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Interferon γ regulates binding of two nuclear protein complexes in a macrophage cell line

(class II major histocompatibility complex genes/DNA-binding protein/transcription/lymphokine/Ia antigens)

PATRICIA W. FINN*, CATHERINE J. KARA*†, JOHN DOUHAN III*, TU TRAN VAN*, VIRGINIA FOLSOM‡, AND LAURIE H. GLIMCHER*§

*Department of Cancer Biology, Harvard School of Public Health, Boston, MA 02115; §Department of Medicine, and †Program in Immunology, Harvard Medical School, Boston, MA 02115; and ‡Life Technologies Inc., Gaithersburg, MD 20877

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ABSTRACT **Interferon γ (IFN-γ) is a potent inducer of major histocompatibility complex (MHC) antigens during normal immune responses and in abnormal responses in autoimmune disease. In this report we identify two nuclear factors whose binding to the murine E_β class II MHC β-chain gene is regulated by this cytokine. IFN-γ stimulation of murine macrophages results in the appearance of increased binding of one protein complex, complex A, and decreased binding of a second, faster migrating protein complex, complex B. Although the contact residues for both of these proteins lie within the highly conserved Y-box transcriptional element, their binding specificity differs. The protein in complex B is a CCAAT-box-binding protein that may be similar or identical to NF-Y or YB1, previously identified class II Y-box-binding proteins. The DNA sequence requirements for the binding of the slower migrating complex, complex A, are not limited to CCAAT-box sequences but include sequences upstream of the Y box. These upstream sequences are required both for IFN-γ-induced gene transcription and for IFN-γ-induced modulation of binding activity. These data suggest a model in which upstream sequences contribute to formation of a lymphokine-regulated complex downstream. The IFN-γ-induced binding protein described as complex A in this report differs from the IFN-γ, -α, or -β-induced nuclear factors previously identified.**

The level of class II major histocompatibility complex (MHC) antigens on macrophages can be increased by several stimuli, the most potent of which is the cytokine interferon γ (IFN-γ). This increase in class II results in an increase in the intensity of the T-cell immune response (1). Furthermore, cultured cells from virtually every organ as well as a variety of tumor cell lines can be induced to express class II in response to IFN-γ (2). In some instances these cells then acquire the ability to act as antigen-presenting cells to T lymphocytes (3). There is evidence that abundant *de novo* expression of class II in target organs amplifies autoimmune tissue destruction (4). This induction may well be due to IFN-γ, since astrocytes in experimental allergic encephalomyelitis and synovial cells in rheumatoid arthritis express class II, and IFN-γ has been shown to induce class II on these cells *in vitro* (5, 6). Understanding the mechanism by which IFN-γ induces class II gene expression in its target cells is therefore of importance.

MATERIALS AND METHODS

Cell Line. The P388D1 murine macrophage-like cell line was grown in RPMI 1640 medium (GIBCO) supplemented with 8% fetal bovine serum, 10 mM Hepes, 100 μg of penicillin per ml, 100 μg of streptomycin per ml, 0.1 mM 2-mercaptoethanol, and 2 mM L-glutamine.

DNA Transfection. P388D1 cells were transfected by a modification of the DEAE-dextran method (7). Chloramphenicol acetyltransferase (CAT) assays were performed as described (8) and quantitated by scintillation counting. CAT activity was assayed at 72 hr after transfection, in the presence or absence of recombinant IFN-γ (rIFN-γ; gift of Genentech and the American Cancer Society), 20 units/ml, which was added for the last 48 hr.

Plasmid Constructions and Oligonucleotides. The E_β–CAT constructs were generated by making 5′ serial deletions from the HX_2 fragment of the murine E_β^d class II β-chain gene (9), which contains 2.7 kilobases (kb) of 5′ flanking region with the 3′ boundary at −66 relative to the transcription start site (Fig. 1*A*). 5′192 refers to the 5′ border and was constructed by BAL-31 exonuclease deletion followed by cloning into the *Bgl* II site of $pA10cat_2$ (8). For the constructs 5′159, 5′110, 5′130, and 5′162–3′110, $pA10cat_2$ was digested with *Bgl* II, made blunt at the ends, and then digested with *Sal* I. Synthetic oligonucleotides were synthesized with one blunt end and one *Sal* I end for directional cloning into the vector. 5′110 and 5′162–3′110 have a *Xho* I site inserted before the 3′ blunt end. 5′160–3′130 is a construct in which a synthetic oligonucleotide spanning the conserved W box, which has the 5′ border at position −160 and the 3′ border at position −130, was cloned into the *Bgl* II site of $pA10cat_2$. *Hae* III restriction sites were used to generate the H–H fragment (Fig. 1*A*), which was subcloned into the *Sma* I site of pUC18, resulting in plasmid pH–H. Oligonucleotides containing conserved W, X, and Y motifs (Fig. 1*A*) were synthesized on a Biosearch synthesizer by standard phosphoramidite techniques (14), as were all subsequent oligonucleotides. Gel-purified, annealed oligonucleotides were either used as unlabeled competitors or 5′-end-labeled with [γ-^{32}P]ATP and polynucleotide kinase and purified by electrophoresis in a 12% polyacrylamide gel. DZ_α is an oligonucleotide spanning the X motif of the human HLA-DZ α-chain gene (15), with the sequence CCCAGCAA-CAGATACATTCACTCAGAG. $X(A\alpha)_{45}$ is an oligonucleotide that corresponds to the X motif of the murine A_α gene (10). The E_β-Y oligonucleotides (15) and the other oligonucleotides described below are listed in Table 1. E_α-Y contains the Y box of the E_α gene (16) and TK contains the CCAAT box found upstream of the thymidine kinase gene (17). H-2K^b-IBP-1 (IBP) spans the IFN-responsive sequence (IRS) of the H-2K^b gene (18), and M-IFN spans the IRS of the metallothionein gene (19). BRE-2 spans an interleukin 4-responsive region of the A_α^k gene (20).

Gel Retardation Assays and Footprint Analysis. Nuclear extracts were prepared by a modification (20) of the method of Dignam *et al.* (21) from P388D1 cells that were incubated with or without rIFN-γ (20 units/ml) for 17 hr. Mobility-shift

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Abbreviations: CAT, chloramphenicol acetyltransferase; IFN, interferon; rIFN, recombinant IFN; IRS, IFN-responsive sequence; MHC, major histocompatibility complex.

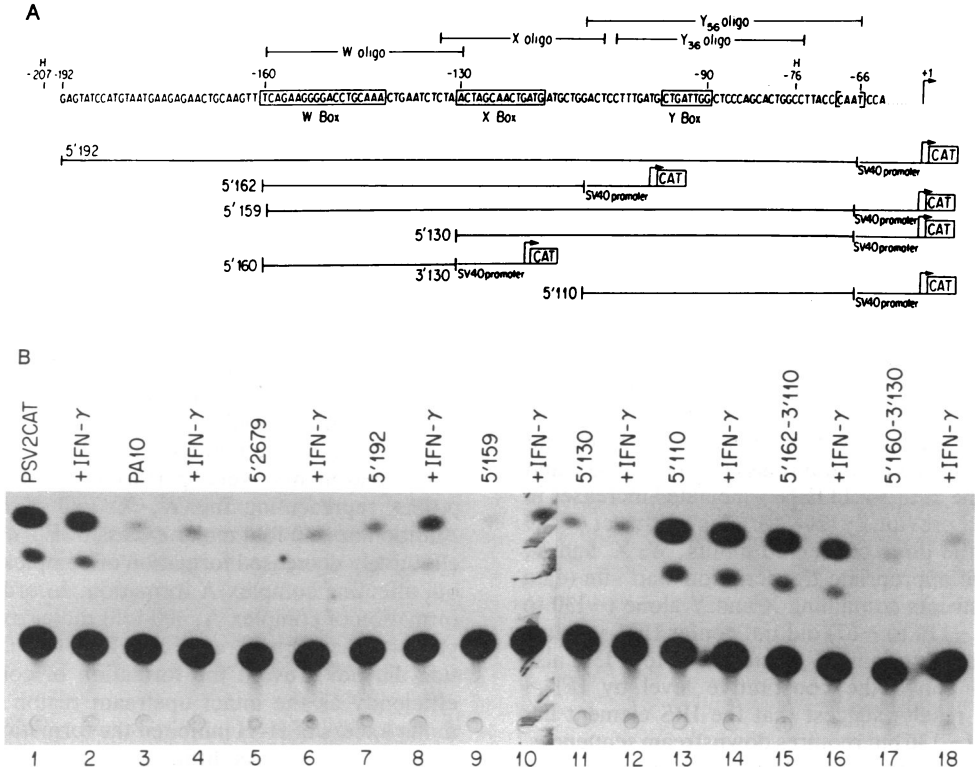

FIG. 1. (*A*) E_β^d–CAT constructs and oligonucleotides. Part of the 5′ flanking sequence of the E_β^d gene (9) is shown, including conserved W, X, and Y motifs (refs. 10–13; boxed). *Hae* III sites (H) are indicated. Bars above the sequence represent synthetic oligonucleotides used in gel retardation analyses. Constructs used in transfection experiments are shown below the sequence and contained the indicated portions of the sequence inserted upstream of the simian virus 40 (SV40) promoter and CAT gene of $pA10cat_2$. (*B*) IFN-γ induction of E_β^d–CAT constructs in the P388D1 macrophage cell line. E_β^d–CAT constructs were assayed for CAT activity in response to rIFN-γ in transient transfection assays. P388D1 cells transfected with pSV2cat (contains SV40 promoter and enhancer; ref. 8) (lanes 1 and 2), $pA10cat_2$ (contains SV40 promoter only) (lanes 3 and 4), and constructs as in *A* (lanes 5–12) were assayed for CAT activity expressed in the absence (odd-numbered lanes) or presence (even-numbered lanes) of rIFN-γ. Each transfection and CAT assay was performed as five independent experiments and a representative result is shown.

assays were performed as described (20). The H–H fragment probe (Fig. 1*A*) was prepared by *Bam*HI cleavage of pH–H (described above) and end-labeling with either [α-^{32}P]dNTP and Klenow fragment of DNA polymerase I or [γ-^{32}P]ATP and polynucleotide kinase, followed by digestion with *Eco*RI and isolation by gel electrophoresis. Approximately 10,000 cpm (≈1 ng) of end-labeled DNA was used for each 10-μl reaction mixture containing 2.4–4.0 μg of protein from nuclear extract. Nuclear extracts were normalized for protein (Bio-Rad protein assay) and equal amounts were added within experiments. As an additional control for protein concentration, extracts were incubated with a 220-base-pair (bp) fragment of the A_α upstream region. This resulted in three retarded bands, none of which was affected by IFN-γ treatment (data not shown). DNA–protein complexes were resolved in a 4% polyacrylamide gel in 45 mM Tris/45 mM boric acid/1 mM EDTA (0.5× TBE) run at 11 V/cm with cooling. The gels were dried and autoradiographed with intensifying screens at −70°C. For competition analysis, nuclear extracts were preincubated with water or competitor DNA for 5 min at room temperature; all other components were then added and the samples were processed as for mobility shift analysis. For footprint analysis, ten identical 10-μl reaction mixtures were pooled into one large lane of a 4% acrylamide gel. After electrophoresis, the gel was subjected to copper cleavage as described (22), except that incubation was for 11.5 min. Bands of interest were excised, eluted, purified, and run in a 6% acrylamide/7 M urea gel. Sequences were aligned by comparison to the G, G+A Maxam–Gilbert ladder (23) of the end-labeled DNA.

RESULTS AND DISCUSSION

Macrophages are sensitive targets for the actions of IFN-γ. A 2- to 5-fold IFN-γ-mediated increase in class II gene transcription has been reported (24–28), and we have found that the cell surface expression of class II MHC antigens in P388D1, a murine macrophage cell line, is induced after 24 hr of treatment with as little as 1 unit of rIFN-γ per ml (data not

Table 1. Sequences of oligonucleotides

Name	Sequence	Ref.
E_β-Y_{36}	CTACCTTTGATGCTGATTGGCTCCCAGCACTGGCCT	15
E_β-Y_{56}	TGGAGACTCCTTTGATGCTGATTGGCTCCAGCACTGGCCTTTACCCAATCTCGAG	15
E_α-Y	GTCTGAAACATTTTTCTGATTGGTTAAAAGTTGAGTGCT	16
TK	GCGTCTTGTCATTGGCGAATTCGAACACGC	17
H-2K^b-IBP-1	CAGGTTAGGTGCAGAAGTGAAACTGTGGAGATGGGGAATCC	18
M-IFN	TTCTCCACCTCGGCAGTTTCTCCTCTCT	19
BRE-2 (A_α)	GATCCCGTGATTACCTTAATATGTTTGCCTAGAAGGAGGCAAA	20

Solid underlining, Y-box or CCAAT-box motif; broken underlining, IFN-responsive sequence (IRS).

shown). To identify the upstream sequences responsible for the transcriptional effects of IFN-γ, P388D1 cells were transfected with plasmid constructs containing 5′ upstream sequences of the mouse E_β gene fused to the CAT gene. Starting with 2.6 kb of upstream sequence, serial 5′ deletions were made and tested for IFN-γ inducibility. Preliminary results identified a 127-bp region required for IFN-γ inducibility (Fig. 1). This region extends from −192 to −66 relative to the start site of transcription and includes three conserved motifs, W, X, and Y. The X and Y motifs are two conserved elements located in the same positions in all class II genes sequenced to date (10–12). W is a conserved motif located just upstream of the X motif in all class II β-chain genes (13). A construct containing residues −159 to −67 also was IFN-γ inducible (Fig. 1*B*). The level of induction averaged 3-fold, which is consistent with data obtained in several other laboratories that define IRS elements upstream of the human DQ_β and DR_α and murine E_α and E_β class II genes (29–32) and is comparable to the 2- to 5-fold IFN-γ-mediated increases in transcription noted previously (24–28). This construct (−159 to −67) contains the three conserved motifs, W, X, and Y, and initiated at the appropriate transcription start site (data not shown). Constructs containing X and Y alone (−130 to −67) or Y alone (−110 to −67) did not confer IFN-γ inducibility. A construct containing W alone (−160 to −130) also was not induced above the constitutive level by IFN-γ treatment. These results suggest that the IRS element lies between −159 and −130 but requires downstream sequences, including X and Y motifs, for full IFN-γ responsiveness. In addition, constructs containing W and X (Fig. 1*B*, lane 15) or Y alone (lane 13) had greater constitutive activity than those containing X and Y alone (lane 11), suggesting the presence of a negative regulatory element between −130 and −110 that also requires downstream elements. Both negative and positive regulatory elements have been demonstrated for the DQ_β and E_α genes (29, 32). Constructs containing X alone (−130 to −110) may further define the boundaries of these elements in the E_β gene.

The activation of transcription by specific DNA sequences is usually accomplished by transcription factors that bind to these sequences. An IFN (αβ)-induced nuclear factor that binds upstream of the class I MHC gene family has been identified by several groups (33); however, there is no obvious homology between the class I MHC IFN consensus sequence and proximal upstream class II sequences (unpublished results) with the exception of a sequence located at −564 upstream of the DR_α gene (19). When nuclear extracts from P388D1 cells treated for 17 hr with rIFN-γ (20 units/ml) were analyzed by mobility-shift analysis with a radiolabeled 130-bp *Hae* III (H–H) E_β DNA fragment containing all three conserved motifs, two distinct retarded bands were observed (Fig. 2*A*). These two DNA–protein complexes are termed A and B. Densitometry showed that complex A (upper band) was increased 6-fold by treatment of P388D1 cells with rIFN-γ for 17 hr (lane 3), whereas complex B was diminished 2-fold. Proteinase K treatment abolished complex formation (data not shown). In 10 separate experiments with different P388D1 nuclear extracts, the increase in complex A ranged from 2.8-fold to 17-fold and the decrease in complex B ranged from 1.3-fold to 2-fold.

To determine the binding site of these proteins, competition experiments were performed with unlabeled DNA probes representing the W, X, and Y motifs (Fig. 2*B*). Addition of a 40-fold molar excess of E_β-Y_{36} oligonucleotide effectively decreased formation of complex B (lane 15) without affecting complex A formation. In order to prevent the formation of complex A, 400-fold molar excess Y-box competitor (lane 17) was required. However, the Y oligonucleotide did not prevent the formation of complex A or B as efficiently as the intact upstream region (H–H); a 50-fold molar excess of H–H inhibited the formation of complexes A and B (lane 4). As little as 10-fold molar excess H–H effectively decreased complex A formation (data not shown). Furthermore, when W or X oligonucleotide alone was used as competitor, no inhibition was seen of either complex A or B (lanes 6–13). When W and X were used together, or added with Y, there was no difference as compared to the use of Y alone (not shown). Interestingly, an oligonucleotide from the upstream region of the A_α gene, BRE-2 (Table 1; this sequence has been shown to be responsive to interleukin 4), behaved in a different way, inhibiting formation of complex A but not of complex B (Fig. 2*C*, lanes 5 and 6). This effect was specific, as X(Aα)45, an oligonucleotide spanning the X motif of the A_α gene (13), prevented the formation of neither complex (lane 7). Thus, the proteins in complexes A and B appear to have different sequence requirements for binding, since BRE-2 competed only with complex A.

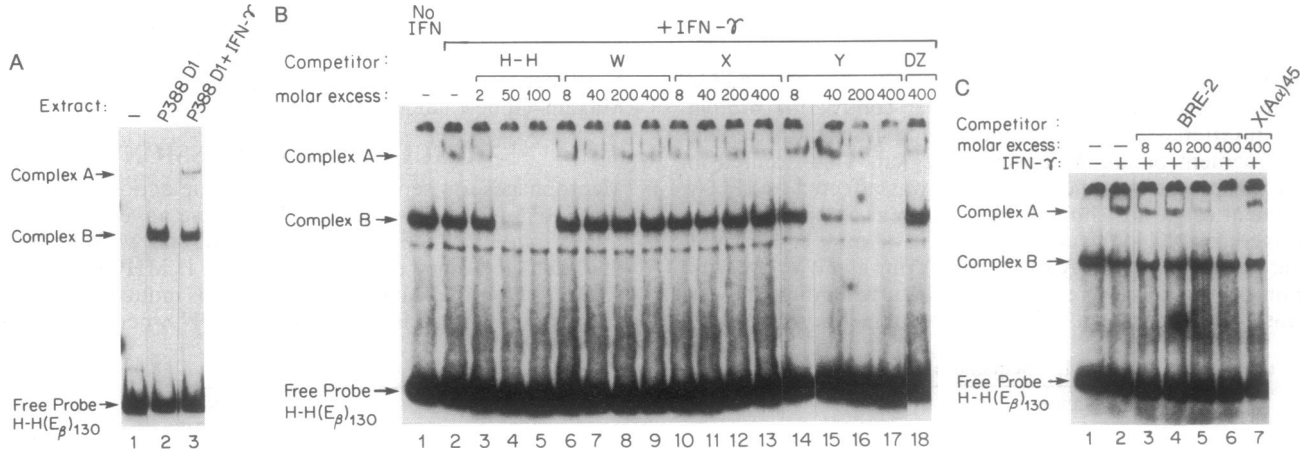

FIG. 2. (*A*) Regulation of binding of nuclear protein to the class II E_β gene by IFN-γ. Mobility-shift assays were conducted with nuclear extracts from P388D1 cells untreated (lane 2) or treated with rIFN-γ at 20 units/ml (lane 3). Lane 1 shows free H–H probe (no extract added). (*B*) Unlabeled Y box preferentially inhibits formation of labeled complex B. Competitors were H–H, the unlabeled 130-bp homologous *Hae* III–*Hae* III fragment (lanes 3–5); W (lanes 6–9), X (lanes 10–13), and a 36-bp Y oligonucleotide (lanes 16 and 17), which are unlabeled oligonucleotides from the E_β upstream region corresponding to conserved W, X, and Y motifs (Fig. 1*A*); and DZ_α (lane 18). The amount of competitor indicated above each lane represents nanograms; 10 ng equals approximately 40-fold molar excess of probe concentration for W, X, and Y. (*C*) BRE-2 inhibits formation of only complex A. Competitors were BRE-2 (lanes 3–6) and X(Aα)45 (lane 7).

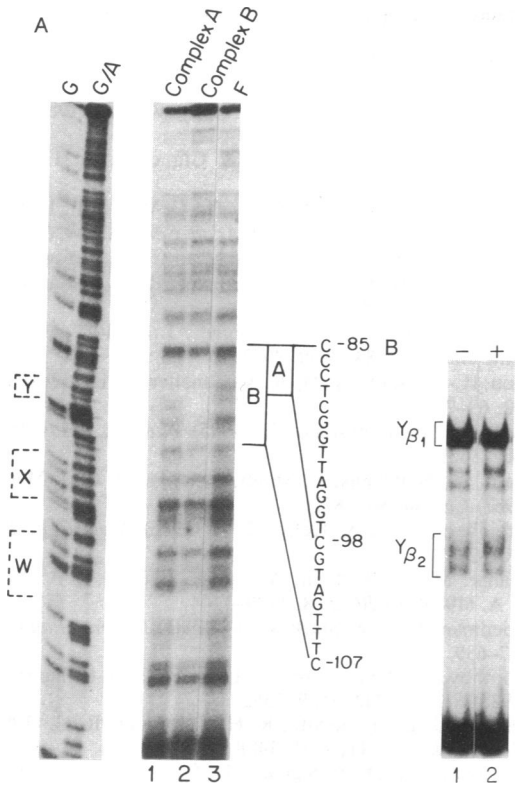

FIG. 3. (*A*) Footprint analysis of binding of nuclear extracts of cells treated with IFN-γ to probe H–H (noncoding strand). Lane 1, complex A; lane 2, complex B; lane 3, free probe. G and G/A represent Maxam–Gilbert ladders. (*B*) DNA-binding activity of P388D1 nuclear proteins to Y boxes as an isolated target sequence. The probe was a radiolabeled oligonucleotide E_β-Y_{36} (Table 1). Lane 1, unstimulated sample; lane 2, IFN-γ treated sample. The mobility-shift assays were performed as in Fig. 2.

To further define the target sequence, footprinting analysis of the noncoding strand was performed on each of the two complexes by using the nuclease activity of 1,10-phenanthroline–copper ion (22). In complex B, which is downregulated by rIFN-γ, the Y motif (positions −90 to −97) and part of the sequence between X and Y (bases −85 to −107) were protected (Fig. 3*A*). In complex A, which is upregulated by rIFN-γ, a more restricted region was protected that lay completely within the B footprint and included only the Y motif (bases −85 to −98). The 3′ border of both A and B protection extended beyond the Y motif to position −85. Identical results were obtained for the coding strand (data not shown). These results suggest the existence of two distinct DNA–protein complexes that are regulated by rIFN-γ. Complex B has a footprint pattern very similar to NF-Y, an E_α Y-box-binding protein described by Dorn *et al.* (11). Of interest in this regard is the isolation by Didier *et al.* (34) of a cDNA, YB1, encoding a DR_α Y-box-binding protein whose expression is negatively regulated by IFN-γ at the mRNA level.

The localization of the footprint of complex A to the Y motif was consistent with our earlier results (Fig. 1*B*) suggesting that downstream elements are necessary for full rIFN-γ responsiveness. The results of others (32) had also demonstrated that the presence of all three motifs, W, X, and Y, was required for rIFN-γ-induced gene transcription. One possibility is that complex A proteins must initially interact with sequences upstream of Y before forming a final lymphokine-responsive complex at the Y-box site. It was of interest therefore to determine whether binding to the Y motif alone was increased by IFN-γ treatment. When the Y-box oligonucleotide was used as probe, two complexes, $Y_{\beta 1}$ (upper band) and $Y_{\beta 2}$ (lower band), were detected (Fig. 3*B*, lane 1) and a third complex, which migrated between these two, was occasionally observed. The Y-box complexes were also present in IFN-γ-stimulated samples but were not increased as evidenced by densitometry (lane 2). Formation of the complexes binding to the Y box was sequence-specific, as demonstrated by competition experiments using homologous and irrelevant DNA probes. A 10-fold molar excess of homologous Y box effectively prevented formation of both $Y_{\beta 1}$ and $Y_{\beta 2}$ (data not shown). Complexes $Y_{\beta 1}$ and $Y_{\beta 2}$ did not behave like complex A, since their formation was not modulated by rIFN-γ and was prevented by a much lower amount of Y-box oligonucleotide than required to inhibit complex A formation (Fig. 2*B*, lanes 16 and 17). This suggests a model in which upstream sequences contribute to formation of a lymphokine-regulated complex downstream.

Recently, an IFN-γ-induced factor, IBP-1, that binds the IRS of the $H\text{-}2K^b$ class I gene was described (18). This sequence controls the stimulation of MHC class I expression by IFN-α, -β, and -γ (18). Similar sequences (Table 1) are found upstream of many IFN-responsive genes [the metallothionein, IFN-β, HSP70 major heat shock protein, immunoglobulin κ chain, 6-16, IP-10, and the human 2′-5′ oligo(A) synthetase E gene are a few examples] (19, 35–40). One of these sequences binds IBP-1 (18). Since there is no homology between the sequence protected by the proteins in complex A or B and the IRS, it was unlikely that the complex A or B proteins were IBP-1. To test this directly, however, competition experiments using the class I IRS and the IRS upstream of the metallothionein gene were performed. Neither of these oligonucleotides at a 25-, 50-, or 100-fold molar excess significantly prevented the formation of complex A or complex B (Fig. 4, lanes 14–16 and 17–19). Thus the IFN-

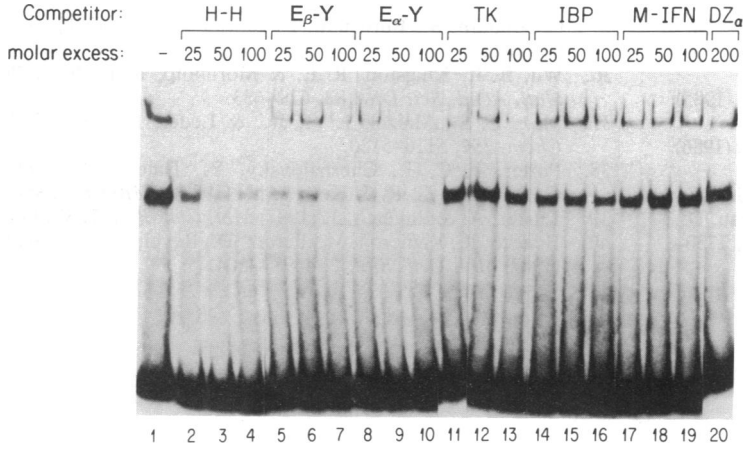

FIG. 4. Relationship of complexes A and B to other IFN-induced binding proteins and to CCAAT-box-binding proteins. Binding of nuclear proteins in IFN-γ-stimulated P388D1 extracts to radiolabeled H–H fragment was assayed in the presence of no competitor (lane 1) or in the presence of unlabeled H–H (lanes 2–4), E_β-Y_{56} (lanes 5–7), E_α-Y (lanes 8–10), TK (lanes 11–13), IBP (lanes 14–16), M-IFN (lanes 17–19), or DZ_α (lane 20) oligonucleotides. The competitors are described in Table 1, except for DZ_α (15).

γ-induced class II-binding proteins appear to represent a species distinct from IBP-1.

The Y-box sequence of all class II genes is highly conserved and contains an inverted CCAAT box. The Y-box motifs of the human DR_α and the murine E_α genes are identical to the E_β Y box but differ substantially in the flanking sequences (Table 1). These DR_α and E_α Y-box motifs have been shown to bind proteins YB1 (34) and NF-Y (11, 16), respectively, which are CCAAT-box-binding proteins distinct from the CCAAT-box-binding proteins previously described (e.g., CBP and CTF/NF-1). To determine the relationship between complex A and B proteins and the YB1 and NF-Y CCAAT-box-binding proteins, competition experiments were performed. Formation of complex B was prevented by a 25-fold excess of oligonucleotide containing the E_α Y box (lanes 8–10) or the E_β Y box (lanes 5–7) but not as well by an oligonucleotide containing the CCAAT motif upstream of the thymidine kinase gene (lanes 11–13). The complex B protein therefore behaves like YB1 and NF-Y and may be similar or identical to one or both of these proteins. Final resolution of this issue awaits the isolation of cDNA clones encoding the complex B protein. Complex A formation can be most efficiently prevented by the DNA fragment containing W, X, and Y motifs (compare Fig. 4, lanes 2–4, and Fig. 2*B*, lanes 3–5, to Fig. 4, lanes 5–7 and 8–10) and therefore does not appear to be a CCAAT-box-binding protein.

This report demonstrates the binding of IFN-γ-modulated proteins to class II gene transcriptional control regions. Overall, our results are most easily explained by invoking two different Y-box-binding proteins with distinct but overlapping target sequences within the Y box. Another possibility is that the different mobilities of the complexes and the distinct footprints result from posttranslational modification of a single binding protein or from formation of a dimeric protein. Indeed, complex A may be generated by the binding of complex B protein multimers or of B protein complexed to another protein. The observation that the W- and X-box regions are also required for IFN-γ-induced gene transcription and sequence-specific binding suggests a cooperative interaction among all three motifs.

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