Expression of the major histocompatibility complex (MHC) class II-associated invariant chain (Ii) is required for efficient and complete presentation of antigens by MHC class II molecules and a normal immune response. The Ii gene is generally co-regulated with the MHC class II molecules at the level of transcription and a shared SXY promoter element has been described. This report defines the proximal promoter region of Ii which may regulate Ii transcription distinct from MHC class II. In vivo genomic footprinting identified an occupied, imperfect CCAAT box and an adjacent GC box in the proximal region. These sites are bound in Ii-positive cell lines and upon interferon-γ induction of Ii transcription. In contrast, both sites are unoccupied in Ii-negative cell lines and in inducible cell lines prior to interferon-γ treatment. Together these two sites synergize to stimulate transcription. Independently, the transcription factor NF-Y binds poorly to the imperfect CCAAT box with a rapid off rate, while Sp1 binds to the GC box. Stabilization of NF-Y binding occurs upon Sp1 binding to DNA. In addition, the half-life of Sp1 binding also increased in the presence of NF-Y binding. These findings suggest a mechanism for the complete functional synergy of the GC and CCAAT elements observed in Ii transcription. Furthermore, this report defines a CCAAT box of imperfect sequence which binds NF-Y and activates transcription only when stabilized by an adjacent factor, Sp1.

One of the central steps in elicitation of an immune response is the presentation of foreign antigenic peptides by the major histocompatibility complex (MHC) class II molecules on the surface of cells (1, 2). Recognition of the peptide-MHC class II complex by the T-cell receptor on class II-restricted T lymphoid cells leads to activation of the T cells and the initiation of the immune response (3, 4). Binding of peptides to the class II molecules is regulated in part by the MHC class II-associated invariant chain (Ii) protein (5, 6). The Ii is an integral membrane protein which associates with class II α/β molecules in the endoplasmic reticulum (7, 8). This association has multiple effects on class II molecules, all of which optimize the binding and presentation of foreign peptides derived from the extracellular environment (reviewed in Refs. 9, 10). The Ii also appears to play a role in targeting the class II molecules to endosomal or endosomal-like compartments where peptide is bound (11–14). The critical role of Ii was demonstrated in mutant mice lacking a functional Ii gene (15, 16). MHC class II function was impaired resulting in inefficient antigen presentation and a deficiency in CD4+ T cells.

The tight functional association of Ii and class II is reflected in their coordinate transcriptional regulation. Both molecules are only expressed in a select group of cells including B cells, activated human T cells, thymic epithelial cells, and macrophages (17–19). Ii and class II are also coordinately induced by interferon-γ (IFN-γ) in several cell types including macrophages and brain glial cells (20–23). The Ii promoter and the class II promoters share a common DNA motif, S/X/Y, which interacts with at least three distinct transcription factors (4, 24–26). Several studies have clearly demonstrated that this motif is responsible for both the constitutive and IFN-γ-induced coordinate regulation (27–30). In class II promoters, this motif is located proximal to the TATA box and is generally sufficient for transcriptional activation. In contrast, the Ii promoter has over 170 base pairs (bp) between the TATA box and the S/X/Y motif. There are several cases of differential regulation between Ii and class II genes (17, 18, 31, 32). The additional Ii promoter sequences between the S/X/Y and TATA domains may harbor elements mediating the discordant transcription. In the murine Ii promoter, an NF-κB site has been identified which mediates basal and TNFα induction of Ii transcription (33). In addition, promoter deletion analysis implicated a GC box as important for the basal activity of the murine gene (30). Recently, two NF-κB sites have been characterized at position −172 and −118 base pairs of the human Ii promoter (34). The function of these elements is dependent on cell type-specific differences in the binding of NF-κB subunits. Despite these studies, little is known about the Ii proximal promoter and the factors bound there which may be required for transcription as well as potentially mediating the effects of the distal S/X/Y motif.

The Y box of the S/X/Y motif is an inverted CCAAT site which binds the heteromeric transcription factor NF-Y (also known as CBF, CP1, and YEBP) (35–38). NF-Y is clearly distinct from the other known CCAAT box-binding proteins, CTF/NF-1 and C/EBP. NF-Y is composed of an A, B, and C subunit of 42, 36,
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and 40 kDa, respectively (39, 40). NF-Y is highly conserved through evolution (41). The yeast transcription factors HAP3, HAP5, and HAP6 are homologs of the A, B, and C subunits, respectively, and are functionally interchangeable in vivo (42). In addition to MHC class II promoters, NF-Y-binding sites are found in many unrelated promoters; e.g., HSV-tk (37), collagen (36), and albumin (43). Typically, the binding sites are located in the proximal promoter region between −60 and −80 base pairs upstream of the transcription initiation site (44). Functional analysis has also shown that NF-Y can be important in transcription reinitiation as well as in activation, suggesting a role in basal transcription (43, 45).

In the MHC class II promoters, NF-Y also functions to stabilize and/or recruit additional transcription factors to the S/X/Y complex. In vitro, NF-Y and the X box-binding protein, RFX, co-stabilize binding to their DNA sites (46). Furthermore, in vivo mutation of the NF-Y-binding site abrogates factor binding to the upstream X1 and X2 sites (47). In addition, NF-Y requires that the DNA stereospecifically align the X and Y boxes for activation (48). NF-Y has also been studied at the serum albumin promoter and in that context functions synergistically with C/EBP to activate transcription (43). The interaction between these factors partially destabilizes NF-Y binding but results in a large synergistic increase in the formation of stable preinitiation complexes. These studies of NF-Y suggest that although found in many different promoters, its mode of function may depend upon the local environment of that specific promoter.

In this report we now describe the in vivo characterization of the elements and factors involved at the proximal promoter of the human Ii gene. Through the use of in vivo genomic footprinting, two adjacent binding sites with homology to a GC box and an imperfect CCAAT box are identified. The GC and CCAAT elements act synergistically to promote Ii transcription and are bound by the transcription factors Sp1 and NF-Y, respectively. NF-Y and Sp1 bind cooperatively at the Ii promoter to mediate, at least in part, the observed functional synergy. Thus, maximal Ii transcription requires the action of two NF-Y factors; one in the distal S/X/Y motif and one downstream (47). In addition, NF-Y requires that the DNA stereospecifically align the X and Y boxes for activation (48). NF-Y has also been studied at the serum albumin promoter and in that context functions synergistically with C/EBP to activate transcription (43). The interaction between these factors partially destabilizes NF-Y binding but results in a large synergistic increase in the formation of stable preinitiation complexes. These studies of NF-Y suggest that although found in many different promoters, its mode of function may depend upon the local environment of that specific promoter.

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RESULTS

In Vivo Genomic Footprinting Reveals an Occupied GC Box and an Imperfect NF-Y Site—Several studies by our laboratory and others have characterized transcriptional elements present in the distal promoter region of the II gene. However, little is known about the identity or requirement for proximal elements. We directly addressed this question by mapping the DNA sequences occupied in intact cells by in vivo genomic footprinting. In vivo footprinting allows the visualization of protein-DNA interactions on endogenous genes in the more physiologically relevant intact cell. Fig. 1 displays the footprints for the first 90 base pairs of the II promoter in cell lines constitutively positive for II expression, Raji and H9 and an II-negative cell line, Jurkat. Two very clear domains of contacts were detected in the B lymphoblastoma cell line, Raji and the T lymphoblastoid cell line H9 (Fig. 1, A and B, lanes 1-4 and summarized in Fig. 1C). The contacts in both of these cell lines and in another II-expressing B cell line, Namalwa (data not shown) were identical. Five protections and two enhancements on the upper strand were clustered over a sequence with high homology to a consensus GC box. The single guanine residue on the lower strand of this GC box was also protected (Fig. 1B). In addition, two guanine residues flanking the GC box were weakly protected. Downstream of the GC box region a second, weaker cluster of contacts is visible. There is a weak protection at position −50 on the upper strand. On the lower strand there is a strong protection at −51 and a second weak protection at −39. These contacts partially overlap with a imperfect NF-Y/CCAAT box-binding site. A consensus NF-Y/CCAAT box has previously been characterized in the distal region of the II promoter and is referred to as the Y box. Thus we named the imperfect, proximal NF-Y/CCAAT homology, Y-proximal (Y-prox).
previous in vivo footprint analysis of other consensus NF-Y-binding sites (49, 54). In addition, protection of the Y-proximal adenine residues at position −45, −48, and −49 was detectable in Raji cells by a modified in vivo footprinting protocol (data not shown, 59). Analysis of the II-negative, T lymphoblastoid cell line Jurkat did not detect any protein-DNA interactions at the GC box and Y-proximal elements on the lower strand and only a single weak protection on the upper strand (data not shown, Fig. 1, A and B, lanes 5 and 6). Our previous in vivo analysis of the distal portion of the II promoter also revealed it to be unoccupied in Jurkat cells. The single protection in Jurkat cells could indicate an extremely weak interaction at the Y-proximal site or more likely reflect an alteration of the DNA structure in that area, since the rest of the promoter is unoccupied. A similar bare promoter was observed for the MHC class II promoter DRA in Jurkat cells even though the transcription factors are available in the nucleus (54). These results suggest that class II and II-negative cells may prevent transcription of these genes by blocking the accessibility of transcription factors to the promoters. Furthermore, the class II and II genes may use a common mechanism to block accessibility despite their location on different chromosomes.

II gene transcription is strongly induced by IFN-γ in a number of non-lymphoid cell types. In the glioblastoma cell line U105-MG, the induction is mediated through a 5–6-fold increase in the rate of II gene transcription (34). In vivo footprint analysis of the II proximal promoter in U105-MG cells revealed no protein-DNA interactions prior to IFN-γ induction (Fig. 2). Four h after the addition of IFN-γ, protections are clearly visible at the GC box and Y-proximal site. The contacts are identical to those observed in the Raji and H9 cells. A similar pattern of interaction was observed at 18 and 48 h post-induction. This region of the II promoter has not been implicated in the IFN-γ response; rather two upstream elements, the ISRE and SXY domain, appear to mediate the induction (27, 29, 60).

Analysis of those IFN-γ-responsive elements as well as the two NF-κB sites displayed in vivo occupancy only after IFN-γ treatment (34, 49). Interestingly, protection at these distal elements did not become maximal until 24 or 48 h post-induction (see "Discussion"). This indicates that the entire II promoter is shielded from transcription factor binding in the uninduced state, and IFN-γ treatment relieves this repression.

The GC Box and Y-proximal Site Synergistically Activate II Transcription—We addressed the functional importance of the newly identified in vivo binding sites by preparing site-specific mutants of the recognition sequences and assaying the effect on transcription from the II promoter. The GC box was altered by changing the core sequence to three successive T residues. This mutation blocks Sp1 binding to GC boxes as shown previously (61) and below. Four residues were altered in the Y-proximal site which eliminated all potential homology to CCAAT boxes. Each of the mutations were assayed in the context of the 790-base pair II promoter fused to the CAT reporter gene. These constructs were first transfected into the constitutively expressing Raji B cell line and assayed by CAT activity. The results are normalized to one for expression from the wild type construct. Each bar represents data from seven independent experiments, and the S.E. is indicated by the error bars.
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FIG. 4. In vitro binding to the Ii proximal promoter detects complexes with the GC box, the Y-proximal site, and both together. A, EMSA analysis with the wild type Ii probe, IiGC/Yprox (−32 to −85 bp). Shaded triangle indicates increasing amounts of Raji nuclear extract (0.2–2 μg) added to the binding reaction and the shaded rectangle represents a constant 1 μg of nuclear extract. Specific complexes are labeled A–D while n.s. indicates the nonspecific band. Oligonucleotide competitors are indicated above lanes 5–10 and are described under “Experimental Procedures.” B, EMSA competition analysis demonstrates that the complex binding to the Y-proximal site is related to NF-Y. The wild type Ii probe (IiGC/Yprox) is used in lanes 1–5, and the high affinity NF-Y site DRA Y-box is used for lanes 6–10.

Namalwa, were used (data not shown). All of the bands increase in intensity as the amount of nuclear extract is increased. However, the slowest migrating band, D, accumulates most rapidly. At the lowest protein concentration band D is the least abundant complex, but at the highest protein concentration band D represents a significant portion of the total shifted probe. This pattern is consistent with band D representing a multimeric complex composed of two or more of the faster migrating complexes. The specificities of the complexes were identified by oligonucleotide competition. Self-competition with the Ii sequence containing both the GC box and Y-proximal sites (lane 5) abrogated all binding except for the weak nonspecific band (n.s.). Bands D and B were competed with an Ii Y-proximal oligonucleotide (lane 6) while the Ii GC box oligonucleotide competed for the D, C, and A bands (lane 7). An oligonucleotide mutated in both the GC and Y-proximal sites failed to compete for any band (lane 8). Therefore, binding at the Y-proximal site is related to the D and B bands while binding at the GC box is related to the D, C, and A bands. This finding is consistent with the D band representing specific, simultaneous binding at both the GC box and Y-proximal site. The GC box has strong homology to a Sp1-binding site. Competition with a Sp1 consensus binding site oligonucleotide removes bands D, C, and A (lane 9). The resulting pattern is identical to competition with the Ii GC box oligonucleotide and further suggests Sp1 is binding at the Ii GC box. Competition with a high affinity OTF-2-binding site from the MHC class II DRA promoter had no effect on any of the complexes (lane 10).

Although the Y-proximal site is divergent at the highly conserved second C residue of the CCAAT box, the remainder of the sequence conforms to an 11-bp NF-Y consensus. The consensus was derived from 17 different published NF-Y-binding sites (Table I). The MHC class II DRA Y box is a high affinity NF-Y-binding site and was used in EMSA competition to access the relationship between the Y-proximal binding activity and NF-Y (Fig. 4B). Competition of the Ii GC/Yprox oligonucleotide probe with the DRA Y box eliminated the D and B bands (lane 3). This pattern of competition is identical to competition with the Ii Y-proximal site as shown in Fig. 4A, lane 6. The unrelated DRA octa oligonucleotide did not compete for any of the bands. The Y-proximal site also has weak homology to the OTF-1 binding consensus TAATGARAT. However, none of the Ii-specific bands were competed for by a TAATGARAT oligonucleotide (lane 5). An identical pattern of competition was observed when a probe corresponding to the DRA Y box was used (lanes 6–10). Both Ii GC/Yprox and DRA Y box oligonucleotides competed the NF-Y band. Neither the DRA octa or TAATGARAT competed for the band. Together, the competition results suggest that NF-Y interacts at the Y-proximal site. Furthermore, both NF-Y and Sp1 can bind simultaneously to the Ii promoter as evidenced by the D band.

A direct test for the presence of NF-Y and Sp1 on the Ii promoter is available through the use of antibodies specific for the two transcription factors. Specific antibodies or preimmune sera are added to the EMSA binding reaction and then resolved by gel electrophoresis. A specific antibody interaction either diminishes the gel shift band (blocking) or shifts the complex higher in the gel (supershift). We first tested the antibodies on the wild type Ii probe (Fig. 5, lanes 1–5). The preimmune sera has no effect on any of the complexes. However, the antisera specific for the A subunit of NF-Y resulted in a supershifted band concomitant with a decrease in the intensity of the D and B bands. This is consistent with the association of the D and B bands with binding at the Y-proximal site. The anti-Sp1 antibody also produced a supershifted complex and diminished the D and C bands. However, the B band was unaffected. This is also consistent with the D and C bands representing GC box binding. An unrelated antibody specific for NF-κB-p50 did not affect any of the bands. The antibodies were also assayed on the NF-Y complex derived from the DRA Y box probe (lanes 6–10). Only the antibody specific for NF-YA subunit supershifted the NF-Y complex. To simplify the gel shift-antibody pattern, the two transcription factors. Specific antibodies or preimmune sera are added to the EMSA binding reaction and then resolved by gel electrophoresis. A specific antibody interaction either diminishes the gel shift band (blocking) or shifts the complex higher in the gel (supershift). We first tested the antibodies on the wild type Ii probe (Fig. 5, lanes 1–5). The preimmune sera has no effect on any of the complexes. However, the antisera specific for the A subunit of NF-Y resulted in a supershifted band concomitant with a decrease in the intensity of the D and B bands. 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A partial list of genes containing NF-Y-binding sites

| Gene                  | CCAAT/flank sequence | Location | Refs. |
|-----------------------|----------------------|----------|-------|
| Human II Y box        | AGCCCAATGGGA         | −205     | 27, 28|
| Mouse II Y box        | AGCCCAATGGGA         | −198     | 29, 30|
| MHC DRA               | GGCCCAATCAGA         | −67 (rev.)| 47    |
| Mouse Eα              | AACCAATCAGA          | −52 (rev.)| 83    |
| Interleukin-4         | GGCCCAATCAGC         | −107 (rev.)| 84    |
| β-Actin               | GGCCCAATCAGC         | −91      | 85    |
| α-Globin              | AGCCCAATGAGC         | −70      | 86    |
| HSV-tk                | CCAGCAATGAGA         | −82 (rev.)| 37    |
| α1(II)collagen        | AGCCCAATCAGA         | −96 (rev.)| 36    |
| α2(I)collagen         | CAGCCCAATCAGA        | −80 (rev.)| 36    |
| Roas sarcoma virus-long term repeat | CACCCCAATCGGC | −129 (rev.) | 87    |
| Lipoprotein lipase    | AGCCCAATAGGT         | −65      | 88    |
| Albumin               | AACCAATGAGA          | −88      | 43    |
| Tyrosine amino trans. | AACCAATGAGA          | −75      | 89    |
| Thrombospondin 1      | GGCCCAATGGGC         | −91      | 90    |
| MDR-1                 | AGCCCAATCAGC         | −75 (rev.)| 73    |
| Consensus             | AGCCCAATCAGC         | −62 to −129|
|                       | GA                   | GG A C   |
| Human II Y-proximal   | GAGCAGAATCAGA        | −51      | this report |

**Fig. 5.** EMSA-antibody supershift analysis identifies NF-Y interacting at the Y-proximal site and Sp1 at the GC box. The antibodies used are indicated above each lane. --, no antibody; PI, preimmune antiserum. The probes used are marked above each gel. Other markings are as described in Fig. 4.
Cooperativity between Sp1 and NF-Y at Ii Promoter

Control of Ii gene transcription plays a critical role in the presentation of antigens by MHC class II molecules and the maintenance of a normal immune response. This report now defines a new region of the Ii promoter required for expression of this gene. Examination of the in vivo occupancy of the Ii promoter in constitutively expressing cell lines identified a bound GC box and imperfect CCAAT box (Y-proximal) at positions −74 and −53, respectively, relative to the transcription initiation site. Only 11 base pairs or one helical turn of the DNA separate these two recognition sites. In addition, binding at the TATA box at position −24 was detected when an alternative in vivo footprinting protocol was utilized to reveal contacts at adenine residues (data not shown). Combined with our previous studies (34, 49), these observations now complete the in vivo footprinting analysis of the entire 300-base pair Ii promoter. There are eight elements clearly bound in vivo: TATA box (−24 bp), Y-proximal (−53 bp), GC box (−74 bp), ISRE (−96 bp), X1 (−118 bp), X2 (−2 Kb−2 bp), Y (−207 bp), and X (−236 bp). Additionally, the S element (−260 bp) has been defined functionally but does not display interaction contacts in vivo. This is consistent with the related S element in the class II DRA promoter which also did not show in vivo contacts (54, 62).

The GC box and Y-proximal sites were occupied in both B and T cell lines which constitutively express Ii. However, neither site was bound in the Ii-negative Jurkat T cell line. The lack of in vivo binding occurs despite the presence of the transacting factors Sp1 and NF-Y in the nuclei of these cells, as detected by in vitro binding assays (34, 54).2 Furthermore, none of the elements within the 300-bp Ii promoter are occupied in vivo in the Ii-negative cell line. However, when the Ii promoter is transiently introduced into these cells, it displays a significant level of transcriptional activity. This finding suggests a higher order inhibition of DNA binding at the endogenous Ii promoter, potentially at the level of chromatin reorganization. This is consistent with the nucleosome hypersensitivity studies done on many genes, which demonstrated that inactive genes are often found in condensed, nucleosome-resistant regions of the chromatin (63–65). Interestingly, the functionally related class II DRA gene promoter is also unoccupied and not expressed in the Jurkat cell line. Although the class II genes

Fig. 6. The DNA binding half-lives of NF-Y and Sp1 are stabilized when both factors are complexed with the DNA together. A, off-rate analysis of NF-Y independently bound to the Ii Y-proximal site, t1/2 = 1.7 min (squares), or bound as a complex with Sp1 at the Ii proximal promoter, t1/2 = 7.5 min (circles). B, comparison of NF-Y binding stability at the consensus DRAY box, t1/2 = 6.0 min (squares), and the Ii NF-Y-Sp1 complex, t1/2 = 7.5 min (circles). C, off-rate analysis of Sp1 bound either independently to the Ii GC box, t1/2 = 3.2 min (squares) or in a complex with NF-Y, t1/2 = 8.9 min (circles). Where the horizontal line at 50 intersects the plot indicates the calculated half-life for that plot. Values were determined from PhosphorImage analysis of EMSA competition gels as described under “Experimental Procedures” and are plotted as the percent of complex shifted over the initial complex formed before competition versus the time of competition. Band D was quantitated for the half-life of Sp1 and NF-Y after competition with either the GC box or a consensus Y-box, respectively (see lane 1, Fig. 5 for example gel prior to competition). Band B of the Ii-Y-proximal probe was quantitated for the half-life of NF-Y alone (see lane 11, Fig. 5 for example of gel prior to competition). Band C of the Ii GC probe was quantitated for the half-life of Sp1 alone (see lane 16, Fig. 5 for example of gel prior to competition). Band “NF-Y” of the DRAY box probe was quantitated for the half-life of NF-Y on the consensus Y box (see lane 6, Fig. 5 for example gel prior to competition). Each point is the average of at least three independent experiments and the S.E. in indicated by the error bars.

analysis, EMSA-binding reactions with DNA probes containing either the Ii GC box or Y-proximal site or both together were allowed to reach equilibrium. Specific competitors in excess of 1000-fold were then added. Aliquots of the binding reaction were subsequently loaded onto a continuously electrophoresing gel at 0, 1, 2, 4, 8, 15, and 25 min after competitor addition. The intensities of the specific bands were quantitated by PhosphorImage analysis and plotted as the intensity of the band after competition for a given time over the intensity of the band prior to competition (see “Experimental Procedures” for details). The time at which only 50% of the complex is remaining is calculated as the half-life and thus is a measure of the stability of the interaction after the complex is formed. As shown in Fig. 6A, NF-Y binding to the Y-proximal site of Ii is very unstable with a half-life of only 1.7 min. However, when NF-Y binds to the Ii promoter in conjunction with Sp1 binding the half-life of NF-Y increases 4.5-fold. To examine whether the increased stability would be significant, a comparison was made to the consensus NF-Y site from the DRA promoter (Fig. 6B). Similar half-lives were detected for both the functional DRA-NF-Y interaction and the Ii-NF-Y-Sp1 complex. Examination of Sp1 binding to the Ii GC box also revealed a stabilization of binding when in an Sp1-NF-Y complex (Fig. 6C). The half-life of Sp1 binding alone was 3.2 min. When present together with NF-Y, the half-life of Sp1 increased to approximately 3-fold. These findings indicate that both factors are stabilized when binding at adjacent sites and suggests a mechanism by which the imperfect Y-proximal element can function as an NF-Y-binding site. Thus, DNA binding cooperativity is one of the underlying sources for the functional synergy observed between Sp1 and NF-Y.

DISCUSSION

2 K. L. Wright, unpublished observations.
Cooperativity between Sp1 and NF-Y at the Ii Promoter

Cooperativity between Sp1 and NF-Y at the Ii Promoter

Interferon-γ is a potent inducer of Ii and class II transcription (23, 66–68). In the IFN-γ-inducible cell line U105-MG, the Ii proximal promoter elements GC and Y-proximal sites are unoccupied prior to the induction. IFN-γ treatment up-regulates binding at both proximal elements by 4 h, and binding continues at 18 and 48 h after addition IFN-γ. Loading of the proximal elements appears to occur prior to loading at the distal elements. The distal X and Y boxes display only the very weak beginnings of interaction at 4 h but by 24 h show maximal binding (49). Loading at the negative Ii element kB-I appears to be delayed even further, until 48 h. This finding suggests that interactions at the Y-proximal site and/or the GC box occur first and may be required to open the promoter for additional factor binding. Thus, the Ii promoter minimizes basal level expression by preventing binding of the transacting factors in the uninduced state. This is in direct contrast to the class II DRA gene in which all elements are bound prior to IFN-γ induction in this cell line (54). Treatment with IFN-γ then induces an up-regulation of the weak binding activity at the X box of the DRA S/X/Y domain to a strong interaction. The Ii promoter also utilizes an S/X/Y domain for the IFN-γ response (27, 29) and to a lesser degree an ISRE element (60). Thus, the induction pathway for Ii and class II are likely to be highly related. However, the mechanism by which the Ii promoter remains unoccupied until IFN-γ treatment is unclear. In vivo analysis of several other promoters has revealed situations analogous to both the all or none effect observed for Ii and the limited effect detected for class II DRA (53, 69–71). It is somewhat surprising to find two coordinately regulated genes involving different mechanisms. Interestingly, an in vivo study of the class II DRA promoter demonstrated a central requirement for NF-Y in the loading of the promoter (47). Stable introduction of a DRA promoter construct with a mutated Y/CCAAT box blocked in vivo binding at all promoter elements in the IFN-γ-induced U105-MG cells. In contrast mutation of the other promoter elements S, X1, and X2 only has a local effect on binding in vivo. The findings from this study implicated the transcription factor NF-Y binding to the Y/CCAAT box as critical for recruitment of the other promoter factors and/or opening the chromatin structure across the promoter region. This has potentially important implications for the Ii promoter since two NF-Y-binding CCAAT boxes are present in this promoter, the distal Y box of the S/X/Y domain and the Y-proximal element. It will be interesting to determine if one or both of these elements modulate accessibility to the Ii promoter.

Synergistic activation by two distinct transcription factors is becoming a common phenomenon and several different mechanisms have been reported (see Ref. 72 and references within). This report defines a synergistic activation of Ii transcription by Sp1 bound at the GC box and NF-Y bound at the Y-proximal site. Neither site was capable of activating Ii transcription in the absence of the other site. However, together they formed a strong transcriptional activator. At least one basis for their synergy is that NF-Y binds to the Y-proximal element with a very short half-life. Sp1 also binds to the Ii GC box with a short half-life. Stabilization of both NF-Y and Sp1 binding occurs when Sp1 and NR-Y bind the Ii promoter together. This suggests that in vivo Sp1 and NF-Y may only bind cooperatively to the Ii promoter and thus are completely dependent on each other to activate transcription. In vivo footprint analysis of mutant Ii promoters indicates both sites must be present for binding to occur at either site. Interestingly, the human multidrug resistance gene MDR has a similar arrangement of GC box and CCAAT box in its proximal promoter (73). These elements were shown to functionally synergize although the mechanism has not been investigated. The MDR CCAAT box matches the consensus NF-Y sequence (Table I), however, stabilization of DNA binding may also play a role in MDR activation.

A number of studies have described cooperative interactions of Sp1 with other factors, but not NF-Y. Examples include OTF-1 (74), C/EBP (75), NF-xB (76), Ets (77), E2, YY1 (78, 79), and Sp1 (80, 81). In at least one case a direct physical interaction between Sp1 and the E2 factor was clearly demonstrated even in the absence of an E2 DNA-binding site (82). In addition, Sp1 can multimerize and thereby synergistically activate transcription (80). However, in most cases the mechanisms are less well defined. NF-Y has been described in two distinct cooperative interactions. The MHC class II DRA promoter has an NF-Y-binding site which must be stereospecifically aligned with the X box to function (48). Reith et al. (46) recently demonstrated that stable binding of NF-Y and the X box factor RFX required a cooperative interaction between the two proteins. NF-Y also binds in the serum albumin promoter adjacent to a C/EBP site. The binding of both proteins is required to promote stable preinitiation complex formation (43). Interestingly, in this case NF-Y DNA binding is weaker in the complex with C/EBP than when alone. Clearly these studies reinforce the hypothesis that synergy can occur through many different mechanisms. In particular, NF-Y appears to have multiple activating functions which are dependent on the local promoter environment.

In conclusion, maximal transcription from the Ii promoter requires a GC box and NF-Y site proximal to the TATA box. These sites function in a synergistic manner, and it is shown that cooperativity in DNA binding is at least one mechanism underlying the synergy. An additional outcome of this study is the identification of a functional, imperfect NF-Y-binding site that would be undetected in homology searches for the consensus core sequence CCAAT or CAAT. This raises the potential for an even greater usage of NF-Y in promoters that do not have a classical CCAAT box. Finally, the Ii gene exhibits all or none in vivo binding across the promoter in response to IFN-γ induction. With the previous observations that NF-Y can control access to the DRA promoter in vivo (47), it will be important to determine if NF-Y plays a similar role for Ii. This could have broad implications for the regulation of multiple genes with an NF-Y/CCAAT box.

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