JFC1 is transcriptionally activated by nuclear factor- κ B and up-regulated by tumour necrosis factor α in prostate carcinoma cells

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The human promoter region of JFC1, a phosphatidylinositol 3,4,5-trisphosphate binding ATPase, was isolated by amplification of a 549 bp region upstream of the *jfc1* gene by the use of a double-PCR system. By primer extension analysis we mapped the transcription initiation site at nucleotide –321 relative to the translation start site. Putative regulatory elements were identified in the *jfc1* TATA-less promoter, including three consensus sites for nuclear factor- κ B (NF- κ B). We analysed the three putative NF- κ B binding sites by gel retardation and supershift assays. Each of the putative NF- κ B sites interacted specifically with recombinant NF- κ B p50, and the complexes co-migrated with those formed by the NF- κ B consensus sequence and p50. An antibody to p50 generated a supershifted complex for these NF- κ B sites. These sites formed specific complexes with nuclear proteins from tumour necrosis factor α (TNF α)-treated WEHI 231 cells, which were supershifted with antibodies against p50

and p65. The *jfc1* promoter was transcriptionally active in various cell lines, as determined by luciferase reporter assays following transfection with a *jfc1* promoter luciferase vector. Co-transfection with NF- κ B expression vectors or stimulation with TNF α resulted in significant transactivation of the *jfc1* promoter construct, although transactivation of a mutated *jfc1* promoter was negligible. The expression of a dominant negative I κ B (inhibitor κ B) decreased basal *jfc1* promoter activity. The cell lines PC-3, LNCaP and DU-145, but not Epstein–Barr virus-transformed lymphocytes, showed a dramatic increase in the expression of JFC1 after treatment with TNF α , suggesting that transcriptional activation of JFC1 by the TNF α /NF- κ B pathway is significant in prostate carcinoma cell lines.

Key words: PI 3-kinase, promoter, secretion, transcription.

INTRODUCTION

JFC1 is a phosphatidylinositol 3,4,5-trisphosphate (PIP₃) binding protein [1] with ATPase capacity [2] that has been recently identified in our laboratory using a yeast two-hybrid system with the C-terminal half of the leucocyte NADPH oxidase cytosolic factor p67^{PHOX} as bait [1]. Although the NADPH oxidase is restricted to leucocytes, JFC1 is expressed in several tissues with a secretory function, with high expression in prostate cells [1]. JFC1 is a tandem C2 domain-containing protein, and its C2A domain is highly similar to the C2B domain present in synaptotagmin I [1], a protein whose expression is limited to the nervous system and which is considered to be a phosphoinositide-dependent calcium sensor that regulates neurotransmitter release ([3]; see also [3a]). The synaptotagmin C2B domain has been shown to interact with *N*-ethylmaleimide-sensitive factor attachment protein (β -SNAP) [4] and is responsible for calcium-dependent synaptotagmin dimerization [5]. Similarities between JFC1 and synaptotagmin suggest that JFC1 could act as a counterpart of synaptotagmin in tissues other than the nervous system. Thus it has been indicated previously that JFC1 could act as an accessory protein between PIP₃ and Src homology 3 (SH3)-domain-containing proteins in an ATP-dependent manner [2], and in particular as an assembling factor during the secretion of NADPH oxidase [1]. However, the enriched expression of JFC1 mRNA in tissues with secretory functions [1] and the ubiquitous distribution of the phosphoinositide 3-kinase (PI 3-

kinase) pathway suggest that the role of JFC1 is not limited to the assembly of the NADPH oxidase [1].

PI 3-kinase and its product PIP₃ have been shown to be extensively involved in signalling pathways related to membrane trafficking, as well as in cell proliferation, survival and cytoskeletal reorganization, in most cell lines. It is now clear that numerous adaptor proteins take part in the differentiation of the signal transduction pathways downstream of PI 3-kinase, and that these proteins determine the final cellular event of the PIP₃-mediated cascade [6–8]. Therefore the activation of the PI 3-kinase pathway could originate several and distinct cellular functions in different cell types, and even in the same cell type under different conditions, depending on the availability of the adaptor proteins in an active form. Consequently, regulation of the expression of JFC1 may be critically important in determining the fate of a cell once the PI 3-kinase pathway is activated.

The transcription factor nuclear factor- κ B (NF- κ B) was first identified as a regulatory molecule indispensable for the activation of gene transcription in B-lymphocytes [9]. Identified NF- κ B family members include NF- κ B1 (p50 or p105), NF- κ B2 (p52 or p100), p65 (RelA), RelB and c-Rel [10]. These proteins share a highly conserved structure called the Rel homology domain (RHD) that allows these factors to form homo- or hetero-dimers. The RHD also functions in DNA binding, nuclear localization and interaction with the inhibitor κ B (I κ B) proteins [11,12]. The major active NF- κ B complex in mammalian cells is a heterodimer containing p50 and p65. This complex binds to a

Abbreviations used: CRE, cAMP response element; EBV, Epstein–Barr virus; I κ B, inhibitor κ B; IKK, I κ B kinase; *jfc1*-LUC, *jfc1* luciferase construct; NF- κ B, nuclear factor- κ B; PI 3-kinase, phosphoinositide 3-kinase; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; RHD, Rel homology domain; TK, thymidine kinase; TNF α , tumour necrosis factor α .

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number of different DNA recognition sequences [13–15] with the consensus DNA motif 5' GGGRNYYYCC 3'.

In resting cells, NF- κ B is sequestered in the cytoplasm in an inactive form bound to the I κ B protein. NF- κ B activity can be induced in most cell types by cytokines such as tumour necrosis factor α (TNF α) and interleukin-1 [9], mitogens and viral proteins [16]. Upon stimulation, I κ B is phosphorylated by I κ B kinases (IKKs) and proteolytically degraded. The degradation of I κ B or the partial degradation of the p105 and p100 precursors activates NF- κ B, which then forms dimers, translocates to the nucleus, and binds to target genes [10,17]. The NF- κ B family have been shown to be involved in the regulation of the transcription of several genes that play an essential role in the inflammatory, immune and apoptotic responses [18,19].

The identification and functional characterization of transcriptional regulatory elements is a critically important step for understanding the tissue specificity of gene expression, and may contribute to our understanding of the function of a particular protein in a physiological perspective. In this work we present the cloning of the *jfc1* promoter region and the characterization of three regulatory elements with NF- κ B binding capacity. Furthermore, the regulation of JFC1 expression in response to TNF α in prostate carcinoma cells is presented.

EXPERIMENTAL

Cloning of the promoter region of JFC1

The promoter of *jfc1* was isolated by the amplification of a 549 bp region upstream of the *jfc1* gene by nested PCR using the GenomeWalker™ human DNA libraries as template, as described by the manufacturer (Clontech). The *jfc1*-specific oligonucleotide GGGAGGGAGGGCAGAGCCCAAGCC (co-ordinate nucleotides +56 to +32) and the outer adaptor primer 1 (Clontech) were used in the primary PCR. The secondary PCR was performed using the *jfc1* oligonucleotide CCTCTTGCGA-TGGGTGGCCCTCTG (co-ordinate oligonucleotides +31 to +7) and the nested adaptor primer 2 (Clontech). The amplified fragment was identified by dideoxynucleotide sequencing. To confirm the sequence of the cloned fragment, we amplified the same upstream region of the *jfc1* gene by PCR using *pfu* polymerase (Promega) and human genomic DNA (Clontech) as template. For this purpose we designed the primer CGA-CGGCTCGGGCTGGTATCGTGCCAA with the co-ordinate nucleotides –549 to –523 according to the sequence identified by the nested PCR described above, and the 3' primer GGGAGG-GAGGGCAGAGCCCAAGCC with the co-ordinate nucleotides +56 to +32 of *jfc1*. The new PCR product was verified by dideoxynucleotide sequencing and its sequence was aligned with that of the former nested PCR product sequence using the sequence analysis software Omega™ 2.0. Both methodologies isolated the exact sequence. The PCR product was cloned into the polylinker of pbasic-LUC (Promega) to generate jfc1-LUC. TK-LUC was generated by cloning the herpes simplex virus thymidine kinase (TK) promoter upstream of the firefly luciferase gene (Promega). The various steps in the cloning of the plasmid constructs were performed by standard techniques [20].

Primer extension analysis

Primer extension was performed as described previously [21]. Briefly, the antisense oligonucleotide TCAAGGTGGGGTGA-GAGAC that co-ordinates nucleotides –165 to –183 of the human *jfc1* gene was phosphorylated at the 5' end with T4 polynucleotide kinase (New England BioLabs) and 30 μ Ci of [γ -³²P]ATP (3000 Ci/mmol). The radiolabelled primer was incu-

bated in the presence of 20 μ g of prostate poly(A)⁺ mRNA (Clontech) as described previously [20] in 15 μ l of hybridization buffer (10 mM Tris/HCl, pH 8.3, 150 mM KCl, 1 mM EDTA). Hybridization was performed for 16 h at 65 °C. Reverse transcription was carried out using avian myeloblastosis virus reverse transcriptase (Promega) at 42 °C for 2 h. Samples were then extracted with phenol/chloroform (1:1, v/v), precipitated with ethanol and resolved by electrophoresis on 6% (w/v) polyacrylamide gels (Stratagene). Standards for sizing the primer extension products were generated using single-stranded M13mp18 DNA and the Sequenase 2 kit (U.S. Biochemical) as described by the manufacturer.

Cell culture, transfections and luciferase assays

The immature B cell lymphoma line, WEHI 231, and the human cervical carcinoma cell line, Hela S3 (A.T.C.C.), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and 0.45% (w/v) glucose (Gibco/BRL), at 37 °C in 5% CO₂/air. WEHI 231 cells were supplemented with 50 μ M β -mercaptoethanol, as described elsewhere [22]. Prior to treatment, WEHI 231 cells were diluted to a density of 3.5×10^5 cells/ml with fresh medium and incubated for 5 h. The prostate carcinoma cell lines PC-3, DU-145 and LNCaP-FGC (referred to here as LNCaP) (A.T.C.C.) were cultured in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (Gemini Bioproducts), 50 units/ml penicillin and 50 μ g/ml streptomycin.

Transfections were performed in 6-well plates using FuGene 6 (Roche) as described by the manufacturer. The transfected DNA mixture comprised 5 μ g of the firefly LUC plasmids (see above) and 250 ng of pCMV β gal (Clontech) or pRL-TK (Promega) as internal control for transfection efficiency. pCMV β gal directs the expression of the *Escherichia coli* β -galactosidase gene by using the cytomegalovirus immediate-early promoter. pRL-TK directs expression of the renilla luciferase (RL) gene using the herpes simplex virus TK promoter. Where appropriate, the DNA mixture also included 100–250 ng of the NF- κ B expression vectors (pCMV-p50 and pCMV-p65), the control vector pCMV-PA, the I κ B dominant negative cDNA pUSE-I κ B (S32A/S36A), or the control vector pUSE (Upstate Biotechnology). Cell extracts were prepared 48 h after transfection and assayed for firefly and renilla luciferase activities using the Stop and Glo kit (Promega) as instructed by the manufacturer. β -Galactosidase activity was measured using a Galacto-light kit (Tropix, Inc.) as instructed by the manufacturer. In some experiments, transfected LNCaP cells were incubated in medium containing 10% (v/v) charcoal-stripped serum overnight before they were stimulated with 1 ng/ml TNF α (Roche) 12 h before harvesting. Lysates were then analysed for firefly and renilla luciferase activity as described above.

Nuclear extracts

Nuclear extracts were prepared from WEHI 231 cells essentially as described [23]. The cells were treated with TNF α (50 ng/ml; Sigma) for 2 h to overnight at 37 °C before preparation of the extracts. Nuclear extracts from unstimulated WEHI 231 cells were used as negative controls.

Electromobility gel shift assays

Electromobility gel shift assays were performed as described previously [21]. ³²P-labelled double-stranded oligonucleotides (1 ng) representing putative NF- κ B sites and controls were incubated with either purified NF- κ B p50 (0.5 gel shift unit; Promega) or 5 μ g of nuclear extract protein, prior to the

resolution of the samples by gel electrophoresis using 4–12% (w/v) polyacrylamide TBE gels (Invitrogen). The electromobility of the samples was detected by autoradiography. When gel retardation competition analysis was performed, the extract or purified protein was preincubated with 1 µg of unlabelled double-stranded competitor oligonucleotide for 15 min prior to the addition of the ³³P-labelled double-stranded oligonucleotides. Supershift analysis was performed by incubation of the nuclear extracts or purified protein with a rabbit affinity-purified polyclonal antibody raised against NF-κB (p50 or p65) (36 µg/µl; Rockland Immunochemicals, Inc.) for 1 h to overnight at 4 °C prior to the addition of the ³³P-labelled double-stranded oligonucleotide.

The oligonucleotide pairs used were as follows: TCGACTGGGGGAACAGCTGT and TCGAACAGCTGTTCCCCAG (*jfc1* promoter site 1; co-ordinates –405 to –390 plus *SalI* overhang); TCGACCAGGAAGCTCCGTAT and TCGAATACGGAGCTTCCTGG (*jfc1* promoter site 2; co-ordinates –42 to –27 plus *SalI* overhang); TCGAGGGGCACAGCCCCAGC and TCGAGCTGGGGCTGTGCCCC (*jfc1* promoter site 3; co-ordinates –18 to –3 plus *SalI* overhang); AGTTGAGGGGACTTTCCCAGGC and GCCTGGGAAAGTCCCCTCACT (consensus NF-κB; Santa Cruz Biotechnology, Inc.), GTTGATGACGTCAAGGC and GCCTTGACGTCATCAACT [control consensus cAMP response element (CRE)]; TCGACACTATTTACACACTCTATG and TCGACATAGAGTGTGTAAATAGTG (hepatitis B virus large surface antigen hepatocyte nuclear factor 3 site plus *SalI* overhang) [24].

Immunoblotting

For JFC1 expression analysis, cells were incubated in medium containing 10% (v/v) charcoal-stripped serum overnight before the addition of TNFα (10 ng/ml). The incubation at 37 °C was then continued for a further 6 h before the cells were lysed and analysed by immunoblotting using a previously described antibody raised against JFC1 that recognizes a specific duplet [1].

Mutagenesis

Mutagenesis of NF-κB binding sites 1, 2 and 3 in the *jfc1*-LUC construct was performed by PCR. For this purpose we utilized the oligonucleotide GTGTCTGCAGGGCCCTTTCTGTTTCAAATTTGTGCATGCGCC, which mutates NF-κB site 1 to TTTCAAATTT and contains an *ApaI* restriction site, and the oligonucleotide GGGGGGCTCGAGGGGTGGCCCCCTCTGGGCATCAGCTAATTTTGAACCCAGCTGGGCATACAATTTTGAAGGGGCAGA, which mutates sites 2 and 3 to TTTCAAATTT and contains an *XhoI* restriction site. The construct *jfc1*M3-LUC was then engineered by the cassette exchange ligation of the PCR product into the deleted *jfc1*-LUC wild-type construct using the restriction enzymes *ApaI* and *XhoI* in the original clone and PCR product.

RESULTS

Cloning of the 5' flanking region of the *jfc1* gene and identification of the *jfc1* transcription start site

We identified and isolated a 549 bp region corresponding to the 5' flanking region of the human *jfc1* gene using the GenomeWalker PCR system (Clontech). This putative promoter sequence was further confirmed from two additional independent PCRs using human genomic DNA as template. An identical sequence was obtained from the three clones, and is presented in Figure 1. Several putative *cis*-acting regulatory elements were

Figure 1 Sequence of the 5'-flanking region of the human *jfc1* gene

The sequence of the promoter region of JFC1 is numbered in relation to the translation initiation site (ATG; in bold), which is designated +1. Arrows indicate the primers used for the PCRs performed using human genomic DNA as a template. The bent arrow indicates the transcription initiation site. The NF-κB binding sites 1, 2 and 3, identified by Omega™ according to the consensus sequence GGGRRHTYYCC and analysed in this work, are underlined. Multiple putative Sp1 and E box sites are also indicated by underlining.

Table 1 Comparison between the NF-κB consensus sequence and the *jfc1* promoter NF-κB binding sites

Lower-case letters indicate the nucleotides that differ from the NF-κB consensus sequence. Identity indicates the number of nucleotides that are identical to the 10-nucleotide NF-κB consensus sequence. Co-ordinates were determined from the sequence presented in Figure 1, with the A nucleotide of the ATG designated as +1. The NF-κB consensus sequence was obtained from [15,36,46,47]. R = A or G; H = A, T or C; Y = C or T.

NF-κB site	Sequence	Identity	Co-ordinates	Strand
NF-κB consensus	GGGRHTYYCC	—	—	—
<i>jfc1</i> site 1	GctGTTCCCC	8/10	–393 to –402	Complement
<i>jfc1</i> site 2	aGGAAGCTCC	8/10	–40 to –31	Lead
<i>jfc1</i> site 2	GGaGCTTCct	8/10	–31 to –40	Complement
<i>jfc1</i> site 3	GGGGCTgTgC	8/10	–6 to –15	Complement

identified in the *jfc1* promoter region sequence, including consensus binding sites for transcription factors NF-κB (Table 1), Sp1 and two E boxes (Figure 1). However, no canonical TATA and CAAT boxes, which are frequently found upstream of transcription initiation sites, were detected. The initiation site of the JFC1 RNA transcribed from the *jfc1* promoter was determined by primer extension analysis using prostate poly(A)⁺ RNA (Clontech) (Figure 2). For this purpose we used a primer complementary to bases –165 to –183 of the JFC1 cDNA. The primer extension reactions yielded a single product of 157 bp, corresponding to an initiation site mapped at –321 relative to the translation start site. No products were observed when yeast tRNA was used as a template, indicating that the primer hybridization is specific.

Figure 2 Identification of the transcription initiation site of the human *jfc1* promoter

The transcription initiation site of the *jfc1* promoter was identified by primer extension analysis using avian myeloblastosis virus reverse transcriptase (Promega) as described in the Experimental section. Lanes 1–4 represent sequencing reactions of single-stranded M13mp18 DNA using the sequencing primer –40 M13 as described in the Experimental section. Lane 5 contains a negative control using yeast tRNA (20 μ g) as template, and lane 6 contains primer extension of prostate poly(A)⁺ mRNA (5 μ g) as template.

Although several genes that lack TATA and CAAT boxes possess many transcription initiation sites [25,26], the identification of a single transcription initiation site in *jfc1* suggests that different elements could be responsible for the regulation of transcription initiation of the *jfc1* promoter. In fact, a number of putative consensus sequence motifs that may play a role in the regulation of the initiation of transcription of *jfc1* have been identified. The transcription initiation site of the *jfc1* promoter is characterized by the presence of a G/C-rich domain, with the sequence CCCGCGTGTGC (Figure 1). Interestingly, GC-rich regulatory sequences have been described in many promoters that do not contain TATA boxes, initiator elements or CAAT boxes [27]. The transcription factor Sp1, that binds to the G/C-rich regulatory element GGCGG, has been found to promote initiation of transcription in TATA-less promoters [28] and to help to correctly position transcription start sites and stabilize complex formation [29]; moreover, a contribution of Sp1 in the regulation of TATA-less promoters has been shown in various studies [21,30]. Since there are six putative regulatory elements for Sp1 in the 5' flanking region of *jfc1* (Figure 1), it would not be unlikely that this factor is involved in the control of transcription of *jfc1* at a basal level. Furthermore, two class A E boxes (CAGCTG) are present in the upstream region of the *jfc1* gene. This is a relevant observation, since E-box-mediated transcription initiation has been described in a human TATA-less promoter [31]. Finally, we identified a downstream core promoter element DPE located 34 bases downstream of the initiation site. DPE elements located in a similar position have been suggested to be a counterpart to the TATA box in some human TATA-less promoters [32], although the elements that determine the transcription initiation site of the *jfc1* promoter remain to be elucidated.

Characterization of NF- κ B transcription factor binding sites in the *jfc1* promoter using recombinant NF- κ B

To analyse the activity of the three putative NF- κ B binding sites identified in the *jfc1* promoter, gel retardation and supershift analysis of these regulatory elements were performed. For this

Figure 3 Gel retardation analysis of *jfc1* promoter regulatory regions with NF- κ B recombinant protein

Double-stranded oligonucleotide probes of the NF- κ B consensus sequence, as well as of *jfc1* promoter sites 1, 2 and 3, were labelled with [³²P]ATP and analysed for their ability to form gel retardation complexes with recombinant NF- κ B p50 (Promega) as described in the Experimental section. Unlabelled double-stranded oligonucleotide specific competitors were included as indicated below the gel. In addition, unlabelled double-stranded oligonucleotide probes for NF- κ B M1 (mutated NF- κ B site 1 of the *bcl-2* p2 promoter [19]), hepatocyte nuclear factor 3 (HNF-3) [24] and CRE [24] were included to show the specificity of the reactions. The main retarded DNA–protein complex showing a similar mobility for consensus, site 1, site 2 and site 3 oligonucleotides is indicated by an arrow. The autoradiograph is representative of three independent experiments.

purpose, double-stranded oligonucleotides corresponding to the putative *jfc1* promoter NF- κ B sites 1, 2 and 3 were evaluated for their capacity to interact with recombinant NF- κ B p50 (Figure 3). Under these experimental conditions, all three of the putative NF- κ B sites in *jfc1* interacted specifically with p50. The DNA–p50 complexes formed co-migrated with the one formed between the NF- κ B consensus sequence and p50 (Figure 3). Moreover, all complexes formed between the double-stranded oligonucleotides and p50 were inhibited by the addition of unlabelled consensus NF- κ B double-stranded oligonucleotide as competitor, but not by the addition of unrelated sequences (Figure 3). Finally, when the unlabelled double-stranded oligonucleotides corresponding to *jfc1* NF- κ B sites 1, 2 and 3 were used as competitors, the formation of complexes was inhibited in each case, supporting the specificity of the DNA–p50 complex for all three of these putative sites. An antibody to p50 generated a supershifted complex for all the NF- κ B sites of *jfc1*, as well as for the NF- κ B consensus sequence (Figure 4), further confirming that the binding is specific. In addition, no complexes were observed when the NF- κ B p50 protein was replaced in the reaction medium by recombinant activator protein-1 (Figure 4). Together, these data indicate that putative NF- κ B sites 1, 2 and 3 in *jfc1* interact specifically with NF- κ B p50.

Characterization of DNA binding proteins in nuclear cell extracts from TNF- α -treated WEHI 231 cells that interact with the *jfc1* promoter putative NF- κ B binding sites

Gel retardation analysis has shown that recombinant p50 binds to all of the NF- κ B binding sites present in the *jfc1* promoter region *in vitro*. To determine the nature of the factors that interact with these sequences *in vivo*, we analysed the ability of the double-stranded oligonucleotide spanning the putative NF- κ B binding sites of *jfc1* to bind nuclear proteins present in the TNF α -treated WEHI 231 immature B cell lymphoma line (Figure 5). WEHI 231 cells are known to be a good source of NF- κ B transcription factor family members [33]. All three putative NF- κ B sites of the *jfc1* promoter interacted specifically with proteins

Figure 4 Electrophoretic mobility supershift analysis of the *jfc1* promoter regulatory regions with NF-κB recombinant protein

³²P-labelled double-stranded oligonucleotide probes for the consensus NF-κB site (A), *jfc1* site 1 (B), *jfc1* site 2 (C) and *jfc1* site 3 (D) were analysed for their ability to form gel retardation and supershift complexes with recombinant p50 (Promega) as described in the Experimental section. The rabbit anti-p50 antibody (Santa Cruz Biotechnology) was incubated in reactions as indicated. Unlabelled double-stranded oligonucleotide probes of the NF-κB consensus sequence or of *jfc1* sites 1, 2 and 3 were used as competitors where indicated. An unrelated unlabelled competitor, hepatocyte nuclear factor 3 (HNF-3), was added as indicated to demonstrate the specificity of the observed complex. Double-stranded oligonucleotide probes of an unlabelled CRE consensus sequence and a mutated NF-κB site 1 of the *bcl-2* p2 promoter (NF-κB M1) were also used to test the specificity of the interaction. In some cases, recombinant protein activator protein 1 (AP1) (Promega) was used in place of NF-κB p50 to corroborate the specificity of the reaction. Retarded and supershifted complexes are indicated by arrows. The autoradiograph is representative of three separate experiments.

Figure 5 Gel retardation and complex supershift analysis of the human *jfc1* promoter regulatory regions with extracts from TNFα-treated WEHI 231 cells and antibodies specific for NF-κB

³²P-labelled double-stranded oligonucleotide probes of the consensus NF-κB site (A) and *jfc1* promoter site 1 (B), site 2 (C) and site 3 (D) were analysed for their ability to form supershifted complexes with DNA binding proteins in nuclear extracts from TNFα-treated WEHI 231 cells. Anti-p50 and anti-p65 antibodies (Rocklands Immunochemicals, Inc) were incubated with the nuclear extracts as indicated and as described in the Experimental section. Unlabelled double-stranded oligonucleotide probes of the consensus NF-κB sequence or of *jfc1* NF-κB sites 1, 2 or 3 were used as competitor DNAs where specified below the gels to demonstrate the specificity of the observed complexes. An unlabelled CRE consensus sequence double-stranded oligonucleotide was also used to test the specificity of the interaction. The arrows indicate the positions of the retarded protein-DNA (p65/p50 heterodimer) and supershifted complexes. Where indicated, a nuclear extract from unstimulated WEHI 231 cells was used in the assay. The autoradiograph is representative of three different experiments.

present in the WEHI 231 cell nuclear extracts (Figure 5). Given that it is known that a p65/p50 heterodimer is the predominant NF-κB complex in cells [34], we further investigated the nature of the DNA-protein complexes formed with *jfc1* putative NF-κB binding sites 1, 2 and 3 by supershift analysis using antibodies raised against p50 and p65. Nuclear extracts from TNFα-treated WEHI 231 cells were incubated with antibody specific for either

p50 or p65. With the addition of the anti-p50 antibody, the DNA-protein complexes were supershifted to the position indicated with the upper arrows (left side of autoradiographs) for the consensus NF-κB site (Figure 5A) and for *jfc1* sites 1, 2 and 3 (Figures 5B, 5C and 5D respectively). Similarly, when the anti-p65 antibody was incubated with the nuclear extracts, a supershifted complex was formed for *jfc1* sites 1, 2 and 3 (Figures 5B,

Figure 6 Transcriptional activity of the *jfc1* promoter in Hela S3 and PC-3 cells

The construct jfc1-LUC contains the *jfc1* promoter sequence from -549 to +60 located directly 5' to the promoterless firefly luciferase (*LUC*) reporter gene in pbasic-LUC (Promega), such that the *jfc1* promoter governs the expression of the *LUC* gene. The TK-LUC construct contains the herpes simplex virus TK promoter upstream of the *LUC* gene. Transient transfections were performed in Hela S3 cells and the prostate carcinoma cell line PC-3. Reporter luciferase activities were measured as described in the Experimental section. Transcriptional activities are reported relative to that of the TK-LUC construct in Hela S3 cells, which is designated as having a relative activity of 1.0. pCMV- β gal was used to correct for transfection efficiency. These results are the means of four separate experiments; error bars indicate S.E.M.

5C and 5D respectively), mimicking the behaviour of the consensus NF- κ B site (Figure 5A). These data suggest that, under these conditions, both p65 and p50 are components of the complex detected. The interactions do appear to be specific, as all complexes formed between the double-stranded oligonucleotides and the nuclear extracts were inhibited by unlabelled consensus NF- κ B oligonucleotide as competitor (Figures 5A–5D). The interactions were also inhibited by unlabelled oligonucleotides corresponding to *jfc1* sites 1, 2 and 3, but not by the addition of unrelated sequence (CRE), supporting their specificity.

Functional activity of the *jfc1* promoter region

To examine if the putative *jfc1* promoter region has transcriptional activity, the jfc1-LUC construct, that contains the luciferase gene downstream of the putative *jfc1* promoter region, was transiently transfected into two different cell lines, Hela S3 and the prostate carcinoma cell line PC-3 (Figure 6). The activities are shown relative to the activity of the herpes simplex virus TK promoter (pTK-LUC), which is a constitutively active but relatively weak promoter in comparison with the cytomegalovirus immediate-early promoter. In both cell lines the putative *jfc1* promoter showed significantly higher transcriptional activity than the herpes simplex virus TK promoter. Furthermore, the *jfc1* promoter appeared to have greater transcriptional activity in the prostate carcinoma cell line PC-3 than in the Hela S3 cells. This observation is consistent with our previous report that JFC1 is highly expressed at the RNA level in the prostate [1].

The gel shift and supershift analysis presented above demonstrated that sites 1, 2 and 3 of the putative *jfc1* promoter can bind to NF- κ B. However, to determine whether NF- κ B can, in fact, transactivate transcription from this region, the *jfc1* luciferase construct (jfc1-LUC) was examined for activity in the presence and absence of exogenously expressed NF- κ B (p65/p50) in Hela S3 cells (Figure 7A). The transcriptional activities are reported relative to that of the jfc1-LUC construct in the absence of NF- κ B, which is designated as having a relative activity of 1.0. Exogenously expressed NF- κ B was found to transactivate tran-

Figure 7 Transactivation of transcription from the *jfc1* promoter by NF- κ B

The constructs jfc1-LUC (described in Figure 6) and jfc1 M3-LUC (containing mutated *Jfc1* NF- κ B sites) were used to evaluate the effect of NF- κ B on the transactivation of transcription from the *jfc1* promoter. (A) The relative activity of the construct in Hela S3 cells was examined in the presence (NF- κ B) and absence (pA) of exogenously expressed p50/p65 by co-transfection with the expression vectors pCMVp50 and pCMVp65. Transcriptional activities are reported relative to that of the jfc1-LUC construct in the absence of NF- κ B, which is designated as having a relative activity of 1.0. pRL-TK was used to correct for transfection efficiency. These results are the means of four separate experiments; error bars indicate S.E.M. (B) The relative activity of the construct jfc1-LUC in Hela S3 cells was examined in the presence (pUSE-IkB) and absence (pUSE) of exogenously expressed dominant negative I κ B using the expression vector pUSE-IkB (Upstate Biotechnology). Transcriptional activities are reported relative to that of the jfc1-LUC construct in the absence of I κ B, which is designated as having a relative activity of 1.0. pRL-TK was used to correct for transfection efficiency. These results are the means of six separate experiments; error bars indicate S.E.M.

scription 9-fold from the *jfc1* promoter. On the other hand, the transactivation by NF- κ B of jfc1M3-LUC, a mutated *jfc1* promoter that lacks the three NF- κ B binding sites, was negligible (Figure 7A). To further demonstrate that transcription from the *jfc1* promoter in Hela S3 cells is transactivated by NF- κ B, the transcriptional activity of the *jfc1* promoter was evaluated after co-transfection with the pCMV I κ B dominant negative construct (Upstate Biotechnology) (Figure 7B). This vector expresses a non-phosphorylatable, non-degradable I κ B. The results show that the activity of the *jfc1* promoter was inhibited by the ablation of NF- κ B signalling by the dominant negative I κ B. Altogether, these data suggest an important role for the NF- κ B pathway in the transcriptional regulation of *jfc1*.

jfc1 promoter activity and protein expression in TNF α -stimulated human prostate carcinoma cells

It is now clear that TNF α treatment leads to the activation of the NF- κ B pathway in a vast range of cell lines, including prostate

Figure 8 Transactivation of the *jfc1* promoter by TNF α

The ability of TNF α to transactivate the *jfc1* promoter was evaluated by transient transfection in LNCaP cells. LNCaP cells were incubated in medium containing 10% (v/v) charcoal-stripped serum overnight before transfection with either *jfc1*-LUC or the control vector pbasic-LUC. Then 1 ng/ml TNF α was used to stimulate the transfected cells, and the incubation at 37 °C was continued for a further 12 h before the cells were harvested. Lysates were analysed for firefly and renilla luciferase activities as described in the Experimental section. These results are the means of four separate experiments; error bars indicate S.E.M.

carcinoma cell lines [35]. Therefore we examined the inducibility of the *jfc1* promoter by TNF α in LNCaP prostate carcinoma cells. Our results (Figure 8) indicate that *jfc1* promoter activity is significantly increased after TNF α treatment, although no effect was observed when the mutated *jfc1*M3 promoter was used in the assays (results not shown). To examine the effect of TNF α on JFC1 expression, the human prostate carcinoma cell lines LNCaP, PC-3 and DU-145, as well as Epstein–Barr virus (EBV)-transformed B lymphocytes, were stimulated with 10 ng/ml TNF α (Figure 9). The expression of JFC1 was dramatically augmented in all prostate cell lines (Figure 9A), but was unaffected in lymphocytes (Figure 9B). These data suggest that TNF α regulates *jfc1* promoter activity and protein expression, and that the TNF α /NF- κ B pathway may be involved in the differential expression of JFC1 under physiological conditions in prostate carcinoma cells.

DISCUSSION

In this work we cloned and sequenced the promoter region of the human *jfc1* gene, identified NF- κ B-responding elements and examined the role of the NF- κ B transcription factor in regulation of transcription from this promoter. In a previous report we demonstrated that JFC1 mRNA expression is tissue-specific [1], indicating that close regulation may take place at the transcriptional level. In the present work, three putative NF- κ B binding sites were identified in the *jfc1* promoter (Table 1). Similar to the NF- κ B sites found in the interleukin-2 gene [36], the pro-interleukin-1 β promoter [37] and the *bcl-2* promoter [19] to cite some examples, these sequences differ to some extent from the consensus NF- κ B binding motif 5' GGGRHTYYCC 3'. However, our experimental data clearly show that they are specific NF- κ B binding sites. Gel retardation analysis demonstrated that all three sites interact specifically with NF- κ B transcription factors. Moreover, supershift analysis performed with nuclear extracts from TNF α -treated WEHI 231 cells and antibodies that recognize either the p50 or the p65 NF- κ B component suggests that, under these conditions, NF- κ B binds to sites 1, 2 and 3 of the *jfc1* promoter in a p65/p50 heterodimer complex configuration. The p65/p50 heterodimer is the most abundant NF- κ B complex in the majority of cell types [34], and is also responsible for transcriptional activation via the p65 transcription-activation domain.

Our data suggest that NF- κ B might play a significant role in activation of the transcription of *jfc1* *in vivo*. The level of transcription from the *jfc1* promoter was evaluated by transient transfection of the *jfc1* promoter luciferase reporter construct into Hela S3 and PC-3 prostate carcinoma cells. The isolated 5' flanking region of *jfc1* was transcriptionally active in the absence of exogenous transcription factors in both cell lines (Figure 6). Overexpression of p50 and p65 increased by 9-fold the promoter activity of *jfc1*, but not that of the mutated *jfc1*M3 promoter, further confirming the involvement of NF- κ B in the transcriptional regulation of *jfc1* (Figure 7A). Co-transfection with *jfc1*-LUC and an expression vector for a dominant negative form of I κ B dramatically reduced the transcriptional activity of the *jfc1* promoter (Figure 7B), confirming that NF- κ B is involved in the regulation of *jfc1* transcription.

Figure 9 Augmented expression of JFC1 in prostate carcinoma cells in response to TNF α

LNCaP, DU-145 and PC-3 human prostate carcinoma cells (A) and EBV-transformed lymphocytes (B) were cultured as described in the Experimental section before the addition of TNF α (10 ng/ml). The incubation at 37 °C was then continued for a further 6 h (A) or for the indicated times (B). Cells were lysed and proteins (100 μ g) were analysed by immunoblotting against JFC1 using an antibody raised against an N-terminal peptide that recognizes a specific JFC1 duplet in various cell types, as described previously [1].

TNF α has been shown to induce programmed cell death in a variety of transformed cells. However, alternative signalling pathways triggered by activation of the TNF receptor have been demonstrated not only not to induce apoptosis but also to promote survival [35]. Indeed, the fate of a cell after TNF α stimulation seems to be determined by the predominance of pro-apoptotic pathways over pro-survival pathways or vice versa. Prostate carcinoma cells do not escape from the conflicting effects of TNF α . TNF α has been described to activate a NF- κ B-dependent pro-survival pathway in prostate cancer cells [19,38]. This mechanism involves activation of PI 3-kinase/Akt signalling by TNF α , which leads to the activation of IKK and NF- κ B. However, when the PI 3-kinase is inhibited pharmacologically, or the NF- κ B pathway is inhibited by an I κ B super-repressor, prostate carcinoma cells are sensitized to TRAIL (TNF-related apoptosis-inducing ligand)-induced [39] or TNF α -induced [40] apoptosis. Therefore NF- κ B is considered to play a central role in the pro-survival effect of TNF α . Moreover, the cytoprotective effect of NF- κ B involves the transcriptional regulation of pro-survival genes [19,41,42]. In a recent report we demonstrated that the TNF α /NF- κ B pathway leads to transcriptional activation of the survival factor Bcl-2 in prostate carcinoma cells, and this effect was only detected when cells were treated in the absence of hormone [19]. In the present work, under similar experimental conditions, we demonstrated that the *jfc1* gene is transcriptionally regulated by NF- κ B in PC-3 prostate carcinoma cells, and that TNF α increases promoter activity (Figure 8) and up-regulates the expression of JFC1 in the prostate carcinoma cell lines LNCaP, PC-3 and DU-145, but not in EBV-transformed lymphocytes (Figure 9).

Interestingly, it has been reported that prostate carcinoma cells undergo neuroendocrine differentiation [43], and that this is a positive indication of prostate tumour progression. Neuroendocrine differentiation is a phenomenon characterized by the appearance of vesicles and the secretion of mitogenic neuropeptides [44]. Moreover, the PI 3-kinase pathway has been shown to induce the neuronal differentiation of LNCaP cells [45], and transcriptional activation of proteins required for the secretion of mitogenic peptides has been suggested to be a necessary event in neuroendocrine transformation [44]. Therefore, since JFC1 possesses a PIP3 binding domain and shares many characteristics with secretory proteins, its overexpression in response to activation of the TNF α /NF- κ B pathway could be physiologically relevant, and may play an important role in the regulation of the autocrine effect triggered by the secretion of mitogenic factors by prostate carcinoma cells.

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