Th2-Specific Protein/DNA Interactions at the Proximal Nuclear Factor-AT Site Contribute to the Functional Activity of the Human IL-4 Promoter

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Th2-Specific Protein/DNA Interactions at the Proximal Nuclear Factor-AT Site Contribute to the Functional Activity of the Human IL-4 Promoter

Min Li-Weber,*‡ Padmini Salgame,† Chenggang Hu,† Ilia V. Davydov,‡ Oskar Laur,* Sibylle Klevenz,* and Peter H. Krammer*

IL-4 is a pleiotropic immunoregulatory cytokine secreted by activated Th2, but not Th1, cells. The proximal IL-4 promoter contains MARE, C/EBP, P0, octamer-like, P1, and activating protein-1 elements. The half c-Maf binding site (MARE), P0, and P1 sites were previously shown to be involved in Th2-specific transcriptional activity. Except the MARE and P1 site, the molecular basis for Th2 specificity of the P0 site has not been analyzed. Here, we provide the first detailed analysis of the P0 binding factors and show that in Th2, but not in Th1, cells, NF-AT and proteins of the activating protein-1 family are involved in cooperative binding to the P0 and the adjacent octamer-like site. In the mouse Th2 D10 cells, Oct-1/Oct-2 are also found to participate in formation of the P0-binding complexes. Mutation, deletion, and methylation interference analysis demonstrate that both the P0 and the octamer-like sequence are required for inducible binding. Furthermore, we provide the first report of the functional relevance of each site in the human IL-4 promoter by mutagenesis/transfection analysis and demonstrate that the octamer-like, P0 and P1 sites are important for the biologic function of the IL-4 promoter. The MARE site, although it was shown to be critical for the function of the murine IL-4 promoter, does not appear essential for human IL-4 promoter activity in Jurkat T cells. These findings suggest that besides c-Maf, another Th2-specific factor(s) may be involved in tissue-specific expression of the IL-4 gene. The Journal of Immunology, 1998, 161: 1380–1389.
does not appear to be critical for transcriptional activity in IL-4-producing Jurkat T cells.

Materials and Methods

Cells

The cell lines used in this study were the human T lymphoblastoid cell line Jurkat J16, the human Th1 (PPD) and Th2 (TT3) cell clones (1, 32), and the mouse Th2 cell clone D10 and the mouse Th1 cell clone (CI) 29. Culture conditions for the cells were described previously (1, 32).

Antibodies

Abs specific for Oct-1 and Oct-2 (33) were supplied by Dr. T. Wirth (Institut für Medizinische Strahlen und Zellforschung, Würzburg, Germany). The anti-NF-ATc mAb (MA3-024) was purchased from Affinity Bio-Reagents (Golden, CO). Abs specific for c-Fos and c-Jun were purchased from Oncogene Science (Dianova, Hamburg, Germany). The anti-c-Jun AP-1 (D, broadly reactive with c-Jun, JunB, and JunD p59 proteins) and anti-c-JunD (329) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ab specific for c-Jun B was supplied by Dr. P. Angel (German Cancer Research Center, Heidelberg, Germany).

Nuclear extract preparation and electrophoretic mobility shift assays (EMSA)

Nuclear extracts from D10, CI 29, human Th1, and Th2 cells were prepared as described previously (32). The pGST-NF-ATc and pGST-NF-ATp bacteriophage expression vectors were supplied by Dr. E Serfling (Institute of Pathology, University of Würzburg, Würzburg, Germany). The pGST-NF-ATp plasmid contains the Nael/SnaI fragment of NF-ATc cDNA spanning the DNA binding and protein interaction domains of NF-ATp (27). The pGST-NF-ATc plasmid contains NF-ATc peptides from amino acids 293 to 716 (36).

The recombinant NF-AT-GST fusion proteins were isolated from the NF-ATc- and NF-ATp-expressing Escherichia coli. Blue slow strains as follows. Twenty-five milliliters of an overnight culture of bacteria was inoculated into 500 ml of Luria Bertani medium containing 100 μg/ml ampicillin and 0.1% glucose. After 3-h incubation at 37°C, 0.4 mM isopropl-

β-D-thiogalactoside (IPTG) were added, and the bacteria were cultured at 37°C for another 30 min. The bacteria were harvested, washed once with ice-cold PBS, and sonified and centrifuged at 10,000 rpm for 20 min. The pellets were resuspended in binding buffer [10 mM reduced glutathione and 50 mM Tris-HCl, pH 8.0, at 4°C for 2 h] and then chilled for 2 h at 4°C. The cell lysates were dialyzed against 10 mM Tris-HCl, pH 8.0, before use. The cell lines used in this study were the human T lymphoblastoid cell line Jurkat J16, the human Th1 (PPD) and Th2 (TT3) cell clones (1, 32), and the mouse Th2 cell clone D10 and the mouse Th1 cell clone (CI) 29. Culture conditions for the cells were described previously (1, 32).

Results

PMA- and PMA/ionomycin-inducible nuclear factors interact with the P0 site

The P0 site was previously shown to be active in Th2 D10 cells but not in a Th1 cell clone (22). Therefore, we chose D10 cells to investigate the Th2-specific P0-binding nuclear factors. An oligonucleotide containing the P0 DNA sequence (−68 to −45) was used as a probe in EMSA. This P0 probe contains two DNA sequences, a NF-AT consensus sequence, P0 (20), and an octamer-like sequence (21). The experiments showed that induction of the Th2 D10 cells by PMA resulted in formation of one inducible complex on the P0 probe (Fig. 2A, lane 3). Activation of the cells with PMA plus ionomycin generated a slower shifting complex (Fig. 2A, lane 4). Stimulation of D10 cells by ionomycin alone did not induce DNA binding activities on the P0 probe. We designated these two inducible complexes NF-P0b and NF-P0a, respectively. A weak, fast shifting complex was also detectable. Since this complex was not consistently present, and its binding activity was not specifically competed by the oligonucleotides tested, it was considered to be due to an unspecific DNA–protein interaction. In contrast to the P0 probe, a P1 probe containing the NF-AT consensus (20), and the same octamer-like sequence (Fig. 1) did not generate a PMA-inducible complex. A PMA/ionomycin-inducible complex was detected by the P1 probe, which was previously shown to contain NF-ATF (and/or NF-ATc) (24) (Fig. 2B). When we used an oligonucleotide containing the binding site for the constitutive protein Sp-1 as a control, the Sp-1 binding activity could be detected in all nuclear extracts (Fig. 2C).

To further investigate the P0 binding proteins, nuclear extracts were prepared from PMA/ionomycin-induced D10 cells in the presence or the absence of CsA. As shown in Figure 2D, formation of the PMA/ionomycin-inducible NF-P0a, but not that of the PMA-inducible NF-P0b, was blocked by treatment of the cells

Plasmid construction and mutations

The CAT reporter plasmid pCAT6-IL-4 (−269/+11) was constructed as described previously (25). The luciferase reporter construct pLuc-IL-4 (−269/+11) plasmid was constructed by cutting the IL-4 promoter fragment −269/+11 from pCAT6-IL-4 (−269/+11) with HindIII and BamHI and recombining into the HindII and BglII sites of pTATAluc vector (a gift from T. Wirth, Institut für Medizinische Strahlen und Zellforschung). The mutated pCAT6-IL-4 (−269/+11) and pLuc-IL-4 (−269/+11) constructs (in Figs. 10 and 11) were generated by oligonucleotide-directed PCR mutagenesis. Mutations were confirmed by sequencing analysis.

Transfections

The CAT reporter constructs were transfected into Jurkat T cells using the DEAE-dextran method as described previously (37). The luciferase reporter constructs (10 μg) were transfected by electroporation. Jurkat cells (4 × 10⁶/ml) in RPMI 1640 medium were electroporated using a Bio-Rad Gene Pulser (Bio-Rad, Munich, Germany) at 700 μF and 240 V. The transfected cells were allowed to recover overnight and divided into aliquots. One aliquot was further treated with 10 ng/ml PMA (Sigma, Deisenhofen, Germany) and 1 μM ionomycin (Calbiochem, La Jolla, CA) for 8 h. Luciferase activity was determined in 10 μl of cell extract using the luciferase assay substrate (Promega, Heidelberg, Germany) with a Dualolumi-Lux Model 9507 luminometer (Berthold, Germany).

RNA isolation and RT-PCR

Total cellular RNA was prepared using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA (1 μg) was reverse transcribed with oligo (dT). PCR amplification was then performed for 35 cycles with specific primers (Stratagene, Heidelberg, Germany) according to the manufacturer’s instructions. RNA (1 μg) was reverse transcribed with oligo (dT). PCR amplification was then performed for 35 cycles.
with a low dose of CsA (100 ng/ml). These experiments demonstrate that the P0 probe interacts with at least two nuclear factors: one, NF-P0b, induced by PMA, and the second, NF-P0a, induced by PMA/ionomycin and sensitive to CsA.

The octamer-like site is required for inducible binding of nuclear factors to the P0 site

MARE was previously identified downstream of the P0 site (29). Using recombinant c-Maf and NF-ATp proteins in EMSA, c-Maf and NF-ATp were shown to bind together to the P0 and MARE sites (29). To investigate whether NF-P0a and NF-P0b can also be formed on the P0 and MARE sites, a Maf probe (from 259 to 227) containing the P0 and MARE sequences (29) and a P0octmu probe (from 268 to 231) containing P0, MARE, and a mutated octamer sequence (Fig. 1) were used for EMSA. The experiments showed that mutation (the P0octmu probe) or deletion (the Maf probe) of the octamer-like sequence abolished binding of NF-P0a and NF-P0b to DNA (Fig. 3A, lanes 3–6). Multiple constitutive complexes were observed when a double amount of nuclear proteins was used in EMSA (Fig. 3A, lanes 7 and 8).

The MARE sequence overlaps with a weak C/EBP binding site (25). It has been suggested that c-Maf and C/EBP, both b-ZIP proteins, may interact to trans-activate the IL-4 promoter (29). To analyze whether C/EBP proteins were involved in binding to the Maf probe, a competition EMSA was performed. The experiment showed that the constitutive complexes formed by using more protein extracts were competed by the unlabeled IL-4 Maf oligonucleotide and by an oligonucleotide containing the C/EBP binding site, but not by the oligonucleotide containing the AP-1 site (Fig. 3B). This experiment indicates that the constitutive complexes detected by the Maf probe may, therefore, be formed by homo- or hetero-dimers of c-Maf and C/EBP proteins. The slowest complex formed by the Maf probe was slightly increased upon PMA/ionomycin induction (Fig. 3A, lane 8, indicated by an arrow). However, the induction obtained with the Maf probe is much less than that seen with the P0 probe. These data indicate that the octamer-like sequence is probably required for a high affinity binding of the inducible factors to the P0 site.

The P0-binding complexes contain octamer proteins

The octamer-like sequence between the P1 and P0 sites was previously shown to weakly bind recombinant Oct-1 and Oct-2 proteins (33). To investigate whether NF-P0a and NF-P0b contain octamer proteins, a competition EMSA was conducted using an

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**FIGURE 1.** The proximal DNA sequence of the human IL-4 promoter. The previously identified elements, AP-1, P1, octamer-like, P0, MARE (in the shaded region), and C/EBP are indicated in the boxes. P1 and P0 contain DNA sequences homologous to the IL-2-NF-AT binding site. The DNA fragments used for EMSA are indicated. Differences in nucleotides between the murine and human IL-4 promoters are indicated above the human IL-4 DNA sequence.

**FIGURE 2.** The P0 site interacts with two inducible factors in Th2 D10 cells. A. A 32P-labeled IL-4 P0 DNA probe (−68 to −45) was incubated with nuclear extracts (10 μg) prepared from Th2 D10 cells noninduced or induced by ionomycin (1 μM), PMA (10 ng/ml), or PMA plus ionomycin. The two inducible complexes are indicated as NF-P0b and NF-P0a, respectively. B. EMSA was conducted using a 32P-labeled P1 DNA probe (−79 to −54) containing the P1 and the octamer-like DNA sequence as in A. C. A 32P-labeled SP-1 oligonucleotide was used as a positive control. The SP-1 protein is indicated. D. The P0 probe was incubated with nuclear extracts prepared from Th2 D10 cells induced by PMA/ionomycin in the presence or absence of CsA (100 ng/ml).
oligonucleotide containing a typical octamer motif from the SV40 enhancer. The experiment showed that formation of both NF-P0a and NF-P0b was specifically competed by the octamer oligonucleotide but not by an unrelated oligonucleotide containing a binding site for AP-3 (Fig. 4A). To further analyze whether octamer proteins are components of NF-P0a and NF-P0b, EMSA was conducted in the presence or the absence of Abs against Oct-1 and Oct-2. As shown in Figure 4B, formation of both NF-P0a and NF-P0b complexes was inhibited by anti-Oct-1 and anti-Oct-2 Abs. Thus, NF-P0a and NF-P0b contain octamer proteins.

We next investigated sequence-specific interaction of NF-P0a and NF-P0b with the IL-4 promoter by a methylation interference analysis. The DNA fragment used in EMSA was partially methylated by dimethylsulfate. After incubation of nuclear extracts with the methylated DNA probe, the bound and free DNA were separated by EMSA and eluted from the gel. Sequence analysis of the eluted DNA showed that methylation of the G and A residues on the octamer-like sequence reduced binding of both NF-P0a and NF-P0b (Fig. 5). Thus, the octamer-like sequence is involved in the formation of both inducible complexes. The experiments also showed that methylation of the G residues on the P0 site (the NF-AT consensus sequence) strongly influenced binding of NF-P0a and NF-P0b (Fig. 5). Therefore, both sites are essential for formation of the inducible complexes. A subtle difference between the interference patterns of the two complexes was observed even though NF-P0a had almost the same DNA contact points as NF-P0b. A slightly stronger influence on the first G residue of the P0 site was shown in the binding of NF-P0a than of NF-P0b (Fig. 5, indicated by a black triangle). This might be due to binding of an additional protein to the P0 site upon coinduction with ionomycin.

The P0-binding complexes contain proteins of the AP-1 family
Since NF-P0b and NF-P0a are induced by PMA and PMA/ionomycin, respectively, we investigated the components of these complexes by competition EMSA using oligonucleotides containing binding sites for known inducible factors. The experiment showed that formation of both NF-P0a and NF-P0b was competed by an excess of the unlabeled octanucleotides containing the AP-1 or the IL-2 NF-AT binding site, but not by the oligonucleotides containing the NF-kB or AP-3 site (Fig. 6A). This indicates that proteins of the AP-1 and of the NF-AT family may participate in the formation of the P0-binding protein complexes. NF-P0b is induced by PMA and is resistant to CsA. However, binding of NF-P0b to the P0 site could be competed by the IL-2 NF-AT binding site. This may be due to the fact that the IL-2 NF-AT binding sequence contains an AP-1 binding site (38).

To further investigate the components of NF-P0a and NF-P0b, EMSA analysis was performed in the presence of Abs against...
c-Fos and c-Jun. As shown in Figure 6B, formation of NF-P0a and NF-P0b was completely inhibited by the Ab specific for c-Fos, but was only slightly interfered by the anti-c-Jun Ab. Conversely, both Abs blocked binding of AP-1 to its own site (Fig. 6C). The specificity of the anti-Fos Ab was controlled by an experiment using SP-1 binding oligonucleotide as the probe (Fig. 6C). To further

FIGURE 5. Methylation interference analysis of the DNA contact points of NF-P0a and NF-P0b. The P0 probe was partially methylated by dimethylsulfate and used for a preparative EMSA. The bound (B) and free (F) DNAs were eluted, piperidine cleaved, and subjected to electrophoresis on a sequencing gel. Bases whose methylation reduces or enhances the formation of NF-P0a and NF-P0b are indicated by circles and triangles, respectively. The first G residue on the P0 site was more enhanced in NF-P0a than in NF-P0b and is indicated by a black triangle.

FIGURE 6. NF-P0a and NF-P0b contain proteins of the AP-1 family. A, Competition analysis of the P0 binding proteins. The 32P-labeled P0 probe was incubated with nuclear extracts from PMA/ionomycin-induced Th2 D10 cells in the presence of different DNA competitors (10 ng) as indicated. — indicates the reaction performed in the absence of DNA competitors. B, EMSA analysis of the P0 binding proteins by anti-Fos and anti-Jun Abs. The 32P-labeled P0 probe was incubated with the Th2 D10 nuclear extract in the presence of anti-Fos and anti-Jun Abs. — indicates the binding reaction with normal serum. C, As controls, a typical AP-1 binding oligonucleotide and a SP-1 binding oligonucleotide were 32P labeled and were incubated with the Th2 D10 nuclear extract in the presence of anti-Fos and anti-Jun Abs as in B. D, EMSA analysis of the P0 binding proteins by an anti-Jun/AP-1 Ab that is broadly reactive with c-Jun, JunB, and JunD p39 proteins. The 32P-labeled P0 probe was incubated with the Th2 D10 nuclear extract in the presence of 1 and 2 μl of anti-Jun/AP-1 Ab as indicated. — indicates the binding reaction with normal serum. E and F, EMSA analysis of the P0 binding proteins by Abs specifically reactive with c-JunB and c-JunD. The 32P-labeled P0 probe was incubated with the Th2 D10 nuclear extract in the presence of 1.5 μl of either anti-c-JunB or anti-c-JunD Abs. — indicates the binding reaction with normal serum.
investigate whether proteins of the Jun family were involved in formation of the P0 complexes, an Ab (anti-Jun/AP-1), broadly reactive with c-Jun, JunB, and JunD p39 proteins, and Abs specific for c-JunB and c-JunD were used in the assay. In these experiments, formation of both complexes was blocked by the anti-Jun/AP-1 Ab (Fig. 6D). Formation of the complexes were also interfered with Abs specific for c-JunB and c-JunD, respectively (Fig. 6, E and F). Thus, proteins of the AP-1 family are components of NF-P0a and NF-P0b.

**NF-P0a contains NF-AT**

The NF-AT binding sequence was first identified as a regulatory element of the IL-2 promoter (34). The IL-2 NF-AT binding site is composed of two DNA sequences, a NF-AT consensus and a weak AP-1 site. By itself, neither NF-AT nor AP-1 can stably bind to the IL-2 NF-AT site. Assembly of NF-AT with AP-1 is required for stable binding of NF-AT to the IL-2 promoter. In contrast, the P1 site of the IL-4 promoter can interact directly with NF-AT (24). Since all P sites of the IL-4 promoter share DNA sequence similarity, it has been proposed that all P sites may interact directly with NF-AT (38). To investigate whether the P0 site can stably interact with NF-AT, recombinant NF-ATc and NF-ATp were used for EMSA. The P1 probe was included as a positive control. The experiments confirmed that NF-ATc/p strongly bind to the P1 probe. In contrast, only a very weak binding activity with rNF-ATcp and no binding activity with rNF-ATc were observed when the same amount of recombinant proteins was used with the P0 probe (Fig. 7A). This indicates that NF-AT has a much lower affinity for the P0 than for the P1 site.

To analyze the components of NF-P0a and NF-P0b, Abs against NF-ATc were added to the nuclear extracts in the EMSA. Formation of NF-P0a, but not that of NF-P0b, was inhibited by anti-NF-ATc Abs (Fig. 7B). Thus, even though NF-ATc alone did not bind to the P0 probe, NF-ATc is detected in NF-P0a by anti-NF-ATc Abs, indicating a cooperative binding of NF-AT to P0. The presence of other factors is suggested by the interference of anti-Fos and anti-octamer Abs with the formation of NF-P0a. Thus, NF-P0a may be composed of three proteins, NF-AT, Fos, and octamer proteins.

**Difference in binding of nuclear factors to the P0 site in Th1 and Th2 cells**

We have shown that in the mouse Th2 D10 cells, AP-1, NF-AT, and octamer proteins may interact with the P0 and the octamer-like site. The P0 site was previously reported to be functionally active in Th2, but not in Th1, cells (22). However, AP-1, NF-AT, and Oct-1/Oct-2 are not restricted to Th2 cells (24). To investigate whether the P0-binding complexes observed in Th2 D10 cells are differentially expressed in Th1 cells, we prepared nuclear extracts from mouse Th1 cell clone 29 that does not produce IL-4 (39). A comparative study showed that the nuclear extract from clone 29 had a much lower level of binding activity on the P0 site than that from D10 cells (Fig. 8A). To further investigate the molecular basis of the Th2 specificity of the P0 site, nuclear extracts were prepared from noninduced and PMA/ionomycin-activated human Th1 and Th2 cell clones. EMSA analysis of the nuclear extracts showed that an inducible P0-binding complex was formed by Th2 but not by Th1 cell-derived nuclear extracts (Fig. 8B). We have independently generated human Th1 and Th2 cells three times. The same observation was obtained. This is not due to a difference in the amount of NF-AT in Th1 and Th2 cells. When the same nuclear extracts were incubated with a 32P-labeled IL-2 NF-AT binding oligonucleotide, a very similar binding of the NF-AT/AP-1 complex to the probe was obtained (Fig. 8C). Equal loading of nuclear extracts was controlled using the SP-1 binding site (Fig. 8D). Formation of this inducible complex was inhibited by CsA. In contrast to extracts from mouse D10 cells, the human Th2 nuclear extracts did not form the CsA-resistant NF-P0b complex (see Fig. 2D). Serologic analysis of the inducible Th2-specific complex showed that anti-Fos, anti-Jun, and anti-NF-ATc Abs blocked formation of this complex (Fig. 9A). Therefore, NF-AT and proteins of AP-1 family are involved in formation of the P0 binding complex. In contrast to the mouse D10 cells (see Fig. 4B), anti-Oct-1/2 Abs did not react with the inducible complex formed by the human Th2 nuclear extracts (Fig. 9A). However, the octamer-like sequence is required for inducible binding. As shown in Figure 9B, the IL-4 Maf probe that lacks the octamer-like sequence did not form the inducible complex. These experiments demonstrate that nuclear proteins of Th1 and Th2 cells may interact differentially with the IL-4 promoter at the P0 and the octamer-like sites.

**Functional relevance of the human P1, octamer-like, P0, and MARE sites**

The P1 site was previously shown to be essential for the functional activity of the human IL-4 promoter (16). However, the octamer-like, P0, and MARE sites of the human IL-4 promoter have not been analyzed. To investigate the functional relevance of these sites on the human IL-4 promoter, we chose a IL-4-producing Jurkat subline, J16, for the functional studies. J16 cells express a high level of IL-4 mRNA upon stimulation with PMA and ionomycin for 4 h. Stimulation of J16 cells with ionomycin alone also induces expression of IL-4 mRNA. However, the production of IL-4 mRNA was significantly dropped after 8-h stimulation (data not shown). Therefore, both the PMA and the ionomycin signal are required for maintenance of high level IL-4 expression.

To investigate the biologic function of the octamer-like, P0, and MARE sites of the human IL-4 promoter, site-directed mutations were generated. The wild-type and the mutated IL-4 promoter fragments were placed in front of the luciferase reporter gene.
Activities of the IL-4 promoter were analyzed by transient transfection of the constructs into Jurkat J16 cells. Mutation experiments showed that a 6-bp exchange at the MARE site did not affect the overall promoter activity (Fig. 10, A and B). The inducibility of the promoter was reduced after mutation at this site (Fig. 10 C). This reduction was mainly due to an increase in the basal promoter activity at the noninduced stage (Fig. 10 A). Mutations of the octamer-like and of the P0 sites reduced the promoter inducibility by approximately 20 and 40%, respectively (Fig. 10 C). In contrast to the MARE site, mutations of the octamer-like and the P0 site greatly reduced the overall promoter activity (Fig. 10, A and B). Also, mutation of the P1 site reduced by 90% the inducible and overall promoter activity, as previously reported (16, 21). These experiments demonstrate that the octamer-like and the P0 site are important for the functional activity of the human IL-4 promoter.

To further define the functional role of the MARE site, we generated two human IL-4 promoter/CAT reporter constructs containing a 7-bp exchange or a 13-bp deletion at the MARE site. Mutations of the MARE site led only to a 25% reduction in promoter activity (Fig. 11, A and B). Deletion of 13 bp at the MARE site reduced by approximately 70% the basal but not the inducible activity of the promoter (Fig. 11). The great reduction of the basal promoter activity was probably due to disruption of the distance between the TATA box and the P0 and other regulatory elements. Thus, the MARE site does not appear to be essential for function of the human IL-4 promoter in Jurkat T cells.

Discussion

Th1 and Th2 cells produce distinct subsets of cytokines. IL-4 is expressed in activated Th2 cells. One of the activation-responsive sequences of the IL-4 promoter, the P0 site, is of particular interest because it was shown to confer Th2-specific activity (22). In line with this finding, we show here binding of inducible transcription factors to the P0 site in activated Th2, but not Th1, cells. Analysis of the components of the P0 binding complexes NF-P0a and NF-P0b revealed the presence of several known transcription factors, such as the PMA-inducible proteins of the AP-1 family and the ionomycin-inducible protein NF-AT. In the mouse D10 cells, Oct-1 and Oct-2 proteins were also found to be involved in formation of the P0 binding complexes.

The P0 probe contains the octamer-like sequence. However, no significant DNA binding to the P0 probe was detected in nonstimulated nuclear extracts. This is in agreement with our observations that by themselves, Oct-1 and Oct-2 bound weakly to the
IL-4 octamer-like sequence (33). The P0 region also does not contain an AP-1 binding site as demonstrated by footprinting analysis using recombinant Fos and Jun proteins (31). Upon induction of D10 cells, NF-P0a and NF-P0b bind to the P0 and the octamer-like sequences. Both NF-P0a and NF-P0b were found to contain AP-1 family and octamer proteins.

**FIGURE 10.** Transient transfections using the human IL-4 (−269/+11)/luciferase constructs. A, Relative luciferase activity from extracts of Jurkat J16 cells transfected with wild-type (wt) and MARE (Mafmu), P0 (P0 mu), octamer-like (octmu), and P1 (P1 mu) mutated constructs of the IL-4/luciferase plasmids. Results are the averages of four independent transfection experiments. B, Results are expressed as relative promoter activity after mutation at each site. C, Results are expressed as the fold induction. D, Schematic representation of the human IL-4 promoter and of the proximal promoter sequence. Differences in DNA sequence between the human and the murine promoters are indicated above the human IL-4 promoter sequence. Constructs containing 4- to 6-bp substitutions within the proximal promoter region are indicated.

**FIGURE 11.** Transient transfections using the human IL-4 (−269/+11)/CAT constructs containing point mutations or deletion at the MARE site. A, CAT activity from extracts of Jurkat cells transfected with wild-type (wt), MARE-mutated (Mafmu), and MARE-deleted (Mafdel) constructs of the IL-4/CAT plasmids. Results are the averages of two independent transfection experiments. B, Results are expressed as relative promoter activity after mutation or deletion at the MARE site. C, Results are expressed as the fold induction. D, Schematic representation of the human IL-4 promoter and of the proximal C/EBP (boxed) and MARE (shaded region) sequences. Constructs containing 6-bp substitutions or a 13-bp deletion at the C/EBP and MARE regions are indicated.
Based on a comparison of the different NF-AT binding sequences identified to date, the NF-AT binding site is present as a 9-bp element positioned next to an AP-1 element. NF-AT and AP-1 are shown to cooperatively bind to many promoters (40). Although all P sites of the IL-4 promoter share DNA sequence similarities, they bind to NF-AT with different affinities (41). In general, the NF-AT sites in which the GGAAA core sequence is preceded by a T rather than by an A bind NF-AT proteins more strongly (40). The NF-AT binding sequence in the P1 site contains four T next to the GGAAA core sequence, while the NF-AT consensus sequence in the P0 site does not (41). This may explain why rNF-ATp binds poorly to the P0 probe and no binding activity was detectable with rNF-ATc in EMSA. Although P0 is a weak NF-AT site, the PMA/ionomycin-induced complex NF-P0a contains NF-ATc, as detected by anti-NF-ATc Abs. These data indicate that proteins of the AP-1 family, octamer proteins, and NF-AT may cooperatively bind to the P0 site, an otherwise very weak binding site for each individual factor.

Interestingly, our experiments show that inducible binding of nuclear factors to the P0 site was detectable with nuclear extracts of normal human Th2 but not Th1 cells. This may partially explain the Th2 specificity of the P0 site observed previously (22). NF-AT and proteins of the AP-1 family are not restricted to Th2 cells. Yet, specific binding of these proteins to the P0 site was observed only in Th2 cells. Similar observations have recently been reported for NF-AT that binds to the P4 and P1 sites only in Th2 cells (32, 35). This suggests that another tissue-specific factor(s) or tissue-specific post-translational modifications of DNA binding proteins may contribute to the Th2-specific interaction of the ubiquitous nuclear factors to the P4, P1, and P0 sites of the IL-4 promoter. This suggestion is supported by the recent finding that the recombinant c-Maf, an oncogene product expressed exclusively in Th2 cells, binds to the MARE and P0 sites in association with the recombinant NF-AT (29). Since the P0 probe used in our experiments and the Th2-specific P4 and P1 binding activities (32, 35) do not contain a MARE site, we assume that c-Maf is not the only factor conferring Th2-specific expression to the IL-4 gene.

In this study, we also investigated the functional role of the P0 and the octamer-like site in regulating the activity of the human IL-4 promoter. We show that the octamer-like site, although not essential for the inducibility of the IL-4 promoter, plays an important role in the overall promoter activity. Binding of NF-AT to the P0 site may have been abrogated by the mutations introduced at the octamer-like site. Since NF-AT may cooperate also with c-Maf to bind to the P0 site (29), mutations at the octamer-like site do not seem to be critical for the inducibility of the IL-4 promoter. We show that the P0 site by itself is a weak binding site for NF-AT. Mutations at the P0 site resulted in an approximately 40% reduction of the promoter inducibility and a 70% decrease in the overall promoter activity. Therefore, the P0 site is important for human IL-4 promoter activity.

Subtle differences between the human and the murine systems were observed. Oct-1 and Oct-2 were identified in the P0 binding complexes formed by the D10 cells. However, the octamer proteins were not found in the P0 binding complex formed by the human Th2 cells. Also, the PMA-inducible CsA-resistant NF-P0b was not detected in extracts from the human Th2 cells. Furthermore, the MARE site has recently been shown to play a critical role in regulation of the murine IL-4 promoter (42). However, nucleotide substitution at the MARE site did not significantly influence the overall activity of the human IL-4 promoter. The 13-bp deletion of the MARE site, although it significantly reduced the basal activity of the human IL-4 promoter, did not influence its inducibility. In EMSA, the DNA probe containing the P0 and the MARE sites did not show significant inducible binding activities (Fig. 3). Instead, multiple constitutive complexes were generated by both noninduced and induced nuclear extracts (Fig. 3). The MARE site was previously identified as a weak NF-IL-6 binding site (25). Thus, c-Maf and NF-IL-6, both b-ZIP proteins, may interact at this site (29). The multiple constitutive complexes observed with the MARE probe in our experiment might be generated by such interactions. CD4+ Th2 cells are generally considered the principal producers of IL-4 in an immune response. However, other cell types, like mast cells, basophils, and CD4+ T cells, are found to produce IL-4 as well (43–49). Although the MARE site does not seem to be essential for the activity of the human IL-4 promoter in Jurkat T cells, the importance of this site for IL-4 transcription in other IL-4-producing cells needs to be examined.

Previously, we showed that NF-IL-6 is involved in trans-activation of the IL-4 promoter and that NF-IL-6 mRNA is detectable in IL-4-producing cells but not in the Th1 clone 29 (25). This indicates that NF-IL-6 may be restricted to only certain T cell subsets. Recently, high mobility group (HMG) I(Y) chromosomal proteins, which by themselves are unable to stimulate or inhibit promoter activity, were found to compete with NF-AT for binding to the P1 site (27). HMG I(Y) was found expressed at different levels in different T cell lines and, therefore, may be another candidate for the differential regulation of the IL-4 promoter activity in different T cell populations. A potential NF-xb binding site was identified that shares the NF-AT binding sequence at the P1 site (26). NF-xb is well known to activate IL-2 gene expression. Opposite effects of the two subunits of the NF-xb heterodimer, p65 (RelA), and p50 (NF-xb1) on IL-2 and IL-4 promoter activities in the human Jurkat T cell line were reported (26). Therefore, NF-xb may also play a role in differential regulation of Th1 and Th2 cytokines. Stat6, the IL-4-induced transcription factor, was found to interact with the P2 site (28). Stat6 activation correlated consistently and uniquely with IL-4-induced Th2 differentiation. Very recently, GATA-3 was found in Th2 cells and was shown to activate the IL-4 promoter in B lymphoma M12 cells. Antisense GATA-3 inhibited the expression of all Th2 cytokine genes in Th2 D10 cells (50). Taken together, we suggest that the Th2-specific expression of the IL-4 gene is controlled by a multifactor system. The findings described here provide additional evidence that differential interaction of transcription factors with promoter/enhancer elements of cytokine genes is one of the mechanisms responsible for the tissue-specific expression of type 1 or type 2 cytokines.

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