

Characterization of a palindromic enhancer element in the promoters of *IL4*, *IL5*, and *IL13* cytokine genes

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Background: The genes encoding the cytokines *IL-4*, *IL-5*, *IL-13*, and *GM-CSF* are located in close proximity on human chromosome 5. We have previously described a motif in the promoters of these genes with a common palindromic sequence: CCAAG...CTTGG. These half sites are variably spaced and, within *GMCSF*, flank a second internal palindromic site. The *GMCSF* palindrome was shown to act as a strong enhancer of transcription when linked to a heterologous promoter.

Objective: We sought to determine whether the related palindromic elements from *IL4*, *IL5*, and *IL13* also act as enhancers of gene transcription.

Methods: Reporter plasmids driven by palindromic elements were transfected into Jurkat T and HeLa cells to determine enhancer activity, and T-cell extracts were used in electrophoretic mobility shift and methylation interference assays to determine the DNA-binding characteristics of palindrome-binding proteins.

Results: Enhancer activity was observed in unactivated T cells and HeLa cells, whereas in T cells the *IL4* palindrome mediated an activation-specific response. Mutational analysis of this element revealed that both halves of the CCAAG...CTTGG palindrome and part of the intervening sequence were essential for mediating interaction with protein complexes. Electrophoretic mobility shift assays suggested that the 4 palindromes bound similar factors because complexes formed between Jurkat nuclear extracts and each palindromic element showed identical mobility, and these elements were able to cross-compete for binding.

Conclusions: These data suggest that constitutively expressed factors are involved in mediating the enhancer function of these elements in T cells and that these factors might either be present or have closely related homologues in other cell types. Also, an activation-dependent factor might be recruited to modulate the function of the *IL4* element. (*J Allergy Clin Immunol* 2003;111:826-32.)

Key words: Palindrome, transcriptional regulation, *IL-4*, *IL-5*, *IL-13*, *GM-CSF*, enhancer

Abbreviation used

EMSA: Electrophoretic mobility shift assay

The cytokine genes encoding *IL-4*, *IL-5*, *IL-13*, and *GM-CSF* are located in a cluster on human chromosome 5 between q23 and 31.¹ One of the most abundant sources of these cytokines is the T_H2 subset of CD4⁺ T-helper lymphocytes, which express the genes after stimulation of the T-cell receptor and activation. The cytokines are involved in mediating the regulation of humoral immune responses, and their overexpression is observed in helminth infections and allergic diseases, such as asthma. *IL-4* and *IL-13* both stimulate immunoglobulin class switching in B cells to produce IgE.²⁻⁴ *IL-13* stimulates eotaxin release from the lung and might play a role in airway remodeling. *IL-5* plays a pivotal role in eosinophil differentiation, activation, recruitment, proliferation, and cell survival.⁵⁻⁷ *GM-CSF* affects both hematopoietic precursors and differentiated macrophages and granulocytes and is expressed more widely than *IL-4*, *IL-5* and *IL-13*, being secreted by both T_H1 and T_H2 cells and a number of other lineages. In view of the role of T_H2 cytokines in the pathophysiology of allergic disease, there has been much interest in the mechanisms of transcriptional regulation of these genes, particularly with respect to whether they are coordinately regulated. Intracellular cytokine staining and FACS analysis has shown the capacity of individual cells to produce a variety of the 5q cytokines; however, it is unclear whether their expression is simultaneous. Although a number of transcription factors, such as GATA3, c-Maf, and JunB,⁸⁻¹⁰ have been proposed to regulate expression of the different cytokines, none have been shown to have activities at all of the promoters.

We have previously identified an enhancer element in the promoter of the *GMCSF* gene (–192 CTTG-GAAAGGTTTCATTAATGAAAACCCCAAG –161), which contains 2 symmetrically nested inverted repeats: a GC-rich outer palindrome and an AT-rich inner palindrome. In transfection assays this region was shown to act as a strong transcriptional enhancer of a linked reporter gene and also as a constitutive enhancer of gene transcription when linked to a heterologous promoter.¹¹ The external inverted repeat contains a core sequence, CTTGG...CCAAG, which, variably spaced, was also found to be present in the murine *GMCSF* gene and in

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GM-CSF tcgaGGTT**CTTGG**AAAGG**TTCATTAATG**AAACCC**CCAAG**CCTG
CCAAG**AACCTTTCC**AAGTAATTACTTTTGGG**GTT**CGGACagct

IL-4 tcgaAGTTT**CCAAG**ATGCCACCTGTACT**TGGA**AAGAA
TCAA**AGGTTCT**TAGGTGGACAT**GAACCTTCT**Tagct

IL-5 tcgaACTCGACCCTG**CCAAGGCTTG**CAGTTTC
TGAGCTGGGAC**GTTCCGAACCGT**CAAAGagct

IL-13 tcgaCC**ACTTG**GCCTATAAAAGCTGCCACAAGAGCC**CAAGCCA**
GGT**GAACCC**GGATATTTTCGACGGTGTCTCG**GTTTCGGT**Tagct

IL-4m1 tcgaAGTTTTAGCTATGCCACCTGTAA**CGTAA**AAGAA
TCAA**AATCGAT**ACGGTGGACATT**GCA**TTTCTTagct

IL-4m2 tcgaAGTTT**CCAAG**ATAGCTACGTAA**CTTGGA**AAGAA
TCAA**AGGTTCTATCGATGCATTGAACCTTCT**Tagct

IL-4m3 tcgaAGTTTTAGCTATGCCACCTGTACT**TGGA**AAGAA
TCAA**AATCGAT**ACGGTGGACAT**GAACCTTCT**Tagct

IL-4m4 tcgaAGTTT**CCAAG**ATGCCACCTGTAA**CGTAA**AAGAA
TCAA**AGGTTCT**ACGGTGGACATT**GCA**TTTCTTagct

IL-4m5 tcgaAG**CGTCCAAG**ATGCCACCTGTACT**TGGA**AAGAA
TC**GCAGGTTCT**ACGGTGGACAT**GAACCTTCT**Tagct

IL-4m6 tcgaAGTTT**GTAA**GATGCCACCTGTACT**TGGA**AAGAA
TCAA**ACATTCT**ACGGTGGACAT**GAACCTTCT**Tagct

IL-4m7 tcgaAGTTTCCA**CT**ATGCCACCTGTACT**TGGA**AAGAA
TCAAAGGT**GAT**ACGGTGGACAT**GAACCTTCT**Tagct

IL-4m8 tcgaAGTTT**CCAAG**ATGCC**GTCTGT**ACT**TGGA**AAGAA
TCAA**AGGTTCT**ACGG**CAG**ACAT**GAACCTTCT**Tagct

IL-4m9 tcgaAGTTT**CCAAG**ATGCCACCTGTAA**CTGGA**AAGAA
TCAA**AGGTTCT**ACGGTGGACATT**GACCTTCT**Tagct

FIG 1. Oligonucleotides for wild-type and mutant palindromes used in the study. Palindromic sequences are in **boldface**. Mutations are underlined.

the promoters of human, but not murine, *IL4*, *IL5*, and *IL13* cytokine genes. This palindromic element was not present in the promoters of other functionally unrelated genes located within the 5q locus nor within the promoters of T_H1 cytokines, such as IFN- γ . Considering the strong positive regulatory effect of the *GM-CSF* palindromic element and the presence of related elements in the promoters of the T_H2 -expressed cytokines, we reasoned that this motif might be involved in the positive regulation of all of these cytokine genes. Here we provide evidence that the conserved palindromic elements from *IL4*, *IL5*, and *IL13* also act as enhancers of transcription and, as determined by means of electrophoretic mobility shift assay (EMSA) analysis, might be recognized by similar factors.

METHODS

Cells and culture conditions

The human T-cell lines Jurkat J6 and J16 were grown in RPMI 1640 medium (Gibco BRL), and HeLa cells were grown in MEM. Both were supplemented with 10% FCS (Sigma), L-glutamine (2 mmol/L), penicillin (100 IU/mL), and streptomycin (100 μ g/mL) at 37°C and 5% CO₂ in humidified air.

Plasmids

Oligonucleotides homologous to the palindromic elements of *IL4*, *IL5*, *IL13* and *GMCSF* (Fig 1) were ligated into the pCAT promoter vector (Promega) by using standard methods. Vectors bearing single-copy inserts of the palindrome in the same orientation were isolated. All recombinant vectors were verified by means of sequence analysis with an Applied Biosystems 377 automated sequencer. Reporter constructs of the *IL4* promoter with and without its palindromes (p-443-

+51.IL-4.CAT and p-371+51.IL-4.CAT) were generated by means of PCR amplification from a human genomic clone containing a 150-kb fragment from the h5q31 locus. The parental *IL5* clone p-517+35.IL-5.CAT (courtesy of Dr D. Cousins) was used as a template for amplification of a shorter fragment lacking the palindrome (−450+35.IL-5.CAT). Oligonucleotides for amplification of the fragments from *IL4* were as follows: 5' catgggatccAGGACAGTTTCCAAGATGCC, 5' catgggatccGATATTACTCTATCTTTCCC (sense), and 5' catgctcagTTGCAGTGACAATGTGAGGC (antisense). For *IL-5*, fragments were as follows: 5' catgggatccTTTCAATCACTGTCTTCC-CAC (sense) and 5' ctctcagAACGGTTCTGCGTTTGC (antisense). The amplimers were digested with *Bam*HI and *Xho*I and ligated into pBL3.CAT. The c-Maf expression vector was prepared by ligating a *Hind*III/*Xba*I fragment from pBS.m.c-Maf (courtesy of Dr J. Morgan) into pcDNA3 (Stratagene). A carboxy terminally Flag-tagged GATA3 expression vector, pCMV.GATA3.Flag, was prepared by means of PCR with the oligonucleotides 5' tcgaattccATGGAGGTGACGGCG-GACCAGC (sense) and 5' gatccctgagatctcactgtcgtcatcgtcctgtatgc-CATGGCGGTGACCATGTCTGGAGG (antisense) by using pmc5b8 (courtesy of Dr J. D. Engel) as a template. The amplimer was digested with *Eco*RI and *Bgl*II and ligated into pcDNA3. GRE.SV.CAT has been previously described.¹²

Transfections

Transfections and CAT assays were carried out as previously described.¹² Jurkat T cells (4×10^6) were transfected with 5 µg of reporter plasmid DNA plus 1 to 5 µg of expression plasmid DNA where indicated. Electroporation was carried out at 300 mV, 960 µF, and ∞ Ohms by using a Gene Pulser (Bio-Rad). Cells were incubated at 37°C and 5% CO₂ in humidified air, either left unstimulated or stimulated with phorbol dibutyrate (100 ng/mL) and ionomycin (1 µg/mL, Calbiochem) 24 hours after transfection, and harvested 24 hours later. Cell extracts were prepared in 200 µL of reporter lysis buffer (Promega), and CAT activity was assayed by means of thin-layer chromatography and quantitated with a phosphorimager and ImageQuant software (Molecular Dynamics). Cells were cotransfected with 0.5 µg of pCMV-β-galactosidase, and β-galactosidase assays were performed by using standard procedures. HeLa cells were transfected by means of calcium phosphate precipitation, as previously described.¹³

Electrophoretic mobility shift assays

Jurkat J6 nuclear extract¹¹ was used in band retardation assays. Oligonucleotide probes were labeled with [γ]³²P-ATP with T4 polynucleotide kinase, according to standard procedures.¹⁴ Five micrograms of Jurkat nuclear extract was incubated with 7.6 fmol of ³²P end-labeled oligonucleotide duplex plus 80 ng of poly (dI,dC) in binding buffer (10 mmol/L Tris-HCl [pH 7.5], 1 mmol/L MgCl₂, 0.5 mmol/L Na₃EDTA, 50 mmol/L NaCl, 0.5 mmol/L dithiothreitol, and 4% glycerol) for 15 minutes at room temperature. Where indicated, unlabeled specific competitor DNA was added to the binding reaction at a 10× molar excess. Complexes were resolved on 5% polyacrylamide gels run in 0.3× Tris Borate/EDTA buffer. EMSA assays were performed at least 3 times.

Methylation interference assays

Methylation interference assays were carried out according to the method of Ausubel et al¹⁴ with minor modifications. Sense and antisense oligonucleotides were separately 5' end-labeled, annealed to the unlabeled complementary oligo, and then incubated in 200 µL of dimethylsulfate reaction buffer (50 mmol/L sodium cacodylate [pH 8.0], 1 mmol/L EDTA [pH 8.0]). One microliter of dimethylsulfate was added for 7 minutes to randomly methylate guanine residues at an average of one site per molecule of probe.

The reaction was terminated by addition of 40 µL of dimethylsulfate stop buffer (1.5 mol/L NaOAc [pH 7.0], 1 mol/L β-mercaptoethanol, and 10 µg of tRNA) before ethanol precipitation. An 8× EMSA reaction was performed with both sense- and antisense-labeled probes. Complexes were separated on 5% native polyacrylamide gels run in 0.3× Tris Borate/EDTA buffer. Positions of free and retarded probes were identified by means of autoradiography and were excised from the gel. DNA was eluted by means of incubation in 300 µL of TE buffer (10 mmol/L Tris-HCl [pH 7.5] and 1 mmol/L EDTA) containing 50 mmol/L NaCl and then ethanol precipitated. DNA pellets were resuspended in 100 µL of 10% piperidine and heated to 95°C for 20 minutes to cleave at methylated guanine nucleotides. Piperidine was removed under vacuum aspiration, and DNA was washed 4 times in 100 µL of H₂O before being separated on 14% denaturing PAGE run in 0.5× TBE. Radioactive bands were revealed by means of autoradiography. Methylation interference assays were performed at least 3 times.

RESULTS

The palindromic elements contribute to basal activity of *IL4* and *IL5* promoters

Human *IL4* and *IL5* promoter constructs with either the palindromes intact (p-443+51.IL-4.CAT and p-517+35.IL-5) or lacking (−371+51.IL-4.CAT and p-450+35.IL-5.CAT) were generated. *IL13* constructs were not synthesized because the palindrome abuts the TATA box, thereby preventing comparative analysis. Expression of both *IL4* and *IL5* constructs in activated Jurkat cells revealed very low baseline promoter activity. To demonstrate the contribution of the respective palindromes to promoter function, it was necessary to co-transfect these cells with previously described transcriptional activators: c-Maf for the *IL4* promoter and GATA3 for *IL5* promoter to increase the basal activity above the threshold of the assay's sensitivity. Although both *IL4* constructs were activated by c-Maf, the shorter (−371) construct showed only 60% of the activity of the palindrome-containing plasmid. Similarly, the shorter *IL5* plasmid was not as active as that containing the palindrome (Fig 2). These data suggest that the palindromes contribute to basal promoter activity.

The palindromic elements act as positive regulators of gene transcription in different cell types

We directly tested whether the different palindromes mediated basal activity by linking them to heterologous promoters and assaying their transcriptional activation capacity in Jurkat cells. Each of the cytokine palindromes acted as a constitutive enhancer. The palindromes showed broadly similar levels of enhancement, although the *IL4* element was also responsive to activation (Fig 3, A). Potent enhancer activity was also observed in Jurkat J16 (data not shown) and HeLa cells (Fig 3, B). In contrast, a dimer of an unrelated sequence, the glucocorticoid response element from the MMTV promoter, had no basal enhancer activity either in Jurkat (Fig 3, A) or HeLa cells (data not shown). Co-expression of c-Maf did not

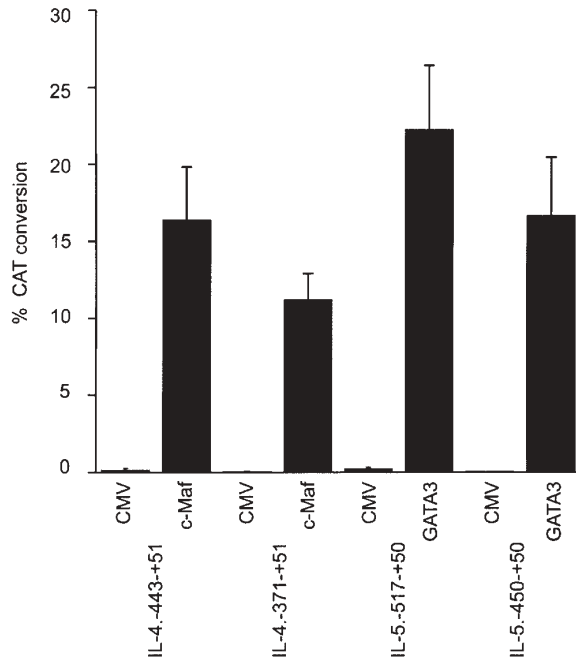


FIG 2. The *IL4* and *IL5* palindromes contribute to basal promoter activity. CAT constructs driven by the *IL4* and *IL5* promoters either with or without their palindromes were transiently transfected into Jurkat J6 cells. Cells were cotransfected with an empty expression vector (cytomegalovirus [CMV]) or with CMV.c-Maf (for *IL4*) or CMV.GATA3.Flag (for *IL5*). Data are expressed as the percentage conversion of CAT substrate and are the mean of 3 independent experiments.

alter the transcriptional activity of the *IL4* palindrome, nor did GATA3 expression affect the *IL5* palindrome's activity (data not shown). In contrast, both of these factors displayed strong activation potential on the intact promoters, which bear consensus response elements (Fig 2). Taken together, these data suggest that the palindromes from *IL4* and *IL5* act similarly to the *GMCSF* element as transcriptional enhancers. The *IL13* palindrome also displayed enhancer activity when coupled to a heterologous promoter. The similar activities of these elements prompted us to analyze the nature of the protein complexes by which they are bound.

The cytokine palindromes are recognized by protein complexes that have similar mobility in EMSA

EMSA analysis was carried out by using the *IL4* palindrome as a probe and Jurkat nuclear extracts and *IL4*, *IL5*, *IL13*, and *GMCSF* palindromes as competitors. Binding of specific factors could be competed with a molar excess of any of the palindromes (Fig 4). These experiments were repeated, replacing the *IL4* probe with the remaining 3 palindromes with similar results (data not shown). These data are strongly suggestive that common factors or families of factors mediate DNA binding

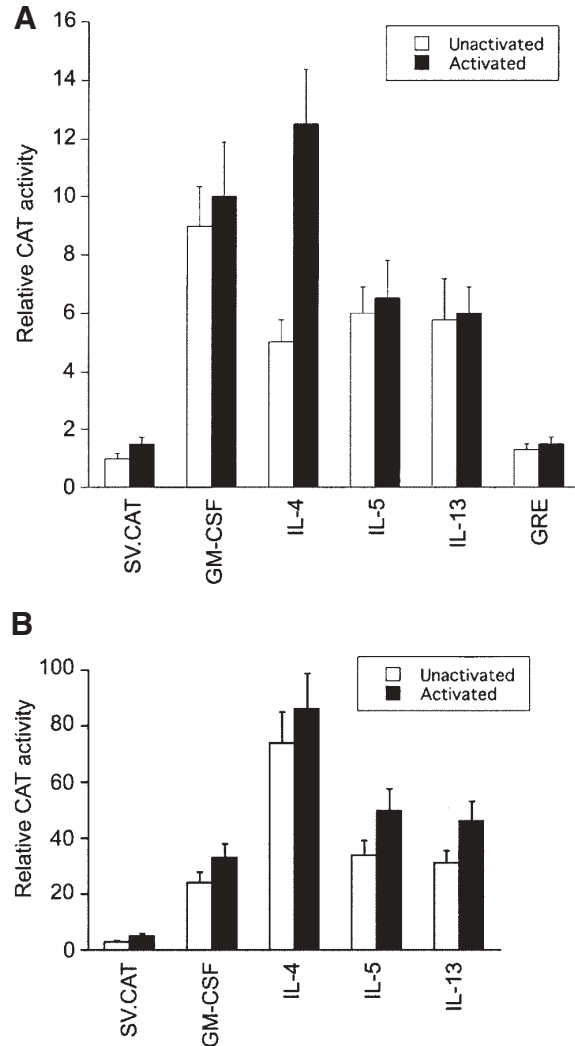


FIG 3. The palindromes from *IL4*, *IL5*, *IL13*, and *GMCSF* act as transcriptional enhancers. CAT constructs driven by the SV40 promoter either alone (SV.CAT) or with single upstream copies of each of the 4 palindromes (*GMCSF*, *IL4*, *IL5*, and *IL13*) were transiently transfected into either Jurkat J6 (A) or HeLa (B) cells. The negative control vector is 2 copies of the TAT GRE upstream of SV.CAT. Data are expressed as CAT activity relative to the unactivated SV.CAT sample and are the mean of at least 3 independent experiments.

of the 4 palindromic sequences. There were differences in the affinity of interactions, as assessed by the capacity of individual palindromes to cross-compete; however, these differences might reflect the effects of variation in the primary sequences and orientation of the palindromic sequences. Use of Jurkat J16 and HeLa extracts demonstrated that these cells contained factors that formed complexes on the palindromic probes that were indistinguishable in term of migration from those from the Jurkat extracts (data not shown). This suggests that Jurkat and HeLa cells contain either identical proteins or factors of similar size and DNA-binding properties.

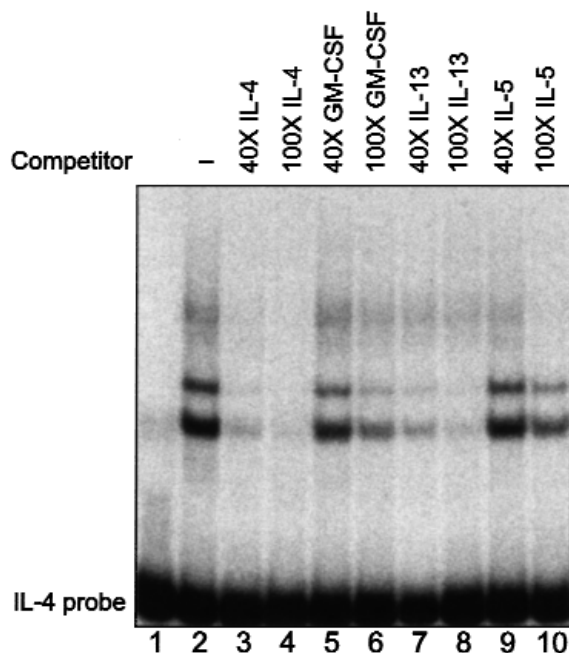


FIG 4. Specific complexes formed between Jurkat J6 nuclear extracts and the *IL4* palindrome can be competed by other palindromic sequences. *IL4* probe was incubated in the absence (lane 1) or presence (lane 2) of Jurkat J6 nuclear extract. The individual palindromes from *IL4* (lanes 3 and 4), *GM-CSF* (lanes 5 and 6), *IL13* (lanes 7 and 8), and *IL5* (lanes 9 and 10) were used as specific competitors at the concentration shown.

Conserved regions of the palindrome mediate factor binding

To clarify the regions of the *IL-4* probe that supported protein binding, the retarded complexes from EMSA, using the partially methylated *IL4* palindrome as a probe, were subjected to methylation interference analysis. Fig 5 demonstrates that methylation of specific guanine nucleotides is sufficient to prevent recruitment of complexes to the DNA probe. Underrepresentation of particular bands in the retarded sample suggests either inability or reduced ability of protein to bind to DNA when that guanine residue is methylated. On the sense strand, methylation of positions 2 and 3 was strongly inhibitory to complex formation, whereas methylation at positions 5, 6, and 7 had a weaker effect, although they still reduced complex formation. In contrast, methylation at sites 1 and 4 had no effect. On the antisense strand, methylation at site 1 caused a weak reduction in binding; modification of positions 2, 3, 4, and 5 were strongly inhibitory, whereas methylation at sites 6 and 7 had no effect on binding. These results suggest that complexes bound to the 2 half sites with different affinities in vitro, and methylation of G residues in the 5' half site was strongly inhibitory to binding; however, modification within the 3' half site caused a more modest reduction. These data suggest that both half sites of the *IL4* palindrome are involved in supporting complex formation. Sites of interaction on the 5' palindromic half site on the *IL4* sequence extended

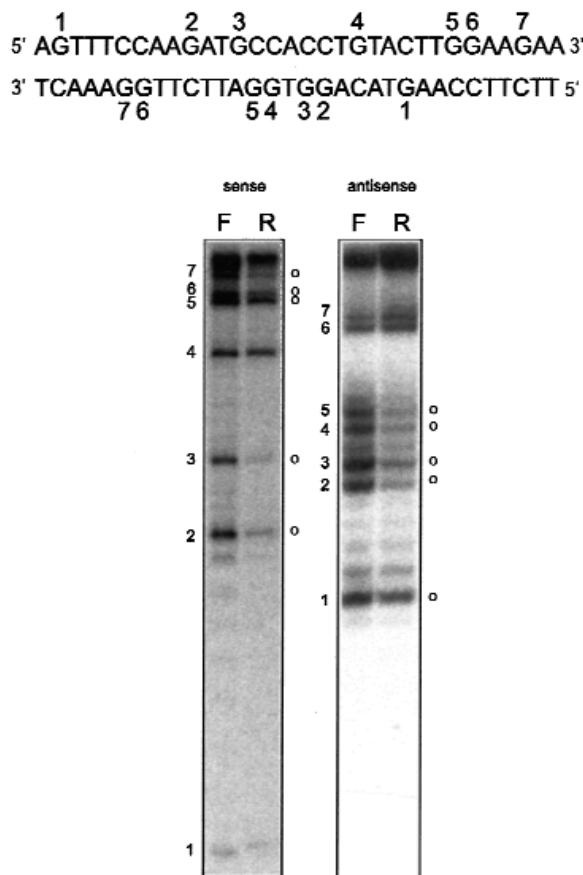


FIG 5. Identification of the sites of protein interaction with the *IL4* palindrome by means of methylation interference. DNA from retarded complexes (*R*) and free probe (*F*) were recovered from a standard EMSA assay and subjected to piperidine cleavage. Equal amounts of radioactivity were loaded to each lane. The influence of a methylated guanine residue in complex formation was assessed by means of comparison of the prevalence of each residue in free and retarded complexes.

beyond the conserved CTTGG motif. It is possible that such contacts impart stability on complex formation and potentially cause a preferential binding to this site rather than to the 3' half site. The different complexes observed in EMSA analysis yielded identical protected regions in methylation interference analysis, suggesting either that factors binding DNA have identical DNA recognition characteristics but different sizes or that additional factors are recruited to the DNA-binding complexes, causing slower migration in EMSAs.

Mutagenesis of the *IL4* palindromic element alters protein interactions in vitro

The mutated *IL4* oligonucleotides described in Fig 1 were used as competitors in EMSA analysis of the capacity of Jurkat J6 nuclear extracts to bind to the wild type *IL-4* palindrome. EMSA analysis demonstrated that there was heterogeneity in the capacity of the mutant oligonucleotides to compete with the wild-type palindrome for factor binding. The negative control competitor (AP-2) was unable to compete (Fig 6). In contrast to

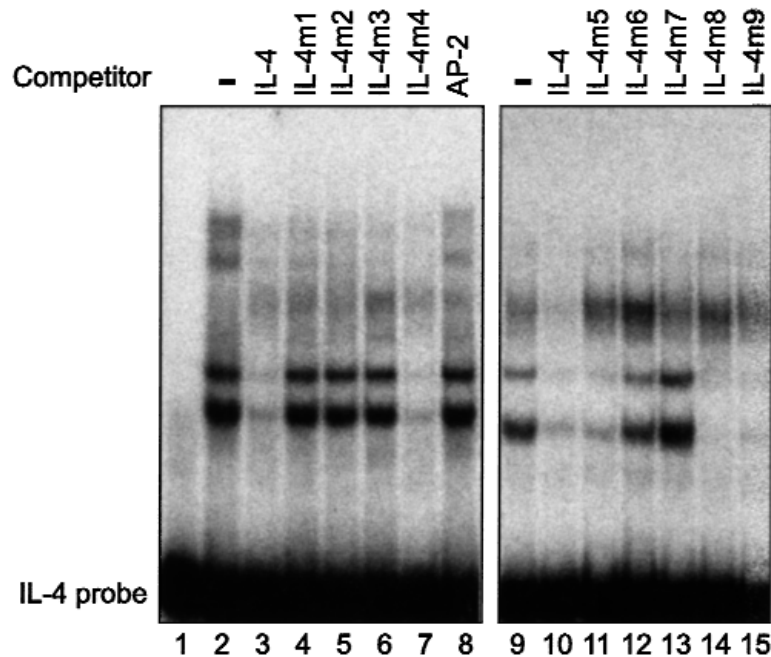


FIG 6. Mutant *IL4* palindromes display different abilities to act as competitors in EMSA. *IL4* probe was incubated in the absence (lane 1) or presence (lane 2) of Jurkat J6 nuclear extract. Wild-type (lane 3) or mutant palindromes described in Fig 1 (lanes 4-7 and 11-15) were used at 40× molar excess as competitors. A second, non-*IL4* competitor (AP-2) was used in lane 8.

the *GMCSF* palindrome, in which the 2 half sites were equally able to act as competitors, the *IL4* palindrome demonstrated a marked preference for factor binding to the 5' half site (see mutants 3 and 4). Mutants within the 5' half site (mutants 6 and 7) prevented competition, whereas mutants 8 and 9, which were present within the central region and 3' half site, competed efficiently. Taken together, these data suggest that although both halves of the *IL4* palindrome are involved in supporting complex formation, there was a binding preference for the 5' half site, and it is possible that recruitment to this half site facilitates interaction with the 3' half site. Whether these in vitro data mirror events in vivo remains to be determined.

DISCUSSION

Transcriptional regulation of the locus encoding *IL-4*, *IL-5*, and *IL-13* has been the subject of detailed analysis in the mouse but limited work in human subjects. Because these cytokines are upregulated in asthma and during allergic reactions, efforts have been made to understand mechanisms of coordinate regulation. To date, a number of factors, such as c-Maf, GATA3, and NF-AT, have been proposed to play important roles in regulation of the murine locus. Of these, the role of c-Maf appears to be restricted to upregulation of *IL-4* expression,⁸ whereas NF-AT mediates activation-dependent responses. GATA3, the expression of which is critical for T-cell differentiation, has been implicated in expression of each of the cytokines,¹⁵⁻¹⁷ yet a role in reg-

ulating GM-CSF has not been analyzed. Furthermore, GATA3 has been implicated as having roles both at distal enhancers, such as in the *IL4* gene¹⁷ and at the proximal promoters of *IL5* and *IL13*.^{15,16} None of the factors so far described has been shown to act at all of the proximal promoters.

We have previously reported that the *GMCSF* palindromic element acts as a potent enhancer of gene transcription.¹¹ Because elements of this palindrome are also present within the human *IL4*, *IL5*, and *IL13* promoters, it is possible that it serves to play a role in coordinate expression. Here we show that the *IL4*, *IL5*, and *IL13* palindromes also act as enhancers. The *GMCSF*, *IL5*, and *IL13* elements all showed similar levels of activity in the cell lines used, with only slight increases evident on cellular activation. In contrast, activity of the *IL4* palindrome was significantly enhanced. This observation suggests the involvement of additional factors that potentiate *IL4* enhancer function in response to activation stimuli. Palindrome enhancer function was not restricted to T cells because transfection of reporter constructs into the human epithelial cell line HeLa also permitted potent transactivation potential. This activity was observed both in unactivated cells and in those treated with phorbol ester and calcium ionophore. These data suggest that the cells constitutively express factors that are either homologous or display similar functional properties to those involved in Jurkat J6 and J16 T-cell enhancer activities.

EMSA analysis supported the hypothesis that the palindromes might mediate coordinate activity. Protein complexes formed on an individual palindrome could be

competed away with a molar excess of the other elements. However, it was in these EMSA assays that differences between the palindromes were observed. Judging by the concentrations of oligonucleotide required for competition, complexes bound more efficiently to the *IL4* and *GMCSF* elements than to *IL5* and *IL13*. These complexes might be stabilized by in vivo interactions with neighboring factors, and furthermore, the conformation of the palindromic elements might be different when in their correct chromosomal locale or within a rigid plasmid backbone. The fact that in transfection assays the palindromes display broadly similar activities lends support to this hypothesis. Despite the fact that the palindromes display different spacing of half sites, the helical structure of DNA predicts that these sites are located on the same face of the DNA strand.¹⁸ Consequently, contacts between protein and DNA are unlikely to be significantly hindered by the length of the intervening sequence.

Functional and biochemical analysis of the *IL4* sequence exposed certain differences between the different T_H2 cytokine palindromes. Cross-competition analysis demonstrated that the *IL4* and *GMCSF* sequences displayed higher affinity for binding of protein complexes than *IL13* and *IL5*. However, all palindromes supported binding of a complex that migrated with similar mobility, and all palindromes were able to compete, suggesting recruitment of common or related factors. Within the *IL4* palindrome, both methylation interference analysis and EMSA suggested half-site preference.

The chromosome 5q palindromic sequences do not bear homology to response elements that have thus far been described. Additionally, the variable distance between the 2 half sites raises a question as to whether the sites are recognized by factors that either homodimerize or heterodimerize or by an individual factor with 2 independent DNA recognition domains. Both scenarios are requisite of tolerance for half-site spacing. Huylenbroeck and colleagues¹⁹ have recently reported a family of factors, including SIP1 and δ EF1, which possess 2 distinct zinc finger DNA-binding domains. These have been shown to contact 2 palindromic half sites within the *Xenopus laevis brachyury* gene and mediate its correct spatial expression. Indeed, this family also displays spacing tolerance. The nature of factors binding to the cytokine palindromes is currently under investigation.

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