Characterization of a Silencer Regulatory Element in the Human Interferon-γ Promoter*

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Although the precise molecular mechanisms underlining the regulation of human IFN-γ gene expression by analyzing both upstream elements has not been fully demonstrated, we selected the BE region for investigation. In this report, we characterize the nuclear protein complexes in Jurkat cells that bind to the BE region specifically and analyze the function of the BE region in transient transfection experiments when linked to the IFN-γ and a heterologous promoter.

Materials and Methods

Oligonucleotides—Oligonucleotides were synthesized by the phosphoramidite method on a DNA/RNA synthesizer (Applied Biosystems, model 392, Foster City, CA). The synthesized oligonucleotides were 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 [32P]dCTP (Amersham Corp.) using the Klenow fragment (Life Technologies, Inc.). Sequences of two specific competitor oligonucleotides are shown as follows: AP2 oligonucleotide (GGTGTGGAAAGTCCCCAGGCTCCCCAGCAC) from the distal 72-base pair repeat region of the SV40 gene (15) and YY1 oligonucleotide (ATGCCTGCAGAAATGGCTTACGCAG) from the upstream conserved region of the Moloney murine leukemia virus gene (16).

Results—Jurkat cells (CD4+ human lymphoblast cell line) were cultured in complete medium (RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 unit/ml penicillin-streptomycin). Purified human peripheral blood T cells (fresh T cells that bind to the BE region specifically and analyze the function of the BE region in transient transfection experiments when linked to the IFN-γ and a heterologous promoter.

MATERIALS AND METHODS

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Cell Lines and Reagents—Jurkat cells (CD4+ human lymphoblast cell line) were cultured in complete medium (RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 unit/ml penicillin-streptomycin). Purified human peripheral blood T cells (fresh T cells, CD3+ > 95%) were cultured in the same medium. Y1 antiernuer was generously provided by Drs. K. Becker and K. Oztat (Laboratory of Developmental and Molecular Immunology, NICHD, National Institutes of Health, Bethesda, MD). Anti-AP2 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Nuclear Extract—Nuclear extracts were prepared as follows (17): 1 x 106 cells were treated with 50 μl of lysis buffer (10 mM KCl, 0.5% Nonidet P-40, 25 mM Hepes, pH 7.8, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 100 μg/ml 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 Nonidet P-40, then were put into a 300-μ1 volume of extraction buffer (600 μM KCl, 10% glycerol with the same concentrations of Hepes, phenylmethylsulfonyl fluoride, 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

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The abbreviations used are: IFN-γ, interferon-γ; CAT, chlorampheni
col acetyltransferase; EMSA, electrophoretic mobility shift assay; YY1, Yin-Yang 1 factor; BE, binding element; DTT, dithiothreitol; TK, thymidine kinase; IL, interleukin.

Interferon-γ (IFN-γ) has diverse biological activities in the immune system. It is predominantly produced by activated T cells and large granular lymphocytes, and it is clear that its production in vivo is tightly controlled and restricted (1, 2). Although the precise molecular mechanisms underlying the strict control of the IFN-γ gene expression have not been fully characterized, a major role of regulated gene transcription in IFN-γ production has been well established, and both positive and negative control over IFN-γ transcription has been demonstrated (3). Our laboratories have been investigating the regulation of human IFN-γ gene expression by analyzing both the gene structure and identifying the cis-acting functional elements. Our earlier results, based on deletion analysis of the hIFN-γ promoter, indicated that there may be at least two enhancer elements and two potential silencer elements located upstream region of the TATA box (3). One of the silencer elements was 36 base pairs in length and located between −251 to −215 (designated as BE, binding element, in this report), as demonstrated by transient transfection studies with human peripheral blood T lymphocytes (3).

Negative regulatory elements have been identified in the promoters of several cytokine genes, including IL-2 (4), IL-3 (5), IL-4 (6), GM-CSF (7), IFN-α (8, 9), IFN-β (10, 11), TNF-α (12), and LD78 (13). This suggests that the negative motif plays an important role in cytokine gene regulation. In order to understand the mechanisms by which the IFN-γ production is tightly controlled, we selected the BE region for investigation. In this report, we characterize the nuclear protein complexes in Jurkat cells that bind to the BE region specifically and analyze the function of the BE region in transient transfection experiments when linked to the IFN-γ and a heterologous promoter.

Because of the heterologous nature of the BE region, we have conducted a series of experiments to characterize BE function and analyze the binding proteins which interact with this region. Transient transfection assays in the Jurkat T cell line revealed that the BE region possesses silencer activity, which is orientation-dependent when reinserted 5' to the IFN-γ core promoter. However, when the BE region was inserted in front of a heterologous promoter (thymidine kinase (TK)), a mild enhancer activity was observed. Utilizing the electrophoretic mobility shift assay, we have identified two major DNA-protein complexes (designated as S and E complexes) which interact with this region. Mutational analysis indicated that the silencer activity observed with the IFN-γ promoter correlated with the S complex and the enhancer activity correlated with the E complex. Preliminary characterization of these two DNA-protein complexes has demonstrated the presence of multiple proteins in each complex. We have found that the S protein complex has a recognition sequence similar to the nuclear factor AP2, and we have identified the nuclear factor Yin-Yang 1 (YY1) as one of the proteins in the E complex.

Previous analysis of the human interferon-γ (IFN-γ) promoter indicated that the region of DNA from −251 to −215 (designated here as BE (binding element)) possessed silencer activity, as deletion of this region caused an increase in promoter activity. Based on this finding, we have conducted a series of experiments to characterize BE function and analyze the binding proteins which interact with this region. Transient transfection assays in the Jurkat T cell line revealed that the BE region possesses silencer activity, which is orientation-dependent when reinserted 5' to the IFN-γ core promoter. However, when the BE region was inserted in front of a heterologous promoter (thymidine kinase (TK)), a mild enhancer activity was observed. Utilizing the electrophoretic mobility shift assay, we have identified two major DNA-protein complexes (designated as S and E complexes) which interact with this region. Mutational analysis indicated that the silencer activity observed with the IFN-γ promoter correlated with the S complex and the enhancer activity correlated with the E complex. Preliminary characterization of these two DNA-protein complexes has demonstrated the presence of multiple proteins in each complex. We have found that the S protein complex has a recognition sequence similar to the nuclear factor AP2, and we have identified the nuclear factor Yin-Yang 1 (YY1) as one of the proteins in the E complex.

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determined by BCA (Pierce Chemical Co.).

Electrophoretic Mobility Shift Assay (EMSAs)—The protein-DNA binding reaction (18) was conducted in a 20-ml reaction mixture with 0.5 μg of Poly(dI-dC) (Sigma), 2 μg of nuclear protein extract, 5 x 10^6 cpm [32P]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) or 2× Y buffer (24% glycerol, 24 mM Hepes, pH 7.8, 8 mM Tris-HCl, pH 7.9, 2 mM EDTA, 2 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 Co.). The film was developed after overnight exposure at −70°C.

UV Cross-linking—The bromouridine-substituted BE oligonucleotide was synthesized on the same DNA/RNA synthesizer and labeled with [α-32P]dCTP using a Klenow fragment (Life Technologies, Inc.). UV-cross-linking was carried out according to a published procedure (18) with the following modification: UV irradiation was performed on the gel after EMSA with 305-m UV light generated by the UV Transilluminator 400 (Stratagene, La Jolla, CA) at a distance of 0 min, 60 min. The specific bands were cut out and ground, then eluted with TBS buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% SDS). 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.

Immunoprecipitation—The UV-cross-linked DNA-protein complex obtained from the last step was boiled for 5 min in 1× reducing buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 1% SDS, and 0.5% 2-mercaptoethanol), then diluted with 500 μl of TNN buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 1% Triton X-100). 10 μl of antibody and 20 μl of protein A-Sepharose CL-4B (100 μg of resin (Pharmacia Biotech Inc.) in 750 μl of fresh TNT buffer) were then added to the DNA-protein complex. After overnight rotation at 30 rpm, the resin was spun down and washed three times with 0.5 ml of TNT buffer, then boiled in 40 μl of 1× reducing buffer for 5 min and centrifuged again. The supernatant that contained the immunoprecipitated salt-resistant probe-complex was loaded onto a 10% SDS-PAGE mini-gel and electrophoresed at 140 V for 1 h. The gel was dried and exposed to the x-ray film overnight.

Reporter Gene Construction—Two kinds of reporter gene vectors were used in this study: 1) plasmid 108, 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 human IFN-γ promoter fragment (upstream of the IFN-γ promoter). To test enhancer activity, we inserted the BE fragment 108, 108 (vector with inserted BE in proper orientation), BEp108 (vector with inserted BE in reverse orientation), and BEr108 (BE in reverse orientation). After electroporation in the presence of the reporter plasmids, the viable cells were separated from dead cells after 3 h and cultured in the absence of stimulation for 21 h, then in the presence of 10 ng phorbol myristate acetate and 1 μg of ionomycin/ml for 24 h. Each of the bars represents the mean value (X ± S.D.) of β-galactosidase activity from four independent experiments. B, enhancer activity of the BE region in the TK promoter. Functional activity of the BE region was examined in the TK promoter in the plasmid pBCTKp-CAT, TK (parental vector), BEpTK (vector inserted BE in proper orientation), and BErTK (vector inserted BE in reverse orientation). After electroporation in the presence of the reporter vector, the viable cells were separated from dead cells after 3 h and cultured in the absence of stimulation for 45 h. Each of the bars represents the mean value (X ± S.D.) of the CAT activity from three individual experiments.

RESULTS

Activity of the BE Region on the IFN-γ Promoter—In order to determine if BE could affect promoter function, we examined the activity of the BE region on the core human IFN-γ promoter (Fig. 1A). In plasmid 108, the human IFN-γ promoter fragment (−108 to +64) was inserted in front of the human IFN-γ promoter (BEpl108) and in reverse orientation (BErl108) did not show any inhibitory effect on the promoter activity. Interestingly, the same BE fragment in reverse orientation (BEr108) did not show any inhibitory effect on the IFN-γ promoter, implying that the silencer activity of the BE region was orientation-dependent.

Activity of the BE Region on a Heterologous Promoter—When the BE fragment was inserted at the 5′ end of the TK promoter (Fig. 1B), the promoter activity was increased by about 5-fold as measured by CAT activity, and this effect was orientation-independent as both orientations of the BE region (BEpTK and BErTK) had the same effect on the promoter. This result indicated that the BE region also possessed a weak enhancer ac-

FIG. 1. Bifunctional activities of the BE region. 8 x 10^6 Jurkat T cells were transfected with 20 μg of the reporter gene vector as described under “Materials and Methods.” A, silencer activity of the BE region in the IFN-γ promoter. Functional activity of the BE region was examined in the IFN-γ promoter in the plasmid 108, 108 (parental vector), BEp108 (vector with inserted BE in proper orientation), and BEr108 (vector with inserted BE in reverse orientation). After electroporation in the presence of the reporter plasmids, the viable cells were separated from dead cells after 3 h and cultured in the absence of stimulation for 21 h, then in the presence of 10 ng phorbol myristate acetate and 1 μg of ionomycin/ml for 24 h. Each of the bars represents the mean value (X ± S.D.) of β-galactosidase activity from four independent experiments. B, enhancer activity of the BE region in the TK promoter. Functional activity of the BE region was examined in the TK promoter in the plasmid pBCTKp-CAT, TK (parental vector), BEpTK (vector inserted BE in proper orientation), and BErTK (vector inserted BE in reverse orientation). After electroporation in the presence of the reporter vector, the viable cells were separated from dead cells after 3 h and cultured in the absence of stimulation for 45 h. Each of the bars represents the mean value (X ± S.D.) of the CAT activity from three individual experiments.
Regulation of IFN-γ Gene Transcription

Fig. 2. Nuclear DNA-binding protein complexes identified in the EMSA assay utilizing G5 buffer. A, specific nuclear protein complexes binding to the BE region. A 32P-labeled BE oligonucleotide was used as a probe in EMSA. Lane 1, no competitor; lane 2, 100 M excess unlabeled BE oligonucleotide; lane 3, 100 M excess SP1 binding oligonucleotide. B, localization of protein binding sites by mutation analysis of the BE region. Four BE mutants were generated by changing nine nucleotides in sequence of BE region (the mutated sequences are shown in C). 32P-Labeled wild type BE and mutated BE were used as probes in the EMSA assay as shown at the top of each lane. C, sequences of the wild type and mutated BE oligonucleotides. In the sequences of the mutated BE, unchanged nucleotides are indicated by the dotted lines.

Fig. 3. Functional analysis of the M2 and M3 oligonucleotides. A, correlation of the S protein complex with the silencer activity of the BE region. M2 and M3 were inserted in the IFN-γ promoter as described under “Materials and Methods.” The transfection assay conditions are described in the legend to Fig. 1A. Each bar represents the mean value (X ± S.D.) of β-galactosidase activity from four separate experiments. B, activities of M2 and M3 in the TK promoter. M2 and M3 were inserted in the TK promoter of the pBCTK-CAT plasmid as described under “Materials and Methods.” The transfection assay conditions are described in the legend to Fig. 1B. Each bar represents the mean value (X ± S.D.) of CAT activity from three separate experiments.
M2 mutant (M2pTK) expressed the same enhancer activity as the wild type BE (BEpTK), but the M3 mutant (M3pTK) failed to exhibit any enhancer activity. This result indicated that there might be another complex(s) which was not revealed by the EMSA analysis conducted above and which should bind to the M2, but not the M3, mutant oligonucleotide.

As in vitro DNA-protein interaction is affected by the binding buffer, we tried several binding buffers with different pH and salt concentrations in order to determine whether we could identify any new specific DNA-protein complex(s) which may be responsible for the enhancer activity of the BE region. In Y buffer, which was distinct from the previous buffer with respect to pH and salt concentration, we found a specific DNA-protein complex designated "E" which was competed by cold BE oligonucleotide in a dose-dependent pattern (Fig. 4A, lanes 2–4), but was not competed by an unrelated oligonucleotide SP1 (A, lanes 5–7). A faster migrating DNA-protein complex, designated L in this figure, was also detected but it could not be reproducibly observed so it was not further investigated.

To determine the DNA binding site of the E complex, we used the same panel of 32P-labeled BE mutant oligonucleotides, M1, M2, M3, and M4, to probe the Jurkat cell nuclear extracts in the Y buffer (B). The results are shown in Fig. 4B. With the exception of M3, all the other mutant BE oligonucleotides were able to form the E complex, indicating that the DNA sequences corresponding to the M3 region were involved in the E complex formation. Combined with the functional data from the TK promoter, these results indicated that the E complex correlated with the enhancer activity of the BE region.

**Characterization of the S Complex**—The mutational study suggested that the binding sequences of the S complex were located in the M2/M3 region of the BE oligonucleotide. A sequence homology search for the known nuclear factors led our attention to the AP2 (15, 21) and the YY1 (14, 22) nuclear factors. As shown in Fig. 5, A and B, the S complex binding site shared high homology with the binding sequences of the AP2 and the YY1 nuclear factors. Based on this homology, we used the AP2 and the YY1 binding oligonucleotides as competitors in EMSA (C). The results showed that the S complex can be specifically competed by the AP2 oligonucleotide, but not by the YY1 oligonucleotide. This indicated that the S complex may contain an AP2-like nuclear protein. In order to investigate that possibility, we carried out UV cross-linking analysis of the S complex using a bromouridine-substituted BE oligonucleotide (D). The experiment demonstrated that the S complex consisted of two subunit proteins (bands a and b) with approximate molecular masses of 50 and 40 kDa (after deduction of the probe molecular mass). The 50-kDa protein had the same molecular weight as the AP2 nuclear factor (15, 21), suggesting the possible involvement of this factor in the suppressive activity of the BE region. However, EMSA experiments utilizing rabbit reticulocyte lysates generated AP2, supershift experi-

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**Fig. 4. Nuclear DNA-binding protein complexes identified utilizing Y buffer.** A, specific nuclear protein complexes binding to the BE region. A 32P-labeled BE was used as a probe in the EMSA assay with the Jurkat cell nuclear extract. Lane 1, no competitor; lanes 2–4, different molar excess of specific competitors; lanes 5–7, different molar excess of nonspecific competitors. B, mutation analysis of the E complex binding site in the BE region. 32P-Labeled BE mutant oligonucleotides (shown at the top of each bar) were used as probes in the EMSA assay under the same conditions as described in A.

**Fig. 5. Characterization of the S protein complex.** A, binding sequence homology between the S complex binding site and the AP2 nuclear factor binding site. B, binding sequence homology between the S complex binding site and the YY1 binding site. C, the AP2 oligonucleotide competes specifically with the BE probe. A 32P-labeled BE oligonucleotide was used as a probe in the EMSA assay with the Jurkat nuclear extract in the GS buffer. –, no competitor; AP2, 100 x excess of AP2 oligonucleotide; YY1, 100 x excess of the YY1 oligonucleotide. D, UV cross-linking analysis of the S complex. The EMSA assay was carried out with 40 μg of Jurkat cell nuclear protein and 7 μg of poly(dI-dC). After treatment with UV light in the gel, the S complex was excised and resolved by 10% SDS-PAGE as described under "Materials and Methods."
Regulation of IFN-γ Gene Transcription

**Fig. 6. Characterization of the E protein complex.** A, the E complex is competed specifically by the YY1 binding oligonucleotide. A 32P-labeled BE oligonucleotide was used as a probe in EMSA with Y buffer. Lane 1, no competitor; lane 2, 100 μ excess of unlabeled BE oligonucleotide; lane 3, 100 μ excess of YY1 oligonucleotide; lane 4, 100 μ excess of SP1 oligonucleotide. B, the E protein binding affinity is influenced by salt concentration. KCl was added to the reaction mixture with Y buffer to generate salt concentrations as indicated at the top of lanes 2 and 3. All other conditions were kept constant. C, the E complex is specifically competed by a YY1 antiserum. 1 μl of rabbit anti-YY1 antiserum (lane 2) and anti-NFκB p50 antiserum (lane 3) was used in the EMSA assay with the radiolabeled BE oligonucleotide in the Y buffer. The reaction mixture was incubated at room temperature for 20 min before addition of the BE probe and resolved by the gel after incubation 20 min with the BE probe.

ments utilizing AP2 antibodies, and cotransfection studies with an AP2 expression vector have all failed to demonstrate any interaction of AP2 with the BE region (data not shown), indicating that subunit a is an AP2-like nuclear protein with respect to DNA binding sequence and molecular mass.

**Characterization of the E Complex—**As demonstrated above, the binding site of the E complex was located in the M3 region of the BE oligonucleotide. Sequence homology analysis (Fig. 5B) revealed that the M3 region shared a high homology with the binding site of the YY1 nuclear factor. The YY1 nuclear factor has been reported to be a multifunctional nuclear factor (14, 16, 23–25), and we wanted to determine if YY1 might be a component of the E complex. In an oligonucleotide competition assay (Fig. 6A), the E complex was competed specifically by the YY1 oligonucleotide (lane 3), but not competed by an irrelevant oligonucleotide SP1 (lane 4). As YY1 binding has been shown to be affected by salt concentration (26), we wanted to know whether salt concentration had any effect on the E complex. As shown in B, the E complex was affected dramatically by salt concentration of the binding buffer. The E complex was reduced in the presence of 30 mM KCl (lane 2) and disappeared completely with 60 mM KCl (lane 3). This result was consistent with the binding characteristics of the YY1 factor as reported by Meier and Groner (26). To further prove that YY1 was in the E complex, we carried out supershift analysis with anti-YY1 antiserum (C) and demonstrated that the E complex was competed by the anti-YY1 antiserum (lane 2), but not by the anti-p50 (a subunit of NFκB) antiserum, a control antiserum (lane 3). We next performed the UV cross-linking analysis of the E complex followed by immunoprecipitation (Fig. 7). The UV-cross-linking analysis showed that the E complex consisted of three proteins (A) with approximate molecular masses of 96, 65, and 48 kDa (after deduction of the probe molecular mass). To determine whether the 65-kDa component of the E complex was YY1 (YY1 molecular mass is 65 kDa), we performed immunoprecipitation of the UV-cross-linked products using the anti-YY1 antiserum. As shown in B, immunoprecipitation with the anti-YY1 antiserum yielded a protein band of molecular mass 65 kDa (lane 2), whereas the unrelated antiserum did not immunoprecipitate any protein from the UV-cross-linked E complex (lane 1). Based on these results, we conclude that the E complex consists of at least three subunit proteins, one of which is the YY1 factor.

**Fig. 7. Identification of the YY1 factor as one of the E complex subunit proteins.** A, the E complex exhibits three subunit proteins in the UV cross-linking assay. UV cross-linking was carried out as described in the legend to Fig. 5, except that Y buffer was used here. B, immunoprecipitation analysis of the E protein subunits. After UV-cross-linking of the E complex, immunoprecipitation was carried out with YY1 antiserum. NFκB p50 antiserum was used as a control. After immunoprecipitation, the precipitated subunit protein was resolved on a 10% SDS mini-gel.

**Modulation of the S and E Complexes—**In order to determine if the appearance of the S and E complexes correlates with the IFN-γ transcriptional activation, we investigated the modulation of the complexes after stimulation of human peripheral blood T cells. First, when we compared the levels of S and E complexes in nuclear extracts from fresh T cells and Jurkat cells by EMSA, comparable levels were observed between the two cells (data not shown). We next analyzed the two complexes in the fresh T cells following combined stimulation for either 4 or 24 h with phorbol myristate acetate plus ionomycin or phytohemagglutinin (Fig. 8), which are known inducers of IFN-γ gene expression in the T cells. The EMSA results indicated that the 4-h treatment did not change the levels of either the E or S complexes (A), but after 24-h treatment, a disappearance of the E complex but no significant change of the S complex was observed (B). These results are consistent with the fact that IFN-γ transcription does not continue after 24-h stimulation (27) and raises the possibility that the S complex may suppress IFN-γ transcription at this time. However, further studies will
be required to verify the importance of the S complex in the regulation of IFN-γ transcription.

**DISCUSSION**

Studies on the regulation of gene transcription often focus on mechanisms of transcriptional activation (28). However, transcriptional repression is also an important factor in the regulation of many genes, including the lymphokine genes (3, 26). Based on deletion analysis of the IFN-γ promoter region, Chrvvia et al. (3) developed a model of cytokine gene transcriptional regulation in which enhancer activity may be modified by a silencer element. In this study, we have characterized one region had a mild enhancer activity that was associated with a complex binding site. A sequence homology search indicated that a sequence homology search indicated that the S complex might have the same binding specificity as the AP2 factor, but suggests that AP2 is not part of the S complex. This conclusion is supported by a recent report characterizing the TNF-α promoter silencer element (12). In the report, Fong et al. (12) identified a novel silencer binding protein that is responsible for the suppressive activity of this silencer element. As the factor also was similar to the AP2 nuclear factor with respect to the homology of DNA binding sequence, they concluded that the repressor factor is an AP2 like nuclear protein, but they failed to demonstrate that its binding could be competed by an AP2 binding oligonucleotide.

The E protein complex, associated with the weak enhancer activity observed with a heterologous promoter, also had specific binding activity to the BE region, but the buffer requirements were different than those used for analysis of the S complex. With the previous binding buffer, we were unable to detect the E complex, because the salt in the previous buffer seriously affected its binding activity. When we switched to a salt free binding buffer, “Y buffer,” the specific binding of the E complex to the BE region was clearly demonstrated. Interestingly, in the Y buffer, the S complex was drastically reduced, indicating the requirement of salt for S complex formation and/or binding.

Several lines of evidence presented here strongly support the possible involvement of the YY1 factor in interacting with the BE region. YY1 (also called CF1, α, NF-E1, or UCRRB) is a 65-kDa DNA-binding protein, belonging to the GLI-Krüppel family, which is expressed in most cells and highly conserved between human and mouse (14, 24, 25). YY1 is a dual functional factor, as it acts as a repressor in some genes, including the adenos-associated virus P5 promoter (14), the immunoglobulin (Ig) k3 enhancer (24, 30), the long terminal repeat of Moloney murine leukemia virus (16, 23), the β-casein gene promoter (26, 31), and the α-actin promoter (32, 33). YY1 also functions as a activator in other genes, including the c-myc promoter (30, 33, 34), the IgH intronic enhancer (24, 30), and the mouse promoter of ribosomal proteins L30 and L32 (25). A recent report demonstrated that the YY1 factor can initiate transcription in combination with TF11B and RNA polymerase (35). However, the YY1 factor has not yet been reported to be involved in cytokine gene expression. The first indication of a possible involvement of YY1 in the complex which binds to the BE region came from the fact that the binding sequence of the E complex had a high sequence homology with the YY1 factor binding site (Fig. 5B). When we used the YY1 binding site as a competitor in EMSA, the E complex was competed by this oligonucleotide (Fig. 6A), and this complex was also competed by the anti-YY1 antiserum, but not by the irrelevant antiserum (Fig. 6C). In addition, the results from UV cross-linking indicated that the E complex consisted of three proteins, one of which had molecular mass of 65 kDa, similar to YY1 (Fig. 7A). To further identify the 65-kDa protein as YY1, we successfully immunoprecipitated the YY1 factor from UV-cross linked E complex by anti-YY1 antiserum (Fig. 7B). Taken together, our data strongly support the fact that the YY1 protein is part of E complex. Our results are supported by the report of Meier and Groner (26), in which they demonstrated that YY1 factor participates in repression of the β-casein gene promoter and binding of the YY1 factor to the promoter DNA is down-
regulated with an increasing salt concentration in the assay system. Their results suggested that binding of the YY1 factor to DNA may be enhanced by decreasing ionic strength in the binding buffer system.

Results from the functional studies of the BE region with two different promoters indicate that the BE region possesses silencer function specific for the IFN-γ promoter. In the IFN-γ promoter, BE exerted a strong silencer activity as only one copy of BE could completely inhibit the inducible IFN-γ promoter activity. This silencer activity is strictly dependent on orientation of the BE insert as an inverted BE region did not exhibit any silencer activity. This feature is in agreement with data obtained on other silencer elements (36). As the mutant forms of BE, M2 and M3, which no longer formed the S complex, did not express silencer activity, we postulate that the silencer activity of BE in the IFN-γ promoter is mediated by the S complex. Furthermore, our data suggests that in the S complex, an AP2-like protein may interact with an as yet unidentified 40-kDa protein and mediate the silencer activity.

In contrast to the silencer activity in the IFN-γ promoter, BE showed modest enhancer activity when placed in front of the TK promoter. Our data from mutation analysis suggest that the enhancer activity of the BE region was mediated by the E protein complex and that the YY1 factor is part of that complex.

It is not clear what functional role, if any, the E complex plays in the IFN-γ promoter, as we did not observe enhancer activity in the IFN-γ promoter with the M2 mutant, which no longer binds to the S complex, but still retains capacity for binding to the E complex. However, although we failed to obtain direct evidence to support a positive role for the E complex in the IFN-γ promoter, we still cannot exclude the possibility that this complex affects IFN-γ transcription. It has been well established that the YY1 factor expresses different activity in different promoters and that the YY1 factor is part of that complex.

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