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Identification of a distal tandem STAT6 element within the CCL17 locus

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Summary

The CC chemokine ligand CCL17 is one of the major chemo-attractants for T_H2 cells. Interleukin-4-induced activation of CCL17 expression was recently demonstrated to result from two STAT6 motifs in the proximal promoter. Here we provide evidence that a distal tandem STAT6 element further elevates expression from the CCL17 locus approximately twofold. This is demonstrated by reporter gene assays using different fragments of the CCL17 promoter and the region 2.5 kb upstream from the transcriptional start site. By electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation experiments, we show that STAT6 binds to the motifs *in vitro* and *in vivo*, respectively. Insertion of nucleotide exchanges into the STAT6 core motifs results in diminished promoter activation and abrogated STAT6 binding, as demonstrated by reporter gene and EMSA studies. Collectively these data reveal an additional element involved in the regulation of CCL17 expression.

Keywords

Human; Chemokines; Allergy; Transcription factor

Introduction

The chemokine ligand and receptor system is regarded as the most important determinant for leukocyte migration and positioning under normal as well as pathologic conditions [1]. Based on the position of two cysteine residues, the chemokine ligands and their corresponding receptors have been divided into four groups: the CC, CXC, C, and CX3C families, of which the last two are less well characterized [2].

CCL17, an 8-kDa chemokine ligand of the CC family, is strongly associated with the T_H2 branch of the immune system, as its receptor, CCR4, is predominantly expressed on T_H2 cells [3]. Other CCR4-positive cell types include cutaneous lymphocyte-associated antigen (CLA)-positive T cells [4], natural killer cells [5], and platelets [6]. The sources of CCL17 are more diverse; monocytes, dendritic cells, endothelial cells, fibroblasts, bronchial epithelial cells, smooth muscle cells [2], keratinocytes [7], and, more recently, T cells themselves [8], have been reported to secrete the chemokine. Most of these cell types produce CCL17 upon stimulation with one or more of the cytokines interleukin (IL)-4, IL-13, tumor necrosis factor- α , and interferon- γ .

The association of CCL17 with various allergic diseases such as atopic dermatitis, allergic asthma, allergic rhinitis [9], and allergic contact dermatitis [10] underscores the importance of the T_H2 cytokines IL-4 and IL-13 as stimuli for its production. The receptor systems for IL-4 and IL-13 are closely related and share STAT6 as their main signaling component [11]. The canonical STAT6 signaling cascade is set off by stimulation of the receptor, leading to Janus kinase-mediated phosphorylation of tyrosine residues within the intracellular tail of the IL-4R α receptor chain. This enables STAT6 to bind IL-4R α via a phosphotyrosine–SH2 domain interaction. This close vicinity to the likewise receptor-attached Janus kinase leads the latter to phosphorylate STAT6 itself, ultimately leading to its dimerization. Active STAT6 dimers translocate to the nucleus, where they bind to sequence motifs and repress or enhance transcription of target genes [11].

Recently two STAT6 motifs were identified in the CCL17 proximal promoter approximately 150 bp upstream of the transcriptional start site (TSS) [8]. Mutations of those two elements abrogated IL-4 induced transcription from reporter constructs and therefore appeared to be necessary to confer IL-4 inducibility of CCL17. In addition a study on the murine CCL17 promoter demonstrated the importance of five STAT6 elements for IL-4–induced CCL17 expression in macrophages [12]. One of these elements is homologous to the 5' STAT6 site (termed “B-site” in the present study) in the human CCL17 promoter. We show here that a tandem STAT6 element located ~2.5 kb upstream of the TSS further adds to IL-4 inducibility of the CCL17 locus.

Subjects and methods

Preparation of human primary T cells

Peripheral blood mononuclear cells were isolated by Ficoll-Paque Plus (Amersham, Buckinghamshire, UK) density gradient centrifugation, washed twice with RPMI 1640 medium, and incubated for 1 hour in plastic tissue culture dishes at 37°C in a humidified atmosphere containing 5% CO₂. Nonadherent lymphocytes were collected and T cells were enriched by subjecting the cells to nylon wool chromatography (Polysciences, Eppelheim, Germany). Purity was assessed by staining for CD3-FITC/CD19-PE (BD Biosciences, Heidelberg, Germany) followed by fluorescence activated cell sorter analysis on a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). A purity of more than 90% was routinely achieved (Figure 1).

Cell culture and enzyme-linked immunoabsorbent assay

Human primary T cells were cultured in RPMI 1640 containing 10% heat-inactivated fetal calf serum (Invitrogen, Lofer, Austria), 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Human embryonic kidney HEK293 cells were cultured in Dulbecco's modified Eagle's medium with the same supplements.

Recombinant human IL-4 was a generous gift from Novartis and was used at a final concentration of 50 ng/ml.

Human TARC/CCL17 levels were measured using a commercially available enzyme-linked immunoabsorbent assay (ELISA; R&D Systems, Minneapolis, MN).

Western blotting

Nuclear extracts (described below) were mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. After denaturing, the samples were run on a precast 4–20% NuPAGE gradient gel (Invitrogen, Lofer, Austria) and blotted onto nitrocellulose (Bio-Rad, Vienna, Austria). We used anti-phosphoSTAT6 or anti-STAT6

(both Cell Signaling Technology, Danvers, MA) as primary antibody and an anti-rabbit-horseradish peroxidase conjugate (Bio-Rad, Vienna, Austria) as secondary antibody. Detection was carried out using enhanced chemiluminescence (Supersignal West Pico Detection Kit; Pierce, Rockford, IL). Stripping was achieved by incubating the membrane in 100 mmol/l mercaptoethanol, 2% SDS, and 62.5 mmol/l Tris-HCl buffer, pH 6.7, for 30 minutes at 55°C.

RNA isolation and real-time polymerase chain reaction

Total RNA from T cells was isolated using the TRIzol reagent (Invitrogen, Lofer, Austria). Total RNA (2–4 µg) was reverse transcribed with RevertAid H Minus M-MuLV reverse transcriptase (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) analysis was done on a Rotorgene 2000 (Corbett Research, Cambridge, UK) using iQ SYBR Green Supermix (Bio-Rad, Vienna, Austria). Primers for detection of CCL17 or the reference gene large ribosomal protein P0 were used as previously published [8]. Induction ratios (x) were calculated using the formula $x = 2^{-\Delta\Delta C_t}$, where C_t represents the threshold cycle of a given gene and ΔC_t represents the difference between the C_t values of the gene in question (CCL17) and the C_t value of the reference gene (large ribosomal protein P0). $\Delta\Delta C_t$ is the difference between the ΔC_t values of the samples induced with IL-4 and the ΔC_t of the noninduced sample. The mean induction ratios of all replicate analyses were calculated.

Transient transfections

The day before transfection, approximately 5×10^4 cells were seeded into 24-well cell culture plates in fresh medium. The cells were transfected using the calcium phosphate coprecipitation method. Briefly 1 µg plasmid DNA (reporter construct) and 0.25 µg of expression vector pcDNA 3.1 (Invitrogen, Lofer, Austria), containing the coding sequence of STAT6, were mixed with 7 µl 2 mol/l CaCl₂ and filled up to a total volume of 50 µl with water. This mixture was added dropwise to 50 µl 2 × HEPES-buffered saline (280 mmol/l NaCl, 1.5 mmol/l Na₂HPO₄, 50 mmol/l HEPES, pH 7.05) while vortexing at the same time. After addition, the mixture was vortexed for another 10 seconds and added to the cells. After overnight incubation of the transfectants, fresh medium was added and the cells were cultured in the presence or absence of 50 ng/ml IL-4 for 24 hours. After the IL-4 stimulation, luciferase activity was assessed using the Promega Luciferase Assay System (Promega, Mannheim, Germany) according to the manufacturer's instructions.

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear extracts from IL-4-induced or noninduced T cells were prepared according to the method described by Andrews and Faller [13]. A 5-µg quantity of the extracts was incubated with oligonucleotide probes that had been end labeled with [³²P]dCTP (Amersham, Buckinghamshire, UK) using Klenow fragment (MBI Fermentas, St. Leon-Rot, Germany). For supershift assays, 2 µg of antibody (all antibodies were obtained from Santa Cruz Biotechnology, Heidelberg, Germany) were added to the binding reactions. The oligonucleotide corresponding to the sequence from –2463 to –2451 bp relative to the transcriptional start site (TSS) was generated by annealing 5'-ATGATTTGTTCCCCAGAACAAA-3' (forward) and 5'-CTGTTTGT-TCTGGGGAACAAAT-3' (reverse). The second oligonucleotide, from –2432 to –2420, was annealed from 5'-CTCAAAGCTTTCTTCAGAAGGAC-3' (forward) and 5'-CTCAAAGCTTTCTTCAGAAGGAC-3' (reverse). One additional oligonucleotide containing a known STAT6 site from the CCL26 promoter [14] was used as a positive control. For oligonucleotide competition experiments, nonlabeled oligonucleotide was added in 50-fold molar excess to the binding reaction 30 minutes before the radiolabeled probe.

Chromatin immunoprecipitation

T cells were crosslinked in 0.4% formaldehyde at a cell concentration of $1.5 \times 10^7/\text{ml}$. Crosslinking was stopped with 125 mmol/l glycine. The cells were then washed in PBS and resuspended in SDS lysis buffer from the EZ ChIP kit (Upstate, Billerica, MA). Shearing was done with a Branson Sonifier 250 sonicator and checked on an agarose gel. The rest of the protocol was carried out as described in the kit. The antiserum used for precipitating was STAT6 M-200 (Santa Cruz Biotechnology, Heidelberg, Germany). Enrichment of the upstream tandem STAT6 motif from the CCL17 locus was checked by real-time PCR using the primers 5'-TGCACCAGCCTTGAAGTGAACCAG-3' (forward) and 5'-GCTACACAAGTCAAGGGACAGCTGATTA-3' (reverse). For the PCR reactions, Failsafe Enzyme Mix and Failsafe Premix C were used (Epicentre, Madison, WI).

Cloning of reporter gene constructs

A fragment containing 2575 bp of sequence from the CCL17 promoter region was amplified by PCR (36 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 3 minutes) using Pfu Polymerase (MBI Fermentas, St. Leon-Rot, Germany) from genomic DNA (Roche, Vienna, Austria) using the primers 5'-ATACGCTAGCCAGCCTGCACCAGCCTTGAA-3' (forward) and 5'-CTCTCGAGCCAGGAGGGAGTCTCTGTGTGC-3' (reverse), which have attached restriction sites for NheI and XhoI (underlined), respectively. This fragment corresponds to the region from -2535 to +40 relative to the TSS [8]. The PCR reaction was purified using the Wizard SV Gel and PCR Cleanup System (Promega, Mannheim, Germany) and cut with NheI and XhoI (MBI Fermentas, St. Leon-Rot, Germany). After digestion, the reaction was purified again and ligated (T4 ligase from MBI Fermentas, St. Leon-Rot, Germany) into the NheI/XhoI cut pGL3Basic vector (Promega, Mannheim, Germany). Shortened constructs were generated by removing the 5' promoter region using endogenous restriction sites for SmaI (at position -1084 to -1078 relative to TSS) or NsiI (at position -378 to -372 relative to TSS) on the 5' and XhoI on the 3' side (enzymes from MBI Fermentas, St. Leon-Rot, Germany). This resulted in vectors covering 1121 bp (SmaI) and 373 bp (NsiI) of CCL17 promoter sequence. Mutations into the STAT6 motifs (TTCNNNNGAA→TATNNNNGAA) of the 2575 bp construct were inserted using the Quikchange Mutagenesis kit (Stratagene, La Jolla, CA). The reporter gene constructs cdWT, cdMC, cdMD, and cdMCD were cloned by cutting the corresponding pGL3Basic construct with VspI and NheI (MBI Fermentas, St. Leon-Rot, Germany), which generated a 243-bp fragment resulting from the endogenous VspI site downstream of the C and D motifs on the CCL17 insert sequence and the NheI site of pGL3Basic. This fragment was blunted using T4 polymerase (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturer's protocol and gel purified as described above. Finally the fragment was ligated into a SalI-cut and blunted pTATA-LUC vector [15].

The insertion sequences of all constructs were verified by DNA sequencing. The plasmids were purified for transfection with maxi-prep kits (Qiagen, Vienna, Austria).

Results and discussion

Human primary T cells were isolated by Ficoll density gradient centrifugation (Figure 1A). Stimulation of the T cells with IL-4 results in phosphorylation of STAT6 (Figure 1B), which in turn leads to increased CCL17 mRNA expression and protein secretion (Figure 1C, D). In light of the T-cell purity (90%), other CCL17 sources (e.g., monocytes) might have contributed to the IL-4 stimulated CCL17 expression. Recently our group identified two STAT6 binding sites in the proximal promoter of CCL17 and showed them to confer IL-4 inducibility to the locus [8].

A region 2.5 kb upstream of the CCL17 TSS confers increased IL-4 inducibility

Our previous study on CCL17 was partially based upon reporter gene experiments on a 913-bp-long fragment of the CCL17 locus, covering the region from -146 to -1044 bp relative to the translational start site in a vector containing a TATA box. In attempts to further study IL-4 inducibility of the CCL17 locus, we cloned different and longer fragments containing the 5' UTR into the pGL3Basic vector. As IL-4 responsiveness is often mediated by STAT6, we chose the human embryonic kidney cell line HEK293 for the transfection assay. HEK293 cells have proven a valuable model for STAT6-dependent gene expression as they lack functional STAT6 but have an otherwise intact IL-4 signal transduction chain [16]. STAT6-dependent effects are therefore absent unless a STAT6 expression vector is cotransfected. The diverse constructs were thus transfected into the HEK293 cells, whereas STAT6-containing or empty expression vectors were cotransfected. The general induction rates of the constructs were lower than those previously published, which is probably caused by use of the endogenous CCL17 promoter in contrast to the TATA box-bearing vector in the earlier study. Interestingly a fragment encompassing roughly 2.5 kb of CCL17 upstream sequence was twice as much IL-4 inducible as fragments of ~1kb or ~400 bp (Figure 2A). The lack of increased luciferase activity in the absence of STAT6 expression vector suggested the involvement of STAT6. Although the distance to the transcriptional start site (TSS) appears large, our finding of an IL-4-responsive region 2.5 kb upstream of the CCL17 gene is reminiscent of a recent report on another IL-4 regulated gene [17]. In the cited study, the authors found that IL-4-inducible expression of Arginase I is regulated by a STAT6-dependent enhancer located ~3kb upstream of the basal promoter. Based on this, we searched the genomic region upstream of the CCL17 locus for a similar element. At positions -2432 to -2420 bp and -2463 to -2451 bp relative to the TSS, we found two canonical STAT6 binding motifs (Figure 2B). The close vicinity of these and a distance between the motifs and the TSS comparable to the Arginase I enhancer suggested an involvement of the two putative STAT6 binding sites in CCL17 locus regulation. To be consistent with the earlier publication on the proximal STAT6 sites, which were denoted A and B in that article, we refer to the upstream ones as C and D (Figure 2B).

STAT6 binds to the distal tandem motif

To test whether STAT6 present in nuclear extracts from IL-4 stimulated T cells could bind to the upstream elements *in vitro*, we performed EMSAs. Two radioactively labeled oligonucleotides, each corresponding to one of the two putative STAT6 sites (sites C and D, Figure 2B), were used. As Figure 3A shows, both oligonucleotide probes resulted in IL-4-inducible bands in the gel. As controls, probes with nucleotide exchanges at the third and fourth positions of the STAT6 motifs were used (TC→AT). This mutation was earlier shown to abrogate STAT6 binding [11]. When the mutated oligonucleotides were used as radioactively labeled probes, the top bands disappeared. As an additional control, the mutated or wild-type oligonucleotides, as well as an oligonucleotide corresponding to the known STAT6 binding motif of the CCL26 gene [14], were added as unlabeled competitor oligonucleotides. A band became visible only in the first of these reactions, whereas in the latter two, the binding reaction could be efficiently quenched by the unlabeled oligonucleotides. The nuclear complex formation could be supershifted by the inclusion of an anti-STAT6 antibody in the binding reaction, while an anti-nuclear factor- κ B p52 antibody had no effect.

To determine whether STAT6 could bind to the motives *in vivo*, we conducted chromatin immunoprecipitation (ChIP) with an anti-STAT6 antibody. A possible enrichment of DNA fragments containing the tandem STAT6 element was analyzed by real-time PCR using the primer pair as indicated in Figure 2B. The results show that from IL-4-stimulated cells, enrichment over the noninduced samples was obtained in two out of three donors (Figure

3B). In light of the difficulty of ChIP from primary cells, the negative result of one donor is probably a technical problem. Thus the ChIP assay, together with the EMSA, demonstrates actual STAT6 binding to the motives.

Mutations of the STAT6 elements reduce IL-4 inducibility of CCL17

To assess the relevance of the two STAT6 motifs in the upstream region, we introduced site-directed mutations into the 2.5-kb promoter reporter construct. The same nucleotide exchanges as in the EMSA experiments were used, which thus result in a block of STAT6 binding to the motifs. To study the importance of the upstream element independently from the proximal promoter, various single mutations of the former were combined with mutations of the known STAT6 sites [8] in the latter. The mutated constructs were identified with the prefix “M” preceding the site letter (Figure 3C). We transfected these constructs into HEK293 cells while cotransfecting STAT6-containing or empty expression vectors. After 24 hours of stimulation with IL-4, the cells were analyzed for luciferase activity. The results are presented in Figure 3D. Although the wild-type 2.5-kb promoter construct (WT) was four- to fivefold inducible upon transfection of STAT6 and IL-4 stimulation, the MAB double mutant was completely noninducible as shown before [8]. Single mutants of the two upstream motifs (MC, MD) decreased the luciferase activity by more than half, whereas a double mutant of C and D (MCD) was, surprisingly, fairly inducible, albeit still less well than the wild type. This unexpected effect was observed using several different preparations of plasmid. A possible explanation could be enhanced occupancies of the A and B sites by unbound STAT6. This will have to be investigated in more detail in further studies. All other constructs featuring at least one of the proximal sites mutated (MA, MB, MABC, MABD, MABCD) had lost inducibility.

To test whether the C and D STAT6 sites alone could drive IL-4-dependent expression in context of a different promoter, we cloned a short stretch from the CCL17 promoter containing the C and D sites into the pTATA-LUC vector [15]. Again we prepared a wild-type construct as well as mutated versions using the same nucleotide exchanges as before. The vectors were named as before, with the additional prefix “cd.” Transient transfections in HEK293 cells demonstrated the potency of the C and D motifs to confer STAT6-dependent IL-4 inducibility to a minimal promoter (Figure 4).

These findings demonstrate that although the proximal sites are indispensable for proper IL-4 activation of the CCL17 promoter, the distal tandem element multiplies IL-4 inducibility by an approximate factor of 2. This agrees with the reporter gene assay of the shortened constructs (Figure 2A).

In conclusion, in the present study we add a distal element to the known regulatory regions of the gene coding for CCL17. As our experiments demonstrate, the novel tandem STAT6 element increases the expression of CCL17 twofold upon IL-4 stimulation. The distal element therefore appears to have a role in fine tuning the expression of CCL17. A study on the murine CCL17 promoter demonstrates that STAT6 motifs with sequences diverging from the strict consensus have a certain role in regulation of locus activation [12]. This might hold true for the human CCL17 and will thus have to be tested in further studies. It also remains to be seen whether murine CCL17 is regulated by similar distal STAT6 elements as we have identified here. Given the clinical importance of the chemokine CCL17 in allergic diseases, better knowledge of its transcriptional regulation might be of value for developing therapeutic approaches.

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ABBREVIATIONS

ChIP	chromatin immunoprecipitation
CLA	cutaneous lymphocyte-associated antigen
EMSA	electrophoretic mobility shift assay
IL	interleukin
TSS	transcriptional start site

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Figure 1.

CCL17 expression by IL-4-stimulated T cells. (A) Fluorescence-activated cell sorter analysis of cell suspension after peripheral blood mononuclear cells were subjected to density gradient centrifugation and nylon wool chromatography. Plot is representative of three independent experiments. (B) Western blot of nuclear extracts from T cells stimulated with IL-4 for the indicated times. (Left) Detection for phosphorylated STAT6; (right) detection for total STAT6 after stripping the membrane. Arrow marks band corresponding to STAT6. Blot is representative of three independent experiments. (C) Detection of CCL17 mRNA by real-time PCR. T cells were left untreated (–) or were stimulated with IL-4 for the indicated times. Data are mean values for two different donors. Data for individual donors have been normalized to the corresponding zero time point. Error bars indicate standard deviations. (D) Detection of CCL17 protein in culture supernatants by ELISA. T cells were left untreated (–) or were stimulated with IL-4 (IL-4) for the indicated times. Data shown are mean values of two separate experiments. Error bars indicate standard deviations.

Figure 2.

Presence of an IL-4-responsive region 2.5 kb upstream of CCL17 TSS. (A) Reporter gene assay with HEK293 cells. Cells were transfected with luciferase vectors driven by CCL17 promoter fragments of the structures as indicated on the left side (positions are relative to TSS; A–D, STAT6-binding motifs). Expression vector for STAT6 (STAT6) or empty vector control (pcDNA) were cotransfected. Luciferase activity was assessed after overnight cultivation of the transfected cells in the presence (IL-4) or absence (–) of IL-4. Data are mean values of two separate experiments. Error bars indicate standard deviations. $*p = 0.087$, $**p = 0.06$. Fold induction and standard deviation of each construct were normalized to the corresponding noninduced, mock-transfected sample. (B) Genomic sequence of CCL17 IL-4-responsive region and promoter. Shown are ~200 bp of the proximal promoter and the upstream IL-4-responsive region as well as ~50 bp of the first exon (shaded black) and 6 bp of the CDS (indicated by large arrow outline). Positions relative to the TSS are given on the left side. STAT6 motifs are boxed, with the corresponding denominations (A–D) in bold italic type left of sequence block. Sequences of the primers used in the ChIP assay are indicated.

Figure 3.

STAT6 binds to motifs unless they are mutated. (A) EMSA on nuclear extracts from STAT6 transfected HEK293 cells that were incubated in absence or presence of IL-4 as indicated. Radioactively labeled oligonucleotide corresponding to motifs C (C) and D (D) or mutated versions (MC, MD) of the former were used as probes. Unlabeled competitor oligonucleotides corresponding to wild-type or mutated C and D motifs or to the STAT6 site in the CCL26 promoter (E3) were added as indicated. Supershift antibodies were included as shown. Arrows mark STAT6 bands and supershifted bands. (B) Chromatin immunoprecipitation from primary T cells that were stimulated with IL-4 (+ IL-4) or were left untreated (-). The samples precipitated with STAT6 antiserum (STAT6-) as well as the input controls (Input) are shown. Data represent signals obtained by real-time PCR for the CCL17 upstream STAT6 element-containing region. Results were normalized to mean of STAT6 samples. Data for three different donors are shown. (C) Schematic representation of the mutation constructs used in the transfection assay. Genomic organization of the CCL17 locus is sketched on top, with numbers indicating positions in base pairs relative to TSS (+/- 0). Arrow marks beginning of CDS; black boxes labeled A-D represent STAT6 sites. Approximate CCL17 locus sequence coverages of various constructs from WT to MABCD are shown with regard to top scheme. Mutations in the STAT6 sites are indicated by "x." LUC represents beginning of luciferase CDS. (D) Reporter gene assay on HEK293 cells. Cells were transfected with luciferase vectors driven by the various CCL17 promoter fragments as indicated. Expression vector for STAT6 (STAT6) or empty vector control (pcDNA) were cotransfected. Luciferase activity was assessed after overnight cultivation of the transfected cells in the presence (IL-4) or absence (-) of IL-4. Data shown are mean values of four separate experiments. Error bars indicate standard deviations.

Figure 4.

Motifs C and D drive IL-4-dependent expression of a minimal promoter. HEK 293 cells were transfected with pTATA-LUC vectors containing a 200-bp stretch of the CCL17 promoter with the C and D STAT6 binding motifs. The wild-type construct (cdWT) as well as mutated versions (cdMC, cdMD, cdMCD) were transfected, whereas STAT6 expression vector or empty vector control (pcDNA) were cotransfected. The structures of the used constructs are indicated on bottom. Mutations in the STAT6 sites are indicated by “x.” Luciferase activity was assessed after overnight cultivation of the transfected cells in the presence (IL-4) or absence (–) of IL-4. Data are mean values of four separate experiments. Error bars indicate standard deviations. Statistical significance of noninduced vs induced wild-type construct was calculated (* $p = 0.079$).