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Transcriptional repression and DNA looping associated with a novel regulatory element in the final exon of the lymphotoxin-β gene

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Transcriptional regulation has a critical role in the coordinate and context-specific expression of a cluster of genes encoding members of the tumour necrosis factor (TNF) superfamily found at chromosome 6p21, comprising TNF, LTA (encoding lymphotoxin-α) and LTB (encoding lymphotoxin-β). This is important, as dysregulated expression of these genes is implicated in susceptibility to many autoimmune, inflammatory and infectious diseases. We describe here a novel regulatory element in the fourth exon of LTB, which is highly conserved, localises to the only CpG island in the locus, and is associated with a DNase I hypersensitive site and specific histone modifications. We find evidence of binding by Yin Yang 1 (YY1), cyclic AMP response element (CRE)-binding protein (CREB) and CCCTC-binding factor (CTCF) to this region in Jurkat T cells, which is associated with transcriptional repression on reporter gene analysis. Chromatin conformation capture experiments show evidence of DNA looping, involving interaction of this element with the LTB promoter, LTA promoter and TNF 3' untranslated region (UTR). Small interfering RNA (siRNA) experiments demonstrate a functional role for YY1 and CREB in LTB expression. Our findings provide evidence of additional complexity in the transcriptional regulation of LTB with implications for coordinate expression of genes in this important genomic locus.

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Introduction

The tumour necrosis factor (TNF) superfamily has a critical role in immunity and the inflammatory response, with dysregulation of these proteins and associated signalling pathways implicated in the pathogenesis of a number of important autoimmune, infectious and inflammatory diseases. Three key members of the superfamily are encoded by tandemly arranged genes found in a short region of the major histocompatibility complex (MHC) on chromosome 6p21: *TNF* (encoding tumour necrosis factor), *LTA* (encoding lymphotoxin α, LTα) and *LTB* (encoding lymphotoxin-β, LTβ). Expression of these genes is tightly regulated, notably at a transcriptional level, with evidence of stimulus and cell-type specificity in regulation of the *TNF* gene involving promoter and 3' untranslated region (UTR) elements.[1–4] Recently, we highlighted a number of putative regulatory elements lying outside the promoter regions of these genes based on chromatin profiling for DNase accessibility and histone modifications. These included an intergenic site located at 3.5 kb upstream of *LTA*,[5] which is also found in mice.[6] Our analysis demonstrated that

the sequence spanning the final exon of *LTB* showed evidence of DNase I hypersensitivity and acetylation of histones H3 and H4, together with trimethylated K4 of histone H3, raising the hypothesis that the final exon of *LTB* contains a regulatory element that has a role in the transcriptional regulation of *LTB* or neighbouring genes in the *TNF* cluster.[5]

Studies to date characterising the regulation of *LTB* gene expression have defined an inducible core promoter, with evidence of a role for a number of transcription factors, including nuclear factor κB (NFκB), Ets, Egr-1 and Sp1.[7–9] The regulation of *LTB* is physiologically important, as increasing numbers of studies highlight the biological significance of LTβ, and of the LTβ receptor to which it binds as a heterodimer with LTα.[10–14] During embryogenesis, mouse studies have shown a critical role for LTβ in the development of lymphoid organs, and LTβ continues to have an important function in these tissues during adult life. The role of LTαβ is, however, recognised to be much more extensive involving recruitment and activation of diverse cell types, including lymphocytes and T cells. High levels of LTβ are noted in lymph nodes, and specific cell types such as CD4 + T cells from patients with chronic inflammatory conditions, such as tuberculosis and inflammatory bowel disease.[15,16]

Cells of lymphoid lineage, including T-, B-, natural killer cells and lymphoid tissue-inducer cells are important sources of LTβ.[17] Jurkat T cells have been widely

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used as a model to characterise transcriptional regulation of *LTB*, with strong inducibility on stimulation with the phorbol ester phorbol 12-myristate 13-acetate (PMA) and ionomycin.[5,7,8,18] Here, we characterise a putative regulatory region within the final exon of the *LTB* gene and show evidence of recruitment of Yin Yang 1 (YY1), cyclic AMP response element (CRE)-binding protein (CREB) and CCCTC-binding factor (CTCF) with important consequences for transcriptional activity and interactions involving *LTB* and other genes in the TNF locus.

Results

A DNase I hypersensitive site with features of a regulatory element

We have previously identified a DNase I hypersensitive site in *LTB*, DHS 56700, which was present in a variety of cell types, including Jurkat T cells, and localises to the only significant CpG island in the *TNF* locus.[5] We extended this analysis using ChIP-chip and ChIP-seq data from the Duke/UNC/UT-Austin/EBI ENCODE analysis for DNase I to confirm striking DNase hypersensitivity in the fourth exon of *LTB* (Figure 1). Analysis of nucleosome accessibility by formaldehyde-assisted isolation of regulatory elements was also consistent with this (Figure 1), highlighting the region's potential regulatory significance. We had previously shown that the site has marked enrichment of histone acetylation (H3 and H4) together with a peak of trimethylated lysine 4 of histone H3 in Jurkat T cells.[5] We investigated whether these epigenetic marks were present in other cell types using ChIP-seq data from the Broad Institute as part of the ENCODE Histone Modifications analysis data set. This supported the results we had observed in Jurkat T cells (Figure 1). We also investigated sequence conservation at this site among vertebrates based on analysis of 44 species (Vertebrate Multiz Alignment & Conservation) using the UCSC Genome Browser database.[19,20] Analysis of *PhastCons* conserved elements using a conservative threshold, highlighting two regions showing a logarithm of the odds ratio (LOD) >100 (Figure 1). One of these falls in the *LTB* promoter region (chr6: 31658240–31658359) (score 555, LOD 197), the other localises to exon 4 of *LTB* (chr6: 31656508–31656599) (score 521, LOD 146).

Complex protein–DNA interactions involving CREB and YY1

In view of these data, we sought to investigate further the regulatory significance of this region, considering first potential protein–DNA interactions in the region of DHS 56700 using Jurkat T cells as a model system. We carried out systematic *in vitro* solid-phase DNase I footprinting studies over exon 4 of *LTB* and flanking sequences (chr6: 31656372–31657330) using nuclear extracts prepared from Jurkat T cells, either resting or following stimulation with PMA and ionomycin. This showed evidence of a number of sites of protein–DNA interactions with four regions of protection noted (denoted LTB1–4) (Figure 2). To complement these data, we carried out an *in silico* analysis of putative transcription factor binding sites using the JASPAR database combined with cross-species comparison to establish phylogenetic footprints using the ConSite algorithm.[21–23] This highlighted a potential CREB binding site within the
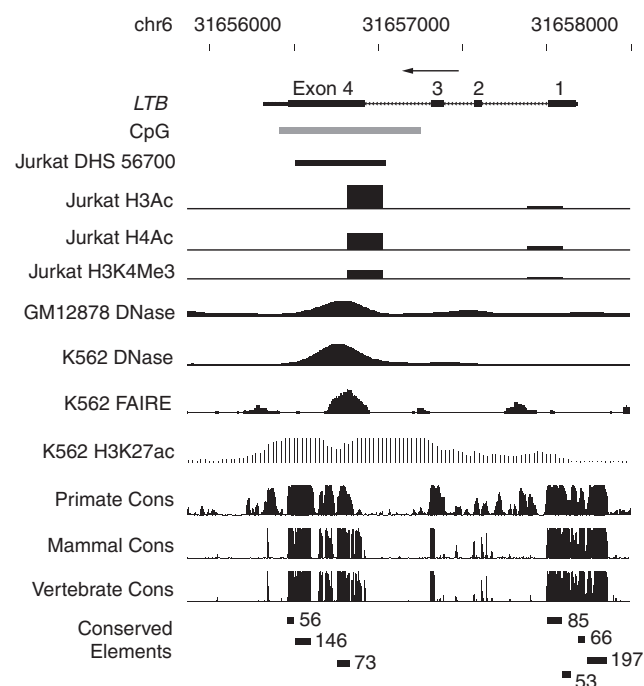

Figure 1 Overview of chromatin accessibility, histone modifications and sequence conservation at *LTB*. A 2.7 kb region spanning *LTB* (chr6: 31655900–31658600) is shown with the location of a CpG island (count 75) indicated. Data for Jurkat T cells show the site of DHS 56700 and histone modifications based on chromatin immunoprecipitation using antibodies to diacetylated histone H3 (H3Ac), tetracetylated histone H4 (H4Ac) and trimethylated histone K4 H3 (H3K4Me3) quantified by real-time quantitative PCR, with enrichment relative to input DNA displayed.[5] Data from the Duke/UNC/UT-Austin/EBI ENCODE group for DNase I (based on DNase-seq and DNase-chip) and formaldehyde assisted isolation of regulatory elements (FAIRE)[52] are shown for the lymphoblastoid cell line GM12878 and chronic myelogenous leukaemia cell line K-562 downloaded from the UCSC Browser. ChIP-seq data for H3K27Ac is shown for K562 cells using data generated by the Broad Institute as part of the ENCODE Histone Modifications analysis. Sequence conservation based on Vertebrate Multiz Alignment and Conservation analysis of 44 species is shown together with conserved elements based on *PhastCons* for placental mammal species.[19]

first DNase I footprint LTB1 at chr6: 31656531–31656542 together with a consensus binding site for YY1 at chr6: 31656514–31656519 (Figure 2). Within the second DNase I footprint LTB2, we noted a potential NFκB (REL) binding site at chr6: 31656743–31656753, although this sequence was not conserved in the mouse. No other *in silico*-predicted transcription factor binding sites corresponded to our experimentally derived DNase I footprinting data for Jurkat T cells.

We proceeded to analyse the regions highlighted by DNase I footprinting using electrophoretic mobility shift assays (EMSAs). A radiolabelled probe spanning the region of protection LTB1 showed strong constitutive binding of at least two protein–DNA complexes, which appeared specific on competition with molar excess of shorter unlabelled probes corresponding to the putative transcription factor binding sites for CREB and YY1 (Figure 3). Complexes I and II show a supershift effect on incubation with anti-CREB and with anti-YY1 antibodies, respectively, consistent with CREB and YY1 binding
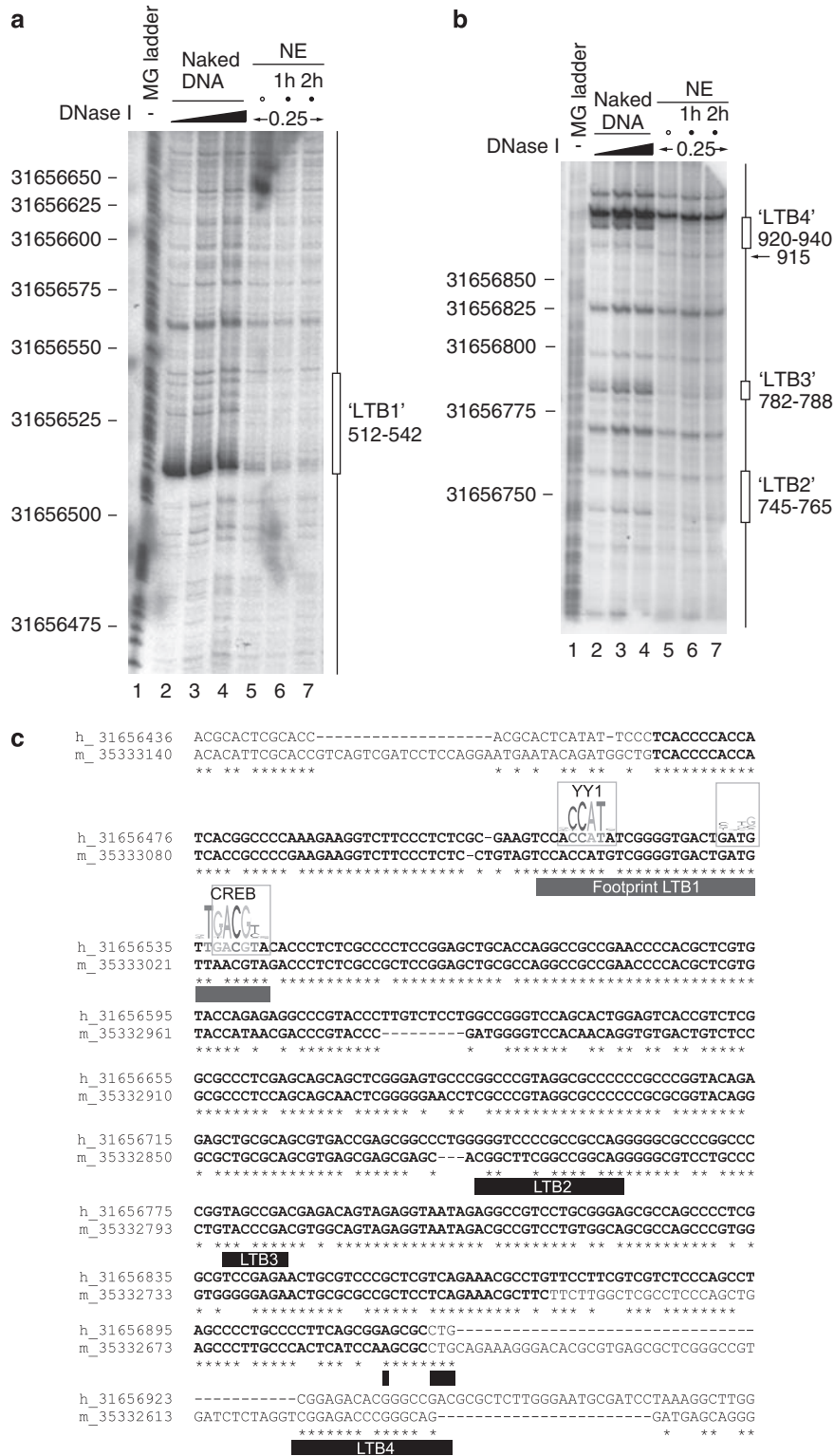
Figure 2 Solid-phase DNase I footprinting of putative regulatory element at *LTB* exon 4 using no nuclear extract (lanes 2–4) or nuclear extract prepared from unstimulated Jurkat T cells (○) (lane 5) or cells stimulated with PMA/ionomycin for times indicated (●) (lane 6–7). Regions of protection are indicated by open boxes, increased DNase sensitivity by arrows with nucleotide position based on a Maxam–Gilbert sequencing ladder (lane 1). (**a**) Footprinting analysis chr6: 31656372–31656725 showed protection at chr6: 31656512–31656542 denoted LTB1. (**b**) Footprinting analysis chr6: 31656634–31656999 demonstrated protection at chr6: 31656745–31656765 (LTB2); chr6: 31656782–31656788 (LTB3); and chr6: 31656920–31656940 (LTB4) with increased DNase sensitivity at chr6: 31656915 flanking this latter region of protection. (**c**) DNA sequence spanning exon 4 of *LTB* showing human-mouse alignment, summarising results of DNase I footprinting experiments and putative transcription factor binding sites. DNA sequences for human (hg18) (March 2006 NCBI Build 36.1) chr6: 31656436–31656971 and mouse (mm9) (July 2007 Build 37) chr17: 35332577–353333140 aligned using the CLUSTAL W (version 1.81).[53] Coding sequence corresponding to exon 4 is shown in bold with footprinted regions indicated by black boxes. Putative transcription factor binding sites are indicated by sequence logos incorporating data from JASPAR nucleotide frequency matrix.[22,54]
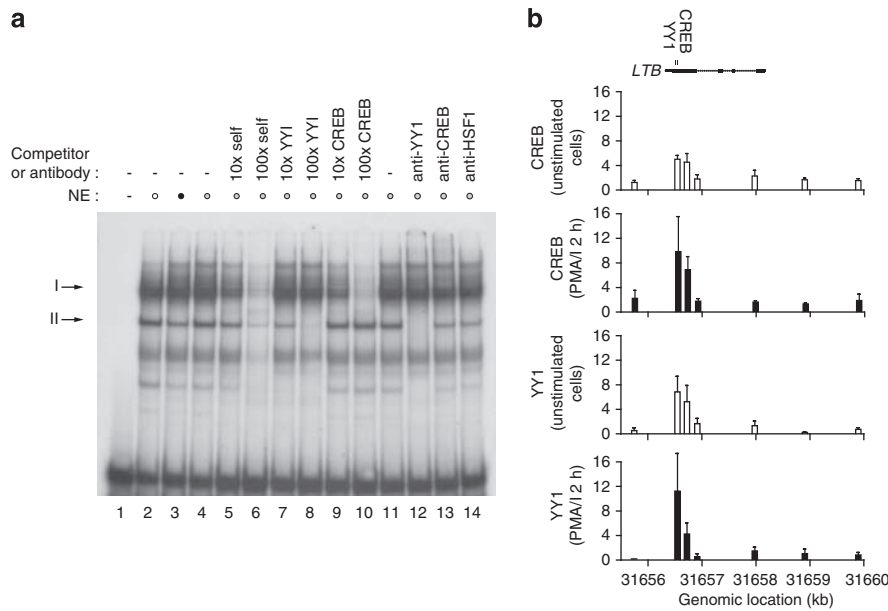
Figure 3 Protein–DNA interactions in LTB1 footprinted region. (**a**) EMSA using a radiolabelled probe spanning the footprinted region run without (lane 1) or with nuclear extracts from Jurkat T cells either unstimulated (○) (lane 2) or following stimulation with PMA and ionomycin for 2 h (●) (lane 3) or 6 h (◐) (lane 4–14). Binding in the presence of molar excess of unlabelled competitor probe is shown (lanes 5–10) and specific antibodies as indicated (lanes 12–14). (**b**) ChIP experiments using antibodies to CREB and YY1 for Jurkat T cells, either resting or collected at 2 h after induction with PMA and ionomycin. Immunoprecipitated DNA was analysed using quantitative real-time PCR using seven amplicons to interrogate the region spanning *LTB*. On the *y* axis, the mean fold difference (± s.e.m.) in enrichment of each of the PCR amplicons is expressed relative to input DNA for three ChIP experiments. Genomic location is shown on the *x* axis.

(Figure 3). These results were also seen on competition and supershift EMSA experiments using shorter radiolabelled probes containing each site individually (data not shown). We then performed chromatin immunoprecipitation assays in Jurkat T cells to investigate whether these protein–DNA interactions were occurring *in vivo*. This confirmed that YY1 and CREB bind to this region in the absence of stimulation and that binding increases during mitogen stimulation (Figure 3). The second region showing protection on DNase I footprinting (LTB2) contained a potential NFκB-binding site but chromatin immunoprecipitation experiments failed to demonstrate binding by p50 or p65 *in vivo* (data not shown). The other two footprinted regions LTB3 and LTB4 did show evidence of specific protein–DNA interactions on EMSA (data not shown), however, the identity of the proteins involved in these complexes remains to be defined.

DNA looping involving LTB *exon 4, the* LTB *and* LTA *promoter, and* TNF 3′ *UTR*
The location of the putative *LTB* regulatory element within the final exon of the gene is relatively unusual, and it was unclear whether any regulatory effects were specific to *LTB* or of more global significance. This promoted us to investigate whether this sequence showed evidence of interaction with other sites in the *TNF* locus. We used a chromatin conformation capture technique[24] to test this hypothesis for a 48 kb region encompassing *NFKBIL1* (encoding nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1), *LTA*, *TNF*, *LTB*, *LST1* (encoding leucocyte specific transcript 1) and *NCR3* (encoding natural

cytotoxicity triggering receptor 3). The 3C assay was developed in which unstimulated or mitogen-induced Jurkat T cells were treated with formaldehyde to crosslink protein–DNA and protein–protein interactions; restriction enzyme digested, diluted and ligated such that intramolecular ligations between crosslinked fragments are favoured over intermolecular ligation between random DNA fragments. Quantitative PCR using Taq-Man technology was performed to analyse the ligation products generated by 3C,[25] including a PCR control template of a restriction enzyme digested and ligated BAC clone spanning the *TNF* locus. This showed evidence of interaction between *LTB* exon 4 and the *LTB* promoter, the *LTA* promoter and *TNF* 3′UTR (Figure 4). This was reproducibly seen for three biological replicate experiments and across different time points of stimulation, although was most striking in the unstimulated state. In HeLa cells, which express *LTB* at very low levels,[5] there was evidence of interaction with the *LTB* promoter but not at other sites (Figure 4).

CTCF binding at LTB *exon 4*
Our findings of accessible chromatin, histone acetylation, recruitment of YY1 and DNA looping interactions across the TNF locus led us to hypothesise that CTCF may be involved. CTCF is an 11 zinc-finger protein with diverse roles in transcriptional regulation, notably not only as an insulator element but also involved in transcriptional repression and activation.[26,27] There is flexibility in the precise DNA-binding motifs recognised by CTCF but recent genome-wide analyses of CTCF-binding sites have defined a 20-mer motif.[28] We analysed the DNA sequence spanning exon 4 of *LTB*, using the
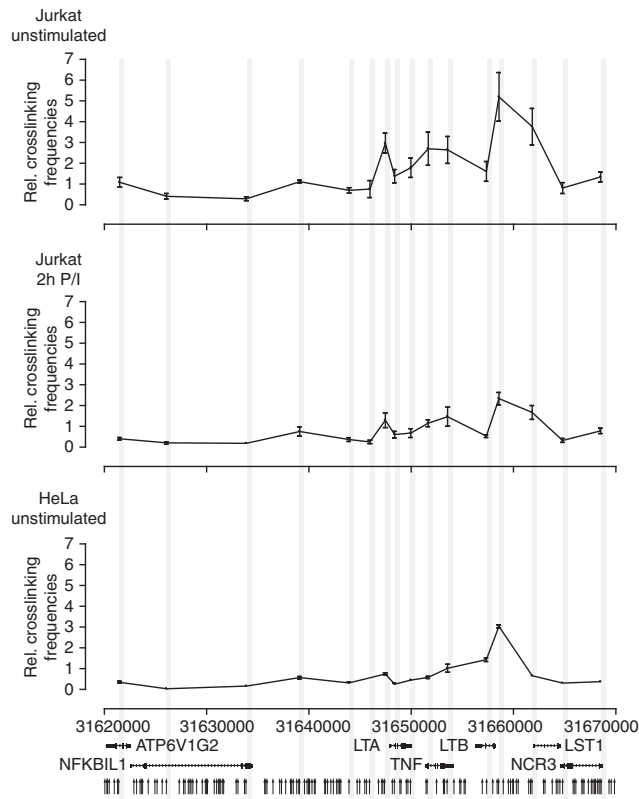
Figure 4 3C assays show sites of interactions across the *TNF* locus with *LTB* exon 4. Relative crosslinking frequencies are shown for different ligation products quantified using a constant primer and TaqMan probe in *LTB* exon 4 and primers tiled over the *TNF* gene locus (chr6: 31621496–31669390) shown by arrowheads and shaded vertical bars. Vertical arrows denote the location of restriction enzyme sites for *Dpn*II. The mean ± s.e.m. is shown for three biological replicate experiments using HeLa cells or Jurkat T cells, either unstimulated or induced with PMA/ionomycin.

CTCF-binding site database.[29] This showed a high-position weight matrix score of 16.7 (greater than 3.0 is a suggestive match), and the sequence, which includes DNase I footprint LTB2, was a match for the CTCF consensus motif (Figure 5). We proceeded to investigate further the potential binding by CTCF to this site in *LTB* exon 4 by EMSA using nuclear extracts from Jurkat T cells. This showed evidence of constitutive binding by specific complexes on competition EMSA, which were competed by unlabelled probes corresponding to a known CTCF-binding site in the chicken β-globin gene insulator (HS4),[30] but not by the same probe containing specific point mutations disrupting the CTCF-binding site (Figure 5). We then investigated whether CTCF was binding *in vivo* by chromatin immunoprecipitation. These experiments showed evidence of constitutive CTCF binding to this region in Jurkat T cells (Figure 5).

We analysed a number of different data sets mapping CTCF binding across the human genome.[28,31,32] This provided robust evidence of CTCF binding in *LTB* exon 4, across a range of cell types, including primary CD4 + T cells (Supplementary Figure S1). Looking more broadly across a 60-kb window that includes *LTB*, we noted two other CTCF-binding sites in regions flanking the TNF locus, located at *NFKBIL1* and *LST1*.

Evidence of transcriptional repression

The complex protein–DNA interactions involving CTCF suggest this site may be functioning as a boundary element, insulating *LTB* from the activity of the neighbouring *TNF* and *LTA* genes; or modulating gene expression through DNA looping, as suggested by the 3C experiments. We therefore engineered reporter gene constructs in which sequences corresponding to the region were placed downstream of the luciferase gene in a pGL3 reporter construct, driven by the SV40 promoter. Transient transfection experiments using a construct containing a 1 kb sequence spanning *LTB* exon 4 showed significant repression of transcriptional activity, by 3.3-fold (95% confidence intervals 1.7–4.9) in resting cells and by 5.4-fold (4.0–6.7) following mitogen stimulation (Figure 6). This effect was reduced by 50% when a shorter sequence, spanning the CTCF site but not the YY1 or CREB-binding sites, was inserted downstream of the luciferase gene in pGL3-SV40Prom.

A functional role for YY1 and CREB in LTB *gene expression*

We specifically investigated the effects of knockdown of *YY1* and *CREB* for *LTB* expression by transient transfection of Jurkat T cells using small-interfering RNAs (siRNAs). At the mRNA level there was efficient knockdown of *YY1* and *CREB* at 72 h after transfection, whereas a cocktail of non-targeting control siRNAs had no effect (Figure 7). Significant suppression of *LTB* expression was observed only when both *YY1* and *CREB* were knocked down (Figure 7).

Discussion

The *TNF* locus within the MHC class III region encodes three important members of the TNF superfamily whose expression is tightly regulated at the transcriptional level. For *LTB*, attention has focused on the role of proximal promoter elements, notably NFκB, Ets and Egr-1.[7,8,18] Our data suggests an additional layer of complexity involving sequences in the fourth exon of *LTB*. We note that this region contains the only significant CpG island in the *TNF* locus, and is associated with histone modifications and an open chromatin configuration based on DNase hypersensitivity mapping. We find evidence of a novel regulatory element in this region, which involves CTCF binding together with YY1 and CREB, and is associated with multiple sites of interaction across the *TNF* gene cluster, notably with the *LTA* and *LTB* promoter regions, and *TNF* 3′ UTR.

Further work is needed to investigate more fully the functional implications of these interactions, but the evidence of DNA looping may prove highly significant. Coordinate expression of LTα and LTβ is important, both during development and induction of the adaptive immune response. These proteins bind to the LTβ receptor as a heterodimer,[33] regulating diverse processes ranging from secondary lymphoid organogenesis to T-cell differentiation, with dysregulation important in immunity and other disease processes.[12–14]

Our finding of transcriptional repressor activity on reporter gene analysis for a region associated with CTCF binding is consistent with the original isolation and cloning of this protein at the chicken and human *c-myc*
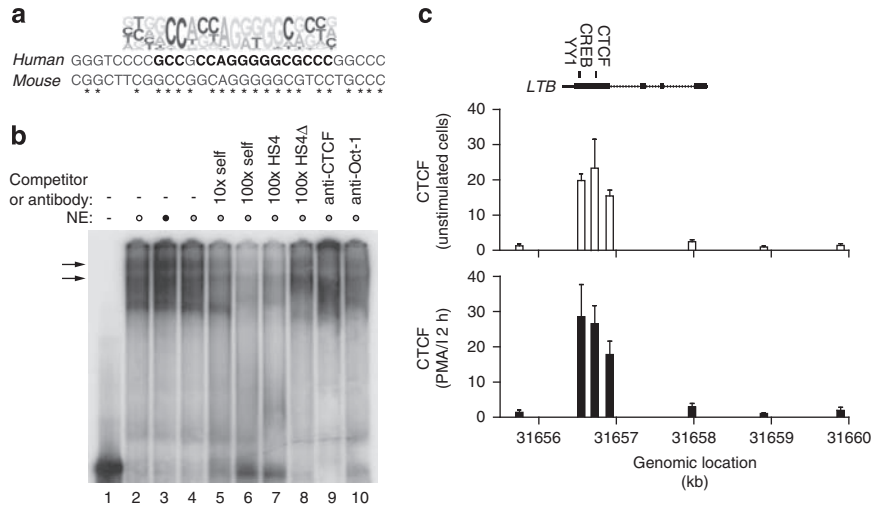
Figure 5 CTCF binding at *LTB* exon 4. (**a**) Human (hg18 chr6: 31656745–31656774) and mouse (mm9 chr17: 35332794–35332823) sequence alignment in *LTB* exon 4 at site of putative CTCF-binding site aligned to consensus sequence logo.[28] (**b**) EMSA investigating protein–DNA interactions using a radiolabelled probe spanning the putative CTCF binding site in *LTB* alone (lanes 1) or incubated with nuclear extracts from Jurkat T cells either unstimulated (○) (lane 2) or following stimulation with PMA and ionomycin for 2 h (●) (lane 3) or 6 h (grey circle) (lanes 4–10). The results of competition using molar excess of unlabelled competitor probe corresponding to the *LTB* exon 4 sequence is shown (lanes 5–6), together with data relating to a probe corresponding to the chicken beta globin insulator HS4 (lane 7) or the same HS4 probe with key CTCF-binding residues mutated (HS4Δ) (lane 8). The effect of specific antibodies is also shown (lanes 9–10). (**c**) ChIP experiments using antibodies to CTCF are shown for Jurkat T cells, either resting or collected at 2 h after induction with PMA and ionomycin. The immunoprecipitated DNA was analysed using quantitative real-time PCR using seven primer pairs to interrogate the region spanning *LTB* (chr6: 31655630–31660002). The mean fold difference (± s.e.m.) in enrichment of each of the PCR amplicons is expressed relative to input DNA for three ChIP experiments.
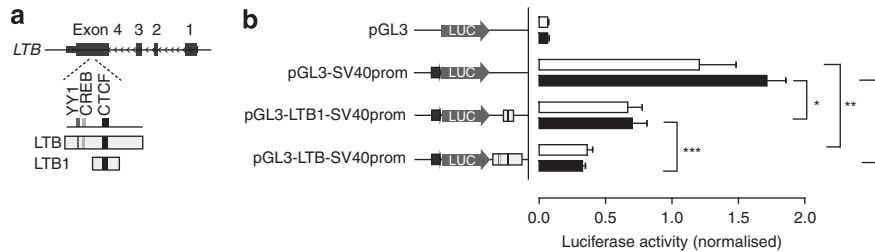

Figure 6 Effects of *LTB* exon 4 sequences on reporter gene expression in Jurkat T cells. (**a**) Reporter gene design. (**b**) Jurkat cells were transiently transfected with different pGL3 reporter constructs as indicated, either pGL3 basic, pGL3 driven by SV40 promoter alone or in the presence of sequence spanning the YY1/CREB/CTCF sites (ch6:31656365–31657327) or the CTCF-binding site without the YY1/CREB-binding sites (chr6: 31656631–31656999). The mean ± s.e.m. of luciferase expression values are shown (normalised by pRL-TK) for five independent transfection experiments, each performed in duplicate. Open bars show expression for unstimulated cells, black bars values following mitogen stimulation (PMA/ionomycin). *$P < 0.0001$, **$P = 0.03$, ***$P = 0.02$ on paired *t*-test (two tailed).

oncogene,[34,35] although it rapidly became clear that the effects of CTCF were pleiotropic. This includes transcriptional activation, enhancer blocking activity and more recently an appreciation of the key role of CTCF in intra- and inter-chromosomal looping.[26,27] The latter can have remarkably complex and specific functions, notably at imprinted gene loci such as *H19/Igf2*, which are critical to allele-specific expression, insulator activity and maintenance of specific DNA-methylation patterns.[36,37] Indeed, it is now thought that the primary role of CTCF is related to chromatin architecture, functioning as the 'master weaver of the genome',[27] critical to regulation of gene expression.

The presence of a YY1-binding site within 250 bp of the CTCF-binding element at *LTB* is potentially highly significant, given the known role of YY1 as a cofactor interacting with CTCF, involved for example in X-chromosome inactivation.[26,38] YY1, like CTCF, is a zinc finger protein involved in diverse biological processes, including transcriptional repression, as seen for example through recruitment of polycomb group proteins leading to methylation of histone H3 K27.[39] There is evidence of direct interaction between YY1 and the N-terminus of CTCF with paired CTCF/YY1 sites noted in the X-inactivation centre, and evidence of a synergistic and essential role for CTCF and YY1 in transactivation of *Tsix*.[38] Both YY1 and CTCF have critical roles at imprinting control regions characterised by differentially methylated CpG islands, and tandem arrays of CTCF- or YY1-binding sites, for example, at *H19/Igf2* and *Peg3*.[40] Although none of the genes in the *TNF* locus are imprinted, there is evidence of allele-specific expression[41] and epigenetic regulation is important.[42] It will be interesting to see in future work whether allele-specific differences relate to CTCF and YY1 binding in the final exon of the *LTB* gene.
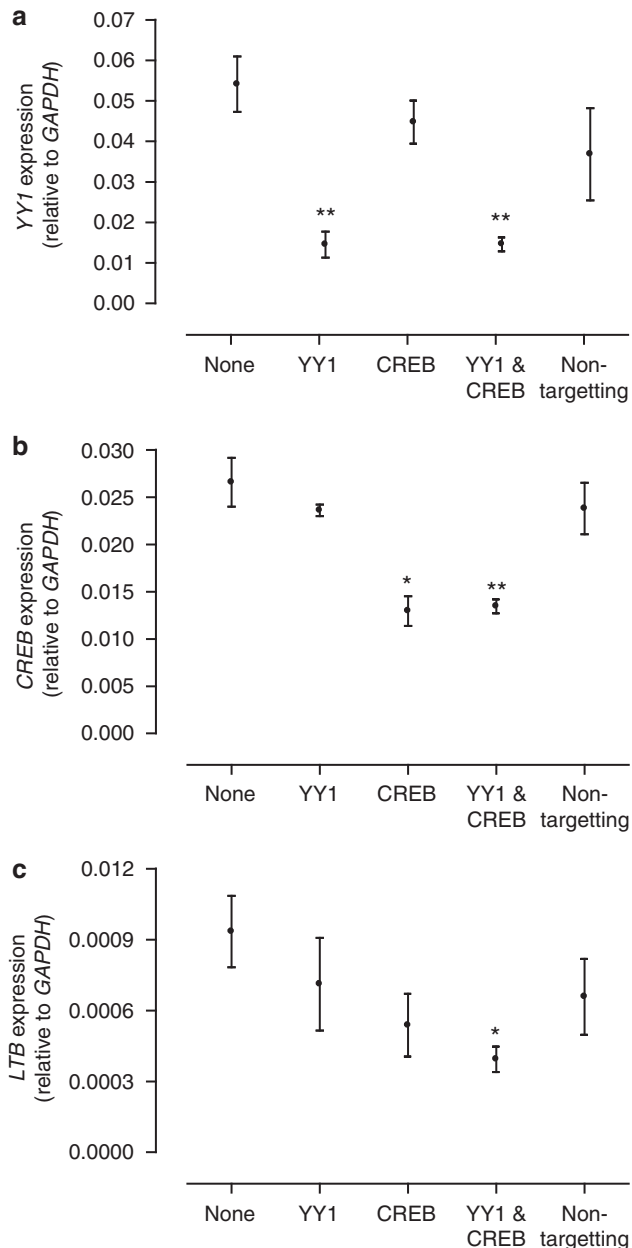
Figure 7 Silencing of *YY1* and *CREB* suppresses expression of *LTB*. Jurkat T cells were transfected with a pool of siRNAs targeting the 3'UTR and open reading frame of *YY1*, *CREB*, *YY1* and *CREB*, or a cocktail of non-targeting control siRNAs, and collected after 72 h. Expression of endogenous (**a**) *YY1* (**b**) *CREB* (**c**) *LTB* was quantified in duplicate by quantitative real-time PCR. The mean ± s.e.m. of three biological replicate experiments is shown with each experiment comprising two transfections. *$P < 0.05$, **$P < 0.01$ on unpaired *t*-test (two tailed).

We also note the evidence of CREB binding in this region of *LTB*. YY1 and CREB have been shown to be involved in the activation and repression of gene expression in many different genes, both alone and in some cases together as noted at *c-fos*.[43,44] siRNA experiments demonstrate a functional role for YY1 and CREB in *LTB* gene expression, with reduced expression of *LTB* when these transcription factors are knocked down. However, further work is required to define whether there are additional YY1- and CREB-binding sites

important in the regulation of *LTB* or whether the effect is directly mediated by the binding sites in the fourth exon of *LTB*. Given the complex interactions involving CTCF at this site, it is also difficult to establish the functional consequences of YY1 and CREB binding when they are studied in isolation. The binding sites are within a region showing striking evidence of histone acetylation and the recruitment of chromatin remodelling activity associated with CREB binding protein and p300 by both YY1 and CREB might prove important but further work is required to characterise this.[45–47]

In summary, we have characterised a novel regulatory element in the final exon of *LTB*, which involves complex protein–DNA interactions, with recruitment of YY1, CREB and CTCF. We find evidence of transcriptional repression and DNA looping whereby this region interacts with other sites in the TNF locus, notably the *LTA* promoter, *LTB* promoter and *TNF* 3′ UTR, highlighting the complexities of transcriptional regulation in this locus and the previously unrecognised role of CTCF.

Materials and methods

Cell culture

Jurkat T cells were grown in RPMI 1640 (Sigma-Aldrich, Dorset, UK) supplemented with 2 mM glutamine (Sigma), 100 U ml^{-1} penicillin (Sigma), 0.1 mg ml^{-1} streptomycin (Sigma) and 10% fetal calf serum (Sigma) at 37 °C in 5% CO_2 and collected in mid-log phase. Mitogens used for cell stimulation were 125 nM ionomycin (Sigma) and 200 nM PMA (Sigma) (final concentration).

Nuclear extracts, electrophoretic mobility shift assays

Nuclear extracts were prepared from Jurkat T cells as previously described.[48] Oligonucleotide probes were radiolabelled with ^{32}P dCTP (Perkin-Elmer, Beaconsfield, UK) and EMSA performed as previously described.[49] For supershift analysis, antibodies to YY1 (sc1703) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CREB (sc-186), HSF1 (sc9144), and CTCF (07–729) (Millipore, Billerica, MA, USA) were used. Probes were generated by annealing forward and reverse oligonucleotides. The sequences are shown in the Supplementary Table; the sequences for CTCF HS4 and HS4Δ (see ref. 30) are as previously reported.

Solid-phase DNase I footprinting

This was performed as previously described.[5] Three radiolabelled DNA probes designed to tile across *LTB* exon 4 were generated by PCR using one biotinylated primer and one primer end labelled with γ32P by T4 polynucleotide kinase. Genomic DNA from the PGF cell line was used for PCR amplification; PGF provides the reference sequence for human genome and is homozygous over the MHC.[50] The sequences are shown in the Supplementary Table. Radiolabelled DNA probe adsorbed onto magnetic Dynabeads M-280 Streptavidin (Invitrogen, Paisley, UK) was incubated in a binding reaction alone (naked DNA) or with nuclear extract from Jurkat T cells. Following DNase I digestion, DNA-binding reactions were analysed on a 7% acrylamide urea gel (7 M) and areas of protection localised using a Maxam-Gilbert sequencing ladder.

Chromatin immunoprecipitation

For a given assay, 1×10^8 Jurkat T cells were crosslinked with formaldehyde and chromatin isolated, sonicated and immunoprecipitated as previously described.[5] Sheep anti-rabbit IgG coated Dynabeads, preincubated with specific primary antibodies, were used for immunoprecipitation. For quantitative chromatin immunoprecipitation analysis by real-time PCR, we followed the methodology described by De Gobbi[51] with the amount of DNA immunoprecipitated by a specific antibody quantified relative to that of non-immunoprecipitated (input) DNA, and normalised relative to a control sequence in the 18S ribosomal RNA gene.

Quantitative chromatin conformation capture (3C-qPCR) assay

3C-qPCR was performed as previously described[25] with minor modifications. Briefly, 1×10^7 Jurkat T cells or HeLa cells were collected for a given condition, crosslinked for 10 min using 2% formaldehyde and lysed using ice-cold lysis buffer (10 mM Tris pH 8, 10 mM NaCl, 0.2% NP-40, $1 \times$ complete protease inhibitor (Roche, Burgess Hill, UK)) and nuclei resuspended in restriction enzyme buffer, incubated at 37 °C with SDS (0.3% final) for 1 h then Triton-X 100 (2% final) for 1 h before restriction enzyme digestion with 400 U DpnII (NEB, Hitchin, Hertfordshire, UK) overnight at 37 °C. Samples were then incubated with SDS (1.3% final) for 20 min at 65 °C, placed in ligation buffer with Triton-X 100 (Roche) (1% final) for 1 h at 37 °C before ligation using high-concentration T4 ligase (Fermentas UK, York, UK) at 16 °C for 4 h followed by 30 min at room temperature. Samples were digested with proteinase K (Roche) and incubated at 65 °C overnight to reverse crosslinks and digest the proteins. Following RNase digestion and phenol–chloroform extraction, DNA was precipitated and resuspended in water. DNA concentration was determined using SybrGreen quantitative PCR and a reference sample of genomic DNA with an internal primer set that did not span a restriction site, and volumes adjusted to normalise concentrations across samples. TaqMan real-time PCR quantification for 3C samples was determined including standard curves of serially diluted control template for each primer set to normalise amplification efficiency. For this BAC RP11-184 (chr6: 31545955–31736849) (BAC PAC Resources, Children's Hospital Oakland Research Institute) was used after digestion with Sau3A1 (isoschizomer of DpnII), ligation and purification. The probe and primer sequences are shown in the Supplementary Table.

Reporter gene assays

DNA fragments spanning LTB exon 4 were synthesised by PCR amplification of genomic DNA from the PGF cell line. PCR primer design introduced restriction sites to clone into BamHI/SalI sites downstream of the luciferase gene in the pGL3prom vector (Promega, Madison, WI, USA): for pGL3-LTB-SV40prom, Forward 5′-AGCTGG ATCCTCAATTTCCAAACAGTCTCCTACA-3′, Reverse 5′-TCGAGTCGACGCCCACCTCATAGGTAAGGA-3′ (chr6: 31656365-31657327); for pGL3-LTB1-SV40prom, Forward 5′-AGCTGGATCCAGCACTGGAGTCACCGT CTC-3′, Reverse 5′-TCGAGTCGACGCTAAAAGCCGC CACTTCC-3′ (chr6: 31656631-31656999). All constructs were verified by sequencing. Jurkat T cells were transiently transfected using Lipofectamine LTX and PLUS reagent (Invitrogen) according to the manufacturer's instructions, allowed to recover after transfection for 1 h, then in which indicated were stimulated with PMA and ionomycin. Cells were collected after 24 h and luciferase assays performed following the manufacturer's protocol. Firefly luciferase constructs were co-transfected with pRL-TK (Promega) to allow normalisation of transfection efficiencies. Two independent endotoxin-free preparations of all constructs were analysed in transfection experiments.

siRNA knockdown assay

siRNA knockdown was performed according to the manufacturer's instructions using Accell SMARTpool siRNA duplexes (Dharmacon, Lafayette, CO, USA) targeting the 3′UTR and open reading frame of YY1, CREB, YY1 and CREB, or a cocktail of non-targeting control siRNAs. Briefly, 2×10^4 cells/well were resuspended in Accell delivery media (Dharmacon) and incubated with 1 μM of each siRNA duplex for 72 h. Each transfection was carried out in duplicate for three replicate experiments. Total RNA was collected for each sample using an RNeasy mini kit (QIAGEN, Crawley, W Sussex, UK) including an on-column genomic DNA digestion step and cDNA prepared using a Superscript III kit (Invitrogen) primed with random hexamers. Expression of endogenous (1) YY1 (2) CREB (3) LTB was quantified in duplicate by SybrGreen quantitative real-time PCR using transcript-specific primers.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies the paper on Genes and Immunity website (http://www.nature.com/gene)