Characterization of an Interleukin 4 (IL-4) Responsive Region in the Immunoglobulin Heavy Chain Germline ε Promoter: Regulation by NF-IL-4, a C/EBP Family Member and NF-κB/p50

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Summary
A large body of data indicate that antibody class switching is directed by cytokines by inducing or repressing transcription from unrearranged, or germline, C\textsubscript{\textit{\textit{\i}}} genes. Interleukin 4 (IL-4) induces transcription of the germline Ce genes in activated B cells and subsequently, cells in this population will undergo switch recombination to immunoglobulin E. Furthermore, the data suggest that transcription of germline Ce genes is required for class switching. In this paper we define DNA elements required for induction of transcription of the germline Ce genes by IL-4. To do this, segments of DNA from the 5' flank of the initiation sites for germline ε RNA were ligated to a luciferase reporter gene and transfected into two mouse B cell lines, one of which can be induced to switch to IgE. By analysis of a series of 5' deletion constructs and linker-scanning mutations, we demonstrate that a 46-bp segment (residing at -126/-79 relative to the first RNA initiation site) contains an IL-4 responsive region. By electrophoretic mobility shift assays, we find that this segment binds three transcription factors: the recently described NF-IL4, one or more members of the C/EBP family of transcription factors, and NF-κB/p50. Mutation of any of the binding sites for these three factors abolishes or reduces IL-4 inducibility of the ε promoter. A 27-bp segment within this IL-4 response region containing binding sites for NF-IL4 and a C/EBP factor is sufficient to transfer IL-4 inducibility to a minimal c-los promoter.

A map of the mouse germline ε DNA segment and transcript is shown (see Fig. 1 A). The DNA sequences which regulate transcription of the mouse and human germline ε RNAs are currently being studied. Initial characterizations of DNA sequences which regulate induction by IL-4 have been published (13-15). In this report we characterize an additional IL-4 responsive region (IL-4RR) in the mouse germline ε promoter that overlaps with the previously characterized IL-4RR of the human germline ε promoter (14). This ε IL-4RR resides within a DNA sequence highly conserved between human and mouse and also has elements similar to those within an IL-4RR within the promoter for mouse germline γ1 transcripts (16, 17).

Materials and Methods
Cell Lines. Two mouse B lymphoma cell lines were used in this study: 22A10, a clone of the slgM\textsuperscript{+} I.29\textmu B cell lymphoma line (18, 19) and M12.4.1, an Ig-negative, class II\textsuperscript{+}, HGPRT-
deficient variant of M12.4 (20), received from Dr. Paul Rothman (Columbia University, New York).

**Cell Culture.** 22A10 cells were cultured as described for 1.29 µ cells (21). M12.4.1 cells were cultured at 37°C in an atmosphere of 5% CO₂ in RPMI 1640 medium supplemented with 10% FCS (Hyclone Laboratories, Logan, UT), 50 µM 2-ME, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, 200 U/ml penicillin, 200 µg/ml streptomycin, and 0.1 mg/ml kanamycin sulfate. Cells were treated with murine IL-4 (a gift from Dr. Steven Gillis, Immunex Corp., Seattle, WA and the Sterling Research Group, Malvern, PA) at 1,000 U/ml, unless otherwise specified. In initial experiments, LPS (055:B5; Sigma Chemical Co., St. Louis, MO) dissolved in RPMI, was added at 25 µg/ml of culture.

**Splenic B Cell Cultures.** Splenic B cells were purified and cultured as described (22), except total B cells were used rather than only small B cells, and cells were cultured for 12 h with IL-4 at 1,000 U/ml and LPS at 25 µg/ml, as indicated.

**Isolation of RNA and RNA Blot Analysis.** Total cell RNA was prepared by the guanidinium isothiocyanate-CæCl method (23) and RNA blots were prepared and hybridized as described (21). Quantitation of hybridization was performed by densitometry on a Begascope 630 blot analyzer (Betagen, Waltham, MA). The Ce probe was a 2.1-kb BamHI/HindIII genomic DNA fragment (24). Hybridization to a GADPH probe (25) or densitometry of 18S rRNA normalized the difference in loading.

**RNase Protection Assay.** To determine the initiation site of germline e RNA, RNase protection assays were performed (26). Total cell RNA was prepared from 22A10 cells induced with an IL-4-containing supernatant from X63Ag8-653-IL-4 for 48 h (27). The X63 supernatant was titrated for germline e RNA induction and used at an optimal dose. The RNase protection probe was transcribed from a 512-bp HincII/PstI genomic DNA fragment encompassing the 1e exon (28, 29) and cloned in Bluescript KS-.

**Promoter-Luciferase Plasmids.** To create the 5'-deletion constructs of the germline e promoter, a series of fragments containing various lengths of the promoter segment were ligated into the plasmid pXP2, which has a polynucleotide but not a promoter upstream of the luciferase (Luc) reporter gene (32). The 3'-border of these promoter fragments was created by PCR amplification of genomic e DNA, using the primers, 3'e (+33/+53 relative to the first start site) and 5'e (+788/-773) (33/). Nuclease +53 is within the 1e exon and is located 5' to the potential protein synthesis initiation codon at +65. The 5'-borders were created by restriction enzyme digestion at BamHI (1.8-kb promoter) or BglII (1.2-kb promoter), cloned into the BglII site of pXP2, or by digestion with BstXI (640-bp promoter), HincII (115-bp promoter), or AvrII (15-bp promoter), and cloned into pXP2 digested with Smal and BglII. BstXI digestion followed by Bal3I digestion created additional smaller promoters that were cloned into pXP2 digested with Smal and BglII. The nucleotide sequences of all plasmids having 640 bp or less of the promoter were verified by sequencing from the 5' direction using a primer complementary to the polynucleotide of pXP2 (pXP2 primer) and also from the 3' direction, using the Luc primer, extending as far as possible.

**Linker-scanning Mutations.** 8-bp Xho linker substitutions were introduced into the full-length promoter-luciferase reporter construct, -162Luc, by using Bal3I to generate a nested set of 5' and 3' deletion mutants, bounded by Xhol linker. The deletion end points were determined by nucleotide sequencing. 5' and 3' deletion clones with matching end points were chosen as best as possible to attempt to preserve the length of the wild-type promoter. Ligation of a BamHI (in polynucleotide) Xhol fragment from a 5' deletion clone into a 5' deletion plasmid digested with BamHI and Xhol resulted in the regeneration of an approximately full-length promoter (in most cases) containing an 8-bp Xhol linker substitution. All constructs were sequenced across the sites of mutation using the pXP2 or Luc primers.

**pFL Reporter Plasmids.** Plasmid pFL contains the mouse c-Fos 71-bp promoter segment from pfosCAT (33) (given by M. Lenardo, National Institutes of Health, Bethesda, MD) ligated 5' to the luciferase gene (34). Double-strand oligonucleotides containing BamHI sticky ends at the 5' end and BglII sticky ends at the 3' end were phosphorylated with γ-[32P]ATP with T4 polynucleotide kinase at 1 U/µl (Boehringer Mannheim, Indianapolis, IN), 2% wt/vol PEG 8000, 75 µg/ml BSA, and 1 µM ATP at 37°C for 30 min. The phosphorylated oligonucleotides were multimerized and ligated into the BamHI site of plasmid pFL. Recombinant clones were screened by PCR amplification using the same e oligonucleotides as primers individually in combination with a primer complementary to the c-Fos promoter. PCR products obtained using the top strand oligonucleotides indicated that the insert was in the correct orientation whereas PCR products obtained using the bottom strand.
oligonucleotides indicated that the insert was in reverse orienta-
tion. Insert-containing clones were sequenced using the Fos primer
fidelity of the BamHI and BgllI restriction sites.

**DNA Sequencing.** Nucleotide sequences of CslCl-purified or of
mini-preparations of plasmids were determined by the dideoxy chain
termination method using Sequenase Version 2 kits (United States
Biochemical Corp., Cleveland, OH).

**Transfection.** Transfection was performed by electroporation
using Cell ZapII (Anderson Electronics, Brookline, MA). Briefly,
RPMI 1640 was used to wash and resuspend an appropriate number
of cells. 50 x 10^6 cells was the maximum transfected in 1 ml. In
the experiments involving transfection of the 5' deletion constructs
and the linker-scanning mutations, the internal control plasmid
pSV2CAT (35) was added to the resuspended cells, mixed well,
and 0.9 ml of the cell mixture was pipetted into sterile cuvettes.
CsiCl-purified plasmid DNAs were added in a volume of 100 ml
to the cuvettes. The cells were electroporated at 1250 #F/300 V,
rested at room temperature for 10 min, and pipetted into complete
medium at ~0.25 x 10^6 cells/ml. After addition of inducers, cells
were incubated for 8-18 h and then assayed for luciferase or chloro-
amphenicol acetyl transferase (CAT) activity.

**Luciferase Assays.** Luciferase assays were performed as described
(36). Cells were washed in PBS, transferred to Eppendorf tubes,
and lysed in 200 ml Triton X-100 lysis buffer (1% Triton X-100,
25 mM glycyglycine, pH 7.8 [Sigma Chemical Co.], 15 mM
MgSO4, 4 mM EGTA, and 1 mM dithiothreitol [DTT]) at room
temperature. The reaction mixture contained 100 ml cell lysate,
350 ml luciferase assay buffer (25 mM glycyglycine, pH 7.8,
15 mM MgSO4, 4 mM EGTA, 15 mM KH2PO4, 2 mM ATP,
and 1.27 mM DTT), and 100 ml of 1 mM luciferin (Analytical Lumines-
cence Laboratory, San Diego, CA) dissolved in distilled H2O. The
level of luciferase activity was determined with a Monolight 2010
luminometer (Analytical Luminescence Laboratory) using plasmid
pSV2Luc (34) as positive control. The values obtained from mock
transfected or lysis buffer control were subtracted as background.

**CAT Assays.** The activity of cotransfected pSV2CAT was as-
sayed to control for transfection efficiency by the diffusion-based
assay (37). 50 ml of cell lysates was heated at 70°C for 10 min be-
fore mixing with 200 ml of 120 mM Tris, pH 7.8, 1 ml chloro-
amphenicol (Sigma Chemical Co.), and 0.1 #Ci [3H]-acyetyl CoA
(200 mCi/mmol) (DuPont NEN Research Products, Wilmington,
DE). The reaction mixture was added to a scintillation vial and
5 ml of Econofluor (DuPont NEN Research Products) was
overlaid. The acetylated chloramphenicol was measured at the end of
a 3-h incubation at 37°C.

**Oligonucleotide Probes for Electrophoretic Mobility Shift Assay.**
Double-stranded oligonucleotides were generated by annealing a
complementary pair of oligonucleotides. The reaction mixture con-
tained 100 ng/ #l of each oligonucleotide in 100 ml NaCl, 10 mM
Trts-HCl, pH 8, and 1 mM EDTA. The DNA was incubated at
95°C for 10 min to disrupt secondary structures and incubated at
10°C below the melting temperature for 1 h and then slowly cooled
to room temperature to anneal. The annealed oligonucleotides were
ethanol precipitated and purified on a 12% polyacrylamide gel.
Double-stranded oligonucleotides were 5' end labeled with Klenow
enzyme and d[32P]dCTP (3,000 Ci/mmol) at 5 x 10^{6} cpm/ #g, and
then gel purified as above.

**Preparation of Nuclear Extracts.** For small scale preparations, the
method of Schreiber et al. (38) and for large scale preparations that
did not express germline e transcripts constitutively, and this
can be further increased 5-20-fold by treatment with IL-4
(Fig. 1 B, right). Addition of LPS does not further increase
the level of transcripts. The other B cell lymphoma used for
these studies is M12.4.1, a class II+, Ig-negative variant that
was derived from the M12 B lymphoma (20). M12.4.1 cells
do not express germline e transcripts constitutively, but germ-
line e transcripts can be induced by treatment with IL-4 plus
LPS (Fig. 1 B, right) (13). Unlike, I.29, IL-4 alone does not
induce detectible germline e transcripts in M12.4.1 cells.

To begin to characterize DNA sequences that regulate trans-
scription of germline e RNA, we verified by a RNase pro-
tection assay the initiation sites of transcription of germline e
RNA that were previously determined in 18-81A20 cells by
Rothman et al. (28). RNA from IL-4-treated I.29 cells
was hybridized with a radioactive RNA probe transcribed from
a 512-bp HindIII/PstI e genomic DNA fragment, digested with
RNase, and the products were electrophoresed on a DNA
sequencing gel. Three protected fragments of 118, 100, and
90 nucleotides were detected, which were not detected when
the probe was hybridized with yeast RNA (Fig. 1 C). As-
suming that the 1e splice donor defined previously by analysis
of cloned germline e cDNA from 18-81A20 cells is also
used in I.29 cells, we identified three transcription start sites
very near the cluster of initiation sites previously identified
(28). The first is located six nucleotides 5' to the most 5'
Figure 1. Structure of the mouse Ig heavy chain germline $\gamma$ gene and $\gamma$ transcript. (A) Restriction map of unrearranged Ce gene and germline $\gamma$ transcript with location of exons and switch region (Se) marked. (B) Blots containing total cell RNA from I.29$\mu$ (left) and M12.4.1 (right) cells that had been cultured for 2 d, as indicated, with medium alone, IL-4 (2,000 U/ml), LPS (25 $\mu$g/ml), or with both IL-4 and LPS, hybridized with Ce probe. (C) Products of an RNase protection experiment using a RNA probe transcribed from the 512-bp HindIII/PstI genomic DNA fragment encompassing the Ie, hybridized with total cell RNA from I.29$\mu$ cells induced with X63-11:4 supernatant at an optimal dose. Hybridization was at 42°C (two left lanes) or at 40°C (two right lanes). Three RNase resistant bands of the indicated lengths (determined by alignment with a DNA sequencing ladder) are detected on the DNA sequencing gel, and the positions of the corresponding RNA initiation sites are indicated on the sequence of the promoter-Ie-Luc construct in Fig. 2 A. We were unable to detect any band corresponding to a start site at -123, relative to our first start site, which was detected in I.29$\mu$ cells by primer extension experiments (53).
not (Fig. 3 B). These results suggest there are several DNA elements residing between -136 and the start site of transcription that are necessary for normal levels of basal expression of the germline e promoter in both cell lines, as assayed in the luciferase plasmid. Consistent with this is the fact that the nucleotide sequence between -144 and -14 is highly conserved, as it is almost identical to the sequence of the human germline e promoter sequence (14, 41).

Localization of an IL-4RR with Linker-scanning Mutations. The sequences in the germline e promoter which control induction by IL-4 appear to be much more localized than those required for basal expression, since only four of the linker-scanning mutations abolish or markedly reduce IL-4 inducibility in both I.29μ and M12.4.1 cells, mutant (mt) 13: at -120/-112, mt 20: at -106/-98, mt 27: at -87/-79, and mt 63: at -65/-39 (Fig. 3, C and D).

The sequence of the -126/-79 segment which contains three of the linker-scanning mutations that abolish or reduce IL-4 inducibility (mts: 13, 20, and 27) is shown in Fig. 4 A. Interestingly, these three mutations altered potential binding sites for three transcription factors. Mt 13 alters a consensus binding site for the C/EBP family of transcription factors (42-45). Mt 20 alters a consensus element for a newly described IL-4 inducible transcription factor, NF-IL4 or IL-4NAF (46, 47). Mt 27 alters a consensus binding site for NF-xB (48). This third mutation also abolishes constitutive expression. The fourth linker-scanning mutation mt 63 (-65/-38) reduces IL-4 inducibility in I.29μ cells by only 60% and basal expression by 90%, although it completely abolishes inducibility and basal expression in M12.4.1 cells. This mutation should abolish the binding site for the B cell–specific DNA binding protein BSAP, which binds to the germline e promoter at -42/-15 and is required for IL-4 plus LPS inducibility of a CAT reporter gene driven by the mouse germ-line e promoter in M12.4.1 cells (13, 15). Note, however, that four other mutations covering the region from -55 to -15 also mutated the BSAP binding site, but have little or no effect on induction by IL-4, although they all eliminate basal activity in M12.4.1 (but only slightly reduce it in I.29μ cells).

A DNA Segment Containing Binding Sites for C/EBP and NF-IL4 Is Sufficient to Confer IL-4 Inducibility. We wished to determine whether the DNA sequences shown by the linker-scanning mutations to be necessary for IL-4 induction cells. The first 53-bp of the le exon does not have a translation initiation ATG codon. Regions indicated above the sequence are: (Flu 4RE) region necessary for induction of the human germline e promoter by IL-4 (14); (complex 1 & 2) a region that binds nuclear factors in B cells/B cell lines stimulated by IL-4 (13); (complex 3) the binding site for BSAP (15, 54) and necessary for expression of the germline e promoter-CAT constructs after LPS plus IL4 induction (13, 15). (EPSILON CONSERVED SEQUENCE) The segment is highly homologous (>80%) between human and mouse sequences. This sequence differs at two sites from that previously reported (28): at +40/+43 we find GGGG instead of GGG and at -15/-19 CCCC instead of CCCC. (B) Sequences of the -162Luc plasmids with linker scanning mutations. Names are given on the left, followed by nucleotides that are either substituted or deleted in the various mutants. These sequence data are available from EMBL/GenBank/DDBJ under accession number U17387.

Figure 2. (A) Sequence of 5' flank of germline e RNA and the first 53 nucleotides of le exon present in the e promoter -162Luc plasmid. This plasmid contains all three transcription start sites detected in I.29μ
Figure 3. Results of transient transfection experiments of germline Ig e promoter-luciferase reporter plasmids having 5' deletions: -162Luc, -127Luc, and -115Luc, or -162Luc with the indicated linker scanning mutations. (A) Basal expression in 1.29µ. Luciferase activity is reported in light units after subtraction of background (no cell extract) of 250-350 light units. Results are normalized to the activity of pSV2CAT which was cotransfected along with the luciferase plasmids. Results from three experiments plus standard deviations of the means are plotted. (B) Basal expression in M12.4.1, as in A. (C) IL-4-inducible expression in 1.29µ. Transfected cells from experiments shown in A were split into two aliquots, one of which was treated with IL-4 for 12 h. Fold induction indicates the luciferase activity in IL-4-treated cells relative to that in untreated cells. (D) IL-4-inducible expression in M12.4.1, as in C.

of the germline Ig e promoter are also sufficient to confer IL-4 inducibility upon a heterologous promoter. To test this, we inserted a series of double-stranded oligonucleotides containing wild-type or mutated sequences of the IL-4RR shown in Fig. 4 A into a luciferase reporter plasmid driven by a minimal c-fos promoter. Some of the plasmids have multiple copies of the oligonucleotides in sense and antisense orientations (indicated in Fig. 4 B). The plasmids were transiently transfected into 1.29µ cells, and the cells were stimulated with IL-4. Only the wild-type oligonucleotide A, which contains both the consensus binding sites for C/EBP and NF-IL4, is able to confer IL-4 inducibility upon the c-fos promoter (Fig. 4 B). The activity of a plasmid with one copy of the A oligo is induced threefold by IL-4, and plasmids with two or three copies are induced about 20-fold. If either the C/EBP or the NF-IL4 site is mutated, the plasmid is not inducible by IL-4. Therefore, both sequence elements are required for IL-4 inducibility, consistent with the linker-scanning anal-
Electrophoretic Mobility Shift Assays Demonstrate that the IL-4RR Does Indeed Contain Binding Sites for NF-IL4, Ig/EBP-1, and NF-kB/p50. To determine if these consensus binding sites bind the predicted proteins, a series of electrophoretic mobility shift assays (EMSAs) were performed using the double stranded oligonucleotides shown in Fig. 4 A as probes and/or as competitors. When wild-type oligonucleotide A is incubated with nuclear extracts from unstimulated or IL-4-treated I.29µ cells, a low mobility complex is induced by IL-4 treatment (Fig. 5 A). Competition experiments demonstrate that this complex binds to a site that matches the consensus sequence for a complex induced by IL-4 (NF-IL4/IL-4NAF) in human monocyte and B cell lines (46, 47). This complex is competed by wild-type oligo A, by A mt 13, and by oligo D, but not by mt 20, which has nucleotide substitutions in the putative NF-IL4 binding site, or by oligo E (Fig. 5 A). Oligo C does not compete (data not shown), apparently because it lacks the first T of the binding site, which has been shown to be important for binding of NF-IL4 (46). A kinetic experiment showed that the IL-4 inducible complex is detected after 30 min of IL-4 treatment, is maximally induced by 4 h, and is maintained for 24 h (data not shown). No later time points were examined. The kinetics of induction are slower and more sustained than that found for the binding activity in monocytes (47).

The IL-4 inducible complex is also detected in splenic B cells treated with IL-4 alone for 12 h and LPS has no additional effect (Fig. 5 C). Thus, although LPS is required for induction of germline e transcripts in splenic B cells, NF-IL4 can be induced by IL-4 alone, indicating that induction of NF-IL4 is not sufficient to induce transcription of the endogenous germline e RNA.

MT 13 disrupts a consensus sequence for the C/EBP family of transcription factors. This family of proteins possesses a basic region and leucine zipper, and all members bind the same DNA sequence, although not all cells have all family members. B cells express two members of the family: C/EBPβ (originally called Ig/EBP-1), which is most abundant in pre-B cells, and C/EBPα (also called NF-IL6, LAP, AGP/EBP, CRP-2, or IL6DBP), which is expressed in mature B cell lines and in splenic B cells induced with LPS (45). To determine if C/EBP family members bind the consensus C/EBP site, we tested binding of recombinant mouse Ig/EBP-1 (49). Both wild-type oligonucleotide A and mt 20 bind Ig/EBP-1, whereas mt 13 does not (Fig. 5 B). Thus, oligonucleotides with a wild-type, but not a mutated, consensus site for C/EBP bind Ig/EBP. The arrow on the left side of Fig. 5 A indicates a complex that might correspond to a C/EBP family member present in I.29µ, since it is competed with both oligos A and mt20, but not with mt 13 or oligonucleotide D. Furthermore, this complex is competed by a multimerized C/EBP binding site from the Ig µ intron enhancer (data not shown) (49). However, it is not competed by oligo E, which includes only the C/EBP site, suggesting that binding may require additional nucleotides (Fig. 5 A). We demonstrated that the putative kB site binds a member of the NF-kB/REL family of transcription factors by performing EMSAs using oligonucleotide B as a probe with nuclear extracts from untreated...
Figure 5. EMSAs of the IL-4RR within the germline Ig ε promoter. Left-most lane in each panel or each set of lanes contains probe alone. (A) EMSA of oligonucleotide A (sequence given in Fig. 4 A) incubated with 5 μg nuclear extracts from 1.29μ cells untreated or induced for 12 h with IL-4 (+). Competitor oligonucleotides were added at 100-fold molar excess. (Arrow) A complex that appears to be due to binding at the C/EBP site (see text). (B) Complexes formed after incubation of recombinant Ig/EBP-I(C/EBP'y) (+) with the wild-type A oligonucleotide, with mt 13 or with mt 20. (C) Oligonucleotide A incubated with nuclear extracts (2 μg) from splenic B cells (Control) or splenic B cells treated with IL-4, IL-4 plus LPS for 12 h. (D) EMSAs with oligonucleotide B demonstrate that NF-κB/p50 binds to the wild-type B oligonucleotide. (Left) Nuclear extracts from untreated or IL-4-treated 1.29μ cells (+) were incubated without or with the indicated oligonucleotides as competitors. κB is a 27-bp oligonucleotide containing the κ enhancer κB site. (Right) Supershift experiment using three antisera. Antiserum to NF-KB/p50 causes a supershift, but antisera for c-Fos and NF-KB/p65 do not (Santa Cruz Biotechnology, Santa Cruz, CA). Antisera specific for p50 and p65 from Dr. Nancy Rice (Frederick Cancer Center, Frederick, MD) gave identical results (data not shown).

or IL-4-treated 1.29μ cells, competing with a DNA fragment containing the NF-κB site from the Ig κ enhancer (Fig. 5 D). Specificity of binding was also demonstrated by competition with wild-type B or with mt 27, which contains the mutated κB site. All three complexes formed with oligo B can be competed with the wild-type B oligo or with a κB binding site, but not with mt 27. In addition, when mt 27 is labeled and used as a probe it fails to bind any complex (data not shown). The binding is not induced by IL-4. This is consistent with the finding that oligo B does not transfer IL-4 inducibility to the c-fos promoter, although the κB site contributes to the IL-4 response, since the linker-scanning
nucleotides from the 3'4 gene are according to the published sequence (41). The positions of the C/EBP site in the 3'4 promoter has been demonstrated to bind C/EBP3' and C/EBP/3 in nuclear extracts (17). The positions of the nucleotides from the 3'4 gene are according to the published sequence (41). To determine which member of the NF-IL4 and C/EBP sites in the mouse germline 3'1 promoter with sequences upstream of the human 3'4 switch region. The C/EBP site in the 3'1 promoter has been demonstrated to bind C/EBPγ and C/EBPβ in nuclear extracts (17). The positions of the nucleotides from the 3'4 gene are according to the published sequence (41). (C) Alignment of the C/EBP consensus sequences located at the start sites of transcription of the mouse germline 3'1 and 3'4 RNAs. The 3'1 sequence has been shown to bind C/EBPγ in nuclear extracts (17).

mutation at this site reduces the response to IL-4 by three-fold in both 1.29μ and M12.4.1 cells. Different members of the NF-kB/Rel family differ somewhat in their DNA sequence requirements for binding (48). To determine which member of this family binds to the putative binding site at -90/-81, we tested a series of NF-kB antisera for their effect in EMSAs using oligo B as the probe. An antisemur to the p50 homodimer superhifts the complex, whereas antisera to p65 or p50B do not (Fig. 5 D, right, and data not shown). As a further control, we show that anti-c-Fos antibody does not supershift the complexes. Note that linker-scanning mutation 39, which mutates -100/-91, has no effect on expression of the promoter in these assays, suggesting that there is no additional transcription factor binding site between the sites for NF-IL4 and for NF-κB/p50.

Discussion

Results Reported Here Extend Previous Studies of the Regulation of the Ig Germline ε Promoter in Mouse and Human. The regulation of the mouse germline ε promoter by LPS plus IL-4 has been previously analyzed using a CAT reporter plasmid transiently transfected into M12.4.1 cells (13). Rothman et al. (13) found that a 5' deletion that deleted the C/EBP site did not reduce inducibility of their reporter gene, whereas we find that mutation of the C/EBP site abolishes induction by IL-4 when used in the absence of LPS. Furthermore, they found that the BSAP binding site is essential for LPS plus IL-4 inducibility in M12.4.1 cells (15), whereas we find that four out of five mutations that affect the BSAP binding site in the promoter do not reduce IL-4 inducibility in either M12.4.1 or 1.29μ cells. Thus, it is possible that induction by IL-4 alone may utilize different transcription factor binding sites than the combination of IL-4 plus LPS. Our findings agree with preliminary results on the IL-4RR of the promoter for the human germline ε transcripts (14).

Characterization of an IL-4RR in the Germline ε Promoter. We have identified three transcription factors that appear to regulate basal and IL-4 induction of transcription of the mouse germline ε promoter: one or more members of the C/EBP family of transcription factors, the p50 subunit of NF-kB, and a newly described IL-4-inducible transcription factor termed NF-IL4 or IL-4 NAF (46, 47). We demonstrate that mutation of any of the sequence elements that bind these factors, within the context of the germline ε promoter, abolishes or reduces induction by IL-4. A fragment containing binding sites for C/EBP and NF-IL4 is sufficient to transfer IL-4 inducibility to a minimal c-fos promoter, but a fragment containing only the C/EBP consensus element or only the NF-IL4 element is not sufficient. The binding sites for these three factors and their positions relative to each other are conserved within the human germline ε promoter. Fig. 6 A shows an alignment of the C/EBP and NF-IL4 consensus elements in the mouse and human germline ε promoters. Furthermore, substitutions in the C/EBP consensus element of the human germline ε promoter eliminate IL-4 responsiveness (14). Effects of mutations in the NF-IL4 or NF-κB consensus elements of the human promoter have not been tested.

Consensus binding sites for the IL-4-inducible complex NF-IL4/IL-4NAF have been identified in various IL-4-inducible promoters (46, 47). Although Kohler and Rieber (46) demonstrated that mutation of the binding site reduced the IL-4 inducibility of a CD23 promoter transfected into a human B cell line, no evidence has been presented to indicate that the binding site for this factor by itself can transfer IL-4 responsiveness to another promoter. Interestingly, binding sites for C/EBP and NF-IL4 are also found in the promoter for mouse germline γ1 RNA at about the same distance from the first RNA initiation site (-122/-108) as in the ε promoter. The segment containing these binding sites is necessary for induction of the promoter-luciferase reporter plasmids by phorbol ester and IL-4 (16). Furthermore, both C/EBPβ and C/EBPγ have been shown to bind the C/EBP site in the germline γ1 promoter (17). Unlike the ε promoter, the germline γ1 promoter is not induced by IL-4 alone, although IL-4 synergizes with phorbol ester to activate this promoter. Fig. 6 B shows that in the γ1 promoter the C/EBP and NF-IL4 elements overlap and have a different position relative to each other than in the ε promoter. Switching to IgG4 in humans is inducible by IL-4, but the germline γ4 promoter and transcription start site have not been defined. These same two consensus elements are present within an evolutionarily conserved region 5' to the Sy4 tandem repeats, which has been postulated to encode the Igγ4 exon and its promoter (41) (Fig. 6 B). Thus, it appears possible that the proteins that bind the C/EBP and NF-IL4 sites interact and function together to effect IL-4 inducibility in all four of these promoters. Although we have been unable to detect a complex of these factors in the EMSAs with oligo A, results suggestive of such an interaction are shown in Fig. 5. In Fig. 5 B it appears that the mt 20 A oligo binds Ig/EBP-1 less well than the wild-type A oligo, although the nucleotides mutated in this oligonucleotide do
not overlap the C/EBP consensus site. This suggests that Ig/EBP may also bind the NF-IL4 site. This is consistent with the finding in Fig. 5A that oligo E, which contains only the C/EBP site, does not compete with the putative C/EBP complex formed with oligo A.

An additional C/EBP consensus element is present at the start site of transcription in both the mouse e and γ1 germline promoters (17) (see Fig. 6C). Lundgren et al. (17) have shown that C/EBP proteins bind at this second C/EBP site in the γ1 promoter. The second C/EBP site in the e promoter is located where an IL-4-inducible complex, termed STF-IL4, has been shown to bind by footprinting and by EMSA competition analyses (13, 50). Unlike the C/EBP sites located upstream in each of these promoters, no obvious consensus binding sites for NF-IL4, as previously defined (46, 47), are nearby. This is consistent with our finding that linker-scanning mutations in this region do not reduce IL-4 inducibility, although they do reduce or abolish basal activity in I.29μ or M12.4.1 cells, respectively.

C/EBPβ/NF-IL6 has been demonstrated to interact (via its leucine zipper) with RelA (NF-κB/p65) and to synergistically activate transcription when binding the IL-8 promoter. By contrast, NF-κB/p50, when bound to the same κB site, had little or no ability to synergize with C/EBPβ (51, 52).

We have been unable to detect binding of p65 to the κB site in the e promoter, although binding of p50 is readily detected. An attractive hypothesis is that induction of NF-IL-4 by IL-4 aids the interaction between C/EBPβ and p50, thus creating an effective transcriptional activator involving C/EBPβ, NF-κB/p50, and NF-IL4. Our data suggest that p50 may interact with a C/EBP protein and/or with NF-IL4, since we have shown that mutation of the κB/p50 site reduces IL-4 responsiveness by threefold, although fragment B, which contains the κB site but not the C/EBP or NF-IL4 sites, is not sufficient to transfer IL-4 inducibility to a minimal c-fos promoter.

In conclusion, we have defined and characterized an IL-4RR in the promoter for germline e RNA which is necessary for induction of the promoter by IL-4 when assayed in a luciferase reporter plasmid. This IL-4RR is sufficient to transfer IL-4 inducibility to another promoter as long as binding sites for NF-IL4 and C/EBP transcription factors are both intact. Binding sites for these same two transcription factors are also closely spaced in promoters of other germline transcripts inducible by IL-4. Future studies will be directed towards determining if these factors form a complex that effects IL-4 induction.

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In addition, the protein IL-4 Stat, the cDNA for which has recently been cloned, appears to correspond to the complex we refer to as NF-IL4 in our manuscript. The reference is Hou, J., U. Schindler, W. J. Henzel, T. C. Ho, M. Brasseur, and S. L. McKnight. 1994. An interleukin-4-induced transcription factor: IL-4 Stat. Science (Wash. DC). 265:1701-1706.

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