Multiple Transcription Factors Are Required for Activation of Human Interleukin 9 Gene in T Cells*

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The genetic elements and regulatory mechanisms responsible for human interleukin 9 (IL-9) gene expression in a human T cell leukemia virus type I-transformed human T cell line, C5MJ2, were investigated. We demonstrated that IL-9 gene expression is controlled, at least in part, by transcriptional activation. Transient expression of the luciferase reporter gene linked to serially deleted sequences of the 5′-flanking region of the IL-9 gene revealed several positive and negative regulatory elements involved in the basal and inducible expression of the IL-9 gene in C5MJ2 cells. An AP-1 site at −146 to −140 was shown to be involved in the expression of the IL-9 gene. A proximal region between −46 and −80 was identified as the minimum sequence for the basal and inducible expression of the IL-9 gene in C5MJ2 cells. Within this region, an NF-κB site at −59 to −50 and its adjacent 20-base pair upstream sequence were demonstrated to play a critical role for the IL-9 promoter activity. DNA-protein binding studies indicated that NF-κB, c-jun, and potentially novel proteins (around 35 kDa) can bind to this important sequence. Mutations at different sites within this proximal promoter region abolished the promoter activity as well as the DNA binding. Taken together, these results suggest that the cooperation of different transcription factors is essential for IL-9 gene expression in T cells.

Interleukin 9 (IL-9) is a T cell-derived cytokine with pleiotropic activities on various cell types (1). The expression of IL-9 is detectable mainly in activated CD4+ helper T cells (2, 3). The targets of IL-9 include T cells (1, 4), B cells (5, 6), mast cells (7, 8), erythroid and myeloid precursors (9, 10), and fetal thymocytes (11). It has been shown that IL-9 can promote the proliferation of activated and transformed T cells, the production of immunoglobulins by B cells, the proliferation and differentiation of mast cells, and erythroid progenitors. The involvement of IL-9 in tumorigenesis has also been suggested, since: 1) the preferential expression of IL-9 was found in cell lines derived from patients with Hodgkin’s disease and analyses large cell lymphoma (12, 13), 2) IL-9 was shown to be a proliferation inducer and a major anti-apoptotic factor for mouse thymic lymphomas (14, 15), and 3) the ratio of occurrence of lymphoma by mutagen or x-ray irradiation increased dramatically in transgenic mice with dysregulated expression of IL-9 (16). Therefore, it is important to unveil the mechanisms of IL-9 gene regulation in the hematopoietic system. Although it has been shown that the production of IL-9 in T cells can be induced by mitogen phorbol ester or anti-CD3 antibody (3), little is known about the control mechanisms of IL-9 expression at the transcriptional level. Human IL-9 gene has been mapped to the long arm of chromosome 5 at q31–32 (17, 18). Interestingly, human IL-3, IL-4, IL-5, GM-CSF, and IL-13 gene clusters have also been localized to the same locus (19, 20). The expression of IL-3, IL-4, GM-CSF, and IL-5 has been well studied in a variety of systems. Certain portions within the promoter regions of these genes were found to be highly conserved, and a common regulatory mechanism was suggested to be involved in the coordinated expression of these cytokines in certain cell types (21, 22). It was noted that the 5′-flanking region of the IL-9 gene contains several putative transcriptional elements, some of which are also identified in the promoter regions of other cytokine genes mapped to the same chromosomal location (3, 18). However, unlike other cytokines, up to now, the expression of the IL-9 gene has only been detected in T cells. To understand the basis of the T cell-restricted expression of IL-9, we undertook the characterization of IL-9 upstream regulatory sequences.

Our previous studies have demonstrated that a 0.9-kilobase SmaI-Sad fragment of the 5′-flanking region of the IL-9 gene contains the DNA sequences required for the basal and inducible expression of the IL-9 gene in a human T cell leukemia virus type I-transformed T cell line, C5MJ2 (18). In the present study, we further defined the cis-acting DNA elements and characterized the trans-acting transcription factors involved in IL-9 expression following 12-O-tetradecanoylphorbol-13-acetate (TPA)/phytohemagglutinin (PHA) stimulation. By using deletion and site-directed mutagenesis, we identified several DNA sequences in the 5′-flanking region of the IL-9 gene as important regulatory elements. Several transcription factors, including AP-1, NF-κB, and potentially novel DNA-binding proteins (around 35 kDa), were found to play critical roles in the regulation of IL-9 gene expression in C5MJ2 cells.

MATERIALS AND METHODS

Northern Blot Analysis and Nuclear Run-on Assays—Total RNA from C5MJ2 cells were prepared at various time points after stimulation with TPA (5 ng/ml) and PHA (10 μg/ml) (23). 10 μg of total RNA was used for Northern blot and hybridized with a 32P-labeled IL-9 cDNA probe. A human β-actin cDNA probe was hybridized to the same filter to ensure equal loading in each lane. The expression level of the IL-9 message was quantitated by densitometric scanning of IL-9 signal.
relative to that of the β-actin internal control.

For nuclear run-on assays, the labeled run-on transcripts from 3 × 10^5 C5MJ2 cells were prepared as described previously (24). 5 μg of plasmids containing the inserts for human GM-CSF (as a positive control), human IL-9, human β-actin, and pBR322 (no insert) were slotted-blotted onto nylon membranes and hybridized with the same amount of labeled run-on products.

**Primer Extension, RNase Protection, and DNA Sequencing—The transcriptional initiation site of human IL-9 in C5MJ2 cells was determined by both primer extension and RNase protection methods as described (25). For the primer extension assay, 50 μg of CSM2 total RNA was hybridized to 1 × 10^6 cpm of a 32P-labeled oligonucleotide primer. The sequence of the synthetic 35-base oligonucleotide primer is 5′-TGCCCTGACCAAGAAGACGGAAGCACGGAGGT-3′, which is complementary to a region within the first exon of the human IL-9 gene (18). The antisense RNA probe for the RNase protection assay was generated by in vitro transcription of ScI-linearized pS928 (18) using T7 RNA polymerase as described previously (26). 1 × 10^6 cpm of antisense RNA probe was hybridized to 50 μg of CSM2 total RNA. The RNA-RNA hybrids were treated with RNase A and RNase T1, followed by proteinase K digestion, and finally fractionated on an 8% urea sequencing gel. A sequencing reaction was run in parallel to serve as a reference for the protected region. The gel shift assay was performed as described previously with 4 μg of the nuclear extract and 2 × 10^4 cpm of the probe (29). In gel supershift experiments, the empirically determined amounts of antibodies (Abs) were preincubated, in parallel with the same amount of preimmune serum, for 40 min at 4°C with the nuclear proteins before the addition of probes. The following Abs were used for gel supershift assays: anti-c-jun un(AP-1) and anti-c-Fos/AP-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p50/NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p50/NF-κB, anti-c-Rel/NF-κB (30), and anti-CREB (a gift of Dr. Chou-Zen Giam). In peptide and oligonucleotide competition experiments, 2 μg of competitor peptides (30) or a 50-fold molar excess of various competitor oligonucleotides were preincubated with Abs or nuclear extracts for 10 min before the binding reactions. The sequences of AP-1, NF-κB, NF-AT, and CRE competitors are: AP-1, 5′-CGCTGAGCTGAGCCCGA-3′ (Promega); NF-κB, 5′-ACAGAGGAGTCTTCCCCGTCGAC-3′; consensus NF-κB p65 binding motif, 5′-GATCATTTCATGATC-3′ (31); CRE, 5′-GATCTGGGCGT-3′.

**DNA/Protein Binding Studies—Nuclear protein extracts were prepared as described (28). The DNase I footprinting assay was performed according to the standard procedure with 20 μg of nuclear extract and 2 × 10^4 cpm of a 32P-5′-end-labeled fragment covering –125 to +8 of the 5′-flanking region of the IL-9 gene. After DNase I partial digestion and phenol/chloroform extraction, samples were electrophoresed through an 8% urea sequencing gel. A sequencing reaction was run in parallel to serve as a reference for the protected region.

**Translational Regulation of IL-9 Gene Expression**

**Fig. 1.** Kinetics of IL-9 mRNA accumulation and transcriptional activation in C5MJ2 cells. Cells were stimulated with TPA (5 ng/ml)/PHA (10 μg/ml) for the indicated time period. Total RNAs were isolated and run on Northern analysis, and the nuclei were isolated for nuclear run-on assay. A, Northern blot analysis. 10 μg of RNA from CSM2 cells was used to hybridize to a 32P-labeled IL-9 cDNA probe, and then to a human β-actin probe. B, nuclear run-on assay. 3 × 10^5 CSM2 nuclei were prepared as described under "Materials and Methods." 5 × 10^4 cpm transcripts were hybridized to 5 μg of denatured plasmid DNAs containing specific and nonspecific inserts immobilized on nylon membranes. C, kinetics of Northern blot and run-on analysis. Induction folds were calculated according to the densitomeric scanning of IL-9 signals versus β-actin signals.

**Fig. 2.** 5′ mapping of the IL-9 mRNA. The 5′-end of the human IL-9 mRNA was determined by primer extension and RNase protection assays as described under "Materials and Methods." DNA ladders (lanes 1–4) were generated by sequencing IL-9 genomic DNA. The same primer used for sequencing was hybridized to unstimulated (lane 5) or TPA+PHA-stimulated (lane 6) CSM2 total RNA and extended with reverse transcriptase. The antisense RNA probe transcribed from IL-9 genomic DNA was hybridized to the total RNA from unstimulated (lane 7) or stimulated (lane 8) CSM2 cells. Following digestion with RNase, the samples were fractionated on a 8% sequencing gel. The mapped transcription start point, the overextended primer extension products, the RNA fragments protected from RNase digestion, and the DNA sequences around the start site are indicated. The restriction enzyme SacI site for constructing IL-9-luciferase plasmids is underlined.

**Table 1.** Kinetics of IL-9 mRNA accumulation and transcriptional activation in C5MJ2 cells. Cells were stimulated with TPA (5 ng/ml)/PHA (10 μg/ml) for the indicated time period. Total RNAs were isolated and run on Northern analysis, and the nuclei were isolated for nuclear run-on assay. A, Northern blot analysis. 10 μg of RNA from CSM2 cells was used to hybridize to a 32P-labeled IL-9 cDNA probe, and then to a human β-actin probe. B, nuclear run-on assay. 3 × 10^5 CSM2 nuclei were prepared as described under "Materials and Methods." 5 × 10^4 cpm transcripts were hybridized to 5 μg of denatured plasmid DNAs containing specific and nonspecific inserts immobilized on nylon membranes. C, kinetics of Northern blot and run-on analysis. Induction folds were calculated according to the densitomeric scanning of IL-9 signals versus β-actin signals.

**Table 2.** Kinetics of IL-9 mRNA accumulation and transcriptional activation in C5MJ2 cells. Cells were stimulated with TPA (5 ng/ml)/PHA (10 μg/ml) for the indicated time period. Total RNAs were isolated and run on Northern analysis, and the nuclei were isolated for nuclear run-on assay. A, Northern blot analysis. 10 μg of RNA from CSM2 cells was used to hybridize to a 32P-labeled IL-9 cDNA probe, and then to a human β-actin probe. B, nuclear run-on assay. 3 × 10^5 CSM2 nuclei were prepared as described under "Materials and Methods." 5 × 10^4 cpm transcripts were hybridized to 5 μg of denatured plasmid DNAs containing specific and nonspecific inserts immobilized on nylon membranes. C, kinetics of Northern blot and run-on analysis. Induction folds were calculated according to the densitomeric scanning of IL-9 signals versus β-actin signals.
**Fig. 3.** Promoter activities of IL-9-luciferase deletion constructs in CSMJ 2 cells. Deletion mutants of the IL-9 5'-flanking region were constructed using a luciferase-based vector, pXP2, and transfected into CSMJ 2 cells as described under "Materials and Methods." The numbers given at the 5' end of each construct represent the positions in base pairs relative to the initiation site of transcription. The result represents the mean of six to seven identical experiments.

**Fig. 4.** Analysis of AP-1-like site at position -146 to -140 of the 5'-flanking region of the human IL-9 gene. A. functional analysis. The sequences of 5' primers for generating the wild type AP-1 construct (pAP-1w) and the AP-1 mutant (pAP-1m) are shown with AP-1-like binding motif underlined. pAP-1w and pAP-1m were constructed by the PCR method and were transfected into CSMJ 2 cells as described under "Materials and Methods." The mean values of three independent experiments are shown. B. protein binding study. Gel shift and gel supershift assays were performed with synthesized double-stranded oligonucleotides harboring wild type (AP-1w) or mutated AP-1 (AP-1m) sites. The sequences of these oligonucleotides are identical to the sequences of 5' primers shown in (A). The nuclear extracts from unstimulated (uNE) or TPA/PHA-stimulated (sNE) CSMJ 2 cells were incubated with a 32P-labeled AP-1w or AP-1m probe in the absence or presence of antibodies against c-Jun or c-Fos. The position of the specific binding complex is indicated by an arrow.
Transcriptional Regulation of IL-9 Gene Expression

5′-Mapping of the IL-9 mRNA—Transcription initiation site for the IL-9 gene in C5M2 cells was determined by primer extension and RNase protection methods. In primer extension experiments, two bands with different densities were observed by using total RNA from activated C5M2 cells (Fig. 2, lane 6). The major band (82%) was 80 bases in length, indicating IL-9 gene transcription mainly initiated at 24 bases upstream from the translation start codon ATG and 25 bases downstream from the TATA box. The minor band (18%), 3 bases longer than the main band, was probably an overextended product. In RNase protection assays, one protected band was obtained with total RNA from both unstimulated and stimulated cells (Fig. 2, lanes 7 and 8) following RNase digestion. The size of the protected fragment corresponds to the size of the major band derived from the primer extension experiment, suggesting that transcription initiates at base 880 (according to the numbering system in the published human IL-9 genomic sequence; Ref. 18). This base is therefore considered as the transcription initiation site of the IL-9 gene and all the IL-9 sequences in this paper will be numbered accordingly. Our 5′-mapping results were slightly different from the ones mapped in stimulated PBMC cells (3), which probably reflect the differences in the control mechanisms of IL-9 gene expression in different cell lines.

Positive and Negative Regulatory Regions in the IL-9 Promoter—We have demonstrated previously that a 0.9-kilobase 5′-flanking region of the human IL-9 gene was able to direct the reporter gene expression in response to TPA/PHA stimulation in C5M2 cells (18). To further identify regions of possible functional significance within this 0.9-kilobase sequence, a series of deletion mutants were constructed for transient expression in C5M2 cells. As shown in Fig. 3, the expression level of the luciferase reporter gene was steadily increased when the DNA sequence between −878 and −379 was deleted, implying the existence of negative control elements in this region. The luciferase activity fluctuated between −379 and −143: the expression level reached the maximum with construct pIL-9-D4 (−379) and decreased about 2-fold with construct pIL-9-D5 (−299); After deletion of another 86-base pair sequence (−213, pIL-9-D6), the reporter gene expression increased again, especially after TPA/PHA induction; When an additional 70-base pair sequence was deleted (−143, pIL-9-D7), the luciferase activity decreased 5–6-fold. These results suggested that there are several positive and negative regulatory elements present between −379 and −143. Through computer search, several DNA sequences within −379 to −143 were found to be similar to the binding sites of certain known transcription factors. These sites included a xB-like binding motif at −331 to −317, an Sp1-like site at −317 to −307, an AP-3-like site at −306 to −298, and an AP-1-like site at −146 to −140. Except the AP-1-like site, none of the other sites were demonstrated to be important in controlling IL-9 gene transcription by mutagenesis studies (data not shown). The AP-1-like site at −146 to −140 was demonstrated to be functional in IL-9 gene expression, since both basal and TPA/PHA-inducible luciferase activities decreased about 2–3-fold when this site was mutated (Fig. 4A). This AP-1-like site differs from the AP-1 consensus sequence by only one nucleotide. The nuclear proteins binding to this site were found to be inducible by TPA/PHA stimulation in the gel shift assay (Fig. 4B, lane 2). Mutations at this AP-1 site abolished formation of the DNA-protein complexes (Fig. 4B, lane 3). These DNA-binding proteins were suggested to be c-Jun and c-Fos by the gel supershift assay, since anti-c-Jun antibody supershifted the retarded band and anti-c-Fos antibody inhibited the formation of the DNA-protein complexes (Fig. 4B, lanes 5 and 6).

RESULTS

Up-regulation of IL-9 Gene Expression in TPA/PHA-stimulated CSMJ2 Cells—The kinetics of TPA/PHA-induced expression of the human IL-9 gene in CSMJ2 cells was examined by Northern blot analysis. As shown in Fig. 1A, the steady-state level of the IL-9 mRNA was very low in unstimulated cells and can be transiently increased following TPA/PHA stimulation. The induction of the IL-9 mRNA by TPA/PHA reached the maximum level 11 h after stimulation and slowly decreased thereafter. 24 h after stimulation, the steady-state level of IL-9 mRNA was still about 3 times higher than that in unstimulated cells. To determine the control mechanisms of IL-9 gene expression after TPA/PHA stimulation, nuclear run-on assays were performed to measure the transcription rate of the IL-9 gene in CSMJ2 cells following TPA/PHA treatment (Fig. 1B). In agreement with the Northern blot analysis, the rate of IL-9 gene transcription was low in unstimulated cells. The transcription rate of the IL-9 gene was drastically increased (7-fold) 2 h after stimulation, reached the peak (10-fold) at 5 h, and then gradually decreased. These results, together with the observation that the steady-state level of the IL-9 mRNA peaked at 11 h of induction (15.5-fold) in Northern blot analysis, suggest that different mechanisms are involved in the accumulation of the IL-9 message. The mRNA induction observed at the first 5 h resulted mainly from the transcriptional activation since both Northern blot and nuclear run-on analyses showed identical induction folds of the IL-9 mRNA (Fig. 1C). In contrast, a predominantly posttranscriptional event was evidenced at 11 h of activation, suggesting a shift in the mechanisms regulating IL-9 mRNA accumulation from the transcriptional to the posttranscriptional level, most likely through increasing the stability of the message.
The sequence between −125 and −46 appeared to be essential for the constitutive and TPA/PHA-inducible expression of the IL-9 gene in C5M2 2 cells (Fig. 3). The construct pIL-9-D9 (−46) showed very low basal level reporter gene expression and failed to respond to TPA induction. An 8-fold (uninduced) and 24-fold (TPA/PHA-induced) increase in promoter activity was noted when −125 to −47 sequence was added. To localize the DNA sequences interacting with nuclear proteins, DNase I footprinting was performed using a 5′-end-labeled probe covering −125 to +7. As indicated in Fig. 5, the region from position −45 to −80 was protected from DNase I digestion, suggesting that DNA-protein interactions exist within this region.

Minimum Sequences for the Basal and Induced Expression of the IL-9 Gene in C5M2 2 Cells—To identify the minimum sequence that mediates the basal and TPA-induced expression of the IL-9 gene, the deletion mutants within the proximal region from −90 to −63 of the IL-9 promoter were constructed. They were designated as phIL-9-D10 to phIL-9-D13, beginning at −90, −80, −74, and −63, respectively. Transient expression of these constructs in C5M2 2 cells showed that phIL-9-D10 and phIL-9-D11 generated luciferase activities comparable to phIL-9-D8 (−125), but a 5-10-fold decrease in luciferase activity was observed in transfections with constructs phIL-9-D12 and IL-9-D13, respectively (Fig. 6A). Since phIL-9-D9 (−47) had very low level of luciferase expression (Fig. 3), DNA cis-elements were proposed to be localized in the region between −47 and −80. This result is consistent with that of the DNase I footprinting assay. A closer inspection of the sequences between −47 and −80 revealed two sites highly homologous to the consensus sequences for NF-κB and cAMP response element (CRE). The NF-κB-like binding motif (GGGGTTTTCC) is located at −59 to −50, and the CRE-like motif (TGATGTC) is immediately upstream from this NF-κB site. To investigate whether these two sites and their neighboring sequences contribute to the promoter activity, mutations were introduced into these two potential binding sites as well as their adjacent upstream sequences. As shown in Fig. 6B, any mutation within the NF-κB-like binding motif (phIL-9-M3), the CRE-like site (phIL-9-M2) or its adjacent 6-base pair upstream sequence (phIL-9-M1) caused a 90% decrease in the basal level and TPA/PHA-induced expression of the reporter gene. Simultaneous mutations at these three sites (phIL-9-M4) almost completely abolished the promoter activity. These results indicated that the three different sites are very important for IL-9 gene expression and they are likely to regulate transcription of the IL-9 gene in a cooperative manner in C5M2 2 cells.

Interactions of Nuclear Proteins with the Proximal Region between −47 and −80 of the IL-9 Promoter—To determine whether NF-κB, CREB, and other transcription factors are involved in the binding to the proximal region between −47 and −80, the gel shift assays were performed with DNA fragments spanning from −47 to −90 (Fig. 7A). As shown in Fig. 7B, nuclear extracts from both unstimulated and stimulated cells formed DNA-protein complexes with this sequence. It was noticed that TPA/PHA stimulation increased the intensity of the major DNA-protein complexes. To identify individual binding sites, competition experiments were carried out with DNA fragments bearing different binding motifs of several transcrip-
Fig. 7. Characterization of trans-acting proteins binding to the sequence between −80 and −47 of the 5′-flanking region of the IL-9 gene. A, the sequences of upper strand of duplex oligonucleotides used in the gel shift or supershift assay. The mutated nucleotides are underlined.
Transcriptional Regulation of IL-9 Gene Expression

Cowman and Farren

Transcription factors. Our data showed that proteins binding to this sequence are specific and the binding could be competed by unlabeled DNA fragment containing human immunodeficiency virus NF-kB motif, but not by unlabeled DNA fragments containing the binding motifs for NF-AT p/c, AP-1, and CREB (Fig. 7B). To further characterize proteins binding to this region, gel supershift assays were performed using specific antibodies against NF-kB, CREB, c-jun, and c-Fos. As shown in Fig. 7C, NF-kB was demonstrated to be one of the components in the DNA-protein complexes since antibodies raised against both Rel and p50 of NF-kB could supershift the complexes (lanes 5 and 7) and the supershift pattern could be reversed by the addition of excess amount of NF-kB peptides (lanes 6 and 8). The anti-c-jun antibody was also shown to be able to shift the DNA-protein complexes in the supershift assay (lane 2), but anti-c-Fos and anti-CREB antibodies failed to shift the retarded band. The CRE-like motif 5’TGACGTCA 3’ at -67 to -60 differs from CRE consensus sequence 5’TGACGTC 3’ by a single base substitution. This variant CRE sequence has been shown to have a markedly reduced binding affinity for CREB (32). Our gel supershift assays and competition experiments also suggested that CRE and CREB may not be involved in the regulation of IL-9 expression.

To characterize proteins binding to the upstream region of the NF-kB site, the UV-cross-linking assay was performed to identify proteins binding to the sequence between -77 and -58. As shown in Fig. 7D, several proteins (around 92, 40, and 35 kDa) from nuclear extract of stimulated cells were cross-linked to the DNA probe (lane 2). UV-cross-linking performed with oligonucleotides mutated at two sites demonstrated to be important by functional studies resulted in the disappearance or decrease in intensity of proteins around 40 and 35 kDa, suggesting that the proteins of 40 and 35 kDa are specific DNA-binding proteins (lane 3). The 40-kDa protein was inducible and was demonstrated to be c-jun by immunoprecipitation of the UV-cross-linked proteins with anti-c-jun antibody (lane 4).

To further investigate whether proteins binding to the proximal region correlates with regulation of the IL-9 gene expression, we used oligonucleotides with mutations within the NF-kB and its adjacent upstream region as the probes in the gel shift assay (Fig. 7, A and E). As compared with wild type sequence, mutations within the NF-kB motif or its adjacent 20-base pair upstream sequence hindered the DNA-protein complexes formation (Fig. 7E, lanes 2-4). Simultaneous mutations of NF-kB and its adjacent 20-base pair upstream sequence completely abolished the formation of DNA-protein complexes (lane 5). These results correlated well with the functional study (Fig. 6B), in which IL-9 promoter constructs containing the same mutations generated much lower luciferase activities as compared to the wild type construct, demonstrating that synergistic binding of these different transcription factors to the same promoter region is crucial for the expression of the IL-9 gene in C5M2 J 2 cells.

DISCUSSION

In this study, the up-regulation of IL-9 gene expression in C5M2 2 cells was shown to be regulated, at least in part, by transcriptional activation. Functional analysis of the 5’-flanking sequence of the IL-9 gene revealed several positive and negative regions controlling IL-9 gene expression. One positive region includes an AP-1 site at -146 to -140, which was demonstrated to be involved in the expression of the IL-9 gene in C5M2 J 2 cells. Members of the AP-1 protein family have been reported to be involved in the expression of IL-2, IL-3, IL-4, IL-5, and GM-CSF in T cells by working independently or cooperatively with other transcription factors, such as NF-AT and Oct1 (33-37). In this study, AP-1 protein was found not only to bind to the AP-1 site at -146 to -140, but also to participate in the formation of DNA-protein complexes binding to another important proximal region (-80 to -47) of the IL-9 promoter. Therefore, AP-1 appears to be an important general transcription factor involved in the up-regulation of a large number of cytokines in activated T cells.

The second positive regulatory region resides within positions -80 to -47 of the IL-9 promoter. Our study revealed that this region is absolutely required for IL-9 gene expression. The DNA sequence within this proximal region is highly conserved in evolution (identical between human and mouse IL-9 promoters). Within this region, an NF-kB motif at -59 to -50 as well as its 20-base pair adjacent upstream sequence were demonstrated to be critical since mutations at one or both sites drastically decreased the promoter activity. The direct interaction of NF-kB with this region was also confirmed by competition experiments and the gel supershift assay. NF-kB or related proteins have been shown to play important roles in the expression of several cytokines (37-41). NF-kB is required for the activation of GM-CSF gene by various mitogens in T cells (37, 41). Unlike GM-CSF gene, the position of this NF-kB motif in IL-9 promoter is located much closer to the TATA box. Compared with this NF-kB site in the IL-9 promoter, a stretch of DNA sequence referred to as the conserved lymphokin element 0 (CLE0) was found in the promoter regions of the GM-CSF, IL-4, and IL-5 genes (22). CLE0, regarded as an essential cis-element, is required for the expression of GM-CSF, IL-4, and IL-5 genes in activated T cells and contains composite binding site for AP-1/Ets or NF-AT factors (22, 31, 37, 42-43). The binding sequences for NF-kB, Ets and NF-AT proteins are quite similar, all of which have the AAAGG motif. The similarity suggests that promoters of those cytokines on chromosome 5 may be evolutionarily related and also raises the possibility that the T cell-restricted expression of IL-9 may be attributed to the unique feature of its promoter sequence.

It should be pointed out that in addition to NF-kB binding to the proximal region between -80 and -47, c-jun and the proteins of 35 kDa have also been shown to specifically bind to this functional region. c-jun may bind to the CRE-like site at -67 to -60, since probes with mutations within this CRE-like motif failed to give rise to the supershifted band using anti-c-jun in our gel supershift assay (data not shown). The nature of 35-kDa proteins remains unclear. It was noticed that a GATA binding motif 5’TGATAC 3’ exists at position -76 to -71 on the minus strand of the IL-9 promoter, and mutations containing this GATA binding site (pIL-9-M1) reduced reporter gene expression. At present, four members of GATA family tran-

B, gel shift assay with oligonucleotide competitors. 50-fold molar excess of competitors were preincubated with nuclear proteins for 5 min before addition of labeled wild type probe (PW1). The position of the specific binding complex is indicated by an arrow. C, gel supershift assay with antibodies and peptide competitors. For the gel supershift assay, empirically determined amount of Abs and same amount of preimmune serum were preincubated with the nuclear proteins before adding probes (PW1). In the corresponding peptide competition assay, 2 μg of the specific peptide was preincubated with Abs before binding reactions. D, UV-cross-linking studies. The sequences of upper strand of wild type and mutation probes used in UV-cross-linking study are shown with mutated nucleotides highlighted. After UV irradiation of DNA-protein complexes, equal volumes of the same sample were treated either with anti-c-jun or preimmune IgG, followed by the addition of anti-rabbit-IgG agarose. The UV-cross-linked proteins and immunoprecipitates were fractionated on a 7.5% SDS-polyacrylamide gel. E, gel shift assay with DNA fragments containing substitution mutations. The position of the specific binding complexes is marked by an arrow.

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scription factors have been characterized (44–45). Among them, only GATA-3 is predominantly present in T cells and is involved in the regulation of several T cell-specific genes (46–48). Recently, IL-5 was shown to be activated by GATA-3 in T cells (43). Since the molecular masses of GATA-3 proteins are around 45 kDa, it is unlikely that the 35-kDa UV-cross-linked proteins are GATA-3.

In summary, our results suggest that synergistic interactions of known and unknown transcription factors are likely to play a critical role in the regulation of IL-9 expression. As indicated by our study, several proteins can bind to the proximal region between –80 and –47. Mutations at different sites within this region not only decreased reporter gene expression but also abolished the formation of specific DNA-protein complexes. In addition to this important proximal region, we have also demonstrated that the AP-1 site at –146 to –140 can be regulated by AP-1 proteins to enhance IL-9 promoter activity, implying “cross-talk” and cooperation may exist between these two regions and among the different transcription factors. The cooperation of multiple transcription factors has been shown to be involved in the expression of IL-3, IL-5, and GM-CSF in T cells (37, 49). For IL-9 gene expression, its T cell-restricted transcription factors have been characterized (44–45). Among

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