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Structural Constraints within a Trimeric Transcriptional Regulatory Region

CONSTITUTIVE AND INTERFERON-γ-INDUCIBLE EXPRESSION OF THE HLA-DRA GENE*

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Constitutive and inducible transcription of the major histocompatability class II HLA-DRA gene involves the upstream S element and the conserved X and Y elements. In this report we have addressed the roles of spatial constraints and stereospecific alignment between the upstream S and X elements, and the X and Y elements, in both constitutive and interferon-γ (γ-IFN)-induced expression. Analysis of the constitutive expression in B cell lines (B-LCL) has previously shown that the X and Y elements must be stereo-aligned. Further study reveals that any spacing changes between S and X, regardless of the helical alignment of these two elements, is not tolerated. These same restraints are involved in an inducible system, because the response to γ-IFN treatment requires both stereo alignment between the X and Y elements and precise spacing between the S and X elements. Neither constitutive nor inducible expression can be restored by correcting the distance and spacing between only the S and Y elements with misalignment of X. These results reveal a common pathway for constitutive and inducible expression that may require either direct or indirect protein complex formation among proteins bound to three highly conserved regulatory elements. We have also evaluated the role of the A/T-rich sequence located immediately 5′ of the Y element and show that it exerts little effect on constitutive and γ-IFN induced DRA expression.

Transcription of mRNA encoding eukaryotic genes requires RNA polymerase II and an array of general transcription factors (reviewed in Ref. 1). Transcription is further modulated by gene-specific transcription factors which bind upstream regulatory elements and intermediary molecules such as coactivators or adapters which target components of the preinitiation complex through protein-protein interactions (2-6). Thus the signal for RNA polymerase II to transcribe a particular gene is likely to be a coordinated interaction between proteins binding core promoter elements, upstream promoter elements, and ternary complex formation involving proteins which do not bind recognition sequences. Although studies of protein-protein interactions at the minimal promoter TATA and initiator elements (INR) are rapidly revealing multiprotein complexes, characterization of essential protein-protein interactions involving multiple upstream control elements is not as advanced despite the many regulatory elements and sequence-specific transcription factors which have been identified. In this report, an analysis of the class II MHC[1] gene, DRA, reveals structural and spatial constraints in three promoter elements that likely reflect restraints of protein-protein interactions.

The regulatory mechanisms controlling the expression of the MHC class II genes allow us to use a biologically significant intact promoter to study mechanisms of cell type-specific, coordinate, and inducible gene expression. These genes represent a large family whose gene products form cell surface heterodimers which are essential in mounting a normal immune response (reviewed in Refs. 7 and 8). The primary immunologic function of the MHC class II molecules is in shaping the T-cell repertoire during thymic development and in presenting processed foreign peptide to T cells during the immune response (9-11). Proper immune function depends not only on the polymorphic structure of the α/β heterodimer, a feature necessary in its ability to bind a multitude of antigenic peptides, but also on the regulated expression of the class II molecule on the cell surface (7).

The complex regulatory mechanisms underlying MHC class II gene expression result in a unique pattern of constitutive and inducible gene regulation (reviewed in Ref. 12). The three forms of the human MHC class II molecules, DP, DQ, and DR, are constitutively expressed on B lymphocytes, macrophages, activated T-cells, and dendritic cells, whereas most somatic cell types do not express, nor can they be induced to express, MHC class II antigens (13, 14). In contrast, endothelial cells, macrophages, fibroblasts, and some glial cells, which normally express low levels of class II MHC molecules, can be induced to express high levels of these molecules following γ-IFN stimulation (15-19). These complex cell type-specific and inducible patterns of MHC class II expression are essential in normal immune function as evidenced by the immunocompromised state of Bare Lymphocyte Syndrome patients who lack class II expression and by the autoimmune state of patients with aberrantly high MHC class II antigen expression on glial cells of the central nervous system, on islet cells of the pancreas, etc. (20-22).

A number of upstream regulatory elements, including the Y box, the X box, and the S element, are important in the

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[1] The abbreviations used are: MHC, major histocompatability; bp, base pair(s); HLA-DRA, human leukocyte antigen-DRα.

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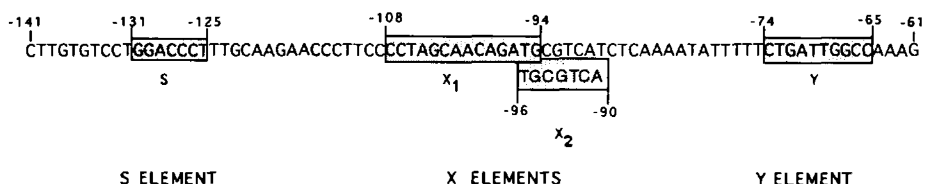

FIG. 1. **DNA sequences of the S, X, and Y elements of the HLA-DRA promoter.** The X_2 element, encompassing nucleotides −96 to −90, extends into the conserved 19-bp spacer region.

expression of the MHC class II gene, DRA (see Fig. 1) (reviewed in Refs. 23–26). The Y element was identified as a cis-acting sequence important in both the constitutive and γ-IFN-induced expression of DRA as well as all other class II MHC genes studied (27–30). This element contains an inverted CCAAT sequence and binds several nuclear proteins, including affinity-purified Y element binding protein (YEBP), NF-Y, and YB-1 (31–33). Although affinity-purified YEBP functions as a transcriptional activator and may be homologous to the recombinant murine NF-Y protein, YB-1 appears to repress γ-IFN-induced transcription of DRA.[2]

The X element is also important in both constitutive and γ-IFN-induced expression of DRA as well as other class II MHC genes. This element is further divided into the X_1 sequence encompassing the 5′ nucleotides of the X box, and an AP-1 like sequence, X_2, which overlaps X_1 and expands into the interspace region. The X_1 sequence binds RF-X, a nuclear protein identified by Southwestern probing of a λgt11 library (34). The X_2 sequence binds to a nuclear protein, hXBP-1, X2BP, as well as to Jun/Fos[3] (35–37). Both the X_1 and X_2 elements are functionally necessary in the constitutive expression of DRA, whereas the hXBP-1 contact sites do not appear to be necessary for γ-IFN-induced expression (38, 39).

Further analysis of a 30-bp region 5′ of the X element reveals a 7-base pair sequence, the S element, as another critical element in both γ-IFN-induced and constitutive expression of DRA (30, 40–43). This element was first identified by Servenius *et al.* (44) to be conserved among many MHC class II genes.

Although many upstream regulatory elements have been defined in the MHC class II genes, the knowledge of protein-protein interactions over this promoter is limited. As a prelude to the analysis of protein complex formation over the DRA promoter, we have previously evaluated the role of stereospecific alignment between the highly conserved X and Y elements which are separated by a spacer of approximately two turns of the DNA helix (45). Specifically, we found that an addition of one or two helical turns in the spacer region between X and Y did not affect constitutive expression, while half-helical turns disrupted expression. These studies reveal that constitutive DRA expression requires stereo-aligned X and Y boxes, implicating indirect or direct protein-protein interactions between the proteins that bind to these two elements. In this report we have evaluated the spacing constraints between the S and X elements, the X and Y elements, and the S and Y elements in both constitutive and γ-IFN induced expression. We show that stereo-alignment between the X and Y elements is also required for γ-IFN-induced expression, indicating that γ-IFN induction is dependent on interactions between elements required in the constitutive expression of DRA. Furthermore we show that a precise distance is required between the S and X elements, as any spacing change, regardless of helical alignment, abolishes constitutive expression and γ-IFN inducibility. These results reveal a unique system where the spatial arrangement of a trimeric regulatory region is critical in the constitutive and inducible modes of gene expression. We have also evaluated the role of the A/T rich sequence found immediately upstream of the Y element. Mutation of this sequence did not significantly affect constitutive or γ-IFN-inducible gene expression.

MATERIALS AND METHODS

Cell Culture and Transfection—Raji is a human Epstein-Barr virus-positive Burkitt's lymphoma cell line that expresses high levels of DR protein. These cells were grown in RPMI 1640 supplemented with 8% fetal calf serum and 2 mM glutamine. The U-373-MG cell line is a glioblastoma multiform cell line which expresses low levels of DR molecules but can be induced by γ-IFN to express high levels of DR (46). These cells were maintained in McCoys 5A with 10% fetal calf serum, 2 mM glutamine, and 100 units of penicillin and 100 μg/ml streptomycin. Transient transfections were performed by electroporation as described previously (47). Following transfection, the U-373-MG cells were treated with recombinant γ-IFN at 500 units/ml for 24–48 h prior to harvest.

Plasmids—Plasmids pD164-2 and 5′Δ-56 have been described previously (29). Briefly, pD164-2 is the parent chloramphenicol acetyltransferase (CAT) reporter construct which lacks DRA-specific promoter sequence. 5′Δ-56 was derived by inserting 56 bp of the DRA promoter sequence and an *Xba*I linker upstream of the CAT gene in pD164-2. The DRA-specific sequences of this construct include 56 bp 5′ of the CAP site, the CAP site, and 27 bp 3′ of the CAP site. 5′Δ-56X+Y was constructed by inserting wild type X and Y sequences into the *Xba*I site of 5′Δ-56. pSpacer +20, +15, +10, +5, −5, and −10 are variants of 5′Δ-56X+Y which have been described previously and are shown in Fig. 2*A* (45).

The 5′Δ-56SXY and its derivatives were made as follows. An oligonucleotide corresponding to the −141 to −98-bp region of DRA (including S and X) was annealed to the complementary strand which contained the region from −109 to −61 bp of the DRA promoter (including X and Y). These annealed overlapping oligonucleotides were made double-stranded with reverse transcriptase (Life Sciences) and then cloned into the *Xba*I site of 5′Δ-56 to create the constructs 5′Δ-56SXY shown in Figs. 3 and 4. Oligonucleotides containing insertions or deletions in the spacer region between S and X, and between X and Y, were used to generate the mutants. The sequences of the insertions or deletion are shown in Fig. 4. Likewise pSpacer SXY-AT1 and pSpacer SXY-AT2 were generated using strand 2 oligonucleotides containing mutations within the A/T-rich region. The exact changes made within the A/T-rich region are shown in Fig. 5. Dideoxynucleotide sequencing was performed directly from the double-stranded plasmid to confirm the sequences of all constructs.

Chloramphenicol Acetyltransferase Assays—The quantitation of acetylated *versus* unacetylated chloramphenicol was performed as described previously (47). -Fold induction was calculated by dividing the percent acetylation of γ-IFN-treated cultures by the analogous untreated controls.

In Vitro Run-off Transcription Assay—The *in vitro* transcription assay was performed as described previously using *Nco*I-linearized templates and a Namalwa nuclear transcription extracts (33).

RESULTS

Stereospecific Alignment between the X and Y Elements Is Critical for DRA Function in a B-cell in Vitro Transcription System—Using *in vivo* transient transfection analysis, we showed previously that stereo alignment between the X and Y elements is absolutely required for DRA promoter function in B-cells (45). The addition of 1.0 or 2.0 helical turns maintained or slightly increased promoter activity compared with the wild type construct. In contrast, insertion of an additional 0.5 or 1.5 helical turns as well as the deletion of 0.5 turn drastically reduced promoter function. When 1.0 turn of the DNA helix was deleted, promoter function was slightly re-

[2] J. P.-Y. Ting, N. J. Zeleznik-Le, A. Painter, T. M. Moore, A. Brown, and B. D. Schwartz, submitted for publication.

[3] B. Vilen and J. P.-Y. Ting, personal observation.

duced, probably due to the deletion of critical residues within the X_2 element.

As an additional approach to assess the role of DNA alignment in DRA promoter function, the abovementioned constructs were analyzed in an *in vitro* transcription assay (Fig. 2). The wild type construct 5′Δ-56X+Y with 19 bp separating the X and Y elements was active in an *in vitro* transcription assay (Fig. 2*B, lane 6*). When integral turns of the DNA helix were inserted into the spacer region, as in pSpacer +20 and pSpacer +10, expression was slightly reduced compared to the wild type control (*lanes 2, 4,* and *6*). However, when half-integral turns were inserted or deleted, as in pSpacer +15, pSpacer +5, and pSpacer −5, the activity was markedly diminished (*lanes 3, 5,* and *7*). As expected, the 5′Δ-56 construct, which lacks the X and Y elements, produced little DRA transcript (*lane 1*). The pSpacer −10 construct, which functioned poorly in *in vivo* transient transfection, also showed a significant loss of function by *in vitro* transcription analysis, most likely due to the deletion of residues within X_2. These data confirmed the general requirement for stereo-alignment between X and Y in an *in vitro* transcription assay and indicate that the distance between these two elements can be altered as long as helical orientation between X and Y is maintained.

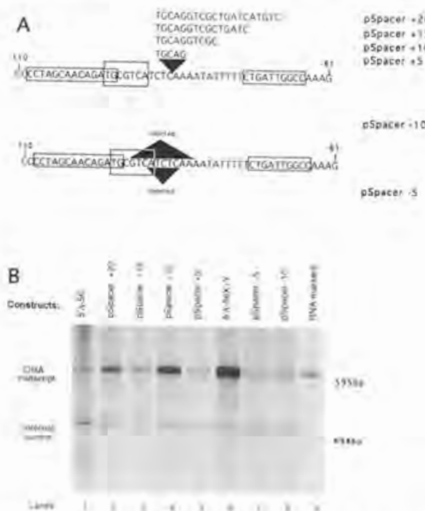

FIG. 2. *A*, plasmid constructs of the HLA-DRA promoter containing altered spacing between the X and Y elements. The mutated oligonucleotides used to create the various spacing mutants, and the position of the insertions or deletions are shown above the wild type sequence. These were inserted in the *Xba*I site of 5′Δ-56 as described under "Materials and Methods" to recreate 110 bp of DRA promoter. The names of the mutated plasmids are shown to the *right*, and the X_1, X_2, and Y elements are *boxed*. *B*, *in vitro* transcription assay using linearized spacer templates and Namalwa B-cell nuclear extracts demonstrates the role of the conserved 19-bp spacer in DRA function. The spacer constructs were linearized with *Nco*I to yield a 595-bp run-off transcript. The 494-bp fragment represents a radiolabeled RNA to control for sample loss during processing.

Precise Spacing between S and X Is Required for Enhanced Transcription Contributed by the S Element—In cells constitutively expressing DRA, the X_1, X_2, and Y elements are required. The S element confers an additional 5-fold enhancement of constitutive DRA expression. Previously, we noted that the distance between the S and X elements of various class II MHC genes and the invariant chain gene is relatively conserved at 15–17 bp (23, 48). To test whether the helical orientation or precise distance between S and X or S and Y affects promoter function in B-cells, various mutants containing alterations in both the distance and the helical alignment between the S and X elements were made and transfected into Raji cells (Fig. 3). pSpacer-S+5XY and pSpacer-S+10XY were designed to increase the distance between S and X by 5 or 10 bp, respectively, representing one-half or one integral turn of the DNA double helix, while maintaining wild type spacing between X and Y. The site of insertion was chosen at −112 bp relative to the start site of transcription, because previous studies showed the absence of any regulatory element in this region (38).

The 5-fold enhancement of transcription in the presence of the S element was observed as reported when comparing 5′Δ-56X+Y (*lane 2*) which lacks the S element to 5′Δ-56SXY, which contains the S element (lane 3). In contrast, pSpacer-S+10XY and pSpacer-S+5XY, reduced the constitutive expression of DRA in B-cells by 5-fold, which essentially negated the function of the S element (*lanes 4* and *5*). pSpacer-SX+10Y with an insertion of 10 bp between X and Y functioned similarly to the wild type 5′Δ-56SXY, whereas insertion of 5 bp between X and Y abolished function (*lanes 6* and 7) as would be expected.

To address the possibility that the function of the DRA promoter may occur independent of the alignment of the X

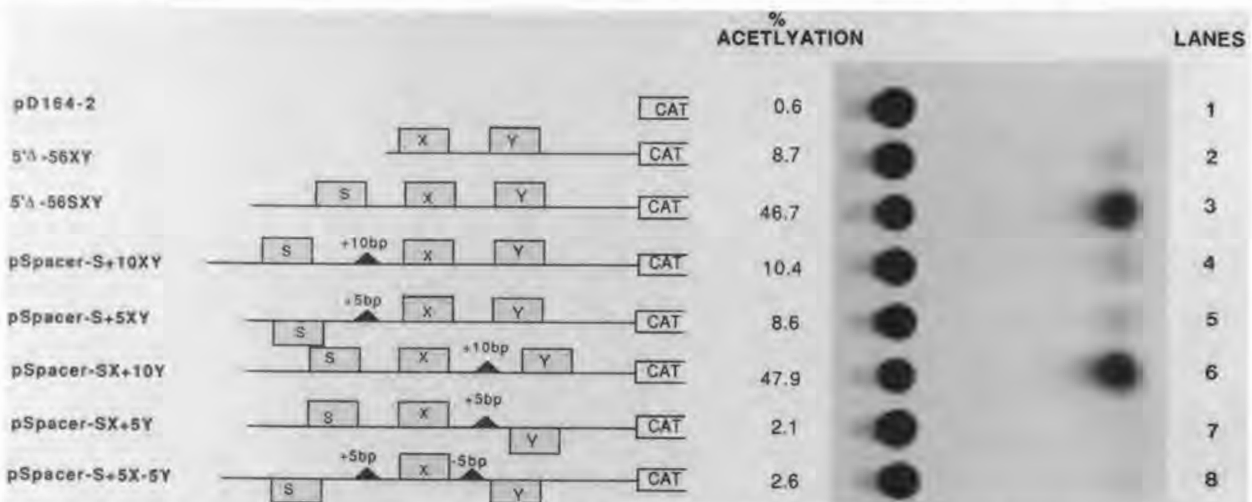

FIG. 3. **Promoter activity of the wild type 5′Δ56-SXY and the corresponding spacer mutants in Raji cells.** The schematics to the *left* depict helical and half-helical turn insertions and deletions between the S, X, and Y promoter elements. The relative stereospecific alignment of the S and Y elements compared with the X element is shown by the orientation of the element above or below the line. The percent acetylation of the [^{14}C]chloramphenicol substrate is shown for each construct. Statistical analysis of three independent Raji transfections using at least two different preparations of plasmid DNA showed a standard error of the mean (S.E.) less than 0.09.

element, and thus only require that S and Y be aligned, an additional construct was made. In this construct, pSpacer-S+5X-5Y, the distance between the S and X elements was increased by 5 bp, and the distance between the X and Y elements was decreased by 5 bp, yet the absolute distance between S and Y was maintained. These manipulations abolished function as shown in *lane 8*, indicating that alignment of S and Y alone is not sufficient for promoter function.

Proper Helical Alignment between the X and Y Elements Is Required for the IFN-γ Induction of DRA—Induced expression of the DRA gene by γ-IFN requires the S, X_1, and Y elements (Fig. 1) (41, 42). All three elements are necessary for an γ-IFN response, yet each individual element or any two of these elements are insufficient to elicit a response. To address the involvement of spatial constraints between these three γ-IFN-responsive elements, the following analysis was performed. We predicted that the misalignment of the X and Y elements in the presence of the S element would abolish γ-IFN inducibility if the γ-IFN induction is dependent on the stereospecific interaction of X- and Y-binding proteins, implying similar or overlapping mechanisms for constitutive and γ-IFN-induced gene expression. If γ-IFN inducibility proceeds independent of an X/Y alignment, then the mechanisms of γ-IFN inducibility and of constitutive expression would be considered different.

The constructs pSpacer-SX+10Y and SX+5Y were transfected into U-373-MG, a cell line which expresses low levels of MHC class II transcripts but can be induced to express high levels of DR upon γ-IFN treatment. In the chloramphenicol acetyltransferase assay shown in Fig. 4, the data were represented as -fold induction, a calculation required to normalize alterations in basal gene expression and reflect only those effects attributed to γ-IFN inducibility. -Fold induction was calculated as the percent acetylation of the γ-IFN-treated culture divided by the percent acetylation of the untreated control. An integral turn insertion between X and Y, represented by pSpacer-SX+10Y, yielded a -fold induction which was slightly less than the wild type, pSpacer-SXY construct (Fig. 4, *lanes 7* and *8*). In the absence of γ-IFN, basal gene expression from pSpacer-SX+10Y was higher compared with the wild type control. This result corroborates the result found in B-cells and could account for the slight reduction in γ-IFN inducibility from this construct. In contrast, insertion of a half-integral turn in pSpacer-SX+5Y produced markedly reduced basal and γ-IFN-induced activity (*lanes 5* and *6*). Identical results have been observed in primary rat astrocytes and in a γ-IFN-inducible melanoma cell line (data not shown). These findings confirm that the γ-IFN induction of DRA occurs through elements required for basal gene expression and reveals that the X and Y elements must be stereo-aligned for γ-IFN inducibility. This suggests that protein-protein interactions which may occur in constitutive gene expression are likely required in γ-IFN inducibility.

Precise Distance Is Required between the S and X Elements to Produce the γ-IFN Induction of DRA—γ-IFN inducibility of DRA has been previously mapped to the conserved S element, although this region alone cannot confer inducibility to a heterologous promoter, suggesting that other elements are required (41, 42). Recent studies have further defined that the X_1, but not the X_2 site, in addition to the Y element is required for the γ-IFN induction of DRA (38, 42).

To analyze the potential spacing constraints between S and

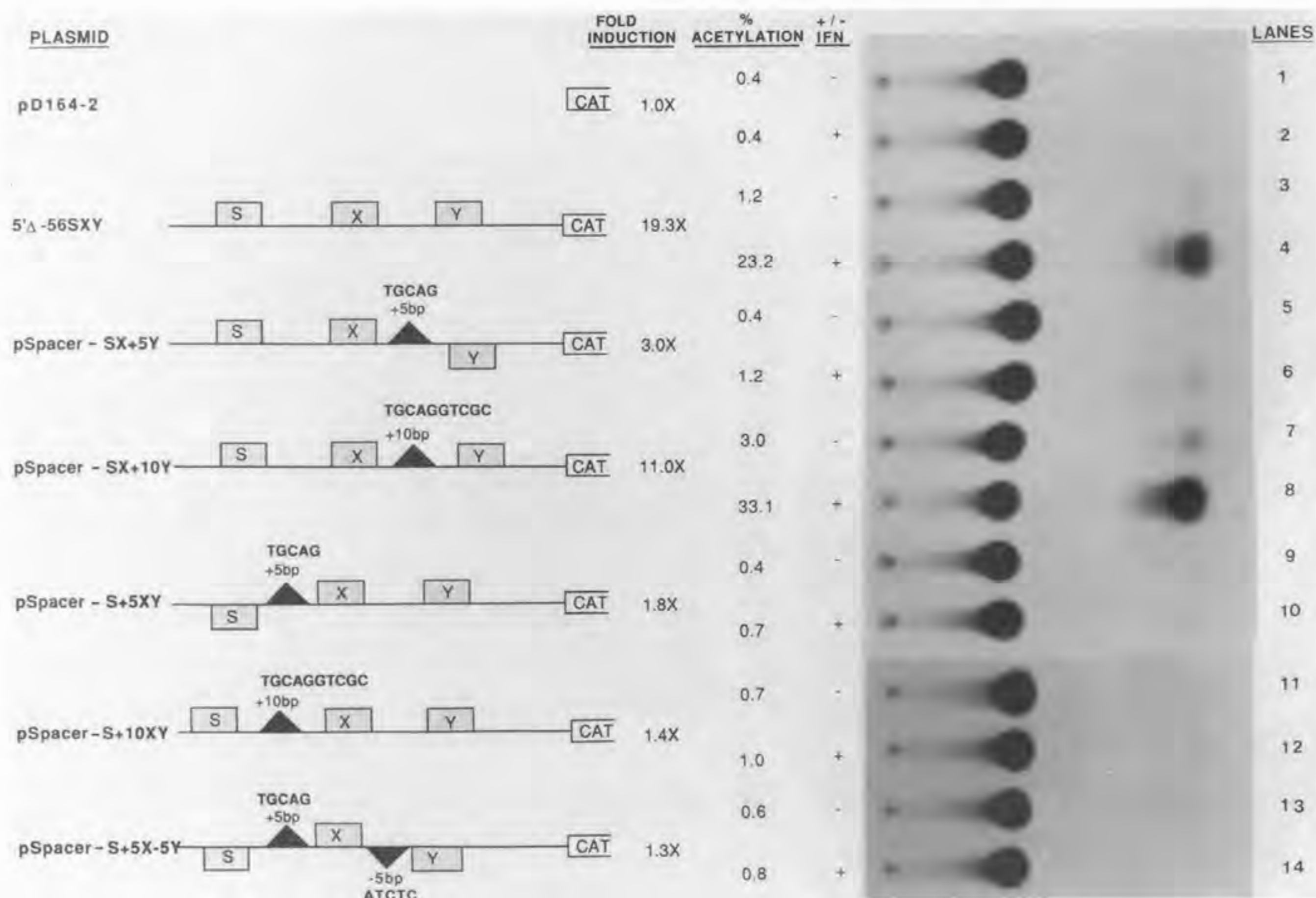

FIG. 4. **Schematic representation of SXY spacer constructs and their promoter activity in the γ-IFN-inducible U-373-MG cell line.** For details of the schematic, see the legend to Fig. 3. The -fold induction was determined by dividing the percent acetylation of the [^{14}C]chloramphenicol substrate in the γ-IFN-treated samples by the untreated samples.

X, mutants were tested which contain alterations in both the distance and the helical alignment between these two elements. The function of the altered promoters was assessed by transient transfection into the γ-IFN-inducible U-373-MG cell line (Fig. 4). Both pSpacer-S+5XY (*lanes 9* and *10*) and pSpacer-S+10XY (*lanes 11* and *12*) produced no γ-IFN induction compared with the wild type control (*lanes 3* and *4*). This indicates that regardless of stereospecific alignment, any change in the distance between S and X abolishes the γ-IFN inducibility of DRA. To address the possibility that γ-IFN induction could be maintained as long as the spacing between S and Y is maintained, pSpacer-S+5X-5Y (described earlier) was tested. This construct when transfected into U-373-MG cells also resulted in the absence of γ-IFN inducibility (Fig. 4, *lanes 13* and *14*). This result confirms the importance of proper helical alignment between the X and Y elements and proper distance between S and X in γ-IFN induction and is consistent with a model of protein-protein interactions involving a trimeric regulatory region for both constitutive and γ-IFN-induced gene expression.

Mutation of the α-Specific Sequence between the X and Y Elements has Minimal Effect on Either Constitutive or Inducible Expression—The boundaries of the Y element are confined in a 10-bp sequence found in all murine and human MHC class II, α- and β-chain genes. A comparison of the α-chain genes with the β-chain genes showed that the sequence homology of the Y element can be extended to nucleotides 5′ of the Y element. In the α-chain genes, this extended conserved sequence, referred to as the α-specific sequence, conforms to ATTTTT (23, 43). Within the β-chain genes, the β-specific sequence is moderately conserved as GATG. In both DRA and the murine homologue, EA, the conserved region is extended to AAAATATTTTT. It is possible that this A/T-rich region may confer some unique three-dimensional structure to the DNA, which signals protein binding or induces DNA bending to facilitate an X/Y interaction.

To demonstrate any functional significance of the α-specific sequence in DRA regulation, constructs were made which contain mutations in the A/T-rich sequence, both in the context of 109 or 141 bp of DRA promoter sequence (Fig. 5). The first set of mutations contain complete substitution of the A/T-rich segment to random sequence which is slightly G/C-rich. These are pSpacer XY-AT1 and pSpacer SXY-AT1. The second mutation, pSpacer SXY-AT2, maintains the same A/T composition as wild type but alters the nucleotide order so as to disrupt a tract of A residues followed by a tract of T residues. Transient transfection of these constructs into Raji cells allowed us to assess the role of the α-specific sequence in constitutive expression of DRA. As shown in Table I, mutation of the A/T stretch to a random sequence in pSpacer XY-AT1 construct resulted in promoter activity slightly higher than the wild type 5′Δ-56X+Y construct (*line 3* compared with *line 2*). In the context of the S element, mutation of the A/T stretch to a random sequence resulted in promoter activity which was slightly reduced compared with the wild type 5′Δ-56SXY (*line 5* compared with *line 4*). The opposite effect of this same mutation in the presence or absence of the S element may reflect a requirement for a different DNA configuration in the presence of two *versus* three regulatory elements. Mutation of the A/T region to an altered order of A/T residues did not result in a significant change, suggesting the composition and not the order of A/T residues is important (*line 6* compared with *line 4*).

To assess the role of the A/T region in the γ-IFN response, we performed transient transfection of wild type and mutated A/T spacer constructs into the glioblastoma multiforme cell line U-373-MG. When random nucleotide sequence replaced the A/T sequence, or when the A/T composition was maintained, yet the order of nucleotides was disrupted, γ-IFN inducibility of DRA was maintained near wild type levels (*lines 5* and *6* compared with *line 4*). These results suggest that the A/T-rich region only has a slight effect on constitutive gene expression in Raji cells but no effect on γ-IFN activation.

DISCUSSION

Understanding the interactions among proteins that bind to upstream regulatory elements and identifying cofactors or adaptors that function in large protein complexes are crucial events in understanding how upstream regulatory elements direct transcription by RNA polymerase II. Although much work has been done with many genes to identify cis-acting elements and their sequence-specific DNA binding proteins, little is known about how these upstream DNA elements interact to signal transcription. This report reveals the spatial constraints placed on a trimeric regulatory region within the proximal promoter region of a class II MHC promoter which contains three positive regulatory elements, the S, X, and Y elements. The data presented demonstrate that two different forms of constraints are imposed on this DNA structure. Stereospecific alignment is required between the X and Y element for both basal expression in B-cell lines and for IFN-γ-induced expression in a glioblastoma line, whereas a distance constraint is placed between the S and X elements for both modes of gene expression. This represents a novel example of a nonviral eukaryotic promoter that is greatly dependent on the spatial relationship of three separate promoter elements.

The most straightforward interpretation of this observation based on the recent literature is the formation of a large transcription complex involving three upstream elements (rel-

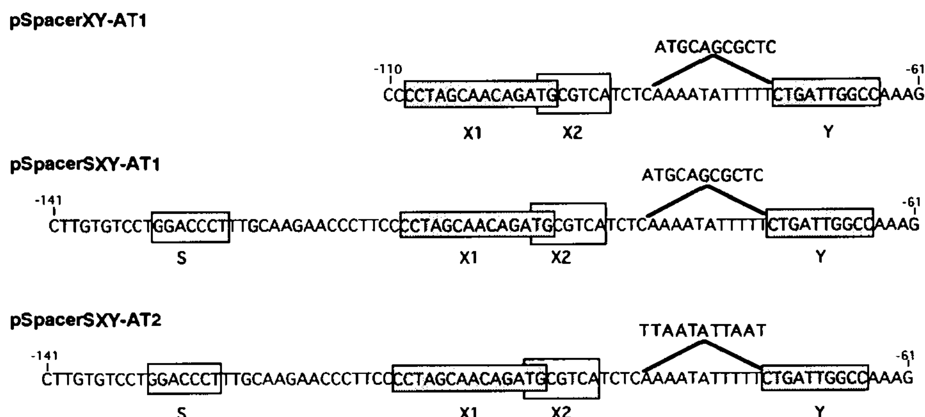

FIG. 5. **Plasmid constructs of the HLA-DRA promoter containing mutations within the α-specific sequences.** The oligonucleotides shown above were cloned into the *Xba*I site of 5′Δ-56 to recreate the DRA promoter to −110. The mutations made in the α-specific sequence are shown above the wild type sequence. The construct name is shown to the *left*.

TABLE I

Role of α-specific sequence in constitutive and inducible DRA expression

Normalized mean and p values of the top three constructs are calculated relative to the 5′Δ-56X+Y control. Normalized mean and p values of the bottom three constructs are calculated relative to the 5′Δ-56SXY control. The data are presented as normalized mean ± S.E. of the mean as in Table I. p values are calculated using two-sided t test.

Construct	Constitutive expression Raji[a]	Inducible expression U-373-MG[b]
	mean ± S.E.	*mean-fold induction ± S.E.*
pD164-2	0.02 ± 0.0	0.06 ± 0.01
5′Δ-56X+Y	1.00	NA
pSpacer XY-AT1	1.40 ± 0.16 ($p < 0.03$)	NA
5′Δ-56SXY	1.00	1.00
pSpacer SXY-AT1	0.66 ± 0.12 ($p < 0.02$)	1.60 ± 0.28 ($p < 0.14$)
pSpacer SXY-AT2	0.75 ± 0.14 ($p < 0.12$)	1.10 ± 0.21 ($p < 0.70$)

[a] Mean of at least nine independent transfections.
[b] Mean of at least four independent transfections.

ative to the TATA box) which require strict structural constraints. Stereo alignment between X and Y as well as a distance requirement between X and S may reflect restraints imposed by protein-protein interactions that are necessary for the engagement of the transcription machinery. The stereospecific alignment between X and Y can be most simply interpreted to reflect the requirement for proteins binding the X and Y elements to bind to the same side of the DNA helix for proper interaction. The strict distance requirement between S and X regardless of helical orientation may reflect two possible situations; one is that an X box binding protein may have sequence specificity for both S and X, and hence this protein can only engage these two sites when a rigid distance requirement is met. Interestingly, *in vitro* gel shift analysis has shown that S and X mutually cross-compete for protein-DNA complex formation (49). Furthermore, recombinant RF-X can interact with both of these sites.[4] Since RF-X is a relatively large protein which can form homodimers, it is possible that each partner of the dimer separately binds to the S and X sites, resulting in a rigid spatial requirement. An alternative explanation is that the proteins which interact with S and X are unique, yet exhibit rigid protein-protein interaction requirements which are highly dependent on their spanning distances.

It is noteworthy that the observations made here are reminiscent of the classical study performed with the SV40 promoter/enhancer (50). It was noted that the helical orientation between enhancer A and the 21-bp GC-rich repeats was critical for optimal gene expression, whereas changes in the distance, and to a lesser degree helical orientation, between the TATA box and the GC box diminished transcription from some start sites. Considering recent indirect evidence for putative interactions between the TATA box binding protein TFIID (TBP), and the GC box binding protein SP1 in other systems, direct protein-protein interaction could explain such constraints (5). Stereospecific alignment between two elements has also been observed in other eukaryotic systems, *e.g.* the *Xenopus* ribosomal RNA genes and the prolactin gene (51, 52). Most interestingly, the role of spacing has been implicated in determining whether the U6 and 7SK RNA genes are transcribed by RNA polymerase I or II (53).

The distance requirement between the S and X region is noteworthy as in most promoter systems tolerate large distance variations. An exception is the adenovirus type 2 Elb promoter where the proper function of the GC boxes and an upstream enhancer sequence is dependent on the distance between these two elements (54). Insertion of an additional 5 bp between these two elements was tolerated, whereas insertion of 10 bp was not. In our system, neither one of these variations are tolerated, representing an even stricter requirement.

The finding that both basal expression and γ-IFN induction require identical spacing and stereospecific alignment is consistent with our previous hypothesis that the γ-IFN induction of class II MHC genes utilizes transcription factors that are important for basal gene expression. Studies from a number of groups have shown that the γ-IFN-responsive elements in the class II MHC promoter are indistinguishable from those required for basal transcription. The only difference is that for basal gene transcription, X and Y are sufficient, whereas the S element enhances the transcription by 5-fold. For an γ-IFN response, all three are required and mutation of any one negates a response. Two recent observations are also consistent with the notion that γ-IFN induction utilizes factors or mechanisms that are involved in basal gene transcription. First, we have recently identified an X box binding protein, IFNEX (IFN-γ-enhanced protein), whose DNA binding activity appears to be enhanced by γ-IFN (38). By UV-cross-linking, IFNEX binding activity is indistinguishable in γ-IFN-treated cells compared with untreated cells.[5] Second, *in vivo* genomic footprint analysis revealed weak occupancy of the X box in an γ-IFN-responsive cell line prior to induction but complete occupancy of the X box after γ-IFN induction (56). Taken together, these findings are consistent with the hypothesis that γ-IFN modifies an existing X-binding protein, resulting in enhanced affinity for the X box. Although there are many other possibilities, this model is also consistent with the central role the X box plays in the present analysis.

The above finding is important because the regulation of MHC class II genes by γ-IFN represents the best defined system for genes that are induced by IFN-γ but not but IFN-α. Class II MHC gene regulation by γ-IFN differs significantly from genes which are induced by both α-IFN and γ-IFN in that 1) three separate promoter elements required for basal gene transcription are required for class II MHC gene induction by γ-IFN, whereas the "classical" α-IFN consensus sequence defined in the promoter regions of the metallothionein and class I MHC genes are not involved; 2) different DNA-binding proteins appears to be involved; and 3) the kinetics of induction is different. Class II MHC promoter may represent a prototype of γ-IFN-inducible and α-IFN-noninducible promoters as evidenced by the recent delineation of similar S, X, and Y elements in the Invariant chain gene promoter and of S and X elements in the FcγR1 promoter (48, 55). It would be of interest to determine if similar constraints are placed on these homologous elements found in other promoters.

In conclusion, these studies revealed tight spatial and/or helical constraints in the class II MHC promoter. This is true of both constitutive and γ-IFN-induced gene expression, suggesting that the two responses involve common pathways that may require direct or indirect protein complex formation over three promoter elements. In contrast, an A/T-rich region within the spacer has little effect on gene expression.

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[4] B. Matija Peterlin, personal communication.

[5] B. Panek and E. Benveniste, personal communication.

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