Detailed delineation of an interferon-γ-responsive element important in human *HLA-DRA* gene expression in a glioblastoma multiform line

(gene regulation/class II major histocompatibility complex gene expression in brain)

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ABSTRACT   In this report, we determined that induction of the DR α-chain gene by recombinant human interferon-γ (IFN-γ) in a human glioblastoma multiform cell line is transcriptionally regulated and showed that protein synthesis is not necessary for this to occur. The regions of the DR α-chain gene that are responsible for basal and recombinant IFN-γ-induced gene transcription have been determined by gene transfer of a series of 5' deletion mutants in which the upstream region of the DR α chain was linked to a reporter gene, chloramphenicol acetyltransferase. Chloramphenicol acetyltransferase transcript and protein levels were determined by S1 nuclease protection and chloramphenicol acetyltransferase enzyme assays, respectively. By using these deletion mutants, we were able to draw the following conclusions. (*i*) One hundred and nine base pairs of upstream sequence contains the basic DR α-chain gene promoter and represents the minimal amount of sequence necessary for basal gene expression. (*ii*) An additional 9 base pairs of upstream sequence can mediate recombinant IFN-γ induction. (*iii*) Maximal recombinant IFN-γ induction requires at most an additional 23 base pairs of upstream sequence. (*iv*) The sequence between positions −267 and −141 does not appear to contain any additional positive or negative regulatory elements. These results suggest that the region between positions −141 and −109 contains a critical IFN-γ-responsive element. Substitution mutagenesis was performed to confirm this suggestion.

Class II major histocompatibility complex (MHC) antigens are crucial to the overall function of the immune system by virtue of their role in antigen presentation and lymphocyte interaction (1, 2). The quality of an immune response is determined in part by the timing and extent of class II antigen expression; therefore, the molecular regulation of these genes is of primary importance.

The class II MHC genes are regulated in a complex fashion and provide one of the most interesting systems to study gene regulation. Expression patterns of class II antigens are extremely diverse and tightly regulated (3–8). It is assumed, therefore, that complex mechanisms must be in place that control the tissue-specific, differentiation-dependent, and inducible expression of class II genes.

Several laboratories including our own have begun to examine these controlling mechanisms and to identify cis-acting DNA sequences responsible for class II MHC gene regulation (9–16). The present report centers on the detailed delineation of interferon-γ (IFN-γ)-responsive regions in the DR α-chain (DRα) gene by using a human glioblastoma multiform line as a model system. We have been particularly interested in the basal and inducible expression of these antigens on glioblastomas because class II antigen expression

in the brain has been linked to the initiation and propagation of autoimmune-like diseases of the central nervous system.

In this paper, we have shown that DRα induction by IFN-γ is transcriptionally regulated. In addition, we have mapped the IFN-γ-responsive regions of the gene encoding DRα to two short DNA segments, each containing a single copy of a heptamer sequence. The significance of this finding to class II MHC gene expression and to the mechanism of IFN-γ-induced gene expression will be discussed.

MATERIALS AND METHODS

Cell Lines and Reagents. The U-373-MG human glioblastoma multiform cell line (17) was obtained from D. Bigner (Duke University, Durham, NC). Recombinant human IFN-γ (rIFN-γ) was generously provided by Biogen (Boston). S1 nuclease was purchased from Bethesda Research Laboratories. RNase-free DNase was purchased from Pharmacia.

Plasmids. Plasmids 5'Δ-1028 and 5'Δ-267 contain 1028 and 267 base pairs (bp) of the upstream sequence for the gene encoding DRα and are identical to pDR1000 and pDR300, respectively, as described (9). Plasmids in the nested deletion series 5'Δ-177 to 5'Δ-53 were derived from pDRA1000 as described (10). All of the aforementioned plasmids will be designated pDRα-CAT deletion mutants.

An additional deletion mutant, 5'Δ-56(xbaI), was prepared in which an *Xba* I linker was placed immediately upstream of the sequence encoding DRα. This plasmid was used to construct a substitution mutant in the putative IFN-γ-responsive region. After linearization with *Xba* I and blunt-ending with the Klenow fragment of DNA polymerase I, two 81-bp oligonucleotides were cloned into the *Xba* I site of 5'Δ-56(xbaI). The first oligonucleotide corresponds to the region of the gene encoding DRα from position −141 to position −61, 5'-CTTGTGTCCTGGACCCTTTGCAA-GAACCCTTCCCCTAGCAACAGATGCGTCATCT-CAAAATATTTTTCTGATTGGCCAAAG-3'. This construct was designated pwt-IRE-γII. The second oligonucleotide corresponds to the 5' sequence of the gene encoding DRα from position −141 to position −61; however, the sequence from position −141 to position −109 was randomly mutated to 5'-AGGTGTGAAGTTCAAAGGGTACCTC-CAAAGGCC-3'. This construct has been designated pmut-IRE-γII.

The negative control plasmid pD164-2 contains the chloramphenicol acetyltransferase (CAT) gene and was derived as described (9). Plasmid pSV2CAT is identical to that de-

Immunology: Basta *et al.*
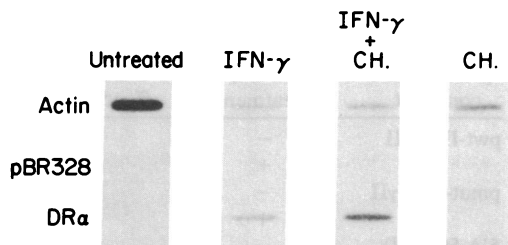
FIG. 1. IFN-γ induction of DRα-specific RNA in the glioblastoma U-373-MG is transcriptionally regulated and does not require *de novo* protein synthesis, as measured by a nuclear run-on analysis. [^{32}P]UTP-labeled nuclear transcripts were prepared from untreated, rIFN-γ- (500 units/ml), rIFN-γ- plus cycloheximide- (CH, 10 μg/ml) treated, or cycloheximide-treated U-373-MG cells. Equivalent numbers of nuclei were used to prepare [^{32}P]UTP-labeled nascent transcripts, and the total cpm were not further equilibrated. These labeled transcripts were then used to probe plasmid DNA specific for γ-actin, DRα, and pBR328 nucleotide sequences.

scribed by Gorman *et al.* (18). The plasmid used to produce an antisense RNA probe for S1 nuclease analysis is pGEM-DRα-CAT. It was constructed by excising an *Xba* I–*Eco*RI fragment from 5'Δ-1028 that contains 295 bp of the upstream sequence of the gene encoding DRα and 266 bp of the CAT structural gene. This fragment was cloned into the *Xba* I and *Eco*RI polylinker sites of pGEM3-Blue (Promega, Madison, WI).

Nuclear Run-On Transcription Assay. Nuclei were prepared after 24 hr from untreated, rIFN-γ-treated (500 units/ml), rIFN-γ- and cycloheximide-treated (10 mg/ml), or cycloheximide-treated U-373-MG cells. From these nuclei, nascent transcripts were radiolabeled, isolated, and used as hybridization probes against plasmid DNA immobilized on nitrocellulose as described (19, 20). Plasmid p34-RI-3 (21), provided by Sherman Weissman (Yale University), was used to identify DRα-specific transcripts. Plasmid pHF1 (22) coding for human γ-actin served as a standardizing control and plasmid pBR328 was used to control for nonspecific binding.

Transfection. Transfection of the glioblastoma cell line was performed by the calcium phosphate method as described by Graham and van der Eb (23) or through electroporation with a Bio-Rad gene pulser under the following conditions. Briefly 3 × 10^6 subconfluent U-373-MG cells in 300 μl of complete medium were pulsed at 200 V at a capacitance setting of 960 μF. Exact specifications of rIFN-γ treatment have been described (9).

CAT Assay. CAT activity produced in transfected cells was assayed according to the method of Gorman *et al.* (18). Acetylated products were visualized by autoradiography of TLC plates. Quantitative measurements were obtained by cutting out both the acetylated and unacetylated spots and measuring radioactivity separately in a scintillation counter; from these data percent acetylation was calculated.

RNA Isolation and S1 Nuclease Protection Analysis. RNA was prepared from transfected cell cultures 48 hr after

transfection as described (24) and was used in RNA protection analyses according to the method of Winter *et al.* (25).

RESULTS

IFN-γ Induction of DRα-Specific RNA in the Glioblastoma U-373-MG Is Transcriptionally Regulated and Does Not Require *de Novo* Protein Synthesis. To determine if IFN-γ induction of the human class II gene DRα in U-373-MG cells is transcriptionally regulated and to determine if protein synthesis is necessary for this induction, we performed nuclear run-on experiments. The results from a representative experiment are shown in Fig. 1. A definite increase in DRα-specific transcripts (row 3) was observed from IFN-γ- and IFN-γ-plus cycloheximide-treated cells. Densitometric measurements of DRα-specific transcripts in the untreated and cycloheximide-treated cells (columns 1 and 4) compared to their respective IFN-γ-treated counterparts show that the induction of nascent DRα transcripts is >4-fold in the absence of cycloheximide and >7-fold in the presence of cycloheximide. It has been demonstrated that steady-state DRα mRNA is induced 4- to 8-fold by IFN-γ in this cell line (26). Therefore in the present system, the majority, if not all, of the regulation of IFN-γ induction of DRα is due to an increased rate of transcription. In addition we have shown that this increase does not require protein synthesis.

Description of a Nested Series of 5' pDRα-CAT Deletion Mutants. To analyze regions of the DRα gene that are important in the IFN-γ-induced gene transcription, a series of deletion mutants of the 5' regulatory region of the gene encoding DRα was prepared and characterized as described (10) and are illustrated in Fig. 2. These DRα sequences were all cloned upstream of the indicator gene CAT in pD164-2 (9). The nine deletion mutants illustrated in Fig. 2 range from positions −1028 to −53 of the DRα gene and all contain a putative CAAT box, a TATA box, and the transcriptional start site. Constructs 5'Δ-1028 through 5'Δ-109 all contain the class II boxes (X + Y), which are two conserved upstream sequences present in all class II MHC genes (27). 5'Δ-91 contains only the 3' class II box, and 5'Δ-53 contains neither.

DRα Upstream Sequences Important in Basal and rIFN-γ-Induced Gene Expression. The deletion mutants illustrated in Fig. 2 were used in DNA transfection experiments to analyze the ability of various upstream regions of DRα to mediate basal and rIFN-γ-induced expression of the CAT gene. CAT activity was determined by measuring the ability of transfected cell extracts to acetylate the substrate [^{14}C]chloramphenicol as described (9). Table 1 represents a summary of more than 13 experiments in which percent acetylation was determined. Each value is based on the average of various numbers of transfection experiments. Inherent variability of transfection and CAT assays necessitated the performance of a large number of experiments with each construct and the performance of statistical analysis. The fold induction was calculated by dividing the average percent acetylation values of rIFN-γ-treated cultures by the average values of untreated cultures. The significance of the induced values versus the uninduced values was determined by a two-sided *t* test and
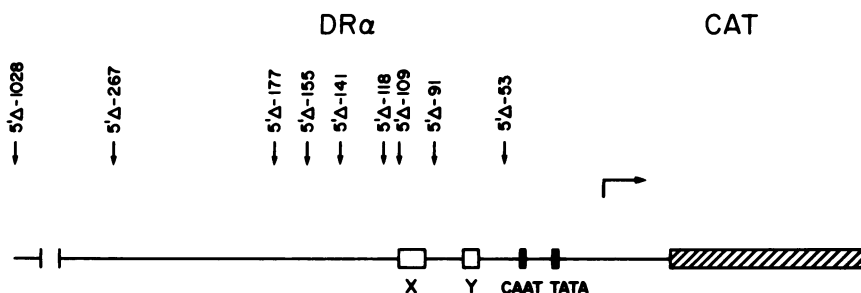

FIG. 2. A map of 5' pDRα-CAT deletion mutants used in transfection experiments. Nucleotide numbers in the pictured constructs (↓) are numbered relative to the DRα cap site. The DRα sequences were all cloned upstream of the CAT structural gene ▨. Boxes labeled X and Y designate two conserved upstream sequences found in all class II genes.

Table 1.   Basal expression and IFN-γ induction potential of 5′ DRα sequences

pDRα-CAT construct	Exp., *n*	IFN-γ treatment	% acetylation	Fold induction	*P*
5′Δ-267	13	−	0.7	13	<0.001
		+	9.2		
5′Δ-177	7	−	0.6	9	<0.001
		+	5.6		
5′Δ-155	2	−	0.4	7	<0.05
		+	2.9		
5′Δ-141	5	−	0.9	8	<0.01
		+	7.6		
5′Δ-118	3	−	0.9	2	<0.1
		+	2.1		
5′Δ-109	8	−	0.7	0	NS
		+	0.7		
5′Δ-91	2	−	0.1	0	ND
		+	0.1		
5′Δ-53	4	−	0.2	0	ND
		+	0.2		
pD164-2	12	−	0.3	0	NS
		+	0.3		

For *P*<, mean acetylation values from both treated and untreated samples were compared in a two-sided *t* test. ND, not done; NS, not significant; −, no IFN-γ treatment; +, IFN-γ treatment.

these *P* values are also shown. The composite of these data illustrates the following two points. (*i*) The basal DRα promoter is intact and contained in construct 5′Δ-109 and in all other constructs with at least this much of 5′ DRα sequence. Further 5′ deletions (constructs 5′Δ-91 and 5′Δ-53) cause a loss of basal expression to the baseline value of the negative control plasmid pD164-2. The higher level of basal expression seen with construct 5′Δ-109 versus 5′Δ-53 or pD164-2 was very consistent. (*ii*) Unlike basal promotor activity, rIFN-γ responsivity is not contained in 5′Δ-109; however, an additional 9 bp of upstream sequence (construct 5′Δ-118) results in some rIFN-γ induction, although the *P* value only shows significance at the 0.1 level. For maximal rIFN-γ induction, however, sequences between bp −118 and −141 are necessary (*P* values ranging from 0.001 to 0.01).

Sequences further upstream of bp −141 (constructs 5′Δ-155, 5′Δ-177, and 5′Δ-267) do not appear to contain additional rIFN-γ-responsive elements as shown by additional statistical analysis (Table 2). These data indicate that the fold-induction values obtained with constructs 5′Δ-177, 5′Δ-155, and 5′Δ-141 are not significantly different from construct 5′Δ-267, whereas the fold-induction value for construct 5′Δ-118 is only different from 5′Δ-267 at a significance value of 0.1.

Substitution Mutation of the MHC Class II IFN-γ-Responsive Element (IRE-γII) Results in Loss of IFN-γ Inducibility. To better define the functional role of DNA sequences between bp −141 and −109 in IFN-γ induction, a substitution mutant (pmut-IRE-γII) was used in transfection experiments. A control construct with wild-type sequences (pwt-

Table 2.   Lack of additional IFN-γ-responsive elements upstream of bp −141 in DRα

| pDRα-CAT construct	Exp., *n*	Induction of test construct/induction of 5′Δ-267, fold/fold	*P*
5′Δ-267	9	1	
5′Δ-177	5	1	NS
5′Δ-155	2	0.8	NS
5′Δ-141	5	0.9	NS
5′Δ-118	2	0.19	<0.1

For *P*, normalized induction ratios were compared in a two-sided *t* test.

Table 3.   Loss of rIFN-γ inducibility by substitution mutations in the IRE-γII

pDRα-CAT construct	rIFN-γ treatment	% acetylation
pwt-IRE-γII	−	0.45
	+	5.8
pmut-IRE-γII	−	0.25
	+	0.25
5′Δ-56(xbaI)	−	0.2
	+	0.2

For % acetylation, each value is an average of two experiments.

IRE-γII) was also examined. In addition the original deletion mutant 5′Δ-56(xbaI), into which either mutated or wild-type sequences were cloned, was included as a baseline control. Results from transfection experiments with these constructs are shown in Table 3. The results demonstrate that mutating the DRα region from bp −141 to bp −109 (pmut-IRE-γII) abolishes IFN-γ-inducible CAT expression. However, when the wild-type control plasmid pwt-IRE-γII was used, IFN-γ inducibility was preserved. This definitively demonstrates the importance of this region in IFN-γ-regulated DRα gene expression. Hence we have designated this region of the DRα gene as a class II IRE-γII.
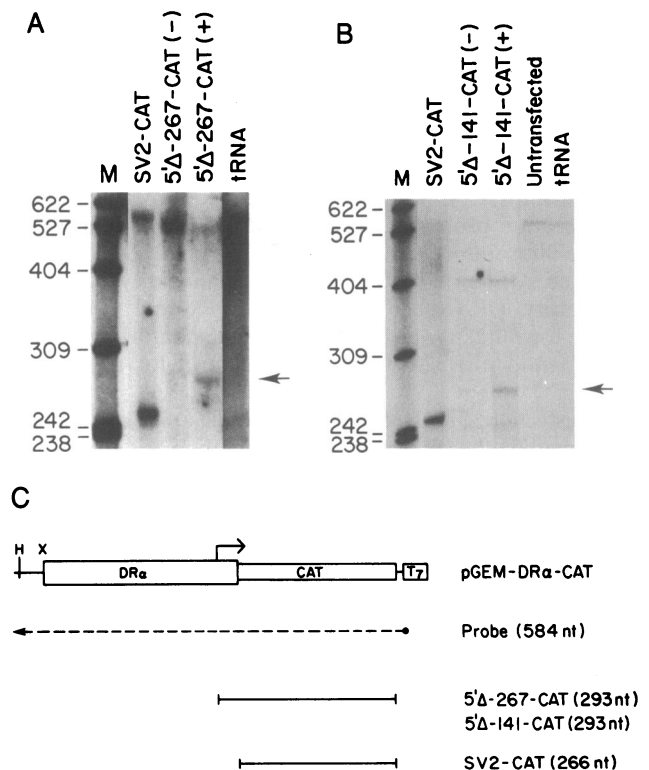
FIG. 3.   S1 nuclease protection of DRα-CAT RNA. A S1 nuclease protection analysis of total cellular RNA isolated from the U-373-MG glioblastoma transfected with the plasmids SV2-CAT, 5′Δ-267 (*A*), and 5′Δ-141 (*B*) is shown. Cells transfected with 5′Δ-267 and 5′Δ-141 were either untreated (−) or treated (+) with rIFN-γ (500 units/ml). Total cellular RNAs isolated from pSV2-CAT transfected cells (35 μg), 5′Δ-267 and 5′Δ-141 transfected cells (70 μg), untransfected cells (70 μg), or control tRNA (70 μg) were hybridized with a ^{32}P-labeled antisense transcript initiated from the T7 promoter of pGEM-DRα-CAT by the procedure of Melton *et al.* (29). This antisense probe contained 266 nucleotides of the CAT structural gene, 295 nucleotides of DRα sequence, and 23 bp of pGEM[3]-Blue sequence. Hybrids were then digested with 5 units of S1 nuclease and electrophoresed on a denaturing 6% polyacrylamide gel. In the marker lane M, ^{32}P-labeled *Hpa* II-digested pBR322 was electrophoresed for size determination. Predicted sizes of protected fragments are shown (*C*).

To determine whether the 5' DRα region from bp −141 to bp −109 could cause IFN-γ induction by itself, we cloned an oligonucleotide corresponding to this region into a pDRα-CAT vector containing only the DRα TATA box and a putative CAAT box but lacking the class II box sequences. This oligonucleotide was also cloned into the vector PA10CAT in which CAT is promoted only by the simian virus 40 promoter region (28). Neither of these constructs was able to promote IFN-γ-induced expression of CAT (data not shown). This suggests that the IRE-γII defined here needs to be in the context of other class II sequences (potentially the class II boxes) to exert its effect.

S1 Nuclease Protection Analysis of RNA Obtained from pDRα-CAT Deletion Mutant Transfections. To determine the transcriptional start site and the levels of CAT-specific RNA in U-373-MG cells transfected with pDRα-CAT, RNA was isolated and subjected to S1 protection analysis. Fig. 3A represents the results from such an experiment where RNA samples obtained from cells transfected with a positive control plasmid, pSV2-CAT, and with 5'Δ-267 untreated or treated with rIFN-γ were analyzed. Fig. 3B shows the results for cells transfected with 5'Δ-141 that were untreated or that were treated with rIFN-γ. The experiment in Fig. 3A was performed with RNA from calcium phosphate transfected cells, whereas the experiment in Fig. 3B was performed with RNA from electroporated cells.

The results illustrate that RNA from cells transfected with pSV2CAT and cells transfected with either 5'Δ-267 or 5'Δ-141 that have been treated with rIFN-γ yielded protected fragments of the predicted sizes (see Fig. 3C and ref. 30). RNA from uninduced cells that were calcium phosphate transfected with 5'Δ-267 showed little protection. This is in accord with the extremely low levels of CAT protein produced by these cells. In contrast, RNA from cells that had been electroporated with 5'Δ-141 in the absence of IFN-γ yielded a significant signal at both the protein level (CAT assay not shown) and here at the RNA level (Fig. 3B), probably because of enhanced transfection efficiency. Despite using RNA from cells transfected by different procedures, CAT transcripts from the correct initiation site of DRα were utilized in both cases as shown by the presence of a 293-bp protected fragment, and the level of accurately initiated transcripts is enhanced by IFN-γ. Negative controls,

including cells that were not transfected with plasmids or tRNA, did not yield any protected fragment.

DISCUSSION

The mechanism(s) by which IFN-γ enhances MHC class II antigen expression is not well understood, and the literature on this subject is sometimes confusing. The question as to whether or not the rIFN-γ-induced changes in class II expression are transcriptional, posttranscriptional, or translational has been raised for some time. In the present paper we have determined that, in a glioblastoma multiform cell line, the majority if not all of the IFN-γ induction of DRα is due to an increased rate of transcription that does not require *de novo* protein synthesis. This is in accord with a report where IFN-γ induction of class II gene expression in the mouse WEHI-3 cell line is shown to be transcriptionally induced (31). However, this report did not determine if the entire IFN-γ-induced response could be accounted for by increased transcription. Rosa and Fellous (30) provided evidence that the majority of the IFN-γ-induced DRα expression in both a melanoma cell line and a skin fibroblast cell line was posttranscriptional in nature. This raises the possibility that IFN-γ-induced class II gene expression may occur by different mechanisms.

This study also finely mapped the IFN-γ-responsive region of DRα in the glioblastoma cells. As summarized in Fig. 4, sequences upstream of the class II boxes are required for IFN-γ induction. Some induction is observed when an additional 9 bp of upstream sequence are included. Interestingly, the border of box X, as defined by Kelly and Trowsdale (32), includes these additional 9 bp. Maximal IFN-γ induction, however, requires another 23 bp of upstream sequence (construct 5'Δ-141). Substitution mutagenesis, which represents a superior approach to deletion mutagenesis, confirms the importance of a region from bp −141 to bp −109. Future site-specific mutational analyses will elucidate the precise nature of the IRE-γII consensus sequence.

In the DQ β chain gene, a sequence between bp −159 and bp −128 has been implicated in IFN-γ induction (14). This region and the maximal IFN-γ-responsive region identified for DRα (bp −141 to bp −119) are ≈50% identical. A conserved septamer defined by Servenius *et al.* (33) is found in both of these regions and in most other class II genes. The
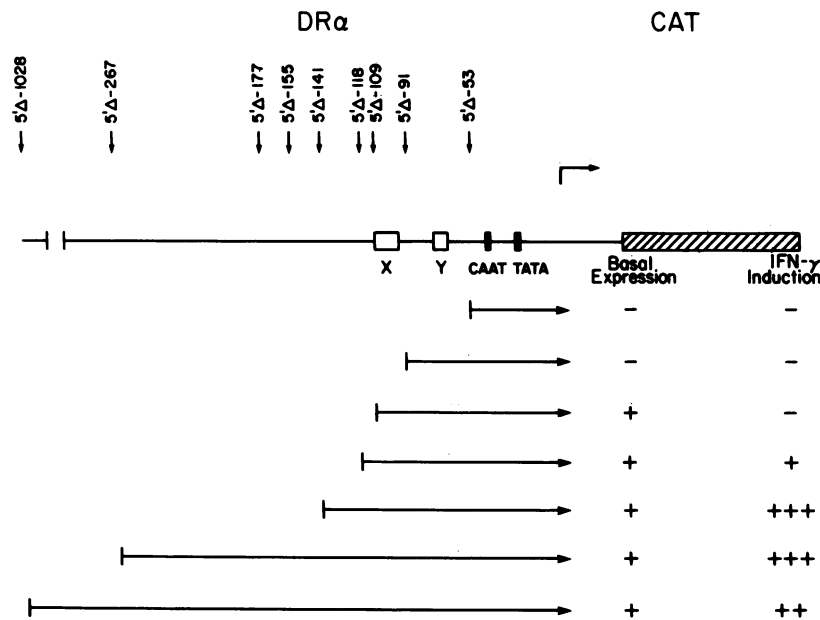

FIG. 4. Basal expression and IFN-γ induction potential of pDRα-CAT constructs. See Table 1 for more information.

Conserved
Septamer   G/A G/A A T/C C T T

DRα   GGACCCTTT GCAAGAACCCTTCCCCTAGCAACAGATGC
       |_Septamer_| |_Septamer_| |_box X_|

E_a^d   GGACCTCGTTGCAAGGAACCCTTTCCTAGCAACAGATGT
       |_Septamer_| |_Septamer_| |_box X_|

E_β^d   GGACCTGCAACT GAATCTCTAACTAGCAACTGATGA
       |_Septamer_| |_Septamer_| |_box X_|

DQβ   GAACCTTCACAAAAAAAAAATCTGCCCAGAGACAGATGA
       |_Septamer_| |_box X_|

A_β^b   GAGCCTTTGTAAACAATG TCTACCCAGAGACAGATGA
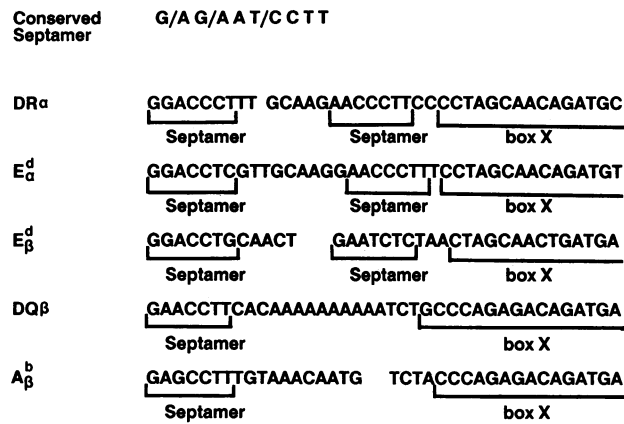 |_Septamer_| |_box X_|

Fig. 5. Conserved septamer sequences in class II upstream sequences. A consensus septamer as defined by Servenius *et al.* (33) is shown in the context of a number of class II MHC genes. The regions shown here containing the conserved septamer are all immediately upstream of the class II X box. Upstream sequences of the genes shown here were obtained from the following references: DRα (21), E_α^d (34), E_β^d and A_β^b (33), and DQβ (14).

consensus sequence of this septamer in the context of a number of class II genes is shown in Fig. 5. Within DRα, a septamer containing 6 of the 7 nucleotides is present between bp -131 and -125, where maximal rIFN-γ responsivity is mapped. Interestingly, another 6/7 septamer is found in the region of DRα (from bp -117 to bp -111), where minimal rIFN-γ responsivity was mapped. Other class II genes such as the murine E_α^d and E_β^d also have a second copy of this sequence in their 5' region. In contrast, the homologous region of the gene encoding the DQ β chain or a number of other class II genes—such as the β chain genes for DP, DO, and mouse A^b—only has one copy of this septamer. Whether these sequence differences reflect functional differences in rIFN-γ induction potential remains to be determined.

The present fine analysis of the upstream regions of the DRα gene has identified a class II IFN-γ-responsive element. This element is clearly different from the IFN-α,β element described by Friedman and Stark (35). Interestingly, in MHC class I genes, the IFN-γ- and IFN-α,β-responsive elements overlap significantly (36–40). The data presented here demonstrate to the best of our knowledge that this is clearly not the case in MHC class II genes that possess an IFN-γ-responsive element that is distinct from any IFN-α,β or -γ consensus sequences previously described.

Interestingly, we had identified (9) a conserved nonamer present in the upstream region of the gene encoding DRα (from bp -265 to bp -257) and of other rIFN-γ-inducible genes, including class I and II MHC genes and the β_2-microglobulin gene. The present detailed analysis of this 5' region of DRα was unable to ascribe any functional significance to this sequence (see Table 1 and Fig. 4, construct Δ-267 vs. Δ-177). This supports the above results, other work (36), and our earlier proposal (9) that the IFN-γ induction of class I and II genes may occur by different pathways.

The data presented here in combination with other reports have provided strong evidence that class II genes contain a number of regulatory regions both upstream to, and within, the gene (9–16). These regulatory elements in various cellular environments seem to account for the differential expression of class II genes. The mechanisms by which certain regulatory elements take precedence over others in different cell types await further elucidation.

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