

Binding of YY1 and Oct1 to a novel element that downregulates expression of IL-5 in human T cells

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Background: IL-5 controls development of eosinophilia and has been shown to be involved in the pathogenesis of allergic diseases. In both atopic and nonatopic asthma, elevated IL-5 has been detected in peripheral blood and the airways. IL-5 is produced mainly by activated T cells, and its expression is regulated at the transcriptional level.

Objective: This study focuses on the functional analysis of the human IL-5 (hIL-5) promoter and characterization of *cis*-regulatory elements and transcription factors involved in the suppression of IL-5 transcription in T cells.

Methods: Methods used in this study include DNase I footprint assays, electrophoretic mobility shift assays, and functional analysis by mammalian cell transfection involving deletion analysis and site-directed mutagenesis.

Results: We identified 5 protein binding regions (BRs) located within the proximal hIL-5 promoter. Functional analysis indicates that the BRs are involved in control of hIL-5 promoter activity. Two of these regions, BR3 and BR4 located at positions -102 to -73, have not previously been described as regulators of IL-5 expression in T cells. We show that the BR3 sequence contains a novel negative regulatory element located at positions -90 to -79 of the hIL-5 promoter, which binds Oct1, octamer-like, and YY1 nuclear factors. Substitution mutations, which abolished binding of these proteins to the BR3 sequence, significantly increased hIL-5 promoter activity in activated T cells.

Conclusion: We suggest that Oct1, YY1, and octamer-like factors binding to the -90/-79 sequence within the proximal IL-5 promoter are involved in suppression of IL-5 transcription in T cells. (*J Allergy Clin Immunol* 1999;103:1125-35.)

Key words: IL-5, promoter, transcription, nuclear factors, T cells, gene expression

IL-5 plays a central role in the control of eosinophilia and in the pathogenesis of eosinophil-associated disorders such as asthma and allergy.¹ The critical role of IL-5 in mediating tissue damage through activation of eosinophils *in vivo* has been shown in a mouse asthma model,² and therefore understanding the control mecha-

Abbreviations used

AP:	Activator protein
BR:	Binding region
BR3o:	Binding region 3 oligonucleotide
CLE0:	Conserved lymphokine element 0
DTT:	Dithiothreitol
EMSA:	Electrophoretic mobility shift assay
NF-AT:	Nuclear factor of activated T-cells
PMA:	Phorbol 12-myristate 13-acetate

nisms for IL-5 production could provide important targets for the treatment of allergic disease. However, in spite of the importance of IL-5 in the development of eosinophilia and allergic disease states, a clear picture of the regulation of the gene has not yet emerged.

IL-5 is produced mainly by activated T lymphocytes and, in common with other lymphokines, is regulated at the transcriptional level.³ The 5'-flanking region of the human IL-5 RNA initiation site contains a regulatory domain that is composed of conserved lymphocyte element 0 (CLE0), Oct, and GATA elements. CLE0 is located immediately upstream of the TATA box. The role of this site in human IL-5 gene expression remains controversial. Mori et al⁴ showed CLE0 to be critical for IL-5 expression; however, investigations conducted by Stranick et al⁵ indicated that it may not be essential. The binding of Oct⁶ and GATA⁷ proteins at 2 sites immediately upstream of the CLE0 element has been reported. Mutations in the GATA element have been shown to abolish IL-5 expression, whereas the functional role of the octamer motif in this region is not clear. An additional Oct element is located at position -244 to -237 of the human IL-5 (hIL-5) promoter and forms complexes with factors antigenically related to members of the Oct family. A study of the human gene in mouse EL-4 cells indicated that mutation of this element reduced promoter activity by 90%.^{6,8}

In addition to these sites, a transcriptionally active nuclear factor of activated T cells (NF-AT) binding site around position -110 in the hIL-5 promoter has been reported. This region contains sequences with homology to NF-AT, NF-Y, and activator protein 1 (AP1) binding sites and has been shown to be critical for expression of hIL-5 constructs in the mouse T-cell clone D10.G4.1.⁵

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Colin J. Sanderson is the recipient of a National Health and Medical Research Council Fellowship.

Received for publication Nov 25, 1998; revised Feb 22, 1999; accepted for publication Feb 22, 1999.

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0091-6749/99 \$8.00 + 0 1/1/98191

TABLE I. Sequences of oligonucleotides used in EMSA

Oligonucleotide	Sequence
BR3o	5'-CGATATGCCATTATTAGGCATTCT-3'
CC mutant	5'-CGATATGtATTATTAGGCATTCT-3'
TAT mutant	5'-CGATATGCCATcgTAGGCATTCT-3'
GC mutant	5'-CGATATGCCATTATTAGgATTCT-3'
NF-κB consensus	5'-AGTTGAGGGGACTTTCCCAGGC-3'
Oct-1 consensus	5'-TGTCGAATGCAAATCACTAGAA-3'
YY1 consensus	5'-CGCTCCGCGGCCATCTTGGCGGCTG- GT-3'

BR3o is the wild-type oligonucleotide for BR3, spanning nucleotides -97 to -74 of the hIL-5 promoter. Substitution mutations in the mutant oligonucleotides of this region are indicated by lower case letters. BR3o and mutant oligonucleotides were synthesized by using standard phosphoramidite chemistry provided by Macromolecular Resources (Colorado State University). The NF-κB and Oct-1 consensus oligonucleotides were obtained from Promega, and the YY1 oligonucleotide was obtained from Santa Cruz Biotechnology, Inc.

The NF-AT site has been found to cooperate with the downstream GATA consensus site to regulate the hIL-5 promoter in the mouse mast cell line CPlI.⁹ Upstream of the NF-AT site, another GATA consensus site has been identified.⁹ No functional significance of binding was determined in this study.

Negative regulatory elements have also been identified in the hIL5 promoter. Expression of the human gene in mouse cells indicated that 2 negative regulatory elements lie between positions -312 and -404⁶ and -127 and -172.⁵ The activity of these elements is dependent on activation of the cells, and deletion of regions containing these elements results in a marked increase in inducible promoter activity. Nuclear proteins that interact with these negative regulatory elements have not been characterized.

In our study we used a human T-cell line, PER-117,¹⁰ which inducibly expresses IL-5 and other cytokines, including IL-2 and IL-4. As in primary T cells, there is no detectable constitutive expression of IL-5, but expression can be induced with phorbol 12-myristate 13-acetate (PMA) and further enhanced by cAMP, ionomycin, and anti-CD28 antibodies. IL-5 production is inhibited by dexamethasone and cyclosporin A, and therefore the gene appears to be functioning in a physiologic fashion in this cell line (Mordvinov V et al, unpublished data). Analysis of results must, however, take into consideration the fact that PER-117 cells do not express the T-cell receptor and as such represent a highly suitable, but not perfect, system for evaluating stimulation of IL-5 gene expression.

In this study we examined *cis*-regulatory elements and nuclear proteins that are involved in induction of hIL-5 transcription. We show the presence of a previously unidentified negative regulatory element between positions -90 to -79 of the hIL-5 promoter. Using specific antibodies and consensus oligonucleotides, we also show that the transcription factors Oct1 and YY1 and octamer-like proteins bind to this region of the promoter. We suggest that Oct1, YY1, and octamer-like factors are involved in negative regulation of IL-5 gene expression in human T cells.

METHODS

PER-117 cells were grown in RPMI 1640 medium supplemented with 7.5% FCS (Trace), 100 mmol/L minimum essential media nonessential amino acid solution (Gibco BRL), 1 mmol/L sodium pyruvate (Gibco), 2 mmol/L l-glutamine (Sigma), 75 mmol/L monothiolglycerol (Sigma), and 10 mmol/L Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.3; Gibco).

A λFIX clone containing an 18.3-kb segment of human chromosome 5q31 (kindly provided by Dr E. Frolova) was used to amplify a fragment of the hIL-5 gene from position -2044 to +45 by means of PCR. This 2089-bp fragment was inserted into the vector pCR2.1 (Invitrogen). The probe used in DNase I protection assays was obtained by subcloning the 351-bp AccI-EcoRI fragment, covering the sequence -306 to +45 of the hIL-5 gene, in the SmaI site of the vector pSP72 (Promega). The protection assays of the coding strand were performed by using a fragment removed by EcoRI and PstI, and the protection assays of the noncoding strand were performed by using a fragment removed by SalI and KpnI. After dephosphorylation of the cleavage products, the fragment was isolated by separation on 1.5% agarose gel, and the resulting excised band was purified by using QiaQuick gel extraction kit (Qiagen). The fragment was 5' end labeled with γ-³²P adenosine triphosphate by using T4 polynucleotide kinase and subsequently purified from unincorporated nucleotides by centrifugation through a G50 column (Boehringer). Nuclear extracts from unstimulated and 6-hour PMA/cAMP-stimulated PER-117 cells were prepared as described later in this section.

DNase I protection assays were performed essentially as described,¹¹ with the following modifications. Nuclear extract was equilibrated in 50 μL of binding buffer (8% glycerol, 1 mmol/L MgCl₂, 0.5 mmol/L EDTA, 0.5 mmol/L dithiothreitol (DTT), 50 mmol/L NaCl, 10 mmol/L Tris-Cl [pH 7.5]) with 2 μg of poly(dIdC) for 20 minutes at room temperature. Forty femtomoles of 5' end-labeled probe were added and incubated for 20 minutes at room temperature. The reaction was stopped, phenol extracted, and ethanol precipitated. The samples were loaded on 6% acrylamide-8M urea gels and electrophoresed. After drying, the gels were exposed to x-ray film overnight.

To position the protected region, the template was sequenced by using a 19-mer oligonucleotide complementary to the 5' extremity of the labeled strand used as the template in the protection assay. Table I lists the oligonucleotides that were used as probes or competitors in electrophoretic mobility shift assays (EMSAs). Nuclear extracts used in EMSAs were prepared by using the method described by Schreiber et al,¹² with the following modification. Protease inhibitor cocktail (Complete, Boehringer Mannheim), 1 mmol/L Na₃V0₄ (Sigma), and 0.5 mmol/L DTT (Sigma) were added to the reaction buffers just before lysis. Protein concentration was determined by using the DC Bio-Rad protein assay. Standard binding reactions contained 3 μg of nuclear extract, 60 mmol/L KCl, 8 mmol/L MgCl₂, 12 mmol/L Hepes (pH 7.9), 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.5 μg poly(dIdC), 12% glycerol, and 25 fmol of ³²P end-labeled oligonucleotide probe. Probe preparation, protein-DNA binding reactions, and PAGE were performed as described by Karlen and Beard.¹³

Polyclonal anti-Oct1, anti-Oct2, and anti-YY1 antisera (Santa Cruz Biotechnology, Inc) were added 20 minutes after the probe in supershift experiments.

The EMSA gel image was generated by using a Kodak DC200 Digital Camera Micrografx Picture Publisher 5.0 and printed on Tektronix Phaser IISDX (printer model 4684). Levels of formation of EMSA complexes were quantified by autoradiography and densitometric scanning of autoradiograms (UMAX Vista-S6, UMAX Data System Inc) with exposures within the linear range.

A λ FIX clone containing an 18.3-kb segment of human chromosome 5q31 was used to construct the hIL-5–luciferase plasmid. The fragment covering the promoter region from nucleotide –506 to +18 was amplified by PCR, blunt ended, and inserted into the SmaI site of the pGL3-Enhancer Vector (Promega). This plasmid was designated the –506/+18 construct. The –281/+18 construct was generated by PCR amplification of this region in –506/+18. The PCR product was purified by means of Qiaquick spin columns (Qiagen Inc) and ligated into the vector pGEM-T (Promega). The IL-5 promoter was then isolated as a NcoI-SacI fragment and ligated into NcoI-SacI digested pGL3-enhancer to generate the –281/+18 construct. Deletion constructs of the hIL-5 promoter were generated by BsaAI/Bal31 digestion of the –506/+18 construct by using standard protocols.¹⁴

Site-directed mutagenesis was performed by using the Altered Sites II in vitro Mutagenesis System (Promega), and mutations were confirmed by DNA sequencing.

In transfection studies 10 μ g of DNA was electrophoresed at 960 μ Fd and 300 V into 10^7 PER-117 cells in 400 μ L of RPMI by using the Bio-Rad gene pulser. After electroporation, the cells were incubated at 37°C for a period of 6 hours before activation. For T-cell activation, PMA (Sigma) and cyclic-AMP (Bt₂cAMP, Sigma) were used at concentrations of 10 ng/mL and 1 mmol/L, respectively. After an additional incubation period of 16 hours, the cells were harvested and resuspended in 100 μ L of reaction buffer containing 50 mmol/L Tris-HCl (pH 7.8), 15 mmol/L MgSO₄, 33.3 mmol/L DTT, 0.1 mmol/L EDTA, 250 μ mol/L LiCoA (Sigma), 500 μ mol/L Na-Luciferin (Molecular Probes), and 0.5% Triton X-100. Luciferase activity was measured in a Victor 1420 multilabel reader (Wallac).

RESULTS

To identify potential sites for sequence-specific interactions of the hIL-5 gene promoter with nuclear proteins from PER-117 cells, footprint experiments with DNase I digestion were performed. We used a 351-bp DNA fragment covering the promoter sequence from position –306 to +45. The DNA probe was incubated with different amounts of nuclear extract protein prepared from unstimulated or PMA/cAMP-stimulated cells. Five footprinted regions were observed on coding and noncoding strands of the hIL-5 promoter. These regions were designated binding regions (BRs) 1 to 5 (Fig 1). The footprints can be classified into 3 types.

The first type can be characterized as constitutive. This was observed in the region located between positions –59 and –43 (BR1) on the noncoding strand of the promoter (Fig 1, B). Nuclear proteins from unstimulated or stimulated cells almost equally protected the sequence in a dose-dependent manner. This footprint has been identified in previous studies and appears to be related to the CLE0 element located at positions –56 to –42 on the hIL-5 promoter.⁵

The second type of protection was PMA/cAMP induced. This footprint, located on the coding strand between positions –107 and –98 (BR5), was observed when the probe was incubated with nuclear proteins from stimulated cells (Fig 1, A). BR5 overlaps with a previously described protein BR of the hIL-5 promoter and may be related to the AP1/NF-AT element.⁵

The third type of footprint could be seen when the

probe was incubated with nuclear proteins from unstimulated cells. In these footprint sequences, located between positions –91 and –73 (BR3) on the coding strand and between positions –78 and –61 (BR2) and positions –102 and –85 (BR4) on the noncoding strand (Fig 1, A and B), were protected. BR2 and BR3 were highly protected with 10 μ g of protein, whereas BR4 was only partially protected with the same amount of protein. In contrast to footprints with protein from unstimulated cells, these 3 BRs were only slightly protected when the incubation was performed with nuclear proteins from PMA/cAMP-stimulated cells. Whereas BR2 may be related to the GATA protein-binding site located around position –70,⁷ BR3 and BR4 represent previously uncharacterized protein-binding sequences of the hIL-5 promoter.

Alignment of footprinted regions from coding and noncoding strands indicates that the sequences that lose protein-binding ability after cell activation overlap (Fig 1, C). Interestingly, both strands of the hIL-5 promoter from position –91 to –73 are almost completely protected in unstimulated cells. Cell stimulation eliminates the protection, suggesting that activation of IL-5 expression alters the protein-binding activity of the region. A surprising observation was the presence of a cleavage between positions –76 and –75 when the probe was incubated with nuclear proteins from unstimulated cells (Fig 1, A). This DNase I cleavage was absent within both the naked DNA and the DNA incubated with protein prepared from stimulated cells. This suggests that a change of local conformation of the DNA induced by binding factors might expose a cleavage site to the DNase I.

Regulation of transcription

To define the functions of the promoter regions, which were found to be protected in DNase I footprint assays, a series of deletion constructs of the 5' hIL-5 promoter was made and transiently expressed in PER-117 cells. No significant differences were observed in basal activities of the different hIL-5 promoter constructs and the promoterless vector pGL3 Enhancer (Promega). Typical luciferase activity levels produced in unstimulated cells transfected with hIL-5 promoter constructs or promoterless vector were 100 to 300 relative light units. Significant luciferase activity was detected in cells transfected with the construct –281/+18 and stimulated with PMA/cAMP. Typical activity levels produced in PMA/cAMP-stimulated cells transfected with this construct were in the range of 20,000 to 50,000 relative light units. This correlates well with the inducible character of hIL-5 expression in PER-117 cells (Mordvinov V et al, unpublished data) and suggests that the hIL-5 promoter contains inducible response elements downstream of position –281.

Deletion of the promoter from position –281 to –91 (construct –91/+18, removing BR5 and one half of BR4) exhibited a moderate effect on expression of the reporter gene (Fig 2). Compared with the construct –281/+18, construct –91/+18 had about 70% promoter activity.

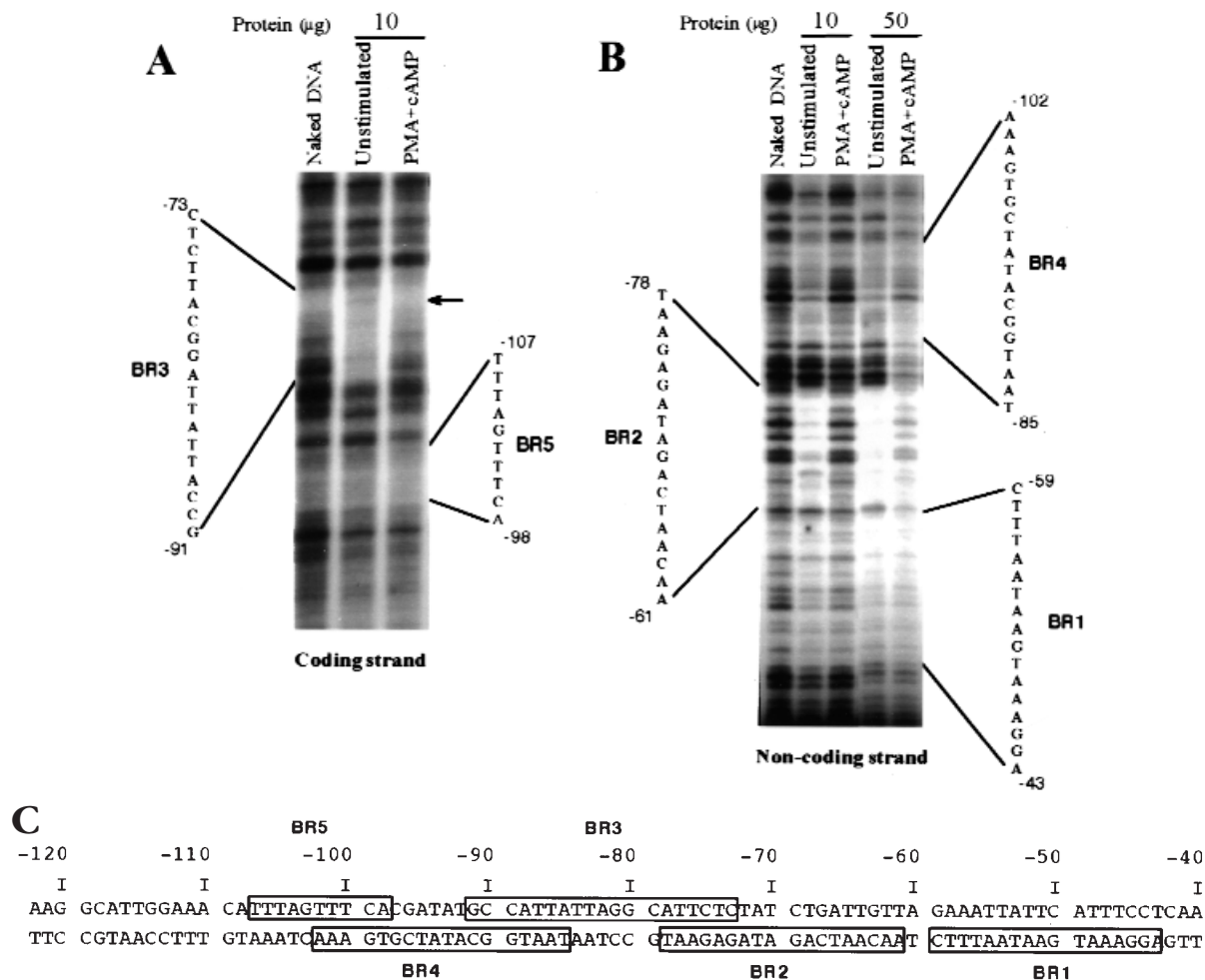


FIG 1. Five regions of the proximal hIL-5 promoter are protected in DNase I footprinting assays. **A**, DNase I footprinting assay with a probe extending from +45 to -306 of the coding strand of the hIL-5 gene. Both unstimulated and PMA/cAMP-stimulated PER-117 nuclear extracts are used, and protected sites and sequences are indicated. *Arrow* indicates a unique cleavage. **B**, DNase I footprinting assay with a probe extending from +45 to -306 of the noncoding strand of the hIL-5 gene. Both unstimulated and PMA/cAMP-stimulated PER-117 nuclear extracts are used, and protected sites and sequences are indicated. **C**, Double-stranded sequence of the proximal hIL-5 promoter from positions -122 to -39 is shown in 5' to 3' orientation. The numbering of residues is defined relative to the transcription initiation site at -1.²⁹ The locations of each BR within the hIL-5 promoter are indicated. The actual bases on each strand that are protected from digestion are contained within the boxes.

Further deletion of 11 nucleotides from construct -91/+18 to yield construct -80/+18 (removing BR4 and one half of BR3) resulted in a 2-fold increase in promoter activity, suggesting that the function of a negative regulatory element had been disrupted. IL-5 promoter activity was significantly decreased on deletion of 5 nucleotides from construct -80/+18 yielding construct -75/+18 (removing BR3 and one half of BR2). Compared with construct -281/+18, the promoter activity of construct -75/+18 fell to 30%. Deletion of the nucleotides between -75 and -47 (construct -47/+18, removing BR2 and one half of BR1) further decreased the promoter activity, indicating the presence of another positive regulatory element.

Taken together, data from deletion analysis indicate that the hIL-5 promoter regions that were found to be protected in DNase I footprint assays are involved in regulation of PMA/cAMP-induced transcription. A new potential negative regulatory element is located within the BR3 sequence, and a positive regulatory region lies downstream of this negative regulatory element. These regions include the BR1, BR2, and partial BR3 sequences and may be related to the CLE0/GATA domain. Both CLE0 and GATA elements have been shown to be essential for hIL-5 promoter activity.^{4,7} The region located upstream of the negative regulatory element may also be involved in positive regulation of the hIL-5 promoter. This region includes the BR5 sequence,

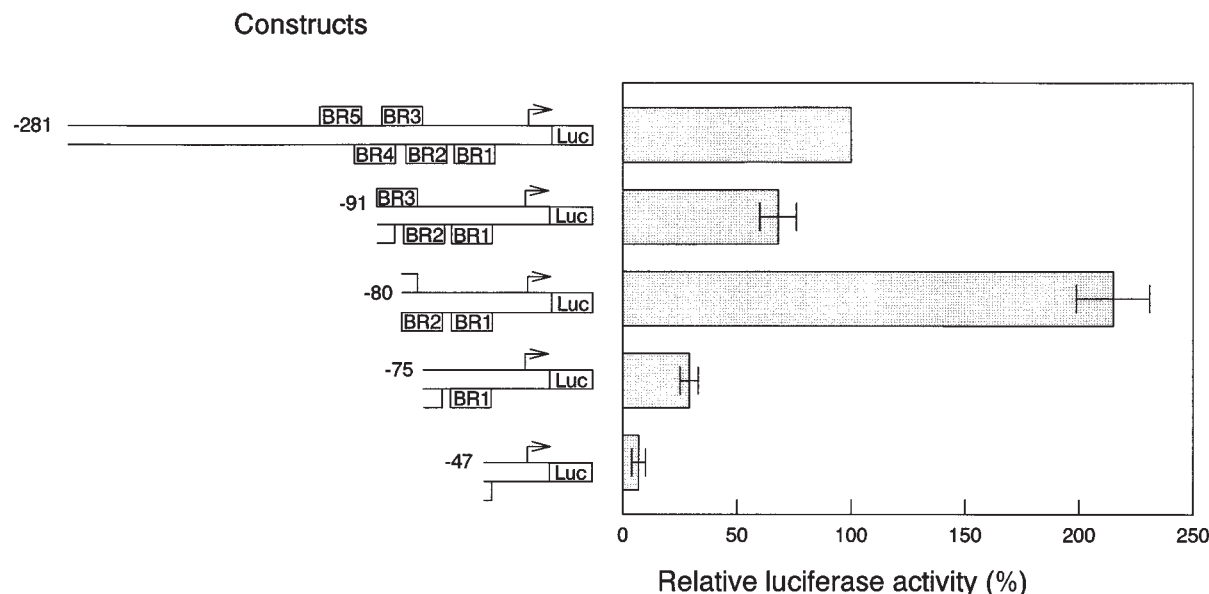
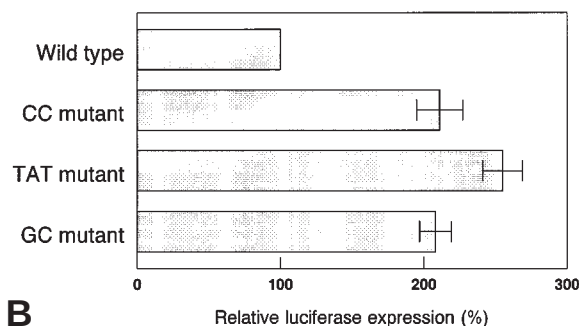


FIG 2. Expression of hIL-5 promoter deletion constructs in PER-117 cells. PER-117 cells were transiently transfected with the -281/+18 hIL-5 promoter luciferase reporter gene construct and with the following 5' deletion constructs schematically represented on the left side of the figure: -91/+18, -80/+18, -75/+18, and -47/+18; locations of each BR within the hIL-5 promoter are indicated. Half of the cells were left as uninduced control and the other half were activated with PMA/cAMP for 16 hours. The luciferase activity for each construct is expressed relative to the construct -281/+18 ($\pm$ SD). Mean values of 5 independent experiments are given.

and half of the BR4 sequence and may be related to the AP1/NF-AT element.⁵

To determine the borders of the negative regulatory element located within the BR3 sequence, substitution mutations were introduced into construct -281/+18. The CC at positions -90 and -89 were replaced by TT (CC mutant), the triplet TAT at positions -86 to -84 was substituted by the triplet CGC (TAT mutant), and the GC at positions -80 and -79 were replaced by AG (GC mutant) (Fig 3, A). The wild-type (construct -281/+18) and mutated constructs were transiently expressed in PER-117 cells, and luciferase activity was measured for each construct in cellular extracts from noninduced and PMA/cAMP-induced cells. No significant luciferase activities were observed in unstimulated cells transfected with the hIL-5 constructs. After cell stimulation, the activities of the mutated IL-5 promoters were always 2- to 2.5-fold higher than the activity observed with the wild-type construct (Fig 3, B). Changing the CC to TT at positions -90 and -89 (CC mutant) led to a 2-fold increase in promoter activity in comparison with the wild-type construct. Replacement of the triplet TAT at positions -86 to -84 with GCG resulted in a 2.5-fold increase in promoter activity (TAT mutant). Substitution of GC at positions -80 and -79 with AG also led to a 2-fold increase in hIL-5 promoter activity (GC mutant). These data are consistent with the results of deletion analysis, indicating that the sequence CCAT-TATTAGGC at positions -90 to -79 is involved in negative regulation of the hIL-5 promoter.

A BR3 -91 GCCATTATTAGGCATTCTC -73
CC mutant -91 --TT----- -73
TAT mutant -91 -----CGC----- -73
GC mutant -91 -----AG----- -73



B **FIG 3.** Effect of mutations of the BR3 sequence on hIL-5 promoter activity. **A**, DNA sequences of the wild type (BR3) and mutants containing substitutions within BR3 are shown. **B**, PER-117 cells were transiently transfected with the -281/+18 hIL-5 promoter luciferase reporter gene construct and the substitution mutant constructs indicated on the left side of the figure (CC, TAT, and GC mutants). Half of the cells were left as an uninduced control, and the other half were activated with PMA/cAMP for 16 hours. The luciferase activity for each construct is expressed relative to the construct -281/+18 ($\pm$ SD). Mean values of 5 independent experiments are given.

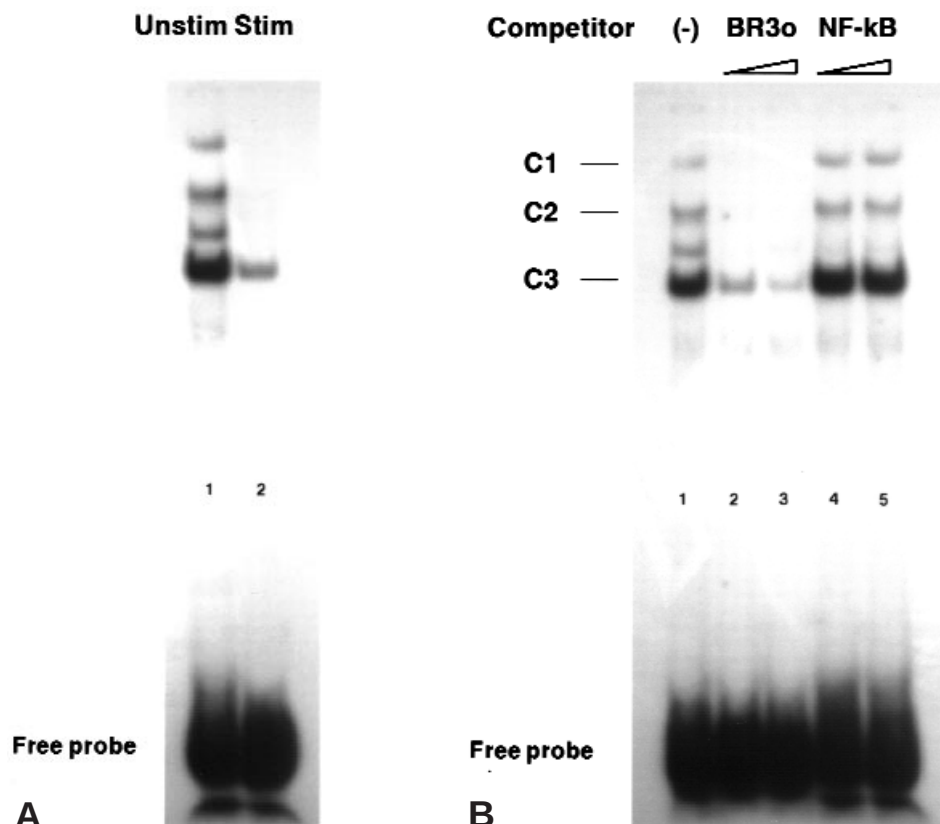


FIG 4. Effect of cell activation on protein-binding activity of the BR3 oligonucleotide and specificity of BR3 oligonucleotide protein binding. **A**, Nuclear extracts from unstimulated (*Unstim*) and PMA/cAMP-induced (*Stim*) PER-117 cells were prepared as described in the Methods section. The probe was BR3o, and free probe is shown at the bottom of each lane. **B**, Nuclear extracts from unstimulated PER-117 cells were analyzed in competition EMSA by using radiolabeled BR3 oligonucleotide. Three BR3o protein complexes are designated C1, C2, and C3. Competition experiments contained 50- and 100-fold molar excess of unlabeled double-stranded BR3o or NF- κ B oligonucleotides. Free probe is shown at the bottom of each lane.

Nuclear proteins binding the BR3 sequence

To examine which transcription factors associate with the BR3 sequence of the hIL-5 promoter, we conducted an EMSA using nuclear extracts from PER-117 cells and a 24-bp oligonucleotide covering BR3 from position -97 to -74. Nuclear extracts from unstimulated cells and radiolabeled BR3 oligonucleotide (BR3o) form 4 DNA-protein complexes (Fig 4, A, lane 1). As in footprint assays, cell activation greatly reduced protein-binding activity of BR3o (lane 2). To exclude potential nonspecific degradation of nuclear proteins in extracts from PMA/cAMP-induced cells, we used radiolabeled SP1 consensus oligonucleotide as probe in a control EMSA. Cell activation had no effect on SP1 protein-binding activity (data not shown), indicating that reduction of BR3o binding activity with extracts from PMA/cAMP-induced cells is unlikely to have resulted from nonspecific protein degradation.

The binding specificity of nuclear proteins to BR3o was initially characterized in binding reactions with unlabeled BR3o and an unrelated oligonucleotide (consensus NF- κ B) as competitor sequences with unstimulat-

ed PER-117 nuclear extracts. As shown in Fig 4, B, unlabeled BR3o inhibited the binding of all DNA-protein complexes in a dose-dependent manner (lanes 2 and 3). In contrast, the NF- κ B consensus oligonucleotide inhibited the binding of only 1 complex and had no effect on formation of the other 3 complexes (lanes 4 and 5). The same results were obtained when SP1 and AP2 consensus oligonucleotides were used in competition EMSA as unrelated competitors (data not shown). This observation suggests that BR3o and nuclear extracts from PER-117 cells form 3 specific DNA-protein complexes, which have been designated C1, C2, and C3 (Fig 4, B).

The BR3o sequence from both strands was then analyzed for sequence homology to consensus binding sites for previously identified transcription factors by using the program TFSEARCH (Searching Transcription Factor Binding Sites, version 1.3).¹⁵ The result of this search indicated that the sequence contains homology to the Oct1-binding site, which overlaps a CCAT box (Fig 5) reported to be the core sequence of the binding motif for transcription factor YY1.¹⁶

To identify BR3-binding nuclear proteins, we used

CCAT	Core sequence of the YY1 binding motif
-93 ATGCCATTATTAGGCAT -77	
TACGGTAATAATCCGTA	} hIL5 promoter
RTAATNA	Oct1 binding motif

FIG 5. DNA sequence homology between the BR3 sequence of the hIL-5 promoter, the Oct1-binding motif (R = A or G), and the core sequence of the YY1-binding motif.

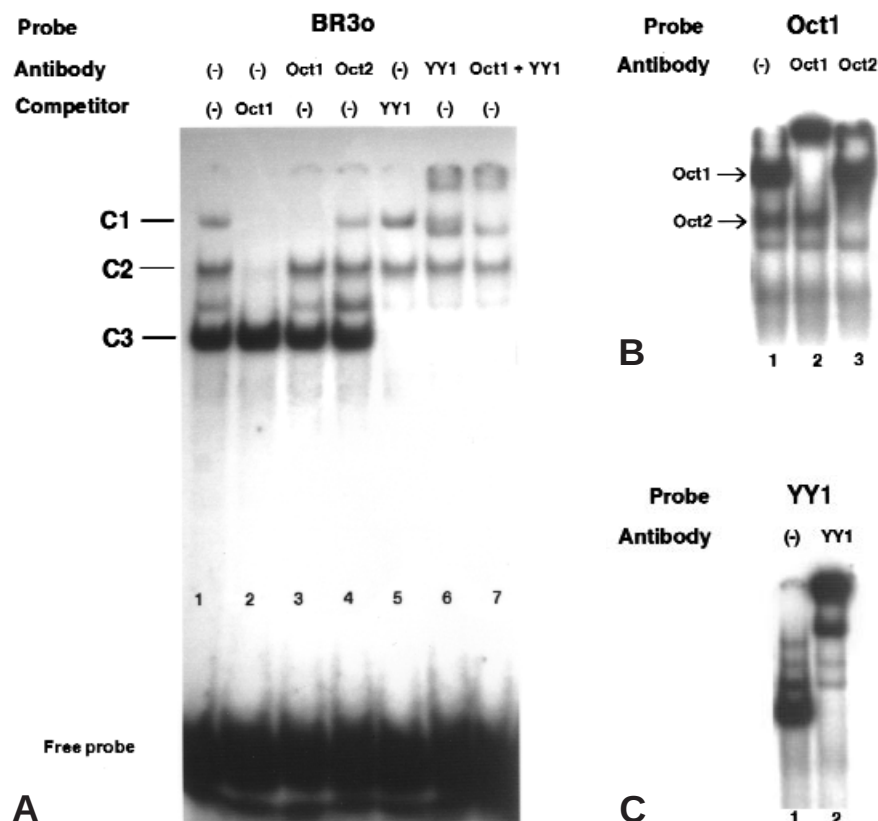


FIG 6. Identification of nuclear factors binding to the BR3 oligonucleotide. **A**, Radiolabeled BR3 oligonucleotide was used as a probe with PER-117 nuclear extracts. Competitor oligonucleotide (50- and 100-fold molar excess) or antibodies (4 μ g protein) were used in the assay as indicated above each lane. Free probe is shown at the bottom of each lane. **B**, Radiolabeled Oct1 consensus oligonucleotide was used as a probe with unstimulated PER-117 nuclear extracts. Anti-Oct1 and anti-Oct2 antibodies (4 μ g protein) were used in the assay as indicated above each lane. The DNA-Oct1 and DNA-Oct2 complexes are indicated by arrows. **C**, Radiolabeled YY1 consensus oligonucleotide was used as a probe with unstimulated PER-117 nuclear extracts. Anti-YY1 antibodies (4 μ g protein) were used in the assay as indicated above each lane.

Oct1 and YY1 consensus oligonucleotides and specific antibodies. Unlabeled Oct1 consensus oligonucleotide used as a competitor in the binding reaction abolished formation of the C1 and C2 complexes (Fig 6, A, lane 2). Incubation with anti-Oct1 antibodies resulted in the loss of C1 complex (lane 3). In contrast, anti-Oct2 antibodies had no effect on BR3o protein binding (lane 4). These results indicate that the Oct1 transcription factor binds to BR3 to form the C1 complex. Although formation of the C2 complex could be inhibited by the Oct1 consensus oligonucleotide, it did not react with the octamer factor-specific antibodies. On the basis of this data, we con-

clude that the C2 complex may contain octamer-like nuclear proteins.

Unlabeled YY1 consensus oligonucleotide abolished formation of the C3 complex (Fig 6, A, lane 5), and anti-YY1 antibodies also resulted in the shift of this complex (lane 6). These results demonstrate that the C3 complex contains YY1. When anti-Oct1 and anti-YY1 antibodies were used simultaneously, C1 was lost, and C3 was shifted (lane 7). This confirms previous data indicating that Oct1 and YY1 transcription factors bind to the BR3 sequence.

As a control experiment, we performed EMSA with

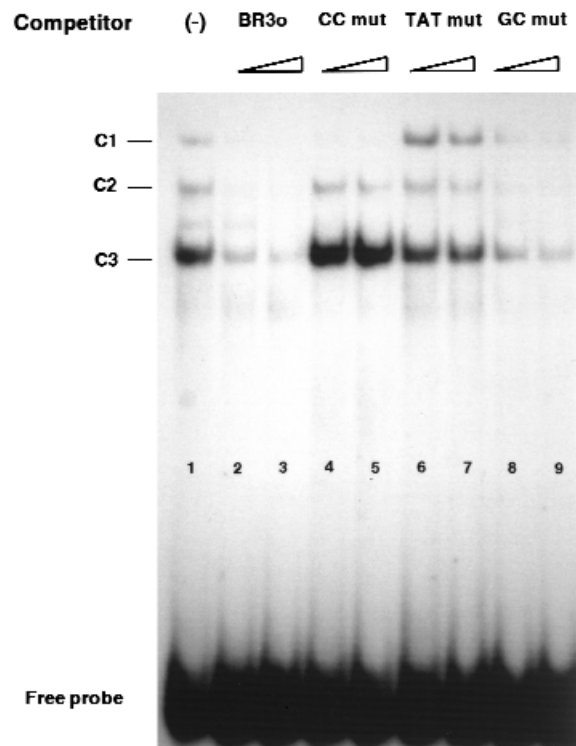


FIG 7. Identification of nucleotides critical for BR3 protein binding. Nuclear extracts from unstimulated PER-117 cells were analyzed in competition EMSA by using radiolabeled BR3 oligonucleotide. Competition experiments contained 50- or 100-fold molar excess of unlabeled double-stranded BR3 oligonucleotide or CC, TAT, and GC mutant oligonucleotides (CC *mut*, TAT *mut*, and GC *mut*, respectively). Free probe is shown at the bottom of each lane.

nuclear extracts from PER-117 cells with Oct1- and YY1-labeled consensus oligonucleotides. As shown in Fig 6, *B* (lane 1), 2 major DNA-protein complexes were detected in nuclear extracts from unstimulated PER-117 cells with the radiolabeled Oct1 consensus oligonucleotide. The DNA-protein complex with lower electrophoretic mobility was shifted by anti-Oct1 antibodies (lane 2), whereas anti-Oct2 antibodies resulted in loss of the complex with higher electrophoretic mobility (lane 3). We conclude that the low-mobility complex contains the Oct1 transcription factor, and the high-mobility complex contains the Oct2 transcription factor. Interestingly, the electrophoretic mobility of the Oct1 complex was similar to the mobility of the C1 complex. The electrophoretic mobility of the Oct2 complex was different to that of any of the BR3o protein complexes.

The YY1 consensus oligonucleotide and nuclear proteins from unstimulated PER-117 cells form 1 major and 3 minor DNA-protein complexes (Fig 6, *C*, lane 1). Interestingly, the electrophoretic mobility of the major YY1 complex was similar to the mobility of the C3 complex. Both the major complex and the slowest migrating complex were shifted by anti-YY1 antibody (lane 2), indicating that these complexes contain the YY1 transcription factor. The electrophoretic mobilities of the YY1 consensus protein-antibody complexes were similar to the mobility of C3 complex shifted by anti-YY1 antibodies.

To determine whether the residues that play a functional role in hIL-5 promoter activity are involved in the formation of the BR3 protein complexes, oligonucleotides containing substitutions in the BR3 sequence were designed. These are displayed in Table I. These unlabeled ds-oligonucleotides were used as competitors in binding reactions with radiolabeled BR3o and nuclear extracts from unstimulated PER-117 cells. As shown in Fig 7, we found that the CC mutant oligonucleotide could not inhibit formation of C2 and C3 complexes (lanes 4 and 5), whereas the TAT mutant oligonucleotide could not inhibit formation of any of the BR3 protein complexes (lanes 6 and 7). Compared with the wild-type competitor (lanes 2 and 3), the GC mutant could partially inhibit formation of C1, C2, and C3 complexes (lanes 8 and 9). According to densitometric analysis, mutation of the GC in BR3o reduced the efficiency of the competitor in binding reactions to 50%. Taken together, these results suggest that the CC at positions -90 and -89, the triplet TAT at positions -86 to -84, and the GC at positions -80 and -79 within the BR3 of the hIL-5 promoter are necessary for protein binding. Formation of complex C1 requires the TAT and GC nucleotides, whereas formation of complexes C2 and C3 requires the CC, TAT, and GC nucleotides (Fig 7). These data correlate well with computer analysis of BR3 and results of protein identification experiments indicating that the sequence

located between positions –90 and –79 of the hIL-5 promoter contains overlapping protein binding sequences for Oct1 and YY1 transcription factors. Combining the functional studies with our EMSA data, we suggest that Oct1, YY1, and octamer-like nuclear proteins compose complexes C1, C2, and C3 and may be involved in the negative regulation of IL-5 transcription in human T cells.

DISCUSSION

The 5'-flanking region of the hIL-5 RNA initiation site was examined for the ability to respond to T-cell activation signals and to regulate transcription of this gene. Using nuclear extracts from PER-117 cells in DNase I footprint assays, we have identified 5 protein BRs within the proximal hIL-5 promoter. Protection of the BR1 sequence by PER-117 nuclear proteins was constitutive and did not depend on cell activation. However, protection of the BR5 sequence was PMA/cAMP induced. In contrast to the BR1 and BR5 sequences, the BR2 to BR4 sequences were highly protected only in unstimulated cells. Protection of these sequences was reduced when nuclear proteins from PMA/cAMP-induced cells were used in the assays. These differences suggest that the complexes bound to the proximal hIL-5 promoter include at least 2 groups of nuclear proteins that are affected by cell activation. The first group includes factors induced by PMA/cAMP. These proteins may be newly synthesized in response to stimulation, or they may be modified by nascent proteins. Alternatively, the relative concentration of the proteins that bind to the BR5 sequence or their binding affinities may be altered in response to activation of the cells. The proteins belonging to the second group are factors showing a reduction in their DNA-binding ability after cell activation. This may be due to modifications of these proteins, such as phosphorylation, dephosphorylation, or acetylation. Alternatively, stimulator-dependent changes in local concentration or potential interaction of these factors with PMA/cAMP-induced proteins may lead to reduction of their DNA-binding activity.

Significantly, the 5 protein BRs identified by DNase I footprinting were also defined functionally as regulatory elements in transfection experiments. The BR1, BR2, and BR5 sequences appear to be related to previously described positive regulatory elements CLE0, GATA, and AP1/NF-AT, respectively. However, the BR3 and BR4 sequences represent previously uncharacterized protein BRs of the hIL-5 promoter. Interestingly, analysis of these regions of the hIL-5 promoter revealed the presence of the palindromic sequence ATGCCAT-TATTAGGCAT located between positions –93 and –77. This suggests that this fragment of the hIL-5 promoter may play a role in the local conformation of the DNA. Comparative analysis of the palindrome by using GenBank indicated that this sequence is not present in the promoter region of any other gene on the database and thus appears to be unique to IL-5. This suggests that a

unique combination of nuclear proteins involved in IL-5-specific transcription bind to this region of the promoter.

Using human IL-5 promoter reporter constructs for functional analysis, we have identified a region between positions –90 and –79 involved in suppression of IL-5 transcription in human T cells. This sequence, located within BR3, has not previously been identified as an important regulatory region in the IL-5 promoter. We have shown that partial deletion of this element or substitution mutations introduced into this sequence significantly increase hIL-5 promoter activity in PER-117 cells. The suppressor activity associated with the –90/–79 sequence in PER-117 cells could also be found in freshly isolated T cells. The activity of the deletion or site-directed mutant constructs transiently expressed in primary human T cells was always 2- to 2.7-fold higher than the activity observed for the wild-type construct (data not shown). This indicates that the –90/–79 sequence located within BR3 and involved in negative regulation of the hIL-5 promoter in PER-117 cells has the same function in primary T cells.

By using EMSA with nuclear extracts from unstimulated PER-117 cells, we have found that 3 specific protein complexes (C1 to C3) bind to a 24-bp oligonucleotide covering BR3 from position –97 to –74. Computer analysis of this region of the hIL-5 promoter for sequence homology to previously identified transcription factors indicated that BR3 has homology to the Oct1-binding site. Using an Oct consensus oligonucleotide and anti-Oct-specific antibodies as competitors in EMSA, we have shown that complex C1 contains Oct1. Oct1 is a ubiquitously expressed transcription factor belonging to the POU homeodomain nuclear protein family.¹⁷ This factor has mainly been characterized as an activator of transcription; however, Oct1 has also been found to exert a suppressive effect on promoter activity in some instances.^{18,19}

Complex C2 appears to bind octamer-like factors because it was inhibited by an Oct1 consensus oligonucleotide but not affected by either anti-Oct1 or anti-Oct2 specific antibodies.

Located within BR3 is a CCAT box that has been reported to be a core sequence for binding of the transcription factor YY1. When consensus oligonucleotide and antibodies specific for YY1 were used in competition EMSA studies, we found that complex C3 contains YY1. The BR3o and nuclear proteins from PMA/cAMP-induced cells form one DNA-protein complex with an electrophoretic mobility similar to C3. Using specific antibodies and consensus oligonucleotide, we have shown that YY1 also binds to BR3o in reactions with nuclear proteins from activated cells (data not shown). YY1 is a zinc finger transcription factor that, depending on its promoter context, can exhibit either activator or repressor activity.²⁰ Regarding its role in cytokine gene regulation, YY1 has been shown to be a repressor of human IFN- γ and human GM-CSF transcription.^{21,22}

Using mutant oligonucleotides as competitors, we

have shown that the -90/-79 sequence involved in suppression of hIL-5 promoter activity is critical for the protein-binding activity of BR3 α . This suggests that Oct1, YY1, and the octamer-like proteins that bind to this sequence may be involved in negative regulation of IL-5 transcription. Importantly, this is the first report to show that YY1 binds to the IL-5 promoter and that it plays a functional role in IL-5 promoter activity.

Whereas mutation analysis showed that binding of complexes C1, C2, and C3 to the -97 to -74 sequence of the hIL-5 promoter is involved in suppression of promoter activity on cell activation, no difference in the promoter activity of uninduced cells on transfection of the wild-type or mutant reporter constructs was observed. One explanation for the lack of effect of the deletion and substitution mutations in unstimulated cells may be that an activator is required for any expression of IL-5. Indeed, it has been shown that AP1 binding to CLE0 is inducible, and mutations of this site abolish IL-5 promoter activity. (Thomas M et al, unpublished data).²³ Therefore the absence of IL-5 expression in unstimulated cells may be dependent on (1) binding of suppressor proteins to the promoter and (2) lack of binding of activator proteins. Cell activation leads to the release of suppressor proteins from the DNA and the formation of activator complexes initiating IL-5 transcription. The Oct1, octamer-like, and YY1 nuclear proteins binding to the BR3 sequence may act as suppressors of IL-5 transcription. This hypothesis is supported by the fact that cell activation alters affinity of nuclear proteins to the BR3. It has been observed that on cell activation, the protection of BR3 and binding of protein complexes to this sequence are reduced. We hypothesize that this may occur through modifications of binding proteins, such as phosphorylation, which leads to alteration of their DNA-binding ability. The difference in poststimulation activity of the wild-type and deletion/substitution mutant hIL-5 constructs could be accounted for by residual binding of the proteins to the BR3 sequence. This is supported by partial protection of the BR3 in footprint assays with nuclear extracts from PMA/cAMP-induced cells and YY1 binding to this sequence in reactions with proteins from activated cells. Thus residual binding of proteins to the BR3 sequence in activated cells may serve as a brake to IL-5 transcription, whereas the elimination of binding sites for suppressor proteins in deletion or substitution mutant constructs leads to maximal promoter activity.

Alternatively, changes in the phosphorylation pattern of BR3-binding proteins may lead to their interaction with other transcription factors that are necessary for IL-5 promoter activity. This interaction may lead to a down-regulation of IL-5 gene expression. This is consistent with the observation that inhibiting the binding of these proteins to DNA leads to an increase in IL-5 promoter activity. Possibly, the Oct1, octamer-like, and YY1 factors may interact with proteins binding to CLE0, GATA, or AP1/NF-AT sites, all of which are located in close proximity to the -90/-79 sequence, and all of which have been shown to be necessary for IL-5 expression. Indeed,

interaction of octamer factors with AP1 and NF-ATp in the IL-2 and IL-4 promoters, respectively, has been reported.²⁴ YY1 has also been shown to interact with transcription factors, and this interaction has been shown to suppress promoter activity.²⁵⁻²⁹

In summary, we have identified 2 previously uncharacterized protein BRs within the proximal hIL-5 promoter. The sequence covering these regions from position -97 to -74 forms 3 specific complexes with nuclear proteins from unstimulated PER-117 cells. Our results indicate that complex C1 contains Oct1, complex C2 contains an octamer-like factor, and complex C3 contains the YY1 nuclear protein. The exact mechanism by which Oct1, YY1, and octamer-like proteins mediate suppression of hIL-5 promoter activity is currently being investigated.

We thank Dr Ursula R. Kees for the PER-117 cell line, and Dr Carolyn Williams and Ms Sandra Stevenson for advice and help with deletion analysis.

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