Identification of a transcription factor that binds to the S box of the I-A β gene of the major histocompatibility complex

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Class II genes of the MHC show a striking homology upstream of the transcription start site that is composed of three conserved sequences (S, X and Y boxes, each separated by 15–20 bp). The presence of the S-box sequence in the mouse MHC class II gene I-A β was examined for its influence on the expression of this gene. Deletion or mutation of the S box decreased the induction of chloramphenicol acetyltransferase (CAT) activity in B lymphocytes by 32 %. In macrophages, deletion or mutation of the S box abolished interferon- γ (IFN- γ) inducibility of CAT activity. Using a gel-retardation assay, we have identified a nuclear factor

whose binding site overlaps the 7-mer conserved sequence of the S box. This factor is present in lymphocytes, macrophages, mastocytes and fibroblasts. Surprisingly, binding of this nuclear factor to DNA was induced by IFN- γ in bone-marrow-derived macrophages, but not in macrophage-like cell lines. The binding site for this factor was defined by DNase I footprinting and partially purified by using an affinity column containing double-stranded oligonucleotides containing a sequence of the S box. A prominent protein of 43 kDa was found that bound specifically to the S-box sequence.

INTRODUCTION

The MHC class II genes are expressed in a cell-type-specific manner [1] and their expression is regulated by the lymphokines interferon- γ (IFN- γ) [2,3] and interleukin-4 [4,5]. The study of the regulation of eukaryotic gene expression has focused on both the sequences required for gene expression and the cellular factors that interact specifically with these controlling elements [6,7]. During the past few years, a number of *cis*-acting regulatory elements have been identified, both upstream and within the introns of several MHC class II genes [8,9]. The definition and characterization of the cellular factors that interact with these

transcriptional regulatory elements is a necessary step to understand the regulation of transcription of these genes.

Upstream of all MHC class II genes there exist three relatively conserved sequences that play an essential role in the transcriptional regulation of these genes [8,9]. They have been referred to as the S, X and Y boxes, and they are separated from each other by 15–20 bp (Figure 1). The Y box contains an inverted CCAAT sequence, and recently a number of proteins that footprint to this CCAAT sequence have been described [9]. The binding of this CCAAT factor to DNA depends on two proteins [10]. The genes for these proteins have recently been cloned and are called NF-Ya and NF-Yb [11]. The X box consists of about

Figure 1 DNA sequence upstream of the I-A β gene

The conserved sequences S, X and Y are indicated by boxes. The sequence covered by the oligonucleotides S, X and Y is indicated. Numbering is from the major transcription start site. The ATG codon in the first exon is boxed.

Abbreviations used: CAT, chloramphenicol acetyltransferase; IFN- γ , interferon- γ ; BMM, bone-marrow-derived macrophages.

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13 bp of loosely conserved sequence, to which the binding of multiple proteins has been reported [9]. Some of the genes for these proteins have been recently cloned: RF-X, whose product binds to the DR α promoter [12]; hXBP-1, whose product interacts with the DR α and DP β promoters [13]; and mXBP, whose product binds to the A α promoter [14]. An X-box-binding protein is thought to be defective in patients with combined immunodeficiency, a disease in which there is no MHC class II expression [12,15].

The S box consists of 7 bp of conserved sequence located at -110 to -130 bp from the transcription start site of MHC class II genes [16,17]. Recent reports have indicated that the S box is important for the IFN- γ -regulated expression of the DRA class II MHC gene [16-19], as well as the human class-II-associated invariant-chain gene [20]. In the mouse A α gene the S box has been referred as H, and the deletion leads to a diminution of constitutive and IFN- γ -mediated expression [21]. In the E α gene the S box contributes only slightly to IFN- γ inducibility [22,23]. Finally, for the DP α gene the IFN- γ -response region has been mapped to a region that contains the X and Y boxes [24]. Trans-acting cellular factors presumed to interact with the S box have been identified by using electromobility shift assays and probes containing the DRA promoter [18,19] and the A α promoter [25]. Our purpose is to study the mechanism of I-A β gene regulation. In this regard, we characterized previously the proteins that bind to the Y and X boxes in the I-A β promoter [10,26,27]. It is the purpose of the present paper to characterize the transcription factor that binds to the S box of the I-A β promoter.

MATERIALS AND METHODS

Nuclear-extract preparation

Nuclear extracts were obtained as described previously [26]. In these studies, the cell lines used included: A20-2J (mouse B lymphocyte), BE 5147 and EL4 (mouse T lymphocytes), P815 (mouse mastocyte), L cells (clone 929, mouse fibroblast, WR19 M.1, IE, PU5-1.8, P388D1 and IC 21 (mouse macrophages at different degrees of differentiation), RAJI (human B lymphocyte) and U937 (human macrophage). All the cell lines were obtained from the A.T.C.C. collection. Mouse bone-marrow-derived macrophages (BMM) were produced *in vitro* by using L-cell-conditioned medium as described [28]. For interferon-stimulation experiments, 300 IRU of murine IFN- γ (*Escherichia coli*-derived recombinant IFN- γ ; Genentech Inc., South San Francisco, CA, U.S.A.)/ml was added to the medium for 24 h. Under these conditions, IFN- γ induces the expression of mRNA for I-A, and I-A surface expression [29]. In some experiments, macrophages were treated for 24 h with recombinant murine IFN- α or - β (1000 U/ml; Sigma, St. Louis, MO, U.S.A.).

Plasmid and DNA

The chloramphenicol acetyltransferase (CAT) reporter constructions were made by using the KS⁺-SV2 CAT [30] vector in which the SV40 enhancer was removed by digestion with *Sph*I and *Pst*I. This new vector, which retained the SV40 promoter, was called KS1. The constructions used in these studies, KS1-SXY (-146 to -26 bp), KS1-XY (-110 to -26 bp) and KS1 mutant (-146 to -26 bp with a mutation of the S box indicated in Figure 3) contained DNA sequence from the mouse class II gene promoter I-A β [26]. The constructions were generated by using specific oligonucleotides and the PCR to generate the appropriate fragment from the I-A β promoter [26] for insertion into the vector. The DNA fragments were ligated directionally into *Xba*I + *Sma*I-digested KS1 vector.

Plasmid 12.33.2, containing 320 bp of 5' flanking region of the I-A β gene, was described previously [26]. Plasmids H1 and H2 were obtained by digestion of the 12.33.2 plasmid with *Hind*III, treating the DNA with restriction enzyme Bal 31, adding *Hind*III linkers and re-ligating [26]. Plasmid 362.8 contains 116 bp of a fragment located about 1600 bp upstream of the I-A β gene [26]. Plasmid PM2.2 contains the V_H region from MOPC 167 [31]. A 615-bp fragment containing the promoter and upstream sequences was isolated from this plasmid. The double-stranded oligonucleotides were synthesized on a model 380 A Applied Biosystems DNA synthesizer (Foster City, CA, U.S.A.).

Transfection assays

WR19 M.1 cells were transfected by the DEAE-dextran method, as previously described [32]. A 15 μ g sample of DNA from the CAT construct and 3 μ g of the plasmid pCH110, a β -galactosidase expression plasmid (Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.) used to measure the efficiency of transfection, were added to each 10 cm plate of cells. A20-2J cells were transfected in suspension in 1 ml, by using 15 mg of DNA and 30 mg of DEAE-dextran. CAT assays were performed as described [33]. Acetylation was quantified performed with the AMBIS Radioanalytic Imaging System (AMBIS, San Diego, CA, U.S.A.).

Gel-electrophoresis DNA-binding assay

The synthetic oligonucleotides were labelled with polynucleotide kinase and [γ -³²P]ATP by standard procedures [34]. For gel-electrophoresis DNA-binding assays, binding was performed in a total volume of 20 μ l in 12% glycerol containing 12 mM Hepes (pH 7.9), 60 mM KCl, 5 mM MgCl₂, 0.12 mM EDTA, 0.3 mM PMSF, 0.3 mM dithiothreitol, 10000 c.p.m. of ³²P-labelled DNA (0.1 ng), 2 μ g of poly(dI-dC) and the indicated amounts of nuclear extract. Binding was allowed to proceed for 30 min at 22 °C. Samples were then applied to a 3.5%-polyacrylamide (acrylamide/bisacrylamide 30:1) 0.25 $\times$ TBE gel (1 $\times$ TBE = 89 mM Tris, 89 mM boric acid, 2 mM EDTA) containing 5% glycerol, and electrophoresis was carried out for 1 h at 4 °C at 250 V (10 V/cm). After electrophoresis, gels were dried and placed in contact with an X-ray film (XAR-5 from Kodak).

DNase I protection assay

The 12.33.2 probe was prepared by digesting the plasmid 12.33.2 with *Hind*III or *Sal*I, followed by end-labelling using the large fragment of DNA polymerase (Klenow) plus [α -³²P]dATP and [α -³²P]dCTP [25]. For DNase I footprinting, the indicated amount of crude nuclear extract was incubated in the presence of 2 μ g of poly(dI-dC), 2 μ l of 100 mM CaCl₂ and 20000 c.p.m. of the end-labelled 12.33.2 fragment, in the incubation buffer (10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA and 5% glycerol) for 1 h at room temperature. Then 4 μ l of diluted DNase I (500 μ g/ml) was added, and the mixture was incubated at room temperature for 2.5 min. The reaction was stopped by addition of 40 μ l of the stop solution (1% SDS, 20 mM EDTA, 40 mM Hepes, 100 mM NaCl and 5 μ g of tRNA). The samples were run on a 6%-acrylamide/8 M urea gel in 1 $\times$ TBE buffer. Gels were autoradiographed at -70 °C with Kodak XAR-5 film and DuPont intensifying screens.

Purification of the binding factor

For this, 18 litres of A20-2J cells were grown, and 75 g wet wt. of cells was obtained; 160 mg of nuclear protein was obtained in

a volume of 31 ml. The protein was applied to a column containing 12 ml of heparin-agarose (Affi-Gel heparin; Bio-Rad, Richmond, CA, U.S.A.). The column was washed with 40 ml of buffer A containing 50 mM NaCl, and the sample was eluted with 0.4 M NaCl in buffer A containing 20 mM Tris/HCl, pH 7.9, 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, 0.1% Nonidet P40, 0.5 mM dithiothreitol, 0.5 mM PMSF, 1 μ g/ml leupeptin and 1 μ g/ml pepstatin A. The flow-through fractions were pooled and diluted 8-fold with NaCl-free buffer A and applied to a 3 ml DNA-affinity column. This column consisted of a double-stranded oligonucleotide (oligo S box) covalently linked to CNBr-activated Sepharose-4B by the method of Kadonaga and Tjian [35]. The sample on the affinity column was washed with 100 ml of buffer A (NaCl-free) and eluted with 10 ml of buffer A containing different concentrations of NaCl. Fractions were desalted and concentrated with a Centricon 10 microcentrator (Amicon, Danvers, MA, U.S.A.).

Molecular-mass determination of S-box DNA-binding protein

For this, total extract or the fraction eluted with 1 M salt from the affinity column was separated by gel electrophoresis. After electrophoresis, the gels were cut into 5 mm slices, and protein was extracted as described previously [10] and the resulting preparation was used in the gel-electrophoresis DNA-binding assay.

Other techniques

Protein concentrations were determined with the Bio-Rad protein assay. SDS/PAGE was performed as described by Laemmli [36] and gels were silver-stained with the Bio-Rad Silver Stain kit. SDS/PAGE molecular-mass standards were from Bio-Rad or Amersham (Arlington Heights, IL, U.S.A.).

RESULTS

It has been reported that the upstream region of class II genes containing the S, X and Y boxes acts as an enhancer [8,9]. In order to determine whether these boxes isolated from the mouse I-A β gene have enhancer activity, we inserted a fragment containing these sequences into a vector (KS1) which contains

the SV40 early promoter but no enhancer. Similar constructions have been made by using the mouse E α and human DRA genes [22,37,38]. A 124 bp fragment from the promoter of the I-A β gene was inserted into the CAT vector, KS1. This 124 bp fragment contained, in addition to the S box, two additional boxes, X and Y, both of which have been shown to be important for the expression of several other MHC class II genes [8,9]. When this CAT construct (KS1-SXY) was transfected into A20-2J B lymphocytes, a CAT activity of 12% was obtained (Figure 2). This was in contrast with a CAT activity of 2% when the KS1 vector alone was transfected into the same cells. A substantial fall in CAT activity was observed when the S box was either deleted (KS1-XY) or mutated (KS1-mutant). When the S-box sequence was deleted or mutated (KS1-mutant), CAT activity fell to 8.5% (32% decrease) or 8% (36% decrease) respectively.

In A20-2J cells, IFN- γ treatment did not modify the expression of I-A β mRNA (results not shown) or the expression of CAT activity (Figure 2). In the macrophage cell line WR19 M.1 the expression of I-A β mRNA and I-A cell-surface expression is IFN- γ -dependent [29,39]. When the KS1-SXY CAT construct was transfected into these macrophages, significant CAT activity was obtained only after the cells were treated with IFN- γ (6.5-fold increase). No significant CAT activity was found when macrophages were transfected with the KS1-XY (S box deleted) or KS1-mutant (S box mutated) constructions even after treating the cells with IFN- γ . No significant CAT activity was observed when macrophages transfected with the KS1-SXY CAT construct were treated with either IFN- α or - β .

These results indicated that the region of the I-A β promoter encompassing the S, X and Y boxes stimulated the expression of the CAT gene in B lymphocytes. In macrophages the expression of the CAT gene in the same construction was IFN- γ -dependent. Based on these results, the S box appears to be an important element for the expression of I-A β in B lymphocytes and macrophages.

A gel-electrophoresis DNA-binding assay was used to screen for nuclear factor(s) that could bind to the S-box sequence of the I-A β gene. In this assay, DNA-protein complexes migrate slower than free DNA. To decrease non-specific binding of proteins to the labelled fragment, the synthetic co-polymer, poly(dI-dC) duplex, was included in the binding reaction. Nuclear extract prepared from murine A20-2J cells (B lymphocytes) was incu-

Figure 2 The S box is an important *cis*-acting element for expression from the I-A β promoter

A CAT reporter plasmid (KS1) was used for the insertion of various fragments of the I-A β promoter. The constructions were: KS1 (vector); KS1-SXY (—146 to —26); KS1-XY (—110 to —26) and KS1 mutant (—146 to —26, with a mutation of the S box indicated in Figure 3). The constructions were transfected into A20-2J (B lymphocytes) and WR19 M.1 (macrophages) cells, and after 48 h CAT activity was assayed. After transfection, cells were treated (black bars) or not treated (hatched bars) with 300 IRU of IFN- γ /ml. CAT activity was quantified by using an AMBIS Radioanalytic Imaging System. The data shown are means $\pm$ 1 S.D. of four experiments.

Figure 3 Electrophoretic mobility shift assay using the S-box oligonucleotide and competition experiments

Nuclear extracts were prepared from the B cell lymphoma A20-2J, and used in a binding assay as described in the Materials and methods section. Reactions contained 1 μ g of nuclear extract, 2 μ g of poly(dI-dC) and either no competitor or competitor at 100 times molar excess, except for the S-box oligonucleotide, which was at 10 times molar excess. The control is the labelled oligonucleotide in the absence of nuclear extract. At the bottom of the Figure we show the DNA sequence of one strand of the double-stranded oligonucleotides used in the study. The conserved sequence for each oligonucleotide is underlined. The lower-case letters refer to bases that are not in the sequence of the I-A β promoter, but have been included in the sequence of the oligonucleotide. The orientation of each X box in respect to the gene is as shown, except for oligo X' box, which is reversed.

bated with labelled double-stranded oligonucleotide (33 bp) covering the area of the S box (Figure 1). One retarded band was observed by this method (Figure 3). The specificity of the complex detected was tested by a competition assay using unlabelled double-stranded oligonucleotides of DNA fragments that contained or did not contain the putative binding site. A 10-fold molar excess of the unlabelled S-box oligonucleotide competed well for binding of the factor for the labelled S-box oligonucleotide (Figure 3). To demonstrate that the binding occurred within the conserved 7 bp of the S box, we mutated the 7 bp within the S box and tested this double-stranded oligonucleotide in a competition experiment. This mutated oligonucleotide at 100-fold molar excess did not inhibit the binding of the nuclear factor to the S-box oligonucleotide. When additional competition experiments were performed, we observed that those oligonucleotides or fragments from the promoter having the S-box sequence (12.33.2, a 320 bp from -245 to +75, and H1, a 152 bp from -221 to -69) competed for binding, but not those oligonucleotides or fragments that lacked the S box, such as the promoter fragment H2 (155 bp from -80 to +75), PM 2.2, a 615 bp fragment containing the IgH promoter, or pUC-13 linearized plasmid (Figure 3). We therefore conclude that a specific complex is formed between a factor(s) in the nuclear extract of A20-2J and the conserved 7 bp containing the S box.

To delineate the factor-binding site further, a DNase I footprint was determined on both the upper and lower strands. Complexes

Figure 4 DNase I footprint of the I-A β promoter

The end-labelled 12.33.2 probe was incubated with BSA (15 μ g) or increasing concentrations of total extracts (A20-2J cells) and footprinted with DNase I. The locations of the S-, X- and Y-box binding sites are indicated at the side. The base-specific sequencing reaction of the probe for G was run in parallel as a marker. The lane containing BSA had a pattern equivalent to that obtained in the absence of protein.

formed between the radio-labelled 12.33.2 fragment and nuclear proteins were digested with DNase I, and resolved by gel electrophoresis. Comparison of the pattern produced by DNase I digestion of complexed DNA with the pattern produced in the presence of BSA revealed several areas of protection (Figure 4). Two of these areas overlap the X and Y boxes and probably correspond to proteins previously described [8,9]. A third protected area overlapped the sequence corresponding to the S box. About 20 bp below the Y box on the upper strand, and above it on the lower strand, there is an additional area of DNase I protection. No differences of the pattern of DNase I protection were found when nuclear extracts of macrophages treated or untreated with IFN- γ were used.

Figure 5 Electrophoretic mobility shift assay of nuclear extracts from different murine and human cell lines

A20-2J is a mouse B lymphocyte; BW 5147 and EL4 are mouse T lymphocytes; P815 is a mouse mastocyte; cell line L929 is mouse fibroblasts; RAJI are human B lymphocytes; and U937 are human macrophages. The probe used is the 33-bp double-stranded oligonucleotide S. For each reaction, 1 μ g of nuclear extract was used.

Figure 6 Electrophoretic mobility shift assay of nuclear extracts from bone-marrow-derived macrophages treated with IFN- γ

The probes used were the 33-bp oligonucleotides S and X; 1 μ g of nuclear extract was used for each reaction. Macrophages were incubated for the indicated periods of time with 300 IRU of IFN- γ /ml before the preparation of nuclear extracts. This treatment induces maximal I-A surface expression, as well as specific mRNA induction [28,37].

To determine the tissue distribution of the nuclear factor that binds to the S-box sequence, extracts from a panel of mouse and human tumour cells that represent cells of the B lymphocyte, T lymphocyte, mastocyte, fibroblast and macrophage lineages were assayed for binding activity. All extracts were found to contain a factor that generated retarded band with the S box containing oligonucleotide (Figure 5). The mobility of the retarded band obtained with each extract was identical with that generated with the A20-2J extracts. Two additional bands were present in Raji and U937 cells. Unlabelled oligonucleotides did not compete with the lower-molecular-mass band, which suggests that it was not specific for the S box.

Since treatment of macrophages with IFN- γ results in the induction of I-A expression, and the S box is an IFN- γ -responsive sequence, nuclear extracts were prepared from BMM before and

Figure 7 Electrophoretic mobility shift assay of nuclear extracts from cells treated with IFN- γ

The different macrophage cell lines were incubated for the indicated periods of time with 300 IRU of IFN- γ /ml before the preparation of nuclear extract. The assay was carried out with 1 μ g of nuclear extract.

after IFN- γ treatment. When we compared the same amount of total protein extract (1 μ g), the retarded band seen with the oligonucleotide containing the S box appeared to be induced in BMM treated with IFN- γ (Figure 6). In order to exclude the possibility that this induction was due to a general decrease in the amount of nuclear protein in IFN- γ -treated cells, we used a double-stranded oligonucleotide containing the X box in the retardation assay. The retarded band, using the X-box oligonucleotide, had almost the same intensity both before and after IFN- γ treatment. In both cases, the retarded bands were specific for the S and X boxes respectively, as demonstrated by competition experiments using unlabelled oligonucleotides with the S and X sequences, as well as the mutants of both. In another set of experiments, extracts from five different macrophage cell lines were tested before and after IFN- γ treatment. In each of these cell lines, IFN- γ induced I-A expression [29,39]. This treatment did not greatly influence the amount of the retarded band in the cell lines studied (Figure 7).

Partial purification of the factor that bound to the S-box sequence was undertaken. The crude nuclear extract was initially fractionated by heparin-agarose chromatography. DNA-binding activity was eluted from the heparin-agarose column with buffer containing 0.4 M NaCl. The binding activity was then passed over a DNA affinity column that was prepared by using a double-stranded oligonucleotide containing the S-box sequence from the I-A β gene. The column was washed with buffer containing NaCl in increasing concentrations from 0.1 to 2.0 M. Each fraction was concentrated, and 0.01 μ g of protein was assayed by the gel-electrophoresis DNA-binding assay. The fractions that contained the most enriched activity were eluted at 0.8 and 1.0 M NaCl. The activity in the 1.0 M NaCl fraction was estimated to have been purified at least 1800-fold over the activity present in the crude nuclear extract.

To determine the molecular size of the S-box binding factor, a sample from each of the fractions collected from the DNA

Figure 8 SDS/PAGE (12% gels) of nuclear proteins from A20-2J cells

Material was applied to the gel in the following amounts: total nuclear extract, 20 μ l; fractions eluted from the second DNA affinity column, 50 μ l. The gel was stained with silver after electrophoresis. Molecular-size markers were run on the same gel and are indicated (in kDa) at the left. The arrow on the right indicates the position of 43 kDa band that may be related to the DNA-binding activity of the S-box binding protein.

affinity column was run on a SDS/12%-polyacrylamide gel and stained with silver (Figure 8). The fraction eluted with 1 M NaCl contained a prominent protein band with a molecular size of 43 kDa. To establish a molecular size for the activity associated with S-box binding protein, the 1 M NaCl wash and total extract were fractionated on a preparative SDS/12%-polyacrylamide gel, the gel was cut into slices, and the protein extracted and renatured. The DNA-binding activity of the protein eluted from each gel slice was tested by using the gel-retardation assay and the S-box-containing oligonucleotide. The retarded band obtained with the renatured proteins was specific for the S box, as demonstrated by competition experiments using the oligonucleotides containing the S box or the mutant described in Figure 3. On the basis of the mobility of standard molecular-size markers, an apparent size of 43 kDa was calculated for the S-box binding activity, both in the purified fraction and in total nuclear extract (Figure 9). Estimates based on the quantity of protein within a band on the silver-stained gel and the quantity of material required to give a retarded band in the gel-electrophoresis DNA-binding assay strongly suggest that the protein

Figure 9 Determination of the molecular size of the activity corresponding to the S-box binding protein

Total nuclear extract or the 1.0 M NaCl fraction eluted from the DNA affinity column was fractionated on a SDS/12%-polyacrylamide gel. The gel was cut into 5 mm slices, and the proteins were eluted, renatured and assayed for binding activity to the S oligonucleotide. Molecular-size makers were run on the same gel and are indicated (in kDa) at the top.

band identified on the silver-stained gel at 43 kDa is the S-box binding protein.

DISCUSSION

In the present study, we characterized the S box in the I-A β gene as a *cis*-acting regulatory element in a B-lymphocyte cell line that also provides IFN- γ inducibility in macrophages. This observation in the I-A β gene confirms the regulatory role of the S box in other human and murine genes. By using 5' deletion analysis in the DR α gene, it has been shown that the inclusion of approx. 30 bp upstream of the X box leads to greater constitutive B-cell expression [38]. Constructs extending only to the X box were not induced by IFN- γ [16,17]. Mutation of the S box decreases reporter-gene expression in B cells, and IFN- γ -mediated expression in non-lymphoid cells is also severely affected [18]. In the murine class II genes, in A α , progressive 5' deletion or mutation of the S box decreases expression in B cells and eliminates IFN- γ -mediated expression on macrophages [35]. Also, for E α , deletion [40] or mutation [22] of the S box leads to a loss of IFN- γ or mutation [22] of the S box leads to a loss of IFN- γ inducibility. Finally, for the E β gene, a greater B-cell expression was observed when the S-box sequence was included in the constructions [41], and IFN- γ -mediated induction in macrophages was lost by 5' deletion of the S element [42].

We have used a gel-electrophoresis DNA-binding assay and DNase-I protection assay to identify a nuclear factor that binds to a region upstream of the I-A β gene. The binding region overlaps a 7 bp sequence that is present upstream of all class II MHC genes [16,17] and has been called the S box. The nuclear factor with a recognition site that overlaps the S box was detected in a wide range of cells, and therefore does not appear to be tissue-specific. DNA fragments or oligonucleotides containing the same sequence competed for the binding of the nuclear factor, but unrelated DNA fragments did not. Moreover, an oligonucleotide with the conserved 7 bp mutated did not compete for binding. This implies that the binding site is sequence-specific. DNase-I protection experiments were used to identify the binding site further. Nuclear factors protected an area overlapping the S box, as well as the X and Y-CCAAT boxes already described [8,9]. These results strongly implicate the 7 bp conserved S-box sequence in the binding of the nuclear factor described here.

Using electromobility shift assays it has been shown *in vitro* that proteins interact with the S region, both in the human DR α promoter [18,19] and in the murine A α promoter [25]. However, the protein has not been well characterized or cloned. The protein is found in class II positive and negative cells. In lymphoid cells, a second complex, WB2, has been described [19]. Moreover, different complexes may bind to the S box of the A α promoter in B cells, macrophages and fibroblasts [25]. The treatment of macrophages with IFN- γ does not induce modifications of the S-complex formation [19].

In the DNase-I protection assay, at about 20 bp in the 3' side of the Y box a protected area was found. In the I-A β gene, this area was not reported previously. In the I-A α , I-E α , DR α and DQ α genes a different consensus sequence located in the same region and called a T box was described [21]. This sequence seems to play a functional role in transcription induced by tumour necrosis factor α , but not by IFN- γ [21]. In the DQ β and E β gene promoters a forward CCAAT motif close to the TATA box was described, but this is not functionally important [8]. The protected area of the I-A β includes a CCAAC motif, but its functional relevance is unknown.

Perhaps the major interest in the S box is that, in contrast with the X and Y boxes, it is an IFN- γ -response region for the genes encoding class II antigens. Therefore we were interested in any changes that could be detected before and after cells were treated with IFN- γ . Interestingly, in BMM the S-box binding activity was inducible by treatment of the cells with IFN- γ . However, when various macrophage cell lines were tested, we could not find any quantitative change in the level of this binding activity. At present we do not have an explanation for the difference observed between BMM and the cell lines. There are several possible reasons, however, that may help to explain the observed inducibility of the S-box binding activity in BMM. First, in contrast with the cell lines, BMM are resting, non-dividing, cells. In addition, it has been demonstrated that the expression of I-A β is cell-cycle regulated [43,44], and several examples have been reported in which the induction of a DNA-binding protein is cell-cycle related [45]. If cell-cycling was the only factor which affected the induction of the W-box binding protein, then in the cell lines treated with IFN- γ , which is a potent anti-proliferative agent [46], some quantitative modifications of the retarded band should have been seen. This, however, was not the case, suggesting that the variations in the cycling of the cells may not be the only factor responsible for the induction of the S-box binding activity in BMM. Second, before IFN- γ was added, the BMM were cultured for 4 days in the absence of L-cell-conditioned media (as a source of macrophage colony-stimulating factor). This treatment may have affected the inducibility of the S-box binding activity. Finally, we cannot exclude the possibility that the inducibility of the S-box binding activity is related to the degree of cell maturation/differentiation, although we use different cell lines with different degrees of differentiation.

It has been shown that in BMM the Y-CCAAT binding protein was induced by treating the cells with lipopolysaccharide [47], a factor that acts as an anti-proliferative agent. In primary astrocytes, but not in a glioblastoma cell line, an IFN- γ -enhanced protein with specificity for the X element was observed [48]. An IFN- γ -inducible complex (A) that binds to the Y box has been described by using the E α promoter [42]. Although some nuclear binding factors have been described which are induced by various stimuli, this is not usually the case [7]. It is possible that IFN- γ induces an allosteric modification in the S-box binding protein, by altering the phosphorylation and/or the glycosylation pattern of the protein. These modifications appear to occur in other nuclear proteins, and may be required for the binding of the S factor to

the DNA. Modification of DNA-binding proteins has been shown to be necessary for both DNA binding and transcriptional activation [49]. These possibilities are currently under investigation in our laboratory.

The S, X and Y boxes are separated from each other by a distance of around 20 bp, suggesting that the proteins that interact with these boxes are on the same side of the DNA [50]. If this is the case, then it is likely that protein-protein interactions are occurring as well. This interaction is not related to the heterodimeric DNA binding activity seen when two proteins interact to obtain efficient binding to DNA, such as Jun/Fos or some of the CCAAT binding factors [51,52], but it is an interaction between proteins that already bind to the DNA separately. In some cases, synergistic effects on transcription are dependent on strict spacing between adjacent *cis* elements [53–56]. Although the interplay between the various nuclear factors that bind to the S, X and Y boxes of the I-A β gene is not yet understood, it is believed that combinations of *cis* elements arranged in unique configurations confer on each gene an individualized spatial and temporal transcription program.

Recently, the class II *trans*-activator (CIITA) has been isolated and characterized as a transcriptional co-activator for the expression of MHC class II genes [57]. The mechanism of action of this *trans*-activator remains elusive. However, CIITA is necessary and sufficient for the IFN- γ -inducible expression of class II genes [58,59]. The expression of CIITA itself is IFN- γ inducible, and its induction does not require protein synthesis *de novo* [58,59]. CIITA also regulates the expression of other genes involved in antigen presentation, such as the invariant chain or the HLA-DM molecule [60]. Although CIITA activates the transcription of class II genes, it does not seem to have DNA-binding activity, suggesting that it may act through other DNA-binding proteins [57]. In this context, one of the targets of CIITA may be the complex that binds to the S box. This activation could be mediated through protein-protein interaction. Obviously, in order to assess the molecular mechanism of class II expression, a mandatory step is the cloning and characterization of the protein(s) that bind to the S box.

This work was supported by grants from CICYT (PM92-0211) and FISS (93/0589) to A.C. and from the U.S. Public Health Service (AI20194) to R.A.M. We thank John Knight for the synthesis of the oligonucleotides, and Ginger Pierce for preparation of the manuscript. We also acknowledge Genentech, South San Francisco, CA, U.S.A., for the gift of IFN- γ , and Martin Cullell for the reading of the manuscript.

REFERENCES

- Flavell, R. A., Allen, H., Burkly, L. C., Sherman, D. H., Waneck, G. L. and Widera, G. (1986) *Science* **233**, 437–443
- Wong, G. H. W., Clark-Lewis, I., McKimm-Breschkin, J. L., Harris, A. W. and Schrader, J. W. (1983) *J. Immunol.* **131**, 788–795
- King, D. P. and Jones, P. P. (1983) *J. Immunol.* **131**, 315–318
- Noelle, R., Kuziel, W. A., Maliszewski, C. R., McAdams, E., Vitetta, E. S. and Tucker, P. W. (1986) *J. Immunol.* **137**, 1718–1723
- Polla, B. S., Poljak, A., Geier, S. G., Nathenson, S. G., Ohara, J., Paul, W. E. and Glimcher, L. H. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4878–4882
- Mitchell, P. J. and Tjian, R. (1989) *Science* **245**, 370–378
- Johnson, P. F. and McKnight, S. L. (1989) *Annu. Rev. Biochem.* **58**, 799–843
- Benoist, C. and Mathis, D. (1990) *Annu. Rev. Immunol.* **8**, 681–715
- Glimcher, L. H. and Kara, C. J. (1992) *Annu. Rev. Immunol.* **10**, 13–49
- Celada, A. and Maki, R. A. (1989) *Mol. Cell. Biol.* **9**, 3097–3100
- Hooft van Huijsduijnen, R., Li, X. Y., Black, D., Matthes, H., Benoist, C. and Mathis, D. (1990) *EMBO J.* **9**, 3119–3127
- Reith, W., Barras, E., Satola, S., Kobr, M., Reinhart, D., Herrero Sanchez, C. and Mach, B. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4200–4204
- Liou, H.-C., Boothby, M. R., Finn, P. W., Davidon, R., Nabavi, N., Zeleznik-Le, N. J., Ting, Y. P.-Y. and Glimcher, L. H. (1990) *Science* **247**, 1581–1584
- Liou, H.-C., Boothby, M. R. and Glimcher, L. H. (1988) *Science* **242**, 69–71

- 15 Reith, W., Satola, S., Herrero Sanchez, C., Amaldi, I., Lisowska-Groszpiere, B., Griscelli, C., Hadam, M. R. and Mach, B. (1988) *Cell* **53**, 897–906
- 16 Basta, P. V., Sherman, P. A. and Ting, J. P.-Y. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8618–8622
- 17 Tsang, S. Y., Nakanishi, M. and Peterlin, B. M. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8598–8602
- 18 Tsang, S. Y., Nakanishi, M. and Peterlin, B. M. (1990) *Mol. Cell. Biol.* **10**, 711–719
- 19 Cogswell, J. P., Austin, J. and Ting, J. P.-Y. (1991) *J. Immunol.* **146**, 1361–1367
- 20 Brown, A. M., Barr, C. L. and Ting, J. P.-Y. (1991) *J. Immunol.* **146**, 3183–3189
- 21 Freund, Y. R., Dedrick, R. L. and Jones, P. P. (1990) *J. Exp. Med.* **171**, 1283–1299
- 22 Viville, S., Jongeneel, V., Koch, W., Mantovani, R., Benoist, C. and Mathis, D. (1991) *J. Immunol.* **146**, 3211–3217
- 23 Kouskoff, V., Mantovani, R. M., Candeias, S. M., Dorn, A., Staub, A., Lisowska-Groszpiere, B., Griscelli, C., Benoist, C. O. and Mathis, D. J. (1991) *J. Immunol.* **146**, 3197–3204
- 24 Yang, Z., Sugawara, M., Ponath, P. D., Wessendorf, L., Banerji, J., Li, Y. and Strominger, J. L. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9226–9230
- 25 Dedrick, R. L. and Jones, P. P. (1990) *Mol. Cell. Biol.* **10**, 593–604
- 26 Celada, A., Shiga, M., Imagawa, M., Kop, J. and Maki, R. A. (1988) *J. Immunol.* **140**, 3995–4002
- 27 Celada, A. and Maki, R. A. (1989) *Mol. Cell. Biol.* **9**, 5129–5222
- 28 Celada, A., Gray, P. W., Rinderknecht, E. and Schreiber, R. D. (1984) *J. Exp. Med.* **160**, 55–74
- 29 Celada, A. and Maki, R. A. (1989) *Eur. J. Immunol.* **19**, 205–208
- 30 Tsonis, P. A., Manes, T., Millan, J. L. and Goetinck, P. F. (1988) *Nucleic Acids Res.* **16**, 7745
- 31 Clarke, C., Berenson, J., Gorman, J., Boyer, P. D., Crews, S., Siv, G. and Calame, K. (1982) *Nucleic Acids Res.* **10**, 773–776
- 32 Celada, A., McKercher, S. and Maki, R. A. (1993) *J. Exp. Med.* **177**, 691–698
- 33 Gorman, C. M., Moffat, L. F. and Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051
- 34 Maniatis, T., Fritsch, E. F. and Sambrook, J. (1992) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor
- 35 Kadonaga, J. T. and Tjian, R. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5889–5893
- 36 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 37 Sherman, P. A., Basta, P. V. and Ting, J. P.-Y. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4254–4258
- 38 Cogswell, J. P., Basta, P. V. and Ting, J. P.-Y. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7703–7707
- 39 Celada, A., Klemsz, M. J. and Maki, R. A. (1989) *Eur. J. Immunol.* **19**, 1103–1109
- 40 Thanos, D., Mavrothalassitis, G. and Papamatheakis, J. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3075–3079
- 41 Finn, P. W., Kara, C. J., Grusby, M. J., Folsom, V. and Glimcher, L. H. (1991) *J. Immunol.* **146**, 4011–4015
- 42 Finn, P. W., Kara, C. J., Donhan, J., Van, T. T., Folsom, V. and Glimcher, L. H. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 914–918
- 43 Lanier, L. L. and Warner, N. L. (1981) *J. Immunol.* **126**, 626–632
- 44 Broxmeyer, H. E. (1982) *J. Clin. Invest.* **69**, 632–639
- 45 Knight, G. B., Gudas, J. M. and Pardee, A. B. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8350–8354
- 46 Goldberg, M., Belkowski, L. S. and Bloom, B. S. (1989) *J. Cell Biol.* **109**, 1331–1340
- 47 Shakhov, A. N., Collart, M. A., Vassali, P., Nedospasov, S. A. and Jongenell, C. V. (1990) *J. Exp. Med.* **171**, 35–42
- 48 Moses, H., Panek, R. B., Benveniste, E. N. and Ting, J. P.-Y. (1992) *J. Immunol.* **148**, 3643–3651
- 49 Hunter, T. and Karin, M. (1992) *Cell* **70**, 375–397
- 50 Vilen, B. J., Cogswell, J. P. and Ting, J. P.-Y. (1991) *Mol. Cell. Biol.* **11**, 2406–2415
- 51 Curran, T. and Franza, B. R. (1988) *Cell* **55**, 395–397
- 52 Lamb, P. and McKnight, S. L. (1991) *Trends Biochem. Sci.* **16**, 417–422
- 53 Akerblom, I. E., Slater, E. P., Beato, M., Baxter, J. D. and Mellon, P. L. (1988) *Science* **241**, 350–353
- 54 Comb, M., Mermod, N., Hyman, S. E., Pearlberg, J., Ross, M. E. and Goodman, H. M. (1988) *EMBO J.* **7**, 3793–3805
- 55 Schule, R., Muller, M., Kaltschmidt, C. and Renkawitz, R. (1988) *Science* **242**, 1418–1420
- 56 Strahle, U., Schmid, W. and Schutz, G. (1988) *EMBO J.* **7**, 3389–3395
- 57 Steimle, V., Otten, L. A., Zufferey, M. and Mach, B. (1993) *Cell* **75**, 133–146
- 58 Chang, C.-H., Fontes, J., Peterlin, M. and Flavell, R. A. (1994) *J. Exp. Med.* **180**, 1367–1374
- 59 Steimle, V., Siegrist, C. A., Mottet, A., Lisowska-Groszpiere, B. and Mach, B. (1994) *Science* **265**, 106–109
- 60 Chang, C.-H. and Flavell, R. A. (1995) *J. Exp. Med.* **181**, 765–767