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Identification of a DNA binding site for the nuclear factor YY1 in the human GM-CSF core promoter

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ABSTRACT

It has been well documented that the repeated CATT(A/T) sequence, localized between −64 and −35 in the human GM-CSF promoter, is required for the promoter activity, and this region likely serves as a core recognition sequence for a cellular transcription factor. However, the transcription factor that interacts with this site was not identified. Here, we report that this element contains a binding site for the nuclear factor YY1, which has not been reported to play a role in the regulation of cytokine gene transcription. Results from transient transfection assays of the Jurkat T cell line revealed that this repeated CATT(A/T) element exhibited enhancer activity when linked to both the human IFN-γ promoter and the TK promoter. Mutation of the YY1 binding site eliminated about 60% of the enhancer activity of the element. We have found that the YY1 binding site could form two specific DNA–protein complexes, A and B, with Jurkat nuclear proteins in the electrophoretic mobility shift assay and that the binding of these complexes correlates with the enhancer activity. UV cross-linking analysis revealed that the A complex is a multi-protein complex and in addition to YY1, other proteins are required for formation of the protein complex. Cotransfection assays with a YY1 expression vector revealed that overexpression of YY1 resulted in an inhibitory effect on the repeated CATT(A/T) element, indicating that in addition to YY1, cofactors also are required for the activator function of the A complex.

INTRODUCTION

GM-CSF (granulocyte–macrophage colony–stimulation factor) is a glycoprotein cytokine involved in hematopoiesis and host defense [1]. Human GM-CSF stimulates the formation of neutrophil, neutrophil–macrophage, macrophage, and eosinophil colonies from normal human bone marrow. GM-CSF can be produced by both immune cells (T cell, macrophage, NK cell) and non-immune cells (fibroblast and endothelial cells), but its production is tightly controlled. With regard to the transcription control of GM-CSF gene expression, the distal and proximal promoter regions of the GM-CSF gene have been under characterization for several years [2–5]. In the distal region (about 3 kb upstream of the GM-CSF encoding site), there is a CsA sensitive enhancer region that can interact with AP1 and NFAT [2]. In the proximal region, several functional regions have been identified [1], including two cytokine consensus region CK-1 (CLE-1) and CK-2 (CLE-2), a GC-rich region immediately downstream of the CK2 element, and a repeated CATT(A/T) region. A NFκB binding site was localized in the overlapping sequence of the CK-2 and GC-rich region.

YY1 is a zinc finger transcription factor [6] that, depending on its promoter context, can display either activator or repressor activity on binding to the promoter DNA. Independently discovered by several laboratories, this protein also has been named CF-1 [7], NF-E1 [8], δ [9], or upstream conserved region-binding protein (UCRBP) [10]. It has been reported that, combined with the general transcription factor IIB and RNA polymerase II, YY1 is able to direct basal transcription on a supercoiled DNA template [11].

It has been well established that the repeated CATT(A/T) region, which is within 68 bp upstream of the mRNA cap site, is very important for the GM-CSF promoter activity [3–5]. Results from deletion and point mutation analysis of the GM-CSF promoter indicated that this repeated CATT(A/T) sequence is required for both inducible and constitutive activity of the GM-CSF promoter. DNase I footprinting revealed that the sequence between −52 and −34, which was encompassed in the repeated CATT(A/T) region, may serve as a core recognition sequence for a cellular transcription factor [3], but the nuclear protein that interacts with this region was not well characterized. Here we report that a DNA binding site for the nuclear factor YY1 has been identified in the repeated CATT(A/T) region, and YY1 is one of the proteins that interacts with this promoter region.

MATERIALS AND METHODS

Oligonucleotide

Oligonucleotides were synthesized by the phosphoramidite method on a DNA/RNA Synthesizer (Applied Biosystems, Model 392, Foster City, CA). The synthesized oligonucleotides were purified through a Pure Pak Cartridge column (Applied

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Biosystems) and treated at 50°C overnight. Complimentary strands were denatured at 80°C for 5 min and annealed at room temperature. The double-stranded probe was labeled with ^{32}P-dCTP (Amersham, Arlington Heights, IL) using Klenow fragment (BRL, Gaithersburg, MD). The YY1 oligonucleotide (ATGCCTTGCAAAATGGCGTTACTGCAG) was derived from the upstream conserved region of the Moloney murine leukemia virus gene [10].

Cell lines and reagents

Jurkat cells (CD4$^+$ human lymphoblast cell line) and YT cells (human NK cell line) were cultured in complete medium (RPMI-1640 supplemented with 10% fetal calf serum [FCS], 2 mM glutamine and 1000 U/ml penicillin-streptomycin). Purified human peripheral blood T cells (fresh T cells, CD3$^+$ >95%) were cultured in the same medium. YY1 antibody and AP2 antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA 95060). Recombinant YY1 protein was generously provided by Drs K.Becker and K.Ozato (Laboratory of Developmental and Molecular Immunology, NICHHD−NIH, Bethesda, MD).

Nuclear extraction

Nuclear extracts were prepared as follows [12,13]: 1×10^8 cells were treated with 500 μl lysis buffer (50 mM KCl, 0.5% NP-40, 25 mM Hepes pH 7.8, 1 mM PMSF, 10 μg/ml Leupeptin, 20 μg/ml Aprotinin, 100 μM DTT) on ice for 4 min. After 1 min centrifugation at 14,000 rpm, the supernatant was saved as a cytoplasmic extract. The nuclei were washed once with the same volume buffer without NP-40, then were put into a 300 μl volume of extraction buffer (500 mM KCl, 10% Glycerol with the same concentrations of Hepes, PMSF, Leupeptin, Aprotinin and DTT as the lysis buffer) and rotated 20 min at 40 rpm. After centrifugation at 14,000 rpm for 5 min, the supernatant used as the nuclear protein extract was harvested, dialyzed against the same buffer with 50 mM KCl and stored at −70°C. The protein concentration was determined by BCA (Pierce, Rockford, IL 61105).

Electrophoretic mobility shift assay (EMSA)

The protein−DNA binding reaction [12,13] was conducted in a 20 μl reaction mixture with 0.5 μg Poly dI.dC (Sigma), 2 μg nuclear protein extract, 5×10^4 cpm ^{32}P-labeled oligonucleotide probe and 10 μl of 2×GS buffer (40 mM Tris pH 7.4, 120 mM KCl, 8% Ficoll, 4 mM EDTA, 1 mM DTT). In some cases the indicated amount of double stranded oligomer was added as a cold competitor. This mixture was incubated at room temperature for 10 min before and 30 min after addition of probe, then loaded on a 5% acrylamide gel (National Diagnostics, Atlanta, GA) that had been pre-run at 210 V for 2 h with 0.5×TBE buffer. The loaded gel was run at 210 V for 90 min, then dried and placed on Kodak X-OMAT film (Eastman Kodak, Rochester, NY). The film was developed after overnight exposure at −70°C.

UV-cross-linking

The repeated CATT(A/T) element oligonucleotide was synthesized on the same DNA/RNA synthesizer and labeled with α^{32}P-dCTP using Klenow fragment. UV-cross-linking was carried out according to a published procedure [14] with the following modification: UV irradiation was performed on the gel after EMSA with 305 nm UV light generated by the UV-Transilluminator 400 (Stratagene, La Jolla, CA) at a distance of 5 cm for 60 min. The specific bands were cut out and ground, then eluted with TES buffer (10 mM Tris−HCl pH 8.0, 1 mM EDTA, 0.1% SDS) at 37°C overnight with 250 rpm rotation. The DNA−protein complexes were precipitated from the elution buffer with 2 volumes of cold acetone, then resolved on a 10% SDS gel at 100 V for 12 h.

Reporter gene construction

Two kinds of reporter gene vectors were used in this study: (1) Plasmid 108 [15], a β-galactosidase expression vector in which the β-galactosidase gene is controlled by the human IFN-γ promoter fragment from −108 to +64 (kindly provided by Dr Christopher Wilson, Department of Pediatrics and Immunology, University of Washington); (2) Plasmid pBCTKp-CAT, a CAT expression vector in which the CAT gene is under control of the Herpes simplex virus thymidine kinase (TK) promoter [16]. The repeated CATT(A/T) element was inserted at the *Hin*dIII site of both plasmids (upstream of the IFN-γ promoter or the TK promoter). The cloned inserts were verified by DNA sequencing. A CMV promoter controlled YY1 expression vector was generously provided by Drs K.Becker and K.Ozato (Laboratory of Developmental and Molecular Immunology, NICHHD-NIH, Bethesda, MD).

Transfection assay

The Jurkat T cells and YT cells were grown in the complete medium as described earlier. 8×10^6 cells were transiently transfected by electroporation in 0.4 ml of complete medium with 20 μg plasmid vector at 260 V and 960 μFD, using a Bio-Rad (Richmond, CA) electroporation device, then diluted into 10 ml complete medium and incubated at 37°C for 3 h. Viable cells were isolated by centrifugation over LSM lymphocyte separation medium (Organon Tekinka, Durham, NC) at 2000 rpm for 20 min, cultured for an additional 45 h in the absence or presence of inducers and then harvested for CAT or β-galactosidase analysis. An RSV LTR driven CAT vector was used to normalize transfection efficiency and CAT activity was measured as described previously [17]. The β-galactosidase assay was carried out according to the published method [18]. β-galactosidase activities were normalized against protein amount loaded and transfection efficiency at each point, and data from three individual experiments was analyzed by the student's T test.

RESULTS

Enhancer activity of the repeated CATT(A/T) element in a heterologous promoter

Enhancer activity of the repeated CATT(A/T) element in a heterologous promoter previously has been demonstrated using the TK promoter [12]. This enhancer activity was confirmed by similar experiments in our laboratory using the TK promoter (data not shown). As the GM-CSF gene is a member of the cytokine gene family, and in lymphocytes, GM-CSF gene expression often parallels that of IFN-γ, we chose the core IFN-γ promoter for studying enhancer activity of the repeated CATT(A/T) element. This IFN-γ core promoter does not contain the CATT(A/T) repeats. In the IFN-γ promoter, the repeated CATT(A/T) element expressed a weak enhancer activity in the absence of stimulation (Fig. 1), and expressed a strong enhancer activity in the presence of PMA/ionomycin stimulation. This enhancer activity is independent of DNA sequence orientation as the reverse orientation showed identical enhancer activity (Fig. 1). Although

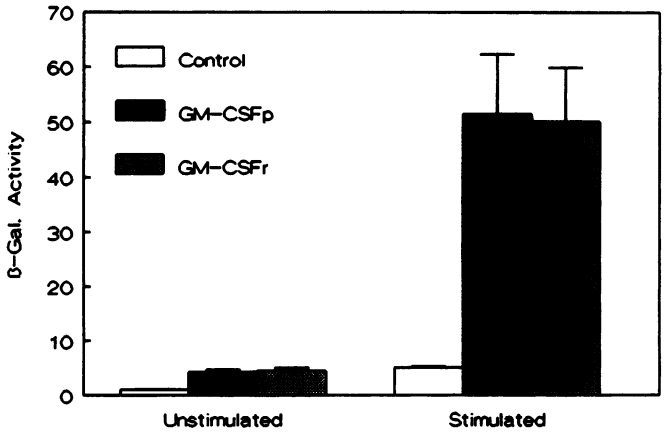

Figure 1. Enhancer activity of the repeated CATT(A/T) region in the IFN-γ promoter. Control: plasmid 108. GM-CSFp: plasmid 108 with the repeated CATT(A/T) region of the GM-CSF promoter in proper orientation. GM-CSFr: plasmid 108 with the repeated CATT(A/T) region of the GM-CSF promoter in reverse orientation. Stimulation was carried out by addition of PMA (10 ng/ml) plus Ionomycin (1 μg/ml) to the culture 24 h before the cells were harvested. Each bar represents the mean value of relative β-galactosidase activities from three independent transfection assays in Jurkat T cells.

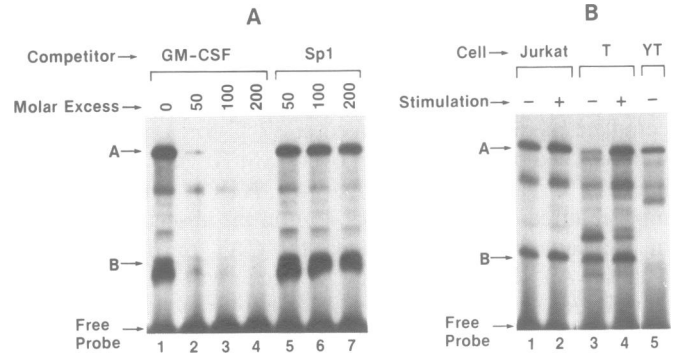

Figure 2. Specific nuclear binding complexes with the repeated CATT(A/T) region. **A.** A radiolabeled repeated CATT(A/T) region oligonucleotide was used as a probe to detect nuclear binding proteins in a nuclear extract from the Jurkat T cell line. Unlabeled and Sp1 oligonucleotide were used as competitors in different molar excess against the radiolabeled oligonucleotide. **B.** Levels of the specific protein complexes were compared in the nuclear extracts from unstimulated and stimulated Jurkat T cells and fresh T cells, as well as in unstimulated YT cells. −: unstimulated; +: stimulated with PMA (10 ng/ml) plus Ionomycin (1 μg/ml) for 3 h.

these experiments were conducted using the IFN-γ promoter, the results are comparable with that from previous studies on the GM-CSF promoter in which this region expressed a similar inducible enhancer activity and mutation of this element eliminated almost all inducible promoter activity [4]. Thus, the IFN-γ promoter provides a suitable model for analysis of the *cis*-acting activity of the repeated CATT(A/T) region of the GM-CSF promoter.

Specific DNA−nuclear protein complexes formed with the repeated CATT(A/T) region

To identify nuclear proteins that can interact with this region, we conducted an electrophoretic mobility shift assay using a

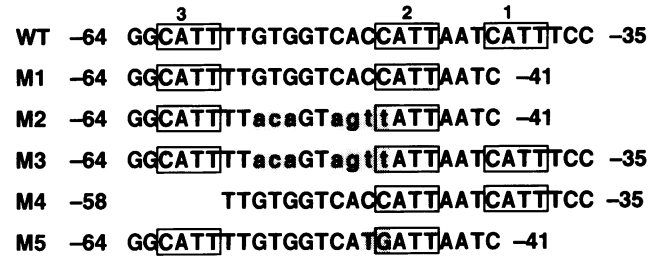

Figure 3. Wild type and mutant CATT(A/T) region oligonucleotide sequences. M1 was generated from the wild type sequence by deletion of 6 nucleotides at the 3′ region. M2 was derived from M1 by mutation of the highlighted sequences. M3 was derived from the wild type by the same mutation as M2. M4 was derived from WT by deletion of box 3. M5 was derived from M2 by mutation two nucleotides as highlighted.

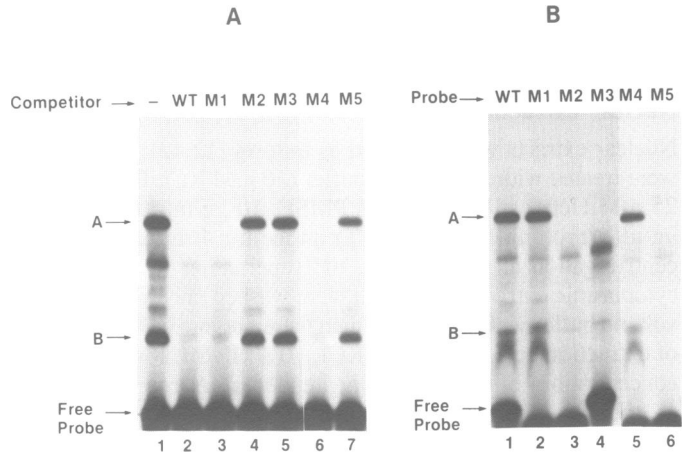

Figure 4. Identification of the nuclear protein binding site. **A.** Radiolabeled wild type (WT) oligonucleotide was used as a probe with the Jurkat T-cell nuclear extract. 100 fold molar excess of unlabeled wild type M1, M2, M3, M4 and M5 oligonucleotides were used for competition. **B.** Radiolabeled WT, M1, M2, M3, M4 and M5 oligonucleotides were used as probes with a Jurkat T-cell nuclear extract.

radiolabeled repeated CATT(A/T) oligonucleotide and nuclear protein from Jurkat T cells, YT cells and human peripheral blood T cells (Fig. 2). There were two specific DNA−protein complexes (A and B) formed with Jurkat nuclear protein (Panel A), and these complexes were competed by unlabeled probe in a dose-dependent pattern (lanes 2−4), but were not competed by an unrelated oligonucleotide Sp1 (lanes 5−7).

The levels of these two complexes were not significantly different when unstimulated and stimulated Jurkat cells were compared (Fig. 2, panel B, lanes 1−2). In contrast, the A complex level was induced by stimulation in the fresh T cells (Panel B, lanes 3−4). YT cells showed some differences from the Jurkat cells and fresh T cells: they lacked the B complex (Panel B, lane 5). As YT cells also can produce GM-CSF, these data indicated that the B complex may not be important for GM-CSF expression.

Mutation analysis of the A complex binding site

In order to localize the DNA binding region of the A complex, we designed five mutant oligonucleotides (M1, M2, M3, M4 and

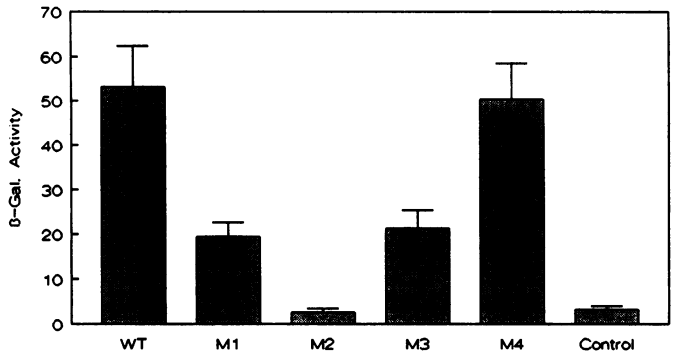

Figure 5. Functional activities of the M1, M2, M3 and M4 oligonucleotides in the IFN-γ promoter. The M1, M2, M3 and M4 oligonucleotides were inserted in the same position as the WT oligonucleotide in the IFN-γ promoter, and these DNA constructs were assayed alone with the WT construct. The promoter activity was tested 48 h later after transfection, and the cells were stimulated with PMA plus Ionomycin for 24 h before harvesting of the cells. Each bar represents the average relative β-galactosidase activity from three independent experiments.

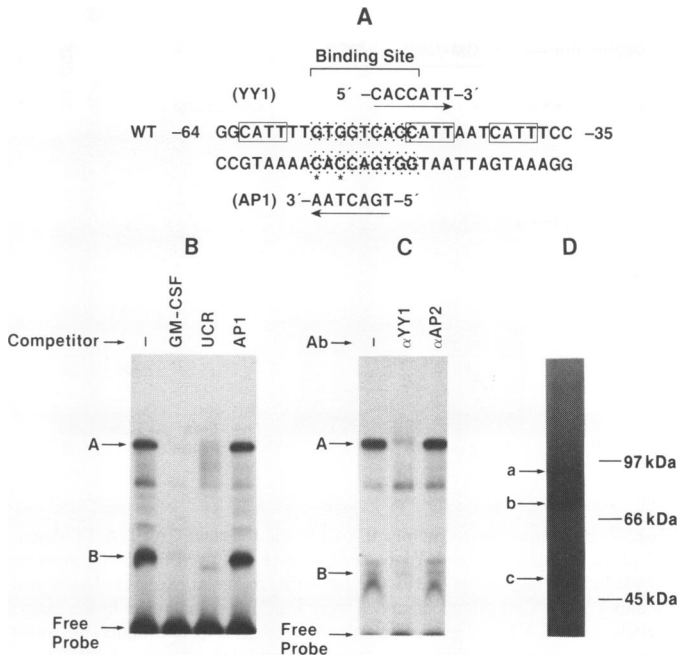

Figure 6. Protein composition of the A complex. **A.** Sequence homology in the A and B complexes binding site to a YY1 or AP1 consensus binding sequence. **B.** A competition assay was conducted using the authentic YY1 and AP1 binding oligonucleotides as competitors against the wild type GM-CSF oligonucleotide in the presence of the Jurkat T cell nuclear extract. 100-fold molar excess of each competitor oligonucleotide was used in the assay, and the unlabeled WT GM-CSF oligonucleotide was used as a positive control. **C.** 3 μg YY1 antibody was used in a regular EMSA as described above. The same amount of the AP2 antibody was used as a negative control. **D.** UV-crosslinking analysis of the A complex.

M5) of the repeated CATT(A/T) element as shown in Figure 3. Results from a competition assay against the wild type radiolabeled oligonucleotide probe indicated that complexes A and B shared the same DNA binding site (Fig. 4, panel A), as the M1 oligonucleotide competed both complexes (lane 3), while the M2 and M3 oligonucleotides failed to compete either complex (lanes 4 and 5). These results were confirmed by EMSA with radiolabeled M1, M2 and M3 oligonucleotides as probes (Fig. 4, panel B, lanes 2–4). As the sequence between −48 and −56 was mutated in both M2 and M3, these results indicated that sequence 5′-GTGGTCACC-3′ (from −48 to −56) is a target for the binding site of both complex A and complex B. To further characterize the nuclear protein binding site, M4 and M5 were generated and used in the competition assay. As shown in lanes 6 and 7 (Fig. 4 panel A), M4 retained the capacity for competing the complexes, whereas M5 did not. Since binding of the nuclear proteins was only abrogated by mutation in the second CATT(A/T) box, these results implied that the second CATT box is critical for formation of the DNA–protein complexes, and it might serve as a center of the DNA sequences in the protein binding site. The binding capacity of M4 and M5 was confirmed in EMSA with probes of radiolabeled M4 and M5 (Fig. 4, panel B, lanes 5–6).

Correlation of the binding of the A and B complexes with the enhancer activity of the repeated CATT(A/T) region

Since we were able to localize the binding site of the A and B complexes, we next carried out a functional analysis utilizing reporter constructs containing mutations in the binding region. The mutant type oligonucleotides were inserted at the same site as the wild type oligonucleotide in the IFN-γ promoter, and functional activities of the constructs containing these mutants were analyzed (Fig. 5). The DNA constructs containing M1 or M3 lost about 60% of enhancer activity compared to that containing the wild type insert, while constructs containing M2 lost all of the enhancer activity. There was no change in enhancer activity of M4. These results indicated that the first CATT(A/T) and the second CATT(A/T) plus its immediately upstream sequence are important for the enhancer activity as deletion or mutation of these sites reduced the enhancer activity approximately 60% respectively (Fig. 5, M1 and M3). Mutation of both sites removed all the enhancer activity of the wild type oligonucleotide (Fig. 5, M2). Since the second CATT(A/T) and its immediately upstream sequence are the binding site for the A and B complexes, these data indicated an appreciable correlation between binding of the A and B complexes and the enhancer activity of the repeated CATT(A/T) element.

Although no DNA–protein complex binding to box 1 was detected in the EMSA assay, these data suggest that box 1 is equally important for the enhancer activity. We propose that box 1 might be involved in formation of a DNA–protein complex that was not identified in our assay system. Thus, we conclude that the enhancer activity is contributed by two CATT(A/T) boxes; the nuclear protein complexes A and B are responsible for the activity of box 2 and an unidentified nuclear protein might be responsible for the activity of box 1.

Characterization of nuclear proteins involved in the A and B complexes

A binding sequence homology search to known DNA binding proteins revealed that the binding site of the A and B complexes contains a sequence which shares 100% homology with the YY1 consensus binding sequence [19], and another sequence that shares 72% homology with an AP1 consensus binding sequence [20] (Fig. 6, panel A). A oligonucleotide competition assay revealed that formation of the A and B complexes was abolished

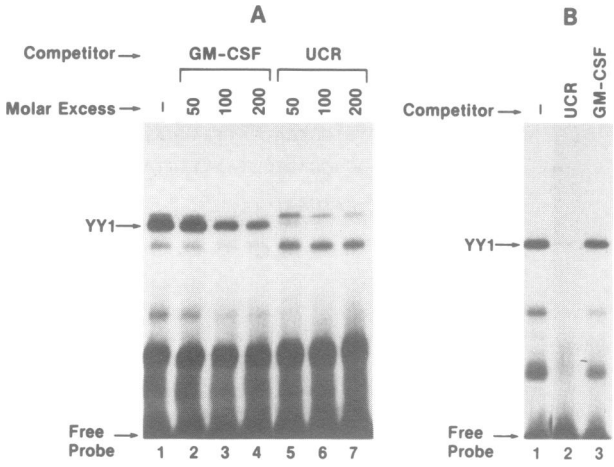

Figure 7. Binding of the recombinant YY1 to the repeated CATT(A/T) region. **A.** 1 μl of the recombinant YY1 bacteria extract was used in EMSA utilizing radiolabeled WT oligonucleotide as a probe. Different molar excesses of unlabeled WT and YY1 (UCR) oligonucleotides were used for competition in the assay. **B.** Radiolabeled YY1 oligonucleotide was used as a probe under the same conditions as A, and 100-fold molar excess of unlabeled YY1 and the WT GM-CSF oligonucleotides were used as competitors.

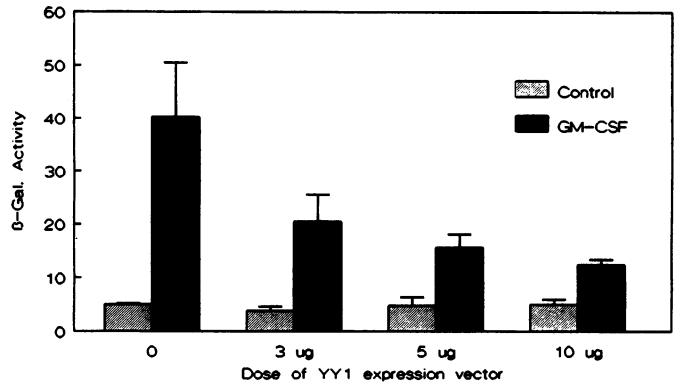

Figure 8. Cotransfection assay with YY1 expression vector. 10 μg reporter gene vector containing the wild type GM-CSF insert and variable amounts (as indicated in the fig.) of the YY1 expression vector were used in the cotransfection assay. pCEP plasmid DNA was used to adjust the DNA amount to 20 μg at each point. The plasmid 108 without the GM-CSF insert was used as a control. The promoter activity was tested 48 h later after transfection, and the cells were stimulated with PMA plus Ionomycin for 24 h before harvesting of the cells. Each bar represents the mean value from three independent experiments.

by a YY1 binding oligonucleotide (UCR) (Fig. 6, panel B, lane 3), but not affected by an AP1 binding oligonucleotide (lane 4). This result suggested that nuclear factor YY1, but not AP1, is involved in formation of the A and B complexes. This hypothesis was verified by a supershift assay utilizing an YY1 antibody (Fig. 6, panel C): the A and B complexes were removed by the YY1 antibody (lane 2), but not affected by a control AP2 antibody.

In order to further characterize the protein complex composition, an UV crosslinking analysis of the A and B DNA–protein complexes was performed. Our results revealed that there are three bands, a, b and c, derived from the A complex (Fig. 6, panel D). Although band c was not clearly observed, it was consistently present; this result has revealed that complex A contains at least three protein components. Band a migrated a little faster than a 97 kDa marker, while band b migrated at a molecular weight level of approximately 65 kDa (after deduction of the probe molecular weight), identical to mobility of the YY1 protein [6]. Band c migrated at a level of approximately 50 kDa. Combined with the results from the supershift experiment, these data indicated that the A complex is a multiprotein complex and YY1 is one of the protein components.

A cofactor is required for formation and function of the A complex

To investigate the role of the other proteins in the formation of the A complex, a recombinant YY1 protein [21] was utilized in a binding assay with the radiolabeled wild type GM-CSF probe (Fig. 7, panel A). The result indicated that affinity of the YY1 protein to the GM-CSF element is lower than that of the complex A, as the same molar excess of unlabeled GM-CSF oligonucleotide could not abolish the YY1–DNA complex (lanes 2–4 of Fig. 7A compared with lanes 2–4 in Fig. 2A), but a YY1 binding oligonucleotide (UCR) did compete very well (lanes 5–7). This result is supported by a parallel experiment with a radiolabeled YY1 oligonucleotide (UCR) as a probe (Fig. 7, panel B) in which the GM-CSF oligonucleotide did not compete against the YY1 oligonucleotide for binding to the recombinant YY1 protein (lane 3), but unlabeled YY1 oligonucleotide did compete (lane 2). Combined together, these results indicated that proteins a and c (Fig. 6, panel D) are required along with YY1 to form a high affinity complex capable of binding to the GM-CSF promoter.

In order to examine the effects of overexpression of YY1 on the enhancer activity of the repeated CATT(A/T) region, a YY1 expression vector was cotransfected with the CATT(A/T) IFN-γ promoter construct into the Jurkat T cell line (Fig. 8). The cotransfection results showed that overexpression of YY1 reduced the enhancer activity of the repeated CATT(A/T) region. The overexpression of YY1 was verified by Western blot assay of the transfected cell lysate with YY1 antibody (data not shown). Although the inhibitory effect was dose dependent between 3 μg and 10 μg of the YY1 expression vector, it could not suppress the enhancer activity completely. Combined with the DNA binding data, these results indicate the cofactors are required not only for formation of the A complex, but also for the activator function of the complex.

DISCUSSION

The role of GM-CSF in promoting the growth and differentiation of hematopoietic progenitor cells is well established [1]. Characterization of the GM-CSF promoter structure and function is necessary to define the molecular events involved in the induction of GM-CSF gene expression. Deletion analysis of the proximal promoter region (−626 to +37) indicated that a region as short as 90 bp (−53 to +37) is sufficient for the promoter activity [3]. It is interesting that within a 64 bp sequence upstream of the cap site, there is an repeated CATT(A/T) region localized between −64 and −35 that contains 3 CATT(A/T) sequences (Fig. 3). Earlier point mutation analysis of the proximal promoter (−626 to +37) indicated that these 3 CATT(A/T) sequences are required for GM-CSF promoter activity [4]. A recent report has revealed that the third CATT sequence is related to a silencer activity, and sequences between the third and second CATT(A/T)

box also are required for promoter activity [5]. DNase I footprinting revealed that this repeated CATT(A/T) region could serve as a core recognition site for a cellular transcription factor [3]. Recently, it was reported that a 45 kDa protein from AML cells and K562 CML cells could interact with this region and act as a repressor [5], but nuclear proteins that are responsible for the enhancer activity of this repeated CATT(A/T) region have not yet been identified.

Our functional data confirmed the findings from other laboratories. In this study, we used Jurkat T cells as a model and confirmed the enhancer activity of the repeated region not only in the TK promoter (data not shown), but also in a heterologous cytokine (human IFN-γ) promoter. This enhancer activity dose not show DNA orientation dependence. These data along with previous reports indicate that the repeated region of the GM-CSF promoter is a typical enhancer element.

By mutation analysis of the repeated region, we have found that the first CATT(A/T) and second CATT(A/T) as well as its immediately upstream sequence are important for the enhancer activity of the repeated region as deletion or mutation of either of the sites decreased enhancer activity approximately 60%, but did not eliminate all activity (Fig. 5). The results are consistent with those obtained from mutation analysis of the proximal GM-CSF promoter [4,5]. Our data showed that mutation of both sites eliminated all the enhancer activity. Taken together, these data suggested that the first CATT box and second CATT box as well as its immediately upstream sequence are important for the enhancer activity of the repeated CATT(A/T) region.

In the Jurkat and fresh T cell nuclear extracts, we identified two specific protein complexes, A and B, and only the A complex in the YT cell nuclear extract, which binds to the repeated CATT(A/T) region. The A complex was inducible after combined stimulation of PMA and Ionomycin in the fresh T cells. In the Jurkat cells, the basal level of the A complex is obviously higher than that in the fresh T cells and YT cells, and the complex was not inducible (Fig. 2B). These results are supported by Western blot analysis of the Jurkat and fresh T cell nuclear proteins with YY1 antibody (data not shown). The high basal level of the A complex may be used to explain why, in the absence of stimulation, the repeated CATT(A/T) region expressed appreciable enhancer activity (Fig. 1, and ref. 12), and the GM-CSF core promoter (−68 to +37) expressed a high basal activity in the Jurkat cells [3]. While the activity of the repeated CATT(A/T) region could be upregulated by stimulation (Fig. 1, and ref. 12), these data suggest that in the Jurkat T cells, stimulation is not required for expression of the nuclear proteins that interact with the repeated CATT(A/T) region, but stimulation could upregulate their activity. The mechanism of this effect is not clear: stimulation may induce other factors that are involved in formation of the initiation complex or induce phosphorylation of YY1, as has been reported by Becker and coworkers [21].

The binding site of complexes A and B is related to the sequence between −56 and −45 (-GTGGTCACC-), which includes part of the second CATT box and its immediately upstream sequence. This result confirms previous results from DNase footprinting analysis [3]. The sequence between −57 and −24 was protected by nuclear proteins from crude nuclear extracts of unstimulated S-LB-I cells or MLA 144 cells in the DNase footprinting assay, indicating this region is the binding site for the nuclear proteins. In our experiments, formation of the A complex was not affected by deletion of the first and third CATT boxes, indicating that these sequences may not be involved in the binding of the A complex. Since the first box is associated with part of the enhancer activity, it may be involved in the binding of other nuclear proteins that were not revealed in our analysis. In the mouse GM-CSF promoter, the CLE0 element shares a homology with the 3′ end of the repeated CATT(A/T) region in the human GM-CSF promoter [22], and contains an AP1/NFAT like binding site [23], but the function of the AP1 binding site was not confirmed in a cotransfection assay with an AP1 expression vector [24]. As mutation of the sequence between −56 and −48 not only abolished formation of both the A and B complex, but also eliminated 60% of the enhancer activity of the repeated CATT(A/T) region, these data suggested that the second box and its immediately upstream sequence is the binding site of the A and B complexes, and these two complexes are likely responsible for the function of this region.

YY1 is a member of the GL1-Kruppel transacting factor family and was first isolated by Shi and colleagues [6]. Members of this family have the unique ability to act as transcriptional activators or inhibitors, depending on the context of the binding site [9] or local concentration of the factor [25]. For example, YY1 activates the c-myc gene in terminally differentiated B cells [26], but represses the adeno-associated virus P5 promoter [6] and skeletal α-actin gene [27−29]. YY1 also can direct basal transcription in combination with TFII and polymerase II [11,30,31]. YY1 binding sites have been described in at least 20 genes [6−11], but have not been reported in the cytokine genes. YY1 has been reported to interact with a variety of other transcription factors, such as adenovirus E1A [6], Sp1 [31−33], B23 [34], c-Myc [26], TATA-binding factor [11] and compete with SRF for DNA binding [19,28]. The interaction of YY1 with E1A reverses its inhibitory effect on the P5 promoter [6], and interaction of YY1 with c-Myc abolishes its active effect on the c-myc gene [26]. Thus, the interaction of YY1 with other transcription factors may be particularly important in regulating promoter activity.

Several lines of evidence support the finding that YY1 is a component of the A complex. We found that the A complex binding site shows 100% homology with YY1 binding sequence, and formation of the complex was abolished by addition of a YY1 binding oligonucleotide (UCR). To verify that YY1 is involved in formation of the A complex, we used YY1 antibody and recombinant YY1 protein in the EMSA assay. Our results indicated that the A complex was abrogated specifically by YY1 antibody (Fig. 6C), and the recombinant YY1 protein could bind specifically to the repeated CATT(A/T) oligonucleotide (Fig. 7A). In addition, UV cross-linking analysis of the A complex indicated that one of the components in the A complex has a mobility identical to YY1 (Fig. 6D) and immunoprecipitation analysis of this component with YY1 antibody demonstrated it is YY1 (data not shown). Thus, these results indicated that complex A is a multiprotein complex and YY1 is one of the protein components. As no band was revealed by the UV cross-linking analysis of the B complex (data not shown), and its mobility in EMSA is similar to that of a previously reported derivative derived from protease digestion of a YY1 complex [35], our data also suggests that the B complex may be a derivative of the A complex.

To investigate the role of the cofactors in DNA binding activity of the protein complex A, an EMSA assay was performed with the recombinant YY1. Compared with the A complex, the recombinant YY1 expressed a lower affinity for the enhancer DNA, but it showed high affinity to the control YY1 binding

oligonucleotide (UCR) (Fig. 7). Since flanking sequences in the enhancer and control DNA are different and they can affect protein binding affinity, we suggest that this difference in the recombinant YY1 and complex A may be due to a difference in flanking regions of YY1 binding sites in the two DNAs. As complex A is a multiprotein complex that showed a high affinity to the enhancer DNA, we concluded that cofactors are required for YY1 to form this high affinity protein complex.

The interaction of YY1 with the enhancer DNA was not only demonstrated by *in vitro* analysis, but also by *in vivo* experiments utilizing cotransfection of Jurkat cells with a YY1 expression vector. This analysis indicated that overexpression of YY1 in fact suppressed the enhancer activity. Thus, YY1 alone could not function as an activator protein, indicating that the functions of YY1 and complex A are different. As the enhancer activity could not be totally inhibited by the transfected YY1, this data suggested that YY1 may not be an active suppressor and the remaining enhancer activity may be due to the presence of the first CATT(A/T) box. While it is not clear how the enhancer activity is suppressed by the overexpressed YY1, we hypothesize that overexpressed YY1 may compete with the complex A for DNA binding, and therefore block part of the enhancer activity that was due to complex A. It is suggested that in complex A, the cofactors not only contribute to DNA binding affinity of YY1, but also to expression of the activator function. This hypothesis is supported by a model from the adeno-associated virus promoter. In the adeno-associated virus P5 promoter, there are two YY1 binding sites located upstream and downstream of the TATA box [6]. At the downstream site, YY1 can initiate basal transcription [30,32], while at the upstream site, YY1 functions as a repressor by itself. This repressor effect of YY1 can be changed into an activator effect in the presence of the adenovirus E1A protein. More experiments are required to test this model and further characterize the role of YY1 in GM-CSF promoter function. Thus, this experimental system may be another example demonstrating the significance of cofactors in regulating the functional roles of YY1.

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