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The G-protein coupled receptor CMKLR1/ChemR23: Studies on gene regulation, receptor ligand activation, and HIV/SIV co-receptor function

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The mouse chemerin receptor gene, *mcmklr1*, utilizes alternative promoters for transcription and is regulated by all-trans retinoic acid

Keywords: G-protein coupled receptor, Transcriptional regulation, All-trans retinoic acid, Luciferase reporter gene, CCAAT element

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Abbreviations: CMKLR1, chemoattractant-like receptor 1; GPCR, G-protein coupled receptor; EMSA, electrophoretic mobility shift assay; AP-1, activator protein 1; AP-4, activator protein 4; GATA-1/-2/-3, GATA-binding factor -1, -2 or -3; CREB, cAMP-responsive element binding protein; PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide; cDNA, complementary DNA; RA, retinoic acid; RARE, RA-responsive element; RAR, retinoic acid receptor; ATRA, all-trans retinoic acid; PCR, polymerase chain reaction; RT, reverse transcription; TBE, Tris borate/EDTA; bp, base pair(s); kb, kilobase(s).

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Abstract

CMKLR1 (chemoattractant-like receptor 1) is a G-protein coupled receptor implicated in cartilage and bone development and is expressed in organs like the parathyroid gland, brain and lung. The receptor is also expressed in dendritic cells and in macrophages where it acts as a co-receptor for entry of HIV/SIV isolates into human CD4⁺ cells. Recently, a protein named “chemerin” (also known as TIG2) was isolated from human inflammatory fluids and hemofiltrate and found to be the endogenous ligand for CMKLR1. We have previously described the genomic organization of the *cmklr1* gene and characterized its promoter in mouse neuroblastoma NB4 1A3 cells. In the present study we identify a second transcript, *cmklr1b*, in mouse microglia BV2 cells. *cmklr1b* is transcribed from an alternative promoter with a transcription start site located 6,780 bp downstream of the previously identified exon 1 (*cmklr1a*). The *cmklr1b* promoter lacks a TATA box but contains two CCAAT boxes in opposite directions. 5' Deletion analysis of the promoter region in BV2 cells using a luciferase reporter gene assay indicates two regions, between 623 - 755 bp and 56 - 125 bp upstream of transcription start site, to be important for promoter function. The proximal promoter region includes both CCAAT boxes, and site-directed mutagenesis separately within these elements revealed that only the forward CCAAT element was important for transcription. Although the forward CCAAT element is essential for transcription electrophoretic mobility shift and super-shift assays demonstrated that both CCAAT elements actually bind nuclear proteins from BV2 cells and identified the binding factor as NFY. Real-time reverse transcriptase-PCR experiments of *cmklr1b* expression in all-trans retinoic acid (ATRA)- stimulated BV2 cells showed strong up-regulation of receptor transcript. Luciferase reporter gene assay of the promoter in ATRA-stimulated BV2 cells confirmed that transcriptional activity of the *cmklr1b* promoter is increased by ATRA. However, deletion analysis could not identify an ATRA-responsive element within the promoter region suggesting that gene activation is likely to occur through alternative mechanisms. The results emphasise a possible role of *cmklr1* in bone modelling.

1. Introduction

The human chemoattractant-like receptor 1 (hCMKLR1) was first described as a functionally unknown (orphan) G-protein coupled receptor (GPCR) (Gantz *et al.*, 1996). The receptor, also named ChemR23, was shown to be expressed in dendritic cells and macrophages (Samson *et al.*, 1998). It was found to function as co-receptor for select isolates of simian immunodeficiency virus (SIV) and human immunodeficiency virus-1 (HIV-1) (Samson *et al.*, 1998) facilitating viral entry into CD4⁺ cells, thereby assisting the major co-receptors, CCR5 and CXCR4. Recently, Wittamer *et al.* (2003) showed that a (chemotactic) protein isolated from human inflammatory fluids could activate CMKLR1 (Wittamer *et al.*, 2003) and the same year Meder *et al.* (2003) isolated the ligand from a hemofiltrate (Meder *et al.*, 2003). The chemotactic protein, named “chemerin” or “TIG2”, is secreted as a precursor, “pro-chemerin,” which upon proteolytic cleavage, removing six to nine amino acids in the COOH-terminal, becomes able to activate CMKLR1. Although CMKLR1 thus seems to be identical to the chemerin/TIG2 receptor, its ultimate naming awaits to be established. TIG2 (“tazarotene-induced gene 2”) has earlier been described to be implicated in psoriasis and has been reported to be up-regulated by the synthetic RA analogue, tazarotene (Nagpal *et al.*, 1997). The ligand also seems to play an important role in chemotaxis of immature dendritic cells and macrophages (Wittamer *et al.*, 2003) as well as in the mechanisms of bone modelling (Adams *et al.*, 1999).

The expression of the mouse orthologue mCMKLR1, designated DEZ by Methner *et al.* (1997), was shown to be differentially regulated during embryonic development, displaying high expression in cartilage and bone, whereas the main expression in the adult mouse was seen in the parathyroid glands, lung, and brain (Methner *et al.*, 1997). In order to clarify the molecular mechanisms underlying the mCMKLR1 expression we previously isolated the *mcmklr1* gene, mapped its genomic structure, and characterized its promoter (Mårtensson *et al.*, 2004). In this study we present a new splicing variant of *mcmklr1*, *mcmklr1b*, which has an identical coding sequence compared to *cmklr1a* but differ in its

non-coding region and is transcribed by an alternative promoter. In view of the effect of tazarotene, a RAR-specific retinoid (Chandraratna, 1996), on chemerin/TIG2 ligand expression (Nagpal *et al.*, 1997), we also investigated whether the *mcmklr1* gene is similarly regulated. This was performed by studying the effects of the RAR-specific activator, ATRA, on *mcmklr1* gene function.

2. Materials and methods

2.1 Cell culture

The mouse microglia cell line, BV2, was kindly provided by Prof. Adriano Fontana (Department of Internal Medicine, University Hospital Zürich, Switzerland) and the mouse embryonic fibroblast cell line, 3T3 clone A31, was obtained from ECACC (no. 86110401). Both cell lines were grown in Dulbecco's modified Eagle's medium with Glutamax I (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 µg/ml streptomycin. The cells were incubated at 37°C in 7% CO₂.

2.2 Northern blot analysis

Northern blot analysis was performed on mRNA from the mouse cell lines BV2, NB4 1A3, and 3T3 clone A31. Total RNA was isolated from the cells by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987), mRNA was selected using a commercial kit (Amersham Biosciences), and 6 µg was used for the blot. Northern blotting was also performed on mRNA isolated from BV2 cells treated for 20 h with different concentrations of ATRA, 100 nM PMA, and 500 ng/ml LPS all from Sigma. Total RNA and mRNA were isolated as above and 4 µg was used for the blots. A 1.2 kb *EcoRI/BamHI*-fragment containing the complete coding region of *mcmklr1* was radioactively labelled with [α ³²P] dCTP (NEN) using the Megaprime DNA labelling kit

(Amersham Biosciences). The blots were hybridized and washed according to standard procedures (Sambrook, 1989) and exposed to X-ray film (KODAK) overnight at -70°C.

2.3.1. RNA isolation and reverse transcription for real-time PCR

BV2 cells (1.2×10^5 /well), NB4 1A3 cells (2.3×10^5 /well), and 3T3 clone A31 cells (1.4×10^5 /well) were seeded into 24-well plates. The day after, 5 μ M ATRA was added to some wells and the plates were incubated for 4 h and 16 h, respectively. Cells were lysed in 200 μ l lysis buffer (100 mM Tris-HCl pH 8, 500 mM LiCl, 10 mM EDTA, 1% lithium dodecyl sulfate, and 5 mM DTT in each well) and the cell lysate was further processed with a QIAshredder (Qiagen) to reduce viscosity. PolyA⁺ RNA was captured from the cell lysate with 50 μ g SeraMag Oligo dT₁₄ paramagnetic beads (Serva). The beads were washed twice in 200 μ l washing buffer I (100 mM Tris-HCl pH 8, 150 mM LiCl, 1 mM EDTA and 0.1% lithium dodecyl sulfate), and once in 200 μ l washing buffer II (100 mM Tris-HCl pH 8, 150 mM LiCl and 1 mM EDTA). PolyA⁺ RNA was eluted in 10 μ l water, and first strand synthesis was carried out by reverse transcription (RT) for 50 min at 50°C in a final volume of 20 μ l containing 20 mM Tris-HCl pH 8.4, 50 mM KCl, 10 mM DTT, 5 mM MgCl₂, 0.5 mM dNTP, 0.5 μ g Oligo (dT)₁₂₋₁₈, 30 units RNaseOUT (Invitrogen), and 50 units Superscript III RT (Invitrogen). To generate first strand cDNA from mouse tissues (skeletal muscle, spleen, brain, kidney, liver, lung, and heart), total RNA was isolated as described in section 2.2. Approximately 10 μ g was mixed with 100 μ g SeraMag Oligo dT₁₄ paramagnetic beads in 100 mM Tris-HCl pH 8, 500 mM LiCl, 1 mM EDTA and subsequent PolyA⁺ RNA isolation and first strand synthesis was carried out as described above for cell culture derived samples.

2.3.2. Real-time PCR

Real-time quantitative PCR was performed in a LightCycler system (Roche) using the Sybr Green I detection method. The reactions were performed in a total volume of 10 μ l

containing 2 µl of diluted (1:20) cDNA or external PCR standard, 50 mM Tris-HCl pH 8.3, 10 mM KCl, 50 mM (NH₄)₂ SO₄, 3 mM MgCl₂, 200 µM of each dNTP, 0.5 µg/µl BSA, 1:30 000 dilution of SYBR Green I, 0.5 µM of each primer (Table 1), and 0.5 units FastStart Taq DNA polymerase (Roche). Following denaturation at 95°C for 10 min, a total of 45 cycles were run (each 10s at 95°C, 10s at 55°C and 10s at 72°C). Melting curve profiles were recorded and the specificity of the amplification product was further verified by electrophoresis on a Visigel (Stratagene) using GelStar (FMC) DNA staining. To generate external standards, specific PCR products for each gene were gel purified using QIAquick gel extraction kit (Qiagen). The copy-number was calculated based on the measured concentration at 260 nm, and serial ten-fold dilutions were made in ultra-pure water.

2.4. Rapid amplification of cDNA ends (5'-RACE)

Total RNA and mRNA were isolated from BV2 cells as described in section 2.2. 5'-RACE was performed using the Marathon™ cDNA Amplification kit (Clontech). The adaptor-specific primers (provided in the kit) together with the *mcmklr1* specific primers, 5'-GACCCAGACGGCCGAGCAGGTCATGTAG 3', and 5'-GACCAGACGGGGAGCAGCACGGAGATGC 3', were used in the primary and secondary PCR rounds, respectively. Amplified 5'-fragments were subcloned into pBluescript (SK-) for sequence analysis using the BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems).

2.5. Mapping of the novel splicing variant of the *mcmklr1* gene

Isolation and mapping of the genomic region of *mcmklr1* have previously been described (Mårtensson *et al.*, 2004). Mapping of the new splicing variant, *mcmklr1b*, was achieved by PCR-based genome walking (Universal GenomeWalker™ kit, Clontech) using BAC

DNA (mouse strain 129/SvJ) as template (sequence data from exon 1 of *cmklr1b* has been deposited in GenBank, accession no. [AY681348](#)).

2.6. Construction of reporter plasmids for analysis of promoter activity

The region immediately upstream of the *mcmklr1b* transcription start site was isolated by genome walking (Universal GenomeWalker™ kit, Clontech) using BAC DNA as template. In the primary and secondary PCR the adaptor-specific primers (provided in the kit) were used together with the *mcmklr1* primers, 5'CCCTCCTCCACGGTGCCAGCGCCAG3', and 5'GCCAGCGTCTCGAGCTGAGAC3', respectively. The amplified products were subcloned in front of the luciferase gene in the (promoterless) reporter plasmid, pGL3-Enhancer (Promega). Sequence data from the 5' region of *cmklr1b* has been deposited in GenBank, accession no. [AY681348](#).

2.7. 5' Deletions of the promoter region

5' Deletions of the -1,389 bp promoter construct (c1) were obtained by exonuclease III digestion using the Erase-A-Base kit (Promega). Briefly, the promoter plasmid was linearized with *Mlu*I and *Kpn*I and digested with exonuclease III at 30°C for different lengths of time. The reaction was stopped with S1 nuclease and Klenow DNA polymerase were added to blunt the ends which was followed by re-ligation of the plasmid with T4 DNA ligase (Henikoff, 1984). The deletions were confirmed by sequence analysis.

2.8. Mutated reporter plasmids

Site directed mutagenesis (QuickChange™, Stratagene) by inversed PCR was used to introduce 2 bp-mutations in the forw.CCAAT and the inv.CCAAT elements separately. Sixteen cycles of PCR were run using the plasmid c10 (containing 125 bp upstream transcription start site) as template, and the primers forw.CCAATmut

5'GAACACAGTATAGAAAATGAGCACAGATGTGGG3' and inv.CCAATmut 5'CTCTTCCTTGTGATTtCTCTGAGGAACACAG3', respectively. After digestion with *DpnI* for 3 h at 37°C, products were transformed into competent *E. coli*, and positive clones were identified by PCR screening using the primers 5'CTCTGAGGAACACAGTATAGAAA3' and 5'CCATTTCTCTTCCTTGTGATTt3', respectively. Introduced mutations were confirmed by sequence analysis.

2.9. Luciferase reporter assay

The day before transfection, BV2 cells (4×10^4 /well) and 3T3 clone A31 cells (2×10^4 /well) were seeded into 48-well (Nunc) or white 96-well (Costar) plates, respectively. Deletion and mutation luciferase constructs, 180 ng (BV2) and 500 ng (3T3), were co-transfected with pRL-TK (6 ng for BV2 and 17 ng for 3T3) (Promega) as internal control. Cells were transfected using 0.8 µl/well of the transfection agent, *TransIT-LT1* (BV2, Mirus) or 5 µl/well of *SuperFect* (3T3, Qiagen). The day after transfection media were exchanged with fresh medium, and 24 h after transfection 5 µM ATRA was added to cells to be stimulated; the cells were incubated for another 16 h. Forty hours after transfection cells were harvested in reporter lysis buffer (40 µl for BV2 and 20 µl for 3T3) (Promega) and stored at -70°C until analysis. Firefly and *Renilla* luciferase activities of the lysates were analysed in a dual-luciferase assay (Promega) using a BMG Lumistar Galaxy microplate luminometer. Firefly luciferase signals were compensated for differences in transfection efficiency using the internal control (*Renilla*) signal.

2.10. EMSA experiments

Nuclear extracts were prepared from BV2 cells essentially as described by Andrews and Faller (1991) (Andrews and Faller, 1991) with the following modifications: 0.5 mM DTT (dithiothreitol) + 0.2 mM PMSF (phenylmethylsulphonyl fluoride) in buffer A and C were replaced by protease inhibitor tablets (Roche), and buffer A was supplemented with 10%

Nonidet P40 (Roche). Protein concentrations were determined using the BCA Protein Assay Reagent kit (Pierce). Synthetic oligonucleotides (Table 2) were used as probes in the gel-shift and super-shift assays. Sense strands were labelled with [γ -P³²]ATP (Amersham Biosciences) using the 5' end labelling kit (Amersham Pharmacia Biotech) and purified on Microspin™ G-25 columns (Amersham Pharmacia Biotech). The labelled sense strands were mixed with 2 × molar excess of antisense strands in 10 mM Tris-HCl (pH 7.6). After denaturation by boiling for 2 min in a water bath, the strands were allowed to anneal by cooling the bath to room temperature. Binding was performed in a 15- μ l reaction mixture containing 10 fmol P³² labelled probe, 10 mM Tris-HCl (pH 7.5), 7 mM MgCl₂, 8% glycerol, 1 μ g poly-(dI-dC) (Amersham Biosciences), and 8 μ g of nuclear extract. The mixture was incubated at 25°C for 30 min. Unlabelled DNA competitors were mixed with nuclear extracts and pre-incubated at 25°C for 30 min before labelled probe was added and incubated at 25°C for another 30 min. The DNA-protein complexes formed were resolved on a 4% non-denaturing polyacrylamide gel. Electrophoresis was carried out in 1 x TBE buffer at 150 V for 3 h at 4°C. The dried gel was exposed to X-ray film (Kodak) overnight at -70°C. For super-shift assays, labelled probe and extracts were incubated for 20 min at 25°C, whereafter 2 μ g of NFY-A or NFY-B antibodies (Santa Cruz Biotechnology) was added and the incubation extended for an additional 45 min at 25°C.

2.11. Data analysis

Luciferase reporter experiments were performed as triplicates where each experiment was repeated three times. Error bars are depicted as standard error of the mean (SEM). For the real-time reverse transcription-PCR (real-time RT-PCR) experiments, error bars are shown as standard deviations (SD).

3. Results

3.1. Detection of a novel transcription start site of the *mcmklr1* gene

BV2 cells, endogenously expressing *mcmklr1* (Fig. 1), were used for 5'-RACE analysis (Fig. 2A). The results revealed three main products, which were subcloned into pBluescript (SK-) and sequenced. The 600-bp fragment turned out to be related to *cmklr1* whereas the two larger bands, >1,018 bp in size, were shown to be non-specific amplified fragments. The 600-bp fragment was 132 bp shorter than the cDNA fragment that was amplified in the previous 5'-RACE revealing the *cmklr1a* transcript (Mårtensson *et al.*, 2004). Sequencing of several bacterial clones of the 600-bp construct revealed four possible transcriptional start sites located 124 to 144 bp upstream of the translational start codon (Fig. 2B). We suggest the putative transcription start site to be located 144 bp upstream of the translational start codon in the cDNA sequence; it is designated +1 in figure 2B and 4. The other shorter sequences may represent truncated fragments.

3.2. Genomic organization of the *mcmklr1* splicing variants

PCR-based genome walking was performed in order to map the genomic structure of the new splicing variant, designated *cmklr1b*. An isolated BAC DNA clone containing the *cmklr1* gene was used as template (Mårtensson *et al.*, 2004). The two splicing variants, *cmklr1a* and *b*, differed in their exon 1 structure, which in *cmklr1b* was shown to be located 6,780 bp downstream of exon 1 in *cmklr1a* (Fig. 3). Sequence data from exon 1 of *cmklr1b* has been deposited in GenBank, accession no. [AY681348](#). The 3' end of the mRNA was assumed to be identical with the corresponding sequence described by Methner *et al.* (1997) (GenBank accession no. [U79525](#)).

3.3. Characterization of the 5' region of the *mcmklr1b* gene

The 5' flanking region of *cmklr1b* was obtained by genome walking upstream of the transcription start site using the same BAC clone as in section 3.2 (Mårtensson *et al.*, 2004). In order to identify the putative promoter region, a 1,389 bp long fragment upstream of the transcription start site was searched for any presence of transcription consensus sites using the TRANSFAC database (Heinemeyer *et al.*, 1999). The region does not contain a TATA box, but other sites – including two consensus sites for CCAAT binding proteins, AP-1-, AP-4-, GATA-1-, GATA-2-, GATA-3- and CREB- motifs – were identified (Fig. 4). Sequence data from the 5' region of *cmklr1b* has been deposited in GenBank, accession no. **AY681348**.

3.4. Northern blot analysis

Since it has been reported that tazarotene, a synthetic RA derivative, up-regulates the chemerin/TIG2 ligand gene (Nagpal *et al.*, 1997) we wanted to investigate if ATRA also effects *cmklr1* gene regulation. BV2 cells were treated for 20 h with ATRA and, for comparison, PMA and LPS. It was found that ATRA strongly up-regulates the receptor transcript (Fig. 5A) while stimulation with PMA and LPS had no effect on *cmklr1* expression (Fig. 5B).

3.5. Real-time RT-PCR

Expression of *cmklr1b* was quantified in skeletal muscle, spleen, brain, kidney, liver, lung and heart mouse tissue using real-time RT-PCR (Fig. 6). The results were compared with the previously described expression of the *cmklr1a* transcript (Mårtensson *et al.*, 2004). With the possible exception of brain, the overall expression levels of *cmklr1b* were too low to allow for proper quantification. The *cmklr1a* gene was primarily expressed in lung and heart tissue while in brain, skeletal muscle and kidney the expressed levels were lower. Expression was also studied in untreated or ATRA-treated BV2 cells and in 3T3 clone A31 cells (used as negative control, data not shown). The *cmklr1b* expression was greatly

increased upon stimulation with 5 μ M ATRA for 4h and 16h, respectively, (Fig. 5C) indicating that *cmklr1b* is an inducible gene. For comparison, the levels of *cmklr1a* were also investigated: no difference was found between non-stimulated and ATRA-stimulated BV2 cells (Fig. 5D).

3.6. Functional activity of the *mcmklr1b* promoter

In order to functionally localize the promoter region of *mcmklr1b* expression, two fragments of 1,000 and 1,389 bp size upstream of the *cmklr1b* transcription start site were subcloned in front of the luciferase reporter gene in the reporter vector, pGL3-E. These constructs, together with the deletion constructs, were transiently transfected into BV2 cells for evidence of transcriptional activity (Fig. 7). The results showed that two shifts in luciferase activity occurred between 755 and 623 bp as well as between 56 and 125 bp upstream of the transcription start site, indicating the presence of two transcriptionally important regions. Sequence analysis of the first region indicated the presence of a GATA-1, -2, or -3 binding site and an AP-1 consensus site. The second region contained two opposing CCAAT binding motifs (Fig. 4). The role of these two elements was elucidated by 2-bp mutations within the CCAAT binding elements (Fig. 9). The construct with the mutated forward CCAAT box resulted in a decreased transcriptional activity compared to the wild-type construct (c10), indicating that this element may increase the transcription of *cmklr1b*. Base-pair changes within the inverted CCAAT element had no significant effect on the transcription activity.

In an attempt to localize a possible ATRA-responsive element in the *cmklr1b* promoter, the deletion constructs were transiently transfected into BV2 cells, which subsequently were stimulated with ATRA (Fig. 7). Each deletion construct gave a higher transcriptional activity in ATRA-stimulated BV2 cells compared to non-stimulated cells. However, no ATRA-responsive element could be identified.

The cell specificity of the *cmklr1b* promoter was analysed by transient transfection of the deletion and mutation constructs into mouse embryonic fibroblast 3T3 (A31) cells not expressing *cmklr1* endogenously (Fig. 1, 8, 9). A transcriptional activity appeared also in these transfected cells, where constructs c1 to c10 gave rise to a similar transcriptional activity pattern. On the other hand, the activity decreased using the c11 and c12 constructs. However, pre-treatment with ATRA decreased the transcriptional activity.

3.7. Interactions between DNA-specific nuclear protein factors and promoter elements

Since different kinds of nuclear proteins are able to bind CCAAT boxes – for example NFY, NF1, C/EBP, and CP2 proteins – EMSA was performed to determine whether the two identified CCAAT boxes were able to bind nuclear proteins, and also to identify the nature of the binding proteins.

The radio-labelled double-stranded forw.CCAAT and inv.CCAAT probes (Table 2) were incubated with nuclear extracts isolated from BV2 cells. A clear-cut shift appeared when incubating the radio-labelled forw.CCAAT probe with nuclear BV2 extract (Fig. 10A), suggesting that a protein was binding to the probe. The shift was abolished by addition of cold “self” competitor (forw.CCAAT probe) but not with a mutated “self” competitor, indicating that the binding was specific (Fig. 10A). In order to examine the nature of the binding protein, different cold probes containing motifs of various CCAAT binding proteins (Table 2) were used in a competition analysis. Only the NFY-binding motif was able to compete for the binding to the forw.CCAAT probe (Fig. 10B).

In order to investigate the identity of the protein interacting with the inverted CCAAT element, radio-labelled inv.CCAAT probe was mixed with BV2 extract. A shift was obtained which disappeared by competition with cold self competitor (inv.CCAAT probe). This was in contrast to the mutated inv.CCAAT probe which had not effect on the shift (Fig. 11A). Cold NFY, NF1, C/EBP or CP-2 probes were used in the further competition

assay. Only cold NFY competitor interfered with the binding, indicating that this protein also binds to the inverted CCAAT element (Fig. 11B).

A super-shift assay was performed using antibodies directed against the NFY subunits A and B, in order to confirm that the obtained shifts with the two CCAAT probes resulted from binding to the NFY protein (Fig. 12). Super-shifts were attained for both types of CCAAT probes, suggesting that NFY is the protein binding to both CCAAT elements.

Since the observed effects of pre-treatment with ATRA on the transcriptional activity may be explained by an increased expression of NFY, an attempt was made to titrate the EMSA shift by adding increasing amounts of extracts from ATRA-stimulated and non-stimulated cells. However, no difference in shift intensity was observed between stimulated and non-stimulated cells (data not shown).

4. Discussion

The receptor, CMKLR1, was recently “de-orphanized” when the natural ligand was isolated from human inflammatory fluids by Wittamer *et al.* (2003) and from a human hemofiltrate by Meder *et al.* (2003). The ligand has been named “TIG2” (Nagpal *et al.*, 1997; Meder *et al.*, 2003) or “chemerin” (Wittamer *et al.*, 2003), and is a protein that plays a pivotal role in the chemotaxis of immature dendritic cells and macrophages (Wittamer *et al.*, 2003). Chemerin/TIG2 also has been implicated in keratinocyte differentiation and psoriasis (Nagpal *et al.*, 1997).

We have previously reported the genomic organization of the mouse *cmklr1* receptor gene and the basal transcriptional regulation of mCMKLR1 expression (Mårtensson *et al.*, 2004). In the present study we describe the identification of an additional splicing variant of *mcmklr1*, designated *mcmklr1b*, which is transcribed from an alternative promoter. 5'-RACE experiments using cDNA from the mouse microglia cell line, BV2, revealed the new transcript to be of a slightly shorter size than the one previously identified in the

neuroblastoma cell line, NB4 1A3 (Mårtensson *et al.*, 2004). The novel transcript turned out to have an alternative exon 1 (51 bp in size) located 6,780 bp downstream of the previously described exon 1 (183 bp in size) (Mårtensson *et al.*, 2004). The finding of an additional splicing variant of the mouse *cmklr1* gene was not entirely unexpected in view of the existence of two splicing variants in the human gene (GenBank accession nos. U79526 for *hcmklr1a* and U79527 for *hcmklr1b*).

The expression of *mcmklr1a* and *b* in different mouse organs (skeletal muscle, spleen, brain, kidney, liver, lung, and heart) were investigated using real-time RT-PCR. Generally, the levels of *cmklr1b* were too low to allow for a reliable conclusion, whereas high levels of *cmklr1a* were found in lung and heart. Although the highest level of the *cmklr1b* transcript was found in brain, it represented less than 10% of the corresponding *cmklr1a* expression. On the other hand it cannot be ruled out that *cmklr1b* is expressed in specific cell populations or may be subjected to up-regulation upon proper stimuli. That *cmklr1b* is an inducible transcript was confirmed when we showed that the expression is greatly increased by ATRA-stimulation in BV2 cells. The levels of the *cmklr1a* transcript was clearly unaffected by ATRA. This indicates that the *cmklr1* gene is bi-functional, giving rise to one ATRA-inducible transcript (*cmklr1b*) and one constitutively expressed transcript (*cmklr1a*). It is notable that RA plays a crucial role in differentiation and development processes of many organs, including the skeleton. In developing bone RA affects various types of cells, such as chondrocytes, during their differentiation (Underhill *et al.*, 2001), a process where mCMKLR1 has been suggested to be involved (Methner *et al.*, 1997). RA has also been shown to be an effective agent in the treatment of diseases, such as acute promyelocytic leukemia (Soprano *et al.*, 2004).

Deletion analysis of the 5'-flanking region of *cmklr1b* indicates that there are two regions of importance for transcription. Within the distal region, located between 623 - 755 bp upstream of the transcription start site, a GATA-1, -2 or -3 as well as an AP-1 consensus motif were identified and may function as enhancer elements. The proximal region,

situated between 56 - 125 bp upstream of the transcription start site, contains a forward and an inverted CCAAT-binding motif and is essential for transcription. Mutational analysis within each CCAAT box separately suggests that, while the forward CCAAT element stimulates transcription, the inverted element is without effect. The deletion constructs were also analysed following transfection into 3T3 clone A31 cells, a cell line not expressing *cmklr1b* endogenously. The results indicated that only the proximal region was important. Mutations of the two CCAAT elements within this region gave similar results also when 3T3 (A31) cells were tested. Taken together, this indicates that while the proximal region seems to be controlled by a common mechanism, present in both BV2 and 3T3 (A31) cells, the cell specificity may reside in the distal region.

The observation that ATRA up-regulates *cmklr1b* gene expression, along with its reported effect on chemerin/TIG2 expression, indicates a possible co-regulation of receptor and ligand genes. Although ATRA stimulation resulted in an overall increase in transcriptional activity, deletion analysis of the *mcmlr1* promoter region did not reveal any well-defined RA-responsive element, also known as RARE. Since the RA signalling cascade is complex it is possible that the activation occurs through an indirect mechanism. Another possibility is that the RARE element is not present in the 1,389 bp fragment but is located further upstream, or even downstream, in the genome. In general, RA affects gene transcription by binding to the nuclear RA receptor families of which the particular RARs specifically bind the trans-RA isomer (Chambon, 1996). The RARs function as ligand-activated transcription factors binding specific DNA sequences, the RAREs, thereby regulating the RA-responsive gene (Chambon, 1996). Besides this classical way of action, RARs also indirectly affect transcriptional regulation by interacting with other transcription factors, such as AP-1 and GATA-2, thereby regulating gene transcription from other motifs in the DNA (Pfahl, 1993; Tsuzuki *et al.*, 2004). This can be a possible way of action for RA in up-regulating the *cmklr1b* transcript. Activation of the *cmklr1b* gene might be part of a consorted activation of several genes involved in related functions, as previously indicated by the above-mentioned co-regulation of CMKLR1 and its ligand. The chemerin/TIG2

ligand has been shown to be up-regulated by the synthetic RA analogue, tazarotene, which also binds to the RARs (Nagpal *et al.*, 1997).

Although the forward CCAAT element is essential for transcription, mobility shift- as well as super-shift assays showed that both of the identified CCAAT boxes bind the same transcriptional protein. This binding protein was identified as NFY. It consists of three subunits and is a common factor in the functional regulation of transcriptional activity (Mantovani, 1999).

The present study provides the first description of an alternative splicing variant of the mouse *cmklr1* gene, further increasing the complexity of the chemerin receptor (CMKLR1) regulation and maybe also its tissue-related functional significance. The emerging picture of the regulation of the *cmklr1* gene indicates a bi-functional mode of action including an inducible, possibly tissue specific, and a basal transcription control. The results from the functional experiments with ATRA emphasises the potential involvement of this receptor in, i. a., bone modelling.

Acknowledgments

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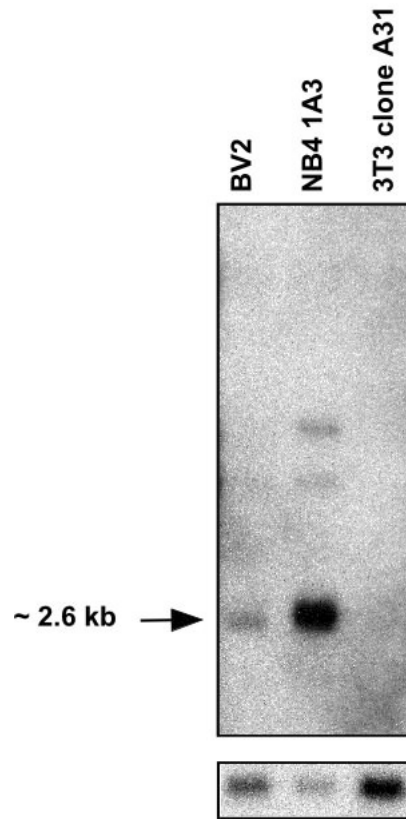


Fig. 1. Northern blot analysis of *mcmklr1* expression in the mouse cell line BV2 (microglia), NB4 1A3 (neuroblastoma), and 3T3 clone A31 (embryonic fibroblast). The previously studied NB4 1A3 cell line is used for comparison. The 3T3 clone A31 cell line represents a negative control because it does not express *cmklr1* endogenously. Six μ g mRNA were loaded into each well. The hybridisation probe consisted of the full coding region of *mcmklr1*. Transcripts are indicated with arrow. Signals from the β -actin probe are shown below.

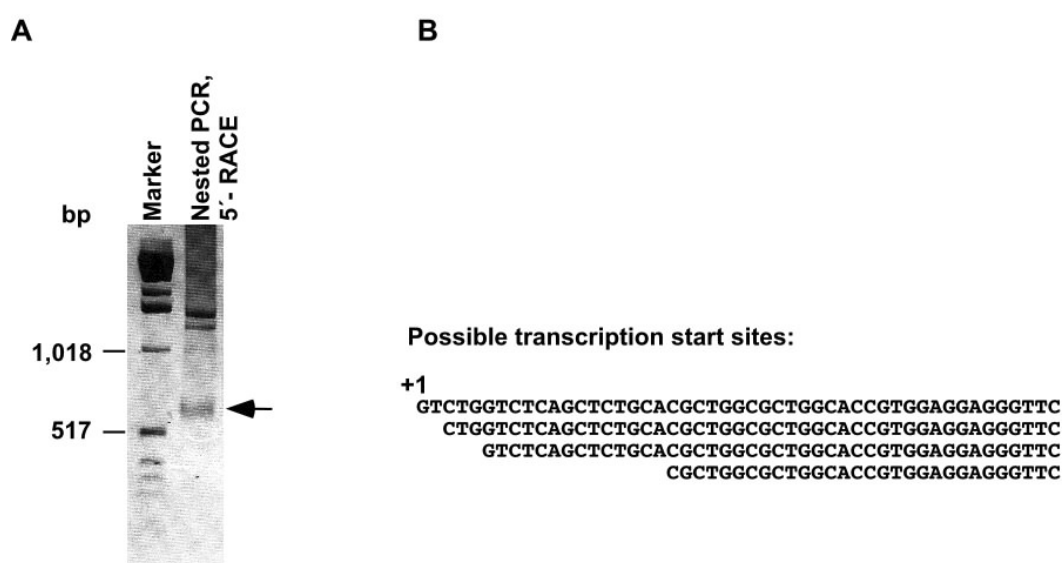


Fig. 2. 5'-RACE analysis. (A). Identification of the transcriptional initiation site of *mcmklr1b* by 5'-RACE using cDNA from BV2 cells. The RACE resulted in 3 fragments that were subcloned and sequenced. The relevant PCR-product is indicated by arrow whereas the two larger unmarked fragments, >1,018 bp in size, represent unspecific amplified bands. The size marker lane with two band sizes is shown. (B). Sequencing of different subclones of the relevant 600-bp fragment revealed 4 possible transcriptional start sites.

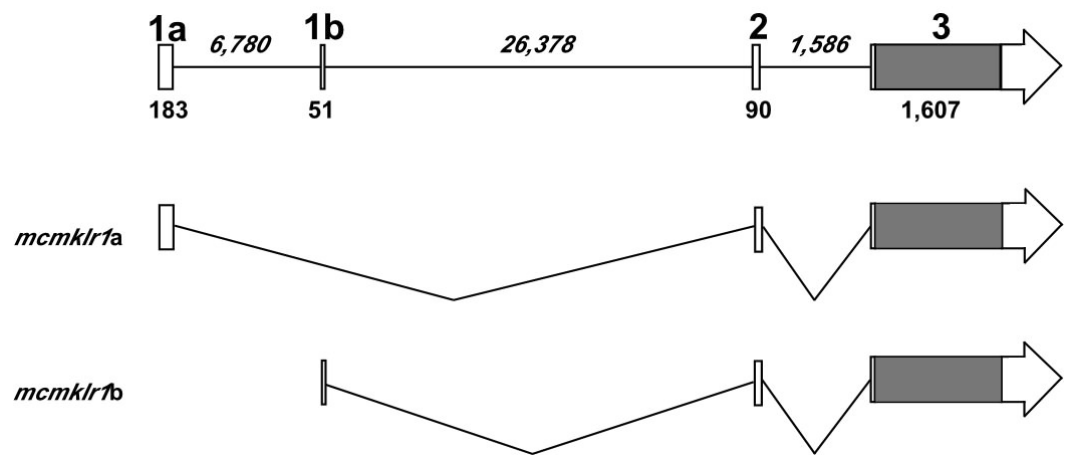


Fig. 3. Genomic organization of the mouse *cmklr1* gene and the alternative splicing transcripts *mcmklr1a* and *b*. Sequence data of *cmklr1a* is found in GenBank with the accession nos. [AY342407](#), [AY342408](#), [AY342409](#) and [U79525](#) whereas sequence data of *cmklr1b* has been deposited with GenBank accession no. [AY681348](#). The receptor gene is located to mouse chromosome 5, region F. The exons (1-3, lengths in roman figures) are shown as boxes. Shaded boxes display the coding region while open boxes indicate untranslated regions. Intron lengths are shown in italic figures. Sizes of exons and introns are given in number of base pairs.

```

-1389 GGTGACGGC CCGGGCTGGT CCTCAAGTAA GATTGGAAAG AGTGAGATT
-1339 TTGGTGCTGC GGGGTGGAGA GCAGTGGGTT TCCATGGGCG TGCCGATGAA
-1289 AGAAATGTTT TTGACAGAAG CCAGTTGGCA GTGGCCAGTG ATCCCAAAGC
-1239 CAGCTGCTCT GTCCGGGGAG TCAGCTTCTA GCCTTGCCCC TTGGCCTCCT
AP-4
-1189 CATCCAGGCC TCCTTCATCTGCTTGGGCA GATCTGAGCA GCCTTTGGCT
GATA-1
-1139 GCAGCCTGGG TCCTATGTCT GAGCTCTGGT GGGGGTGCCT GGGCGTCCCC
-1089 TGTCATGTG CTGTGTAAT GTTGAAGTA CCCATTGTC ATTCAGCAGG
AP-1
-1039 GCCCAGGGTC ACTTCTGCTT TCTCGGGATA CAGGTTCTGA GATCTCAGCC
-989 CAGGCTGGTG ATGCAGTAGT TCTTGTGCAA GGAGGTGTGT GGTGAGGGAG
-939 ACTGAGCGGA GGGCACACGC AGTGTTCCTT GGTGGCAGG GGTGTGAGC
-889 AGACAAGGAG ACCCTGCTGA GCCACGTGGG ACAAGTCCCG CAGCTCTGGT
-839 TTGGCTCCTG AGTCTCCTCA GTCTATCCT CTCTCAGAGT TCGTAGGAT
-789 GTTGTGGTCT TTGGCCTGTA GTCCCTGCCC AGTGAGTGTC AGGTTGTGG
-739 TTAAGGACAG ATGTCAAAG CTATCCCT GTTGATGAC CTGTCTGAG
GATA-1/-2/-3
-689 AGTCTGCAGA GTCTCCGAGG GTCTTGTGTG AGAGGGACAA GTTCACACTG
-639 GGCTAGTTTC CTTTCTGTG CTGTGGACCA CAGCCTGACTAAAAGCCGAG
AP-1
-589 GAGAGGGTTT GTTCAGCTCA CAGTTCCTGT CACAGTCAAT CACTGAGGAG
-539 AAGTCAAGGC AGAACTCAA GCACCTAGTC ACGTCACAGC CGCAGTCGGG
CREB
-489 AGCAACAGT AAAGCTGTCC TGGCTGCCTG CAGGCTCCCG CAGCGGGGTG
-439 GGTCTTCTCA CACACACACA CACTCACACA CACACACACA CACACACACA
-389 CACACTCTCA CACACACACA CACACACACT CTCTCACACA CACACACACA
-339 CACACACACA CTCGTGATAA ATTAGCAGTT ACTACAGCTT CAGGATTACA
-289 TCCTCCCAAC CTCCCTCACC CCCACCCCA CGCAGTTCCC GTAATTTTCT
-239 CATTGATCAC AAATATTGAG TGACTTTTCG CTGTGTCAAT TCTGGGAGTC
-189 AGGATGCGGC CATCTTTGAG GGTCTGTCCA TCATTGCAAG CGACAGTGAG
-139 GGCAATGCA CGTGTGAGA GGTTCAGACC ATTCTCTTCCTTGATTG
inv.CCAAT
-89 CTCTGAGGAACACAGTATA GCAATAG CACAGATGTG GGTGTGGCCA
forw.CCAAT
-39 CAGCTGCA GGCTGTGCCA GGCATAAGAA GTCTTCCCGG TCTGGTCTCA
AP-4 +1
12 GCTCTGCACG CTGGCGCTGG CACCGTGGAG GAGGGTTCAG GTATTTCAG
62 TCACGCGCAG TAACAGACCA GCCAAGGACC AGGACTGGAG TTCTGTTCTA
112 CAACGGTGGA ACAGTGAACG GTCTCCAAG AGATG
+144

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Fig. 4. Nucleotide sequence of the 5'-flanking region of the *mcmklr1b* gene. The fragment was isolated from a BAC clone, subcloned, sequenced (GenBank accession no. [AY681348](#)) and analysed for the presence of transcription consensus motifs. The putative transcription initiation site is shown in bold (+1) and indicated by arrow. Downstream of the transcription start site, cDNA sequence is shown where the translation initiation codon

is in italics. Potential transcription binding motifs are boxed and presented in bold letters. Underlined and indicated sequences represent probes used in EMSA.

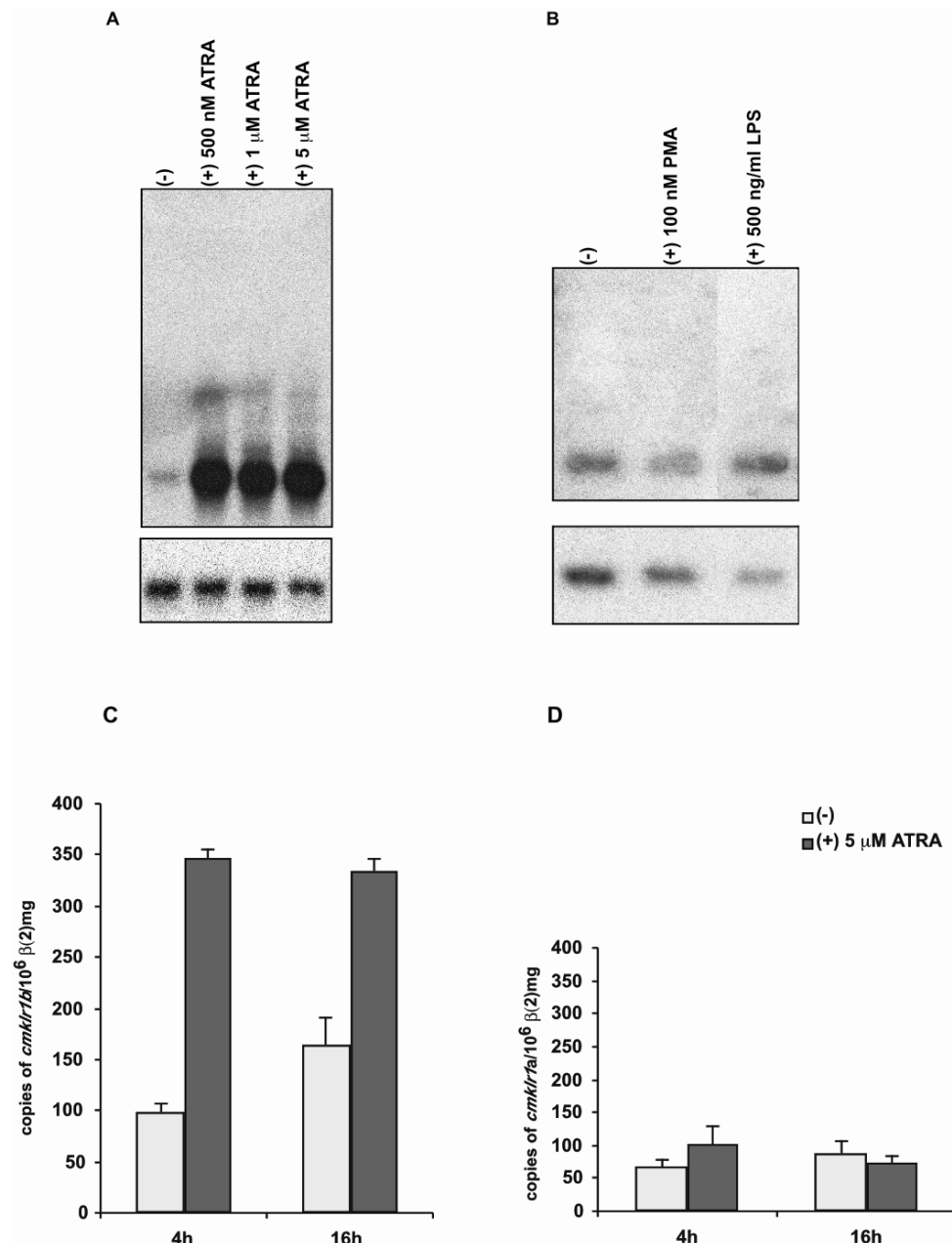


Fig. 5. Effects of different stimulating substances on *cmklr1* mRNA expression. (A) BV2 cells were treated with different concentrations of ATRA and *mcmklr1* mRNA expression was analysed after 20 h using northern blot analysis (the hybridisation-probe consisted of the full coding region of *mcmklr1*). Wells were loaded with 4 µg mRNA of BV2 cells without stimulation (lane 1), or stimulated with 500 nM (lane 2), 1 µM (lane 3) or 5 µM ATRA (lane 4). Signals from the β-actin probe are shown below. (B) Four µg mRNA from BV2 cells without stimulation (lane 1), and stimulated for 20 h with 100 nM PMA (lane 2), or 500 ng/ml LPS (lane 3). The hybridisation probe consisted of the full coding region of *mcmklr1*. Signals from the β-actin probe are shown below. (C) BV2 cells with/without pre-treatment with 5 µM ATRA for indicated time lengths followed by *mcmklr1b* mRNA expression analysis using real-time RT-PCR. (D) As for (C) followed by *mcmklr1a* expression analysis. Copy-numbers of *mcmklr1a* and b are normalised to the copy-numbers of the house-keeping gene, β(2)-microglobulin (β(2)mg). Bars depict means + SD from duplicate samples.

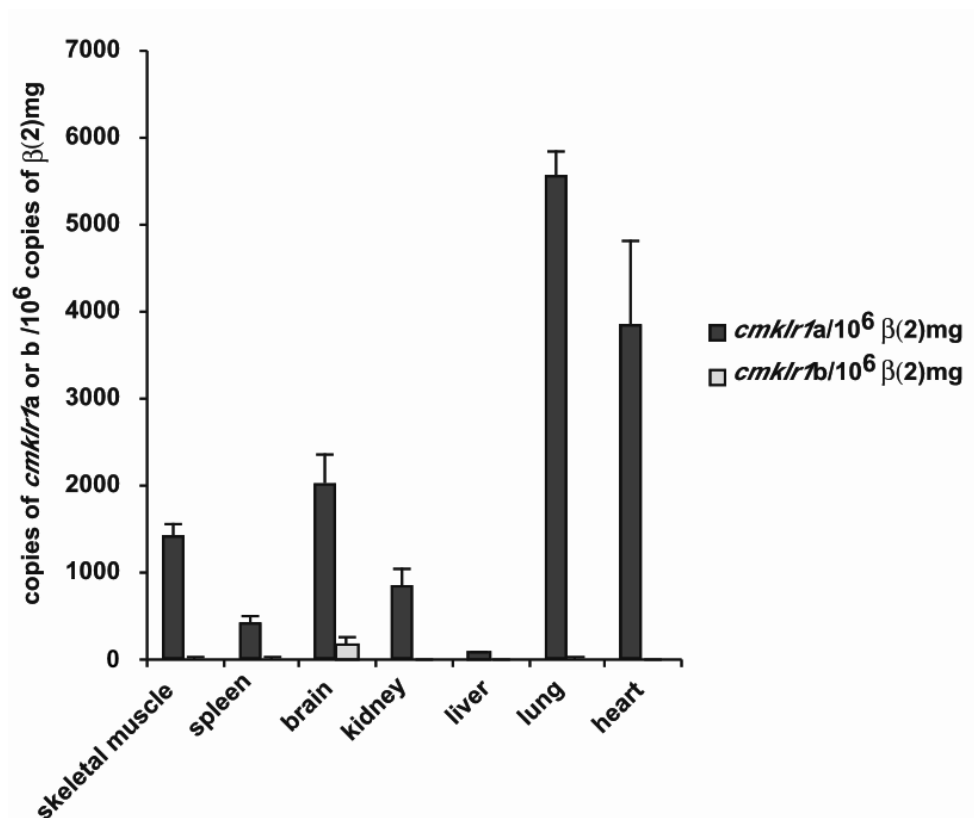


Fig. 6. The distribution of *cmklr1a* and *b* transcripts in different mouse tissues. Real-time RT-PCR analysis was performed on cDNA obtained from mRNA isolated from skeletal muscle, spleen, brain, kidney, liver, lung, and heart. Copy-numbers of *cmklr1a* and *b* are normalised to the copy-numbers of the house-keeping gene, β(2)-microglobulin (β(2)mg). Bars depict means + SD from duplicate samples.

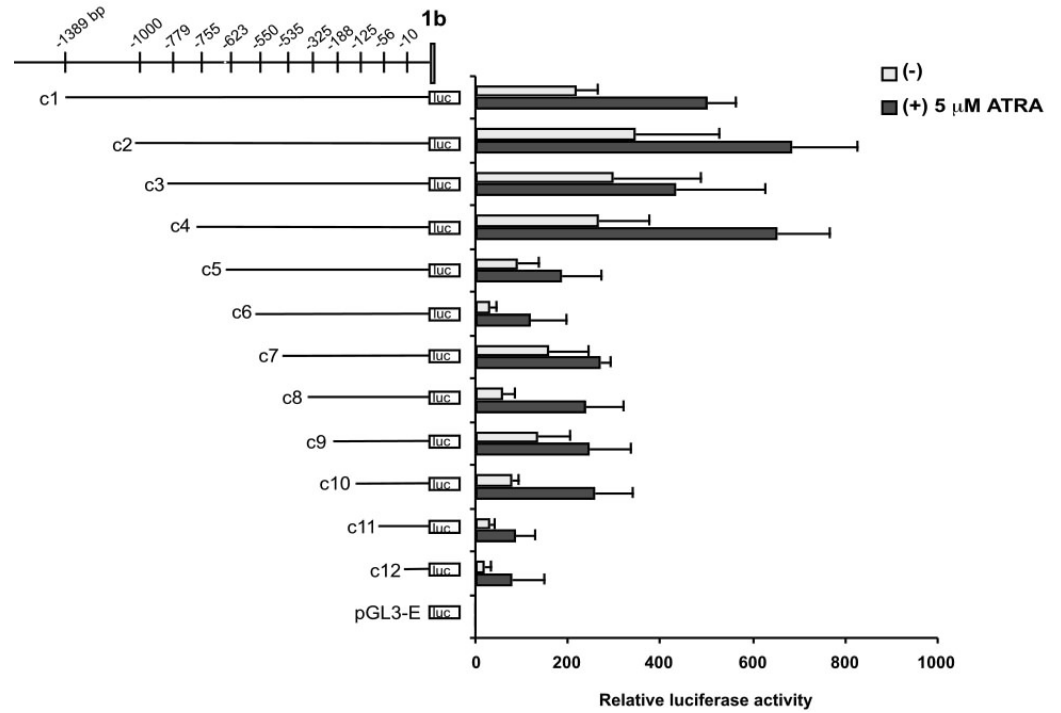


Fig. 7. Transcriptional activity of 5' deletion constructs of the *mcmklr1b* promoter region in non-stimulated and ATRA-stimulated BV2 cells. Deletion constructs (c1-12), generated with exonuclease III digestion, were transiently transfected into BV2 cells. Twenty-four hours after transfection, 5 μ M ATRA was added to cells for stimulation. Cells were harvested after 40 h and assayed for luciferase activity. Bars represent means + SEM (n=3) of the luciferase activity relative to activity from the empty vector (pGL3-E). A pGL3-Enhancer vector, containing an SV40 promoter, was used as positive control in the assay (not shown).

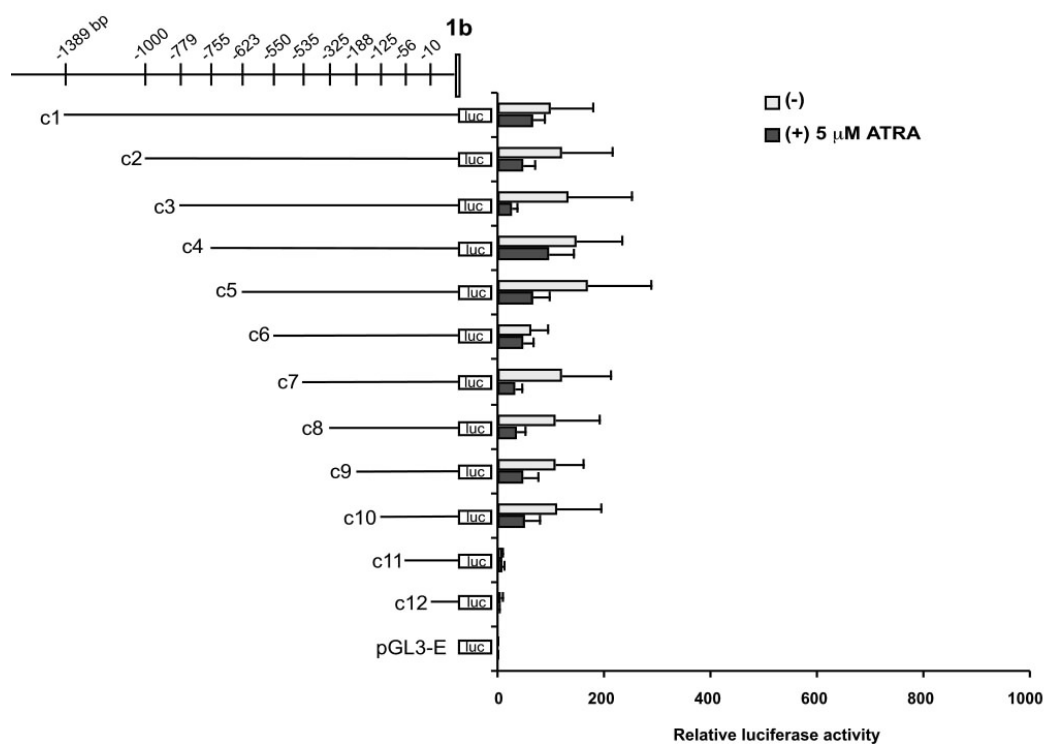


Fig. 8. Transcriptional activity studied in 3T3 clone A31 cells. The same protocol as for the BV2 cells illustrated in Figure 7.

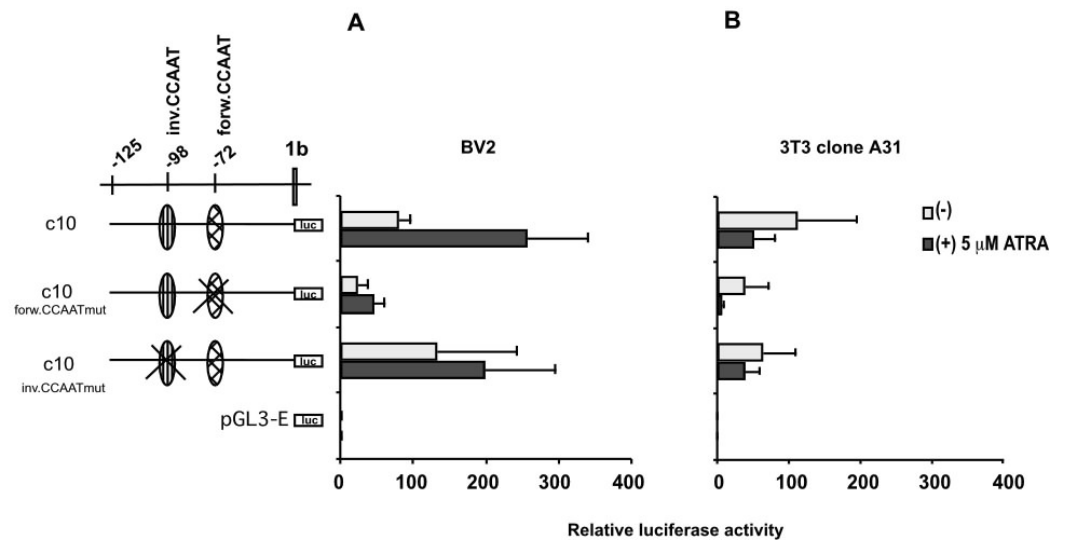


Fig. 9. Transcriptional activity of constructs where the forward (c10forw.CCAATmut) and the inverted (c10inv.CCAATmut) CCAAT boxes were mutated separately and are illustrated as crossed-out ovals. The constructs were transiently transfected into (A) BV2 and (B) 3T3 clone A31 cells. Twenty-four hours after transfection cells were stimulated with 5 μ M ATRA and after 40 h assayed for luciferase activity. Bars represent means + SEM (n=3) of the luciferase activity relative to activity from empty vector (pGL3-E). A pGL3-Enhancer vector, containing an SV40 promoter, was used as positive control in the assay (not shown).

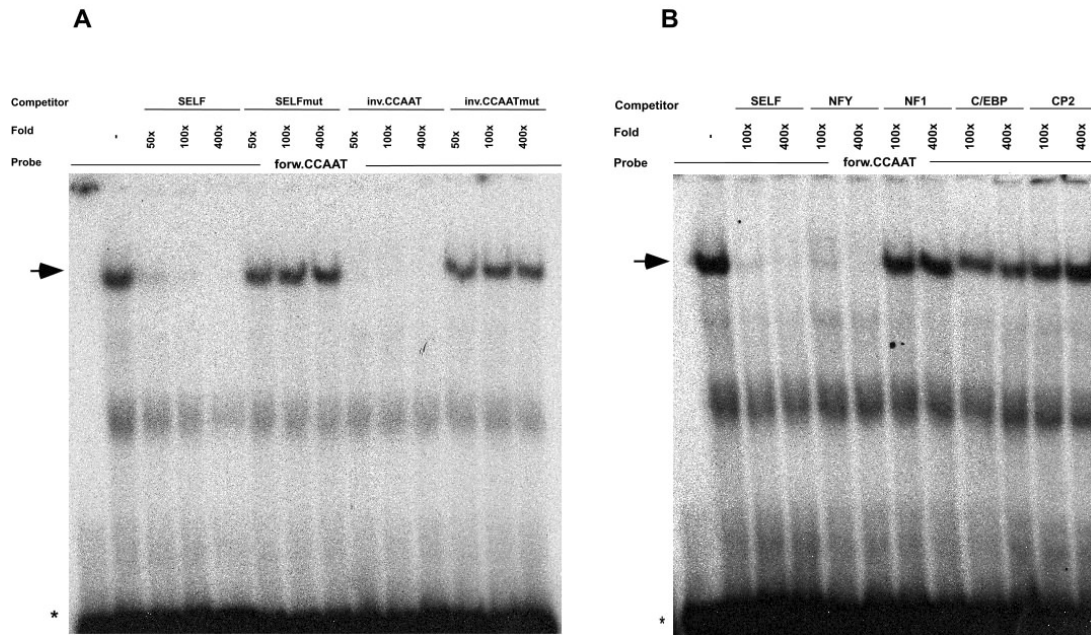


Fig. 10. Identification of the transcriptional factor binding the forward CCAAT element using competition EMSA. (A) Radioactively labelled forw.CCAAT probe incubated with nuclear BV2 extract (8 μ g) and excess of different cold competitors (SELF, SELFmut, inv.CCAAT, and inv.CCAATmut). (B) Radioactively labelled forw.CCAAT probe incubated with nuclear BV2 extract (8 μ g) and excess of different cold competitors (SELF, NFY, NF1, C/EBP, and CP2). Gel-shift is indicated by arrow and free probe with asterisk.

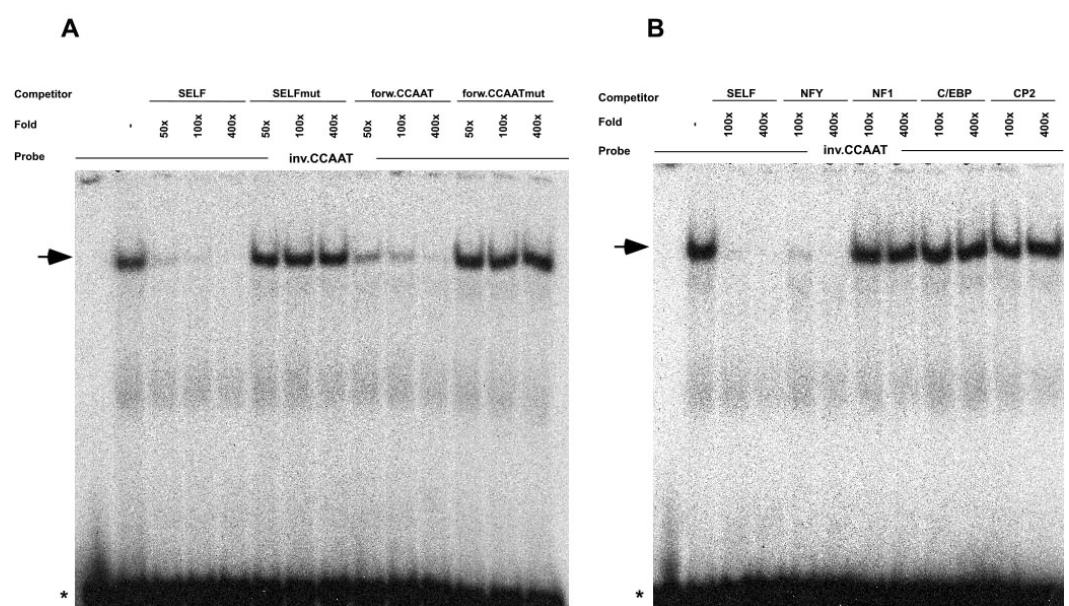


Fig. 11. Identification of the transcriptional factor binding to the inverted CCAAT element using competition assays. (A) Radioactively labelled inv.CCAAT probe was incubated with nuclear BV2 extract (8 μ g) and excess of different cold competitors (SELF, SELFmut, forw.CCAAT, and forw.CCAATmut). (B) Radioactively labelled inv.CCAAT probe was incubated with nuclear BV2 extract (8 μ g) and excess of different cold competitors (SELF, NFY, NF1, C/EBP, and CP2). Gel-shift is indicated by arrow and free probe with asterisk.

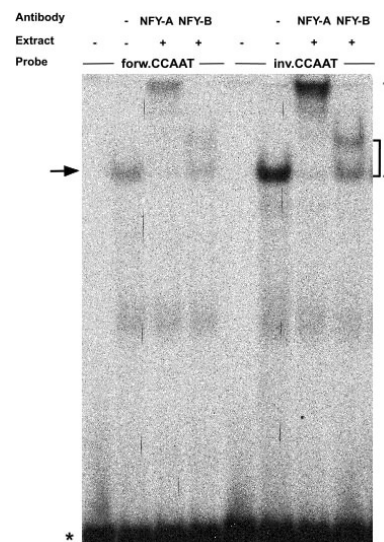


Fig. 12. Identification of the protein binding to the forward and inverted CCAAT elements using super-shift assay with antibodies specific to NFY subunits A and B. Radio-labelled probes were incubated with BV2 nuclear extracts (8 μ g) in the presence or absence of NFY antibodies (2 μ g). Gel-shift is indicated by arrow, super-shift with bracket, and free probe is marked with asterisk.

Table 1. Oligonucleotides^a used in real-time RT-PCR.

Name	Sequence in 5' to 3' direction
mouse β (2)-microglobulin	ATGGGAAGCCGAACATACTG CAGTCTCAGTGGGGGTGAAT
<i>mcmklr1a</i>	CAAGCAAACAGCCACTACCA TAGATGCCGGAGTCGTTGTAA
<i>mcmklr1b</i>	CACCGTGGAGGAGGGTTCA TAGATGCCGGAGTCGTTGTAA

^aOligonucleotides were synthesized by MWG Biotech.

Table 2. Oligonucleotides^a used as probes in gel-shift and super-shift assays.

Name	Sequence in 5' to 3' direction (sense strand) ^b	Location
forw.CCAAT	GAACACAGTATAG CCAAT GAGCACAGATGTGGGT	-81/-47
forw.CCAATmut	GAACACAGTATAG ^{aaa} AAT GAGCACAGATGTGGGT	
inv.CCAAT	TTTCTCTTCCTTGTG ATTGG CTCTGAGGAACACAG	-108/-74
inv.CCAATmut	TTTCTCTTCCTTGTG ATTt tCTCTGAGGAACACAG	
CBF/NFY cons	AGACCGTACGTG ATTGG TTAATCTCTT	
C/EBP cons	TGCAGATTG CGCAAT CTGCA	
NF1 cons	TTTTGGATTGA AGCCAAT ATGATAA	
CP2 cons	GTTTTACTCGGTAGAGCAAG CACAA ACCAG	

^aAll oligonucleotides were synthesized by MWG Biotech.

^bDNA binding motifs are indicated in bold letters. Mutations relative to the corresponding wild-type sequence are shown in lower-case letters.