Supplementary Materials and Methods

Gene expression analysis

mRNA was prepared using the Trizol™ method (Sigma) as outlined by the manufacturer. cDNA was synthesised using Mo-MLV reverse transcriptase and oligo (dT) and mRNA levels were measured by Real Time quantitative PCR using the (ABI Prism 7700 sequence detection system, Perkin Elmer) with SYBR Green. Relative mRNA levels were calculated using cDNA standard and the input was normalised according to GAPDH expression.

DNaseI hypersensitive site (DHS) mapping

DHS mapping was performed as described (Cockerill, 2000). Essentially, nuclei prepared from cells were incubated with increasing amount of DNaseI (Worthington) for 15 min on ice. Following DNA extraction, 20 µg of DNA for each sample was digested with BamHI or PstI. After complete digestion DNA was then separated by electrophoresis on an 0.8% agarose gel in TAE and subjected to Southern blot analysis. A PvuII-BamHI fragment (+497 bp to +848 bp) was used as a probe for Southern blot analysis as described in (Himes et al., 2001).

Plasmid construction

All reporter gene constructs in this study were derived from pGL2 (Promega). pGL2-promoter vector carrying the SV40 promoter (Promega) was used as control. Different lengths of c-fms promoter regions and intronic enhancer regions of c-fms (FIRE, FIRE-1kb, FIRE+FIRE-1kb) were amplified from BAC clone (RPCI23-232H21) using primers starting from the indicated sequence and inserted into the upstream and downstream of luciferase gene as indicated in Results. The (–201bp) *c-fms* promoter plasmid was constructed by deleting SmaI-XbaI fragment from a larger *c-fms* promoter plasmid (–425bp). The promoter region with mutations were amplified from the (–135 bp) c-fms promoter plasmid. The sequence integrity of all constructs was examined by sequence analysis.

Reporter gene assays

Transient transfections into RAW264 cells were performed in triplicate by using jetPEI™ transfection reagent (Polyplus-transfection, Strasbourg, France) according to manufacturer's instructions. The jetPEI reagent was employed at 3μl/μgDNA to obtain the optimum transfection efficiency and DNA amounts were kept constant by adding empty expression vector (control). The consistency in transfection efficiency was monitored by measuring Renilla luciferase encoded by pRL-CMV. 10^6 of RAW cells were transfected with 100 μl reaction mix containing 0.13 pmole reporter, 0.03 pmole PU.1/control, 0.02 pmole Pax5/control, 0.5 fmole pRL-CMV and jetPEI reagent or 100 µl reaction contained 0.13 pmole reporter, 0.04 pmole effectors, 0.22 fmole pRLCMV and jetPEI reagent, respectively. 10^7 of A20 (germinal centre B) cells were transfected with plasmid DNA (2.5 pmol reporter, 0.56 pmol PU.1/control, 2.4 pmol Pax5/control and 0.96 fmol pRL-CMV) by electroporation (400V, 550µF) (BioRad, Gene Pulser II). Cells were harvested after 24 hr incubation at 37˚C and lysed in 1x passive lysate buffer (Promega). Luciferase activity was measured by using Dual-Glo™ Luciferase Assay System (Promega). All DNA used for transfection was purified by two rounds of caesium chloride equilibrium centrifugation.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described previously (Dyer RB, Herzog NK, 1995). In brief, 10^8 wild type pro-B cells were lysed in a buffer consisting of 0.32 M Sucrose, 50 mM KCl, 3 mM $CaCl_2$, 2 mM magnesium acetate, 10 mM Tris pH 8.0, 0.1% NP-40, 0.15mM spermine, 0.5 mM spermidine, 10 mM NaF, 1 mM DTT, 0.5 µM PMSF and 0.1% protease inhibitor cocktail (Sigma) and nuclei were pelleted by centrifugation for 5 minutes at 400 g. After pelleted nuclei were lysed in a buffer consisting of 20 mM HEPES pH 7.9, 25% glycerol, 1.5m M $MgCl_2$, 0.176 M KCl, 0.2% NP-40, 0.2 mM EGTA, 10mM NaF, 1 mM DTT, 0.5 µM PMSF and 0.1% protease inhibitor cocktail. Protein content was measured by a Bradford assay (BioRad) using BSA as a standard. To prepare probes, the complementary oligonucleotides described in Supplementary table 1 were annealed and labelled with (g-^{32}P) ATP by T4 polynucleotide kinase (New England Biolabs).

Binding assays were performed as described previously (Cockerill et al., 1993). To detect the binding of recombinant Pax5 DBD protein to the wild type or mutated c-fms promoter probes, 50 fmoles of probe were incubated with 10 ng of Pax5 protein in 16 μl binding buffer (15 mM HEPES pH 7.9, 50 mM KCl, 50 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DTT) in the presence of 12.5 ng poly-dIdC for 30 minutes at room temperature. To investigate the effect of recombinant Pax5 to the binding of wild type pro-B cell nuclear proteins to the c-fms promoter, 50 fmoles of probe were incubated with increasing amounts of recombinant Pax5 (3-100 ng) in the binding buffer for 10 minutes on ice, then nuclear proteins from pro-B cells in the binding buffer were added followed by 10 minutes incubation on ice. When a super-shift assay was performed, 4 μg PU.1 Ab (Santa Cruz, sc-352x), 6 μg Pax5 Ab (Santa Cruz, sc-1975x) or 6 μg control IgG were added to the proteins before adding to the probe. Protein-DNA complexes were resolved on a 4% polyacrylamide gel and exposed to a PhosphorImager screen.

To measure the affinity of Pax5 protein to each promoter sequence, an EMSA with 50fmoles of oligonucleotide probes (CD19-1, Mb-1, and c-fms promoter) and increasing amounts of Pax5 (10 – 200 ng) was performed. The signal intensity of shifted bands and free probes was measured by using Quantity One software and the amount of recombinant Pax5 which gives 50% binding was calculated.

Western blot analysis

Transfected cells were washed with PBS and lysed in standard reducing SDS-running buffer. The proteins in cell lysates were resolved on a 8% SDS-PAGE gel and electrotransferred onto a nitrocellulose membrane (Pierce). The membranes were blocked and incubated with Pax5 c-terminus antibody (Ab) (1 μg/ml, BD Biosciences), Pax5 N-terminus Ab (2–5 μg/ml, Chemicon International) or β-actin Ab (1μg/ml, Sigma) at 4˚C overnight followed by an incubation with HRP conjugated goat anti rabbit Ig or goat anti mouse Ig (1:20000 – 1:10000, Jackson Immunoresearch) for one hour at room temperature. Proteins were visualised by Chemiluminescence substrate (Pierce). Blocking and antibody incubation were performed in StartingBlock™ blocking buffer (Pierce) and washing was performed in PBS containing 0.1% Tween.

Protein expression and purification

The human Pax5 paired domain (residues 1-149) was expressed in E. coli C41 (DE3) under T7 promoter control from plasmid pRSET. Protein purification was performed as described (Garvie et al. 2001).

References

Cockerill PN, Shannon MF, Bert AG, Ryan GR, Vadas MA. (1993) The granulocyte-macrophage colony-stimulating factor/interleukin 3 locus is regulated by an inducible cyclosporin A-sensitive enhancer. *Proc Natl Acad Sci U S A*. **90**.2466-70.

Cockerill, P.N. (2000) Identification of DNaseI hypersensitive sites within nuclei. *Methods Mol Biol,* **130**, 29-46.

Dyer RB, Herzog NK. (1995) Isolation of intact nuclei for nuclear extract preparation from a fragile B-lymphocyte cell line. *Biotechniques*. **19**.192-5.

Garvie, C.W., Hagman, J. and Wolberger, C. (2001) Structural studies of Ets-1/Pax5 complex formation on DNA. *Mol Cell*, **8**, 1267-1276.

Himes, S.R., Tagoh, H., Goonetilleke, N., Sasmono, T., Oceandy, D., Clark, R., Bonifer, C. and Hume, D.A. (2001) A highly conserved c-fms gene intronic element controls macrophage-specific and regulated expression. *J Leukoc Biol*, **70**, 812-820.

Supplementary Table 1

probes for EMSA		
wild type	upper strand	AAAAAAAAAAGGGGAAGAGGAGCCAGTGCAACAGACAGGAACGTGTTCATC
	lower strand	GATGAACACGTTCCTGTCTGTTGCACTGGCTCCTCTTCCCCTTTTTTTTTT
mutant 1	upper strand	AAAAAAAAAAGGGTCAGAGGAGCCAGTGCAACAGACAGGAACGTGTTCATC
	lower strand	GATGAACACGTTCCTGTCTGTTGCACTGGCTCCTCTGACCCTTTTTTTTTT
mutant 4	upper strand	AAAAAAAAAAGGGGAAGAGGACGCAGTGCAACAGACAGGAACGTGTTCATC
	lower strand	GATGAACACGTTCCTGTCTGTTGCACTGCGTCCTCTTCCCCTTTTTTTTTT
mutant 5	upper strand	AAAAAAAAAAGGGGAAGAGGAGCCAGTCGAACAGACAGGAACGTGTTCATC
	lower strand	GATGAACACGTTCCTGTCTGTTCGACTGGCTCCTCTTCCCCTTTTTTTTTT
mutant 7	upper strand	AAAAAAAAAAGGGGAAGAGGAGCCAGTGCGGCAGACAGGAACGTGTTCATC

	lower strand	GATGAACACGTTCCTGTCTGCCGCACTGGCTCCTCTTCCCCTTTTTTTTTT
mutant 8	upper strand	AAAAAAAAAAGGGGAAGAGGAGCCAGTGCAACAGACAGCTACGTGTTCATC
	lower strand	GATGAACACGTAGCTGTCTGTTGCACTGGCTCCTCTTCCCCTTTTTTTTTT
mutant 9	upper strand	AAAAAAAAAAGGGGAAGAGGAGCCAGTGCAACATTCAGGAACGTGTTCATC
	lower strand	GATGAACACGTTCCTGAATGTTGCACTGGCTCCTCTTCCCCTTTTTTTTTT
mutant 10	upper strand	AAAAAAAAAAGGGGAAGAGGAGCCAGTGCAACAGAGAGGAACGTGTTCATC
	lower strand	GATGAACACGTTCCTCTCTGTTGCACTGGCTCCTCTTCCCCTTTTTTTTTT
mutant 11	upper strand	AAAAAAAAAAGGGGAAGAGGAGCCTTTGCAACAGACAGGAACGTGTTCATC
	lower strand	GATGAACACGTTCCTGTCTGTTGCAAAGGCTCCTCTTCCCCTTTTTTTTTT

real time PCR primers		
MPO promoter	Forward	CTCCAGGCTCGGGACAAA
	Reverse	TCTGCCTGCTCCGAAATCA
MPO last exon	Forward	CGTGTTCAGTAAACAGCAGAGACA
	Reverse	TCTTCGACACGGTGGTGATG

LM-PCR primers		
rDNA	P01 (+70 - +94)	GCACCGCGACAGACCCAAGCCAGTA
	P02 (+67 - +89)	GACAGACCCAAGCCAGTAAAAAG
	P03 (+57 - +83)	CAGACCCAAGCCAGTAAAAAGAATAGG