Repression of I-Aβ Gene Expression by the Transcription Factor PU.1*

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The PU.1 protein is an ets-related transcription factor that is expressed in macrophages and B lymphocytes. We present evidence that PU.1 binds to the promoter of the I-Ab gene, i.e. a PU box located next to the Y box. Transfection of PU.1 in B lymphocytes or in interferon-γ-treated macrophages represses I-Ab gene expression. The inhibitory effect of PU.1 was obtained with the DNA binding domain of the protein, but not with the activation domain. Using the gel shift retardation assay we found that in vitro transcribed/translated NF-YA and NF-YB bind to the Y box of the I-Ab promoter. When PU.1 was added to the assay, a supershifted DNA band was found, indicating that PU.1 and NF-Y proteins bind to the same DNA molecule. We conclude that I-Ab gene expression is repressed by PU.1 binding to the PU box domain.

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The abbreviations used are: MHC, major histocompatibility complex; CAT, chloramphenicol acetyltransferase; IFN-γ, interferon-γ; bp, base pair(s).

2 A. Celada, S. McKercher, and R. A. Maki, submitted for publication.

3 C. Van Beveren, M. J. Klionsz, A. Celada, S. R. McKercher, and R. A. Maki, submitted for publication.
(Dounce). The crude nuclei were extracted at 4°C with a buffer containing 0.4 M NaCl for 30 min with continuous stirring followed by centrifugation at 10,000 × g for 1 h. The supernatant was dialyzed, and extracts were cleared by centrifugation at 10,000 × g for 10 min. The supernatant (protein concentration of 1-5 mg/ml) was frozen in aliquots and stored at −70°C.

Transfection Assays—A20–2J (mouse B lymphocyte) cells were transfected in suspension in 1 ml using the DEAE-dextran method as described previously (30). 15 μg of the experimental DNA and 3 μg of the plasmid, pCH110, a β-galactosidase expression vector used to measure transfection efficiency and 30 μg of DEAE-dextran were added to each plate. In the cotransfection experiments with PU.1 or the retinoic acid receptor, 2 μg of the indicated plasmid were used (30). Chloramphenicol acetyltransferase (CAT) assays were performed using a standard protocol (31). Briefly, cells were isolated 48 h after the DNA was added and subjected to three freeze-thaw cycles in dry ice/ethanol at 37°C. The extracts (10–60 μl standardized by β-galactosidase activity) were incubated with [35S]chloramphenicol and acetyl coenzyme A for 60 min at 37°C, incubated for 7 min at 60°C, followed by extraction with ethyl acetate. The samples were dried and resuspended in 20 μl of ethyl acetate for thin layer chromatography. Acetylation was quantified using a radioanalytic imaging system (AMBIS, Inc., San Diego, CA). Each experiment was performed at least three times, and the figures represent the mean and 1 S.D.

Plasmid Constructions—The CAT reporter constructions were made using the KS+SV2CAT vector (32) in which the SV40 enhancer region (Spl to PstI) was removed. The different constructions, KS1-WXY (~124 to ~26) and KS1 mutant (mutation in the Y box as indicated in Fig. 3) containing the I-Aβ promoter, were generated using specific oligonucleotides or by polymerase chain reaction using the plasmid 12.33.2, which contains the I-Aβ promoter (10). The DNA fragments were ligated directionally into Xba1–Smal-digested KS1 vector. DNA fragments used for the gel electrophoresis DNA binding assay were generated by cloning. Double-stranded oligonucleotides were prepared using a DNA synthesizer (380 A; Applied Biosystems Inc., Foster City, CA) and cloned in the BamHI site of the vector, pGEM1. The plasmid containing the gene for the retinoic acid receptor (pECE RARα) was obtained from Dr. M. Pfahl (La Jolla Cancer Research Foundation) (33). The plasmids containing NF-YA and NF-YB as described previously (30) were ligated directionally into Xba1–Smal-digested KS1 vector. DNA fragments used for the gel electrophoresis DNA binding assay were generated by cloning. Double-stranded oligonucleotides were prepared using a DNA synthesizer (380 A; Applied Biosystems Inc., Foster City, CA) and cloned in the BamHI site of the vector, pGEM1. The plasmid containing the gene for the retinoic acid receptor (pECE RARα) was obtained from Dr. M. Pfahl (La Jolla Cancer Research Foundation) (33). The plasmids containing NF-YA and NF-YB were obtained by polymerase chain reaction, using oligonucleotides prepared from the sequence of the plasmid vectors containing NF-YA and NF-YB as described previously.

Finally, PU.1 and the PU.1 binding site (170–260) with the CAT construction were as described previously (13). The plasmid containing the activation domain (75–93) was also as described previously (35). The pECE vector contains the SV40 promoter and enhancer (34). In vitro transcription/translation of the NF-YA, NF-YB, and PU.1 was performed as described by the manufacturer (Promega, Madison, WI). RNA was transcribed using T3 RNA polymerase. The RNA template (2 μg) was added to a rabbit reticulocyte lysate (Promega) to generate [35S]methionine-labeled protein in vitro. Protein production was confirmed by SDS-polyacrylamide gel electrophoresis and autoradiography. Gel electrophoresis DNA binding assays were performed directly in vitro translated product. The relative concentration of protein for NF-Y or PU.1 was measured taking into account the amount of radiolabeled methionine incorporated during the in vitro translation, quantified using an imaging system (AMBIS, Inc., San Diego, CA), and then diluted to an equal relative concentration. In some cases the gels were dried and placed into contact with x-ray film (Kodak XAR-5). Radioactivity was quantified using radioanalytic imaging system (AMBIS, Inc.). For dimethyl sulfate protection assays a 320-bp fragment of the I-Aβ promoter was used according to the protocol previously described (10).

RESULTS

The PU.1 protein was cloned using an expression library (λgt 11) and an oligonucleotide covering the Y box binding site that contains a PU.1 box (GAGGGA). To determine whether the PU.1 protein could bind to the oligonucleotide containing the Y box a 320-bp fragment containing the I-Aβ promoter was used for gel retardation assays. When the in vitro transcription/translated PU.1 protein was added to the 320-bp radiolabeled fragment, gel electrophoresis was performed, and a retarded complex was observed (Fig. 1). This complex was effectively displaced by a 50-fold molar excess of a cold oligonucleotide containing the I-Aβ box. Oligonucleotides that contained the Y box and modified PU.1 core sequence did not compete effectively for binding of the PU.1 protein to labeled probe. These results suggest that PU.1 protein recognized the PU.1 box in a sequence-specific manner.

To delineate the binding of PU.1 on the I-Aβ promoter, we performed methylation protection experiments using the 320-bp fragment (Fig. 2). Binding of the PU.1 to the I-Aβ promoter resulted in the protection of two guanine residues on the upper strand within the core of the PU.1 box. No guanine residue on the lower strand was protected. These results confirmed the data obtained with the gel retardation assay, showing that the PU.1 protein binds to the PU.1 box next to the Y box.

To examine the possible role of PU.1 protein in I-Aβ expression, we attempted to determine whether transcription from the I-Aβ promoter is affected by the PU.1 protein. Using the I-Aβ promoter linked to the CAT gene, we observed a low CAT activity when this construct was transfected into the B cell line, A20–2J. As we were interested in the cell type-specific enhancing activity of the I-Aβ promoter, we linked a 124-bp fragment
of the I-\(\alpha\)\(\beta\) promoter containing the W, X, and Y boxes to the SV40 promoter (Fig. 3), which then gave us a better signal in the CAT assay. This type of construct has been used by others to obtain a more efficient expression of other MHC class II genes, including the I-\(\epsilon\)\(\alpha\) and I-\(\alpha\) genes (35–37). Each CAT construct was cotransfected with the \(\beta\)-galactosidase expression plasmid, pCH110, into the B cell line A20–2J. All CAT values were then normalized to the level of \(\beta\)-galactosidase expression to correct any differences in transfection efficiency. The KS1 vector gave a level of CAT activity of \(<1\%\), while KS1 containing the W, X, and Y boxes gave a CAT activity of 19.5% (Fig. 3). When a mutation of the Y box was made, CAT activity was lowered to 13.1%, suggesting that the Y box plays an important role in the I-\(\alpha\)\(\beta\) gene expression. In fact the Y box contributes 33% of the expression of the KS1-WXY construct. The Y box mutation was designed to disrupt the binding of the NF-Y to the Y box as measured by a gel electrophoresis DNA binding assay (10).

To determine the effect of PU.1 protein on I-\(\alpha\)\(\beta\) expression we cotransfected an expression plasmid of PU.1 into A20–2J cells together with the CAT constructs (Fig. 3). There was a decrease in CAT activity from 19.5% to 11.2% with PU.1 (42% reduction). This suggested that PU.1 abolishes the contribution of the Y box-binding proteins to the transcriptional activity of the I-\(\alpha\)\(\beta\) promoter. The reduction in CAT activity seems to be proportional to the amount of PU.1 transfected. Using 2 \(\mu\)g of PU.1 DNA, the CAT activity was 11.2% while with 1 \(\mu\)g, CAT activity was 16.4%. We also tested the effect of plasmids containing either the binding domain or the activation domain of the PU.1 transcription factor. When the plasmid containing the PU.1 binding site was cotransfected with the CAT constructs, the acetylation dropped to 12.2% (37% reduction). However, the cotransfection of the activation domain did not reduce the CAT activity (18.5% acetylation). As a control, we used a plasmid vector that expresses the retinoic acid receptor. Under these conditions, the levels of CAT activity were not modified (20.0% acetylation). When the Y box was mutated in cells cotransfected with PU.1, the CAT expression activity was not modified: 12.5% versus 11.0% (PU.1), 11.3% (binding domain) and 12.0% (activation domain). Thus, PU.1 was able to repress CAT activity in those constructs containing the Y box sequence. This effect seems to be related to the binding activity of PU.1.

To ascertain whether the suppressive effect of PU.1 was specific to the I-\(\alpha\)\(\beta\) promoter and not due to a general down-regulation of transcription, the effect of the plasmid containing PU.1 was tested on a vector containing the PU.1 binding site. For these experiments, we used a fibroblast cell line that did not express PU.1. The vector pBLCAT2, which contains the CAT gene linked to the thymidine kinase promoter, transfected into fibroblasts produced 1.2% acetylation (Fig. 4). Cotransfection of an expression vector with PU.1 (pECE PU.1) did not modify the basic levels of CAT expression. Only if the pBLCAT vectors contained a PU.1 box in the presence of pECE PU.1 did the CAT activity increase 4-fold, suggesting that PU.1 is a transcription factor enhancing this particular promoter, which contains the binding site of PU.1. Transfection of the KS1-WXY vector produced an acetylation of 38.3%. Cotransfections of PU.1 expressing vector with the KSI-WXY vector reduced the CAT activity in this cell type to 24.2% (37% of the original activity) (Fig. 4).

We also tested the effect of the PU.1 protein on I-\(\alpha\) expression induced by IFN-\(\gamma\). Bone marrow derived macrophages were transfected with the KS1-WXY and incubated with 300 IU/ml murine IFN-\(\gamma\) for 24 h. Under these conditions, IFN-\(\gamma\) induced the expression of mRNA for I-\(\alpha\)\(\beta\) and I-\(\alpha\) surface expression (29). In these cells, the KS1 vector containing the W, X, and Y boxes gave a CAT activity of 1.5%, which rose to 15.3% in the presence of IFN-\(\gamma\) (Fig. 5). The transfection of PU.1 showed an inhibitory activity on IFN-\(\gamma\)-induction of CAT activity (43% reduction for the wild type and 39% for the binding domain). The repressive activity of PU.1 protein on the CAT expression of the KS1-WXY construction is probably mediated through the binding to DNA in the PU sequence, because the binding domain, not the activation domain, reduced the CAT activity. Thus, the PU.1 protein was able to repress CAT activity only when it bound to DNA.

To investigate whether there is a competitive binding between PU.1 and NF-Y, the proteins that bind to the Y box of the I-\(\alpha\)\(\beta\) promoter, that could account for the transcriptional repression of the I-\(\alpha\)\(\beta\) gene, a gel electrophoresis DNA binding assay was performed. Using nuclear extracts prepared from the B cell line A20–2J and a 320-bp probe containing the Y box, a gel electrophoresis DNA binding assay was performed, and a retarded band was found. The addition of a cold oligonucleotide covering the Y box displaced the binding of nuclear factor. However, an oligonucleotide with a mutation of two GG within the Y box (CTGATTGG) was not able to compete for binding, showing that the binding to the Y box is specific. We had previously shown that the nuclear factor that bound to the I-\(\alpha\)\(\beta\) Y box was composed of two components, named factor A and B, which were separated by FPLC using a monoQ column (11). These factors correspond to the proteins NF-YA and NF-YB.2
The genes for NF-YA and NF-YB were expressed in vitro using T7 polymerase to generate RNA and rabbit reticulocyte lysate for the preparation of protein. When added individually to a labeled fragment of DNA containing the Y box, none of the proteins bound well to DNA (Fig. 6). When NF-YA was mixed with NF-YB, however, the complex bound to the DNA containing the Y box with high affinity. A retarded band was also found when we used in the assay transcribed/translated PU.1 or the binding domain of PU.1. The retarded complex DNA-NF-Y proteins was at the same position as the retarded complex with PU.1. This is probably due to the large fragment (320 bp) used as a probe. Competition experiments showed that the retarded bands were specific for the Y box or the PU.1 expression vector. The KS1 constructions were also transfected in the absence or presence of PU.1 expression vector.

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In order to characterize the interaction between PU.1 and NFY proteins and the DNA Y box, we used a 33-bp synthetic oligonucleotide containing this area. When we incubated in vitro transcribed/translated PU.1 or NF-YA + NF-YB proteins with the Y box oligonucleotide, the proteins produced a retarded band (Fig. 7). When we included NF-YA + NF-YB and PU.1 proteins a supershift band was observed. The amount of supershifted DNA was proportional to the amount of PU.1.
was a 320-bp fragment of the I-Aβ proteins. In some cases, PU.1 bound to the same DNA molecule as the NF-YA, NF-YB, or PU.1 proteins. Nuclear extracts were prepared from A20-2J cells. The probe was a 320-bp fragment of the I-Aβ promoter that contained the Y and the PU box. Retarded complexes were detected by autoradiography. The relative concentration of protein for NF-Y or PU.1 was measured taking into account the amount of radiolabeled methionine incorporated during the in vitro translation and was quantitated using an imaging system (AMBIS, Inc.) and then diluted to equal relative concentration.

Fig. 6. Gel electrophoresis DNA binding assay of nuclear extracts or in vitro transcribed/translated NF-YA, NF-YB, or PU.1 proteins. Nuclear extracts were prepared from A20-2J cells. The probe was a 70-bp fragment from a vector in which a 33-bp synthetic oligonucleotide containing the Y box sequence was cloned. A supershift band was found when NF-YA, NF-YB, and PU.1 proteins were added together with the probe.

included in the assay. These data demonstrated that, at least in some cases, PU.1 bound to the same DNA molecule as the NF-YA-NF-YB complex.

To further characterize the interaction between NFY and PU.1 proteins, we quantified the binding activities of the factors, both separately and together (Fig. 8). NF-YA alone bound small amounts of DNA, while NF-YB alone bound little or no DNA. When increasing amounts of NF-YA were added to a constant amount of NF-YB (4 μl), a linear relationship between the amount of NF-YA and protein-DNA complex was observed. Under these conditions, NF-YB was apparently in excess, since even when high amounts of NF-YA were added to the reaction there was no evidence that the percentage of probe bound reached a plateau (Fig. 8). These results indicate that NF-YA alone binds to DNA, but that the affinity between DNA and NF-YA is much greater when NF-YA is associated with NF-YB. There was a significant binding when either PU.1 or the PU.1 binding site were added to DNA (Fig. 8). In the presence of a constant amount of NF-YB there was a linear increase proportional to the amount of NF-YA and PU.1 present in the reaction (Fig. 8). At each concentration in the presence of PU.1 or the PU.1 binding domain, the binding of the NF-YA + NF-YB complex was higher than in the absence of PU.1. In contrast, in the presence of the PU.1 activation domain the binding of NF-YA + NF-YB remained unchanged.

DISCUSSION

Transcription is regulated by gene-specific transcription factors that bind to regulatory elements in gene promoters and enhancers, stimulating the intrinsic basal rate of transcription initiation. In addition, transcriptional repression comes from a set of molecules that inhibit transcription in a gene-specific manner (38, 39). Repressors are of two different types, passive and active. Passive repressors inhibit the effect of positively acting transcription factors by, for example, competing for their DNA binding sites or making inactive complexes. Active repressors possess intrinsic repressing activity and inhibit transcription directly.

Depending of the genes and the tissues, different mechanisms have been implicated in MHC class II gene regulation. Numerous studies have reported factors that exert a negative effect on the transcription of a wide range of genes (40, 41). In some cases, a silencing factor might be operative in suppressing the transcription of class II genes in nonexpressing cells. This is suggested by fusion studies that have demonstrated extinction of class II expression in hybrids of class II-negative and -positive cells (42). The fusion of L929 fibrosarcoma cells with splenic B lymphocytes retained the Eβ gene at the genomic level but none of the clones had any detectable basal or inducible class II message or gene product. A more recent study found reactivation of low levels of class II transcription in plasmacytoma cells and human T cells on transient fusion with B lymphoblastoid cells and, to a much lesser extent, with splenocytes (43). These two studies demonstrated the presence of different mechanisms controlling the tissue specificity of MHC class II gene expression.

In cells that do not express MHC class II genes, a cis-acting regulatory element silences expression of I-αβ gene (44). Using competition electrophoretic mobility shift assays, the core protein binding site was localized within a region of an 8–10-bp response element designated AβNRE at −543 to −534 bp. A nuclear extract from B cells does not bind to this element and mutation of this site abrogates the transcriptional silencing activity of this region in epithelial cells. The AβNRE is not found in the Aα gene, but is present 480 bp upstream of the transcription initiation site in the analogous human gene DQβ. These data suggest that the AβNRE repressor acts in a gene-specific and tissue-specific manner.

In our experiments, we show that PU.1 is able to repress the expression of the I-αβ gene. Binding to the PU DNA box, which is next to the Y box in the I-αβ gene, was mediated by PU.1 protein as demonstrated using electrophoretic mobility shift assays and dimethyl sulfate protection assays. One possible mechanism of repression is based on the affinity of different proteins to bind to the same or different DNA sequences (40, 41). In this regard, we can mention that the NF-Y factor and the F2 bind in a mutually exclusive manner to a critical promoter region of the gene for the IE110k protein of herpes simplex virus (45). Moreover, NF-Y factor can act as a negative regulator competing with factor 3 in the promoter of ApoA-I gene (46). Recently it has been reported that YB-1, a protein identified by using a radiolabeled Y box sequence to screen a λgt11 expression cDNA library, represses the IFN-γ activation of MHC class II genes (47). This could explain the inverse relationship between the levels of YB-1 and MHC class II induction by IFN-γ. The data presented show that PU.1 protein is able to repress the expression of the class II I-αβ gene. In our
experiments we show that PU.1 and NF-Y proteins bind to the same DNA molecule.

The PU box is present only in one of the MHC class II gene promoters, the I-Aβ, and therefore the PU.1 protein, may act as a specific gene regulator. Another PU.1 box has been described in the promoter of the TAP gene (46). However, the involvement of this PU box and the PU.1 protein in the regulation of TAP gene expression is unknown. The regulatory role of PU.1 in I-Aβ gene expression in vivo is more difficult to demonstrate. Like Y-B1, PU.1 is down-regulated by IFN-γ, and both proteins may regulate class II expression by different mechanisms (27, 47). Recently, in murine tissue macrophages, it has been shown that IFN-γ induces binding of PU.1 to the I-Aβ gene (49). The binding increases gradually, plateauing at 6-9 h and decaying to basal levels 24 h after stimulation. In IFN-γ-stimulated macrophages, the levels of I-Aβ mRNA can be detected after 8 h of incubation with IFN-γ, increasing gradually up to 24 h (28). The data from these two reports suggest a correlation between PU.1 release from the I-Aβ gene promoter and the expression of mRNA.

Some transcriptional activators are down-regulated by inhibitory proteins with which they form protein complexes with altered or reduced DNA binding activity. One example of such a mechanism in the repression of MHC class II genes is provided by the glucocorticoid receptor. This protein is able to form heterocomplexes with the X box-binding protein and represses MHC class II I-Aβ gene expression (30). It has been reported recently that PU.1 can specifically repress the glucocorticoid-induced activation of promoters carrying a glucocorticoid response element and other nuclear receptors such as the thyroid hormone or retinoic acid (50). The glucocorticoid receptor represses PU.1-mediated transcriptional activation, showing that, in some cases, PU.1 protein can form heterodimers that inhibit gene expression.

The binding of the NF-Y proteins to DNA increases in the presence of the PU.1 protein. This could be due to the binding to different DNA molecules or to the simultaneous binding of NF-Y and PU.1 proteins to the same DNA molecule. In the latter case, the interaction between NF-YA/PU.1 may explain the repression of I-Aβ gene expression when cells were transfected with a vector coding for the PU.1 protein. The activation domain of PU.1 is known to bind to the transcription factor TFIID in vitro (51). Due to the proximity of the PU box to the transcription initiation site, the interaction between PU.1 and TFIID could explain the repressive effect of PU.1. However, the binding domain of PU.1 without the activation domain is able to repress I-Aβ gene expression, suggesting that the hypothetical interaction with TFIID is not an important factor. It is well documented that synergism between different transcriptional activators has a major role in transcriptional activation (52). Regulation of MHC class II gene expression requires three separate elements, the boxes W, X, and Y with stereospecific and distance constraints (53), and it also requires cooperation between the different factors that bind to these boxes (54–56).

In this context, the complex of NF-YA and PU.1 proteins could have an inhibitory effect by disruption of the interactions between the proteins that bind to the W, X, and Y boxes of the MHC class II genes, which would involve interference with the preinitiation complex assembly. It is also possible that active repressors could promote local chromatin changes resulting in repression of transcription.

The results reported here are one more example of how transcription factors can interact with DNA resulting in reduced expression of genes that are activated by other transcription factors. These interactions provide an important opportunity for crosstalk between different signal transduction pathways and allow for modification of the responses of particular genes to specific extracellular stimuli.

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Fig. 8. Titration of DNA binding of recombinant proteins. Gel electrophoresis DNA binding assay was done with increasing amounts of in vitro transcribed and translated NF-YA, NF-YB, and PU.1 proteins. There was binding complementation between NF-YA and NF-YB and cooperative binding with PU.1 or the binding domain of PU.1. The retarded complexes were quantitated using the AMBIS radiographic imaging system. Percentage binding is referred to the total amount of DNA added to the assay. There was binding complementation between NF-YA and NF-YB and cooperative binding with PU.1 or the binding domain of PU.1. In the assays marked as NF-YA + NF-YB, NF-YB was at a constant concentration of 4 μl while the amounts of NF-YA were those indicated in the figure.

Repression of I-Aβ Gene Expression by PU.1

**PU.1 Wild type**

**PU.1 Binding domain**

**PU.1 Activation domain**

| Protein (μl) | Percentage Binding |
|-------------|-------------------|
| NF-YA       | 0%                |
| NF-YB       | 0%                |
| PU.1        | 0%                |
| NF-YA       | 0%                |
| NF-YB       | 0%                |
| PU.1        | 0%                |

**FIG. 8.** Titration of DNA binding of recombinant proteins. Gel electrophoresis DNA binding assay was done with increasing amounts of in vitro transcribed and translated NF-YA, NF-YB, and PU.1 proteins. There was binding complementation between NF-YA and NF-YB and cooperative binding with PU.1 or the binding domain of PU.1. The retarded complexes were quantitated using the AMBIS radiographic imaging system. Percentage binding is referred to the total amount of DNA added to the assay. There was binding complementation between NF-YA and NF-YB and cooperative binding with PU.1 or the binding domain of PU.1. In the assays marked as NF-YA + NF-YB, NF-YB was at a constant concentration of 4 μl while the amounts of NF-YA were those indicated in the figure.
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