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# Functional Analysis of -351 Interleukin-9 Promoter Polymorphism Reveals an Activator Controlled by NF-κB

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## Abstract

Genetic studies have shown linkages for asthma to the chromosomal region 5q31-q33 in humans that includes the IL-9 gene. An A-to-G base substitution has been identified at bp -351 in the IL-9 promoter. The role of this polymorphism in IL-9 promoter function was assessed utilizing CD4+ T cells purified from individuals with one or two of the G alleles in comparison to those homozygous for the wild type A. The presence of an A at -351 (A allele) increased mitogen-stimulated IL-9 transcription 2-fold in comparison to subjects with one or 2 G alleles at this position. Binding of nuclear extract proteins from IL-9-producing human cell lines to DNA sequences including this base exchange demonstrated specific binding of the transcription factor NF-κB. Binding of NF-κB to the IL-9 promoter was confirmed *in vivo* using the chromatin immunoprecipitation assay. Recombinant NF-κB bound to a promoter fragment with the A allele with 5 fold higher affinity than it did to a promoter with the G allele. Individuals carrying the A allele of the IL-9 promoter display increased synthesis of IL-9, which may result in strong Th2 immune responses and a modulation of their susceptibility to infectious, neoplastic, parasitic, or atopic disease.

### Keywords

Interleukin-9; Cytokine; Polymorphism; Functional Genomics; Transcription

## Introduction

Interleukin (IL)-9 is a cytokine that displays pleiotropic effects in immunoregulation and inflammation on a variety of cell types 1. This cytokine was originally described as a mast cell growth factor due to its ability to stimulate IL-6 production and induce survival of mast cells 2 3. Further work demonstrated that IL-9 contributes to mast cell-mediated allergic responses by stimulating production of mast cell proteases and the high affinity IgE receptor α chain 4. Th2-type lymphocytes are the major source of human IL-9 5. A role for IL-9 in the late phase allergic response has been suggested from studies that show IL-9 induces



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influx and maturation of eosinophils in synergy with IL-5 and increases cell surface expression of the IL-5 receptor α chain leading to differentiation and increased survival of the eosinophil 6. These observations are supported by the finding that IL-9 levels and eosinophil numbers were increased in bronchoalveolar lavage fluid of patients with atopic asthma following segmental allergen challenge 7. On airway epithelial cells, IL-9 stimulates production of chemokines such as CCL11 (eotaxin-1) and CCL2 (MCP-1), proteases and mucin which can lead to an increased inflammatory cell infiltrate 8 9. A role for IL-9 in tumorigenesis has been suggested as cell lines derived from patients with Hodgkin's and anaplastic large cell lymphoma's overexpress IL-9 10.

Several genetic studies have linked a major gene for asthma to the chromosomal region 5q31-q33 in humans 11. Within this region is a gene cluster for the cytokines GM-CSF, IL-4, IL-5, IL-13 and IL-9. IL-9 has been considered a strong candidate gene for asthma due to linkage disequilibrium observed between serum total IgE levels and a marker within this gene 12. In support, a genetically determined reduction in IL-9 production in mice leads to bronchial hyporesponsiveness 13. Genetic variations in the nucleotide sequences of promoters can contribute to the altered expression of genes in complex inherited diseases 14 15 16. Previously, we described an A-to-G exchange in the human IL-9 promoter located 351 base pairs upstream from the transcription start site (Figure 1) 17.

The linkage studies can not distinguish direct influences of a polymorphism on gene function from differences mediated by other polymorphisms that may be in linkage disequilibrium. To better understand mechanisms that modulate IL-9 expression, we performed studies to determine the functional significance of the -351 nucleotide exchange in the IL-9 promoter and the basis of its linkage with allergic diseases. We demonstrate that the A-to-G nucleotide exchange results in decreased mitogen-stimulated promoter activity. Specific binding of the transcription factor NF-κB was demonstrated to a region spanning the polymorphism. The region including the NF-κB site functions as an activator, and the A-to-G base exchange decreases the activation mediated by NF-κB. This study demonstrates a functional role for the -351 base exchange in the IL-9 promoter, which has implications for a variety of immunological diseases.

## Results

### Differential production of IL-9 from T cells isolated from individuals with the A or G alleles

T cells are a major source of IL-9, so it was important to determine if the IL-9 polymorphism would alter the levels of IL-9 produced in response to different stimuli. CD4+ T cells were purified from the blood of individuals homozygous for the A allele or, due to the low frequency of the allele in the population (17% in Denver population 17 and 18% in Virginia population (data not shown)), from individuals who carried at least one G allele. Our initial studies examined IL-9 mRNA production from these two groups of subjects (n=3 in each group). Spontaneous IL-9 expression was low in CD4+ T cells from both groups in the absence of stimulation with no differences between the groups (data not shown). Stimulation of the cells with PMA led to a marked increase in IL-9 expression with statistically higher levels being found in cells from individuals homozygous for the A allele (Figure 2) ($p<0.05$). PHA also increased IL-9 expression, but in contrast to PMA stimulation

there was no difference in IL-9 expression between the two genotypes (Figure 2). The combination of the two compounds gave the highest levels of IL-9 stimulation and similar to PMA alone, cells from individuals homozygous for the A allele had higher levels of IL-9 expression than those with the G allele, however this did not reach statistical significance (Figure 2). We extended these studies to look at IL-9 protein production in CD4+ T cells from individuals homozygous for the A allele in comparison to those with 1 or 2 of the G alleles. As shown in Table 1, unstimulated levels of IL-9 were similar between the two groups of individuals (35.0±5.6 pg/ml A/A vs 37.1±7.1 pg/ml G/A or G/G). When the cells were stimulated with both PMA and PHA a trend was observed for individuals homozygous for the A allele to produce higher levels of IL-9 as compared to those with at least one G allele (87.2±27.8 pg/ml A/A vs 58.7±13.1 pg/ml G/A or G/G).

### NF-κB binds to a sequence encompassing the polymorphic residue

Effects of the base exchange in the IL-9 promoter on factor binding were investigated by EMSA. The -351 base substitution is situated within a sequence that has similarity to a NF-κB site (Figure 1). We hypothesized that complexes detected in our EMSA could include NF-κB. To address this hypothesis, EMSAs were conducted using oligonucleotide probes containing the putative NF-κB binding site with either the A or G alleles. Since differences in IL-9 promoter activity were noted in cells stimulated with PMA, EMSAs were conducted with nuclear extracts made from PMA-stimulated Jurkat T cells. Similar results were observed with nuclear extracts derived from unstimulated cells, but binding was substantially less (data not shown). As demonstrated in Figure 3, each probe produced similar patterns of shifted complexes, but binding to the AT probe was always more efficient relative to the GC probe. All of the shifted complexes were specifically competed by excess unlabeled probe DNA (data not shown). Efficient competition was also detected using excess unlabeled NF-κB consensus oligonucleotide, but not by an oligonucleotide with a mutated NF-κB consensus sequences. The presence of NF-κB in the slowest migrating complexes was confirmed by supershifting with both anti-NF-κBp50 and anti-NF-κBp65 antibodies (Figure 4). No supershifting was observed with an isotype matched anti-NFAT antibody. Identical results were observed when nuclear extract was used from purified CD4+ T cells that had been stimulated with PMA/PHA (Figure 5). Again, stronger binding was observed to the AT probe and this binding could be competed by a NF-κB consensus probe, but not a mutated form of the NF-κB probe. Supershifting was observed with both anti-NF-κBp50 and anti-NF-κBp65 antibodies.

### Relative binding of recombinant NF-κB p50 to the two forms of the IL-9 promoter

Specific binding of the site by NF-κB was confirmed by EMSA using the recombinant p50 subunit and labeled probes (data not shown). Using increasing concentrations of recombinant protein, we evaluated the binding affinity of NF-κBp50 to wild type (A) and polymorphic (G) forms of the promoter. Data were evaluated by phosphoimager and relative Ka's for each form of the promoter were calculated using Scatchard analysis (Figure 5). A five fold higher binding affinity of recombinant p50 to the A form (Ka $0.67\times10^{-12}$) of the IL-9 promoter was observed compared to the G form (Ka $3.39\times10^{-12}$) of the IL-9 promoter (Figure 5).

### In vivo detection of NF-κB p50 and p65 binding to the IL-9 promoter

The EMSA experiments indicated that NF-κBp50 and p65 could bind to the IL-9 promoter using *in vitro* binding assays, but these assays may not be indicative of *in vivo* binding of these proteins. To determine if the NF-κBp50 and p65 proteins bind to the IL-9 promoter within a cell, we used the ChIP assay. Jurkat T cells were initially examined since this cell type supports transcription of the IL-9 gene. As shown in Figure 6a, NF-κBp50 and p65 binding to the -351 region of the IL-9 promoter was detected in PMA stimulated Jurkat T cells. In order to confirm specificity of antibody immunoprecipitation, an anti-Stat6 antibody was used as a control. This protein does not interact with this region of the IL-9 promoter as determined by EMSA and the antibody was not able to immunoprecipitate a DNA fragment containing the -351 residue. We subsequently purified CD4+ T cells from a non-allergic individual and repeated the ChIP assay on PMA-stimulated cells. The results shown in Figure 6b confirm the results with the Jurkat T cells in that both the NF-κBp50 and p65 subunits could be detected binding to the -351 region of the IL-9 promoter.

### IL-9 -351 promoter polymorphism results in altered transcriptional activity

To determine whether the A to G polymorphism at position -351 affects IL-9 promoter function, the transcriptional activity of IL-9 promoter fragments was measured. Fragments of the IL-9 promoter were cloned into a luciferase reporter plasmid and the activity measured in the human T cell line Jurkat. In the first set of experiments, basal activity for each plasmid was determined and normalized to that of the -346 construct, which was assigned a relative transcription index of 1.0 (Figure 8A). This construct is deleted just upstream of the polymorphic residue. Addition of 23 bases with the A allele at position -351 resulted in a 10.21±4.81 fold increase in transcription ($p<0.01$). Within this segment, changing the sequence of the A to G allele resulted in a 12.38±4.47 fold increase in transcription ($p<0.01$). In the second set of experiments, transfected cells were stimulated with PMA/PHA for 6 hrs and transcriptional activity measured (Figure 8B). For these experiments, data for each plasmid were normalized to the unstimulated sample (assigned a relative transcription index of 1.0). There was no increase in promoter activity for the -346 construct when PMA/PHA was added (0.97±0.12). In contrast, addition of the putative NF-κB site resulted in a 1.78±0.22 ($p<0.02$) fold increase and a 1.85±0.20 ($p<0.01$) fold in promoter activity from the A and G containing constructs, respectively. These studies have been extended to CD4+ T cells purified from subject either homozygous for the A allele of heterozygous for the G allele. Addition of 10 nM JSH-23 (a NF-κBp65 nuclear transport inhibitor), prior to PMA stimulation, inhibited IL-9 transcription (data not shown) and protein production (Table 1) from purified CD4+ T cells.

## Discussion

Improvements in the ability to sequence genes have led to a large number of single-nucleotide polymorphisms being described. These polymorphisms have been used in association studies to identify whether or not a particular gene is linked with a disease state. Many of these studies only demonstrate a statistical association, but fail to show that the polymorphism has a functional effect. As such, linkage of these polymorphisms to disease often fails to be replicated in subsequent studies. The concept that promoter polymorphisms

may influence transcription levels by altering promoter strength, and thereby produce phenotypic differences, has been shown for numerous polymorphisms including the human IL-4 gene 18, CD14 gene 19, the CCL5 (RANTES) promoter 20, 21 and the IL-10 gene 16. Polymorphisms in the IL-10 locus are associated with allele- and tissue-specific differences in expression of these genes 22, 23.

The current studies were performed in order to examine effects of a base exchange in the IL-9 promoter on the regulation of IL-9 transcription. An influence on gene expression was confirmed with functional assays by comparing expression of IL-9 protein and mRNA from CD4+ T cells treated with various stimuli purified from individuals homozygous for the A allele or carriers of the G allele at position -351. Stimulation of CD4+ T cells with PMA led to a 2 fold increase in IL-9 protein and 1.73-fold increase in mRNA production in individuals homozygous for the A allele (Table 1 and Figure 2). Previous studies in mice have shown that strains that produce lower levels of IL-9 have lower bronchial hyperreactivity following allergen challenge in sensitized mice 13. Our results would suggest that individuals who carry the G allele, either in the homozygous or heterozygous state, would be less likely to respond to allergen provocation, thus having the G allele would be protective against allergy development.

EMSAs were used to characterize specific binding of nuclear protein to sequences encompassing the -351 base substitution. We demonstrated specific binding of the transcription factor NF-κB, specifically NF-κB composed of heterodimers containing the p50 and p65 subunits, to the IL-9 promoter to a site that included the -351 residue (Figures 3 and 4). These results were confirmed *in vivo* using a human T cell line and purified human CD4+ T cells (Figure 6a and b). A role for NF-κB in IL-9 transcription is supported by a previous study in which IL-9 transcription induced by lipopolysaccaride in mouse mast cells was prevented with an inhibitor of NF-κB 24. NF-κB is a cytoplasmic transcription factor that when activated, through multiple signal transduction pathways, is able to translocate to the nucleus and modulate gene transcription 25. NF-κB is composed of a least five family members that have the ability to form homo or heterodimers with each other. Each subunit contains a nuclear localization signal, DNA-binding domain and protein-dimerization regions 26. Examination of the EMSAs, revealed that the binding of NF-κB appeared to be greater on the AT form of the IL-9 promoter. Scatchard analysis using recombinant p50 (the DNA binding subunit of NF-κB) demonstrated 5 fold higher binding affinity of NF-κB for the AT form of the promoter as compared to the GC form (Figure 5). The magnitude of binding may be different when the p50 subunit forms heterodimers with other members of the NF-κB family. However, the increased binding of NF-κB to the AT form is consistent with the level of increased promoter activity observed following stimulation of purified T cells with PMA from individuals homozygous for the AT allele.

Transfection assays were also used to assess the influence of the A to G base exchange. Addition of the promoter region containing the base exchange and the putative NF-κB binding site resulted in increased transcription levels in the basal and PMA/PHA stimulated cells (Figure 8). This indicates that this region functions as an important promoter element for IL-9 transcription. Surprisingly, the basal transcription levels for the A- and G-containing promoters were markedly higher than the IL9-346 promoter that was missing the

NF-κB binding site. Additional factors can bind to this region (data not shown) and it is likely that these influence promoter activity in the basal state. Addition of PMA/PHA further increased transcription of the promoter constructs ∼2 fold for both the A- and G-containing promoters, supporting the concept that NF-κB can bind to this site. With these truncated constructs, a difference in promoter activity was not observed. One explanation for this observation is that these studies do not address the role that upstream promoter elements might have in regulating transcription through the -351 site. With a full-length IL-9 promoter, the site might be important for opening closed chromatin structures and in this case the difference in binding of NF-κB would have more of an effect on transcription. Using purified CD4+ T cells from subjects, the increase in transcription following PMA/PHA stimulation was inhibited by addition of an NF-κBp65 nuclear transport inhibitor, supporting the idea that NF-κB can bind to this region (Table 1).

Overproduction of IL-9 has been linked to many human diseases including asthma 27, sinusitis 28, mastocytosis 29 and Hodgkin's lymphoma 30. Several genetic studies have been performed that have demonstrated linkage for asthma to the chromosomal region 5q31-q33 in humans that includes the IL-9 gene 11, 12. Despite the linkage of this region to disease, very few studies have examined specific polymorphisms within the IL-9 promoter and gene for association with disease. A recent study of a GT repeat in the IL-9 gene demonstrated a gene-environmental interaction with specific IgE to dust mite allergen, however no functional effect of this polymorphism was reported 31. Having demonstrated a functional effect of the -351 polymorphism in our study, examination of this polymorphism in a large association study in multiple populations would be warranted. The polymorphism is unlikely to have a major protective role in Caucasian populations as we have only found an allele frequency of 17% for the G allele. However, the G allele frequency in other populations may be different which may allow an important role for this polymorphism in disease prevention to be identified.

## Materials and Methods

### Subjects

Heparinized venous blood was obtained with informed consent from healthy human volunteers (18-55 years old) using a protocol approved by the Human Investigation Committee at the University of Virginia. Genomic DNA was extracted from anti-coagulated blood using a Wizard Genomic DNA Purification kit (Promega, Madison, WI). A section of the IL-9 promoter containing the -351 site was amplified by PCR. The sequence for the upstream primer was 5′-CGTTAGAACACCCATGAC-3′ and the downstream primer was 5′-TTCTGGTTGTGAGAGTTAG-3′ (Integrated DNA Technologies, Inc., Coralville, IA). The product was gel purified and the sequence of the DNA analyzed on an Applied Biosystems DNA 377 Prism sequencer by the DNA Sciences Core at the University of Virginia.

### Cell culture

Jurkat T cells were purchased from American Type Tissue Culture (Manassas, VA). Jurkat T cells were grown in suspension to mid-log phase in complete RPMI 1640 medium

(Invitrogen; Carlsbad, CA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (Invitrogen), 2 mM L-glutamine and 10% fetal bovine serum (FBS) (HyClone; Logan, UT). Cells were maintained at 37° C in 5% $CO_2$.

## Cytokine determination

IL-9 levels were measured in the supernatants from T cell cultures. PBMCs were isolated through Ficoll-Hypaque (Sigma, St Louis, MO) density centrifugation. $CD4^+$ T cells (99% pure) were enriched from PBMCs using positive magnetic affinity column purification ($CD4^+$; Miltenyi Biotec). The cells were washed and resuspended in complete RPMI-1640 medium containing 1 mol/L HEPES (Invitrogen) and 10,000 U/ml penicillin and 10 μg/ml streptomycin supplemented with 10% autologous serum. T cells were cultured with or without various stimuli before collection of the supernatants or mRNA. Stimuli were as follows: 1.0 μg/ml phytohemagglutinin (PHA) (Sigma) for 48 hrs with 1.0 μg/ml PMA for the final 24 hrs or just 1.0 μg/ml PMA for 24 hrs. For studies in which NF-κB activity was inhibited, the activation inhibitor JSH-23 (Calbiochem, La Jolla, CA) was used at $1\times10^{-10}$M. These concentrations were chosen from preliminary studies involving dose-response curves that demonstrated optimal activity or from other published studies. IL-9 levels were determined using a Bio-Plex bead-suspension assay (Bio-Rad, Hercules, CA). The sensitivity of the assay was 2.08 pg/ml. Total RNA was extracted from cells using a SV Total RNA Isolation® kit (Promega, Madison, WI). Conversion of the mRNA to cDNA was performed using a Taqman Reverse Transcription kit (Roche, Branchburg, NJ) as previously described 32. Briefly, 200ng of RNA were added to each reaction along with oligo dT primers, 5.5 mM $MgCl_2$, 2 mM dNTPs, RNasin and reverse transcriptase. Reactions went through one cycle of 10 min at 25° C, 30 min at 48° C and 5 min at 95° C in a Bio-Rad iCycler thermocycler (Bio-Rad, Hercules, CA). The cDNA was amplified by real-time PCR with sybr-green detection (Bio-Rad) using appropriate primer pairs for IL-9 and the housekeeping gene ß-actin. Primers for ß-actin have been previously described 32. Primers used for detection of IL-9 mRNA are as follows: IL-9 forward 5′-GGGATCCTGGACATCAACTT-3′, IL-9 reverse 5′-CAGAAGACTCTTCAGAAATG-3′. Quantification of changes in receptor expression induced by cytokines was performed using the comparative $C_T$ method. Briefly, the amount of target, normalized to an endogenous reference and relative to a calibrator was calculated by $2^{-\Delta\Delta CT}$ with $\Delta\Delta C_T$ = (threshold cycle unstimulated gene of interest-threshold cycle unstimulated housekeeping gene)-(threshold cycle stimulated gene of interest-threshold cycle stimulated housekeeping gene). The comparative $C_T$ method was validated by showing that the efficiencies of target and reference amplification were equal across a range of mRNA concentrations. Primer pairs and probes for each reaction were synthesized by Integrated DNA Technologies.

## Electrophoretic mobility shift assay

Nuclear protein used was from Jurkat T cells resting or stimulated with phorpol 12-myristate 13-acetate (PMA) (Santa Cruz Biotechnology; Santa Cruz, CA). Additional nuclear extracts were prepared from primary CD4+ T cells purified from peripheral blood as described above using an NXTRACT-1KT nuclear extraction kit as per manufactures instructions (Sigma). AT and GC oligonucleotides representing base pairs -340 to -362 of the IL-9 promoter (from

the transcription start site) were synthesized (Integrated DNA Technologies, Inc.) (base exchange is underlined):

| | |
|---|---|
| AT: | 5′-TCGAGTAAAAAGGGAAAATACAGACCT-3′: |
| | 5′-TCGAAGGTCTGTATTTTCCCTTTTTAC-3′; |
| GC: | 5′-TCGAGTAAAAAGGGAGAATACAGACCT-3′: |
| | 5′-TCGAAGGTCTGTATTCTCCCTTTTTAC-3′. |

Synthesis of $^{32}$P-labeled double-stranded probe DNA and EMSAs were performed as described 33, 34. Labeled probe was electrophoresed on a 6% non-denaturing polyacrylamide gel to separate labeled probe from free nucleotide and unlabeled double stranded oligonucleotides that were incompletely extended. The probe was eluted from the gel and isolated over a centrisep separation column (Princeton Separations; Adelphia, NJ) and each probe was counted to determine the radioactivity. From the counts, the fmol of each probe was calculated. EMSAs were electrophoresed on a 6% non-denaturing polyacrylamide gel. To estimate binding affinity, relative fractions of bound and free probe were quantitated using a Molecular Dynamices phosphoimager. Specific binding of NF-κB to the IL-9 promoter was evaluated by competition with excess unlabeled double-stranded NF-κB consensus oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′: 5′-GCCTGGGAAAGTCCCCTCAACT-3′) and mutant consensus probes (base change is underlined) (5′-AGTTGAGGCGACTTTCCCAGGC-3′: 5′-GCCTGGGAAAGTCGCCTCAACT-3′; Santa Cruz Biotechnology; Santa Cruz, CA). Supershift assays were performed with anti-NF-κB (p50 (sc-7178X) and p65 (sc-7151X) and anti-NFAT antibodies (sc-13033X) (Santa Cruz). Binding of NF-κB was also confirmed using recombinant NF-κB (p50) protein (Active Motif; Carlsbad, CA).

## Chromatin immunoprecipitation assay

Chromatin immunoprecipititation (ChIP) assays were performed using a ChIP assay kit from Active Motif according to manufacturer's instructions. Immunoprecipitation was performed with anti-NF-κB(p50 and p65) or anti-Stat6 (antibody control). Following phenol/chloroform extraction and ethanol precipitation, samples were amplified by PCR (34 cycles) that generated a 100 base pair product surrounding the -351 residue. The primer sequences are the same as the ones used to genotype the subjects in the study.

## Reporter constructs

Reporter constructs were generated by cloning promoter segments of different lengths upstream from the putative translation initiation site of the human IL-9 gene into the luciferase reporter plasmid pGL3-basic (Promega; Madison, WI). Numbering of the constructs is from the transcription start site. A-containing and G-containing plasmids at position -351 were created for both the -369 IL-9 promoter constructs. Promoter DNA was amplified using PCR with genomic DNA (from homozygous donors) and appropriate primers (upstream primers for the IL-9 constructs: -369 5′-TACTCGGTACCATAATGTAAAAAGGGA(A/G)AATAC-3′; -346 5′-TACTCGGTACCAGACCTGGGCGTTCATGGAAAG-3′; downstream primer: 5′-

ACAAGAAGCTTGACAGCGGACTGGAG-3′). Fragments were digested with KpnI and HindIII restriction enzymes and were ligated into the pGL3-basic vector digested with the same enzymes. All constructs were confirmed by sequencing

### 3232 Transient transfection assays

The activity of the IL-9 promoter was determined by measuring the level of expression of the luciferase reporter gene. Briefly, 10 μg of IL-9 promoter-containing plasmid was incubated for 10 min with $1\times10^7$ Jurkat T cells at room temperature. Transfection was performed using electroporation (BTX electroporation system, San Diego, CA) with a single pulse of 25 msec at 250 volts. Cells were resuspended in culture medium and allowed to recover for 16 hrs before stimulation. Stimualtion with PMA/PHA and JSH-23 were as described above for 6 hrs before cells were collected by centrifugation and lysed (20 mM Tris pH 7.6, 150 mM NaCl, 2 mM EDTA, 10% glycerol and 1% Triton X-100). Cellular extracts were assayed for luciferase activity using a MonoLight 2010 (Analytical Luminescence Laboratories, Ann Arbor, MI) by counting and integrating for 30 s. Data were normalized to total protein and calculated as stimulation indexes in comparison with resting (luciferase negative) cells and subsequently normalized in comparison with the activity of the -346 IL-9 promoter construct.

### Statistical analysis

Data are expressed as means ±SEM. Statistical significance was determined by the Mann-Whitney test for nonparametric data comparing IL-9 protein and mRNA levels in the T cell cultures from individuals homozygous (A allele) or heterozygous (A and G allele) for the IL-9 genotype. A value of $p<0.05$ was considered to indicate statistical significance.

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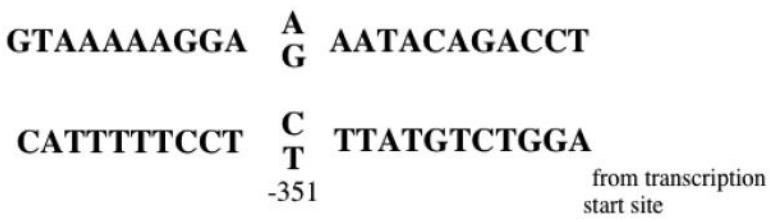


**Figure 1.**

Diagram depicting the IL-9 -351 polymorphism showing the wild type ('AT') and base exchanged ('GC') forms of the promoter.

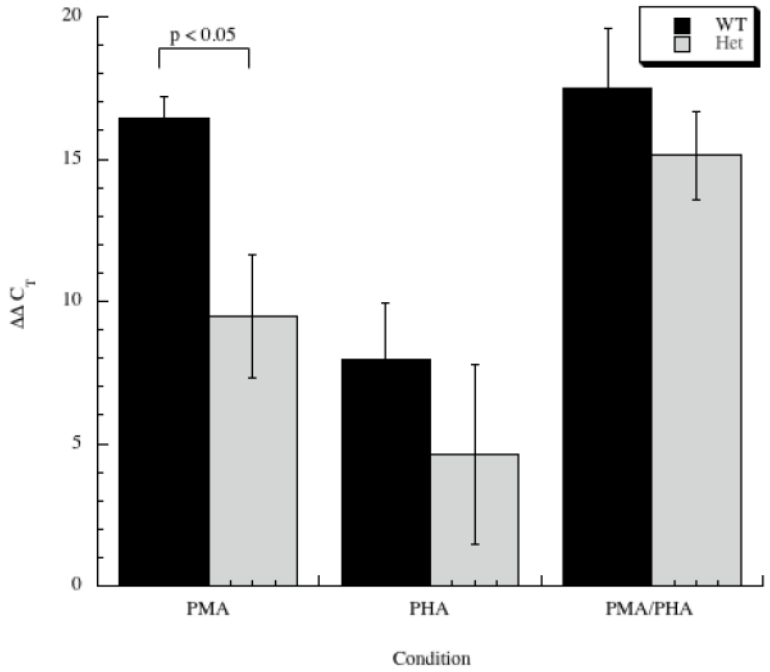


**Figure 2.**

IL-9 mRNA production from purified CD4+ T cells. T cells were enriched from peripheral blood of subjects, containing either the A/A or G/A alleles for the IL-9 -351 polymorphism, using magnetic bead affinity purification and cultured for 24 hrs in the presence or absence of PMA (1.0 μg/ml), PHA (1.0 μg/ml) or both. Cells were collected and RNA isolated. IL-9 mRNA levels were measured using quantitative PCR and separated according to IL-9 genotype: A/A (n=3) and G/A (n=3). P<0.05 is considered statistically significant.

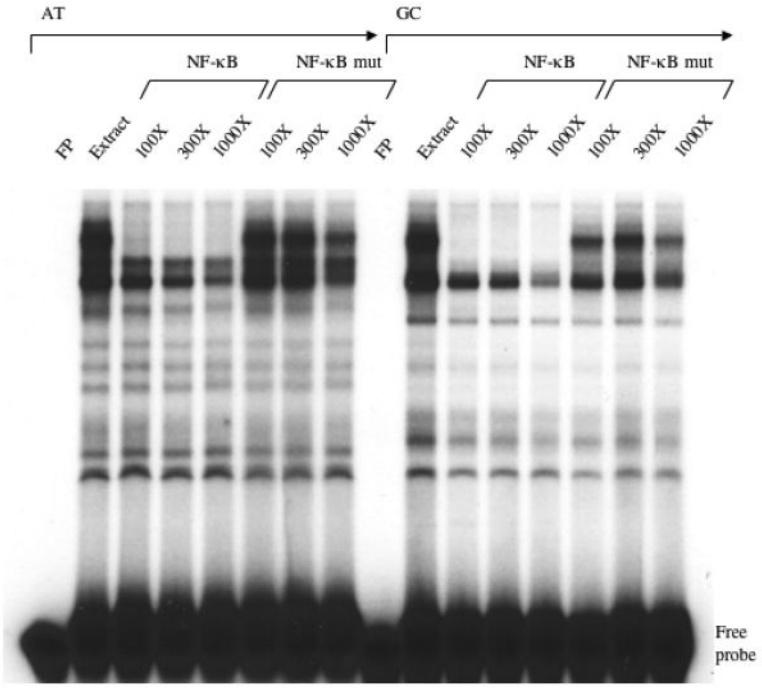


**Figure 3.**

NF-κB-specific consensus competition. Human PMA-stimulated T cell (Jurkat) nuclear extract was allowed to interact with $^{32}$P-labeled oligomers. The labeled probes represent the wild type (‘AT’) and base exchanged (‘GC’) forms of the IL-9 promoter at base -351. Non-specific binding was mitigated by addition of poly dIdC. FP represents free probe without addition of nuclear extract. Competition of shifted bands was performed with increasing concentrations (100-1000 fold) of unlabeled oligonucleotide. Bands were eliminated by competition with excess unlabeled NF-κB consensus probe, but not a mutated form of the NF-κB probe.

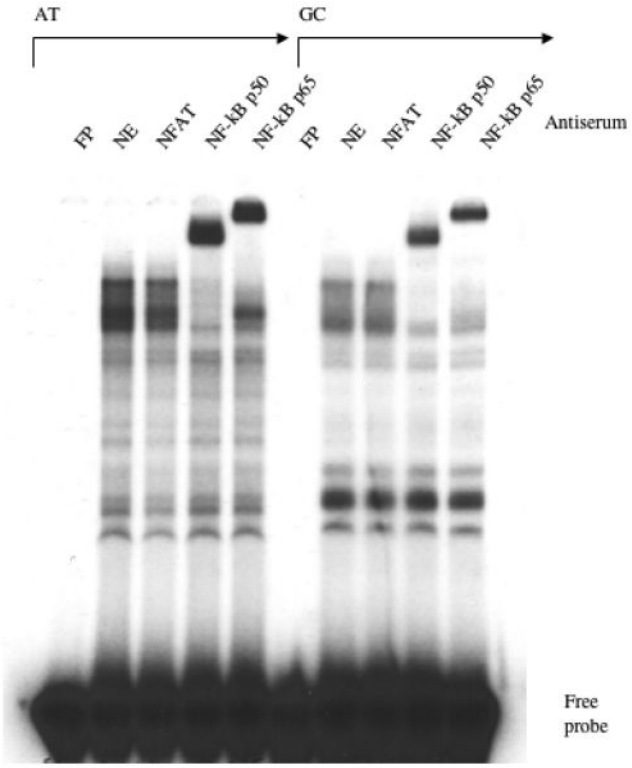


**Figure 4.**

Supershift assay demonstrates both p50 and p65 are part of the NF-κB complex on the IL-9 promoter. Jurkat nuclear extract was allowed to interact with $^{32}$P-labeled oligomers. The labeled probes represent the wild type ('AT') and base exchanged ('GC') forms of the IL-9 promoter at base $^{-}$351. Anti-NF-κBp50 and anti-NF-κBp65 supershift the higher molecular weight band and anti-NF-κBp50 also supershifts the lower molecular weight band. An isotype matched anti-NFAT antibody did not alter any of the shifted complexes.

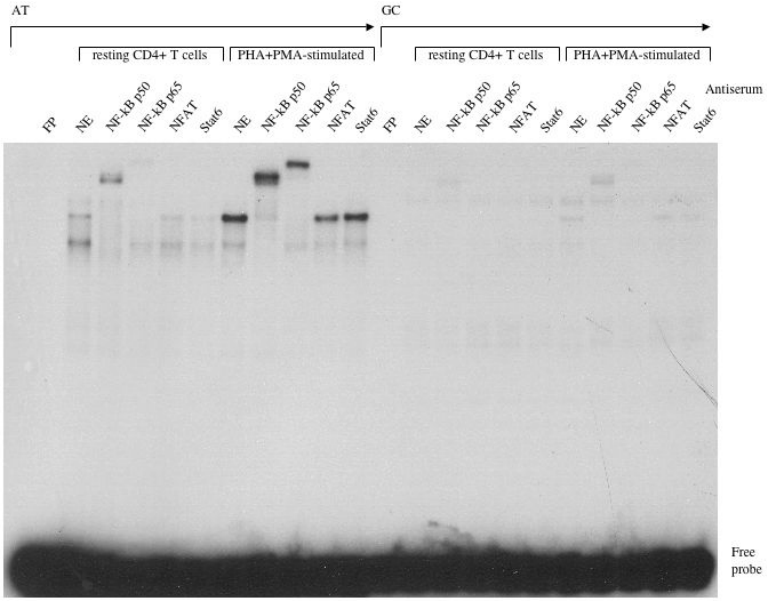


**Figure 5.**

Binding of NF-κB to the IL-9 promoter from extracts derived from CD4+ T cells. CD4 cells were isolated from peripheral blood and stimulated with PMA/PHA and nuclear proteins isolated. Nuclear extract was allowed to interact with $^{32}$P-labeled oligomers. The labeled probes represent the wild type ('AT') and base exchanged ('GC') forms of the IL-9 promoter at base $^{-}$351. Competition of shifted bands was performed with increasing concentrations (100-1000 fold) of unlabeled oligonucleotide. Bands were eliminated by competition with excess unlabeled NF-κB consensus probe, but not a mutated form of the NF-κB probe. Anti-NF-κBp50 and anti-NF-κBp65 supershift the higher molecular weight band and anti-NF-κBp50 also supershifts the lower molecular weight band. An isotype matched anti-NFAT antibody did not alter any of the shifted complexes.

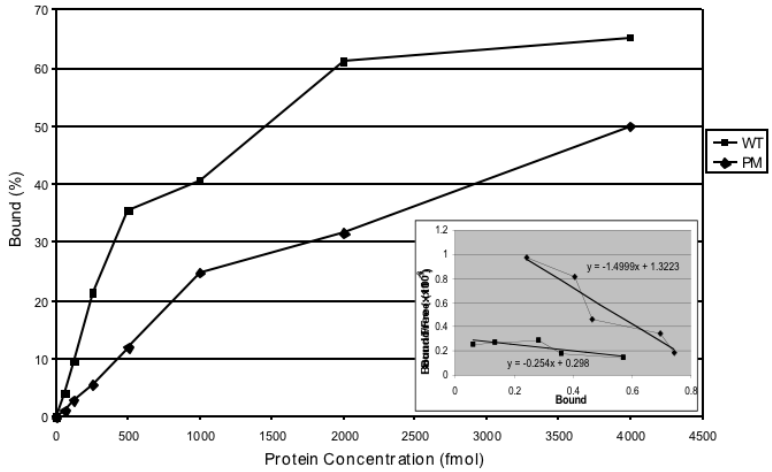


**Figure 6.**

Scatchard analysis of recombinant NF-κB p50 binding to the IL-9 promoter. Labeled probes were used representing the wild type ('AT') and base exchanged ('GC') forms of the IL-9 promoter at base -351. Increasing concentrations of recombinant NF-κB p50 were added to each probe and the percentage of each probe bound was calculated. From this the relative Ka of recombinant NF-κB p50 binding to each probe was calculated.

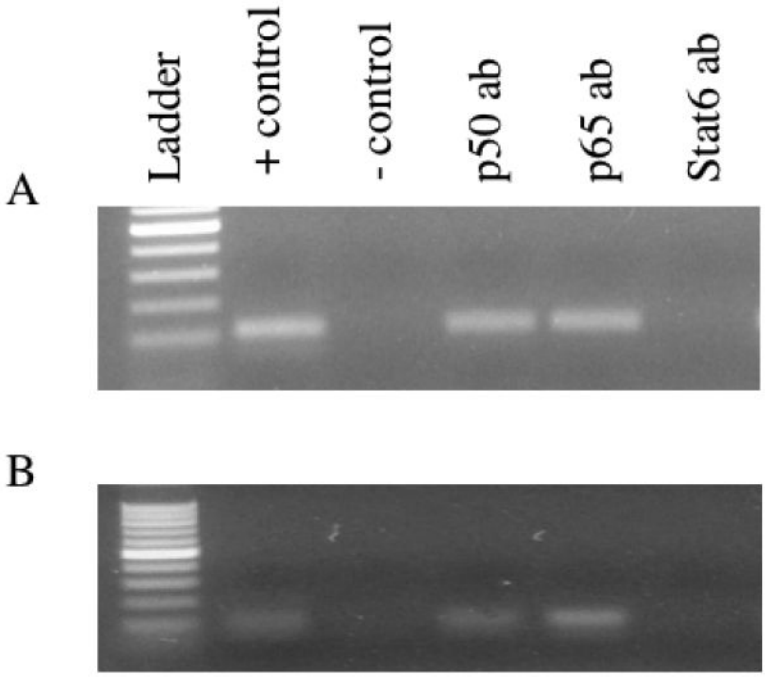


**Figure 7.**

Chromatin immunoprecipitation assay performed on A) Jurkat T cells and B) purified human CD4+ T cells to measure *in vivo* NF-κB p50 and NF-κB p65 binding. Proteins were cross-linked to the DNA with formaldhyde and antibodies directed against NF-κB p50, NF-κB p65 or Stat6 (antibody control) were added to precipitate any protein-DNA complexes. The -control lanes were processed according to the protocol, but did not have any antibody added to the samples. The + control lane was DNA that went through sonication and reversal of the protein-DNA contacts followed by ethanol precipitation. PCR were performed on isolated DNA using primers that span the -351 site in the IL-9 promoter and analyzed by agarose gel electrophoresis.

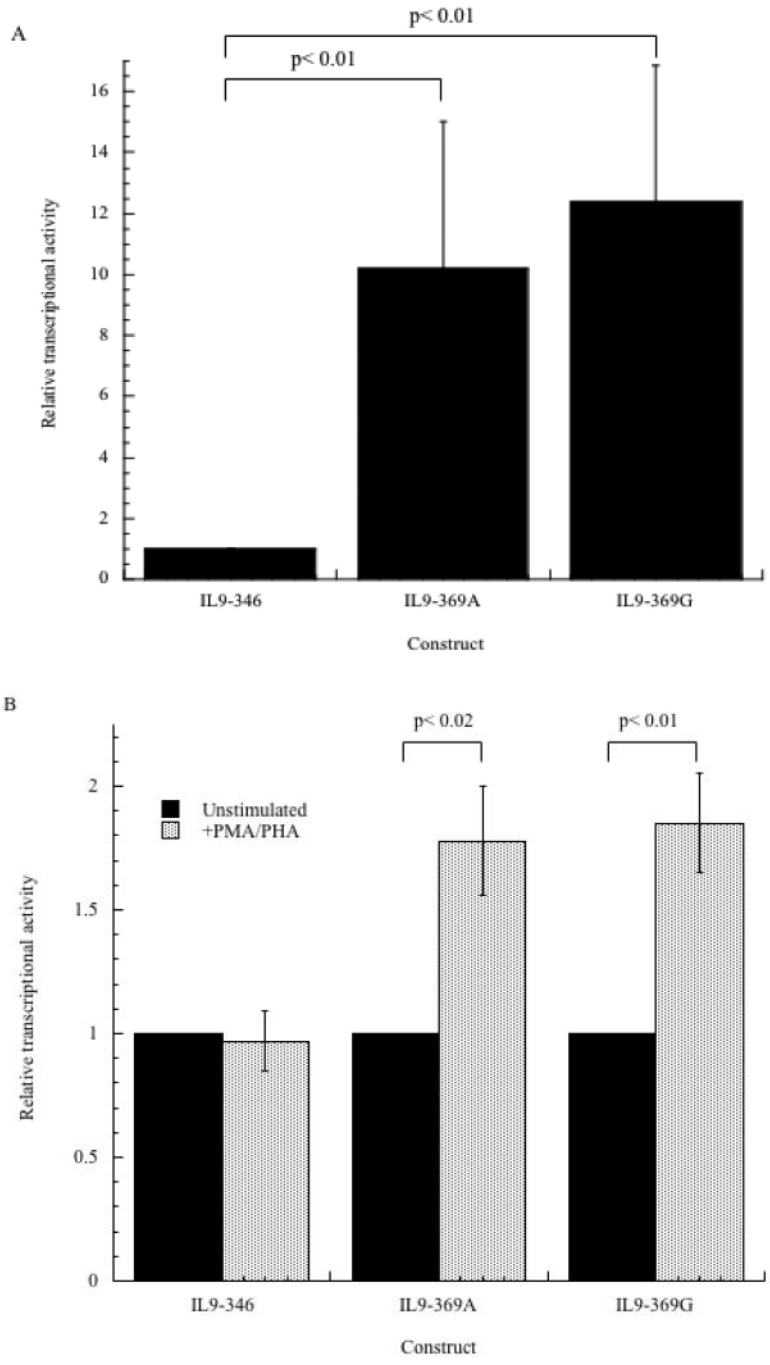


**Figure 8.**

Transient transfection assays were performed in the human T cell line Jurkat. A. Using electroporation, $1\times10^7$Jurkat cells were transfected with 10μg of each plasmid construct. Following incubation for 48 hrs, cells were collected and luciferase activity measured from cell lysates. Basal transcription of each IL-9 promoter template was normalized relative to the activity of the -346 construct. B. Transfection was performed as described above with the exception that Jurkat T cells were stimulated with PMA/PHA for the final 6 hrs to induce IL-9 transcription. Data was normalized to the unstimulated sample for each

construct. Data points represent the average of five independent experiments with error bars being calculated as standard errors of the mean.

## Table 1

| Genotype | Unstimulated | PHA/PMA | Inhibitor |
|---|---|---|---|
| A/A (n=10) | 35.0±5.6 | 87.2±27.8 | 34.1±4.7 |
| G/A or G/G (n=9) | 37.1±7.1 | 58.7±13.1 | 33.5±4.3 |

Concentration is in pg/ml