Two different negative regulatory elements control the transcription of T-cell activation gene 3 in activated mast cells

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T-cell activation gene 3 (TCA3) encodes a β -chemokine that is transcriptionally regulated in mast cells; the gene has a functional NF- κ B element at positions –194 to –185. The 5'-flanking region of this gene is also known to have a negative regulatory region between –2057 and –1342. To characterize the negative regulatory elements (NREs), this region was sequenced and then digested by *Hind*III enzyme into two fragments, NRE-1 (–2057 to –1493) and NRE-2 (–1492 to –1342). Both NRE-1 and NRE-2 in the 5'–3' orientation inhibited chloramphenicol acetyltransferase (CAT)-protein synthesis by a TCA3–CAT construct transfected into mast cells that were then activated. Only NRE-1 inhibited CAT-protein synthesis in the 3'–5' orientation. Further deletion of the 5' region of NRE-1 partially abolished the inhibitory activity. Both NRE-1 and NRE-2 inhibited the activity of a CD20–CAT con-

struct independent of cell activation. Electrophoretic mobility shift assays showed DNA–protein complex formation with subsequences (CCCCATTCT) of NRE-1 (NRE-1a) and (CCATGA) of NRE-2 (NRE-2b). NRE-1a appears to be novel. NRE-2b is identical with a putative silencer motif in the α_{IIb} integrin gene. Site-directed mutagenesis demonstrated that both NRE-1a and NRE-2b are important in the negative regulation of TCA3 promoter activity. *In vivo* ligation-mediated PCR footprinting of the NRE-2 region revealed protection between –1372 and –1354, which contains NRE-2b. The data thus demonstrate identity of a silencer motif, here termed NRE-2b, in both the α_{IIb} integrin gene and the TCA3, and that this silencer region in mast cells is functional both *in vivo* and *in vitro*. Further, evidence is presented that the promoter for TCA3 contains a novel silencer motif, termed NRE-1a, characterized by a CT-rich sequence.

INTRODUCTION

The T-cell activation gene 3 (TCA3) is a member of the β -chemokine family [1], which is uniquely expressed in T-lymphocytes and mast cells, but not in B-lymphocytes, monocytes/macrophages or fibroblasts [2]. This gene was originally isolated from a murine T-cell clone by subtractive hybridization on the basis of its activation-specific message accumulation profile [3]. TCA3 is induced in CD4+ and CD8+ T-cell clones after antigen or mitogen stimulation [2], and both Th1- and Th2-type cells express TCA3. TCA3 message is induced in murine mast cells after stimulation with PMA and the ionophore A23187, or after aggregation of the high-affinity IgE receptor (Fc ϵ RI) [4]. The structure of TCA3 consists of three exons and is similar to the structures of the MIP-1 genes [5]. Recombinant TCA3 causes neutrophil and macrophage chemotaxis *in vivo* [1,6]. TCA3 protein or TCA3-transfected Chinese hamster ovary cells when injected into mice with myeloma tumours induce complete tumour regression [7]. The human homologue of murine TCA3 is believed to be I-309 [8], which has been shown to cause monocyte chemotaxis *in vitro* [9]. Regulation of the expression of TCA3 has been extensively examined in mast cells following aggregation of Fc ϵ RI. Northern-blot analysis has demonstrated that TCA3 message appeared in mast cells within 2 h of mast cell activation, with a $t_{1/2}$ of 30 min. Nuclear run-on experiments have revealed that the induction of TCA3 message

is largely due to an increase in the level of transcription *de novo* [10]. Analysis of the promoter region has demonstrated that inducible gene expression is directed by a region extending –82 nt from the transcription start site, and that expression is enhanced by a region extending further to –1342. Electrophoretic mobility shift assays (EMSAs) revealed that protein in activated mast cells binds to a nuclear factor (NF)- κ B element of TCA3. In these studies a putative inhibitory element was demonstrated between –2057 and –1342. While there is information about negative regulatory elements (NREs) controlling the expression of genes including interleukin (IL)-4 [11,12], the IL-2 receptor [13] and integrins [14], nothing is known about the NREs controlling the expression of chemokines. Limitation of the expression of certain chemokines and thus the influx of specific inflammatory cells would be one mechanism by which an inflammatory response could be modified. This led us to further define the putative suppressor region of the promoter of TCA3.

In order to verify the negative regulatory activity of the –2057 to –1342 region, a series of constructs with TCA3 and CD20 promoters containing NREs were generated. Following comparative sequence analysis, EMSAs were performed, which revealed specific DNA–protein complex formation in the regions –2002 to –1983, containing a NRE designated NRE-1a, and –1377 to –1348, containing a NRE termed NRE-2b. *In vivo* footprinting and site-directed mutagenesis confirmed that both NRE-1a and NRE-2b are necessary for the negative regulatory

Abbreviations used: TCA3, T-cell activation gene 3; NRE, negative regulatory element; Fc ϵ RI, high-affinity IgE receptor; EMSA, electrophoretic mobility shift assay; IL, interleukin; DMEM, Dulbecco's modified essential medium; CAT, chloramphenicol acetyltransferase; LM-PCR, ligation-mediated PCR; CMV, cytomegalovirus; CNRF-1, chemokine negative regulatory factor-1; DMS, dimethyl sulphate; NF, nuclear factor.

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The nucleotide sequence will appear in GenBank Nucleotide Sequence Databases under the accession number U77972.

activity between -2057 and -1342. As will be shown, NRE-2b is identical with the putative silencer motif of the α_{1b} integrin gene and NRE-1a appears to be novel.

MATERIALS AND METHODS

Cells

The cloned mast-cell line Cl.MC/C57.1 (provided by S. Galli, Harvard Medical School, Boston, MA, U.S.A.) was maintained as described [15]. Briefly, cells were cultured in Dulbecco's modified essential medium (DMEM) containing 10% heat-inactivated fetal-calf serum, 50 mM β -mercaptoethanol, 4 mM L-glutamine and 100 mg/ml penicillin/streptomycin (complete DMEM) in a humidified 5% CO₂ incubator. The cell number was adjusted to 5×10^5 cells/ml twice weekly by adding fresh media.

Stimulation conditions

For activation, mast cells were adjusted to a density of 1×10^6 cells/ml in complete DMEM and PMA (stock solution 1 mg/ml in DMSO) added at 50 ng/ml and A23187 (stock solution 10 mM in DMSO) added at 0.5 mM. Next the cells were incubated at 37 °C for 3 h in a humidified atmosphere of 5% CO₂/95% air. Cells were then centrifuged at 400 *g* at 4 °C for 10 min and the cell pellet was resuspended in complete DMEM. The reaction was terminated by centrifugation at 400 *g* at 4 °C and the cells were washed twice by addition of ice-cold PBS.

Chloramphenicol acetyltransferase (CAT) reporter gene constructs

pE1bCAT (provided by D. Margolis, University of Massachusetts, Worcester, MA, U.S.A.) is a 4-kb pSP72 derivative carrying the CAT gene and E1b TATA box. Serial deletions of the 5'-end of the 2 kb CAT construct were made using the Erase-a-Base system (Promega, Madison, WI, U.S.A.). To generate an NRE-1- or NRE-2-TCA3-CAT construct, a promoter region encompassing the -2057 to -1493 region or the -1492 to -1342 region upstream from the transcription start site of TCA3 genomic DNA was isolated and inserted into the 5'-end (*Hind*III site) of a 0.7 kb or a 0.3 kb TCA3-CAT construct. To generate NRE-1- or NRE-2-CD20-CAT constructs, these NRE-1 or NRE-2 fragments were inserted into the *Hind*III site, immediately upstream of the human CD20 promoter (provided by J. Kehrl, NIH, Bethesda, MD, U.S.A.). Clones with 3' to 5' orientations were made for each NRE-TCA3-CAT construct or each NRE-CD20-CAT construct as internal controls. None of the TCA3-CAT constructs had an E1b TATA box.

Nucleotide sequence analysis

To verify the sequence and orientation of each CAT construct, DNA sequence analysis was performed using a Sequenase Version 2.0 kit (United States Biochemical, Cleveland, OH, U.S.A.) [16]. The DNA sequence was obtained by directed sequencing of inserts in the pSP72 vector with primers flanking the SP6 and T7 promoter regions to determine both strands.

Transfection and CAT assay

Transient transfection and CAT assays were performed as described with minor modifications [10].

EMSA

EMSAs were performed as described [17,18] to analyse binding

of nuclear factors to both NRE-1 and NRE-2 sites. Double-stranded oligonucleotides were synthesized and labelled with [γ -³²P]ATP using the T4 polynucleotide kinase (Promega). Radio-labelled probe was incubated with 1 or 5 mg of nuclear extracts of either unstimulated mast cells or cells stimulated for 3 h with PMA and A23187. For competition assays, unlabelled specific oligonucleotides were used. For non-competitive assays, unlabelled unrelated oligonucleotides were employed. Non-denaturing PAGE (4%, w/v) was performed at 100 V for 3 h using a desk-top electrophoresis apparatus, vertical slab gel unit SE 600 (Hoeffer Scientific Instruments, San Francisco, CA, U.S.A.), and the gel was dried and exposed for autoradiography.

In vivo methylation of cells and isolation of genomic DNAs

Cells, (50–100) $\times 10^6$, were methylated *in vivo* as described [19,20]. Subsequently, genomic DNAs were isolated directly from *in vivo* methylated and control cells by suspending them in lysis buffer [10 mM Tris/HCl, pH 8.0/100 mM NaCl/25 mM EDTA/0.5% (v/v) SDS/300 μ g of proteinase K per ml] followed by incubation at 55 °C overnight with gentle agitation. DNAs were extracted by a standard method [21]. *In vitro* methylation and subsequent cleavage of naked DNA with piperidine were carried out as described [22]. A trace amount of piperidine remaining in DNA preparations was removed by freeze-drying in a Speed-Vac (Savant) and by chromatography with a Sephadex G-50 spin column.

In vivo ligation-mediated (LM)-PCR footprinting

LM-PCR footprinting was performed as described with modification [23]. Exponential amplification was 3 min at 95 °C and 20 cycles with 1 min at 95 °C, 2 min at 63 °C and 3 min at 76 °C. The labelling cycle was 3 min at 95 °C and 2 cycles each for 1 min at 95 °C, 2 min at 66 °C and 10 min at 76 °C. For each footprinting experiment, a parallel cyclic PCR sequencing reaction was performed using a Sequitherm cycle sequencing kit with 1 pmol of an end-labelled primer 3. The sequencing method was employed as per the manufacturer's recommended procedures (Epicenter Technologies, Madison, WI, U.S.A.).

Oligonucleotide primers

For top-strand LM-PCR genomic footprinting analysis, specific primer sets were used for the initial extension reaction (primer 1), the exponential amplification reaction (primer 2) and the labelling reaction (primer 3). Primer sets for the top strand of NRE-2 were:

primer 1 GGTTGTGCAGCCAGATAAAAAACAG;

primer 2 TATGCAGTGGGCAGTACTTGGGATAAGC;

primer 3 AGCTCATAGGAGGCCCTCAAGAAAAAGCAG.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the unique site elimination mutagenesis kit (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Briefly, a target mutagenic primer and a selection primer were annealed to both NRE-1- and NRE-2-0.7 kb TCA3-CAT constructs. The selection primer was generated by converting an *Avi*II site into a *Bcl*I site in the *amp* gene carried in the NRE-TCA3-CAT construct. Plasmids with this primer still retained β -lactamase activity and displayed ampicillin resistance. Wild-type NRE-TCA3-CAT constructs, which still contained the *Avi*II site, were linearized by restriction enzyme digestion with *Avi*II. This reduced their ability to transform *Escherichia coli*. A repair-defective (*mutS*) strain of *E. coli* was transformed with NRE-

TCA3-CAT constructs from the mutagenesis reaction. The plasmid DNA from transformed *E. coli* cells was used to transform competent *E. coli* DH5 α cells following a second round of *Avi*III selection. The mutated positive transformants were confirmed by sequencing analysis.

RESULTS

Evaluation of the 5' flanking sequence of TCA3 for negative regulatory activity

We have previously reported that the region between -2 kb and -1.3 kb contains negative regulatory activity [10]. To characterize this 5'-flanking sequence, this region was divided into two fragments, NRE-1 (-2057 to -1493) and NRE-2 (-1492 to -1342) on the basis of *Hind*III sites. These fragments were inserted into two fully functioning -728 and -324 TCA3-promoter-CAT constructs. Both NRE-1 and NRE-2 inhibited CAT-protein synthesis of both -728 and -324 TCA3-CAT constructs in the 5'-3' orientation (Figure 1). NRE-1, but not NRE-2, was able to inhibit CAT-protein synthesis of both -728 and -324 TCA3-CAT constructs when placed in the 3'-5' orientation. Both NRE-1 and NRE-2 were thus distance (from

transcription start site)-independent, but only NRE-1 was orientation-independent.

Determination of the minimal sequence between -2057 and -1493 required the inhibition of the promoter activity of TCA3 in mast cells. Progressive deletions of the 5'-flanking region of TCA3 were next generated and sequenced, and the transcriptional activity of the genomic DNA segment was assessed by CAT assay (Figure 1, constructs 13 and 14). The 5'-deletion construct, -2014 to -1342 , retained the negative regulatory activity of NRE-1. Further deletion of the 5'-end of the 2 kb CAT construct, -1798 to -1342 , resulted in a loss of inhibitory activity of NRE-1. These results are consistent with the conclusion that the core motif for NRE-1 is located between -2014 and -1798 .

Sequence analysis of the 5'-flanking region containing negative regulatory activity

To further investigate the transcriptional regulation of TCA3, we next sequenced the region encompassing -2057 to -1342 (Genbank accession number is U77972). Comparison of this sequence against published silencer motifs known to bind to

Figure 1 Schematic diagram of the TCA3 promoter constructs with or without NREs and CAT protein levels (pg/100 μ g of total cellular protein) expressed in mast cells stimulated with PMA and A23187

A series of constructs containing the sequences encompassing -2057 to -1493 (NRE-1) or -1492 to -1342 (NRE-2) were joined to the fully functioning TCA3 promoter constructs -728 and -324 TCA3-CAT (constructs 1 and 6 in order from top to bottom) in both orientations (constructs 2, 3, 4, 5, 7, 8, 9 and 10). The 5'-deletion mutations, -2014 to -1342 (construct 13) and -1798 to -1342 (construct 14), were generated using 2 kb TCA3-CAT (construct 12); 1.3 kb TCA3-CAT was used as a positive control (construct 11) (see the Materials and methods section). The construct base numbering parallels the position of the upstream region of TCA3 with respect to the upstream sequence from the transcription start site. Suppression of reporter gene expression NRE-TCA3-CAT constructs is determined in mast cells stimulated with PMA and A23187, where equimolar amounts of TCA3-CAT constructs were used for transfection. Induction of reporter gene expression of NRE-CAT constructs in unstimulated mast cells was not detected (results not shown). Data are the average from one experiment performed in duplicate. Similar data were obtained in two additional experiments. Abbreviation used: mcg, μ g.

Table 1 Comparison of TCA3 NRE sequences with known/putative silencer motifs

The numbering parallels the position of the 5'-end of the deletion with respect to the upstream sequence from the transcription start site. Lower-case characters indicate unmatched bases.

Silencer	5'-end base	Sequence
NRE-1a of TCA3	—1998	C c C C Ca T T C T
NRE-1b of TCA3	—1970	C T t C C C T c C T
IL-4 silencer		C T C C C T T C T
NRE-2a of TCA3	—1382	C C Aa C C C a C T g T G T
Lysozyme silencer		C C A C C C T C T C T G T
NRE-2b of TCA3	—1361	C C A T G A
α_{IIb} integrin silencer		C C A T G A

transcription factors resulted in the identification of sequences that are similar to or identical with the silencer motifs associated with IL-4 [11], lysozyme [21] and α_{IIb} integrin [14] (Table 1). The 5' upstream region in NRE-1 contained the subsequence 5'-CCCCCATTCT-3' (—1998 to —1989) (NRE-1a) and the subsequence 5'-CTTCCCTCCT-3' (—1970 to —1961) (NRE-1b), both of which show similarities to the silencer motif of IL-4, 5'-CTCCCTTCT-3' [11]. A region in NRE-2 between —1379 and —1370 was found to have the sequence 5'-AACCCACTGT-3' (NRE-2a), that shows similarity to the silencer motif of lysozyme, 5'-ANCCCTCTCY-3' [24]. A region in NRE-2 between —1361 and —1356 contains 5'-CCATGA-3' (NRE-2b), which is identical with a putative silencer region in the α_{IIb} integrin gene [14].

Evaluation of promoter specificity of TCA3–NREs

To examine the promoter specificity of TCA3–NRE, and to determine whether these NREs have an inhibitory effect on gene expression in both resting and activated mast cells, we analysed the effect of NRE-1 and NRE-2 on the expression of the CAT reporter gene in a CD20–CAT construct containing a human CD20 promoter (Figure 2). The CD20 promoter contains at least

Figure 3 EMSA of the NRE-1 region

(A) Oligonucleotide sequences in the NRE-1 region. Underlining indicates silencer motifs. (B) EMSA with a labelled oligonucleotide containing —1998 to —1989 (NRE-1a) (lanes 1–3) in resting (lane 2) and activated (lane 3) mast cells; or EMSA with oligonucleotide containing —1970 to —1961 (NRE-1b) in resting (lane 5) and activated (lane 6) mast cells. No nuclear extract was added to lanes 1 and 4. (C) Competitor oligonucleotides were added to the labelled oligonucleotide containing NRE-1a at 50-fold molar excess. These include competitors that consist of a specific competitor with unlabelled oligonucleotide containing NRE-1a (lane 3); cross-competitors with unlabelled oligonucleotides containing silencer motif of IL-4 (lane 4) and NRE-1b (lane 5); and unrelated competitor, 5'-ACTGAAGCTTGACTATTGGGAAGAGTTC-TAACTATAG-3' (lane 6), competed with labelled oligonucleotides. Nuclear extracts in lanes 2–6 were from resting cells. No nuclear extract was added to lane 1. Arrow denotes CNRF-1–NRE-1a. All lanes are from the same autoradiograph of a single gel. This experiment was repeated three times with similar results.

Figure 2 Schematic diagram of the CD20–CAT promoter constructs with or without NREs and their CAT protein levels (pg/100 μ g of total cellular protein) in unstimulated mast cells and mast cells stimulated with PMA and A23187

A series of constructs containing the TCA3 upstream sequences encompassing —2057 to —1493 (NRE-1) or —1492 to —1342 (NRE-2) were joined to fully functioning CD20 promoter constructs. The construct base numbering parallels the position of the upstream region of the human CD20 gene with respect to the upstream sequence from the transcription start site. Suppression of reporter gene expression of equimolar amounts of NRE–CD20–CAT constructs is shown in unstimulated mast cells (hatched bars) and mast cells stimulated with PMA and A23187 (solid bars). Data are the average from one experiment performed in duplicate. Similar data were obtained in two additional experiments. Abbreviation used: mcg, μ g.

A Oligonucleotide Sequences for EMSA

Oligo A	ACTGAAGCTT GACTATTGG GAAGAGTCT AACTATCTAG	-1497 to -1458
Oligo B	AACTATCTAG TAGTTACACT CAGTTGGCAA CAACACTGA	-1467 to -1428
Oligo C	CAACACTGA AGGTTTTTCA GTTGACAGG CTAAGGTCAA	-1437 to -1398
Oligo D	CTAAGGTCAA CATAGGTTAG CATAGCCAAC CCACTGTGTA	-1407 to -1368
Oligo E	CCACTGTGTA TAGGCCCCAT GATGCCAATG CCAGAAAGCT	-1377 to -1338
Oligo F	CCAGAAAGCT TAGCTGCTTT TTCTTGAGGG CCTCC	-1347 to -1313

Figure 4 EMSA of the NRE-2 region

(A) Sequences of the overlapping double-stranded oligonucleotides in the NRE-2 region. (B) 1 μ g (lanes 1, 3, 5, 7, 9 and 11) and 5 μ g (lanes 2, 4, 6, 8, 10 and 12) of nuclear extracts from mast cells stimulated with PMA and A23187 were mixed with labelled oligonucleotides A (lanes 1 and 2), B (lanes 3 and 4), C (lanes 5 and 6), D (lanes 7 and 8), E (lanes 9 and 10) and F (lanes 11 and 12). Solid arrow denotes CNRF-2-oligonucleotide D complex and open arrow denotes CNRF-3-oligonucleotide E complex. All lanes are from the same autoradiograph of a single gel. This experiment was repeated three times with similar results.

two positive regulatory elements. These are Oct-1 and Oct-2 [25]. NF- κ B is not known to regulate the transcription of CD20 gene. We have reported that TCA3-CAT constructs show undetectable levels of transcription in resting mast cells regardless of the presence of the NRE region [10]. In contrast, the CD20 promoter-CAT construct has relatively high transcriptional activity in resting cells (Figure 3). Further, both NRE-1 and NRE-2 in either orientation down-regulated the promoter activity of CD20-CAT in both resting and activated mast cells. Both NRE-1 and NRE-2, when used individually, were capable of inhibiting greater than 85% of the promoter activity, suggesting the negative regulatory region of TCA3 has a redundancy of NREs for expression of CAT from the CD20 promoter in resting cells. Neither NRE was able to direct CAT-protein synthesis when inserted into the *Hind*III site immediately upstream of the cytomegalovirus (CMV) promoter in a CMV-CAT construct (results not shown). These results indicate that the activity of NRE-1 and NRE-2 is not specific to the TCA3 promoter, and that the effects of NRE-1 and NRE-2 on at least one promoter (the CD20 promoter) is independent of induction.

Identification of the silencer motif of NRE-1 using EMSA

In order to examine the putative silencer regions identified by functional analysis using CAT assays, EMSAs were performed next. Oligonucleotides containing NRE-1a, NRE-1b and the

A Oligonucleotide Sequences for EMSA

NRE-2a	<u>AGCCAACCCACTGTGTATAG</u>
Silencer of Lysozyme	<u>TCACCACCTCTCTGTGAAG</u>
NRE-2b	<u>TAGGCCCATGATGCCAATGC</u>
Silencer of α_{IIb} Integrin	<u>CTGAAAGCCATGACTCTCC</u>

Figure 5 EMSA of NRE-2a and NRE-2b

(A) Competitors were synthesized containing NRE-2a with the short flanking sequence of TCA3, the silencer motif of lysozyme, NRE-2b with the short flanking sequence of TCA3, the silencer motif of α_{IIb} integrin, mutated (Δ) NRE-2b, and the mutated (Δ) silencer motif of the α_{IIb} integrin. Underlining indicates silencer motifs. (B) To examine nuclear protein(s) binding to NRE-2a, labelled oligonucleotide D was used with or without competitor oligonucleotides at 25- to 75-fold molar excess (lanes 1–9). These included competition with unlabelled oligonucleotide D at 50-fold molar excess (lane 4); cross-competition with unlabelled oligonucleotides containing NRE-2a with the short flanking sequence of TCA3 (lanes 5 and 6) and the silencer motif of lysozyme (lanes 7 and 8) at 25- and 75-fold molar excess (lanes 5, 7 and 6, 8); and unrelated competition with oligonucleotide A (lane 9). Nuclear extracts in lanes 2 and 4–9 were from resting cells and the nuclear extract in lane 3 was from activated mast cells. No nuclear extract was added to lane 1. To identify nuclear protein(s) binding to NRE-2b, labelled oligonucleotide E was used with or without competitor oligonucleotides at 25- to 75-fold molar excess (lanes 10–18). These included competition with unlabelled oligonucleotide E at 50-fold molar excess (lane 13); cross-competition with unlabelled oligonucleotides containing NRE-2b with the short flanking sequence of the TCA3 (lanes 14 and 15) and the silencer motif of the α_{IIb} integrin (lanes 16 and 17) at 25- and 75-fold molar excess (lanes 14, 16 and 15, 17); and unrelated competition with oligonucleotide A (lane 18). Nuclear extracts in lanes 11 and 13–18 were from resting cells and the nuclear extract in lane 12 was from activated cells. No nuclear extract was added to lane 10. Solid arrow denotes CNRF-2-oligonucleotide D complex and open arrow denotes CNRF-3-oligonucleotide E complex. All lanes in Figure 5 are from the same autoradiograph of a single gel. These experiments were repeated three times with similar results.

silencer motif of IL-4 were generated (Figure 3). EMSA using NRE-1a and NRE-1b revealed that nuclear proteins from both resting (Figure 3, lanes 2 and 5) and activated (Figure 3, lanes 3 and 6) cells were able to bind to oligonucleotide NRE-1a (Figure 3, lanes 2 and 3) but not to NRE-1b (Figure 3, lanes 5 and 6). A protein or protein complex binding to NRE-1a, here referred to

Figure 6 Site-directed mutagenesis of NRE-1a and NRE-2b

(A) NRE-1a and NRE-2b mutated constructs. Underlining indicates silencer motifs. Lower-case nucleotides indicate mutated bases. (B) Diagram of the TCA3 promoter constructs without (constructs 1 and 2 in order from top) or with NRE-1 (construct 3) or NRE-2 (construct 5) with base substitution of both NRE-1a (construct 4) and NRE-2b (construct 6) and their CAT protein levels (pg/100 μ g of total cellular protein) in unstimulated mast cells (construct 1) and mast cells stimulated with PMA and A23187 (constructs 2–6). This experiment was repeated three times with similar results. Abbreviation used: mcg, μ g.

as chemokine negative regulatory factor-1 (CNRF-1), was observed both in resting mast cells and in mast cells activated with PMA and A23187 (Figure 3, lanes 2 and 3). Thus we next characterized NRE-1a further but not NRE-1b, which did not show gel retardation.

To verify the specificity of this CNRF-1–NRE-1a complex, competition experiments were performed. Labelled oligonucleotide NRE-1a was incubated with nuclear extracts prepared from unstimulated mast cells (Figure 3, lanes 2–6) in the presence of specific or non-specific competitor oligonucleotides (Figure 3, lanes 3–6). The addition of excess unlabelled competitor containing NRE-1a (Figure 3, lane 3), but not the competing oligonucleotides containing the silencer motif of the IL-4 gene (Figure 3, lane 4), blocked the CNRF-1–NRE-1a complex. Formation of the complex was not blocked by competitor containing NRE-1b (Figure 3, lane 5) or an unrelated heterologous competitor (Figure 3, lane 6). These results demonstrate that the shift of oligonucleotide NRE-1a is not due to the protein interacting with the silencer motif of the IL-4 gene.

Identification of the silencer motifs of NRE-2 using EMSA

In order to characterize NRE-2 further, EMSAs were again performed. Overlapping oligonucleotides (A–F) encompassing NRE-2 were generated (Figure 4). Nuclear extracts (1 μ g and 5 μ g) from mast cells stimulated with PMA and A23187 were incubated with oligonucleotides A–F (Figure 4). Complexes were formed only with oligonucleotide D containing NRE-2a (Figure 4, lanes 7 and 8) or with oligonucleotide E containing NRE-2b (Figure 4, lanes 9 and 10) with a dose-dependent increase in the protein–DNA complex formation. Oligonucleotide D containing NRE-2a was shifted more than oligonucleotide E containing

NRE-2b. These results suggest that oligonucleotides D and E bind different proteins or protein complexes, denoted as CNRF-2 and CNRF-3 respectively.

Comparative analysis of the silencer motifs of NRE-2 of TCA3 with EMSA

To determine the inducibility and specificity of the CNRF-2–oligonucleotide D and CNRF-3–oligonucleotide E complexes in the NRE-2 region, competitors were synthesized that contained NRE-2a with a short flanking sequence (20 bp, whereas oligonucleotide D has 40 bp), the silencer motif of lysozyme, NRE-2b with a short flanking sequence (20 bp, whereas oligonucleotide E has 40 bp), the silencer motif of $\alpha_{1\text{IIb}}$ integrin, deletion mutants of NRE-2b of TCA3, and the silencer motif of $\alpha_{1\text{IIb}}$ integrin (Figure 5). To determine the specificity of the CNRF-2–oligonucleotide D interaction, labelled oligonucleotide D containing NRE-2a was incubated with nuclear extracts prepared from unstimulated (Figure 5, lanes 2 and 4–9) and stimulated (Figure 5, lane 3) mast cells in the presence of specific (Figure 5, lane 4) or non-specific competitor oligonucleotides (Figure 5, lanes 5–9). CNRF-2–oligonucleotide D complex formation did not require treatment of mast cells with PMA and A23187, as shown by the presence of the complexes in lanes 2 and 3 of Figure 5. The specificity of this complex was confirmed by competition experiments. Addition of excess unlabelled oligonucleotide D blocked this complex (Figure 5, lane 4). Addition of unlabelled competitors containing NRE-2a with a short flanking sequence or the silencer motif of lysozyme with increasing concentrations of competitors at a 25- or 75-fold molar excess (Figure 5, lanes 5, 6 and 7, 8 respectively) did not block this complex formation. Formation of this complex was not blocked by unrelated heterologous competitors, such

Figure 7 *In vivo* DMS footprinting of the top strand of NRE-2 of TCA3

Lane 1, *in vivo* DMS-treated DNA (mast cells stimulated with PMA/A23187); lane 2, *in vivo* DMS-treated DNA (resting mast cells); lane 3, *in vitro* DMS-treated DNA (resting mast cells). Solid bar denotes the protected region. All lanes are from the same autoradiograph of a single gel. This experiment was repeated three times with similar results.

as oligonucleotide A (Figure 5, lane 9). The observation that NRE-2a with a short flanking sequence, which oligonucleotide D contains, did not compete with oligonucleotide D indicates that NRE-2a, which is similar to the silencer motif of lysozyme, is not responsible for the gel retardation.

To determine the specificity of the CNRF-3–NRE-2b interaction, labelled oligonucleotide E containing NRE-2b was incubated with nuclear extracts prepared from unstimulated (Figure 5, lanes 11 and 13–18) and stimulated (Figure 5, lane 12) mast cells in the presence of specific (Figure 5, lane 13) or non-specific competitor oligonucleotides (Figure 5, lanes 14–18). CNRF-3–NRE-2b complex formation did not require treatment of mast cells with PMA and A23187, as shown by the appearance of these complexes in both lanes 11 and 12 of Figure 5. The specificity of this CNRF-3–NRE-2b complex was confirmed by competition experiments. Addition of excess unlabelled oligonucleotide E blocked this complex formation (Figure 5, lane 13). Addition of unlabelled competitors at 25- and 75-fold molar excess to either NRE-2b of TCA3 or the putative silencer motif of α_{IIB} integrin (Figure 5, lanes 14, 15 and 16, 17) blocked this complex formation in a dose-response manner. These results suggest that the shift of NRE-2b is due to CNRF-3, which also interacts with the silencer motif of α_{IIB} integrin.

Site-directed mutagenesis of NRE-1a and NRE-2b

To confirm the functionality of the subsequences NRE-1a and NRE-2b, in down-regulating the promoter activity of TCA3, the

activity of NRE-1a and NRE-2b was assessed in NRE-1– and NRE-2–0.7 kb TCA3–CAT constructs respectively. Five bases in either NRE-1a or NRE-2b in NRE-1– or NRE-2–TCA3–CAT constructs were mutated (Figure 6A). The activities of NRE-1– and NRE-2–0.7 kb TCA3–CAT construct with and without a mutation were assayed by measuring the CAT protein expression. The Δ NRE-1–0.7 kb TCA3–CAT construct had less than 20 % of the negative regulatory activity of NRE-1, indicating NRE-1a is essential for the negative regulatory activity of NRE-1. The Δ NRE-2–0.7 kb TCA3–CAT construct had approx. 65 % of the negative activity of NRE-2, suggesting that NRE-2b is necessary but not sufficient to confer the negative regulatory activity of NRE-2, consistent with the possibility that sequences neighbouring such as NRE-2a contained in oligonucleotide D are also involved in negative regulatory activity.

In vivo footprinting of the NRE-2 region of TCA3

To further characterize the extent of NRE-2b, we next identified the regions of direct protein–DNA contact by employing *in vivo* LM-PCR footprinting after dimethyl sulphate (DMS) treatment on the NRE-2 region of TCA3. Oligonucleotides specific for the various upstream regions of TCA3 were used as described in the Materials and methods section. As shown in Figure 7, the top strand of the TCA3 promoter region from position –1372 to –1354 encompassing NRE-2b is protected by protein(s) from both resting mast cells and mast cells stimulated with PMA and A23187. The footprinting gel shows reduced intensity of G and A residues with relatively hypersensitive GG residues (–1365 to –1364) with both *in vivo* resting and stimulated cells. Thus these *in vivo* data are in agreement with the data from EMSA showing a specific CNRF-3–NRE-2b interaction (Figure 5) and site-directed mutagenesis showing that NRE-2b is necessary for the negative regulatory activity of NRE-2 (Figure 6).

DISCUSSION

We have previously demonstrated that transcription was undetectable by CAT assays when a 2 kb TCA3–CAT construct containing the region between –2057 and –1342 was transfected into mast cells [10]. However, induced transcription was restored upon deletion of this region from the 2 kb TCA3–CAT construct. In addition to the potential enhancer elements between –1341 and –136 within the 5'-flanking region of TCA3, these results strongly suggested the existence of silencer elements between –2057 and –1342. As reported in this paper, further analysis with multiple NRE constructs, including 5'-deletion mutants, revealed that multiple regions, rather than a single sequence element, contribute to NRE activity (Figure 1). Both NRE-1 and NRE-2 were able to inhibit approx. 60 % of CAT-protein synthesis of TCA3–CAT constructs, confirming the presence of distinct NREs between –2057 and –1342. This finding suggested that negative regulation of TCA3 involved multiple elements [26], and co-operation of NRE-1 and NRE-2 was required for more efficient down-regulation of the TCA3 promoter.

Both NRE-1 and NRE-2 were distance (from transcription start site)-independent, but only NRE-1 was orientation-independent (Figure 1). Silencers were initially defined as autonomous *cis*-acting NREs that function in a position- and orientation-independent manner [27]. However, silencers that are position-dependent [24,28] or orientation-dependent [29,30] have now been reported, which is consistent with the data on NRE-1 and NRE-2.

When either NRE-1 or NRE-2 was inserted into a CAT construct containing the CD20 promoter, the transcriptional

Figure 8 Schematic representation of possible interactions between proteins and DNA at the 5'-regulatory region of TCA3

The —201 to —181 region containing the NF- κ B regulatory element has previously been shown to be occupied by NF- κ B after activation. The —2002 to —1983, —1407 to —1383 and —1372 to —1354 regions are occupied by transcription factors independent of activation. Boxed sequences represent protected sites identified by footprinting *in vivo*. Solid ellipses represent protein binding demonstrated both by EMSA and footprinting *in vivo*. Broken ellipses represent protein binding demonstrated only by EMSA. Bold characters indicate motif or consensus sequence of the regulatory element.

activity conveyed by the promoter was substantially reduced (Figure 2). Like many other silencers, both NRE-1 and NRE-2 from TCA3 were able to inhibit the activity of a heterologous promoter [24,29,31–35]. Furthermore, both NRE-1 and NRE-2 alone inhibited more than 85% of the promoter activity of CD20–CAT. These results indicate a redundancy of NREs within the TCA3 promoter. Such functional redundancy is observed in the regulatory elements of other genes, including enhancers, silencers and locus control regions [32].

Both NRE-1a and NRE-1b subsequences in the NRE-1 region are similar to a portion of the IL-4 NRE [11] (Table 1, Figure 3). However, NRE-1a only revealed specific DNA–protein complex formation, and this shift of NRE-1a was not due to the protein interacting with the silencer motif of the IL-4 gene (Figure 3B). Deletion of a region containing both NRE-1a and NRE-1b subsequences abolished the negative regulatory activity of NRE-1 (Figure 1). Site-directed mutagenesis experiments revealed that altering NRE-1a resulted in a significant loss of negative regulatory activity (Figure 1). These results suggest that NRE-1a represents a novel silencer region.

NRE-2a is similar to the silencer motif [24] ANCCCTCTCY, identified in *cis*-NREs of genes encoding human collagen type II [36], and lysozyme [24] (Table 1), as well as other genes including human ϵ -globulin [37] ACCCTCTTC. However, an unlabelled oligonucleotide containing NRE-2a with a short flanking sequence failed to inhibit the shift of oligonucleotide D (Figure 5). These results suggest that while NRE-2a is not responsible for the gel retardation, oligonucleotide D may contain a yet to be characterized silencer sequence.

NRE-2b contains CCATGA, which is identical with one of the putative silencer motifs of the α_{ITb} integrin gene (Table 1), and exhibits reverse complementarity to the other putative silencer motif of the α_{ITb} integrin gene, TGATGG. Competitors containing the CCATGA motif as seen in TCA3 and the silencer region of the α_{ITb} integrin gene abolished specific DNA–protein complex formation (Figure 5). Also LM-PCR footprinting *in vivo* on the NRE-2 region revealed protection between —1372 and —1354, which contains the CCATGA motif (Figure 7). These results suggest that both TCA3 and the α_{ITb} integrin gene

share the same silencer protein(s). The silencer motif of the α_{ITb} integrin gene has been reported to correlate with the differentiation-dependent expression of this gene. Site-directed mutagenesis of the CCATGA motif resulted in the partial loss of the negative regulatory activity of NRE-2 (Figure 6). These results are consistent with the conclusion that the CCATGA motif is necessary but not sufficient to confer the negative regulatory activity of NRE-2. Both EMSA and LM-PCR footprinting *in vivo* also demonstrated the binding of a nuclear protein or protein complex to the NRE-2b region in both uninduced and induced mast cells. Although we were not able to correlate results from uninduced mast cells with the data from transient transfection experiments using TCA3 promoter constructs, due to undetectable levels of promoter activity in resting mast cells, we did examine the effect of NRE-1 and NRE-2 on CD20 in CD20–CAT. With this construct, CAT protein synthesis was detectable in resting mast cells (Figure 2). Inhibition of transcription occurred with NRE-1 or NRE-2 in both induced and uninduced mast cells. Thus both NRE-1a and NRE-2b may function as general rather than induction-specific regressors of TCA3 gene activity. The combined control by enhancer(s) and NREs would thus result in balanced TCA3 gene expression.

We have shown that the transcription rate of TCA3 is undetectable in resting mast cells by Northern-blot analysis, nuclear run-on experiments and CAT assays using TCA3–CAT constructs with or without the NRE region. These results suggest that the repression of the basal transcription rate of TCA3 in the resting mast cells is not due to the action of NREs [11]. In contrast with the undetectable CAT protein synthesis by a 2 kb TCA3–CAT construct following mast cell activation, the mRNA level of the endogenous TCA3 gene was elevated [10]. This discrepancy may be due to complex DNA–protein and/or protein–protein interactions requiring additional elements in the upstream region from the NREs [11,12,38], an intronic sequence [39], or a 3'-untranslated region to overcome the negative regulatory activity [40].

In summary, we have demonstrated functional NREs in TCA3 (Figure 8). This information adds to the understanding of the regulation of TCA3 that has been described as having a

functional NF- κ B binding site [10]. NRE-1 is distance-independent and orientation-independent; NRE-2 is distance-independent and orientation-dependent. Neither region is specific to TCA3. Both NRE-1 and NRE-2 function in mast cells independent of cell activation. NRE-1 contains NRE-1a, which binds to the hypothetical CNRF-1; NRE-2 contains NRE-2b, which binds to hypothetical CNRF-3, and a second binding region from -1407 to -1383, which binds to hypothetical CNRF-2 and requires further characterization. Both NRE-1a and NRE-2b participate in the transcriptional regulation of TCA3 gene expression along with enhancers in mast cells upon activation. The NREs may be important *cis*-acting regulators for other genes. Further characterization of nuclear factors binding to these elements and delineation of the 5' upstream region containing these NREs will provide further insight into the role of negative regulation in the expression of chemokines.

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