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IDENTIFICATION OF A PHORBOL ESTER RESPONSIVE REGION IN THE MYELOID-SPECIFIC PROMOTER OF THE c-fgr PROTO-ONCOGENE

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Expression of the c-*fgr* proto-oncogene is activated during differentiation of myeloid cells. We used a luciferase reporter assay to identify sequences that regulate c-*fgr* gene transcription during differentiation of human U937 promonocytic cells, induced by phorbol 12-myristate 13-acetate (PMA) or by tumour necrosis factor-α (TNF-α) and 1,25-dihydroxycholecalciferol (1,25-DHCC). We found that the region from nucleotides -344 to -116, with respect to the transcriptional start site, is required for basal activity of the c-*fgr* promoter in U937 cells, and that the region from nucleotides -1211 to -752 is responsive to PMA. No sequence elements responsive to TNF-α and 1,25-DHCC were found, suggesting that these agents induce c-*fgr* mRNA accumulation by a mechanism differing from that mediating the effects of PMA.

Cells of the monocyte-macrophage lineage play a central role in inflammation and immunity. The differentiation of monoblasts to monocytes to macrophages, and the subsequent ability of the mature cells to phagocytose and/or present antigen, has therefore been the subject of considerable research. Understanding the mechanisms that control this aspect of hemopoiesis would be of benefit in the control of diseases such as myeloid leukemias and rheumatoid arthritis, as well as infectious diseases such as tuberculosis and leprosy. One *in vitro* model for the study of myeloid cell biology is the human monoblastic leukemia cell line U937. Treatment of U937 cells with various modulating agents such as phorbol 12-myristate 13-acetate (PMA) or tumour necrosis factor-α (TNF-α) and 1,25-dihydroxycholecalciferol (1,25-DHCC) will induce the cells to differentiate to a monocyte/macrophage-like phenotype (1).

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Abbreviations:
1,25-DHCC, 1,25-dihydroxycholecalciferol; PMA, phorbol 12-myristate 13-acetate; TNF-α, tumour necrosis factor-α.

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The c-*fgr* proto-oncogene, which encodes a member of the Src family of protein tyrosine kinases (2), is normally expressed in mature peripheral blood monocytes and granulocytes, and in macrophages, but not in their precursors (3). In accordance with this, differentiation of U937 cells in response to the modulating agents listed above is accompanied by the accumulation of c-*fgr* mRNA (3-5). Induction of monocytic or granulocytic differentiation of the human monoblastic HL60 cell line also leads to accumulation of c-*fgr* mRNA and its product, $p55^{c\text{-}fgr}$ (Fgr) (6). The c-*fgr* gene is also expressed in some transformed B-lymphoid cells, but not in normal B-lymphocytes (7,8). The function of Fgr in myeloid cells is unclear. The kinetics of activation of c-*fgr* expression during differentiation suggest that Fgr is not involved in regulating loss of proliferative ability, but rather in regulating the activity of mature cells (5,6). Fgr would then resemble other members of the Src family, which appear to have roles in terminally differentiated, post-mitotic cells (2). In support of this idea, Fgr has recently been demonstrated to be associated with the low affinity $Fc_{\gamma}RII$ receptor in neutrophils, suggesting a role in Fc_{γ}-mediated phagocytosis (9).

We previously isolated and characterised a human c-*fgr* genomic clone (cF2.3) and mapped a cluster of transcriptional start sites used in B-lymphoid cells (Fig. 1A) (10). Transcription from this cluster of start sites gives rise to mRNA containing exons 1a, 1b and 2-12 (Fig. 1A) (10,11). It was subsequently shown that these start sites are used exclusively in B-lymphoid cells and that the major transcriptional start site in myeloid cells lies approximately 10 kb upstream (Fig. 1A) (12). Transcription from this site gives rise to an mRNA containing exons M4, 1b and 2-12 (Fig. 1A), as well as to at least three less abundant alternatively spliced mRNAs (12). In this paper we report an analysis of the promoter activity of the region immediately upstream of the myeloid-cell-specific exon M4 (Fig. 1A). In particular, we have investigated the response of this region to treatment of U937 cells with the modulating agents PMA, TNF-α and 1,25-DHCC.

Materials and Methods

Cosmid library screening. A cosmid library of human genomic DNA was kindly provided by Dr Dimitris Kioussis (National Institute for Medical Research, Mill Hill, UK). This library was constructed in cosmid vector cos202 using partially digested *Sau*3AI fragments of genomic DNA from the human acute lymphocytic leukaemia cell line HPB-ALL (13). A radiolabelled DNA probe specific for c-*fgr* exon M4 was derived from the myeloid cell c-*fgr* cDNA clone pFM2 as described previously (14) and used to screen the genomic library as described previously (10). A number of positive colonies were detected and colony-purified by two further rounds of screening at low density. Cosmid clone c3 was selected for further study and the position of exon M4 was determined by restriction mapping, Southern hybridization and nucleotide sequencing, performed as described previously (10).

Construction of luciferase reporter plasmids. Appropriate restriction fragments were excised from cosmid clone c3 and inserted into the polylinker of plasmid vector pGL-2 Basic (Promega Corporation, Madison, WI, USA), upstream of a luciferase reporter gene.
Culture and transfection of U937 cells. U937 cells were cultured at 37°C in 5% CO_2 in RPMI 1640 medium (GibcoBRL, Paisley, UK) supplemented with 10% (v/v) fetal calf serum (GibcoBRL), 200 μM 2-mercaptoethanol (GibcoBRL), 100 U ml^{-1} penicillin and streptomycin (Sigma, Poole, UK), 10 mM HEPES (GibcoBRL) and 2mM glutamate (Sigma). U937 cells were transfected with luciferase reporter plasmids by electroporation, as follows. Cells in exponential growth phase were washed and resuspended at 10^8 ml^{-1} in PBS (Sigma). Aliquots of 250 μl were transferred to 0.4 cm electroporation cuvettes (Bio-Rad, Hemel Hempstead, UK) and mixed with 50 μg of plasmid DNA. Electroporation was then performed using a Bio-Rad Gene Pulser set at 220 V and 960 μF. Each aliquot of transfected cells was diluted to 10 ml with RPMI 1640 medium containing the supplements listed above, and incubated for 24 h at 37°C in 5% CO_2. Each culture was then split into two. The first half was incubated for a further 48 h in the presence of PMA (Sigma; 5 ng ml^{-1}) or a mixture of TNF-α (Genentech, San Francisco, CA, USA; 50 ng ml^{-1}) and 1,25-DHCC (Hoffman La Roche, Nutley, NJ, USA; 100 nM), as previously described (5). The second half was incubated under the same conditions for 48 h in the absence of any modulating agents.
Luciferase assays. Transfected cells were harvested, washed in PBS, pelleted and lysed by incubation for 15 min at room temperature in 250 μl Lysis Reagent (Promega). Cell debris was removed by microcentrifugation for 30 sec at 6,000 g. An 80 μl aliquot of supernatant was added to 400 μl Luciferase Assay Reagent (Promega) and immediately placed into a luminometer (LKB Pharmacia, Uppsala, Sweden). Light emission was measured over a 1 min period. Transfection efficiency was determined in each case by dot-blot hybridization to transfected plasmids in cell lysates, as described by Abken and Reifenrath (15).

Results and Discussion

Isolation of c-fgr genomic clone c3 and construction of reporter plasmids. The c-*fgr* genomic clone cF2.3, which we isolated previously (10), did not extend upstream as far as exon M4. We therefore screened a cosmid library of human genomic clones (13) with an exon M4-specific DNA probe derived from the myeloid cell c-*fgr* cDNA clone pFM2 (14). This resulted in the isolation of cosmid clone c3, which contained the complete c-*fgr* gene, including exon M4 and approximately 20 kb of upstream sequences (Fig. 1A). Six overlapping restriction fragments were excised from c3 and inserted into the polylinker of plasmid vector pGL-2 Basic, upstream of a luciferase reporter gene. The six inserts shared the same 3' terminus, at position +99 with respect to the major myeloid-specific transcription start site (1), but had staggered 5' termini (Fig. 1B).

Sequences required for basal activity of the c-fgr promoter in U937 cells. The six c-*fgr*-luciferase reporter plasmids, or the pGL-2 Basic plasmid, were transfected into undifferentiated U937 cells by electroporation. The luciferase activities directed by the plasmids are shown in Fig. 2A. The c-*fgr* gene fragment extending from -116 to +99 (pGL-116) did not direct any luciferase expression above the background level seen with pGL2-Basic, but the longer c-*fgr*

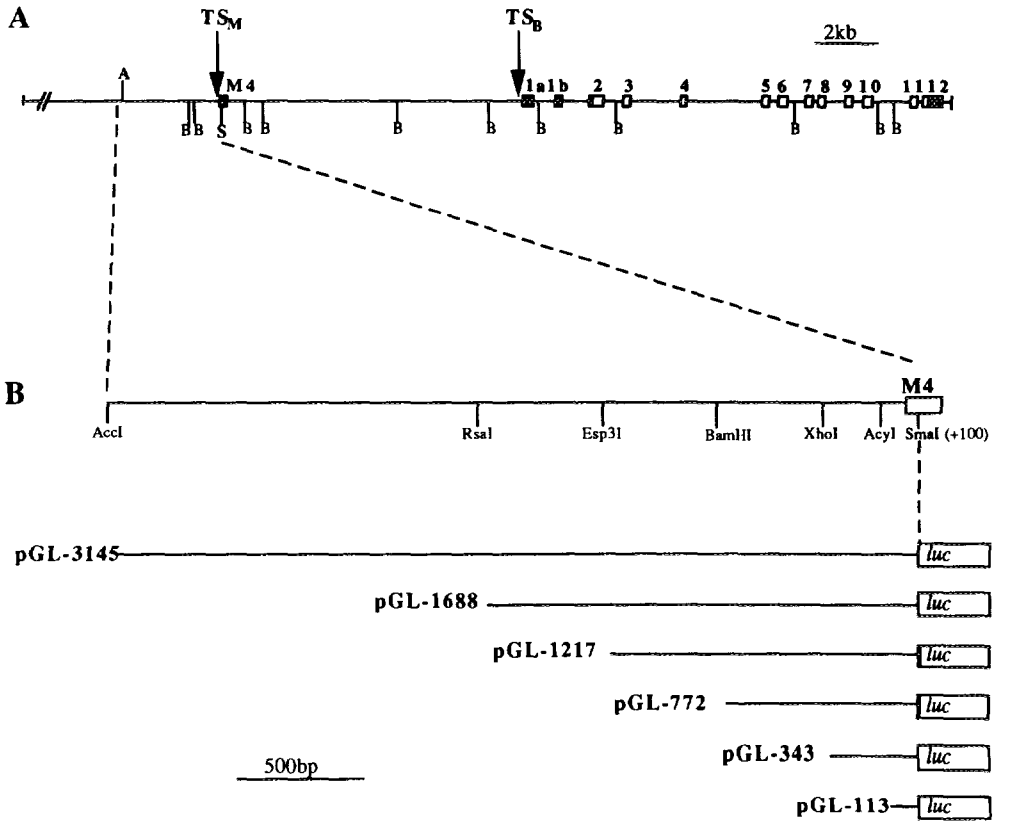

Figure 1. (A) Map of the insert of cosmid clone c3, containing the human c-*fgr* gene. Exons used in the major c-*fgr* mRNA species are shown as white (coding sequence) and black (non-coding sequence) boxes. The gap at the left of the insert accomodates approximately 14 kb of DNA. A, *Acc*I; B, *Bam*HI; S, *Sma*I; TS_B, cluster of transcriptional start sites used in B-lymphoid cells; TS_M, major transcriptional start site used in myeloid cells. (B) Fragments of the c-*fgr* gene used in reporter constructs. Fragments were cloned upstream of the luciferase gene in plasmid vector pGL2-Basic (Promega). The 3' end of each fragment was the *Sma*I site at position +99 with respect to TS_M. The 5' ends of the fragments were the *Acc*I, *Rsa*I, *Esp*3I, *Bam*HI, *Xho*I and *Acy*I sites located at positions -3200, -1686, -1211, -772, -344 and -116, respectively.

gene fragments each directed luciferase expression of approximately 10-12 times background. These data indicate that the region between -344 and -116 is required for c-*fgr* promoter activity in U937 cells. This region contains consensus NFκB and SP-1 binding sites at -304 and -140, respectively (12), which could be involved in mediating promoter activity. There is a consensus SP-1 binding site at -52, and a TATA box at -31 (12), which appear not to be sufficient for c-*fgr* promoter activity.

Sequences required for transcriptional activation of the c-fgr promoter in response to PMA. As shown in Fig. 2B, PMA treatment had no effect on the luciferase expression directed by pGL-

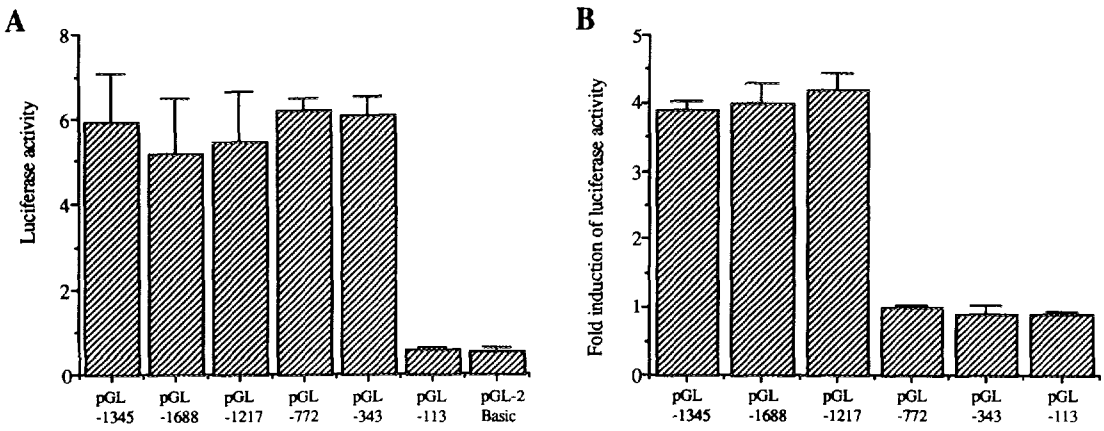

Figure 2. (A) Luciferase activity in untreated U937 cells transfected with c-*fgr* reporter constructs, expressed in arbitrary units. Values represent the mean (with SEM) of three independent experiments. (B) Induction of luciferase activity by PMA in U937 cells transfected with c-*fgr* reporter constructs. Transfected cultures were split into two halves. One half was treated with 5 ng ml^{-1} PMA and the other was left untreated. The value for each construct represents luciferase activity in PMA-treated cells divided by luciferase activity in untreated cells and is the mean (with SEM) of three independent experiments. Transfection efficiencies did not vary significantly between samples, as judged by dot-blot hybridization to the transfected plasmids in cell lysates (15).

116, pGL-343 or pGL-772. However, PMA induced an approximately 4-fold increase in the luciferase expression directed by pGL-1211, pGL-1686 and pGL-3200. These data demonstrate that the 459 bp region between positions -1211 and -772 contains one or more PMA-response elements. Examination of the nucleotide sequence of this region (16) shows that it contains consensus binding sites for AP-2 at -834, and for IRBP at -824 and -1178. Both AP-2 and IRBP have been shown to mediate PMA-responsiveness in other systems (17), but further work will be required to determine whether these particular sites are functional in U937 cells. Our data show that the region from -772 to +99, whilst including sequences required for basal c-*fgr* promoter activity (-344 to -116), is not sufficient to confer PMA-responsiveness upon the c-*fgr* promoter. This contrasts with a previous report by Link et al (12) that the region between -772 and +99 was PMA-responsive. This discrepancy could arise because Link et al (12) assayed promoter activity from integrated plasmid constructs in stably transfected cell lines, whilst we assayed promoter activity in a transient expression assay, which is free from position effects.

In contrast to treatment with PMA, treatment with TNF-α and 1,25-DHCC had no effect on the levels of luciferase expression directed by any of the c-*fgr* reporter constructs (data not shown). Our previous work showed that c-*fgr* mRNA levels do increase in U937 cells in response to these modulating agents, however (5). If they do so by stimulating transcription from the major myeloid promoter, rather than by post-transcriptional mechanisms, they must do so through

sequences lying upstream of position -3200. Such sequences might be responsive to cytokines rather than to phorbol esters. Alternatively, there is a second c-*fgr* promoter, which is used at very low efficiency in PMA-treated U937 cells (12), and it is possible that TNF-α and 1,25-DHCC regulate c-*fgr* mRNA levels by activating this promoter.

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