

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- κ B/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_H genes. Interleukin 4 (IL-4) induces transcription of the germline $C\epsilon$ 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 $C\epsilon$ genes is required for class switching. In this paper we define DNA elements required for induction of transcription of the germline $C\epsilon$ 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- κ B/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-fos promoter.

Antibody class switching is effected by a DNA recombination event that results in the substitution of one Ig heavy chain constant region (C_H) gene for another, thus changing the C_H region and thereby the effector function of the antibody, but keeping the antigen binding specificity unchanged. The specificity of class switching is regulated by cytokines that direct switching by inducing or repressing transcription from unrearranged or germline C_H genes before switch recombination to the same C_H gene (1-7). For example, a large body of data indicate that IL-4 induces transcription of the germline $C\epsilon$ gene in B cells activated by polyclonal activators, e.g., LPS, EBV infection or anti-CD40 antibody, and that subsequently cells in this population will undergo switch recombination to the $C\epsilon$ gene, thereby allowing expression of IgE (7-11). Furthermore, it has recently been shown that if a DNA segment which contains the promoter and first exon (I, or germline exon) of the germline transcript is deleted, switching does not occur on that chromosome (5, 6, 12). Although the sequences necessary and sufficient for switching have not been defined, these gene knockout experiments indicate that transcription of germline transcripts is probably necessary for switching. Therefore, it is important to understand how transcription of unrearranged C_H genes is regulated.

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)¹ 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 γ 1 transcripts (16, 17).

Materials and Methods

Cell Lines. Two mouse B lymphoma cell lines were used in this study: 22A10, a clone of the $sIgM^+$ I.29 μ B cell lymphoma line (18, 19) and M12.4.1, an Ig-negative, class II⁺, HGPRT-

¹ Abbreviations used in this paper: CAT, chloramphenicol acetyl transferase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; IL-4RR, IL-4 response region; mt, mutant.

deficient variant of M12.4 (20), received from Dr. Paul Rothman (Columbia University, New York).

Cell Culture. 22A10 cells were cultured as described for I.29μ 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 mg/ml streptomycin, and 0.1 mg/ml kanamycin sulfate. Cells were treated with murine rIL-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-CsCl method (23) and RNA blots were prepared and hybridized as described (21). Quantitation of hybridization was performed by densitometry on a Betascope 603 blot analyzer (Betagen, Waltham, MA). The Cε probe was a 2.1-kb BamHI/HindIII genomic DNA fragment (24). Hybridization to a GAPDH probe (25) or densitometry of 18S rRNA in photos of ethidium bromide-stained agarose gels was used to normalize the difference in loading.

RNase Protection Assay. To determine the initiation site of germline ε 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 ε 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 Iε exon (28, 29) and cloned in Bluescript KS⁻. The plasmid was linearized with HincII and transcribed in vitro with T7 polymerase. 100 μg of total cell RNA was incubated with 250,000 cpm of the labeled RNA probe in a 30-μl volume containing 80% formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl, and 1 mM EDTA at 80°C for 10 min, and then hybridized overnight at the indicated temperatures. Unhybridized RNA was digested at room temperature for 90 min in 10 mM Tris, pH 7.4, 5 mM EDTA, pH 7, 300 mM NaCl, 2 μg/ml RNase T1 (Sigma Chemical Co.), and 40 μg/ml RNase A (Sigma Chemical Co.). The hybridized RNA was recovered and electrophoresed on an 8-M urea sequencing gel alongside a DNA sequencing ladder.

Sequences of Oligonucleotides. Lowercase letters are nucleotides added to create restriction enzyme sites and are not in the ε promoter sequence. ε promoter oligonucleotides are: A-1 (32 mer): 5' gatcTGCCTTAGTCAACTTCCCAAGAACAGA 3'; A-2 (32 mer): 5' gatcTCTGTTCTTGGGAAGTTGACTAAGGCAG 3'; Amt13-1 (31 mer): 5' gatcTGCCTTCGAGGTTCCCAAGAACAGA 3'; Amt13-2 (31 mer): 5' gatcTCTGTTCTTGGGAACCTTCGAGGGCAG 3'; Amt20-1 (32 mer): 5' gatcTGCCTTAGTCAACTTCCCTTCGAGGA 3'; Amt20-2 (32 mer): 5' gatcTCTCGAGGGGAAGTTGACTAAGGCAG 3'; B-1 (19 mer): 5' gatcCAAGGAACTTCCAA 3'; B-2 (19 mer): 5' gatcTTGGAAGTTCCTTG 3'; Bmt27-1 (19 mer): 5' gatcCAAGGGCCTTCGAGGA 3'; Bmt27-2 (19 mer): 5' gatcTCTCGAGGCCCTTG 3'; C-1 (16 mer): 5' gatcCCCAAGAACAGA 3'; C-2 (16 mer): 5' gatcTCTGTCTTGGG 3'; D-1 (19 mer): 5' gatcTCCCAAGAACAGA 3'; D-2 (19 mer): 5' gatcTCTGTTCTTGGGAAG 3'; E-1 (18 mer):

5' gatcCCTTAGTCAACTTCA 3'; E-2 (18 mer): 5' gatcTGAAGTTGACTAAG 3'; 5'ε (-788/-733) (28 mer): 5'ttagatctGACCTGCAGCTGAGACAGAC 3'; and 3'ε (+33/+53) (28 mer): 5'ttagatctTGTGCAGGCTCCCAGGCGTT 3'. Non ε promoter oligonucleotides are: NFκB1 (27 mer): 5' CAACGGCAGGGGAATCCCCTCTCCTT 3'; NKκB2 (27 mer): 5' AAGGAGAGGGGAATTCCCCTGCCGTTG 3'; Luc (+79/+96) primer (18 mer): 5'GGCCCGGCGCCATCTCTAT 3' (30); Fos (-25/-8) primer (18 mer): 5'GGCGCTCAGCTGGCGCC 3' (31); and pXP2 polylinker primer (17 mer): 5' AGATCCAAGCTTGTCGA 3'. Oligonucleotides were obtained from the University of Massachusetts Medical School nucleic acid facility, Operon Technologies Inc. (Alameda, CA), or DNA International (Woodlands, TX).

ε Promoter-Luciferase Plasmids. To create the 5' deletion constructs of the germline ε promoter, a series of fragments containing various lengths of the promoter segment were ligated into the plasmid pXP2, which has a polylinker 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 ε DNA, using the primers, 3'ε (+33/+53 relative to the first start site) and 5'ε (-788/-773). Nucleotide +53 is within the Iε 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 SmaI and BglII. BstXI digestion followed by Bal31 digestion created additional smaller promoters that were cloned into pXP2 digested with SmaI 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 polylinker 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 Bal31 to generate a nested set of 5' and 3' deletion mutants, bounded by XhoI 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 polylinker)/XhoI fragment from a 3' deletion clone into a 5' deletion plasmid digested with BamHI and XhoI resulted in the regeneration of an approximately full-length promoter (in most cases) containing an 8-bp XhoI 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 ng/μl BSA, and 1 mM 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 ε 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 orientation. Insert-containing clones were sequenced using the Fos primer to confirm the number of multimers, their orientations, and the fidelity of the BamHI and BglII restriction sites.

DNA Sequencing. Nucleotide sequences of CsCl-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. 50×10^6 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. CsCl-purified plasmid DNAs were added in a volume of 100 μ l to the cuvettes. The cells were electroporated at 1250 μ F/300 V, rested at room temperature for 10 min, and pipetted into complete medium at $\sim 0.25 \times 10^6$ cells/ml. After addition of inducers, cells were incubated for 8–18 h and then assayed for luciferase or chloramphenicol 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 μ l Triton X-100 lysis buffer (1% Triton X-100, 25 mM glycylglycine, 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 μ l cell lysate, 350 μ l luciferase assay buffer (25 mM glycylglycine, pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 15 mM KH₂PO₄, 2 mM ATP, and 1.27 mM DTT), and 100 μ l of 1 mM luciferin (Analytical Luminescence 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 assayed to control for transfection efficiency by the diffusion-based assay (37). 50 μ l of cell lysates was heated at 70°C for 10 min before mixing with 200 μ l of 120 mM Tris, pH 7.8, 1 mM chloramphenicol (Sigma Chemical Co.), and 0.1 μ Ci [³H]-acetyl 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 contained 100 ng/ μ l of each oligonucleotide in 100 mM NaCl, 10 mM Tris-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 3' endlabeled with Klenow enzyme and α [³²P]dCTP (3,000 Ci/mmol) to 5×10^8 cpm/ μ g, and 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 of Dignam et al. (39), as modified by Boothby et al. (40) were used. Several protease inhibitors were added to all solutions just

before use: 0.5 mM DTT, 0.5 mM PMSF, aprotinin (2 μ g/ml), chymostatin (25 μ g/ml), and leupeptin (10 μ g/ml) (Boehringer Mannheim). Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Richmond, CA).

Electrophoretic Mobility Shift Assay. DNA binding reactions were performed in 20- μ l reaction volumes containing 0.1 ng (30,000 cpm) labeled DNA probe, 2–5 μ g nuclear extracts, 2–4 μ g poly (dI-dC) (Pharmacia, Piscataway, NJ), 10% vol/vol glycerol, 50 mM KCl, 0.1 mM EDTA, 0.005% NP 40, 1 mM DTT, and 12.5 mM Hepes, pH 7.5. The amount of probe, poly (dI-dC), nuclear extract, and KCl concentration used was optimized for each fragment. The reaction mixture was incubated at room temperature for 30 min and then loaded onto a 4–6% native polyacrylamide gel. All gels were electrophoresed in recirculating 0.5 \times TBE buffer at 100 V for 2–3 h. Supershift experiments were performed by adding 1 μ l of antiserum in a 20- μ l binding reaction volume.

Results

Induction of Germline ϵ Transcripts in Two Mouse B Cell Lines. For these studies, we used two B lymphoma cell lines that inducibly express germline ϵ RNA. The I.29 μ mouse B lymphoma expresses surface IgM and can be induced to undergo class switching to IgA by treatment with LPS. Switching can be increased by treatment with TGF- β , which increases transcription from the unrearranged C α genes. I.29 μ cells will switch to IgE expression if treated with IL-4 plus LPS, although switching to IgE is about 100 times less frequent than optimal switching to IgA. The maximal frequency of cells expressing IgE (both cytoplasmic and surface expression) at day 5 after induction is $\sim 0.1\%$, but this level can increase by day 7 to $\sim 0.5\%$ (18, and data not shown). I.29 μ cells synthesize germline ϵ transcripts constitutively, and this can be further increased 5–20-fold by treatment with IL-4 (Fig. 1 B, left) (2). 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⁺, sIg-negative variant that was derived from the M12 B lymphoma (20). M12.4.1 cells do not express germline ϵ transcripts constitutively, but germline ϵ transcripts can be induced by treatment with IL-4 plus LPS (Fig. 1 B, right) (13). Unlike, I.29 μ , IL-4 alone does not induce detectible germline ϵ transcripts in M12.4.1 cells.

To begin to characterize DNA sequences that regulate transcription of germline ϵ RNA, we verified by a RNase protection assay the initiation sites of transcription of germline ϵ RNA that were previously determined in 18-81A20 cells by Rothman et al. (28). RNA from IL-4-treated I.29 μ cells was hybridized with a radioactive RNA probe transcribed from a 512-bp HincII/PstI ϵ 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). Assuming that the I ϵ splice donor defined previously by analysis of cloned germline ϵ cDNA from 18-81A20 cells is also used in I.29 μ 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'

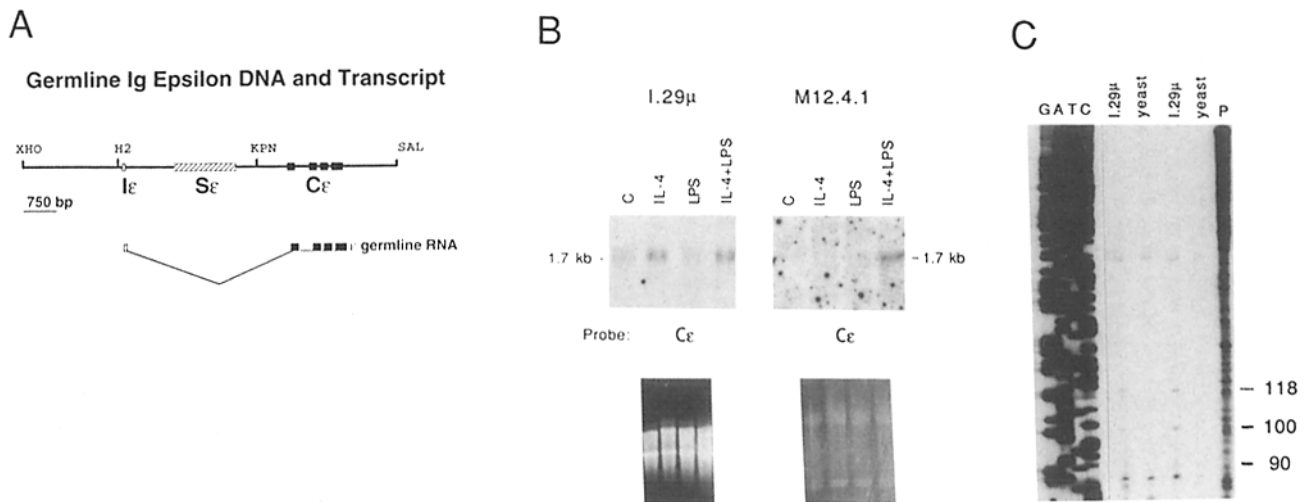


Figure 1. Structure of the mouse Ig heavy chain germline ϵ gene and ϵ transcript. (A) Restriction map of unrearranged $C\epsilon$ gene and germline ϵ transcript with location of exons and switch region ($S\epsilon$) marked. (B) Blots containing total cell RNA from I.29 μ (left) and M12.4.1 (right) cells that had been cultured for 2 d, as indicated, with medium alone, IL-4 (2,000 U/ml), LPS (25 μ g/ml), or with both IL-4 and LPS, hybridized with $C\epsilon$ probe. (C) Products of an RNase protection experiment using a RNA probe transcribed from the 512-bp HincII/PstI genomic DNA fragment encompassing the $I\epsilon$, hybridized with total cell RNA from I.29 μ cells induced with X63-IL-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- $I\epsilon$ -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 μ cells by primer extension experiments (53).

site identified in 18-81A20. The start sites are indicated by arrows on the sequence of the promoter in Fig. 2 A. The nucleotide positions referred to in this manuscript are all given relative to the first RNA initiation site in I.29 μ cells.

Transient Transfection of Luciferase Reporter Plasmids Containing Various Lengths of 5' Flanking Region from the Germline ϵ Segment. To identify DNA segments necessary for regulation of transcription of germline ϵ RNA, a series of luciferase reporter plasmids containing various lengths of the 5' flank of the $I\epsilon$ exon were prepared, varying from having 1.8 kb to only 15 bp of 5' flanking sequences. The ϵ promoter segment present in the plasmid named -162Luc is shown in Fig. 2 A. The plasmids were transiently transfected into I.29 μ or M12.4.1 cells. Optimum luciferase activity was obtained when cells were harvested 12 h after transfection. An internal control CAT plasmid driven by a SV40 promoter/enhancer (pSV2CAT) was used to correct for transfection efficiency when comparing the basal activity of different plasmids. Maximal basal expression is not affected much by deletion of the 5' flanking segment to -127 relative to the RNA initiation sites, but further deletion reduces basal expression in both I.29 μ and M12.4.1 cell lines (Fig. 3, A and B; compare constructs -162LUC, -127LUC, -115LUC; and data not shown).

To test whether expression of these promoter-luciferase plasmids could be induced by IL-4, cells were divided after transfection into two more aliquots and treated with recombinant mouse IL-4 for various times. Optimal responses were obtained with a dose of 240 ng/ml, which is equivalent to 2,500 U IL-4/ml, and we routinely used 1,000 U/ml. The IL-4 inducibility of plasmids with the longest 5' flanks, having

1.8 and 1.2 kb of ϵ promoter segment, is two to four times less than plasmids having from 640 to 162 bp of 5' flank, which are approximately equivalently inducible by IL-4: eightfold in I.29 μ and threefold in M12.4.1 cells. Deletion past -162 reduces inducibility of the luciferase plasmids in both cell lines, and expression of a construct with 115 nucleotides of the ϵ promoter segment cannot be induced by IL-4 (Fig. 3, C and D; and data not shown). Therefore, the DNA segment residing between -162 and the start site of transcription has one or more DNA elements required for IL-4 inducibility of the ϵ promoter luciferase plasmids.

Effects of a Series of Linker-scanning Mutations on Expression of the Germline ϵ Promoter. Linker-scanning mutations were introduced into the -162/+53 promoter segment contained within the -162Luc reporter plasmid in order to identify DNA sequences required for IL-4 inducibility and for basal activity of the germline ϵ promoter. The nucleotides affected by the mutations, either because of substitution or deletion, are shown in Fig. 2 B, and their positions are indicated to the left of each sequence.

Plasmids bearing the linker-scanning mutations were transiently transfected into I.29 μ and M12.4.1 cell in order to test for their ability to drive luciferase expression. Each construct was tested at least three times in both cell lines. Fig. 3 A shows that most of the linker-scanning mutations reduce basal level expression of the promoter relative to that obtained with the wild-type -162Luc plasmid when transfected into I.29 μ cells, although for most mutations, the effect is not dramatic. The effects of the mutations differ in M12.4.1 cells, in that nearly all mutations located 3' to -87 abolish basal expression although mutations located 5' to -92 do

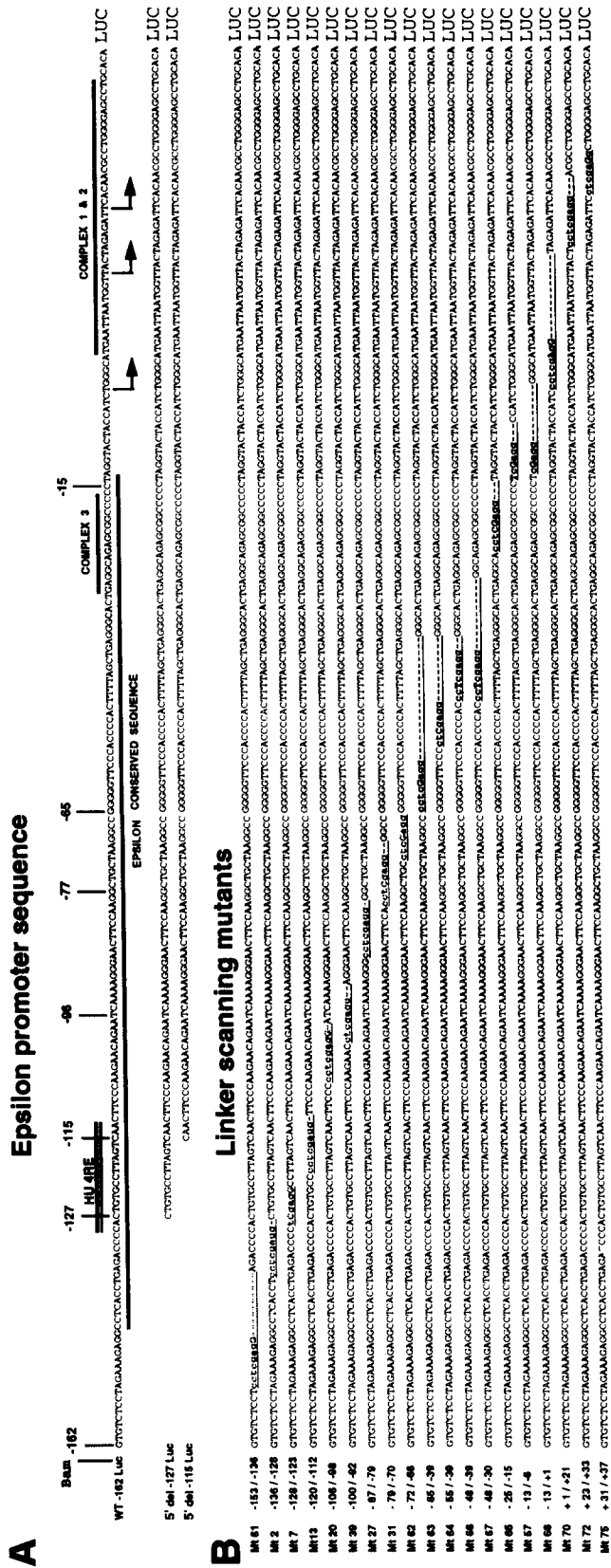


Figure 2. (A) Sequence of 5' flank of germline ϵ RNA and the first 53 nucleotides of Ie exon present in the ϵ promoter -162Luc plasmid. This plasmid contains all three transcription start sites detected in I.29 μ

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 ϵ 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 ϵ promoter sequence (14, 41).

Localization of an IL-4RR with Linker-scanning Mutations. The sequences in the germline ϵ 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.29 μ 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- κ B (48). This third mutation also abolishes constitutive expression. The fourth linker-scanning mutation mt 63 (-65/-38) reduces IL-4 inducibility in I.29 μ 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 ϵ promoter at -42/-15 and is required for IL-4 plus LPS inducibility of a CAT reporter gene driven by the mouse germline ϵ 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.29 μ 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 Ie exon does not have a translation initiation ATG codon. Regions indicated above the sequence are: (Hu 4RE) region necessary for induction of the human germline ϵ 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 ϵ promoter-CAT constructs after LPS plus IL-4 induction (13, 15). (EPILON 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/DBJ under accession number U17387.

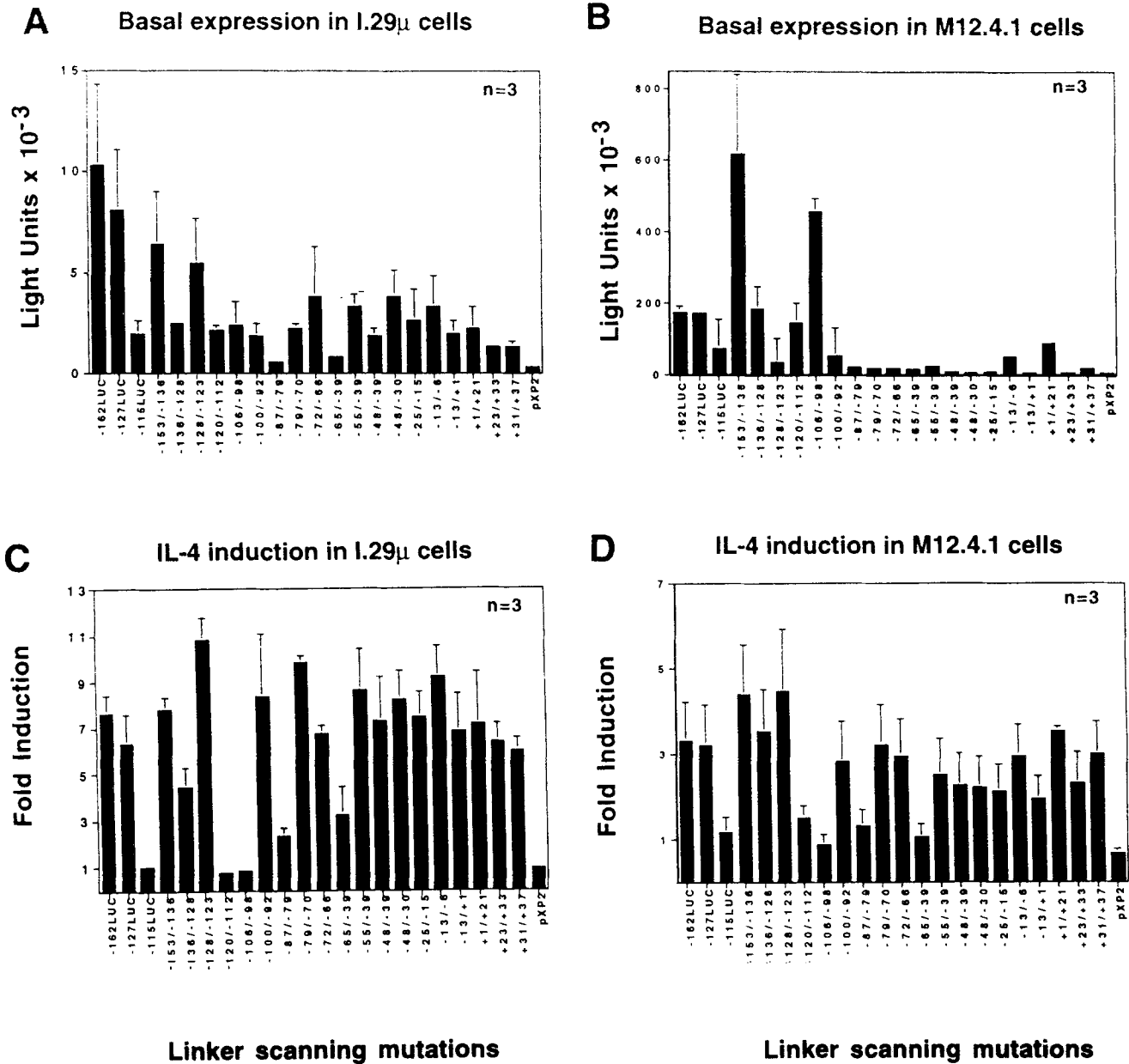


Figure 3. Results of transient transfection experiments of germline ϵ promoter-luciferase reporter plasmids having 5' deletions: -162Luc, -127Luc, and -115Luc, or -162Luc with the indicated linker scanning mutations. (A) Basal expression in I.29 μ . Luciferase activity is reported in light units after 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 I.29 μ . 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 ϵ 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 I.29 μ 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-

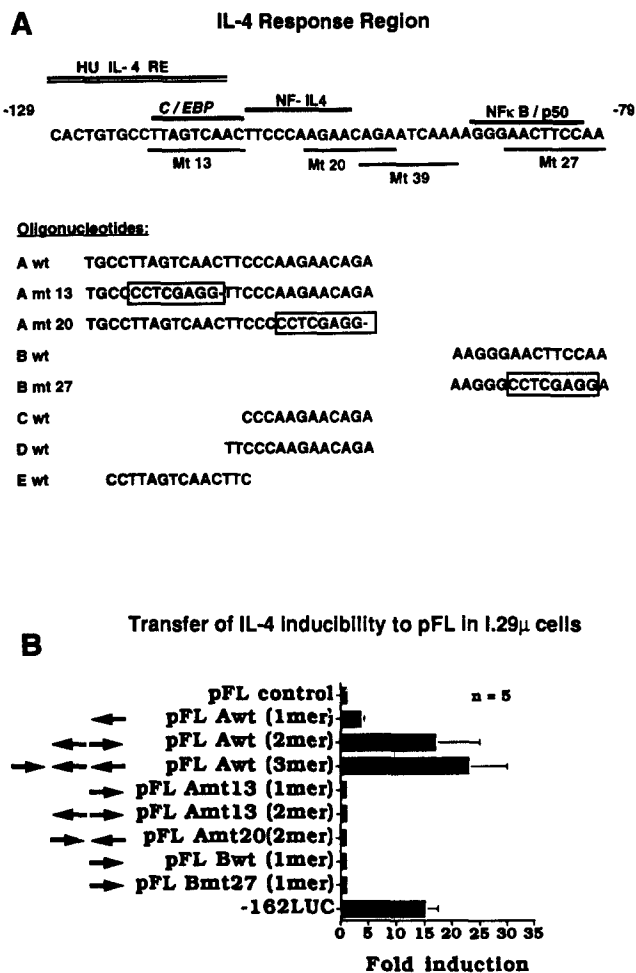


Figure 4. (A) Nucleotide sequence of the IL-4RR in the mouse germline ϵ promoter. Three transcription factor binding sites are detected: a C/EBP consensus element (-120/-112), the sequence defined as an NF-IL-4 site (-111/-102), and a NF- κ B/p50 site (-90/-81). Indicated below the DNA sequence are the positions of the linker scanning mutations tested. (Hu IL4 RE) The segment which when mutated disrupts IL-4 responsiveness of human germline ϵ promoter-CAT constructs (14). Beneath the sequence are the wild-type and mutated double stranded oligonucleotides that were tested for the ability to transfer IL-4 inducibility to a minimal c-fos promoter and/or to bind to nuclear proteins in an EMSA. The mutations of the oligos are the same as those present in the linker scanning mutations. (B) Results of transient transfection experiments in I.29 μ cells, testing the IL-4 inducibility of plasmids in which the indicated oligonucleotides were inserted upstream of the minimal 71-bp c-fos promoter driving a luciferase gene. Transfection and induction conditions were as described above. (pFL control) The vector control, which is not inducible by IL-4. The average and standard deviations of the means of five experiments are plotted. The plasmids tested are indicated by, for example, pFL Awt (1mer) which contains one copy of the wild-type A oligonucleotide. (Arrows, left) The orientations of the oligonucleotide(s) in each plasmid. (-162LUC) The wild-type germline ϵ promoter luciferase reporter plasmid.

ysis, in which it was found that mutation of either of these sequence elements eliminates inducibility. The wild-type B oligo, which has the consensus κ B site, does not transfer IL-4 inducibility to the c-fos promoter. Results are similar when the plasmids are transfected into M12.4.1 cells, although the induction by IL-4 is not as great as in I.29 μ cells (data not shown).

Electrophoretic Mobility Shift Assays Demonstrate that the IL-4RR Does Indeed Contain Binding Sites for NF-IL4, Ig/EBP-1, and NF- κ B/p50. To determine if these consensus binding sites bind the predicted proteins, a series of electrophoretic mobility shift assays (EMSA) 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.29 μ 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 ϵ 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 ϵ 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/EBP γ (originally called Ig/EBP-1), which is most abundant in pre-B cells, and C/EBP β (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.29 μ , 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 κ B site binds a member of the NF- κ B/rel family of transcription factors by performing EMSAs using oligonucleotide B as a probe with nuclear extracts from untreated

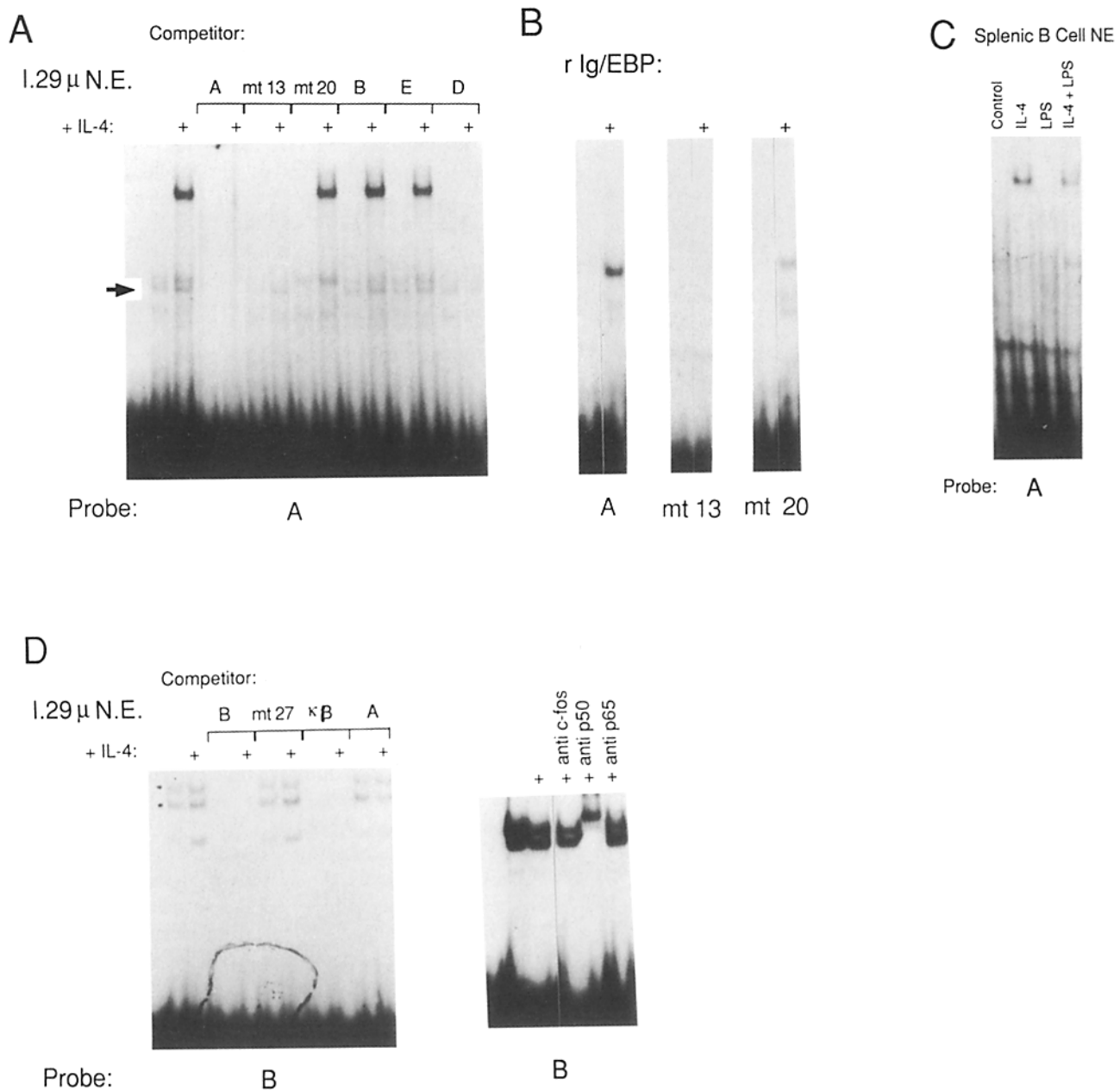


Figure 5. EMSAs of the IL-4RR within the germline ϵ promoter. Left-most lane in each panel or each set of lanes contains probe alone. (A) EMSA of oligonucleotide A (sequence given in Fig. 4 A) incubated with 5 μ g nuclear extracts from I.29 μ cells untreated or induced for 12 h with IL-4 (+). Competitor 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-1(C/EBP γ) (+) with the wild-type A oligonucleotide, with mt 13 or with mt 20. (C) Oligonucleotide A incubated with nuclear extracts (2 μ g) from splenic B cells (Control) or splenic B cells treated with IL-4, LPS, or IL-4 plus LPS for 12 h. (D) EMSAs with oligonucleotide B demonstrate that NF- κ B/p50 binds to the wild-type B oligonucleotide. (Left) Nuclear extracts from untreated or IL-4-treated I.29 μ cells (+) were incubated without or with the indicated oligonucleotides as competitors. κ B is a 27-bp oligonucleotide containing the κ enhancer κ B site. (Right) Supershift experiment using three antisera. Antiserum to NF- κ B/p50 causes a supershift, but antisera for c-Fos and NF- κ B/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 I.29 μ cells, competing with a DNA fragment containing the NF- κ B 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 κ B site. All three complexes formed with oligo B can be competed with the wild-type B oligo or with a

κ B 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 κ B site contributes to the IL-4 response, since the linker-scanning

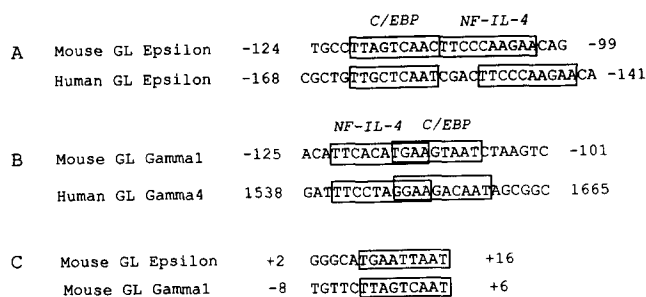


Figure 6. Comparison of DNA sequences 5' to the I exons of IL4 responsive mouse and human germline C_H genes. (A) Alignment of the mouse and human germline ϵ promoters to show homology between the C/EBP and NF-IL4 sites. (B) Alignment of NF-IL4 and C/EBP sites in the mouse germline $\gamma 1$ promoter with sequences upstream of the human $\gamma 4$ switch region. The C/EBP site in the $\gamma 1$ promoter has been demonstrated to bind C/EBP γ and C/EBP β in nuclear extracts (17). The positions of the nucleotides from the $\gamma 4$ 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 ϵ and $\gamma 1$ RNAs. The $\gamma 1$ sequence has been shown to bind C/EBP γ in nuclear extracts (17).

mutation at this site reduces the response to IL-4 by three-fold in both I.29 μ and M12.4.1 cells. Different members of the NF- κ B/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- κ B antisera for their effect in EMSAs using oligo B as the probe. An antiserum to the p50 homodimer supershifts 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- κ B/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 I.29 μ 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- κ B, 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- κ B 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 $\gamma 1$ 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/EBP β and C/EBP γ have been shown to bind the C/EBP site in the germline $\gamma 1$ promoter (17). Unlike the ϵ promoter, the germline $\gamma 1$ 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 $\gamma 1$ 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 $\gamma 4$ promoter and transcription start site have not been defined. These same two consensus elements are present within an evolutionarily conserved region 5' to the S $\gamma 4$ tandem repeats, which has been postulated to encode the I $\gamma 4$ 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. 5 A 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 ϵ and $\gamma 1$ germline promoters (17) (see Fig. 6 C). Lundgren et al. (17) have shown that C/EBP proteins bind at this second C/EBP site in the $\gamma 1$ promoter. The second C/EBP site in the ϵ 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.29 μ or M12.4.1 cells, respectively.

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

We have been unable to detect binding of p65 to the κ B site in the ϵ 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/EBP β and p50, thus creating an effective transcriptional activator involving C/EBP β , NF- κ B/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 κ B/p50 site reduces IL-4 responsiveness by threefold, although fragment B, which contains the κ B 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 ϵ 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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Note added in proof: Since the submission of our manuscript an additional report on the IL-4 response region of the human germline ϵ promoter which is mostly consistent with our results has been published: Albrecht, B., S. Peiritsch, and M. Woisetschlager. 1994. A bifunctional control element in the human IgE germline promoter involved in repression and IL-4 activation. *Int. Immunol.* 6:1143-1151.

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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