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T CELL-SPECIFIC NEGATIVE REGULATION OF TRANSCRIPTION OF THE HUMAN CYTOKINE IL-4

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IL-4 secreted by activated T cells is a pleiotropic cytokine affecting growth and differentiation of diverse cell types such as T cells, B cells, and mast cells. We investigated the upstream regulatory elements of the human IL-4 promoter. A novel T cell-specific negative regulatory element (NRE) composed of two protein-binding sites were mapped in the 5′ flanking region of the IL-4 gene: -511 CTCTCTTCT -303 (NRE-I) and -588 CTGGGTGCTG -300 (NRE-II). A T cell-specific protein Neg-1 and a ubiquitous protein Neg-2 binding to NRE-I and NRE-II, respectively, were identified. Furthermore, a positive regulatory element was found 45 bp downstream of the NRE. The enhancer activity of the PRE was completely suppressed when the NRE was present. These data suggest that IL-4 promoter activity is normally down-regulated by an NRE via repression of the enhancer positive regulatory element. These data may have implications for the stringent control of IL-4 expression in T cells.

The IL-4 secreted by activated T cells plays a key role in the regulation of the immune response by secreting a number of cytokines. One of the cytokines, IL-4, has multiple biologic activities affecting cells of most hematopoietic lineages (1). IL-4 promotes B cell proliferation, regulates Ig class switching, and promotes T cell growth and cytotoxicity (2–5).

IL-4 is secreted by activated but not by resting mature T cells. Typically, the secretion of IL-4 protein after T cell activation is markedly low in contrast to other coordinated expressed cytokines (e.g., IFN-γ and IL-2) (6). In activated T cells, IL-4 mRNA is difficult to detect by conventional Northern hybridization. This indicates that IL-4 production by normal human T cells is stringently regulated. Tight regulation of IL-4 expression may be necessary, as overexpression of IL-4 may induce allergy-like inflammatory diseases (7).

IL-4 limitation of the expression of IL-4 may, therefore, be one of the mechanisms by which activated T cells regulate its production to facilitate an integrated effective immune response. To understand the mechanisms underlying the control of IL-4 gene expression, we investigated the upstream regulatory elements of the human IL-4 promoter. We report here the identification and characterization of a T cell-specific NRE that down-regulates transcription via suppression of a PRE within the IL-4 promoter.

MATERIALS AND METHODS

Cell cultures. Jurkat, EL-4, EJAB, SKW6.4, Hi-60, and K562 cells were cultured in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% FCS, 50 µg/ml gentamicin (GIBCO), 6 mM HEPES (GIBCO, 1 M solution), and 2 mM L-glutamine (GIBCO, 200 mM solution). HeLa cells were cultured in DMEM (GIBCO) containing sodium pyruvate and 1000 µg/ml glucose and replacing the 50 mM 50 µg/ml gentamicin with 150 µg/ml spectinomycin.

PCR analysis of IL-4 mRNA. Total cellular RNA was isolated by using cesium chloride centrifugation (8). cDNA synthesis was performed with 10 µg of total RNA in 50 mM Tris-HCl pH 8.3, 10 mM MgCl₂, 500 µM each of dNTP, 25 pmol 3′ PCR primer, and 7 U AMV reverse transcriptase. The reaction volume was 10 µl. After incubation at 37°C for 1 h, 2 µl of cDNA reaction mixture were used for a PCR in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 µM of each dNTP, 25 pmol of each primer, and 1.25 U of AmpliTaq (Cetus Corp., Emeryville, CA). The reaction volume was 50 µl. Thirty cycles were performed, with 1 min at 93°C (3 min in the first cycle), 1 min at 55°C, and 3 min at 72°C (5 min during the last cycle). PCR primers for IL-4 are from positions 2 to 21 for the 5′ primer and from positions 510 to 529 for the 3′ primer according to IL-4 cDNA sequence (9). The heat shock cognate protein 70 primers are from positions 3992 to 3971 for the 5′ primer and from positions 4571 to 4590 for the 3′ primer (10).

Recombinant plasmids. The −1750 to −650 5′ deletion mutants of the IL-4 promoter were created by S1-nuclease digestion from the SpIhl site of a genomic IL-4 clone, ligation of Sall linkers to the blunt ends, and followed by Sall/BamHI digestion. The Sall/BamHI fragments were inserted into the same sites in πBLCAT3 (11). The −310 construct was made by Alul and HindIII digestion of a −310 genomic clone (12) to generate a −310/+8 (HindIII/Alul) fragment, and the fragment was subcloned into HindIII and HincII sites of pUC18. The −310/+8 fragment was excised from pUC18 by HindIII and BamHI digestion and inserted into the same sites of pBlCAT3. The −270 and −200 deletion mutants were created by either Alul or Alul and Dra I digestion of the −310 genomic clone to generate a −270/+8 (Alul/Alul) and a −200/+8 (Dra I/Alul) fragment. The fragments were subcloned into the HindIII site of pUC18, excised from pUC18 by HindIII and BamHI digestion, and subsequently inserted into the same sites in pBlCAT3. The −250 mutant was made by ligation of a synthesized 50-bp (−250 to −200) oligonucleotide to the −200 Dra I site of the promoter. The deleted HindIII/Alul (−310 to −270) fragment was reinserted into the deleted promoter in either orientation by blunt ligation of the filled-in HindIII sites of the 40-bp fragment and the −270 construct. Internal point mutations were introduced into the −312 IL-4 promoter (see Fig. 3) by replacing the 42-bp (−312 to −270) IL-4 fragment with a series of synthesized mutated 42-bp oligonucleotides. The IL-4 (−270/−200) and (−310/−200)-CAT2 plasmids (see Fig. 4) were constructed by insertion of the Alul (−270 to −200) or the HindIII/Alul (−310 to −270) fragment into the HindIII site of πBLCAT2 (11) (details available upon request). The (−285/−200)-CAT2 construct was created by adding a 26-bp oligonucleotide (−286 to −270) to the −270 Alul site of the promoter. A synthesized 40-bp (−310 to −270) oligonucleotide was phosphorylated by T kinase and

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*Abbreviations used in this paper: NRE, negative regulatory element; PRE, positive regulatory element; PCR, polymerase chain reaction; CAT, chloramphenicol acetyl transferase; tk, thymidine kinase.

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subcloned into the HindII site of pUC18 to generate single or multimerized NRE-CAT2 constructs (see Fig. 5). The single or multimerized inserts were excised by HindIII and BamHI and inserted into the same sites of pBLCAT2. All deletions and mutations were confirmed by DNA sequencing.

Extract preparation and gel shift assays. The nuclear extracts were prepared according to the method of Sealey and Chalkley (13) including a mixture of protease inhibitors: PMSE (0.5 mM), leupentin (0.5 μg/ml), pepstatin (0.7 μg/ml), aprotonin (1 μg/ml), and bestatin (40 μg/ml) in the homogenization buffer.

Gel shift assays were performed essentially as described by Jones et al. (14). Competition experiments were done by mixing the appropriate competitor DNA to the binding reaction before adding the radiolabeled probes. The samples were electrophoresed on a 5% polyacrylamide gel in 0.5X TBE buffer (1X TBE buffer is 89 mM Tris pH 8.2, 89 mM borac acid, and 2 mM EDTA). The synthetic IgV, octamer-containing oligonucleotide used for competition is 5’GAGTATGCAATCATGT3’ (15).

DNA transfections. Fusion genes were transfected into Jurkat and BJAB cells by the DEAE-dextran method and into HeLa cells by the calcium phosphate method as described by Gorman (16). After 24 h of transfection Jurkat cells were stimulated by 20 ng/ml PMA (Sigma Chemical Co., St. Louis, MO) and 10 μg/ml PHA (Sigma) or 0.5 μM ionomycin (Sigma) for 16 to 18 h. CAT assays were performed as described by Gorman (16).

RESULTS

Identification of negatively and positively acting regions. We cloned the human IL-4 gene (12) to identify cis-acting DNA sequences responsible for IL-4 expression. A series of 5’ deletion fragments of the human IL-4 promoter (from -1750 to -200 bp) containing 8 bp of the first exon (+8) were cloned into the plasmid pBLCAT3 (11) to generate fusion genes with the CAT gene. To select proper T cell lines for transfection experiments, we examined several human T cell lines for their expression of IL-4 mRNA. Because conventional Northern hybridization was not sufficient for detecting IL-4 mRNA in T cells, we performed an analysis by PCR. Two primers from different sites of the IL-4 gene were synthesized and the IL-4 mRNA was amplified by 30 cycles of PCR. In this way, one human leukemia T cell line, Jurkat (generously provided by Dr. B. Stadler, Bern, Switzerland) was found to produce IL-4 (Fig. 1). In normal mature T lymphocytes, the IL-4 gene is expressed transiently after Ag or mitogen activation. In contrast, Jurkat cells express IL-4 constitutively (Fig. 1). Upon induction, slightly more IL-4 mRNA was detected. Thus, these cells may represent T cells in an activated state and may be used for studying IL-4 promoter function at such a state.

Transient expression of the various IL-4 fusion gene constructs in Jurkat cells showed that deletions from -1750 to -310 did not have a significant influence on the activity of the promoter. However, further removal of 40 nucleotides from -310 to -270 resulted in an increase in promoter activity by more than 10 times (Fig. 2). Reinsertion of the 40-bp DNA sequence in either orientation into the 40-bp-depleted promoter reduced the activity of the promoter to its original level (Fig. 2). The same results were obtained by transfections without induction. Promoter activity was reduced by 5 to 30% in the absence of induction (data not shown). The weak response to induction by the IL-4 promoter may be explained by the fact that Jurkat cells are already in an activated state. In addition, further deletion from -270 to -200 reduced the promoter activity to the basal level. These data indicate that the 40-bp DNA sequence (-310 to -270) may harbor an NRE, and the region between -270 and -200 a PRE.

Characterization of IL-4 NRE. We focused on the char-
prepared from Jurkat cells resulted in the formation of a single DNA/protein complex for oligo I and three DNA/protein complexes for oligo II (Fig. 3B). We carried out competition experiments to analyze protein binding to these oligonucleotides in detail. The data showed that one of the complexes was formed by the octamer-binding protein Oct-1 (22) [analysis of Oct-1 and Oct-2 mRNA by PCR showed that Jurkat cells contained only Oct-1; data not shown]. Formation of this complex was inhibited only by DNA competitors containing an octamer motif (Fig. 3D, lanes IgHv and II). Two proteins were named NRE-binding proteins 1 and 2 (Neg-1 and Neg-2). The complexes formed by Neg-1 and Neg-2 were in competition with a 50-fold molar excess of oligo I and II, respectively (Fig. 3, C and D). Therefore, Neg-1 and Neg-2 may recognize both oligo I and oligo II sequences. Since the gel shift assay showed only the binding of Neg-1 to oligo I and Neg-2 to oligo II, Neg-1 and Neg-2 may have significantly different affinities to these oligos. Neg-1 and Neg-2 complex formation was not in competition with the unrelated oligonucleotide, oligo III. One protein was named oligo II-binding protein. The formation of the oligo II-binding protein complex was only inhibited by oligo II (Fig. 3D). Previously, a 50-kDa negative regulatory protein, SP-50, was identified to specifically bind to both the IL-2Ra NRE and the long terminal repeat of the HIV-1 NRE core elements (19). The complex formed by the IL-2Ra NRE and SP-50 showed a slightly different electrophoretic mobility than the complexes formed by Neg-1 and Neg-2 (Fig. 3B). The formation of both the Neg-1 and Neg-2 complexes was inhibited by a 50-fold molar excess of the IL-2Ra NRE oligonucleotide. In contrast, the formation of the SP-50 complex was not inhibited by a 50-fold molar excess of oligo I and II. Inhibition of the formation of the SP-50 complex by oligo I and II occurred only at a 300-fold molar excess of DNA (Fig. 3E). Because only a large molar excess of DNA inhibited SP-50 complex formation, we conclude that Neg-1, Neg-2, and SP-50 are distinct proteins. Nuclear extracts prepared from induced Jurkat cells gave the same results. No detectable difference quantitatively as well as qualitatively between induced and noninduced nuclear extracts was observed (data not shown). These data suggest that the DNA-binding proteins are expressed in a constitutive manner.

Neg-1 and Neg-2 are responsible for repression of IL-4 promoter activity. The fact that the IL-2Ra NRE oligonucleotide competed for Neg-1 as well as Neg-2 complex formation raised the possibility that Neg-1 and Neg-2 might be the protein candidates responsible for repression of IL-4 promoter activity. To investigate this possibility, we made a series of mutated NRE promoter-CAT constructs and transfected them into Jurkat cells. The effect of the mutations on blocking of protein binding was tested by competition experiments. As shown in Figure 4, A and B, a mutant oligonucleotide competitor containing a 3-bp substitution (CCT → GAG) in the core element of NRE-I failed to inhibit Neg-1 complex formation. In contrast, mutations outside of the NRE-I core element did not influence the capacity of the oligonucleotides to inhibit complex formation. In oligo II, substitution of the first two nucleotides of the octamer motif (AT → GC) abolished the capacity of the oligonucleotide to inhibit complex formation. In oligo II, substitution of the first two nucleotides of the octamer motif (AT → GC) abolished the capacity of the oligonucleotide to inhibit complex formation.
of IL-4 NRE. Competition experiments were performed to test the mutated oligonucleotides for their protein-binding ability. Mutated (M) nucleotides are shown underneath the tested oligonucleotides for their protein-binding ability. Mutation (NRE-I and NRE-II) abrogated the ability of the oligonucleotides to inhibit Oct-1 as well and Neg-2 complex formation. Mutations outside the octamer motif, but still inside the core element of NRE-II (AAG → CCT), did not impair the oligonucleotide to block Oct-1 complex formation. It abolished, however, its ability to block Neg-2 and oligo II-binding protein complex formation.

The transfection experiments showed that mutations that abolished binding of Neg-1 (Fig. 4A, lane M1) or Neg-2 (Fig. 4B, lanes M2, M3, and M4) to NRE-I or NRE-II, respectively, significantly increased IL-4 promoter activity (Fig. 4C, lanes 2, 4, and 5). Mutations that had no influence on binding of Neg-1 and Neg-2 (including mutations in the oligo III region) (Fig. 4A, lane M2; Fig. 4B, lane M1) did not influence IL-4 promoter activity (Fig. 4C, lanes 3, 6, and 7). Likewise, mutations that eliminated binding of Oct-1 (Fig. 4B, lane M1) had no effect on IL-4 promoter activity (Fig. 4C, lane 3). The exact function of oligo II-binding protein, however, remains unclear. These data clearly demonstrate that interaction of Neg-1 and Neg-2 with the IL-4 NRE plays an important role in negative regulation of IL-4 transcription. We also investigated combinations of mutations in the IL-4 NRE sequences. Single mutations that inhibited binding of both Neg-1 and Neg-2 resulted in an increase in enhancer activity similar to that of Neg-1 or Neg-2 alone (Fig. 4C, lane 9). This indicated that it was sufficient to abolish binding of either Neg-1 or Neg-2 to destroy the negative effect of the IL-4 NRE. Thus, functional repression of IL-4 transcription may require binding of both Neg-1 and Neg-2 to the NRE.

We examined nuclear extracts from different cell lines to further characterize expression of Neg-1 and Neg-2: the T-lymphoid cell lines Jurkat (human) and EL-4 (mouse), the human B-lymphoid cell lines BJAB and SKW6.4, and the human nonlymphoid cell lines HeLa, HL-60 (a promyelocytic leukemia), and K562 (an erythroleukemia). In gel shift assays, Neg-1 was only detected in the human and mouse T cell lines Jurkat and EL-4, respectively, and not in B-lymphoid and nonlymphoid human cell lines (Fig. 5A). These data suggested that Neg-1 may be a T cell-specific factor. In contrast, Neg-2 was specifically identified in BJAB and HeLa cells (Fig. 5, B and C) by the same competitors as the ones used for Jurkat cell extracts. Thus, Neg-2 found in several cell types (Fig. 5B) may be a ubiquitously expressed protein.

**IL-4 NRE functions via repression of PRE activity.** To study the mechanism of repression of IL-4 transcription, we investigated whether IL-4 NRE acts via repression of PRE enhancer activity or directly on the promoter. We cloned the 70-bp PRE enhancer activity-containing fragment (−270 to −200, Fig. 2) in front of the tk-CAT fusion gene, pBLACAT2 (11). As shown in Figure 6, this 70-bp fragment increased the activity of the tk promoter by about 5 times of that of the tk promoter alone. In contrast, the 110-bp NRE- and PRE-containing fragment (−310 to −200) did not show any increase in promoter activity. However, removal of NRE by deletion of 24 nucleotides from −310 to −286 again resulted in an increase of tk promoter activity as in transfection with the 70-bp (−270 to −200)/tk CAT construct (Fig. 6). IL-4 NRE, like the IL-2Rα NRE (19), did not repress the heterologous tk promoter directly. Single and multimerized IL-4 NRE were all equally insufficient (Fig. 6). These data clearly show...
The lymphoid cell lines included the mouse T cell tumor EL-4 and the human B cell tumors BJAB and SKW6.4. The nonlymphoid human cell lines included HL-60, K562, and HeLa. C, competition experiments using extracts from BJAB and HeLa cells at the same conditions used for Jurkat cell extracts.

Figure 5. Cellular distribution of Neg-1 and Neg-2. A and B, gel shift assays performed with oligo I or oligo II and nuclear extracts prepared from different cell types. The lymphoid cell lines included the mouse T cell tumor EL-4 and the human B cell tumors BJAB and SKW6.4. The nonlymphoid human cell lines included HL-60, K562, and HeLa. C, competition experiments using extracts from BJAB and HeLa cells.

Figure 6. The IL-4 NRE acts via repression of the IL-4 PRE enhancer activity. The IL-4 fragments −270 to −200, −310 to −200, −286 to −200, and monomer or multimers of the IL-4 NRE (−310 to −270) were linked in front of the tk promoter of pBlCAT2 to analyze the mechanism of repression of the IL-4 NRE. The constructs were transfected into Jurkat cells. Results are the average of three independent transfection experiments.

Figure 7. Repression of the IL-4 promoter by the IL-4 NRE is T cell specific. The 5' deletion (−310, −270, and −200) of the IL-4 promoter CAT constructs (the same constructs were used in the transfection in Jurkat cells shown in Fig. 2) were transfected into BJAB and HeLa cells. Results are the average of three independent transfection experiments.

Figure 8. The IL-4 NRE has no negative effect on the IL-4 PRE in HeLa cells. CAT constructs containing the tk promoter and the tk promoter linked with the IL-4 −270/−200 and −310/−200 fragments, respectively, were transfected into HeLa cells. The same constructs were used in the transfection in Jurkat cells shown in Figure 6. Results are the average of three independent transfection experiments.
NRE were in cross-competition with each other for Neg-1, Neg-2, and SP-50 complex formations, we do not exclude the possibility that these proteins are closely related. Neg-1 and Neg-2 also differ from the lysozyme silencer binding factor Nep-1. Binding of Nep-1 to the chicken lysozyme silencer requires the full length of the silencer sequence, and NRE-II comprises only about a quarter of this sequence (20).

Different mechanisms of transcriptional repression might be involved in negative regulation. These include competition for DNA-binding sites, quenching, and direct repression (23, 24). Transcriptional repression of IL-4 promoter activity might be the result of competition of binding of Neg-1 and Neg-2 with Oct-1, since the octamer motif is encompassed by NRE-I and NRE-II. This, however, is unlikely for the following reasons. An IL-4 promoter in which the IL-4 NRE was replaced by the IGHV octamer sequence gave an almost identical increase in promoter activity might be the result of competition among Neg-1, Neg-2, and Oct-1.

We also identified a PRE that is located 45 bp downstream of NRE. No sequence similarities between the IL-4 PRE and other known PRE have been found. Our data provide evidence that the IL-4 NRE acts via repression of PRE enhancer activity. IL-4 NRE is located 45 bp 5′ from PRE. Therefore, repression through competition of binding of a positive transcription factor(s) to PRE by Neg-1 and Neg-2 is unlikely. Unlike the chicken lysozyme silencer, the IL-4 NRE does not directly repress the heterologous tk promoter. This indicates that Neg-1 and Neg-2 do not directly interfere with the formation or the activity of the basal transcription complex. To explain negative transcriptional regulation by the interaction of NRE and PRE, we favor a quenching mechanism in which the Neg-1/Neg-2 repressor proteins interfere with the function but not the binding of PRE-activating enhancer protein(s).

The mechanism of negative regulation of IL-4 gene expression appears to be distinct from that of other cytokines, since sequences similar to the IL-4 NRE have not been found in other cytokine genes. Our data indicate that the IL-4 promoter is normally not fully active. Its activity is suppressed by the NRE. The features of IL-4 gene regulation are distinct from all other cytokines known so far. This might partly be explained by the fact that IL-4 gene expression is usually lower than that of other cytokines such as IL-2 and IFN-γ. Overexpression of IL-4 may be dangerous and cause allergic reactions (7). A balanced secretion of IL-4 may, therefore, be of crucial importance. In future studies it remains to be elucidated whether the IL-4 NRE is entirely responsible for the tight control of IL-4 expression in vivo.

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