrow represents the initiation of transcription.

CUS region (−114 to −67 bp) and was expected to respond similarly to MX159. However, from these data the γ-IFN control sequences are affected by the MX128 deletion. An MX159 clone with a *Cla* I linker at its 5′ end (at −159 bp) was still inducible, indicating that the addition of the linkers in the construction of the deletions did not inhibit γ-IFN-mediated induction (data not shown). The failure of MX128, MX107, and MX82, which strongly express MX mRNA, to respond to γ-IFN further demonstrates that γ-IFN-mediated induction acts primarily through transcription of class II genes and not by stabilizing class II mRNA. Moreover, a comparison of the MX159 and MX128 constructions (first four lanes in Fig. 5) suggests that the effect of γ-IFN may be due to a release of negative regulation.

DISCUSSION

The class II genes of the human MHC are expressed in a limited variety of cell types. These include B cells, activated T cells, macrophages, and dendritic cells. All of these cell types participate in the immune response by presenting antigen associated with a class II molecule to T cells. In addition, most cell types that are normally silent with respect to class II gene expression can be induced to express class II genes by treatment with γ-IFN. Once class II molecules are expressed on the surfaces of γ-IFN-induced cells, these cells can participate in antigen presentation. This induction is coordinate for at least six class II MHC genes (the α- and β-chain genes of DR, DQ, and DP) and the invariant chain gene (5). The only class II genes not induced by γ-IFN are those that have not yet been shown to be expressed on the surface of any cell type—i.e., the human DOβ (25), DXα, and DXβ (13, 26) genes and the mouse IAβ2 (27). The expression of the HLA-A, -B and -C genes (class I MHC genes) is increased by γ-IFN as well as by α- or β-IFNs (28). The increased cell-surface density of class I HLA on virally infected cells may make cytotoxic T-cell-mediated killing of the infected cells more efficient. In addition, γ-IFN induces many cellular genes, some of which are active in the anti-viral response, such as oligo 2′,5′ A synthetase (reviewed in ref. 29).

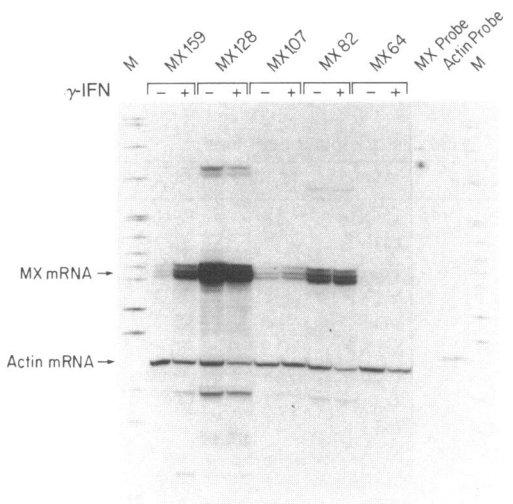

FIG. 5. Protection analysis of RNA isolated from stable pools of M1XP cells transfected with MX159, MX128, MX107, MX82, and MX64 constructions before and after incubation with γ-IFN (12 hr). RNA protection was carried out as described above with the addition of a ^{32}P-labeled anti-sense actin probe to the hybridization. Actin mRNA serves as a control for differences in sample handling. M, as in Fig. 2.

Cis-acting DNA elements have been shown to be important for tissue-specific expression and for induction of many eukaryote genes. A small conserved sequence is responsible for α/β-IFN regulation of class I genes in the mouse (30): a homologous sequence is located ≈600 bp upstream of the DRα gene as well as upstream of the metallothionine gene (31). In the present paper the 5′ flanking sequences of the class II MHC gene DQβ have been examined to determine the location of the cis-acting sequences required for γ-IFN induction, particularly in relation to the CUS that are found 5′ to all class II genes.

A set of 5′ flanking DNA deletions was created in the DQβ gene. The resulting constructions were analyzed for basal expression and their ability to respond to γ-IFN treatment. The data suggest that at least four regions are important for the regulation of the DQβ. First, negative regulatory elements reside upstream from −128 bp as transcription was increased significantly when 5′ flanking DNA sequences were serially deleted. Thus, the largest gene construction, MX2500 (2500 bp of 5′ upstream sequence), was expressed at the lowest level: this may represent the normal silent state of the class II genes in fibroblasts. The MX590, MX159, and MX128 deletions were expressed at higher levels, with the MX128 clones expressing up to five times the amount of DQβ mRNA expressed in established B-cell lines. These deletions place the putative negative control element(s) between the −2500- and −128-bp deletions. The negative regulatory sequences may represent control elements common to all class II genes or they could be unique to the DQβ gene (explaining the lower level of transcription as compared to DRβ). The finding of negative regulatory regions in higher eukaryotic DNAs has been shown in several other systems, such as the β-IFN gene (10, 24) and the rat insulin gene (32).

Two positive regulatory regions located within the CUS region (−114 to −100 and −80 to −67 bp) are necessary for high expression of the DQβ gene. This was demonstrated by the high level of expression in the MX159, MX128, MX107, and MX82 clones. The MX64 clone that does not contain the CUS region still expresses properly initiated MX mRNA but

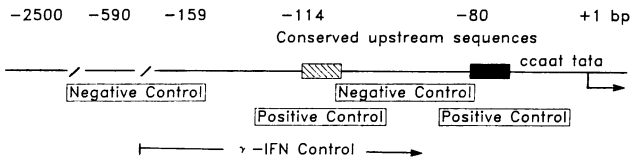

FIG. 6. Schematic representation of DQβ regulation. Four control regions in addition to the CCAAT and TATA elements are highlighted. The hatched box and darkened box represent the highly conserved ends of the CUS from −114 to −100 bp and −80 to −67 bp, respectively. Transcription initiation is indicated by an arrow.

at a very low level. Interestingly, the −80- to −67-bp element is the most conserved of the CUS of class II genes. An additional negative element may reside between −107 and −82, as evidenced by the increase in basal level of transcription of the MX82 clone as compared to the MX107 clone. This region contains the β-chain-specific sequence of the CUS (13). The effect of the −114- to −100-bp element appears to be to override this negative regulation.

Another cis-acting region controls the response to γ-IFN. The induction or increase in transcription of the transfected MX genes by γ-IFN was very strong with the MX159 clones, placing a maximum 5′ end point for the γ-IFN cis-acting sequence at −159 bp upstream of the transcription initiation site. The 3′ end point cannot be well defined with only 5′ end gene deletions. However, the lack of DNA sequence conservation outside of the CUS and loss of γ-IFN induction with the MX128 constructions suggest that the first element of the CUS (−114 to −100 bp) is involved in γ-IFN control. A comparison of the CUS region for the class II genes that are and that are not induced by γ-IFN does not reveal any obvious differences that are important for induction.

A model for DQβ gene regulation can be proposed from these studies (Fig. 6). Transcription may be controlled by at least four elements: high efficiency of transcription elements (−114 to −100 bp and −80 to −67 bp), a γ-IFN induction control element (downstream of −159 bp), and two negative control regions (upstream of −128 bp and −107 to −81 bp). The negative elements would silence the gene in cells that do not present antigen and would be partially overridden in γ-IFN-treated cells. Additional modulation of gene expression in antigen-presenting cells, such as B cells and macrophages, would occur by either derepressing this region or activating a tissue-specific enhancer that would increase transcription. Each of these control regions would act uniquely or in concert with trans-acting regulatory proteins. Evidence from several lines of work has demonstrated that specific trans-acting regulatory factors interact with class II genes. Class II negative human B-cell mutant lines have been created by several groups. These lines have all of the MHC class II genes intact and are reverted by fusion with wild-type human and mouse B cells (33, 34). In addition, patients with severe combined immunodeficiency syndrome (1), like the mutant cell lines, have intact class II genes but fail to express them on their antigen-presenting cells. The factor(s) that is deficient in these mutant cell lines and patients may be either a direct positive activator of class II gene transcription or a factor(s) that derepresses the negative elements described above. The analysis of these gene constructions introduced into B cells and the mutant cell lines rather than fibroblasts and the study of the 5′ flanking DNA in a heterologous gene system will certainly shed more light on the mechanism of DQβ gene regulation.

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