The MHC class II E β promoter: a complex arrangement of positive and negative elements determines B cell and interferon- γ (IFN- γ) regulated expression

Dimitris Thanos^{1,2,+}, Marirena Gregoriou^{1,2}, Dimitris Stravopodis^{1,2}, Katerina Liapaki², Takis Makatounakis¹ and Joseph Papamatheakis^{1,2,*}

¹Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, PO Box 1527, Heraklion 711 10, Crete and ²University of Crete, Department of Biology, Heraklion, Crete, Greece

Received August 9, 1993; Revised and Accepted November 18, 1993

ABSTRACT

The 5' proximal region of the E β gene was studied with respect to B lymphoid expression and responsiveness to cytokines, revealing a complex array of general and cell type specific *cis*-elements and factors. Full lymphoid activity and response to interferon- γ (IFN- γ) is generated by the concerted action of the MHC boxes (H, X and Y) and additional elements. Combinatorial interactions between elements and their cognate factors are indicated by several lines of evidence. Thus, mutations within the X box in the promoter context are strongly deleterious to both B lymphoid activity and IFN- γ regulation. However, the X box alone has minimal lymphoid activity upon heterologous promoters. Data from deletion, insertion and site directed mutagenesis demonstrate that sequences extending ~35 bp 5' of the X box (designated as Cytokine Response Sequence—CRS) have a dual role: they are required for cytokine-regulated expression as well as serving as an enhancer element for cell-specific constitutive expression. A region that carries X and CRS permits both lymphoid activity and IFN- γ response. In contrast, sequences that include X and the downstream Y box are constitutively active in all cell types tested. Combination of the sequences both upstream and downstream of the X box results in a tissue-specific and cytokine-regulated enhancer of full strength. *In vivo* competition studies show that titratable *trans*-acting factors, shared by Class I and Class II promoters, mediate the CRS-dependent IFN- γ response. We report here the identification of novel nuclear complexes that bind to the CRS and recognize sites which correlate with its negative or positive elements. One of these complexes is present in B lymphoid cells only. Three other CRS complexes that are upregulated by either

IFN- α and IFN- γ are competed by a non-Class II, IFN- α stimulated response element (ISRE), providing evidence for the functional interconnection of these cytokines.

INTRODUCTION

The class II genes of the Major Histocompatibility Complex (MHC) encode polymorphic cell surface glycoproteins which are essential for regulating and restricting the immune response; antigen recognition by T cells is mediated by the MHC-antigenic peptide complex. Expression of the class II gene products is required for both antigen presentation in the periphery (1) and the generation of the T cell repertoire in the thymus (2). The expression of class II gene products is restricted to certain cell types such as B cells, macrophages, some activated T cells, dendritic, epithelial cells, Kupffer cells in the liver and Langerhans cells in the skin (3). This specific expression can be further modulated by various positive or negative effectors, interferon γ (IFN- γ) and interleukin 4 (IL-4) (4,5). IFN- γ increases the transcription rate of class II genes in macrophages or in certain epithelial cells, whereas IL-4 stimulates expression in B cells. The molecular mechanisms involved in the tissue and developmental or lymphokine induced expression of class II genes are of additional interest because of the involvement of ectopic class II products in certain autoimmune diseases (6,7) as well as foetal loss (8).

The molecular cloning and sequencing of the proximal promoter regions from diverse mammalian class II genes revealed the presence of two highly conserved sequence motifs, located usually in the region from -40 to -150 relative to the start site of transcription (9,10) but occasionally more distally (11,12). These motifs, called X and Y boxes, play a crucial role in the transcriptional regulation of the class II genes, as shown by

*To whom correspondence should be addressed at: Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, PO Box 1527, Heraklion 711 10, Crete, Greece

⁺Present address: Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138, USA

mutations, internal deletions or 5' deletions assayed in transgenic mice or transfected cells (13–21). Although these elements are required for regulated expression in the context of the class II promoters, they are not sufficient when studied in isolation (16, 19). A third conserved motif, the H box (or S box) has been recognized as a conserved element located 15 to 20 bp upstream of the X box (22). Deletions or mutations in this region strongly interfere with IFN- γ induction or the constitutive expression in B cells (20,23,24).

To understand the molecular mechanisms involved in the regulation of class II genes we have investigated in detail the *cis* acting elements required for B cell specific and IFN- γ induced expression of the mouse E β gene. In this study we provide a detailed description of a cytokine response sequence (CRS) located immediately upstream of the X box. Combination in *cis* of the CRS and the X box reconstitutes a strong IFN- γ inducible and B cell specific enhancer, which is further strengthened by the inclusion of the Y box. The CRS region binds several DNA–protein complexes that are either constitutive or B-cell specific. By competition experiments, we demonstrate for the first time that the regulatory regions of Class I and Class II genes share DNA binding factors, and also that factors are shared between the IFN- γ stimulated Class II genes and unrelated genes which are stimulated by IFN- α .

MATERIALS AND METHODS

Cell lines

Raji, RJ2.2.5, Jurkat, and Ramos, cell lines were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (Seralab) 50 μ g/ml Gentamycin and 50 μ M β -mercaptoethanol. HeLa, LMTK⁻, WEHI-3 and XS63 cells were grown in Dulbeccos' modified Eagle medium (Gibco) supplemented as above, but lacking mercaptoethanol.

Plasmid constructions

All vectors used have been described by Thanos *et al.* (18). Serial 5' deletions of the E β promoter were generated by Bal31 or exonuclease 3' treatment on the –630 to +50 bp fragment of the E β gene and were inserted into the polylinker of the promoterless PLSVOCAT (18). The PstI–NruI promoter region of H-2k^b was also cloned in the above vector (designated H-2k^b CAT).

Insertion constructs were made by cloning restriction fragments or synthetic oligonucleotides from the E α or E β 5' region, in single or multiple copies in the SmaI or XbaI sites of either α GXCAT or pL51CAT respectively. Plasmids used for *in vivo* competition were made as above using the SmaI site of pUC19. All constructions were verified by dideoxy sequencing.

Transfection and enzyme assays

Epithelial and fibroblastic cell lines were transfected by the calcium phosphate method (25). Briefly 1.5×10^5 cells plated the day before were transfected for 14–18 hr with 2 μ g of CAT reporter plasmid, 1 μ g of β -galactosidase expressing plasmid and 5 μ g of salmon sperm carrier DNA. Transfections for *in vivo* competition were done as above but with competitor pUC plasmids carrying various segments in multiple copies and pUC stuffer DNA to give 20 μ g total DNA per plate. WEHI-3 and the lymphoid lines were transfected by the DEAE-Dextran method using 5–15 μ g/ml of plasmid DNA serum free DMEM containing 200 μ g/ml DEAE-Dextran for 3×10^6 exponentially growing

cells. After transfection cells were incubated for 24–36 hrs in the absence or presence 100 u/ml of human or mouse IFN- γ (Genzyme and Holland Biotechnology respectively) or 10 ng/ml TNF- α (Hoffmann La Roche) before harvesting for enzyme assays.

Transfection efficiencies were normalized by β -galactosidase enzyme determinations derived from the cotransfected plasmids pCH110 (26) or pCMV β (27). Chloramphenicol acetyltransferase (CAT) assays were carried out essentially according to Gorman *et al.* (28) with extracts heated up at 65°C for 5min (29).

Site directed mutagenesis

Mutagenized oligonucleotides were synthesized in a Applied Biosystem (Model 380B machine). All oligonucleotides were designed in a way that mutated nucleotides are located in the center of a 29 bases long sequence. The mutated oligonucleotides were annealed with single stranded m13 DNA, carrying the antisense strand of the E β promoter (–200 to +50 bp) and further processed as described by Taylor *et al.* (30). Mutagenized clones were verified by dideoxy sequencing.

DNA mobility shift and methylation interference analysis

Nuclear extracts for mobility shift assays were prepared according to Dignam *et al.* (31) with the addition of protease inhibitors (Leupeptin 1 μ g/ml, Aprotinin 2 μ g/ml, Pepstatin 1 μ g/ml and Antipain 1 μ g/ml). Binding reactions were carried out at room temperature with 5–10 μ g of nuclear extract, 0.1 ng of α -³²P labelled synthetic oligonucleotide or restriction fragment and 1–4 μ g/ml polyd(I: C), unless otherwise specified. Reactions were then subjected to acrylamide gel electrophoresis in 0.4 $\times$ Tris-Borate or 1 $\times$ Tris-Glycine buffer (32). Methylation interference analysis was carried out with DNA terminally labelled by γ -³²PATP, partially methylated with dimethylsulfate (33) and used in a binding reaction, as above. Free and bound probe were eluted, cleaved by piperidine, electrophoresed on 10–20% acrylamide/7.5M urea gel followed by autoradiography.

RESULTS

No more than 200 bp are required for B-cell specific and IFN- γ induced expression of the mouse E β gene

To delineate the *cis* acting elements required for B cell specific and IFN- γ induced expression of the E β gene we constructed a set of closely spaced 5' deletions by Bal31 or exonuclease III digestion, starting from an E β -CAT construct bearing 5' flanking sequences to position –630 (18). The deletion derivatives were transfected into class II positive B cells (Raji, A20), class II negative T and B cells (Jurkat and RJ 2.2.5 respectively), epithelial (HeLa) and fibroblast (LMTK⁻) cells. CAT activities were determined from extracts derived from cells either unstimulated or stimulated with IFN- γ . Results were normalized by β -galactosidase (see Materials and Methods) and were highly consistent: in general the standard deviation was less than 30% of the mean value, and thus differences of 1.5 fold or higher were significant. Fig. 1A tabulates the average results from at least three experiments for each construct in three cell types, including the class II negative Jurkat cells that gave a flat response and served as a control.

We localized the DNA sequences required for full B cell specific expression in Raji (Fig. 1A) or A20 cells (not shown)

Figure 1. Activity of 5' proximal deletions of E β . Numbers indicate the last nucleotide included in the construct. (A) expression in cell lines of class II positive or negative phenotype. Results comparable to those for Raji and untreated (-) or IFN- γ (+) treated HeLa cells shown here, were obtained using A20 and WEHI-3 cells, respectively. Class II negative Jurkat and RJ2.2.5 lines (not shown) gave a flat response. Data show averages from at least three experiments normalised by assaying for β -galactosidase. CAT activity of one unit has been assigned to the 5' deletion -46. The relative position of H, X and Y elements (filled boxes), CAT (open squares) and TATA (filled circles). (B) The functionally critical sequence of the 5' proximal region of E β ; end points of 5' deletions are shown by arrows. Brackets below the sequence delimitate important regions that have been defined by functional and nuclear factor binding studies. ISR₁ and ISR₂ have been defined by 5' deletions ISR₃ by site directed mutations and are all involved in IFN- γ regulation. ISR₁ and ISR₂ are also involved in basal expression exerting negative and positive effects respectively.

to the first 200 bp from the start site of transcription, a region that includes the conserved class II boxes (H, X and Y). Further deletional analysis revealed a combinatorial organization of the E β promoter: sequential addition of regions including the X and H boxes to a minimal Y-containing construct increased the B cell specific expression in more than additive fashion (Fig. 1A, see also below).

When the same constructs were transfected in the class II negative HeLa cells, a cell type dependent usage of the regulatory elements was revealed. Moreover, taken together the results indicated the existence of *cis*-elements other than the previously recognized H, X and Y. A strong negative regulatory region, ISR₁ (Fig. 1B), was mapped between positions -169 and -153, since deletion of this region results in 5 fold increase in the basal level of transcription in HeLa cells; this negative regulatory domain is also active in IFN- γ inducible mouse WEHI-3 but not in class II expressing B cells (Fig. 1A). Deletion of the sequences between -153 to -149 that removes the 5' half of the heptamer motif strongly reduces activity in HeLa (13 fold) but only 1.8 fold in Raji cells. Further deletion of -149 to -141 shows that this regions has a strong positive function in Raji cells only (Fig. 1A). Elimination of sequences between -141 and -132 results in a moderate (2 fold) increase of activity in both Raji and HeLa cells. Further 5' truncation of the E β gene to -111, removing the X box, results in substantial loss of expression, preferentially in B cells. We conclude that the 5'

proximal promoter region of the E β gene is composed of multiple overlapping regulatory domains, positive and negative, which are used differentially by different cell types.

Elements involved in the IFN- γ response region were localized between -200 and -141 by using HeLa cells and the same set of 5' deletions (Fig. 1A). This analysis demonstrates that the above described negative (-169 to -153) and positive (-149 to -141) regions are also involved in IFN- γ response: Deletion of the sequences from -169 to -153 results in a 5 fold decrease in the induction ratio (from 16 fold to 3.1 fold) and deletion of -149 to -141 eliminates the response to IFN- γ altogether (Fig. 1A). Because of their shared phenotype in IFN- γ regulation, we have designated them interferon stimulated region 1 and 2 (ISR and ISR₂) respectively (Fig. 1B). Qualitatively the same results were obtained in the mouse macrophage cell line, WEHI-3 (not shown). These results demonstrate that sequences extending to approximately 35 bp upstream of the conserved X+Y unit of the E β gene, are required for IFN- γ induction. Similar studies of critical 5' deletions have also suggested that, in addition to the X and Y boxes, further upstream sequences are required for IFN- γ induction of the E α , A α , DQ β and DR α promoters (13,17,18,34). Using 5' deletions we have defined within this region two novel regulatory elements. Most interestingly inclusion of ISR₁ limits basal but permits IFN- γ induced promoter activity. A third element (ISR₃) has been defined by site directed mutagenesis as described later.

Figure 2. Enhancer activity and cytokine response of the 5' proximal region of E β . Results shown were derived using the α globin promoter and monomeric inserts (unless as noted by the copy number in parenthesis). Effects comparable to those for Raji and HeLa cells shown here, were also obtained using A20 and LMTK⁻ cells, respectively. The CAT activity of the α -globin vector was taken as one. Enhancer activity in Jurkat and RJ 2.2.5 cells (not shown) never exceeded 2–3 fold even when using the E β , -207/-77 bp segments. Results on IFN- γ inducibility similar to the one shown using HeLa was also obtained in WEHI-3 cells (not shown). Data evaluation and symbols are as in Fig.1.

A 53 bp fragment containing the X box and additional upstream sequences is sufficient for B-cell specific and IFN- γ induced expression

To further characterize the B-cell specific and IFN- γ responsive sequences we cloned oligonucleotides containing overlapping segments of the E β promoter in one or three copies, immediately upstream of the human $\alpha 2$ globin minimal promoter (aGXCAT) or the SV40 early promoter (pL51CAT) (18). We have reported previously that these minimal promoters do not respond to IFN- γ and are unable to direct CAT activities unless enhancer sequences are added (18). All the constructs were used in transient transfection experiments in Raji cells or in HeLa cells in the presence or absence of IFN- γ .

The region spanning all the conserved class II boxes of the E β gene, from -77 to -207, is able to confer IFN- γ inducibility to the minimal $\alpha 2$ globin promoter (Fig. 2; see also ref. 18). The same restriction fragment is a strong B cell specific enhancer (Fig. 2), comparable to the intact E β promoter. Interestingly, a 53 bp oligonucleotide missing the downstream Y box but containing the X box region and upstream DNA is also active in both respects (-162/-110, 1X or 3X; Fig. 2). However, if the upstream DNA is also truncated, the remaining X-box region is unable to confer IFN- γ induction, although it shows a residual 3 fold enhancer effect, exclusively in B cells (-146/-107; Fig. 2). Most oligonucleotides containing sequences exclusively upstream of the X box do not show enhancer activity in B cells or IFN- γ induction in HeLa cells, and even reduce the activity of the $\alpha 2$ globin promoter in resting HeLa cells (e.g. -157/-131; Fig. 2). Nevertheless, this apparently neutral or even negative region, when combined in *cis* with the X box region, strengthens the B-cell specific expression and, most

notably, reconstitutes the IFN- γ inducibility which the X box region by itself lacks. Therefore, we will refer to this region upstream of the X box as a Cytokine Response Sequence (CRS). The results imply a cooperative interaction between these two domains of the E β promoter (see Discussion).

The Y box in isolation is a weak constitutive element in every cell type tested (-109/-77; Fig. 2). The combination of X and Y has an enhancer activity in class II positive cells and in HeLa cells, but not in other class II negative cells (Fig. 2 and data not shown). No induction with IFN- γ is observed with the X plus Y combination, in agreement with our previous results.

Site directed mutagenesis reveals multiple *cis*-regulatory elements in the E β promoter

The deletion and reconstitution analysis emphasized the role of the class II boxes as well as the involvement of additional upstream elements in the regulation of the E β gene. To analyze the functional role of these elements more precisely, we used the site directed mutagenesis method to introduce three or six bp mutations in the E β regulatory region (shown at the bottom of Fig. 3), in the context of a construct bearing sequences of the E β promoter up to -200 bp. The resulting constructs were transfected into HeLa and B cells (Raji and A20). CAT activities were determined for basal level expression in B lymphoid cells and untreated or IFN- γ treated HeLa cells. Fig. 3 presents the results.

Mutations M9 and M10 which disrupt the X box have a strong detrimental effect in B-cell specific expression (Raji and A20; Fig. 3C) and abolish IFN- γ induction in HeLa cells; (Fig. 3A). Another mutation at the 5' end of X box (M8) also reduces both activities, but does not eliminate inducibility (Fig. 3A). Mutations or deletions in the Y box of other class II genes result in loss of expression; the corresponding mutation M12 in the E β promoter severely affects B cell activity, but is still weakly inducible by IFN- γ . An H box mutation (M2) only partially affects IFN- γ inducibility and strongly inhibits B-cell specific expression.

Of the mutations spanning the region between ISR₂ and the X box, mutation M7 preferentially decreases IFN- γ response, as compared with its effect on B lymphoid expression. Of the two other such mutations M5 and M6, only the latter decreases IFN- γ response and B lymphoid activity in A20 but not in Raji cells. These results defined a region centered on mutation M7 (designated Interferon Stimulated Region 3—ISR₃) which is active only in the presence of ISR₁ and ISR₂ as shown by the 5' deletions. The ISR₂ mutations (M3 and M4) significantly decrease induction by IFN- γ in HeLa cells but have little or no effect on B cell specific expression.

Fig. 3B, D shows the results of CAT activities after transfection of a series of 6 bp site directed mutations (MA, MB, MC, MD, ME) spanning the ISR₁, H and ISR₂ regions. Two mutations that alter sequences within or 5' to the H box proper (MB, MC) have little or no effect on B cell activity and IFN- γ responsiveness. Mutations MA, MD and ME strongly affect both B cell and IFN- γ expression patterns. Interestingly, ME combines M3 and M4 which when studied in isolation affect IFN- γ response only. Results similar to those in HeLa were obtained in WEHI-3 cells with the exception of mutations M2 and M7 which marginally affected IFN- γ inducibility and may reflect species specific differences.

These results identify multiple elements within CRS and the X box, which appear to be involved in B cell specific expression,

Figure 3. Effect of site directed mutations within the functionally critical 5' region of E β . The position of mutations is indicated by brackets above or below the sequence shown at the bottom. The activity of three nucleotide (panels A, C) or six nucleotide (panel B, D) mutants was compared to the wild type (wt) construct in HeLa (panels A, B), untreated (filled squares) or following IFN- γ treatment (open squares) and B lymphoid A20 (filled squares) and Raji (open squares) cells (panels C and D).

IFN- γ induction or both. Even the relatively coarse 6 bp substitutions result at most in quantitative rather than qualitative inhibition, suggesting redundancy of the regulatory elements. Nevertheless, the results demonstrate for the first time that a class II gene promoter (E β) is composed of distinct elements which are either used in common (i.e. X box), preferentially in B cells for constitutive expression (i.e. H and Y box), or in HeLa cells after treatment with IFN- γ (i.e. ISR₂ and ISR₃/X junction). Taken together with the results of the 5' deletions and oligonucleotide reconstitution analysis, the results clearly indicate that class II gene transcription is regulated by a combinatorial mechanism. This is exemplified by the observation that each box in isolation is practically inactive. Only when the CRS and X box are combined is a strong enhancer recovered, which is further strengthened by addition of the Y box.

Titrateable *trans*-acting factors mediate IFN- γ response of the E β promoter

We employed an *in vivo* competition assay (35) to detect the presence and function of *trans*-acting factors responsible for the IFN- γ induction of class II genes in HeLa cells. In this assay, a constant amount of an E β promoter-CAT reporter plasmid is cotransfected with increasing amounts of competitor plasmids bearing multiple copies of E β promoter segments in pUC19. The total amount of transfected DNA is kept constant by the addition of pUC19. Typical measurements of CAT activity are shown in Fig. 4A. Increasing amounts of a competitor plasmid containing the combined CRS and X box region strongly decrease the IFN- γ induction of the E β promoter, without significantly affecting the basal level of expression. Plasmids carrying the X

Figure 4. *In vivo* competition (panels A and B) and saturation kinetics (panels C and D) in HeLa cells. Effect of increasing amounts of pUC19 carrying multiple copies of the -162 to -110 (squares) or the -137 to -107 bp (triangles) regions on the expression levels of the cotransfected -630 Eβ CAT (panel A) and -620 H-2K^b CAT (panel B) reporter plasmids. Results are given as % of the uncompeted control (vertical axis) vs molar excess of competitor sequences (horizontal axis) in the presence (filled symbols) or absence (open symbols) of IFN-γ. Saturation of activity of the above Eβ and H-2K^b (panel C and D respectively) reporters in the presence (filled symbols) or absence (open symbols) of IFN-γ.

box (Fig. 4A) or the CRS (not shown) alone are very weak competitors of either basal or inducible expression. The specificity of the competition experiments is demonstrated by the observation that competitors encompassing the Y or TATA boxes are inactive (not shown). These results are in agreement with the *cis*-element analysis confirming that cooperative interactions between the CRS and the X box are required for full IFN-γ inducibility of the Eβ promoter. Eα reporter constructs or oligonucleotide competitors are equally effective in cross-competition studies (not shown), indicating that at least one limiting regulatory factor is shared between the Eα and Eβ genes. Jameson *et al.* (36) have reached similar conclusions using the cAMP responsive chorion gonadotrophin α promoter.

We then performed titration experiments by transfecting increasing amounts of reporter plasmid and assaying the CAT activity from cells treated or untreated with IFN-γ. As shown in Fig. 4C, expression of the Eβ promoter in the presence of IFN-γ reaches a plateau at DNA concentrations lower than those required for saturation of the basal levels of expression. This effect is consistent with higher affinity and/or limiting levels of the DNA binding factors that are involved in IFN-γ induced as compared to basal transcription.

To determine the nature of factors involved in other IFN-γ inducible genes, we have used the same approach to examine whether factors mediating MHC class I and class II induction might be related. This study uses the H-2K^b CAT, which is

Figure 5. Nuclear factor binding of the -207 to -77 bp fragment of Eβ. The C_B, X, Y and non-specific (NS) complexes are indicated on the left. (A) C_B activity in Raji and Ramos cells is shown in the absence of following TNF-α treatment (+) for 6 hrs. (B) Competition analysis. Complex formation is shown in the absence (NO) or the presence of 10 or 20 fold molar excess of self competitor (S10x, S20x). All other competitors were used at 50 fold molar excess and include: a 20 bp long random oligonucleotide (NS), the Eβ X box (-138 to -110 bp), ISR₂+X (-145 to -117 bp), YEα (-83 to -42 bp), X+YEα (-95 to -45 bp), and Y or X+Y of Eβ (-109 to -132 to -77 bp respectively).

IFN-γ inducible in HeLa cells (37). Surprisingly class II competitors effectively decrease IFN-γ response from the class I promoter (Fig. 4B) in a manner similar to the autologous

Figure 6. Nuclear factors that bind at the region 5' to the X box correlate with B lymphoid and Interferon regulated promoter activity. Binding patterns of the -170 to -127 bp CRS probe (panel A), the -170 to -156 bp ISR₁ (panel B), the -162 to -143 bp H+ISR₂ (panel C) and the -145 to -125 bp ISR₃ (panel D) probes (see also Fig. 7B). The source of extracts, free probe (F), various complexes and Interferon treatment are indicated. Competition analysis (panel E) on the ISR₁ and ISR₃ probes was carried out using a 50× excess of the cold competitor as indicated. The sense sequence of the double stranded ISG54-ISRE (38) competitor is: -109 TCACTTTCTAGTTTCACTTTCCCTTTTGTA -80.

combination. Reverse experiments using the Interferon Control Sequences (ICS) of H-2K^b showed that this element is also active as competitor against the E β -CAT plasmid and therefore verify that titration of factors is targeted to the Interferon Responsive Elements (data not shown). Furthermore, titration studies show that the activity of the class I promoter (Fig. 4D) has a saturation pattern similar to that of the E β promoter. These results strongly suggest that some critical factor(s) for IFN- γ regulation are common between the class I and class II genes for IFN- γ regulation.

The E β regulatory domain is recognized by a B cell specific nuclear factor complex

In order to correlate the functional properties of the E β promoter with nuclear factor binding, we carried out Electrophoretic Mobility Shift Assays (EMSA) using as probes various regions of the E β promoter and nuclear extracts from diverse cell types. In initial experiments the -207 to -77 region of the E β gene was used as probe in EMSA with nuclear extracts prepared from the human B lymphoid cell lines, Raji and Ramos. With all extracts a complex pattern was observed, including the previously characterized X (X1 and X2) and Y (Y1 and Y2) box binding complexes (reviewed in ref.3). A slow migrating complex, C $_B$ (Fig. 5A and 5B) is present in Raji and inducible in Ramos cells upon treatment with TNF- α . This complex is very weak in HeLa and WEHI-3 cells, where it is not affected by cytokines (not shown). Thus, the abundance of C $_B$ correlates with the class II expression phenotype of B lymphoid cells only. The DNA sequences involved in the formation of this complex were

Figure 7. Methylation Interference determines distinct nucleotide protection patterns of constitutive and Interferon stimulated nuclear factors that recognize elements within the CRS. (A) Protection patterns of the indicated bound (B) form of the various nuclear complexes on the sense (s) or antisense (a) strands run in parallel with the free (F) probe used. **(B)** Summary of protection patterns of the constitutive C1 (open symbols) and the three Interferon stimulated factors (filled symbols).

Figure 8. Summation of functional regions and nuclear factors that bind to the E β 5' regulatory proximal region (CRS and X). The elements determined by functional analysis are outlined. Nuclear factors in their relative binding topology are shown schematically. Common factors are shown in white except those recognizing the positive regions H and X (black). Two lymphoid specific factors (one present in B and the second in both B and T lymphoid cells) that bind in the ISR₂ are shaded. The factors activated by IFN- γ are indicated by an asterisk.

identified by competition experiments using various segments of the E β or E α promoter. As shown in Fig. 5B, as little as 10 fold molar excess of a restriction fragment identical to the probe competes for the formation of the C_B complex. When individual boxes are used as competitors at the same molar excess no competition is observed. A 50 fold molar excess of the E β X + Y box oligonucleotide (−132 to −107) competes efficiently; the corresponding region of E α is equally effective. Additional, competition experiments showed that C_B is competed in the order self > X + Y > X > ISR₃ + X > > Y; these competitors have uniform ability to compete for the X (X1 and X2) and Y (Y1 and Y2) box binding proteins (Fig. 5B). Methylation interference analysis showed that C_B makes contacts at the X box (not shown). The very slow mobility of the C_B complex and the fact that its formation is most efficiently inhibited by

competitors containing all the conserved boxes indicate that the complex represents multiple DNA binding factors centered at the X box (see Discussion).

Ubiquitous, B cell specific and IFN- γ inducible protein complexes recognize distinct elements within the CRS

To analyse in more detail the protein-DNA complexes in the E β promoter we synthesized various oligonucleotides corresponding to the previously identified regulatory regions (-170 to -127 bp). Initially, an oligonucleotide corresponding to the entire CRS region revealed a complex pattern of ubiquitous or B cell specific (indicated by a dot (.)) nuclear binding activities (Fig. 6A). To facilitate the analysis of the CRS binding factors, smaller oligonucleotides encompassing the ISR₁, ISR₂+H and ISR₃ regions were tested in EMSA and methylation interference experiments. Data are presented in Figs. 6 and 7, and summarized in Fig. 8. An oligonucleotide containing the ISR₁ region is the target for a complex that is present in both Class II positive and Class II negative cells (Fig. 6B). Interestingly, the abundance of this complex is dramatically increased in HeLa cells after treatment with IFN- γ or IFN- α (Fig. 6B). We designated this complex as Interferon Stimulatory Factor-1 (ISF₁). Nuclear extracts prepared from HeLa cells treated with IFNs for various times showed that ISF₁ binding activity is maximally induced within the first hour of IFN treatment and remains constant after 12 hours of treatment. New protein synthesis is not required, since cyclohexamide does not interfere with the induction of ISF₁ binding activity (data not shown). To establish which nucleotides are critical for the formation of the ISF₁ complex we performed methylation interference analysis. Methylation of G residues at positions -157, -163, -167 in the coding strand and at -159, -166, -169 in the non-coding strand interfere with ISF₁ binding on ISR₁ (Fig. 7). One of these G residues (-163) is substituted in the 6-nucleotide mutation, MA, which results in a 5 fold decrease in IFN- γ induced expression of the E β gene (Fig. 3).

When an oligonucleotide containing the H+ISR₂ region was allowed to interact with similar nuclear extracts two complexes were observed. A ubiquitous DNA binding activity (H) with slow mobility (Fig. 6C) makes contacts at the H box as identified by methylation interference analysis (not shown). Complex ISF₂ is more abundant in B cells compared to uninduced HeLa but is rapidly induced after treatment with IFN- γ or IFN- α . Methylation interference analysis revealed that ISF₂ contacts the E β promoter through G residues at -141, -145, -148 and -149 in the non coding strand and -146, -151, -152 in the coding strand. Interestingly, ISF₂ and H box binding activities recognize overlapping sites suggesting mutually exclusive binding (see Discussion). Site directed mutagenesis in the ISF₂ binding site (MD and ME, Fig. 3) reduces both induction by IFN- γ and B-cell specific expression.

Finally an oligonucleotide that includes ISR₃ and extends to the boundaries of ISR₂ and X box, give a complex binding pattern (Fig. 6D). In unstimulated HeLa cells two constitutive binding activities (C1 and C2) are present. The same complexes are observed in Raji or myeloma (XS63) cells. The C1 complex is present in all cell types tested while the C2 activity is very low or absent from Jurkat and LMTK⁻ cells. In B cells, which express the class II genes constitutively, two additional complexes are observed suggesting a role for these proteins in B cell specific expression (B1 and B2, Fig. 6D). Competition analysis verified (not shown) the specificity of those complexes. Treatment of HeLa cells with IFNs results in the induction of a new complex

designated ISF₃ (Fig. 6E). ISF₃ occupies a 3' sub-site of the C1 complex (filled and open circles respectively, Fig. 7) and contacts the DNA from the non-coding strand only, since its binding interferes with methylation of G residues at -129 and -133. The mutation M7 which is located within the ISF₃ site has a strong negative effect on induction by IFN- γ (Fig. 3). The X box binding proteins (as shown by many studies including our unpublished data) also contact the -129 G residue in the non-coding strains in common with the ISF₂ protein. From the above results, we conclude that ISF₁, ISF₂ and ISF₃ inducible binding correlates with stimulation of the E β gene by IFN- γ . To examine the possibility that the ISF complexes may be interrelated we performed gel shift competition experiments by using as probes ISR₁ or ISR₃ regions and various cold oligonucleotides as competitors, with nuclear extracts prepared from HeLa cells induced for one hour with IFN- γ (Fig. 6E). The ISF₃ binding activity is dramatically induced with IFN- γ and is competed by the homologous oligonucleotide. Interestingly, oligonucleotides containing the ISR₁ and H+ISR₂ region also compete for the formation of the ISF₃ complex. A non-specific (NS) oligonucleotide do not compete for ISF₃ formation. As expected, the ISF₁ complex formed on ISR₁ is also competed by ISR₃ and H+ISR₂ oligonucleotides (Fig. 6E), but not by a non-specific oligonucleotide or an X box competitor (not shown). Surprisingly, effective competition for ISF₁ formation is observed with an oligonucleotide corresponding to the Interferon Stimulated Response Element (ISRE) of the ISG54 promoter (38). The above results as well as results from cross-competition experiments using the ISRE as probe and E β competition oligonucleotides (not shown) strongly suggest that similar factors bind to the class II CRS region and other interferon stimulated genes. This apparent sharing of factors is consistent with the *in vivo* cross-competition experiments described above (Fig. 4). Thus, both our *in vitro* binding and *cis*-element analyses show that the CRS region is the target for three related IFN- γ inducible proteins, which recognize the ISR₁, ISR₂ and ISR₃ domains and may play a crucial role in regulated expression of the E β gene (see Discussion).

DISCUSSION

Expression of MHC class II proteins is subject to complex tissue restricted, developmental and cytokine mediated regulation (39). We have shown previously that the 5' flanking region of the E α gene carries multiple control elements, including IFN- γ response elements, that act in a cell type specific manner to regulate promoter activity (18). Here we have studied in detail the proximal 5' flanking region of E β . We have analyzed both the *cis*-elements contained therein and nuclear factors that bind to them in cells of various phenotypes. Our data demonstrate that, besides the earlier described X and Y boxes, multiple elements and factors operate in the region immediately 5' of the E β X box.

The functional importance of various DNA elements for class II gene expression has been documented in earlier studies that emphasized the IFN- γ response (13,14,17,18,19,24,34). Many studies, including our own, have demonstrated that the X and Y boxes are important for both B lymphoid and IFN- γ regulated class II gene expression. Here we show that site directed mutations in either the X or Y boxes of the E β gene are detrimental for B lymphoid activity. The IFN- γ response is eliminated by a mutation in X but not in Y. However, X or Y alone or in combination are unable to confer full B lymphoid and IFN- γ regulated expression upon a heterologous promoter;

the Y or the X plus Y combination have a moderate enhancer activity in various cell types, whereas the X box alone is a weak B-cell lymphoid element. These results are in agreement with the view that the X box is a necessary but not sufficient element. In line with this, earlier reports have shown that the presence of X binding factors does not correlate with B cell specific enhancer activity (40,41,42).

Although by itself the X box of the E β gene only has weak B lymphoid enhancer activity, addition of ~35 bp sequences 5' to it strengthen this activity and also result in IFN- γ regulation. These results are in agreement with earlier studies on the E α (18), DR α (19), and DQ β promoters (43). Thus, the region 5' to the X box, which we designate as the cytokine response sequence (CRS), has a dual role: it serves as an important domain of the enhancer responsible for cell-specific constitutive expression as well as functioning as the minimum region required for IFN- γ response. An adequate regulatory region is generated by the cooperative action of two weak elements combined in cis, namely the X box and CRS.

Site directed and 5' deletion mutants in the CRS define subelements that preferentially regulate B lymphoid or IFN- γ response. One such element is the H box. Linker scanning mutations and/or binding assays have suggested that a region including the H box (also called S, W or Z in different gene contexts) is important for regulating class II expression of the DQ β (13), DR α (24,44), DP α (45) as well as the A α (23,34) and E α genes (20). The importance of this region of E β (designated W in that study) in combination with both the X and Y boxes for proper regulation, has been pointed out previously (15,16), in cell lines different than those of the present study. Site directed mutations show that the contribution of the H box to IFN- γ response is smaller than its role in B lymphoid cells. In addition, our results show that sequences both 5' and 3' to the H box (ISR $_1$ and ISR $_3$ respectively) as well as overlapping (ISR $_2$) with it are critical for IFN- γ response and B cell activity. Interestingly, the binding site of a ubiquitous nuclear complex that binds the H box of E β overlaps with that of the IFN stimulated factor ISF $_2$ in the ISR $_2$ region (see below).

Our results define three additional elements in the CRS, namely ISR $_1$, ISR $_2$ and ISR $_3$, and cognate factors (Fig. 8). The 5' deletion analysis demonstrates negative functions of ISR $_1$. For ISR $_2$ this analysis demonstrates a positive function in B cells only. The substitution mutations indicate that the functions of these elements may be more complex. Thus, clustered 6 bp substitutions in both ISR $_1$ and ISR $_2$ strongly decrease B cell and IFN- γ induced expression; 3 bp substitutions in ISR $_1$ affect IFN- γ response only, indicating distinct protein-DNA interactions in the constitutive and cytokine mediated mode. We note the presence of a TGCA motif in both ISR $_1$ and ISR $_2$ that is included in the region protected by both ISF $_1$ and ISF $_2$. Similar motifs are also present immediately 3' of the H box of the E α and DR α and may represent functionally important elements for IFN- γ regulation of those promoters.

In spite of extensive functional analysis of various class II promoters, little is known about factors involved in IFN- γ mediated activation. An array of constitutive and IFN inducible factors bind to the ISR $_1$, ISR $_2$ and ISR $_3$ regions, in correlation with the phenotype of the various mutants studied. We demonstrate here that the activity of at least three of these factors, ISF $_1$, ISF $_2$ and ISF $_3$, is regulated by either IFN- α or IFN- γ . Two of these factors recognize the TGCA motif in the 5' critical boundaries for IFN- γ response (ISR $_1$ and ISR $_2$) and the third binds in the ISR $_3$. Although the latter sequence does not include

a TGCA motif, cross competition analysis indicates that the three ISFs are very similar, and may belong to the same protein family. Additional data (not shown) suggest that these factors are not related to the X box binding factor as shown for the DR α promoter (46). The functional significance of the resemblance between ISR $_3$ and the J element of DP α and DQ β (47) remains to be established.

ISR $_3$ resembles the conserved ISRE motif of various unrelated IFN regulated genes. Indeed, our competition studies indicate unexpected cross recognition between the E β regulatory region and the ISREs of ISG54 and GBP (48) genes. Previously, various constitutive of IFN induced factors that bind to ISREs have been identified and subjected to extensive analysis (38,49-52). However, the ISREs respond to both IFN- α and IFN- γ (53,54). Their possible relationship to class II elements that usually are thought to respond solely to IFN- γ has not been investigated previously. The picture is further complicated by the fact that usually IFN- α antagonizes class II expression. Although it is not clear if this is an effect at the transcriptional level, it represents a point of convergence for the action of both types of IFNs. Our *in vivo* and *in vitro* results suggest that elements and factors important for IFN- γ regulation of the E β promoter, have binding (but probably not functional) similarities with the ISRE and its cognate factors.

Additional factors that are not affected by IFN treatment recognize a region that overlaps with the ISF $_3$ binding site and extends further 5' to it. Two of these factors are abundant in diverse cell types, while the other two are present only in B-lymphoid cells. One of the lymphoid factors is missing in T-lymphoid cells and may be a candidate for a positive promoter regulator.

Trans-acting factors that regulate class II gene expression have been detected using somatic cell hybrids (47,55-57), but factors binding to the X and Y boxes have not shown clear correlation with the class II expression phenotypes (12,56). We show here that multiple copies of the CRS plus X region of E β are able to eliminate induction, presumably by titrating factors involved in IFN- γ response. The effect does not appear to involve general transcription factors, since no competition is seen for basal expression of heterologous promoters or class II promoters lacking the cognate sequences, or with competitors bearing sequences from -71 to +14 bp of E α (not shown). Interestingly the E β competitor is able to specifically reduce IFN- γ responsiveness of the H-2K b class I promoter, which is mediated by well characterized ISREs (58). This effect, in combination with the results of competition studies on nuclear factor binding, lead us to propose that at least one common factor critically involved in interferon regulation recognizes sites in the E β CRS and the ISRE. It may be pertinent that, according to recent studies on mutant HeLa cells, the IFN- α/β and IFN- γ systems share some signal transduction components (59).

The activity of competitors bearing CRS plus X, contrasting with the inactivity of either element alone, indicates synergistic interaction between factors with different recognition sites. Similar protein interactions may lead to formation of nuclear complex C $_B$ in class II positive cells. A complex that involves the same DNA fragment and is correlated with constitutive and cytokine mediated regulation of class II genes has been described in mouse cells (15). Our present results using both mouse and human cells further show that endogenous class II gene activation and upregulation of this complex are not always correlated. One interpretation is that alternate pathways to activate or sustain constitutive expression operate in different cellular backgrounds.

At present, a scheme to summarize the organization of the proximal regulatory region of the E β gene includes ubiquitous, cell type specific and cytokine response elements (Fig. 8), operating in distinct cellular environments. The data point to important regulatory roles of the CRS and its cognate factors in the expression of class II genes, both in B lymphoid cells and in response to cytokines. A fuller understanding of class II molecular regulation requires cloning and characterization of the pertinent factors and elucidation of their interactions. RF-X (60) and XBP (61) have been cloned already. Recently we have obtained cDNA clones, encoding proteins that recognize sequences within the CRS as well as the related purine motifs within the 5' regulatory region of the E α gene. We anticipate that detailed molecular analysis of those clones will prove valuable towards understanding the subtleties of class II gene regulation.

ACKNOWLEDGEMENTS

We thank Dr F. Kafatos and Dr J. Strominger for critical reading of the manuscript and their suggestions, Dr R. Accolla for making available to us the RJ2.2.5 and its parental Raji cell line. We also thank Ms G. Houlaki for her expert editing and text formatting assistance. This work was supported by the Greek Secretariat General for Research and Technology.

REFERENCES

- Schwartz, R. (1985) *Ann. Rev. Immunol.*, 3: 237–261.
- Blackman, M., Marrack P., and Kappler J. (1989) *Science*, 244: 214–217.
- Benoist, C., and Mathis D. (1990) *Ann. Rev. of Immunol.*, 8: 681–715.
- King, D., and Jones P. (1983) *J. Immunol.*, 131: 315–318.
- Noelle, R., Krammer H., Ohara J., Uhr J., and Vitetta E. (1984) *Proc. Natl. Acad. Sci. USA*, 81: 6149–6153.
- Bottazzo, G., Todd I., Mirakian R., Belfore R., and Pujol-Borrell R. (1986) *Immunol. Rev.*, 94: 137–169.
- Pujol-Borrell, R., Todd I., Doshi M., Bottazzo G., Sutton R., Gray D., Adolf G., and Feldman M. (1987) *Nature*, 326: 304–306.
- Athanassakis-Vassiliadis, I., Thanos D., and Papamatheakis J. (1990) *Eur. J. Immunol.*, 19: 2341–2348.
- Kerr, A., and Trowsdale D. (1985) *Nucleic Acids Res.*, 13: 1607–1621.
- Mathis, D., Benoist C., Williams I.V., Kanter M., and McDevitt H. (1983) *Cell*, 32: 745–754.
- Dorn, A., Fehling J., Koch W., Le Meur M., Gerlinger P., Benoist C., and Mathis D. (1988) *Mol. Cell. Biol.*, 8: 3975–3987.
- Koch, W., Benoist C., and Mathis D. (1989) *Mol. Cell. Biol.*, 9: 303–311.
- Boss, J., and Strominger J. (1986) *Proc. Natl. Acad. Sci. USA*, 83: 9139–9143.
- Dorn, A., Durand B., Marfing C., Le Meur M., Benoist C., and Mathis D. (1987) *Proc. Natl. Acad. Sci. USA*, 84: 6249–6253.
- Finn, P., Kara C., Douhan J., Van T., Folsom V., and Glimsher L. (1990a) *Proc. Natl. Acad. Sci. USA*, 87: 914–918.
- Finn, P., Kara C., Tran Van T., Douhan III J., Boothby M., and Glimcher L. (1990b) *EMBO J.*, 9: 1543–1549.
- Sherman, P., Basta P., Moore T., Brown A., and Ting J. (1989) *Mol. Cell. Biol.*, 9: 50–56.
- Thanos, D., Mavrothalassitis G., and Papamatheakis J. (1988) *Proc. Natl. Acad. Sci. USA*, 85: 3075–3079.
- Tsang, S., Nakanishi N., and Peterlin M. (1988) *Proc. Natl. Acad. Sci. USA*, 85: 8598–8602.
- Viville, S., Jongeneel V., Koch W., Mantovani R., Benoist C., and Mathis D. (1991) *J. Immunol.*, 146: 3211–3217.
- Widera, G., Burkly L., Pinkert C., Botter E., Cowing C., Palmiter R., Brinster R., and Flavell R. (1987) *Cell*, 51: 175–187.
- Servenius, B., Rask L., and Peterson P. (1987) *J. Biol. Chem.*, 262: 8759–8766.
- Freund, Y., Dedrick R., and Jones P. (1990) *J. Exp. Med.*, 171: 1283–1299.
- Tsang, S., Nakanishi M., and Peterlin M. (1990) *Mol. Cell. Biol.*, 10: 711–719.
- Graham, F., and Van de Eb A. (1973) *Virology*, 57: 456–467.
- Hall, C., Jacob P., Ringold G., and Lee F. (1983) *J. Mol. Appl. Genet.*, 2: 101–107.
- McGregor, G., and Caskey D. (1989) *Nucleic Acids Res.*, 17: 23–65.
- Gorman, C., Moffat F., and Howard B. (1982) *Mol. Cell. Biol.*, 2: 1044–1051.
- Mercola, M., Gorman J., Mirrl C., and Kalame K. (1985) *Science* 227: 266–270.
- Taylor, J. W., Ott J., and Eckstein F. (1985) *Nucleic Acids Res.*, 13: 8765–8785.
- Dignam, J., Lebovitz M., and Roeder R. (1983) *Nucleic Acids Res.*, 11: 1475–1489.
- Singh, H., Sen R., Baltimore D., and Sharp P. (1986) *Nature*, 319: 154–158.
- Maxam, A., and Gilbert W. (1980) *Methods in Enzymol.*, 65: 499–560.
- Dedrick, R., and Jones P. (1990) *Mol. Cell. Biol.*, 10: 593–604.
- Schöler, H., and Gruss P. (1984) *Cell*, 36: 403–411.
- Jameson, L., Deutsch P., Gallagher G., Jaffe R., and Habener J. (1987) *Mol. Cell. Biol.*, 7: 3032–3040.
- Kimura, A., Israël A., Le Bail O., and Kourilsky P. (1986) *Cell*, 44: 261–272.
- Levy, D., Kessler D., Pine R., Reich N., and Darnell J. (1988) *Genes Dev.*, 2: 383–393.
- Glimcher, L., and Kara, C. (1992) *Ann. Rev. Immunol.*, 10: 13–49.
- Koch, W., Candéas S., Guardiola J., Accolla R., Benoist C., and Mathis D. (1988) *J. Exp. Med.*, 167: 1781–1790.
- Miwa, K., Doyle C., and Strominger J. (1987) *Proc. Natl. Acad. Sci. USA*, 84: 4939–4943.
- Sloan, J., and Boss J. (1988) *Proc. Natl. Acad. Sci. USA*, 85: 8186–8190.
- Sugawara, M., Ponath P., Shin J., Yang Z., and Strominger J. (1991) *Proc. Natl. Acad. Sci. USA*, 88: 10347–10351.
- Cogswell, J., Austin J., and Ting J. (1991) *J. Immunol.*, 146: 1361–1367.
- Yang, Z., Banerji J., Yi L., Wessendorf L., and Strominger J. (1990) *Proc. Natl. Acad. Sci. USA*, 87: 9226–9230.
- Cogswell, J., Basta P., and Ting J. (1990) *Proc. Natl. Acad. Sci. USA*, 87: 7703–7707.
- Stuart, M., Yarchover J., and Woodward J. (1989) *Cell. Immunol.*, 122: 391–404.
- Decker, T., Lew D., Mirkovich J., and Darnell J. (1991) *EMBO J.*, 10: 927–932.
- Cohen, B., Peretz D., Vaiman D., Benech P., and Chebath J. (1988) *EMBO J.*, 7: 1411–1419.
- Dale, T., Rosen J., Guille M., Lewin A., Porter A., Kerr I., and Stark G. (1989) *EMBO J.*, 8: 831–839.
- Levy, D., Kessler D., Pine R., and Darnell J. (1989) *Genes Dev.*, 3: 1362–1371.
- Rutherford, M., Hannigan G., and Williams B. (1988) *EMBO J.*, 7: 751–759.
- Liew, D., Decker T., Strehlow I., and Darnell J. (1991) *Mol. Cell. Biol.*, 11: 182–191.
- Reid, L., Brasnett A., Gilbert C., Porter A., Gewert D., Stark G., and Kerr I. (1989) *Proc. Natl. Acad. Sci. USA*, 86: 840–844.
- Accolla, R., Carra G., and Guardiola J. (1985) *Proc. Natl. Acad. Sci. USA*, 82: 5145–5149.
- Calman, A., and Peterlin M. (1988) *Proc. Natl. Acad. Sci. USA*, 85: 8830–8834.
- Yang, Z., Accolla R., Pious D., Zegers B., and Strominger J. (1988) *EMBO J.*, 7: 1965–1972.
- Israel, A., Kimura A., Fournier A., Fellous M., and Kourilsky P. (1986) *Nature*, 322: 743–746.
- Loh, J., Chang C.-H., Fodor W., and Flavell R. (1992) *EMBO J.*, 11: 1351–1363.
- Reith, W., Barras E., Satola S., Kobr M., Reinhart D., Herrero Sanchez C., and Mach B. (1989) *Proc. Natl. Acad. Sci. USA*, 86: 4200–4204.
- Liou, H.-C., Boothby M., Finn P., Davidson R., Nabavi N., Zeleznik-Le N., Ting J., and Glimcher L. (1990) *Science*, 247: 1581–1584.