Transcriptional regulation of the human *TRIF* (TIR domain-containing adaptor protein inducing interferon β) gene

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TRIF [TIR (Toll/interleukin-1 receptor) domain-containing adaptor protein inducing interferon β; also known as TICAM-1 (TIR-containing adaptor molecule-1)] is a key adaptor for TLR3 (Toll-like receptor 3)- and TLR4-mediated signalling. We have performed a detailed annotation of the human *TRIF* gene and fine analysis of the basal and inducible promoter elements lying 5′ to the site of initiation of transcription. Human *TRIF* maps to chromosome 19p13.3 and is flanked upstream by *TIP47*, which encodes the mannose 6-phosphate receptor binding protein, and downstream by a gene encoding FEM1a, a human homologue of the *Caenorhabditis elegans Feminisation-1* gene. Using promoter–reporter deletion constructs, we identified a distal region with the ability to negatively regulate basal transcription and a proximal region containing an Sp1 (stimulating protein 1) site that confers approx. 75 % of basal transcriptional activity. *TRIF* expression can be induced by multiple stimuli, such as the ligands for TLR2, TLR3 and TLR4, and by the pro-inflammatory cytokines tumour necrosis factor α and interleukin-1α. All of these stimuli act via an NF-κB (nuclear factor-κB) motif at position -127. In spite of the presence of a STAT1 (signal transduction and activators of transcription 1) motif at position -330, the addition of type I or type II interferon had no effect on *TRIF* activity. The human *TRIF* gene would therefore appear to be regulated primarily by NF-κB.

Key words: innate immunity, lipopolysaccharide (LPS), nuclear factor-κB (NF-κB), stimulating protein 1 (Sp1), Toll-like receptor, TRIF.

INTRODUCTION

TLRs (Toll-like receptors) are a family of cell surface molecules tailored to respond to microbial pathogens, with particular TLRs able to recognize and bind specific PAMPs (pathogen-associated molecular patterns). The best-studied TLRs are TLR4, which senses LPS (lipopolysaccharide), a component of Gram-negative bacteria, and TLR3, which has the ability to sense viral dsRNA (double-stranded RNA) [1,2]. Signalling by TLRs is initiated by the recruitment of a specific set of adaptor proteins (reviewed in [3]). MyD88 (myeloid differentiation factor 88) [4] and Mal (MyD88 adaptor-like protein; also known as TIRAP) [5] were the first of these adaptor proteins to be characterized, with MyD88 acting as a general adaptor for most TLRs and Mal providing specificity to responses mediated by TLR2 and TLR4 (reviewed in [6,7]). The third adaptor to be identified, termed TRIF {TIR [Toll/IL-1 (interleukin-1) receptor] domain-containing adaptor protein inducing IFNβ (interferon 1β)} or TICAM-1 (TIR-containing adaptor molecule-1) [8,9], was closely followed by a fourth called TRAM (TRIF-related adaptor molecule; also termed TIRP or TICAM-2) [10–12]. The putative fifth putative adaptor had been identified previously as SARM (sterile alpha and HEAT-armadillo motifs) [13], although this protein has yet to be shown to have a role in TLR signalling.

TRIF was discovered due to the presence of a TIR domain [8], a region that is conserved in all members of the IL-1 receptor/TLR superfamily [2]. TRIF mRNA is ubiquitously expressed, with its highest mRNA expression found in human liver [8]. In terms of its role, TRIF was first shown to serve as an adaptor for the TLR3-mediated signalling pathway, with studies showing an association of TRIF with TLR3 and the ability of a mutant form of TRIF to inhibit the TLR3-dependent activation of NF-κB (nuclear factor-κB) and the so-called MyD88-independent pathway leading to the activation of the transcription factor IRF3 (IFN regulatory factor 3), which is involved in IFNβ up-regulation [8,9]. Further studies using *Trif*$^{-/-}$ mice [14] demonstrated a key role in both TLR3- and TLR4-mediated signal transduction to both NF-κB and IRF3, with the induction of IFNβ and inflammatory cytokines being severely impaired. A similar phenotype was observed in mice with a frame-shift mutation in the *Lps2* gene [15,16]; this gene was subsequently identified as murine *Trif*. The interaction of TRIF with TLR4 appears indirect, with recent evidence suggesting that another adaptor molecule, TRAM, can act as a 'bridge' between TRIF and TLR4. This is TLR4-specific, since TRAM does not interact with TLR3 [10,12]. The involvement of TRIF with downstream signalling molecules has also been investigated. TRIF has been shown to activate the transcription factor NF-κB; this association is mediated via association of TRAF6 [TNF (tumour necrosis factor) receptor-associated factor 6] binding motifs in its N-terminal portion with the TRAF6 domain of TRAF6 [8,17]. Mutation of these motifs abrogated TRIF-induced NF-κB activation, but had no effect on TRIF-mediated activation of the IFNβ promoter in response to the dsRNA analogue poly(I:C) (double-stranded polyribonucleotide polyinosinic

Abbreviations used: CREB, cAMP response element binding protein; dsRNA, double-stranded RNA; EMSA, electrophoretic mobility shift assay; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney; IκB SR, IκBα super-repressor; IFN, interferon; IL-1, interleukin-1; IRF, interferon regulatory factor; ISRE, interferon-stimulated response element; LPS, lipopolysaccharide; Mal, MyD88 adaptor-like protein; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; ORF, open reading frame; Pam_3/CSK, *N*-palmitoyl-(*S*)-[2,3-bis(palmitoyloxy)-(2*R*,*S*)-propyl/Cys-Ser-Lys; PAMP, pathogen-associated molecular pattern; poly(A)$^+$, polyadenylated; RACE, rapid amplification of cDNA ends; RREB, Ras-responsive element binding protein; Sp1, stimulating protein 1; STAT, signal transducer and activator of transcription; TICAM, TIR-containing adaptor molecule; TIR, Toll/interleukin-1 receptor; TLR, Toll-like receptor; TNF, tumour necrosis factor; TRAF6, TNF receptor-associated factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adaptor protein inducing interferon β; TRIF-DN, dominant-negative version of TRIF.

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Table 1 Oligonucleotides used

Name	Sequence (5′ → 3′)	Used for
TR-1	5′-TGCTCCATCTGTGCCTGGTAGGACAA-3′	5′ RACE
TR-2	5′-TTCTCCTCAGCCAGCAGGTGGTACAA-3′	5′ RACE
TR-3	5′-GAGCCTGAGGAGATGAGCTGGCCGCC-3′	3′ RACE
TR-4	5′-TTGTCCTACCAGGCACAGATGGAGCA-3′	3′ RACE
5T-1	5′-GCGGTACCAACAGCTGCTACATTTATTTCCTGGAAA-3′	Reporter generation
5T-2	5′-GCGGTACCCGAGAGCAGATGCGAGTTCTTCTGG-3′	Reporter generation
5T-3	5′-GCGGTACCCGTGACCGCAAGGGTTGGGAAG-3′	Reporter generation
5T-4	5′-TAGGTACCGCGGGGCGACCCGGGGACTTTCCCGGTG-3′	Reporter generation
5T-4ΔNFκB	5′-TAGGTACCGCGGGGCGACCCGGAGCCTTTAACGGTG-3′	Reporter generation
5T-5	5′-GCGGTACCGTGCTGCGGGGCAGGACTCCGA-3′	Reporter generation
5T-6	5′-GAGGTACCTCCCACACCCACCCGGGACG-3′	Reporter generation
5T-7	5′-TAGGTACCGCGGAAGGGGCGGCGCGCTT-3′	Reporter generation
5T-8	5′-GCGGTACCCTTCCTTCCCAGGGCGCGGG-3′	Reporter generation
5T-9	5′-GAGGTACCCCTCAGCGCGCTACGGTCCG-3′	Reporter generation
5T-10	5′-GCGGTACCCAGAAGGCCCCAGCCCCCAGGA-3′	Reporter generation
3T-1	5′-GCAAGCTTCAGGGATCCGGGGCTGCCGGCT-3′	Reporter generation
ΔNFκB-F	5′-GCGGGGCGACCCGGAGCCTTTAACGGTGCTGCGGGG-3′	Reporter generation
ΔNFκB-R	5′-CCCCGCAGCACCGTTAAAGGCTCCGGGTCGCCCCGC-3′	Reporter generation
GAPDH-F	5′-GGGCTGCTTTTAACTCTGGTAAAG-3′	Northern analysis
GAPDH-R	5′-CTGTAGCCAAATTCGTTGTCATAC-3′	Northern analysis
Sp1 (TRIF)	5′-AAGGGGCGGCGCGCT-3′ 3′-TTCCCCGCCGCGCGA-5′	EMSA
Sp1 (Consensus)	5′-ATTCGATCGGGGCGGGGCGAGC-3′ 3′-TAAGCTAGCCCCGCCCCGCTCG-5′	EMSA
CREB (Consensus)	5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′ 3′-TCTCTAACGGACTGCAGTCTCTCGATC-5′	EMSA
NFκB (Consensus)	5′-AGTTGAGGGGACTTTCCCAGGC-3′ 3′-TCAACTCCCCTGAAAGGGTCCG-5′	EMSA

acid–polycytidylic acid) [17]. This activation requires the association of TRIF and IRF3 independent of Mal or MyD88 [8], and occurs via activation of the IκB kinase-related kinase TBK-1 (Tank-binding kinase-1) [10,17].

Despite the increasing amount of data detailing the signalling events mediated by the TLR adaptor molecules, and in particular TRIF itself, our knowledge of how these adaptors are regulated at the transcriptional level to modulate the innate immune response to microbial pathogens is scarcer. Here we have performed a detailed analysis of the human *TRIF* gene and the factors that regulate its transcription. The *TRIF* gene can be induced by multiple stimuli, including ligands for TLR2, TLR3 and TLR4, as well as the pro-inflammatory stimuli IL-1α and TNFα. *TRIF* expression, however, is unaffected by type I or type II IFN. *TRIF* responsiveness is mediated primarily through NF-κB, since removal of its binding site or its sequestration in the cytoplasm by IκB SR (the IκBα 'super-repressor') can completely abrogate *TRIF* activation. The regulation of the *TRIF* gene would therefore appear to be another important cellular level at which innate immune responses can be modulated.

EXPERIMENTAL

Identification and annotation of human TRIF

The complete nucleotide sequence of the human *TRIF* gene and flanking regions was obtained from approx. 100 kb of human genomic DNA contained within a contig (NT_011255.10) encompassing part of *Homo sapiens* (*HSA*) chromosome 19p13.3. This sequence was obtained from the National Center for Biotechnology Information (NCBI) website at www.ncbi.nlm.nih.gov/ following BLAST analysis of the human genome with a partial cDNA of *TRIF* (GenBank accession no. AB093555). Identification of transcribed nucleotide sequences and repeat sequences in the genomic sequence was performed using the NIX application (http://menu.hgmp.mrc.ac.uk) and the program RepeatMasker (http://searchlauncher.bcm.tmc.edu) [18]. Transcriptional units identified were also compared with the GenBank sequences of known human genes (*FEM1A*, NM_018708; *TIP47*, NM_005817; obtained from www.ncbi.nlm.nih.gov/). Approx. 50 kb of murine genomic DNA containing the murine *Trif* (GenBank accession no. NM_174989), *Fem1a* (NM_010192) and *Tip47* (NM_025836) genes was isolated from another contig (NT_039656.1) localized to *Mus musculus* (*MMU*) chromosome 17C and also obtained from NCBI. Global alignment of the human and mouse genomic sequences of conserved synteny was performed as described previously [19]. A higher-resolution comparison of the conserved non-coding sequences upstream of human *TRIF* and mouse *TRIF* (and other multiple sequence alignments) was carried out using ClustalW (www.ebi.ac.uk/clustalw/). Transcription factor binding site predictions were performed using Signalscan (http://bimas.dcrt.nih.gov/molbio/signal) [20], TFSEARCH (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html) [21] and MatInspector Release Professional (www.genomatix.de/cgi-bin/matinspector/matinspector.pl) [22].

5′ and 3′ SMART™ RACE (rapid amplification of cDNA ends)

5′ and 3′ SMART™ RACE-ready cDNA libraries were generated according to the manufacturer's recommendations (Clontech, Palo Alto, CA, U.S.A) using 1 μg of total human placental RNA or 1 μg of human liver poly(A)$^+$ (polyadenylated) mRNA. 5′ and 3′ RACE reactions and PCR amplifications were performed according to the manufacturer's recommendations (Clontech) as described previously [19] using the gene-specific primers TR-1 and TR-3 respectively (Table 1). To increase the specificity of the RACE reaction, primary RACE PCR products were

diluted 1:50 with 10 mM Tris/EDTA buffer and amplified further using the 'nested' gene-specific primers TR-2 or TR-4 (Table 1) as described previously [19]. PCR products were analysed by agarose gel electrophoresis, gel-purified, cloned into pGEM-T easy (Promega, Madison, WI, U.S.A) and sequenced (MWG-Biotech, Ebersberg, Germany).

Generation of luciferase reporter constructs

In order to analyse the 5′ flanking region of the human *TRIF* gene for promoter activity, the following PCR products were amplified from human genomic DNA (Sigma, St. Louis, MO, U.S.A) using the primers 5T-1 to 5T-10 (forward primers) and 3T-1 (reverse primer) (Table 1): TRIF-348 (nt −348 to +95, where the first nucleotide of *TRIF* exon 1 has been designated +1), TRIF-233 (nt −233 to +95), TRIF-173 (nt −173 to +95); TRIF-138 (nt −138 to +95), TRIFΔ138 [nt −138 to +95 with mutations in four nucleotides within the NF-κB binding site at −116 (C → A), −117 (C → A), −122 (A → C) and −124 (G → A)], TRIF-113 (nt −113 to +95), TRIF-83 (nt −76 to +95), TRIF-64 (nt −57 to +95), TRIF-47 (nt −40 to +95), TRIF-7 (nt +1 to +95) and TRIF+33 (nt +26 to +88). A further promoter–reporter fragment (TRIFΔ348) was also generated which was identical to TRIF-348 except for mutations in nucleotides −116, −117, −122 and −124 identical with those of TRIFΔ138 above.

In the primary PCR reaction, the primers 5T-1/ΔNFκB-R and ΔNFκB-R/3T-1 (Table 1) were used to generate overlapping fragments that were subsequently used as templates for a further PCR reaction using the primers 5T-1/3T-1. Amplification of all PCR fragments was carried out on a PerkinElmer 2400 thermocycler using the following conditions: 2.5 μl of 10 × Vent (Roche Diagnostics, Mannheim, Germany), 1.0 μl of 10 mM dNTPs, 0.5 μl of Vent polymerase (2 units/μl; Roche), 1.0 μl of 200 ng/μl genomic DNA, 1.0 μl each of 5′ and 3′ primers (10 μM) (Table 1) and 18.0 μl of water. The following cycling conditions were then used: one cycle of 95 °C for 1 min, then 25 cycles of 95 °C for 15 s, 69 °C for 15 s and 72 °C for 45 s, followed by a 72 °C extension for 5 min. All *TRIF* constructs were generated with *Kpn*I and *Hin*dIII linkers at their 5′ and 3′ ends respectively; these were subsequently cleaved with their respective restriction endonucleases (New England Biolabs), subcloned directionally into pGL3-Basic and sequenced.

Cell culture and reagents

HEK (human embryonic kidney) 293 cells and U373 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 1% L-glutamine (Sigma). HEK293 cell lines stably transfected with TLR2 (HEK293-TLR2), TLR3 (HEK293-TLR3) or TLR4 (HEK293-TLR4) were gifts from Dr Katherine Fitzgerald (University of Massachusetts Medical School, Worcester, MA, U.S.A), and were grown as above supplemented with 600 μg/ml G418 (Sigma). LPS from *Escherichia coli* serotype O26:B6 and the synthetic bacterial lipopeptide analogue Pam_3/CSK {*N*-palmitoyl-(*S*)-[2,3-bis(palmitoyloxy)-(2*R*,*S*)-propyl/Cys-Ser-Lys} were obtained from Sigma and used at 1 μg/ml. The dsRNA analogue poly(I:C) (Sigma) was used at 2.5 μg/ml. Recombinant human IFNα4, IFNβ and IFNγ (Sigma) were used at 1000 i.u./ml. Recombinant human TNFα was a gift from Dr S. Foster (Zeneca, Alderley Park, Macclesfield, Cheshire, U.K), and IL-1α was a gift from Professor J. Saklatvala (Kennedy Institute of Rheumatology, London, U.K); both were used at 100 ng/ml. The pGL3-Basic and pRL-TK vectors were obtained from Promega. The pGL3-NF-κB construct bearing five repeats of the κB consensus was a gift from Dr R. Hofmeister (Universität Regensburg, Regensburg, Germany), and the pGL3-ISRE reporter plasmid, with five repeats of the ISRE (IFN-stimulated response element) sequence from the ISG15 promoter, was obtained from Clontech. An expression plasmid encoding human TLR3 was obtained from Tularik (San Francisco, CA, U.S.A); plasmids encoding TRIF and TRIF-DN (a dominant-negative version of TRIF; encoding the TIR domain only) were gifts from Shizuo Akira (Osaka University, Osaka, Japan). IκB SR was a gift from Dr Keith Ray (GlaxoSmithKline, Stevenage, U.K).

Transient transfection

Cells were seeded at approx 1×10^5 cells/ml into 24-well plates in complete growth medium, and cultured at 37 °C (in the presence of 5% CO_2) until 50–80% confluent. To 17 μl of serum-free medium was added 3 μl of GeneJuice™ (Novagen, La Jolla, CA, U.S.A); following mixing and a 5 min incubation at room temperature, the solution was added to 400 ng of plasmid DNA, mixed gently and incubated for a further 15 min at room temperature, then added drop-wise to each well of the 24-well plate. This was performed in triplicate for each assay point. The composition of the transfected DNA was typically: 100 ng of *Renilla* luciferase (pRL-TK), 200 ng of luciferase reporter (e.g. pGL3-TRIF) and 100 ng of empty vector (pGL3-Basic) if cells were to be stimulated exogenously. Alternatively, 100 ng of *Renilla* luciferase and 200 ng of luciferase reporter with or without 10, 20 or 30 ng of the TLR3, TRIF or TRIF-DN expression vectors, and/or 10, 25 or 50 ng of IκB SR, was used. The remainder was made up to 400 ng with empty vector. Following transfection, cells were either left untreated at 37 °C (5% CO_2) for 24 h or treated at the concentrations mentioned above with LPS, poly(I:C) or Pam_3/CSK for 16 h; TNFα, IFNα4, IFNβ or IFNγ for 9 h; or IL-1α for 6 h. Optimal treatment times had been determined previously by time-course analysis, and the total incubation time for all transfected cells was 24 h. Cells were then lysed in 300 μl/well 1 × passive lysis buffer (Promega) for 15 min at room temperature, and 25 μl samples of the lysate were analysed in duplicate for firefly luciferase and *Renilla* luciferase activity using a Mediators PhL Luminometer. Reporter activity was then determined as a function of firefly luciferase activity divided by *Renilla* luciferase activity. Differences between means were calculated using Student's *t* test.

EMSA (electrophoretic mobility shift assay)

Double-stranded consensus Sp1 (stimulating protein 1), CREB (cAMP response element binding protein) and NF-κB oligonucleotides (Table 1) were obtained from Promega. A double-stranded *TRIF* Sp1 oligonucleotide (Table 1) was generated by annealing two oligonucleotides (5′-AAGGGGCGGCGCGCT-3′ and 5′-AGCGCGCCGCCCCTT-3′) at 95 °C for 2 min in the following reaction mixture: 10 μl of each oligonucleotide (2 μM), 2.5 μl of 10 × T4 polynucleotide kinase buffer (Promega) and 2.5 μl of water. The reaction was then cooled slowly to room temperature. Double-stranded oligonucleotides were ^{32}P-labelled at 37 °C for 10 min according to the manufacturer's recommendations (Promega), in 2 μl of 1.75 μM double-stranded oligonucleotide, 1 μl of 10 × T4 polynucleotide kinase buffer, 1 μl of [γ-^{32}P]ATP (3000 Ci/mmol at 10 mCi/ml; Amersham Biosciences) and 5 μl of nuclease-free water (Promega). The

reaction was then stopped by the addition of 1 μl of 0.5 M EDTA and the total volume made up to 100 μl with Tris/EDTA buffer.

For the subsequent DNA binding reactions, which were also performed according to the manufacturer's recommendations (Promega), mixtures containing 2 μl of 5 $\times$ gel shift binding buffer (Promega) and 7 μl of nuclease-free water (negative control); 2 μl of HeLa (Promega), U373 or HEK293 cell nuclear extract, 2 μl of 5 $\times$ binding buffer and 5 μl of nuclease-free water (positive control); or 2 μl of HeLa nuclear extract, 2 μl of 5 $\times$ binding buffer, 4 μl of nuclease-free water and 1 μl of 1.75 μM unlabelled competitor oligo (*TRIF* Sp1, consensus Sp1 or CREB Sp1) were incubated at room temperature for 10 min, then 1 μl of ^{32}P-labelled oligonucleotide (*TRIF* Sp1, consensus Sp1 or consensus NF-κB) was added to each reaction. The reactions were incubated for a further 20 min at room temperature, then electrophoresed at 180 V on a 5% native acrylamide/bisacrylamide gel in 0.5 $\times$ TBE (1 $\times$ TBE = 45 mM Tris/borate and 1 mM EDTA). The gel was then dried and analysed by autoradiography.

Isolation and Northern blot analysis of poly(A)$^+$ mRNA

Poly(A)$^+$ mRNA from U373 cells was prepared using the GenElute™ Direct mRNA miniprep kit according to the manufacturer's instructions (Sigma). Cells (2×10^6) were seeded into 10 cm^2 dishes and cultured at 37 °C (5% CO_2) for 24 h, then stimulated for 0–24 h with LPS, poly(I:C), TNFα or IL-1α at the concentrations described above. Following stimulation, cells were washed in PBS and lysed at 65 °C for 10 min in 0.5 ml/sample lysis solution containing 100 μg of Proteinase K (Sigma). To each lysate was then added 32 μl of 5 M NaCl, followed by 25 μl of oligo(dT) beads (Sigma), and the solution was mixed and incubated for a further 10 min at room temperature to allow binding of mRNA to the beads. Each bead–mRNA complex was pelleted by centrifugation at 16000 ***g***, and washed twice in 350 μl of Wash Solution (Sigma) and then once in 350 μl of Low Salt Wash solution (Sigma).

Purified poly(A)$^+$ mRNA was then eluted from the beads by incubation at 65 °C in 50 μl of Elution Solution (Sigma). Northern blots of poly(A)$^+$ mRNA were prepared and hybridized as described previously [23] with a ^{32}P-labelled 2136 bp human TRIF cDNA fragment encoding the entire reading frame, followed by a 904 bp GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA (nt 97–1000; GenBank Accession no. BC026907) to normalize for mRNA loading. The intensities of TRIF mRNA transcripts relative to GAPDH were determined by densitometric analysis using LabWorks™ Image acquisition and analysis software, Version 4.0 (1996–2001; Media Cybernetics), and a mean relative intensity for each time point was calculated by analysing five separate exposures of each blot.

RESULTS

Identification of the human *TRIF* gene

In order to examine the human *TRIF* promoter and its mechanisms of regulation, we first needed to identify and annotate in detail the *TRIF* gene itself. In order to identify the location of the *TRIF* gene in the human genome, a BLAST search of the human genomic DNA database using a partial TRIF cDNA [8] was performed. A region of perfect identity was found on *HSA* chromosome 19p13.3 (Figure 1A). To obtain the position of the putative *TRIF* gene relative to the genes flanking it at its 5′ and 3′ ends, approx. 100 kb of genomic DNA surrounding this region of identity was analysed in detail using the NIX suite of programs (results not shown), and results were confirmed by BLASTN and BLASTX analysis and alignment of the identified cDNA sequences against the human genomic sequence. The results of this analysis are summarized in Figure 1(A). The putative human *TRIF* gene encompasses approx. 16 kb of human genomic DNA and is transcribed towards the telomere of *HSA* chromosome 19p13.3 (Figure 1A). Approx. 6.6 kb 5′ of the *TRIF* gene, and transcribed in the same orientation as *TRIF*, lies the *TIP47* gene (GenBank accession no. NM_005817), a multi-exon gene of approx. 30 kb encoding the mannose 6-phosphate receptor binding protein [24] (Figure 1A). Approx. 20 kb 3′ of *TRIF* lies the *FEM1A* gene (GenBank accession no. NM_018708), a small gene encoding a human homologue [25] of the *Caenorhabditis elegans* sex-determination factor Feminisation-1 or Fem-1 [26], which is transcribed in the opposite orientation to *TRIF* (Figure 1A).

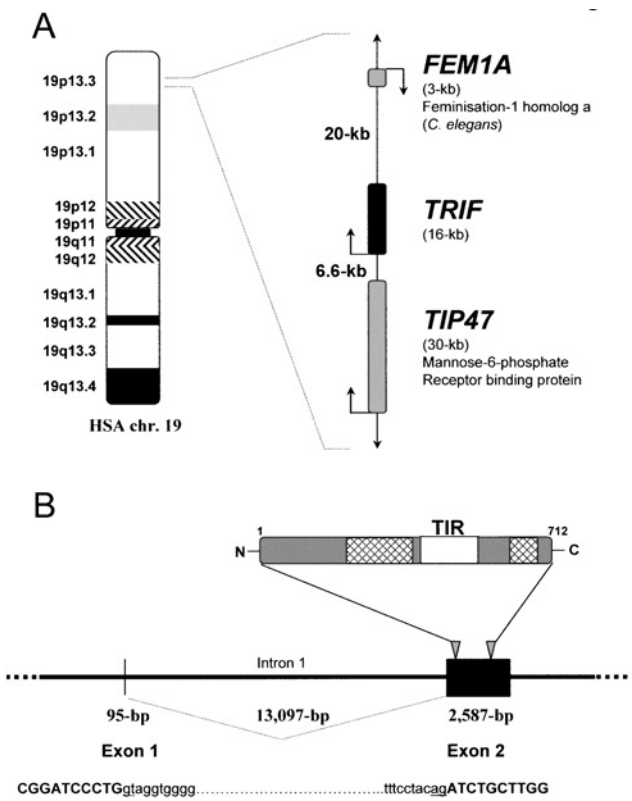

Figure 1 Localization and annotation of the human *TRIF* gene

(**A**) Schematic representation of *HSA* chromosome 19, with an expanded portion of 19p13.3 containing the genes *FEM1A*, *TRIF* and *TIP47* to the right. Each gene is indicated by a box, and the direction of transcription by an arrow. Distances between genes in kb are shown. (**B**) Schematic representation of the genomic structure of the human *TRIF* gene. The sizes and positions of exons 1 and 2 (black boxes) are shown on the genomic sequence (horizontal line), with start and stop codons indicated by inverted triangles. Below are the intron–exon boundaries of *TRIF*, with exonic sequences in bold upper case and intronic sequences in lower case. The mature TRIF protein is represented above (grey rectangle), with N- and C-termini, the TIR domain (white box) and two proline-rich regions (hatched boxes) shown.

Annotation of the human *TRIF* gene and analysis of its 5′ flanking region

Comparison of the available TRIF cDNA sequence against its matching genomic sequence revealed that the entire *TRIF* ORF (open reading frame) is encoded by a single exon (Figure 1B). This ORF of 2136 bp is predicted to encode a 712-amino-acid protein

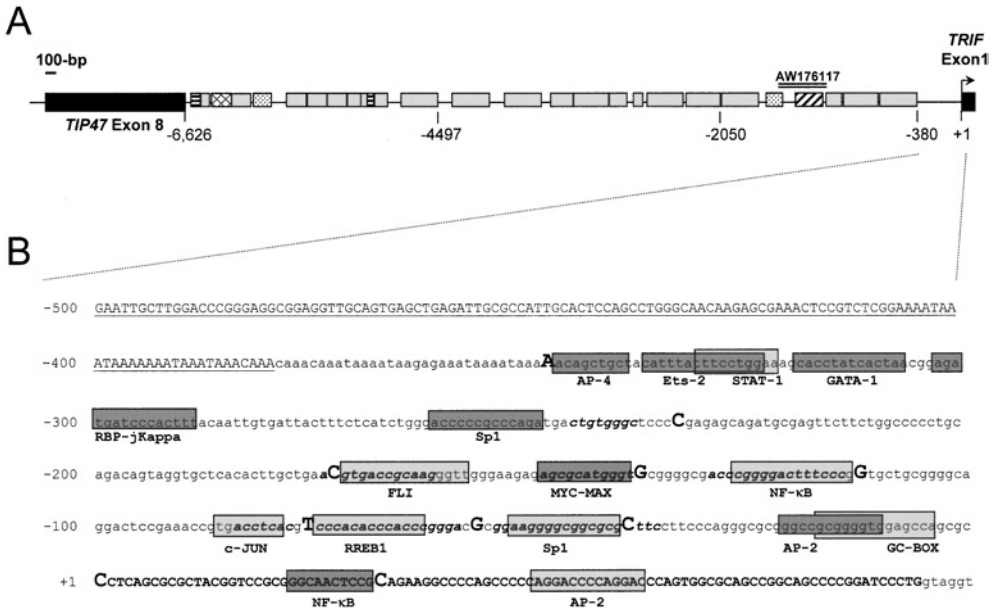

Figure 2 Analysis of the *TRIF* 5′ flanking region

(**A**) RepeatMasker analysis of the 6626 bp genomic region (horizontal line) between exon 8 of the *TIP47* gene and exon 1 of the *TRIF* gene (black boxes). The sizes and relative positions of short interspersed elements (grey boxes), long interspersed elements (dotted boxes), DNA elements (diagonally striped boxes), long terminal repeats (hatched boxes) and simple repeats (horizontally striped boxes) are shown. Also indicated is an orphan EST (double line; GenBank accession no. AW176117). (**B**) Analysis of the 500 bp of 5′ flanking sequence proximal to exon 1 of *TRIF* and of exon 1 itself for the presence of putative transcription factor binding sites. Genomic sequence is in lower case, exon 1 is in bold upper case, and +1 refers to the first nucleotide of exon 1 as determined by 5′ SMART™ RACE. Repetitive sequences are underlined, putative transcription factor binding sites are labelled and shaded (*cis*-elements are in light grey; *trans*-elements are in dark grey), and regions of identity between the human and mouse genes are shown in bold lower case italics. The nucleotide positions of *TRIF* promoter-reporter deletion constructs are indicated in larger font size.

containing a characteristic TIR domain with flanking proline-rich regions of unknown function (Figure 1B). However, in order to identify the entire TRIF cDNA sequence and therefore the structure of the *TRIF* gene and the likely position of the *TRIF* promoter, we first took the genomic sequence between the *TIP47* and *FEM1A* genes and performed BLAST analysis against the EST (expressed sequence tag) database. Two ESTs were identified (GenBank accession nos. BI756610 and BG753364) that indicated that an additional *TRIF* exon lies approx. 13 kb upstream of the exon encoding the *TRIF* ORF. A 380 bp orphan EST was also identified approx. 5.4 kb 3′ of *TIP47* (GenBank accession no. AW176117), but did not appear to be part of the *TRIF* gene itself.

We then sought to confirm these findings by performing 5′ and 3′ SMART™ RACE, which also would definitively identify the 5′ and 3′ ends of the TRIF cDNA and determine the sequence and position of any additional *TRIF* exons. 5′ RACE analysis using TR-1 and TR-2 as primary and nested oligonucleotides respectively (Table 1) yielded a 553 bp PCR product (results not shown) which, when sequenced, confirmed the results of the EST analysis and demonstrated the presence of an additional *TRIF* exon 5′ of the one encoding the ORF. No additional 5′ *TRIF* exons were identified. 3′ RACE using TR-3 and TR-4 as the primary and nested oligonucleotides respectively (Table 1) yielded a 717 bp PCR product (results not shown) which extended the TRIF cDNA sequence 309 bp 3′ of its stop codon. No additional 3′ exons were identified. The structure of the human *TRIF* gene is shown schematically in Figure 1(B). Exon 1 is small (95 bp), is 76% GC-rich and encodes only 5′ untranslated sequences. Exon 1 is separated from the much larger exon 2 (2587 bp) by a single large intron of 13097 bp. Exon 2 encodes the remainder of the 5′ untranslated region, the entire ORF, as well as the entire 3′ untranslated region (Figure 1B). The intron–exon boundaries of the human *TRIF* gene are also shown in Figure 1(B), and conform to the GT–AG rule [27,28].

Having determined the 5′ extent of the TRIF cDNA by RACE, and hence the likely site for the initiation of transcription (designated +1), we next analysed in detail the 6626 bp of the *TRIF* 5′ flanking region for the presence of a functional promoter. RepeatMasker, which analyses genomic DNA for the presence of repetitive elements, was performed and the results showed that, with the exception of a region of 379 bp proximal to *TRIF* exon 1, the majority of the entire *TRIF* 5′ flanking region (75.7%) is composed of repetitive elements, predominantly short interspersed elements (Figure 2A). We therefore considered it likely that the functional *TRIF* promoter is contained within the 379 bp region proximal to exon 1. Further analysis of this region using a number of transcription factor binding site searches revealed putative binding sites for several transcription factors, such as NF-κB [29], AP2 (activator protein 2) [30], RREB1 (Ras-responsive element binding protein 1) [31], Sp1 [32] and the STATs (signal transducers and activators of transcription) [33].

Identification of the murine *Trif* gene and comparative analysis

Examination of the similarities between the human *TRIF* and murine *Trif* genes, in both coding and non-coding sequences, may provide clues to the location of a likely *TRIF* promoter in both species, since comparisons of human and mouse coding and non-coding genomic sequences in regions of conserved synteny have previously been successful in identifying common elements regulating gene expression [34]. To this aim, we identified the murine *Trif* gene by BLAST analysis of the murine genomic and EST databases using the human *TRIF* cDNA sequence, and

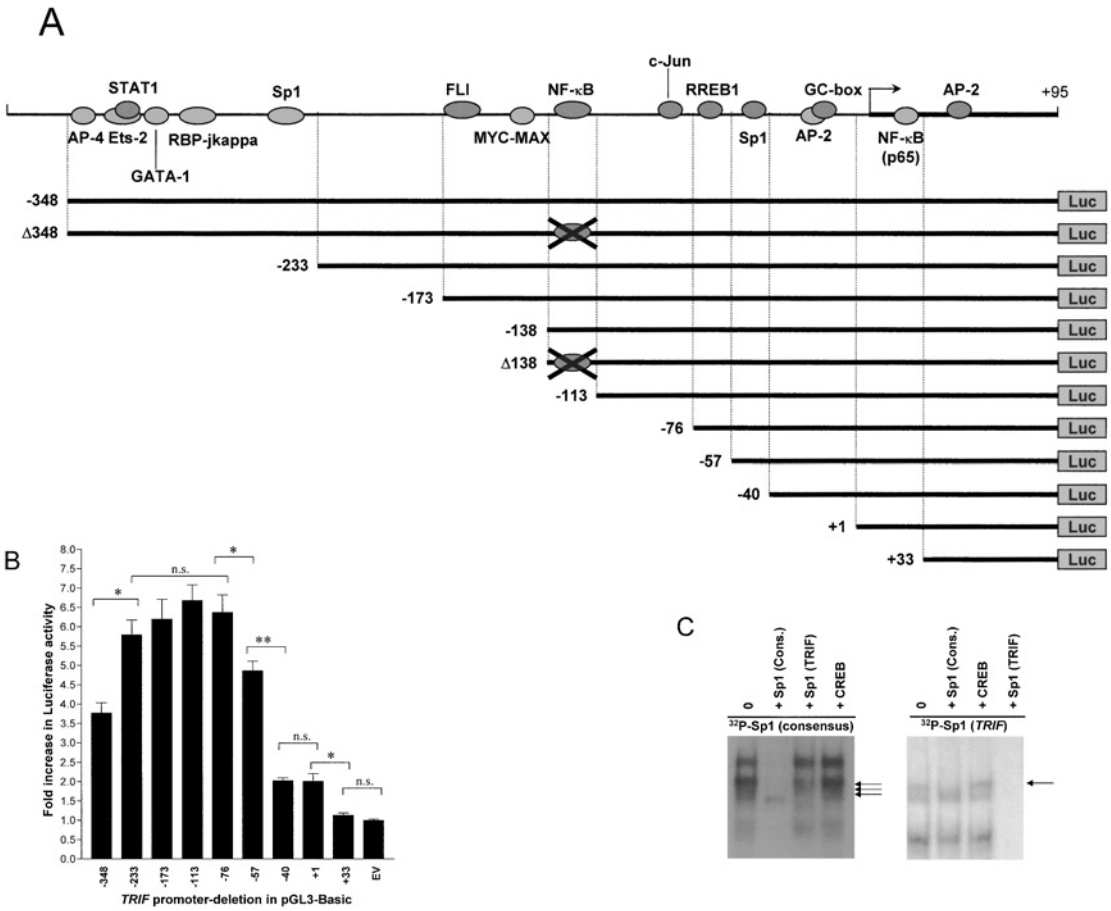

Figure 3 Generation of *TRIF* reporter constructs and analysis of *TRIF* basal activity

(**A**) Representation of the *TRIF* reporter deletion and mutation constructs (in pGL3-Basic) generated for this study. The position of each deletion from the *TRIF* genomic sequence is shown to the left of its respective construct. Constructs containing a mutation in the NF-κB binding site are prefixed by Δ. Above is a diagrammatical representation of the proximal 5′ flanking region of *TRIF* (horizontal line), exon 1 (thicker line) and putative transcription factor binding sites (ovals). The direction of *TRIF* transcription is also shown (arrow). (**B**) Reporter gene analysis of *TRIF* basal activity. HEK293 cells were transiently transfected for 24 h with the relevant *TRIF* reporter gene or with empty vector, then harvested and the luciferase activity determined. Data are expressed as fold increase in luciferase activity relative to empty vector (means $\pm$ S.E.M.; $n = 3$), and are representative of five separate experiments. *P* values were calculated using Student's *t* test ($^{*}P < 0.05$; $^{**}P < 0.005$; n.s., not significant). Similar results were obtained using U373 cells. (**C**) Analysis by EMSA of Sp1–DNA binding. HeLa nuclear extracts incubated with ^{32}P-labelled consensus Sp1 (left-hand panel) or *TRIF* Sp1 (right-hand panel) double-stranded oligonucleotides, with or without a 50-fold excess of unlabelled competitor (double-stranded consensus Sp1, *TRIF* Sp1 or CREB as a negative control), were analysed by native gel electrophoresis and autoradiography. Arrows indicate the competed bands.

subsequently isolated and annotated the murine *Trif* gene. We found that the genomic structure of murine *Trif*, located on *MMU* chromosome 17C, and its position relative to its flanking genes *Fem1a* and *Tip47*, were identical to that of human *TRIF* (results not shown). We then sought to determine, using the programs AVID and VISTA as described previously [19], whether any conserved non-coding sequences exist between human *TRIF* and murine *Trif* that might aid in the identification of a functional *TRIF* promoter. Comparison of the genomic regions containing the *FEM1A–TRIF–TIP47* genes showed that the *FEM1A* and *TIP47* genes are well conserved in their exonic sequences between humans and mice (results not shown). However, although the genomic structures of human *TRIF* and murine *Trif* are similar, only sequences within exon 2 are conserved (results not shown). No conserved non-coding regions between humans and mice in the *TRIF* 5′ flanking region, intron 1 or 3′ flanking region were identified, which at first suggested that this gene might be regulated differently in humans and mice (results not shown). However, a more detailed inspection of the AVID/VISTA alignment and a ClustalW alignment of the human/mouse *TRIF* 5′ flanking regions (results not shown) revealed conservation of certain transcription factor binding sites contained within the proximal human and murine *TRIF* 5′ flanking regions, such as those for FLI (Friend leukaemia integration), Myc-Max, NF-κB, c-Jun, RREB1 and Sp1 (shown in Figure 2B). These data suggest that some or all of these elements may be important for the regulation of the human (and murine) *TRIF* gene.

Constitutive activation of the human *TRIF* promoter

Having determined the area within the 5′ flanking region of human *TRIF* likely to contain a functional promoter, we sought to analyse its basal or constitutive activity and the element(s) controlling this. To this end, we generated 10 promoter–reporter deletion constructs containing various fragment lengths of the *TRIF* 5′ flanking region plus exon 1 placed upstream of the luciferase gene (Figure 3A). We then transfected these *TRIF* reporter gene constructs transiently into either HEK293 cells or U373 cells and examined their ability to drive luciferase expression without

further stimulation of the transfected cells. The results of the analysis for HEK293 cells are shown in Figure 3(B). The 'full-length' pGL3-TRIF-348 promoter construct was able to confer a moderate amount of luciferase expression (approx. 3.8-fold) relative to cells transfected with empty vector alone ($P < 0.005$) (Figure 3B). However, a difference of approx. 40 % in luciferase activity was observed ($P < 0.05$) between cells transfected with pGL3-TRIF-348 or pGL3-TRIF-233 (Figure 3B), which suggested the presence of an element(s) able to negatively regulate *TRIF* basal activity in the 115 nucleotides between positions -348 and -233 of the *TRIF* promoter. Deletion of additional *TRIF* sequence, down to -76, had no further effect on *TRIF*-driven luciferase activity (Figure 3B). However, deletion of sequence down to -57 resulted in a drop of 26.5 % in basal activity ($P < 0.05$) and another deletion, this time to -40, resulted in a further drop of 50 % ($P < 0.005$) in basal *TRIF*-driven luciferase reporter activity (Figure 3B). These data suggest that a 36 bp region lying between positions -76 and -40 within the *TRIF* 5′ flanking region is able to confer approx. 75 % of the basal or constitutive *TRIF* activity in a reporter assay. Similar results were observed in U373 cells (not shown). When we examined which transcription factor binding sites are located within the -76 to -40 region (Figures 2B and 3A), we identified those for Sp1 and RREB1, which suggests that these transcription factors (particularly Sp1) are the likely candidates responsible for the majority of the basal *TRIF* activity observed. Another region located between positions $+1$ and $+33$ conferred a further 15 % of *TRIF* basal activity ($P < 0.05$); the only transcription factor binding site found in this region is a putative NF-κB (p65) element [35], suggesting that this element may play a small role in the basal activation of *TRIF*.

Since the *TRIF* Sp1 binding site located between positions -53 and -41 (AAGGGGCGGCGCG) differs slightly from the consensus Sp1 binding site (NGGGGGCGGGGYN), we tested by EMSA the ability of an unlabelled consensus Sp1 double-stranded oligonucleotide to compete for binding against a ^{32}P-labelled consensus Sp1 oligonucleotide bound to HeLa nuclear extracts (Figure 3C, left panel). Both consensus Sp1 and *TRIF* Sp1 oligonucleotides were able to abrogate or reduce the formation of protein–DNA complexes, whereas an unrelated CREB oligonucleotide had no effect on complex formation. Complexes could only be formed in the presence of nuclear extract (results not shown). When the same unlabelled competitors were tested against ^{32}P-labelled *TRIF* Sp1 (Figure 3C, right panel), similar results were obtained, despite the formation of different protein–DNA complexes. Unlabelled consensus Sp1 competed out the slowest migrating band (as indicated by the single arrow), CREB had no effect, and unlabelled *TRIF* Sp1 fully abrogated protein–DNA complex formation.

Type I or type II IFNs have no effect on the *TRIF* promoter

Previous work, both *in vitro* and *in vivo*, has demonstrated a role for TRIF in the induction of IFNβ expression [8,14]. To determine whether IFN has a positive feedback role in the activation of *TRIF* expression via the STAT element located at positions -330 to -321, we examined the ability of a *TRIF* reporter gene to be induced following stimulation of cells with either type I or type II IFNs. To confirm the efficacy of the IFNs being used, we first transfected into HEK293 or U373 cells an IFN-responsive ISRE–luciferase reporter gene, then stimulated cells with IFNγ, IFNα4 or IFNβ or left the cells untreated. As shown in Figure 4, IFNα4 and IFNβ induced a strong increase in luciferase activity, with IFNγ inducing a more modest response; this demonstrated

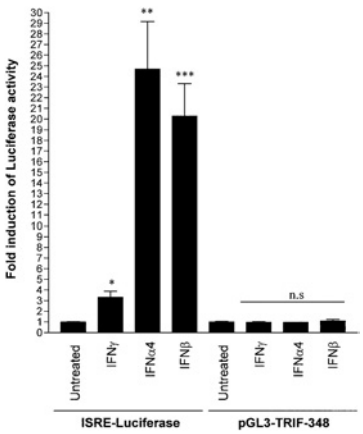

Figure 4 *TRIF* responsiveness to type I or type II IFNs

HEK293 cells were transiently transfected with either an ISRE–luciferase or a pGL3-TRIF-348 construct and then left untreated or treated with 1000 i.u./ml IFNγ, IFNα4 or IFNβ for 9 h. Results are expressed as fold increase in luciferase activity relative to untreated cells for each reporter tested (means $\pm$ S.E.M.; $n = 3$), and are representative of three separate experiments. *P* values were calculated using Student's *t* test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; n.s., not significant). Similar results were obtained using U373 cells.

the efficacy of the IFNs being used. Surprisingly, when we performed a similar experiment on cells transfected with the full-length *TRIF* reporter gene pGL3-TRIF-348, we saw no induction of luciferase activity (Figure 4), suggesting that IFN plays no role in the induction of *TRIF* gene expression.

The TLR3 pathway can regulate the *TRIF* gene

We next determined whether the *TRIF* gene can be positively regulated in a classical feedback mechanism by components of the signalling cascade to which it belongs, namely the TLR3 pathway. We also sought to determine whether NF-κB plays a role in mediating any potential *TRIF* inducibility. As shown in Figure 5(A), poly(I:C), TLR3 and TRIF were all able to induce a similar modest, but significant ($P < 0.005$), activation of the pGL3-TRIF-348 reporter gene, which suggests that the *TRIF* gene can be regulated by its own pathway. Similar results (not shown) were obtained using HEK293-TLR3 cells [for poly(I:C) responses] or HEK293 cells (for TLR3 and TRIF responses). Induction of *TRIF* reporter activity in either cell type by the above stimuli was abrogated by co-transfection of as little as 25 ng of IκB SR (Figure 5A), suggesting that this induction may be mediated by NF-κB. The decrease in *TRIF* activity caused by the addition of the IκB SR was found to be significantly below ($P < 0.005$) the level of activity prior to stimulation (Figure 5A), suggesting that basal NF-κB may also be required. A similar decrease in *TRIF* basal activity was also observed in U373 cells transfected with the IκB SR without any additional stimulation ($P < 0.005$) (Figure 5A), again suggesting a requirement for basal NF-κB. To confirm this, U373 cell nuclear extracts were tested for and found to contain constitutively active NF-κB by EMSA using ^{32}P-labelled consensus NF-κB oligonucleotides (Figure 5B); this NF-κB complex could also be induced following poly(I:C) treatment. Similar results were also obtained with HEK293 cells (not shown).

To demonstrate further that NF-κB is the principal mediator of poly(I:C)-induced *TRIF* activity, we transfected the *TRIF* promoter–reporter deletion and mutation constructs into cells and tested them for their inducibility by poly(I:C). While *TRIF*

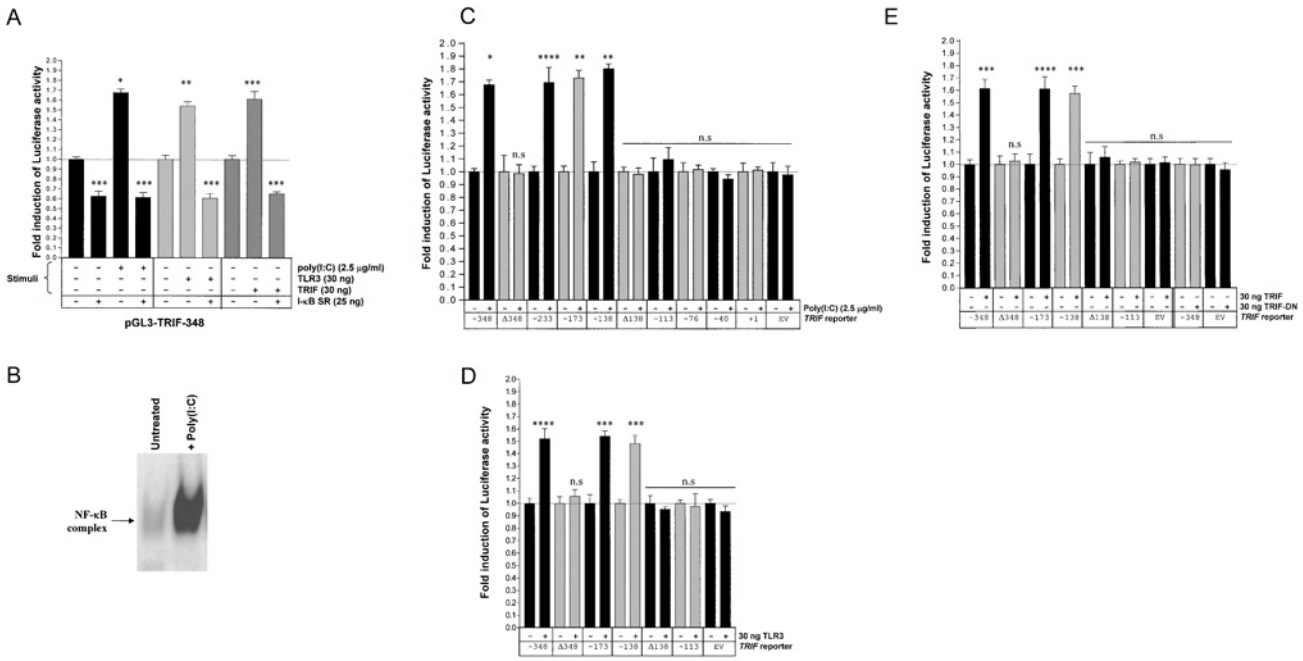

Figure 5 ***TRIF*** **activation by the TLR3 pathway**

(**A**) U373 cells were transfected with pGL3-TRIF-348 with or without 25 ng of IκB SR. Cells were then left untreated, treated with 2.5 μg/ml poly(I:C) for 16 h or co-transfected with 30 ng of TLR3 or TRIF. (**B**) Analysis of NF-κB–DNA binding by EMSA. U373 cells were either left untreated or treated with poly(I:C) for 15 min, and nuclear extracts from these cells were incubated with ^{32}P-labelled NF-κB double-stranded oligonucleotide, then analysed by native gel electrophoresis and autoradiography. An arrow indicates the NF-κB complex. (**C**) U373 cells were transfected with the relevant *TRIF* promoter–reporter deletion/mutation construct (or empty vector), then treated with poly(I:C) as above. Results are expressed as fold increase in luciferase activity relative to unstimulated cells for each reporter tested (means $\pm$ S.E.M.; $n = 3$) and are representative of six separate experiments. pGL3-TRIF-transfected HEK293 cells were also stimulated by co-transfection with (**D**) 30 ng of TLR3 or (**E**) 30 ng or TRIF or TRIF-DN and analysed as above. For all of the above, results are expressed as fold increase in luciferase activity relative to unstimulated cells (means $\pm$ S.E.M.; $n = 3$), and are representative of six separate experiments. Similar results were obtained using HEK293-TLR3 cells [for poly(I:C) responses] or HEK293 cells (for TLR3 and TRIF responses), and P values were calculated using Student's t test (* $P < 0.0005$; ** $P < 0.001$; *** $P < 0.005$; **** $P < 0.01$; n.s., not significant).

reporter constructs containing the NF-κB binding site at positions -127 to -114 (pGL3-TRIF-348, -233, -173 and -138) were all able to induce a significant increase ($P < 0.01$) in luciferase activity following stimulation by poly(I:C) relative to untreated cells (Figure 5C), poly(I:C) had no effect on those constructs with the NF-κB binding site mutated (pGL3-TRIFΔ348 and Δ138) or deleted (pGL3-TRIF-113, -76, -40 and $+1$) or on an empty vector control (Figure 5C). When we tested the ability of TLR3 (Figure 5D) and TRIF (Figure 5E) to activate the same *TRIF* reporter genes, identical results were obtained. In addition, TRIF-DN [8], which is unable to drive NF-κB-luciferase activity due to N- and C-terminal deletions (results not shown), also had no effect on *TRIF*-driven luciferase activity (Figure 5E). Taken together, these data indicate that *TRIF* is positively regulated by components of the TLR3 pathway, principally via binding of the transcription factor NF-κB to the *TRIF* promoter at positions -127 to -114.

Regulation of TRIF by other stimuli

Having determined that *TRIF* gene expression could be induced by activation of the TLR3 pathway, we next examined the ability of *TRIF* to be regulated by other activators of the innate immune system. The construct pGL3-TRIF-348 was transfected into cells that were then treated with Pam_3/CSK (U373 or HEK293-TLR2 cells), LPS (U373 or HEK293-TLR4 cells), TNFα or IL-1α (U373 or HEK293 cells). As shown in Figure 6(A), the pGL3-TRIF-348 reporter gene was able to be significantly induced ($P < 0.005$) relative to untreated cells by all stimuli tested; co-transfection of the IκB SR was able to completely abrogate Pam_3/CSK- and LPS-mediated induction of the *TRIF* reporter gene, and partially abrogate TNFα- and IL-1α-mediated induction (Figure 6A). These data indicate that the *TRIF* gene can indeed be induced by these stimuli via NF-κB. To confirm the role of NF-κB in *TRIF* induction, the *TRIF* promoter deletion and mutation constructs used above were transfected into cells that were then treated with Pam_3/CSK (Figure 6B), LPS (Figure 6C), TNFα (Figure 6D) or IL-1α (Figure 6E). The results show that, no matter what the stimulus used, the NF-κB binding site at positions -127 to -114 was critical for *TRIF* activation, and suggest that cellular responses involving NF-κB activation may all lead to the transcriptional activation of *TRIF* gene expression.

Northern analysis of regulation of TRIF mRNA

The final step in the analysis of the regulation of the *TRIF* gene was to determine whether the results obtained using reporter gene assays could be mirrored by a similar regulation of TRIF mRNA expression, as determined by Northern blot analyses. We therefore treated U373 cells separately for 0–24 h with a number of stimuli, including LPS and poly(I:C), and the pro-inflammatory cytokines TNFα and IL-1α. Northern blot analysis was then performed on poly(A)$^+$ mRNA isolated from these cells using a TRIF cDNA probe. The mRNA transcript encoding TRIF was then quantified relative to that of GAPDH, a housekeeping gene used to normalize mRNA loading. As shown in Figure 7(A), a TRIF mRNA transcript of approx. 2.6 kb was detected in U373 cells that was up-regulated significantly by LPS at 2 h ($P < 0.05$), and to its maximal level (approx. 2.7-fold; $P < 0.005$) after 6 h of LPS

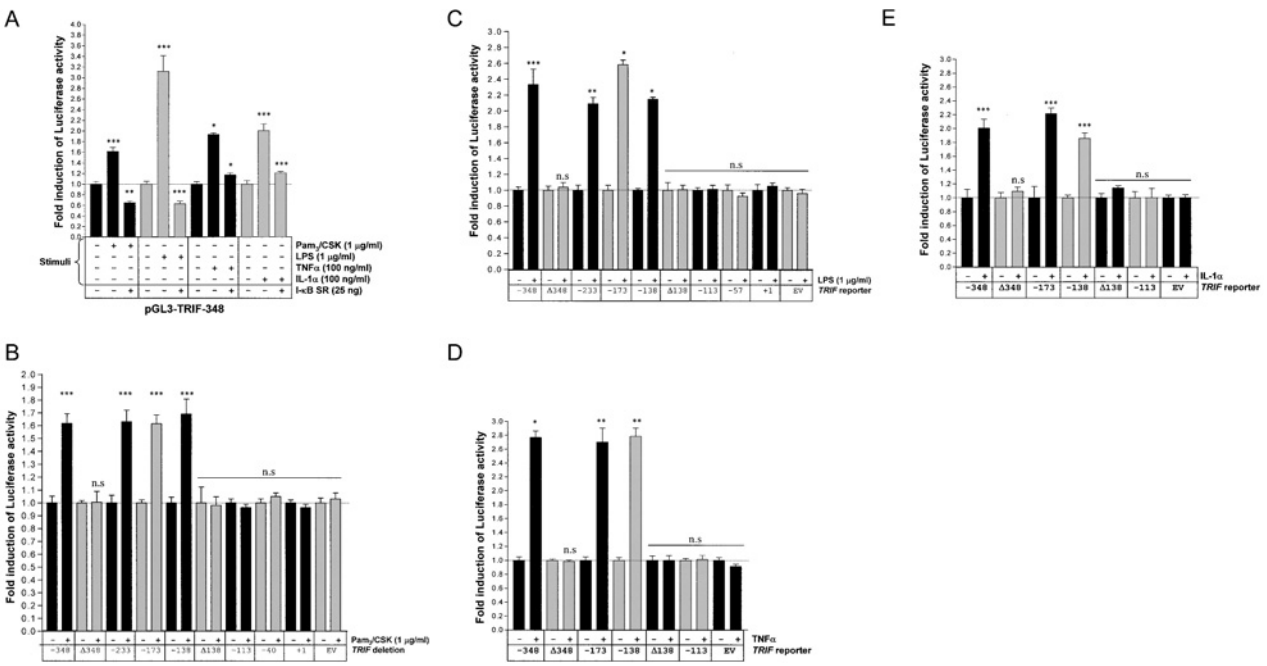

Figure 6 ***TRIF*** **activation by other stimuli**

(**A**) U373 cells were transfected with pGL3-TRIF-348, with or without 25 ng of IκB SR. Cells were then treated with 1 μg/ml Pam_3/CSK or LPS for 16 h, or with TNFα or IL-1α for 6 h. U373 cells were also transfected with the relevant *TRIF* promoter–reporter deletion/mutation construct (or empty vector), then treated with (**B**) Pam_3/CSK, (**C**) LPS, (**D**) TNFα or (**E**) IL-1α as described above. For all of the above, results are expressed as the fold increase in luciferase activity relative to unstimulated cells (means $\pm$ S.E.M.; $n = 3$), and are representative of four separate experiments. Similar results were obtained using HEK293-TLR2 cells (for Pam_3/CSK responses), HEK293-TLR4 cells (for LPS responses) or HEK293 cells (for TNFα and IL-1α responses), and *P* values were calculated using Student's *t* test (* $P < 0.0001$; ** $P < 0.0005$; *** $P < 0.005$; n.s., not significant).

stimulation. The levels of TRIF mRNA then dropped back towards pre-stimulation levels by 24 h of LPS treatment (Figure 7A). These data emulate the induction of the *TRIF*–luciferase reporter gene by LPS observed in Figure 6(C). In addition, U373 cells treated with poly(I:C) or IL-1α showed the same profile of TRIF mRNA induction as that observed after LPS treatment (results not shown).

When TRIF mRNA transcript levels were examined following treatment of U373 cells with TNFα, a somewhat different mRNA profile was observed (Figure 7B). A maximal induction of TRIF mRNA (approx. 2.7-fold) compared with untreated cells was observed after only 1 h of TNFα treatment ($P < 0.005$), and a significant induction of TRIF mRNA by TNFα was still observed after 3 h ($P < 0.05$). However, TRIF mRNA levels fell significantly below pre-treatment levels by 6 h post-stimulation ($P < 0.05$), before returning to normal by 24 h (Figure 7B). Taken together, these data suggest that the *TRIF* gene can be positively regulated by a variety of stimuli. Furthermore, certain stimuli, such as TNFα, are able to evoke a more rapid induction of TRIF mRNA expression.

DISCUSSION

Analysis of the human *TRIF* gene and the mechanisms by which it is regulated was performed because of the key role that TRIF plays in signal transduction by TLR3 and TLR4 [3]. We set out, therefore, to identify the position of *TRIF* in the human genome, and found that it lies on *HSA* chromosome 19p13.3, transcribed towards the telomere. The most interesting thing about the localization of *TRIF* is not the gene itself, but its flanking genes. Downstream of *TRIF* is a gene called *FEM1A* that encodes a human homologue of the sex-determination factor Fem-1 from the nematode *C. elegans* [25,26]. Interestingly, another human *Fem-1* homologue, *FEM1C*, lies immediately downstream on *HSA* chromosome 5q23.1 of *TRAM*, which encodes another TLR adaptor protein whose function is intimately tied up with that of TRIF by acting as a 'bridge' between TRIF and TLR4 [10,12]. This *FEM1A/TRIF–FEM1C/TRAM* alignment is also present in the murine genome, suggesting that these genes are in positions of conserved synteny. However, analysis of the *C. elegans* genome suggests that the emergence of *TRIF* and *TRAM* in evolution occurred after the divergence of the nematode from the lineage leading to vertebrates, since the genes flanking *Fem-1* in *C. elegans* are unrelated, and no genes directly analogous to *TRIF* or *TRAM* were found. In the nematode, *Drp-1*, the gene encoding dynamin-1-related protein, lies upstream of *Fem-1* [36]; downstream lies a gene called *Unc-5* (*Unc*oordinated locomotion-5) [37]. Both of these genes have human homologues on different chromosomes to *TRIF* and *TRAM* [38–41]. Homologues of *TRIF* and *TRAM* were found in the zebrafish *Danio rerio*, however, suggesting that these genes may have emerged with vertebrate life. Unfortunately, there is not enough sequence information to map the *D. rerio Fem-1* gene(s), so their relationship to *TRIF* and *TRAM* is not known as yet. Because *TRIF* and *TRAM*, and *FEM1a* and *FEM1c*, are each related to the other in sequence, it is probable that a duplication event occurred in the region of the genome containing either *TRIF* and *FEM1a* or *TRAM* and *FEM1c*, giving rise to each homologue. Each would then have evolved a new function, implying that TRIF and TRAM both have specific functions. The function of TRAM appears to be in the recruitment of TRIF to the TLR4 signalling complex [10], although why TRIF cannot be recruited directly to TLR4, as is the case with TLR3, is not clear at this stage.

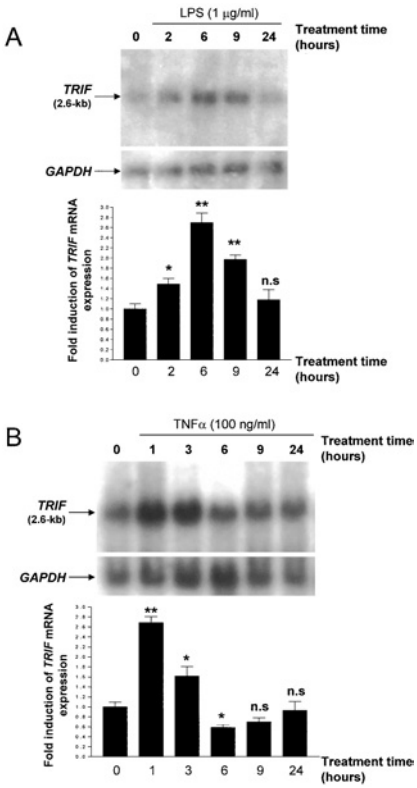

Figure 7 Regulation of TRIF mRNA

Representative Northern blot analyses of poly(A)$^+$ mRNA from U373 cells treated with (**A**) 1 μg/ml LPS or (**B**) 100 ng/ml TNFα for 0–24 h, showing the distribution of the 2.6 kb TRIF mRNA transcript. Blots were sequentially hybridized with a TRIF cDNA encoding the entire ORF and a GAPDH probe. Below each blot is a graph showing the fold induction of TRIF/GAPDH mRNA expression relative to untreated cells (means $\pm$ S.E.M.), as determined by densitometric analysis of five exposures of each hybridizing band. *P* values relative to untreated cells were calculated using Student's *t* test ($^*P < 0.05$; $^{**}P < 0.005$; n.s., not significant).

The identification of a putative *TRIF* promoter was the next step in our analyses. To do this, we first looked for conserved non-coding regions in the orthologous human and murine *TRIF* genes, since comparisons of non-coding sequences in syntenic regions have previously been successful in identifying common elements regulating gene expression [34]. To our surprise, no conserved non-coding regions were identified, suggesting differential regulation of human and murine *TRIF*. However, closer inspection of the two sequences by ClustalW analysis revealed conservation of individual transcription factor binding sites, such as those for Sp1 and NF-κB, in the 5′ flanking region proximal to *TRIF* exon 1. It is interesting that these sites were further shown to be involved in the basal and inducible regulation of human *TRIF*, providing proof-of-concept. We next analysed the 5′ flanking region of *TRIF* for repetitive elements and found that most of it, with the clear exception of the $\sim$400 bp sequence proximal to exon 1, is composed of repeats such as short and long interspersed elements. Although previous studies have shown that repetitive elements can regulate transcription positively or negatively [42,43], the presence of a stretch of continuous elements running from the 3′ end of the *TIP47* gene to within $\sim$400 bp of the *TRIF* gene suggest that these elements probably play no part in the regulation *of TRIF*. The orphan EST identified within the 5′ flanking region of *TRIF* was also found to be composed mostly of repeat sequences, which, combined with the 5′ RACE analysis, suggests that this EST is artefactual.

Analysis of the basal or constitutive function of *TRIF* was carried out using reporter-deletion constructs generated from 350 bp of 5′ flanking sequence proximal to exon 1 plus the 95 bp of exon 1 itself. The results showed that an Sp1 transcription factor binding site found at positions -53 to -41 was responsible for the majority of the basal activity of *TRIF*. The involvement of Sp1 in basal activity was found to be similar to that observed for the murine *Tlr2* gene [44], suggesting that the regulation of genes encoding components of TLR signalling may have common elements. Further experiments which examined the inducibility of *TRIF* by a variety of stimuli such as LPS, poly(I:C) and TNFα showed that another transcription factor, NF-κB, is critical for activation of the gene. This is also similar to murine *Tlr2*, which requires an NF-κB element at -246 for transcriptional activation induced by *Mycobacterium avium* [44]. Removal of the *TRIF* NF-κB site at -127 to -114, either by deletion or by mutation of four critical nucleotides, resulted in a complete abrogation of *TRIF* inducibility by all stimuli tested. Addition of the IκB SR was also able to abrogate *TRIF* induction by all stimuli tested [45,46]. This demonstrates an absolute requirement for NF-κB for *TRIF* activation. The *TRIF* promoter differs from that of *MyD88*, the only other TIR domain-containing promoter to be analysed functionally. The *MyD88* promoter does not contain any NF-κB motifs, and would appear to be regulated via overlapping binding sites for STAT1, IRF1 and IRF2 [47]. This provides a molecular explanation for the induction of MyD88 by IL-6 and other STAT1 activators. TRIF, in contrast, will be induced by inflammatory stimuli that activate NF-κB. *TRIF* would therefore appear to be a more general inflammatory response gene, with *MyD88* being more restricted. Recent work using micro-arrays has indicated that most genes regulated by LPS are on the so-called 'MyD88-independent' pathway. These are presumably regulated by TRIF, making it the more prominent adaptor for TLR4. It is also the primary adaptor for TLR3. This prominence is also reflected in the large number of inflammatory agents that would induce TRIF in comparison with MyD88.

The induction of *TRIF* by the stimuli tested, although clearly evident, was relatively weak compared with that of other promoters, suggesting that basal expression of *TRIF* is adequate for the initial response to TLR3 or TLR4. However, *TRIF* is clearly inducible, and the level of induction of its mRNA by the stimuli was somewhat higher that the induction of the promoter. *TRIF* is likely to be induced during inflammation, promoting responses to ligands for TLR3 and TLR4 during innate immunity. Since TLR3 and TLR4 activate NF-κB, a positive feedback loop is likely to operate, potentiating the response to these TLRs. Another possibility could be that induction of TRIF might skew the signalling pathway activated by TLR4, since TLR4 utilizes four adaptors. Mal and MyD88 regulate the early activation of NF-κB, while TRAM and TRIF regulate late activation, and also activation of IRF3, leading to induction of IFNβ. Induction of TRIF might therefore promote these two signals [9,10,14,15], allowing for a staging of the timing of gene induction during host defence. Since we also identified a putative STAT1 motif in the *TRIF* promoter, we wished to address the question of whether a similar positive feedback system might operate, whereby TLR3 and TLR4 would induce IFNβ, which would in turn induce TRIF expression. The *TRIF* promoter proved insensitive to type I IFNs, however, indicating that this is not the case.

In conclusion, the present study identifies NF-κB as the key transcription factor that regulates *TRIF* gene expression. *TRIF* is therefore likely to be induced during inflammation, where it will potentiate responses to PAMPs sensed by TLR3 and TLR4, and thereby promote innate immunity.

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REFERENCES

1 Akira, S. (2001) Toll-like receptors and innate immunity. Adv. Immunol. **78**, 1–56
2 Dunne, A. and O'Neill, L. (2003) The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. Science STKE (2003), re3
3 O'Neill, L., Fitzgerald, K. and Bowie, A. (2003) The Toll-IL-1 receptor adaptor family grows to five members. Trends Immunol. **24**, 286–290
4 Lord, K., Hoffman-Liebermann, B. and Liebermann, D. (1990) Nucleotide sequence and expression of a cDNA encoding MyD88, a novel myeloid differentiation primary response gene induced by IL6. Oncogene **5**, 1095–1097
5 Fitzgerald, K., Palsson-McDermott, E., Bowie, A., Jefferies, C., Mansell, A., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M. et al. (2001) Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature (London) **413**, 78–83
6 O'Neill, L. (2002) Signal transduction pathways activated by the IL-1 receptor/toll-like receptor superfamily. Curr. Top. Microbiol. Immunol. **270**, 47–61
7 O'Neill, L., Dunne, A., Edjeback, M., Gray, P., Jefferies, C. and Wietek, C. (2003) Mal and MyD88: adapter proteins involved in signal transduction by Toll-like receptors. J. Endotoxin Res. **9**, 55–59
8 Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K. and Akira, S. (2002) Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J. Immunol. **169**, 6668–6672
9 Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T. and Seya, T. (2003) TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat. Immunol. **4**, 161–167
10 Fitzgerald, K., Rowe, D., Barnes, B., Caffrey, D., Visintin, A., Latz, E., Monks, B., Pitha, P. and Golenbock, D. (2003) LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J. Exp. Med. **198**, 1043–1055
11 Bin, L., Xu, L. and Shu, H. (2003) TIRP, a novel Toll/interleukin-1 receptor (TIR) domain-containing adapter protein involved in TIR signaling. J. Biol. Chem. **278**, 24526–24532
12 Oshiumi, H., Sasai, M., Shida, K., Fujita, T., Matsumoto, M. and Seya, T. (2003) TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. J. Biol. Chem. **278**, 49751–49762
13 Mink, M., Fogelgren, B., Olszewski, K., Maroy, P. and Csiszar, K. (2001) A novel human gene (SARM) at chromosome 17q11 encodes a protein with a SAM motif and structural similarity to Armadillo/beta-catenin that is conserved in mouse, Drosophila, and Caenorhabditis elegans. Genomics **74**, 234–244
14 Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K. and Akira, S. (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science **301**, 640–643
15 Hoebe, K., Du, X., Goode, J., Mann, N. and Beutler, B. (2003) Lps2: a new locus required for responses to lipopolysaccharide, revealed by germline mutagenesis and phenotypic screening. J. Endotoxin Res. **9**, 250–255
16 Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S., Goode, J., Lin, P., Mann, N., Mudd, S. et al. (2003) Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. Nature (London) **424**, 743–748
17 Sato, S., Sugiyama, M., Yamamoto, M., Watanabe, Y., Kawai, T., Takeda, K. and Akira, S. (2003) Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappaB and IFN-regulatory factor-3, in the Toll-like receptor signaling. J. Immunol. **171**, 4304–4310
18 Smith, R., Wiese, B., Wojznski, M., Davison, D. and Worley, K. (1996) BCM Search Launcher – an integrated interface to molecular biology data base search and analysis services available on the World Wide Web. Genome Res. **6**, 454–462
19 Hardy, M., Hertzog, P. and Owczarek, C. (2002) Multiple regions within the promoter of the murine Ifnar-2 gene confer basal and inducible expression. Biochem. J. **365**, 355–367
20 Prestridge, D. (1991) SIGNAL SCAN: a computer program that scans DNA sequences for eukaryotic transcriptional elements. Comput. Appl. Biosci. **7**, 203–206
21 Heinemeyer, T., Wingender, E., Reuter, I., Hermjakob, H., Kel, A., Kel, O., Ignatieva, E., Ananko, E., Podkolodnaya, O., Kolpakov, F. et al. (1998) Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. Nucleic Acids Res. **26**, 362–367
22 Quandt, K., Frech, K., Karas, H., Wingender, E. and Werner, T. (1995) MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res. **23**, 4878–4884
23 Hardy, M., Owczarek, C., Trajanovska, S., Liu, X., Kola, I. and Hertzog, P. (2001) The soluble murine type I interferon receptor Ifnar-2 is present in serum, is independently regulated, and has both agonistic and antagonistic properties. Blood **97**, 473–482
24 Diaz, E. and Pfeffer, S. (1998) TIP47: a cargo selection device for mannose 6-phosphate receptor trafficking. Cell **93**, 433–443
25 Krakow, D., Sebald, E., King, L. and Cohn, D. (2001) Identification of human FEM1A, the ortholog of a *C. elegans* sex-differentiation gene. Gene **279**, 213–219
26 Spence, A., Coulson, A. and Hodgkin, J. (1990) The product of fem-1, a nematode sex-determining gene, contains a motif found in cell cycle control proteins and receptors for cell-cell interactions. Cell **60**, 981–990
27 Breathnach, R. and Chambon, P. (1981) Organization and expression of eucaryotic split genes coding for proteins. Annu. Rev. Biochem. **50**, 349–383
28 Shapiro, M. and Senapathy, P. (1987) RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Res. **15**, 7155–7174
29 Grilli, M., Chiu, J. and Lenardo, M. (1993) NF-kappa B and Rel: participants in a multiform transcriptional regulatory system. Int. Rev. Cytol. **143**, 1–62
30 Williams, T. and Tjian, R. (1991) Characterization of a dimerization motif in AP-2 and its function in heterologous DNA-binding proteins. Science **251**, 1067–1071
31 Thiagalingam, A., de Bustros, A., Borges, M., Jasti, R., Compton, D., Diamond, L., Mabry, M., Ball, D., Baylin, S. and Nelkin, B. (1996) RREB-1, a novel zinc finger protein, is involved in the differentiation response to Ras in human medullary thyroid carcinomas. Mol. Cell. Biol. **16**, 5335–5345
32 Nardelli, J., Gibson, T., Vesque, C. and Charnay, P. (1991) Base sequence discrimination by zinc-finger DNA-binding domains. Nature (London) **349**, 175–178
33 Horvath, C., Wen, Z. and Darnell, Jr, J. (1995) A STAT protein domain that determines DNA sequence recognition suggests a novel DNA-binding domain. Genes Dev. **9**, 984–994
34 Loots, G., Locksley, R., Blankespoor, C., Wang, Z., Miller, W., Rubin, E. and Frazer, K. (2000) Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. Science **288**, 136–140
35 Kunsch, C., Ruben, S. and Rosen, C. (1992) Selection of optimal kappa B/Rel DNA-binding motifs: interaction of both subunits of NF-kappa B with DNA is required for transcriptional activation. Mol. Cell. Biol. **12**, 4412–4421
36 Labrousse, A., Zappaterra, M., Rube, D. and van der Bliek, A. (1999) *C. elegans* dynamin-related protein DRP-1 controls severing of the mitochondrial outer membrane. Mol. Cell **4**, 815–826
37 Culotti, J. (1994) Axon guidance mechanisms in *Caenorhabditis elegans*. Curr. Opin. Genet. Dev. **4**, 587–595
38 Shin, H., Shinotsuka, C., Torii, S., Murakami, K. and Nakayama, K. (1997) Identification and subcellular localization of a novel mammalian dynamin-related protein homologous to yeast Vps1p and Dnm1p. J. Biochem. (Tokyo) **122**, 525–530
39 Imoto, M., Tachibana, I. and Urrutia, R. (1998) Identification and functional characterization of a novel human protein highly related to the yeast dynamin-like GTPase Vps1p. J. Cell Sci. **111**, 1341–1349
40 Komatsuzaki, K., Dalvin, S. and Kinane, T. (2002) Modulation of G(ialpha(2)) signaling by the axonal guidance molecule UNC5H2. Biochem. Biophys. Res. Commun. **297**, 898–905
41 Thiebault, K., Mazelin, L., Pays, L., Llambi, F., Joly, M., Scoazec, J., Saurin, J., Romeo, G. and Mehlen, P. (2003) The netrin-1 receptors UNC5H are putative tumor suppressors controlling cell death commitment. Proc. Natl. Acad. Sci. U.S.A. **100**, 4173–4178
42 Hanke, J., Hambor, J. and Kavathas, P. (1995) Repetitive Alu elements form a cruciform structure that regulates the function of the human CD8 alpha T cell-specific enhancer. J. Mol. Biol. **246**, 63–73
43 Bi, S., Gavrilova, O., Gong, D., Mason, M. and Reitman, M. (1997) Identification of a placental enhancer for the human leptin gene. J. Biol. Chem. **272**, 30583–30588
44 Wang, T., Lafuse, W. and Zwilling, B. (2001) NFkappaB and Sp1 elements are necessary for maximal transcription of toll-like receptor 2 induced by *Mycobacterium avium*. J. Immunol. **167**, 6924–6932
45 Traenckner, E., Pahl, H., Henkel, T., Schmidt, K., Wilk, S. and Baeuerle, P. (1995) Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. EMBO J. **14**, 2876–2883
46 Brown, K., Gerstberger, S., Carlson, L., Franzoso, G. and Siebenlist, U. (1995) Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. Science **267**, 1485–1488
47 Harroch, S., Gothelf, Y., Revel, M. and Chebath, J. (1995) 5′ upstream sequences of MyD88, an IL-6 primary response gene in M1 cells: detection of functional IRF-1 and Stat factors binding sites. Nucleic Acids Res. **23**, 3539–3546

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