MOLECULAR AND CELLULAR BIOLOGY, Jan. 1996, p. 157–167 Vol. 16, No. 1
0270-7306/96/$04.00+0

Characterization of the Human Granulocyte-Macrophage Colony-Stimulating Factor Gene Promoter: an AP1 Complex and an Sp1-Related Complex Transactivate the Promoter Activity That Is Suppressed by a YY1 Complex

JIANPING YE,[1]† XIAOYING ZHANG,[1] AND ZIGANG DONG[2]*

Laboratory of Experimental Immunology, Biological Response Modifiers Program, DCT, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702,[1] and The Hormel Institute, University of Minnesota, Austin, Minnesota 55912[2]

Received 13 April 1995/Returned for modification 12 June 1995/Accepted 1 October 1995

It is well documented that a repeated CATT element in the human granulocyte-macrophage colony-stimulating factor (GM-CSF) gene promoter is required for promoter activity. However, the transcription factors that are able to transactivate this enhancer element remain unidentified. Recently, we have found that nuclear factor YY1 can interact with the enhancer element. Here, we report that in addition to YY1, two other nuclear factors have been identified in the DNA-protein complexes formed by the CATT oligonucleotide and the Jurkat T-cell nuclear protein. One of these factors is AP1, and the other one is an Sp1-related protein. Results from transient transfection of Jurkat T cells have revealed that formation of both AP1 and the Sp1-related complex is required for the full enhancer activity of the CATT element. This result is supported by cotransfection of a c-*jun* expression vector and mutational analysis of the AP1 site or the Sp1-related protein binding site. In contrast, formation of the YY1 complex suppresses enhancer activity, since deletion of the YY1 complex induces an augmentation of the enhancer activity and overexpression of YY1 results in an attenuation of the enhancer activity. Results from the mechanism study have revealed that YY1 is able to inhibit transactivation mediated by either AP1 or the Sp1-related protein, and YY1 suppressive activity is DNA binding dependent. Taken together, these data support the ideas that AP1 and the Sp1-related nuclear protein are required for transactivation of the human GM-CSF gene promoter and that YY1 can suppress transactivation of the promoter even under inducible conditions.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein cytokine involved in hematopoiesis and host defense mechanisms (10). GM-CSF is produced by both immune cells (T cells, macrophages, and nature killer cells) and nonimmune cells (fibroblasts and endothelial cells). In an effort to understand the mechanisms by which GM-CSF gene expression is controlled, the promoter region of the GM-CSF gene has been studied extensively (8, 9, 28, 30). It has been reported that there are several functional regions in the proximal region of the promoter (10), including two conserved lymphokine elements (CLE-1 and CLE-2), a GC-rich region, and an NF-κB binding site in the overlapping part of the CLE-2 and the GC-rich region. In the core promoter region, there is a well-established functional region composed of three copies of the CATT sequence (28, 30). This region has some sequence homology with the CLE0 element of the mouse GM-CSF gene promoter.

Of all the identified functional regions, the wild-type CATT element (designated WT in this study) seems to be the most important in regulation of GM-CSF gene promoter activity because mutations generated in this element result in a complete loss of the promoter activity, including constitutive and inducible activity (28). Results from in vitro foot printing revealed that the sequence between −52 and −34 may serve as a core recognition sequence for some nuclear transcription factors (9, 30), but the natures of these factors remain to be identified (9). Recently, we have reported that YY1 is one of the nuclear factors that interact with this element (45).

AP1 is a ubiquitous nuclear protein composed of the *jun* and *fos* gene products (3, 16). AP1 is involved in the regulation of cell proliferation induced by growth factors (1) and tumor promotion induced by phorbol ester (5). More and more studies have revealed that AP1 plays an important role in transcriptional regulation of cytokine genes that contain an AP1 response element in their promoters (12, 34). It has been documented that in cytokine gene regulation, AP1 mediates positive transactivation independently or in association with NF-AT (31).

Sp1 is a zinc finger protein constitutively expressed in various types of cells. Sp1 is involved in transcriptional activation of a variety of cellular genes (4, 19), whether the gene promoter contains a TATA box or not (34). It is generally considered that Sp1 is a proximal promoter factor that activates transcription at a specific binding site within a few hundred base pairs of the transcription start site (6). Recently, three Sp1-related ubiquitous proteins, Sp2, Sp3, and Sp4, have been described (14, 21). To our knowledge, Sp1 activity in regulation of the cytokine gene promoter has not been identified. In contrast to AP1, which mediates an inducible transactivation, Sp1 generally mediates a constitutive transactivation in mammalian cells (6).

Like Sp1, YY1 is also a zinc finger protein constitutively

* Corresponding author. Mailing address: The Hormel Institute, University of Minnesota, 801 16th Ave. NE, Austin, MN 55912. Phone: (507) 437-9640. Fax: (507) 437-9606.

† Present address: Oncology Center, Johns Hopkins University, Baltimore, MD 21231.

expressed in many types of cells (24). YY1 is considered a multifunctional nuclear factor since it can activate, repress, or initiate transcription depending on the gene promoter context (15, 40). YY1 activity has been identified in many cellular and viral genes (40), but its role in cytokine gene regulation remains to be explored. Recently, we have reported that YY1 interacts with a silencer element in the human gamma interferon (IFN-γ) gene promoter (46) and the CATT enhancer element in the human GM-CSF promoter (45).

In an extension of our previous study, we carried out more studies on the natures and functions of the nuclear factors that can interact with the CATT element. We have obtained evidence that AP1 and Sp1-related proteins are two of the nuclear factors that can form protein-DNA complexes with the CATT element. Functional analysis indicates that the formation of both AP1 and the Sp1-related complexes is required for the full activity of the CATT enhancer element. In addition, we have characterized the functional role of YY1 in the regulation of the CATT element activity. Our data indicate that the formation of the YY1 complex can result in suppression of enhancer activity. Further analysis has revealed that YY1 is able to inhibit transactivations mediated by either AP1 or the Sp1-related complex. These results have indicated functional roles for nuclear factors, AP1, Sp1-related protein, and YY1 in the regulation of human GM-CSF gene promoter activity.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides were synthesized by the phosphoramidite method on a DNA/RNA synthesizer (model 392; Applied Biosystems, Foster City, Calif.). The synthesized oligonucleotides were deprotected at 50°C overnight. Complementary 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, Ill.) with Klenow fragment (Bethesda Research Laboratories, Gaithersburg, Md.). A YY1 binding sequence (TGCCTTGCAA AATGGCGTTACTGCAG) was derived from the upstream conserved region of the Moloney murine leukemia virus gene and used to synthesize a YY1 binding oligonucleotide (7). AP1 (CTAGTGATGAGTCAGCCGGATC) and Sp1 (GA TCGATCGGGGCGGGGCGATC) binding oligonucleotides were purchased from Stratagene, Inc. (La Jolla, Calif.).

Cell lines and reagents. Jurkat cells ($CD4^+$ human lymphoblast cell line) were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 U of penicillin-streptomycin per ml (complete medium). Antibodies that recognize nuclear proteins YY1, c-Jun, c-JunB, c-JunD, c-Fos, Sp1, ATF-2, and AP2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.).

Nuclear extraction. Nuclear extracts were prepared as follows (46). Cells (5×10^7) were treated with 500 μl of lysis buffer (50 mM KCl, 0.5% Nonidet P-40 [NP-40], 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.8], 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg of leupeptin per ml, 20 μg of aprotinin per ml, 100 μM DL-dithiothreitol [DTT]) on ice for 4 min. After centrifugation at 14,000 rpm for 1 min, the supernatant was saved as the cytoplasmic extract. The nuclei were washed once with the same volume of buffer without NP-40, then placed into a 300-μl volume of extraction buffer (500 mM KCl and 10% glycerol with the same concentrations of HEPES, PMSF, leupeptin, aprotinin, and DTT as the lysis buffer), and pipetted several times. After centrifugation at 14,000 rpm for 5 min, the supernatant was harvested as the nuclear protein extract and stored at −70°C. Protein concentration was determined with bicinchoninic acid (BCA; Pierce, Rockford, Ill.).

EMSAs. For electrophoretic mobility shift assays (EMSAs), the DNA-protein binding reaction was conducted in a 24-μl reaction mixture including 1 μg of poly(dI · dC) (Sigma), 3 μg of nuclear protein extract, 3 μg of bovine serum albumin, 4×10^4 cpm of ^{32}P-labeled oligonucleotide probe, and 12 μl of 2× Y-buffer (47). In some cases, double-stranded oligomer was added as an unlabeled competitor. This mixture was incubated on ice for 10 min without antibody or 20 min with antibody; then it was incubated for 25 min at room temperature in the presence of a radiolabeled probe and resolved on a 5% acrylamide gel (National Diagnostics, Atlanta, Ga.) that had been prerun at 110 V for 2 h with 0.5× Tris-borate-EDTA buffer. The loaded gel was run at 210 V for 90 min, dried, and placed on Kodak X-Omat film (Eastman Kodak, Rochester, N.Y.). The film was developed after overnight exposure at −70°C. A recombinant YY1 protein has been described before (45).

Reporter vector. Three gene expression vectors were used in this study. (i) Plasmid 108 is a β-galactosidase expression vector controlled by a human IFN-γ promoter fragment from −108 to +64 (33). The repeated CATT(A/T) element was inserted at the *Xba*I and *Pst*I sites upstream of the IFN-γ promoter in the proper orientation. The cloned inserts were verified by DNA sequencing. (ii) A simian virus 40 promoter-controlled luciferase expression vector was used as a transfection efficiency control. (iii) A cytomegalovirus promoter-controlled YY1 or c-*jun* expression vector was used in a cotransfection study (7).

Transfection assay. The Jurkat T cells were grown in the complete medium as described earlier. Cells (5×10^6) were transiently transfected with 10 μg of reporter gene vector with DEAE-dextran (12). A luciferase expression vector (1 μg) was used as an internal control. After being transfected, the cells were washed once in phosphate-buffered saline solution and then cultured in 10 ml of complete medium at 37°C for 48 h. Phorbol myristate acetate (PMA; 10 ng/ml) plus ionomycin (1 μg/ml) was added to stimulate the cell culture 24 h before the cells were collected. The β-galactosidase assay was carried out according to the published method (45). β-Galactosidase activity was determined by hydrolysis of chlorophenol red β-D-galactopyranoside and presented by a reading from a spectrophotometer at 570 nm (Dynatech Laboratories, Inc., Chantilly, Va.). Luciferase activity was determined through a luciferase assay system (Promega) and indicated by a reading from a luminometer. The protein concentration of the cell lysate was determined with the BCA protein assay reagent (Pierce) and indicated by a reading from the spectrophotometer at 570 nm (Dynatech Laboratories, Inc.). The β-galactosidase activity was divided by the luciferase activity and the protein reading at each point. The value resulting from the calculation was used as the normalized β-galactosidase activity. A statistical difference in the results was calculated from the results of three individual experiments by Student's *t* test, with a confidence level of 95% ($P < 0.05$).

RESULTS

Three DNA-protein complexes identified in the Jurkat cell nuclear extract. To confirm the transacting activity of the repeated CATT element, one copy of the WT element was linked to the IFN-γ promoter (Fig. 1A). In a transient-transfection assay of Jurkat T cells, a weak constitutive activity and a strong inducible enhancer activity of the WT element were observed (Fig. 1B). These results are consistent with our previous observations (45).

To investigate the nuclear proteins that interact with this DNA fragment, we conducted an EMSA with the nuclear extracts from unstimulated and PMA-ionomycin-stimulated Jurkat cells. A radiolabeled WT oligonucleotide was used as a probe. The results showed that two or three specific DNA-protein complexes, designated A, B, and C, were formed in the nuclear extracts from unstimulated (Fig. 1C, lanes 1 to 3) and stimulated (Fig. 1C, lanes 4 to 6) Jurkat cells. Since complex A was detected only in the nuclear extract from stimulated Jurkat cells and no change was observed in complexes B and C before and after stimulation, it was obvious that complex A was inducible, while complexes B and C were not inducible. There was an additional specific complex formed in the stimulated nuclear extract, which was located between complex C and the free probe. Our previous data indicated that this was a derivative of complex C (45), so it was not analyzed in the following study.

DNA sequences required for formation of each protein complex. In an effort to know whether formation of the three DNA-protein complexes is associated with the transacting activity of the CATT element, we generated eight mutants that lacked the ability to form one, two, or all three of the complexes through deletion or substitution of base pairs in the WT sequence (Fig. 2A). To determine their protein-binding activities, an oligonucleotide competition assay was conducted, in which mutant oligonucleotides M1 to M7 were used as competitors against the WT probe (Fig. 2B). As indicated at the top of each lane in the EMSA, the ability of each mutant to form the A, B, and C complexes is summarized for each of the competitors. The DNA binding sites of complexes A, B, and C are indicated in Fig. 2C. The binding ability of each mutant oliogonucleotide was confirmed by results from other EMSA experiments in which the mutant oligonucleotides were radio-

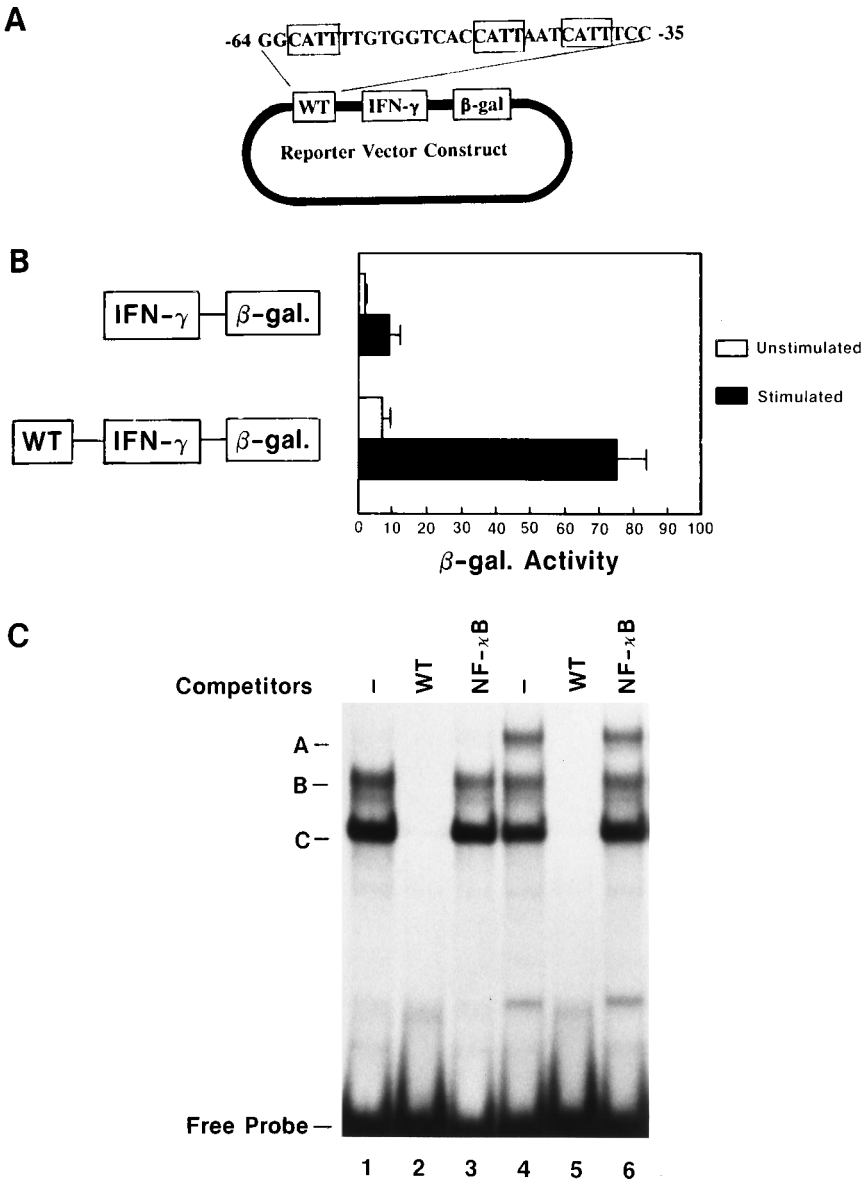

FIG. 1. Enhancer activity and DNA-protein complexes of the CATT element in Jurkat T cells. (A) The β-galactosidase reporter vector was constructed by inserting one copy of the wild-type CATT element (WT) into the *Xba*I and *Pst*I sites upstream of the IFN-γ promoter that controls expression of the β-galactosidase gene. All the reporter vectors with mutant versions of WT were constructed in the same manner in this study. (B) The enhancer activity of WT was determined by transient gene transfection of Jurkat T cells. Each bar represents the mean relative β-galactosidase (β-gal.) activities for three independent transfection assays. 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. The same stimulation was used in the following transfection study. (C) An EMSA was conducted as described in Materials and Methods. The stimulated nuclear extract was made from Jurkat cells that had been treated with PMA (10 ng/ml) plus ionomycin (1 μg/ml) for 3 h. ^{32}P-labeled double-stranded WT oligonucleotide was used as a probe. Unlabeled WT or NF-κB binding oligonucleotide (100 ng) was used as a specific or nonspecific competitor, respectively. The positions of complexes A, B, and C are marked.

labeled and used as probes with stimulated Jurkat cell nuclear extract (data not shown). Oligonucleotide M6 exhibited a reduced ability to form the C complex, but it retained a remarkable binding capacity for the C complex. The DNA-binding ability of M8 is presented in Fig. 5A.

To determine the relationship between formation of the complexes and enhancer activity, reporter vectors M1 and M2 were constructed by inserting M1 or M2 into plasmid 108 at a position upstream of the IFN-γ promoter, and their activities were examined in a transient-transfection assay of Jurkat cells stimulated with PMA-ionomycin. The results showed that M1 lost 60% of the WT enhancer activity and M2 lost all of the WT enhancer activity (Fig. 2D). Since M1 lacks the ability to form complex A and M2 lacks all three complexes, these results suggest that formation of the three DNA-protein complexes is associated with the function of the CATT element.

Complex A contains AP1 protein and accounts for part of the inducible enhancer activity. It has been reported recently that the conserved lymphokine element 0 (CLE0; [−58]CAC CATTAATCATTTCCTC [−40]) in the mouse GM-CSF gene promoter is able to form an AP1 complex (25, 44). Since the CATT element has some homology with CLE0 and complex A exhibits an inducible feature, it was interesting to know whether complex A is an AP1 complex. DNA sequence ho-

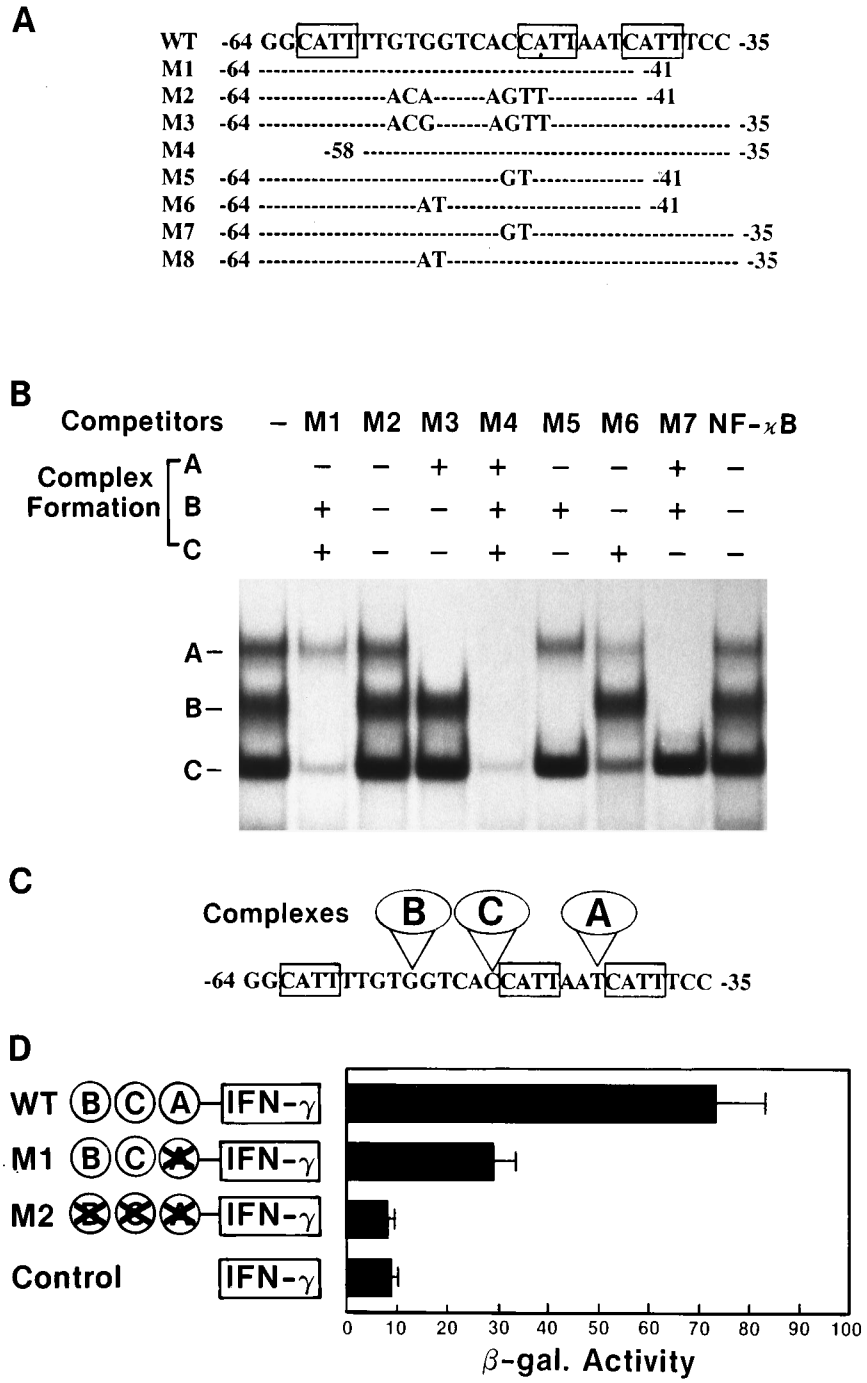

FIG. 2. DNA binding sites and functional relevance of the three complexes. (A) DNA sequences of a variety of mutant versions of the CATT element that were used to synthesize the mutant oligonucleotides. (B) Competition assay with mutants M1 to M7. Stimulated cell nuclear extract was used in the EMSA, with the radiolabeled WT as the probe. Different mutant oligonucleotides were used as the competitor at 100 ng (indicated above each lane). The ability to form each DNA-protein complex is also indicated. (C) Relative position of each complex in the CATT element. (D) The functions of M1 and M2 were determined with stimulation as described in the legend to Fig. 1B. Each bar represents the mean relative β-galactosidase (β-gal.) activities for three independent transfection assays.

mology was found between an AP1 consensus binding sequence and the complex A binding site (Fig. 3A), indicating that complex A may be an AP1 complex. To examine this hypothesis, we conducted oligonucleotide competition and supershift analyses of the complex in the EMSA and collected three lines of evidence. First, we used a radiolabeled AP1 oligonucleotide as a control probe and observed an identical mobility between complex A and the AP1 complex in the EMSA (Fig. 3B). Second, we observed that WT and the AP1-binding DNA cross-competed in an oligonucleotide competition assay by EMSA (Fig. 3B, lanes 2 and 8). Third, complex A was removed by the c-Jun antibody and supershifted by the c-Fos antibody but was not affected by the AP2 antibody in the supershift assay (Fig. 3B, lanes 3 to 5). The same supershift

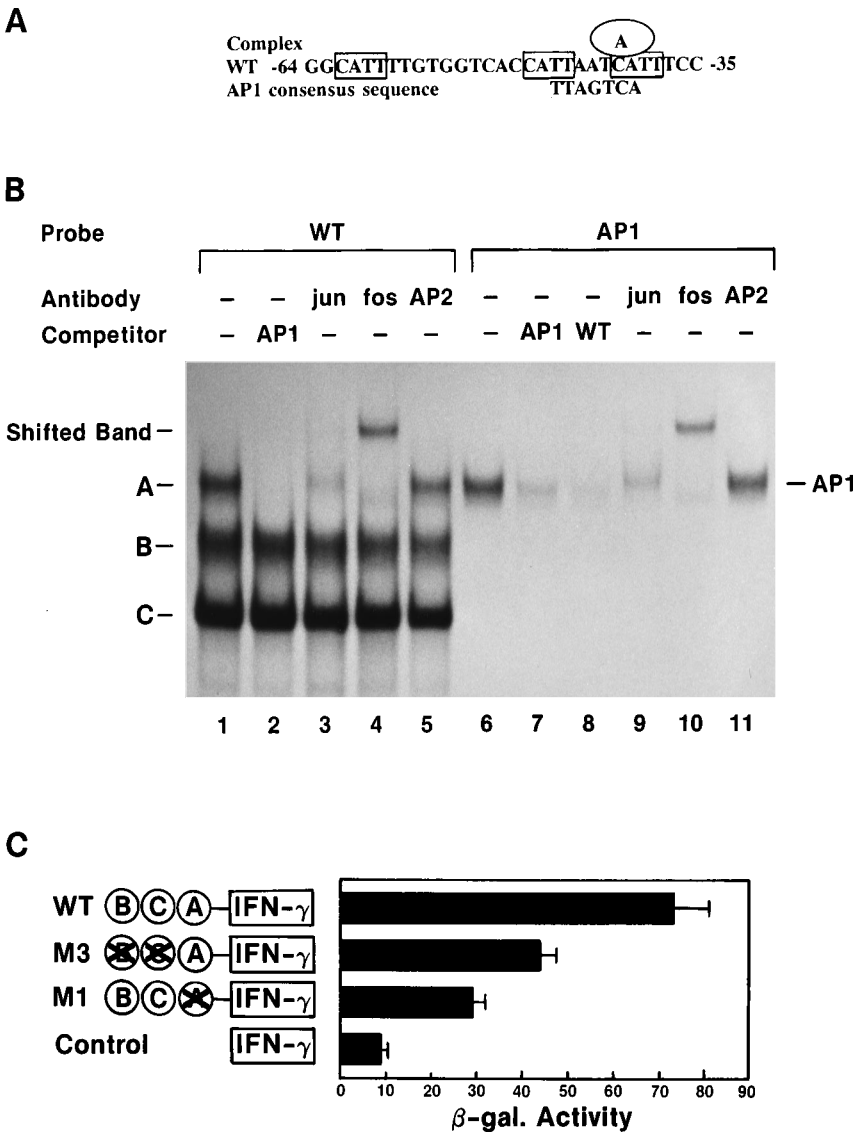

FIG. 3. Characterization of the proteins and functions of complex A. (A) DNA sequence homology in the complex A binding site and AP1 consensus binding sequence. (B) Protein nature of complex A. Radiolabeled WT was used as a probe (lanes 1 to 5) and radiolabeled AP1 binding oligonucleotide was used as a control (lanes 6 to 11) in an EMSA with stimulated Jurkat cell nuclear extract. Competitor oligonucleotide (100 ng) or antibodies (200 ng) were used in the assay, as indicated above each lane. (C) The function of complex A was determined by examining M1 and M3 activities with stimulation as described in the legend to Fig. 1B. Each bar represents the mean relative β-galactosidase (β-gal.) activities for three independent transfection assays.

results were observed for the AP1 control complex (Fig. 3B, lanes 9 to 11). This evidence strongly supports the hypothesis that complex A is an AP1 complex.

To characterize the functional role of the AP1 complex, an M3 reporter that contained only complex A and an M1 reporter that lacked only complex A were assayed in a transient-transfection assay of Jurkat cells stimulated with PMA-ionomycin. Functional results indicate that complex A is required for the inducible enhancer activity, as formation of this complex generated 60% of the full inducible enhancer activity in the absence of complexes B and C (Fig. 3, M3). On the other hand, in the absence of complex A, the CATT element lost 60% of its inducible enhancer activity (Fig. 3, M1). Together, these results suggest that formation of the DNA-AP1 complex in the CATT element is responsible for a significant part of the full inducible enhancer activity.

To further investigate the functional role of nuclear factor AP1 in regulation of the GM-CSF gene promoter, a c-*jun* expression vector was cotransfected with the WT, M1, and M3 reporter genes (Fig. 4). The transfection results showed that overexpressed c-Jun can further upregulate the promoter activity under inducing conditions (Fig. 4, WT). The cotransfection results also indicate that AP1 activity is DNA binding dependent, because the c-*jun* expression vector induces promoter activity only when a complete AP1 binding sequence is present (Fig. 4, WT and M3). It could not activate the promoter when the AP1 binding sequence was mutated (Fig. 4, M1).

Complex B is an Sp1-related protein-DNA complex and contributes to the inducible enhancer activity. Since the formation of complex A did not account for the full enhancer activity, we next investigated what complex was responsible for

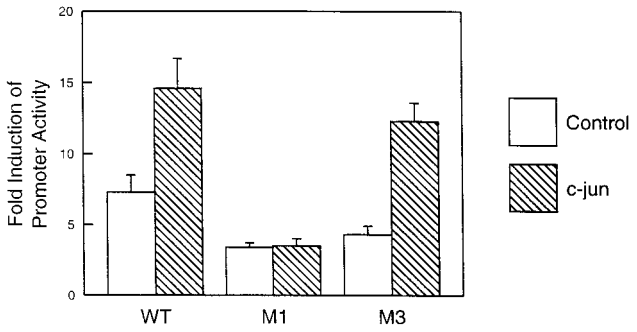

FIG. 4. Cotransfection assay of a c-*jun* expression vector. The c-*jun* expression vector and a reporter gene vector were cotransfected at a ratio of 1:1 with a total of 20 μg of DNA at each point. Promoter activity is expressed as fold induction of β-galactosidase activity after stimulation of the reporter gene. Each bar represents the mean of three individual transfection assays.

the remaining enhancer activity. To address this question, we studied the role of complex B in the activity of the CATT element. We first investigated the protein nature of complex B in an EMSA. In a supershift assay, complex B was removed completely by the Sp1 antibody; in contrast, complexes A and B were not affected at all (Fig. 5A, lane 3). Since complex B was not challenged by other antibodies, including those against AP2 (Fig. 5A, lane 4), c-Jun (Fig. 3B, lane 3), c-Fos (Fig. 3B, lane 4), and YY1 (Fig. 6A, lane 3), these results suggested that removal of the B complex by the Sp1 antibody was a specific effect. Interestingly, in an oligonucleotide competition assay, we could not observe efficient competition between WT and the Sp1 binding oligonucleotides in formation of the B complex (Fig. 5A, lanes 2) or the Sp1 complex (Fig. 5A, lane 7), although there is 78% homology between the complex B binding site and an Sp1 consensus binding site (Fig. 5B). In addition, complex B exhibited a mobility distinct from that of the Sp1 complex (Fig. 5A). These results suggest that the nuclear protein involved in the formation of complex B is different from Sp1 in DNA binding specificity and electrophoretic mobility in an EMSA. The protein could be recognized specifically by the Sp1 antibody, indicating that it may be an Sp1-related nuclear protein.

Since Sp1 plays an important role in the activation of many gene promoters, we then investigated whether complex B accounts for the other part of the enhancer activity. To conduct the functional analysis, M5, which retains only complex B, and M8, whose ability to form complex B is specifically eliminated (Fig. 5A, lane 11), were inserted upstream of the IFN-γ promoter in plasmid 108. The activities of the M5 and M8 constructs were examined by transfection of Jurkat cells followed by stimulation with PMA-ionomycin. The results showed that in the absence of complexes A and C, complex B acted as an activator, and its formation resulted in an enhancer activity equal to 60% of the full enhancer activity (Fig. 5, M5). Alternatively, in the presence of complexes A and C, deletion of complex B resulted in a 60% loss of the full enhancer activity (Fig. 5, M8). Taken together, these results indicate that in addition to complex A, complex B is another activator that contributes to the full enhancer activity of the CATT element.

Complex C contains YY1 protein and mediates a negative transactivating activity. Previously, we observed one complex with the WT probe in a different EMSA system and demonstrated that this complex contains YY1 protein (45). In this study, we used an improved EMSA system and identified three complexes with the same probe. Formation of complex C requires the YY1 binding site (Fig. 2C); this suggests that complex C may be the YY1 complex that we described previously (45). To examine this hypothesis, we conducted further analysis of complex C. In an EMSA, complex C exhibited a mobility identical to that of a control YY1 complex formed by the radiolabeled YY1 binding oligonucleotide (Fig. 6A). Additionally, complex C was reduced specifically by competition with the YY1 binding oligonucleotide (Fig. 6A, lane 2), and similarly, the control YY1 complex was reduced by competition with the WT oligonucleotide (Fig. 6A, lane 7). Moreover, in a supershift assay, the C complex was reduced specifically by the YY1 antibody, and part of complex C was supershifted to a position above complex A (Fig. 6A, lanes 3). To confirm the specificity of the YY1 antibody, a recombinant YY1 protein was used in a supershift assay (Fig. 6B). This recombinant protein forms a specific complex with the WT probe. This complex was reduced by the YY1 antibody in a dose-dependent pattern (Fig. 6B, lanes 2 to 4) but was not changed by the AP2 antibody (Fig. 6B, lane 5). Taken together, these results demonstrated that complex C exhibited identity to the YY1 complex in DNA binding specificity, electrophoretic mobility, and antigenicity. This indicates that complex C is a YY1 complex and may be identical to the one that we characterized earlier (45).

To characterize the functional role of complex C, the transacting activities of an M6 reporter that retains only complex C and an M7 reporter that lacks the ability to form complex C were analyzed in a transient-transfection assay of Jurkat cells followed by stimulation with PMA-ionomycin. Our results showed that in the absence of complexes A and B, complex C did not exhibit any transacting activity in the IFN-γ promoter (Fig. 6C, M6); however, in the presence of complexes A and B, deletion of complex C induced a boost in the enhancer activity of the CATT element by 100% (Fig. 6C, M7). Since elimination of complex C did not decrease but rather augmented the enhancer activity, the data suggest that a negative effect is generated on the CATT element when YY1 binding occurs. This conclusion is supported by the fact that overexpression of YY1 resulted in a decrease in the enhancer activities of all the constructs examined that retained YY1 binding activity (Fig. 7B). Additionally, in the absence of the A and B complexes, the YY1 complex failed to exert any inhibition on the promoter, indicating that the inhibitory effect of the YY1 complex is specific to the positive transactivation mediated by complex A or B.

Mechanism of the suppressive activity of YY1. Since both the A and B complexes mediate positive transactivation in the CATT element, we tried to determine whether the YY1 complex can inhibit only one or both of these complexes. To do this, the effect of complex C on either complex A or B was examined by transfection of WT, M1, M3, M5, M7, and M8 reporter vectors into Jurkat cells followed by PMA-ionomycin stimulation (Fig. 7A). Our results showed that in the absence of the B complex (M3 and M8), the presence of complex C decreased the enhancer activity mediated by complex A (M8). This result implies that YY1 can suppress AP1 in the CATT element. Similarly, in the absence of complex A (M1 and M5), the presence of complex C decreased transactivation by complex B (M1), indicating a negative effect of YY1 on the Sp1-related protein in the CATT element.

To confirm these results, we conducted a cotransfection assay with the YY1 expression vector and the WT, M1, and M8 reporter vectors. Although both M1 and M8 are able to form the YY1 complex, they differ in that M1 forms complex B (Fig. 2B), while M8 generates complex A (Fig. 5A, lane 11). Cotransfection was conducted in Jurkat cells, followed by PMA-

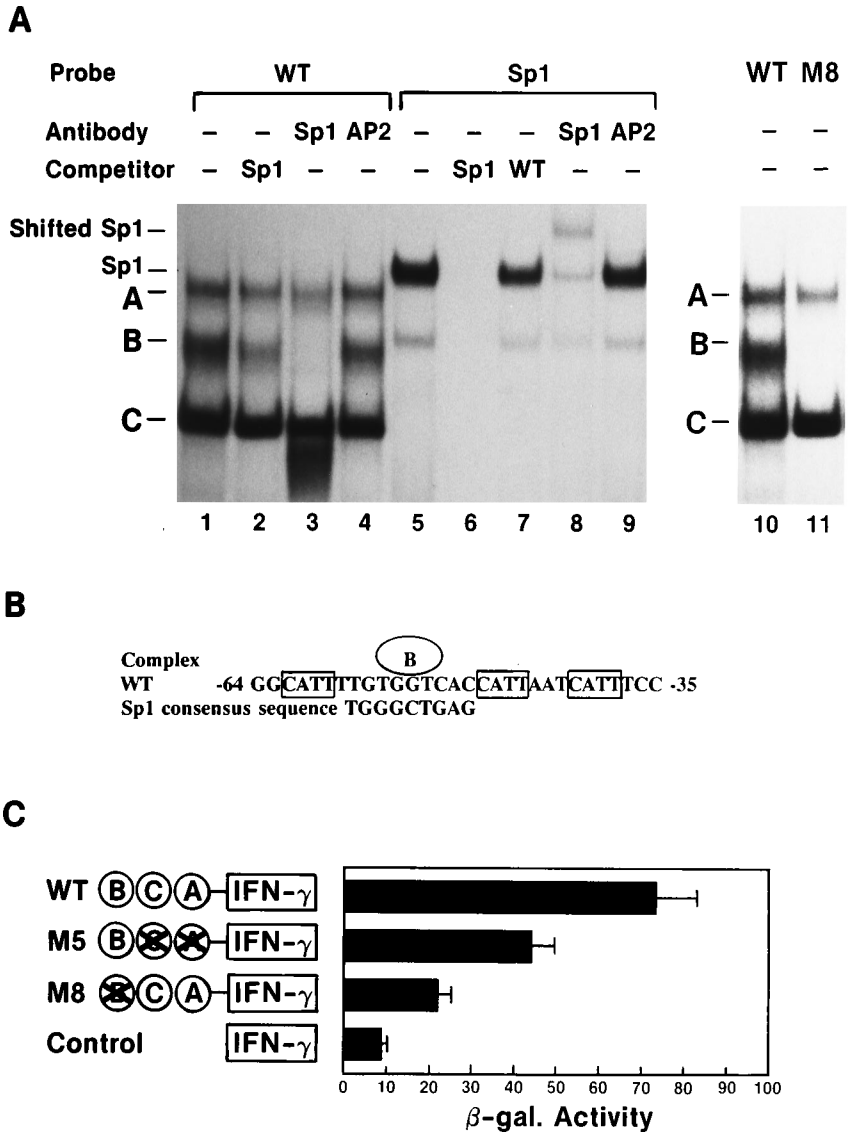

FIG. 5. Characterization of the protein and function of complex B. (A) Protein nature of complex B. Radiolabeled WT (lanes 1 to 4) and radiolabeled Sp1 binding oligonucleotide (lanes 5 to 9) were used as probes in an EMSA with stimulated Jurkat cell nuclear extract. Competitor oligonucleotides (100 ng) or antibodies (200 ng) were used in the assay, as indicated above each lane. Radiolabeled M8 was used in the EMSA to determine its capacity for forming DNA-protein complexes (lane 11) along with radiolabeled WT as a control (lane 10). (B) DNA sequence homology in the complex B binding site and Sp1 consensus binding sequence. (C) The function of complex B was determined by examining M5 and M8 activities with stimulation as described in the legend to Fig. 1B. Each bar represents the mean relative β-galactosidase (β-gal.) activities for three independent transfection assays.

ionomycin stimulation. The results showed that overexpression of YY1 suppressed the enhancer activities of all three reporters, WT, M1, and M8 (Fig. 6A).

Functional data suggest that the inhibitory activity of YY1 is DNA binding dependent (Fig. 7A). To examine this hypothesis, we cotransfected Jurkat cells with a YY1 expression vector and the M7 reporter vector, which lacks YY1 binding activity. The cotransfection result showed that YY1 could suppress WT promoter but not M7 promoter activity (Fig. 7C). This result supports the idea that YY1 activity is DNA binding dependent.

Together, these data indicate that in the CATT element, the YY1 complex can inhibit transactivation mediated by both AP1 and Sp1-related complexes and that the inhibition by YY1 is dependent on DNA binding. Since the DNA binding sites for complexes A, B, and C are clustered together in the CATT element, the inhibitory effects might result from competition between YY1 and AP1 or YY1 and Sp1-related protein for overlapping sequences at their binding sites.

DISCUSSION

GM-CSF is produced not only by lymphocytes but also by nonlymphocytes, such as fibroblasts (29), endothelial cells (20), and epidermal cells (43). This suggests that GM-CSF gene transcription may be controlled by ubiquitous nuclear transcription factors. AP1, Sp1, and YY1 are three ubiquitous nuclear factors that have been identified in the transcriptional control of various genes. The CATT element in the human GM-CSF gene promoter has some sequence homology with the CLE0 element in the mouse GM-CSF gene promoter. Although it has been reported that the enhancer activity of the

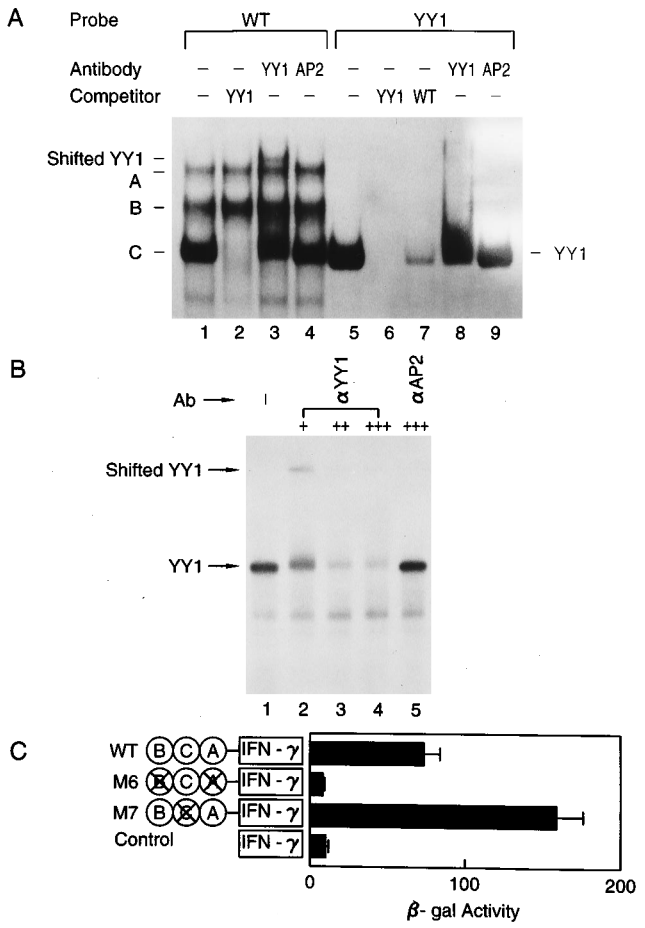

FIG. 6. Characterization of the protein nature and function of complex C. (A) Protein nature of complex C. Radiolabeled WT was used as a probe (lanes 1 to 4) and radiolabeled YY1 binding oligonucleotide was used as a control (lanes 5 to 9) in an EMSA with stimulated Jurkat cell nuclear extract. Competitor oligonucleotides (100 ng) or antibodies (200 ng) were used in the assay, as indicated above each lane. (B) Supershift assay of the recombinant YY1 protein. Bacterial extract that contains recombinant YY1 protein (1 μl) was used in an EMSA with the WT probe. Each + represents 100 ng of antibody. (C) The function of complex C was determined by examining M6 and M7 activities with stimulation as described in the legend to Fig. 1B. Each bar represents the mean relative β-galactosidase (β-gal) activities for three independent transfection assays.

CLE0 element requires cooperative binding of nuclear factors AP1 and Elf-1 (44) or AP1 and NF-AT (25, 41), it remains unknown whether the same binding is required by the CATT element (9). Recently, we reported that YY1 is a nuclear protein that can interact with this element (45). Here, we present the first piece of evidence that AP1 and an Sp1-like protein are two important nuclear factors responsible for the full enhancer activity of the CATT element.

In this study, we identified three DNA-protein complexes, A, B, and C, in the activated Jurkat nuclear extract by using an improved EMSA system which used undialyzed cell nuclear extract and a no-salt reaction buffer (46). The results from a protein characterization study showed that complex A is an AP1 complex that contains c-Jun and c-Fos, complex B is an Sp1-related complex that contains a nuclear protein specifically removed by the Sp1 antibody in the supershift assay, and complex C is a YY1 complex.

It has been reported that the mouse CLE0 element can form an AP1 complex and that the complex can be removed or supershifted by JunB and JunD antibodies (44). In our study, we used JunB and JunD antibodies along with c-Jun and c-Fos antibodies in the supershift assay, but we did not observe any change in the A complex in the presence of either JunB or JunD antibodies (data not shown). It also has been reported that c-Jun is able to dimerize with ATF-2 protein (42). We used an ATF-2 antibody in a supershift assay but did not observe any change in complex A (data not shown). Taken together, these results suggest that complex A is composed of nuclear factors c-Jun and c-Fos and does not involve JunB, JunD, or ATF-2 protein.

We have found that AP1 is responsible for only part of the inducible enhancer activity of the CATT element. We observed that a mutation that abolished AP1 binding resulted in a 60% loss of the enhancer activity and that formation of the A complex generated 60% of the enhancer activity in the absence of the B and C complexes. In the mouse CLE0 element, Elf-1/NF-AT is responsible for part of the enhancer activity (25, 41, 44). To determine whether the enhancer activity is related to Elf-1/NF-AT, the interleukin-2 Elf-1/NF-AT binding oligonucleotide was used as a competitor against the WT probe, but we did not observe any reduction in the three complexes (data not shown). The absence of Elf-1/NF-AT binding might be due to the fact that the CATT element does not contain a complete Elf-1/NF-AT binding sequence. Thus, our data do not indicate that Elf-1/NF-AT contributes to the enhancer activity of the CATT element.

Our results provide the first evidence that the CATT element can form a complex with an Sp1-related nuclear protein and that formation of this complex is responsible for part of the enhancer activity of the CATT element. Although complex B was recognized by the Sp1 antibody, its full nature remains to be characterized. It is possible that complex B contains several nuclear proteins and that Sp1 is one of these proteins. It has been reported that YY1 can physically interact with Sp1 protein (23, 36), so it is possible that complex B is formed by a dimer of YY1 and Sp1. Although we did not see any effects of the YY1 antibody and YY1 binding DNA on complex B in the EMSA (Fig. 5B), we cannot exclude this possibility. The lack of effects may be due to a blockage of the YY1 epitope by the Sp1 protein and alteration of the DNA binding specificity of YY1 after formation of the dimer. To examine this hypothesis, antibodies that recognize different epitopes of YY1 factor are required for the supershift assay. We conclude that complex B is an Sp1-related protein-DNA complex.

Since formation of both complex A and B correlates well with expression of the full enhancer activity of the CATT element, we conclude that cooperative binding of AP1 and the Sp1-related nuclear protein is required for expression of the full enhancer activity. Under the present EMSA conditions, we failed to detect a third complex that contains both the A and B complexes, but this proposal is supported by observations from other groups, in which the complex A and B binding sites were protected by activated nuclear factors in an in vitro footprinting assay (9, 30). Although the level of complex B did not change in the nuclear extract from the stimulated cells, it did account for part of the inducible enhancer activity of the CATT element. The mechanism(s) associated with this activity is not clear. We suggest that expression of complex B's function is dependent on a stimulation that may induce phosphorylation of the Sp1-related protein (18) or result in the availability of a cofactor of Sp1 (33).

It is very interesting that YY1 can suppress transactivation mediated by either AP1 or the Sp1-related protein in the CATT element. YY1 is a multifunctional transcription factor that acts as a positive or negative regulator as well as a tran-

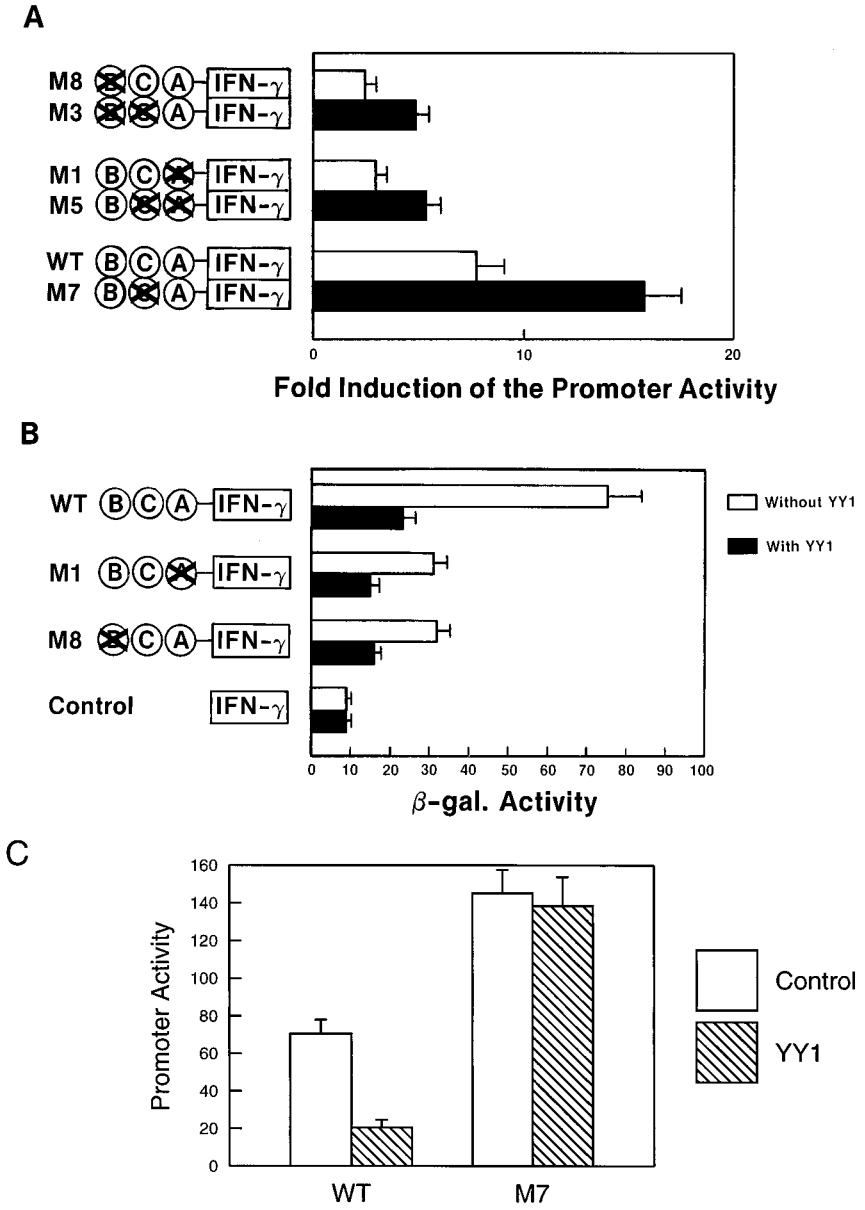

FIG. 7. Characterization of the inhibitory activity of complex C. (A) The suppressive effects of complex C on both the A and B complexes were examined in the system described in the legend to Fig. 1B. Fold induction of each reporter vector construct versus the parental vector was used to express the functional activities of each CATT element. Each bar represents the mean of three independent transfection assays. (B) Cotransfection assay with YY1 expression vector. YY1 expression vector (5 μg) was cotransfected with 10 μg of reporter vector in Jurkat T cells followed by stimulation, as described in the legend to Fig. 1B. Each bar represents the mean relative β-galactosidase (β-gal.) activities for three independent transfection assays. (C) DNA binding dependence of YY1 inhibitory activity. The cotransfection assay was conducted as for panel B. Each bar represents the mean relative β-galactosidase activities for three independent transfection assays.

scription initiator in many mammalian and viral genes (15, 40). It has been shown that in a TATA-less promoter (2, 11) or in a region downstream of the TATA box (37, 38), YY1 can act as a transcription initiator. In the region upstream of the TATA box, some studies suggest that YY1 acts as a repressor in a specific promoter context (40). The suppressive effect of YY1 can be obtained through the following possible mechanisms. (i) YY1 competes with a transcriptional activator for overlapping sequences at the DNA binding site; these activator proteins include the serum response factor (13, 23), NF-κB (24), and lactation-associated complex (26, 35). (ii) YY1 acts as a DNA-bending protein that turns the bound activator protein away from the initiation complex to prevent interaction between the activator and the initiation complex (27). In the c-*fos* gene promoter, in addition to competition with the serum response factor (13), YY1 could also turn CREB away from the initiation complex (27). (iii) YY1 may sequester an activator protein through formation of a protein-protein complex. It has been reported that YY1 can physically interact with some transacting nuclear proteins, like Sp1 (22, 36) and c-Myc (39), but this inhibitory activity of YY1 remains to be demonstrated.

In a previous study, we observed that overexpression of YY1 could inhibit the enhancer activity of the CATT element (45). In this study, we have obtained evidence that YY1 suppresses transactivations mediated by both AP1 and Sp1-related protein. The suppressive mechanism(s) remains to be investigated,

but we propose that a competition for the binding sequence at the DNA binding site might be a mechanism, because the YY1 binding sequence overlaps the binding sequences of either AP1 or an Sp1-related protein. This hypothesis is supported by the fact that YY1 complex alone did not express inhibitory activity in this system.

To detect competitions between YY1 and AP1 and YY1 and Sp1-related protein, more experiments with purified recombinant AP1 and YY1 proteins in an EMSA are required.

Since YY1 can form a protein-protein complex with Sp1, YY1 may also intervene in the activity of complex B through a protein-protein interaction with the Sp1 protein, but our cotransfection data do not support this possibility. In the absence of the YY1 binding sequence, overexpressed YY1 did not exhibit any effect on promoter activity (Fig. 7C).

In summary, we report here that the transcription factors AP1, YY1, and an Sp1-related protein are involved in regulation of the CATT enhancer activity. Both AP1 and Sp1-related protein are required for expression of the full enhancer activity of the CATT element, while YY1 has an inhibitory effect on the enhancer activity. The inhibition effect of YY1 is DNA binding dependent and specific to AP1 and the Sp1-related protein. These data may help us to outline the biological activities of AP1, YY1, and Sp1-related protein in regulating human GM-CSF gene expression.

ACKNOWLEDGMENTS

We thank Howard A. Young and John R. Ortaldo for providing generous experimental supplies. We also thank Carmen Perleberg for secretarial assistance.

This work was supported in part by the Hormel Foundation.

REFERENCES

1. **Angel, P., and M. Karin.** 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim. Biophys. Acta **1073:**129–157.
2. **Basu, A., K. Park, M. L. Atchison, R. S. Carter, and N. G. Avadhani.** 1993. Identification of a transcriptional initiator element in the cytochrome c oxidase subunit Vb promoter which binds to transcription factor NF-E1 (YY-1, δ) and Sp1. J. Biol. Chem. **268:**4188–4196.
3. **Chiu, R., W. J. Boyle, J. Meek, T. Emeal, T. Hunter, and M. Karin.** 1988. The c-Fos protein interacts with c-Jun/AP1 to stimulate transcription of AP-1 responsive genes. Cell **54:**541–552.
4. **Courey, A. J., and R. Tjian.** 1988. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell **55:**887–898.
5. **Dong, Z., M. J. Birrer, R. G. Watts, L. M. Matrisian, and N. H. Colburn.** 1994. Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. Proc. Natl. Acad. Sci. USA **91:** 609–613.
6. **Dynan, W., and R. Tjian.** 1985. Control of eukaryotic messenger RNA synthesis by sequence-specific DNA binding proteins. Nature (London) **316:** 774–778.
7. **Flanagan, J. R., K. G. Becker, D. Ennist, S. L. Fleason, H. P. Driggers, B. Z. Levi, E. Appella, and K. Ozato.** 1992. Cloning of a negative transcription factor that binds to the upstream conserved region of Moloney murine leukemia virus. Mol. Cell. Biol. **12:**38–44.
8. **Fraser, J. K., J. J. Guerra, C. Y. Nguyen, J. E. Indes, J. C. Gasson, and S. D. Nimer.** 1994. Characterization of a cell-type-restricted negative regulatory activity of the human granulocyte-macrophage colony-stimulating factor gene. Mol. Cell. Biol. **14:**2213–2221.
9. **Fraser, J. K., S. Tran, S. D. Nimer, and J. C. Gasson.** 1994. Characterization of nuclear proteins that bind to a critical positive regulatory element of the human granulocyte-macrophage colony-stimulating factor promoter. Blood **84:**2523–2530.
10. **Gasson, J. C.** 1991. Molecular physiology of granulocyte-macrophage colony-stimulating factor. Blood **77:**1131–1145.
11. **Gaston, K., and M. Fried.** 1994. YY1 is involved in the regulation of the bi-directional promoter of the Surf-1 and Surf-2 genes. FEBS Lett. **347:** 289–294.
12. **Gottschalk, L. R., D. M. Giannola, and S. G. Emerson.** 1993. Molecular regulation of the human IL-3 gene: inducible T-cell-restricted expression requires intact AP-1 and Elf-1 nuclear protein binding sites. J. Exp. Med. **178:**1681–1692.
13. **Gualberto, A., D. Lepage, G. Pons, S. L. Mader, K. Park, M. Atchison, and K. Walsh.** 1992. Functional antagonism between YY1 and the serum response factor. Mol. Cell. Biol. **12:**4209–4214.
14. **Hagen, G., S. Muller, M. Beato, and G. Suske.** 1992. Cloning by recognition site screening of two novel GT box binding proteins: a family of Sp1 related genes. Nucleic Acids Res. **20:**5519–5525.
15. **Hahn, S.** 1992. The yin and the yang of mammalian transcription. Curr. Biol. **2:**152–154.
16. **Halazonetis, T. D., K. Georgopoulos, M. E. Greenberg, and P. Leder.** 1988. c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. Cell **55:**917–924.
17. **Inouye, C. J., and E. Seto.** 1994. Relief of YY1-induced transcriptional repression by protein-protein interaction with the nuclear phosphoprotein B23. J. Biol. Chem. **269:**6506–6510.
18. **Jackson, S. P., J. J. MacDonald, S. Lees-Miller, and R. Tjian.** 1990. GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase. Cell **63:**155–165.
19. **Kadonaga, J. T., K. R. Carner, F. R. Masiarz, and R. Tjian.** 1987. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell **51:**1079–1090.
20. **Kaushansky, K.** 1989. Control of granulocyte-macrophage colony-stimulating factor production in normal endothelial cells by positive and negative regulatory elements. J. Immunol. **143:**2525–2529.
21. **Kingsley, C., and A. Winoto.** 1992. Cloning of GT box-binding proteins: a novel multigene family regulating T-cell receptor gene expression. Mol. Cell. Biol. **12:**4251–4261.
22. **Lee, J.-S., K. M. Galvin, and Y. Shi.** 1993. Evidence for physical interaction between the zinc-finger transcription factors YY1 and Sp1. Proc. Natl. Acad. Sci. USA **90:**6145–6149.
23. **Lee, T.-C., Y. Shi, and J. Schwartz.** 1992. Displacement of BrdUrd-induced YY1 by serum response factor activates skeletal α-actin transcription in embryonic myoblasts. Proc. Natl. Acad. Sci. USA **89:**9814–9818.
24. **Lu, S.-Y., M. Rodriguez, and W. S.-L. Liao.** 1994. YY1 represses rat serum amyloid A1 gene transcription and is antagonized by NF-κB during acute-phase response. Mol. Cell. Biol. **14:**6253–6263.
25. **Masuda, E. S., H. Tokumitsu, A. Tsuboi, J. Shlomai, P. Hung, K.-I. Arai, and N. Arai.** 1993. The granulocyte-macrophage colony-stimulating factor promoter *cis*-acting element CLE0 mediates induction signals in T cells and is recognized by factors related to AP1 and NFAT. Mol. Cell. Biol. **13:**7399–7407.
26. **Meier, V. S., and B. Groner.** 1994. The nuclear factor YY1 participates in repression of the β-casein gene promoter in mammary epithelial cells and is counteracted by mammary gland factor during lactogenic hormone induction. Mol. Cell. Biol. **14:**128–137.
27. **Natesan, S., and M. Z. Gilman.** 1993. DNA bending and orientation-dependent function of YY1 in the c-fos promoter. Genes Dev. **7:**2497–2509.
28. **Nimer, S., J. Fraser, J. Richards, M. Lynch, and J. C. Gasson.** 1990. The repeated sequence CATT(A/T) is required for granulocyte-macrophage colony-stimulation factor promoter activity. Mol. Cell. Biol. **10:**6084–6088.
29. **Nimer, S. D., M. J. Gates, H. P. Koeffler, and J. C. Gasson.** 1989. Multiple mechanisms control the expression of granulocyte-macrophage colony-stimulating factor by human fibroblasts. J. Immunol. **143:**2374–2377.
30. **Nimer, S. D., E. A. Morita, M. J. Martis, W. Wachsman, and J. C. Gasson.** 1988. Characterization of the human granulocyte-macrophage colony-stimulation factor promoter region by genetic analysis: correlation with DNase I footprinting. Mol. Cell. Biol. **8:**1979–1984.
31. **Nolan, G. P.** 1994. NF-AT-AP-1 and Rel-bZIP: hybrid vigor and binding under the influence. Cell **77:**795–798.
32. **Penix, L., W. M. Weaver, Y. Pang, H. A. Young, and C. B. Wilson.** 1993. Two essential regulatory elements in the human interferon γ promoter confer activation specific expression in T cells. J. Exp. Med. **178:**1483–1496.
33. **Pugh, B. F., and R. Tjian.** 1990. Mechanism of transcriptional activation by Sp1: evidence for coactivators. Cell **61:**1187–1197.
34. **Rao, A.** 1994. NF-ATp: a transcription factor required for the co-ordinate induction of several cytokine genes. Immunol. Today **15:**274–281.
35. **Raught, B., B. Khursheed, A. Kazansky, and J. Rosen.** 1994. YY1 represses β-casein gene expression by preventing the formation of a lactation-associated complex. Mol. Cell. Biol. **14:**1752–1763.
36. **Seto, E., B. Lewis, and T. Shenk.** 1993. Interaction between transcription factors Sp1 and YY1. Nature (London) **365:**462–464.
37. **Seto, E., Y. Shi, and T. Shenk.** 1991. YY1 is an initiator sequence-binding protein that directs and activates transcription in vitro. Nature (London) **354:**241–245.
38. **Shi, Y., E. Seto, L.-S. Chang, and T. Shenk.** 1991. Transcriptional repression by YY1, a human GLI-Kruppel-related protein, and relief of repression by adenovirus E1A protein. Cell **67:**377–388.
39. **Shrivastava, A., S. Saleque, G. V. Kalpana, S. Artandi, S. P. Goff, and K. Calame.** 1993. Inhibition of transcription regulator yin-yang-1 by association with c-Myc. Science **262:**1889–1892.
40. **Shrivastava, A., and K. Calame.** 1994. An analysis of genes regulated by the multi-functional transcription regulator yin yang-1. Nucleic Acids Res. **22:** 5151–5155.

41. **Tsuboi, A., E. S. Masuda, Y. Naito, H. Tokumitsu, K.-I. Arai, and N. Arai.** 1994. Calcineurin potentiates activation of the granulocyte-macrophage colony-stimulating factor gene in T cells: involvement of the conserved lymphokine element 0. Mol. Biol. Cell **5:**119–128.
42. **Van-Dam, H., M. Duyndam, R. Rottier, A. Bosch, L. D. Vries-Smits, P. Herrilich, A. Zantema, P. Angel, and A. J. Van der Eb.** 1993. Heterodimer formation of c-Jun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus E1A protein. EMBO J. **12:**479–487.
43. **Vasunia, K. B., M. L. Miller, A. Puga, and C. S. Baxter.** 1994. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is expressed in mouse skin in response to tumor-promoting agents and modulates dermal inflammation and epidermal dark cell numbers. Carcinogenesis **15:**653–660.
44. **Wang, C.-Y., A. G. Bassuk, L. H. Boise, C. B. Thompson, R. Bravo, and J. M. Leiden.** 1994. Activation of the granulocyte-macrophage colony-stimulating factor promoter in T cells requires cooperative binding of Elf-1 and AP-1 transcription factors. Mol. Cell. Biol. **14:**1153–1159.
45. **Ye, J., H. Y. Howard, J. R. Ortaldo, and P. Ghosh.** 1994. Identification of a DNA binding site for the nuclear factor YY1 in the human GM-CSF core promoter. Nucleic Acids Res. **22:**5672–5678.
46. **Ye, J., P. Ghosh, M. Cippitelli, J. Subleski, K. J. Hardy, J. R. Ortaldo, and H. A. Young.** 1994. Characterization of a silencer regulatory element in the human interferon-γ promoter. J. Biol. Chem. **269:**25728–25734.