The Regulation of Gene Expression During Differentiation of the Human Monoblastic Cell Line U937

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Summary

Differentiation of the myeloid cell lineage towards a monocyte/macrophage has been studied using the monoblastoid cell line U937 as a model system. Treatment of these cells with various chemical agents, including retinoic acid (RA), 1α,25-dihydroxycholecalciferol (D3), phorbol ester (PMA) and cytokines, results in cessation of proliferation and the appearance of characteristics consistent with the monocyte/macrophage phenotype. Numerous changes in gene expression are observed, including upregulation of c-fgr mRNA and p55\(^{\text{c-fgr}}\), which is thought to have a role in the mature monocyte/macrophage. In the first part of this work I have analysed the mechanisms by which c-fgr gene expression is regulated during U937 cell differentiation. In the second part I have explored the roles of members of the RXR class of transcription factors in U937 cell differentiation.

Following induction of differentiation, c-fgr gene transcription is activated from a myeloid-specific promoter upstream of an unmapped exon, M4. By isolation of a human genomic cosmid clone, exon M4 was mapped 11.1kb upstream of the c-fgr coding exons. The myeloid promoter was characterised using a luciferase reporter gene in transient transfection assays which established that basal levels of transcription required sequences in the region -343 to -128 with respect to the transcriptional start site. Furthermore, the region -1211 to -772 was found to be responsive to PMA, inducing a 4-fold increase in luciferase activity. No promoter sequences responsive to TNF\(\alpha\) in combination with D3 were found, suggesting that these agents direct transcription of the c-fgr gene via a mechanism that differs from the PMA response.

It was determined that both RXR\(\alpha\) and RXR\(\beta\) mRNAs are expressed in undifferentiated U937 cells, and that their expression is upregulated 8-fold and 3-fold, respectively, following PMA-induced differentiation. To study the role of the RXR\(\alpha\) and RXR\(\beta\) in regulating gene expression during myeloid differentiation, U937 cells were stably transfected with
mammalian expression vectors that directed synthesis of either sense or antisense human RXRα RNA or antisense RXRβ RNA.

It was shown that RXRα-antisense transfected cells were resistant to 9-cis RA, all-trans RA and D3-induced inhibition of proliferation. Furthermore, RXRα-sense transfected cells displayed increased sensitivity to RA. However, RXRα-sense expressing cells did not show increased sensitivity to D3, possibly as a result of limiting levels of vitamin D receptor. Both these novel cell lines could be induced to differentiate to the same extent as control cells by treatment with either TNFα or PMA, suggesting that these agents signal differentiation via different pathways to RA and D3. In contrast to these findings, RXRβ-anti-sense cells displayed no such resistance to any of the above treatments, suggesting that RXRβ performs different functions, possibly in more mature cells. The levels of β2-integrin cell surface antigens, CD11a, CD11b, CD11c and CD18, were not affected by expression of antisense RXRα mRNA compared to control cells, whereas sense expression of sense RXRα mRNA resulted in increased levels of the antigens. It was concluded that RXRα, but not RXRβ, plays an important role in signalling the cascade of events that result in cessation of proliferation but not necessarily other aspects of monoblastic differentiation.

Both aspects of this thesis have helped to delineate mechanisms of gene regulation that function during monoblastic differentiation.
Table of Contents

Title 1
Summary 2
Table of contents 4
Acknowledgements 9
List of Tables 10
List of Figures 11
Chapter 1 Introduction 16
1.1 The myeloid cell lineage 18
  1.1.1 Myeloid development 18
  1.1.2 Monocyte/macrophage function 20
  1.1.3 The U937 cell line 22
    1.1.3.1 General characteristics of the U937 cell line 22
    1.1.3.2 The switch from the proliferative to the non-proliferative state of U937 cells during differentiation 23
    1.1.3.3 U937 cells as a model for monoblastic differentiation 24
  1.1.4 Differentiation of the U937 cell line 25
    1.1.4.1 PMA-induced differentiation of U937 cells 25
    1.1.4.2 Retinoic acid- and 1α,25-dihydroxycholecalciferol-induced differentiation of U937 cells 26
    1.1.4.3 Cytokine-induced differentiation of U937 cells 27
1.2 The c-fgr proto-oncogene 30
  1.2.1 The c-src family of non-receptor protein tyrosine kinases 30
    1.2.1.1 Structural features 30
    1.2.1.2 Regulation of kinase activity 41
    1.2.1.3 Expression and function of the Src family proteins 45
    1.2.1.4 Signal transduction mechanisms involving Src family proteins 53
1.2.1.5 C-src gene family transcription 61
1.2.1.6 Post transcriptional modification of c-src family genes 63
1.2.1.7 Conclusions 64
1.2.2 Structure, expression and function of the c-fgr proto-oncogene 64
  1.2.2.1 Isolation of the c-fgr gene 64
  1.2.2.2 Structure of Fgr 64
  1.2.2.3 Expression of c-fgr mRNA and its protein product Fgr 65
  1.2.2.4 Function of Fgr in myeloid cells 69
1.2.3 Regulation of c-fgr gene expression 71
1.3 The retinoid X receptors 75
  1.3.1 Isolation of the retinoid X receptors 75
  1.3.2 Retinoid metabolism 79
  1.3.3 Structure/function relationships of the nuclear receptor superfamily 79
    1.3.3.1 DNA binding 79
    1.3.3.2 Ligand binding 84
    1.3.3.3 Receptor dimerisation 85
    1.3.3.4 The autonomous transactivation functions AF-1 and AF-2 88
    1.3.3.5. Interactions with non-receptor proteins 88
  1.3.4 Expression of retinoid X receptors in foetal and adult tissues 89
  1.3.5 Expression of retinoid X receptors in haemopoietic cells 91
1.4 Aims of the thesis 95

Chapter 2 Materials and methods 96
  2.1 Screening a genomic library 97
    2.1.1 Primary screen 97
    2.1.2 Secondary screen 98
  2.2 Restriction mapping of cosmid clones 99
  2.3 DNA probes 99
  2.4 Oligo-labelling of DNA 103
  2.5 Sub-cloning of DNA fragments 103
2.6 Production of competent *E. Coli* cells

2.7 Transformation of competent *E. Coli* cells

2.8 DNA sequencing

2.9 Plasmid preparation
   - 2.9.1 Small scale plasmid preparation
   - 2.9.1 Large scale plasmid preparation

2.10 Culture of U937 cells

2.11 Electroporation of U937 cells

2.12 Estimation of electroporation efficiency

2.13 Chloramphenicol acetyl transferase assay

2.14 Luciferase assay

2.15 Isolation of mRNA from U937 cells

2.16 Northern blotting and hybridisation

2.17 Synthesis of RNA probes

2.18 Establishment of stably transfected U937 cells

2.19 Cell proliferation assay

2.20 Receptor binding assays
   - 2.20.1 Saturation assay
   - 2.20.2 Displacement assay

2.21 Analysis of cell surface antigens by flow cytometry

Chapter 3 Regulation of c-fgr expression in U937 cells during differentiation

3.1 Results
   - 3.1.1 Isolation of a cDNA clone containing exon M4
   - 3.1.2 Isolation of genomic clones containing exon M4
   - 3.1.3 Restriction digest mapping of genomic clones c1 and c3
   - 3.1.4 Sequencing of exon M4
   - 3.1.5 Promoter analysis using a chloramphenicol acetyl transferase reporter gene
   - 3.1.6 Promoter analysis using a luciferase reporter gene
3.2 Discussion

3.2.1 Isolation and mapping of exon M4-containing cosmid clones 150
3.2.2 Sequencing of the c-fgr myeloid promoter 151
3.2.3 The identification of PMA-responsive regions of the c-fgr promoter 152
3.2.4 D3 and TNFα do not induce c-fgr promoter-luciferase activity 155

Chapter 4 The role of the retinoid X receptors in U937 cells during differentiation

4.1 Results

4.1.1 Analysis of RXRα and RXRβ expression during differentiation of U937 cells 158
4.1.2 Construction of RXRα and RXRβ expression plasmids
   pMEP-RXRαA, pMEP-RXRαS and pMEP-RXRβA 158
4.1.3 Production of pooled transfectant U937 cells and isolation of clones 169
4.1.4 Preliminary cell proliferation assays 171
4.1.5 Characterisation of RXRα expression in U937 clones expressing
   sense RXRα mRNA (αG2S) and antisense RXRα RNA (αB5A) 180
   4.1.5.1 Northern hybridisation analysis 180
   4.1.5.2 Receptor binding assays 180
   4.1.5.3 Transient transfection of a luciferase-retinoic acid response element reporter construct 184
4.1.6 Phenotypic characterisation of transfectant U937 clones expressing
   sense RXRα mRNA (αG2S) and antisense RXRα RNA (αB5A) 187
   4.1.6.1 Cell proliferation assays 187
   4.1.6.2 Analysis of the expression of the β2-integrins CD11a, CD11b, CD11c and CD18 196

4.2 Discussion

4.2.1 RXRα and RXRβ mRNAs are expressed in U937 cells 200
4.2.2 Production and characterisation of stably transfected U937 cells 201
4.2.3 RXRα is involved in the proliferative response of U937 cells 200
to RA and D3 206

4.2.4 RXRα influences the expression of the β2-integrins 210

**Chapter 5** Conclusions and future objectives 212

5.1 Control of c-fgr gene expression during U937 cell differentiation 212

5.2 Regulation of gene expression by RXRα during U937 cell differentiation 213

**Appendices** 217

1 Abbreviations 218

2 Solutions 222

3 Cloning strategies 226

4 Transfectant cell lines 244

5 Publications arising from this thesis 245

**References** 246
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To the memory of my father.
List of Tables

1.2.1 Predicted protein structure and chromosomal location of c-src family genes  31
1.2.2 Transgenic mice with targeted disruption of src family genes (and csk), and a summary of the effects 46
1.2.3 Members of the c-src gene family whose expression is restricted to haemopoietic lineages 50
1.2.4 Homology of predicted human c-fgr amino acid sequence of the unique N-terminal domain (amino acids 1 to 75) and the remaining molecule (76 to 529) with other c-src family members 66
1.3.1 DNA binding domain and ligand binding domain sequence homologies of human RXRα with other nuclear receptors 78
3.1.1 Table of putative transcription factor consensus binding sequences found in the c-fgr myeloid promoter region 133
3.1.2 Representative c-fgr promoter analysis experiment 144
4.1.1 Transfected cell pools and related clones used in experiments 170
4.1.2 Mean fluorescence intensity and percent positive expression of the β2-integrins CD11a, CD11b, CD11c and CD18 in MEP, αG2S and αB5A cells in the absence or presence of 1μM 9-cis RA 198
List of Figures

1.1.1 Schematic representation of myeloid development and the relative positions of differentiative block of U937 and HL60 cells 19
1.2.1 General structure of members of the Src family of protein tyrosine kinases 32
1.2.2 The N-terminal region of Src family proteins and Ga subunits 35
1.2.3 Structure of the catalytic domain of Src (a), and amino acid sequences of each domain (b) 37
1.2.4 Proteins that contain SH2, SH3 or SH1 (tyrosine kinase) domains 39
1.2.5 Conformational changes of repressed and activated Src protein by phosphorylation, and energy profiles of the conformational states 43
1.2.6 Model for the interaction of Src protein with the PDGF-R 55
1.2.7 General structure of the T-cell antigen receptor, B-cell antigen receptor, FcεRI and FcγRIII 58
1.2.8 Putative pathways of T-cell antigen receptor signalling involving protein tyrosine kinases Lck, Fyn, Csk and ZAP-70 60
1.2.9 Phylogenetic trees of the c-src gene family of tyrosine kinases 67
1.2.10 Differential splicing of c-fgr RNA in myeloid cells and EBV transformed B-lymphocytes 74
1.3.1 Chemical structures of all-trans retinoic acid and 9-cis retinoic acid 77
1.3.2 General structure of RXRα 81
1.3.3 Schematic representation of the RXRα DNA binding domain 82
1.3.4 Variously spaced response element/dimer interactions involving RXR, RAR, VDR, TR and ligands required to activate DNA-bound complexes 83
1.3.5 Model for receptor dimerisation via DNA binding domains, and transactivation via AF-1, AF-2 and non-receptor proteins 86
2.1 Specificity of probes 1 to 6 101
3.1.1 Representative autoradiograph from the primary screen 119
3.1.2 Representative autoradiographs from the secondary screen 120
3.1.3 Restriction digests of cosmid clone isolates II (c1), III, I, 2I, 2II, 2III and 3I (c3)

3.1.4 Restriction digest of cosmid c1 (a), and hybridisation to Probe 1 (b)

3.1.5 Restriction digest of cosmid c1 (a), and hybridisation to Probe 6 (b)

3.1.6 Restriction digest of pSK-Eco4.5 (a), and Southern hybridisation of the AccI digest with Probe 6 (b)

3.1.7 The relative overlaps of c3, cF2.3 and c1 (a), and a restriction map of c3 (b)

3.1.8 Restriction digest of cosmid clones c1, cF2.3 and c3, indicating the BamHI fragment that was cloned into pB1-1

3.1.9 Restriction digest (a) and Southern hybridisation (b) of pB1-1 to Probe 1

3.1.10 Diagram of the insert in pB1-1, showing the location of promoter binding sites used for sequencing

3.1.11 The sequence of the c-fgr promoter region (+124 to -1688), including the sequence of exon M4

3.1.12 Diagram of pGCAT constructs

3.1.13 Representative autoradiograph showing CAT activity in transfected U937 cells following 48 hour treatment with 10ng/ml PMA

3.1.14 Luciferase activity in U937 cells transfected with pGL-2basic, pGL-BSK and pGL-2control, following 48 treatment with 10ng/ml PMA

3.1.15 Restriction digest of positive isolates (during cloning of pGL-BSK) with Xhol showing correct construct

3.1.16 Diagram of pGL constructs

3.1.17 Basal luciferase activity in transfected U937 cells (a), and induction of luciferase activity following 48 hour treatment with PMA (b)

3.1.18 Fold induction of luciferase activity in transfected U937 cells following 48 hour treatment with 10ng/ml PMA

3.1.19 Representative experiment showing the lack of induction of luciferase activity pGL-772 following 48 hour treatment with 10ng/ml PMA

3.1.20 Luciferase activity in transfected U937 cells following 48 hour treatment
with 10ng/ml TNFα in combination with 100nM D3

3.2.1 Rare c-fgr mRNA myeloid transcripts

4.1.1 Northern blot analysis of the time-dependent expression RXRα and RXRβ mRNA in U937 cells following treatment with 10ng/ml PMA

4.1.2 Graphic representation of RXRα and RXRβ mRNA, determined by densitometry

4.1.3 Map of pMEP-4

4.1.4 The gene maps of plasmids pSK(7-3)-RXRα, pSK(3-7)-RXRα and pSK-RXRβ

4.1.5 Confirmation of the orientation of the RXRα cDNA insert in pSK(3-7)-RXRα and pSK(7-3)-RXRα by SacI digestion

4.1.6 Confirmation of the orientation of the RXRα cDNA insert in pMEP-RXRαS and pMEP-RXRαA by SacI digestion

4.1.7 Confirmation of the orientation of the RXRβ cDNA insert in pMEP-RXRβA by XhoI digestion

4.1.8 Proliferation assay of MEP, UαS and UαA cells following treatment with 1µM 9-cis RA, 1µM all-trans RA or 100nM D3 (a) and with 10µM CdCl2 (b)

4.1.9 Proliferation assay of MEP, UαS and UαA cells following treatment with 10ng/ml PMA or 100u/ml TNFα (a) and with 10µM CdCl2 (b)

4.1.10 Proliferation assay of MEP, and UαA clones αB5A, αD9A and αH6A upon treatment with 1µM 9-cis RA or 100nM D3

4.1.11 Proliferation assay of MEP, and UαS clones αG2S and αF7S upon treatment with 1µM 9-cis RA or 100nM D3

4.1.12 Proliferation assay of MEP and UβA upon treatment with 1µM 9-cis RA, 100nM D3 or 10ng/ml PMA

4.1.13 Proliferation assay of MEP, and UβA clone βF9A upon treatment with 1µM 9-cis RA or 100nM D3

4.1.14 Northern hybridisation of αG2S, αB5A and MEP cells, in the presence or absence of CdCl2, with a RXRα-specific probe
4.1.15 Representative displacement plots showing the inhibition of $^3$H-9-cis RA binding to MEP, αB5A and αG2S cells 183

4.1.16 Diagram showing the DR-5 RARE of the human RARβ2 gene promoter in pR140-luc 185

4.1.17 Luciferase activity in MEP, αB5A and αG2S cells transfected with pR140-luc and treated with 1µM 9-cis RA, in the absence or presence of CdCl2 186

4.1.18 Time-dependent growth of MEP, αB5A and αG2S cells 189

4.1.19 Dose-dependent proliferation of MEP and αB5A cells treated with 1µM 9-cis RA 190

4.1.20 Dose-dependent proliferation of MEP and αG2S cells treated with 1µM 9-cis RA 191

4.1.21 Dose-dependent proliferation of MEP and αG2S cells treated with 1µM all-trans RA 192

4.1.22 Proliferation of MEP and αB5A cells in the presence of all-trans RA and 100nM D3 (a). Dose-dependent proliferation of MEP and αG2S cells treated with D3 (b) 193

4.1.23 Proliferation of MEP, αB5A and αG2S cells treated with 0.1µM 9-cis RA and 100nM D3 alone or in combination 194

4.1.24 Proliferation of MEP, αB5A and αG2S cells treated with 30u/ml TNFα or 10ng/ml PMA 195

4.1.25 Flow cytometry of MEP, αB5A and αG2S cells, in the presence or absence or 9-cis RA, stained with antibodies for CD11a, CD11b, CD11c, CD18 and CD45 197

4.1.26 Change in mean fluorescence intensity and percentage of MEP, αB5A and αG2S cells, in the presence or absence or 9-cis RA, expressing CD11a, CD11b, CD11c, CD18 and CD45 199

6.1 Cloning strategy for the construction of pB1-1 227

6.2 Cloning strategy for the construction of pGCAT-113 228

6.3 Cloning strategy for the construction of pGCAT-359 and pGCAT-MR 229

6.4 Cloning strategy for the construction of pGCAT-772 230
<table>
<thead>
<tr>
<th>Section</th>
<th>Cloning strategy for the construction of pGL-BSK</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td></td>
<td>231</td>
</tr>
<tr>
<td>6.6</td>
<td>Cloning strategy for the construction of pSK-Eco4.5</td>
<td>232</td>
</tr>
<tr>
<td>6.7</td>
<td>Cloning strategy for the construction of pGL-128</td>
<td>233</td>
</tr>
<tr>
<td>6.8</td>
<td>Cloning strategy for the construction of pGL-343</td>
<td>234</td>
</tr>
<tr>
<td>6.9</td>
<td>Cloning strategy for the construction of pGL-752</td>
<td>235</td>
</tr>
<tr>
<td>6.10</td>
<td>Cloning strategy for the construction of pGL-772</td>
<td>236</td>
</tr>
<tr>
<td>6.11</td>
<td>Cloning strategy for the construction of pGL-911</td>
<td>237</td>
</tr>
<tr>
<td>6.12</td>
<td>Cloning strategy for the construction of pGL-1137</td>
<td>238</td>
</tr>
<tr>
<td>6.13</td>
<td>Cloning strategy for the construction of pGL-1211</td>
<td>239</td>
</tr>
<tr>
<td>6.14</td>
<td>Cloning strategy for the construction of pGL-1688</td>
<td>240</td>
</tr>
<tr>
<td>6.15</td>
<td>Cloning strategy for the construction of pGL-3200</td>
<td>241</td>
</tr>
<tr>
<td>6.16</td>
<td>Cloning strategy for the construction of pMEP-RXRαA and pMEP-RXRαS</td>
<td>242</td>
</tr>
<tr>
<td>6.17</td>
<td>Cloning strategy for the construction of pMEP-RXRβA</td>
<td>243</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
Chapter 1 Introduction

The aim of this thesis was to examine the cell and molecular biology of the myelomonocytic cell lineage using a monoblastic cell line, U937, as an in vitro model. The first objective was to examine regulation of the \textit{c-fgr} proto-oncogene which has been shown to be useful as a marker of the differentiated form. The second objective was to define the role of the retinoid X receptor-\(\alpha\) in the same system. The introduction will therefore cover three topics: the U937 cell line, \textit{c-fgr} proto-oncogene expression and function, and retinoid X receptor expression and function.
Chapter 1.1 The myeloid cell lineage

The development of a pluripotent haemopoietic stem cell in the bone marrow to generate a peripheral phagocytic tissue macrophage depends on several exogenous signals being delivered to the developing cell which influences gene expression at both mRNA and protein levels. The changes in gene expression within these cells govern both their proliferation and development, as with all forms of cellular differentiation, towards a mature phenotype. This section will give an overview of the myelomonocytoid lineage, from the point of view of haemopoietic stem cell development. Particular attention will be given to the monoblastic cell line U937, which is committed to monocytic differentiation that can be induced in vitro. The advantages and disadvantages of using U937 cells as a model of monoblastic differentiation will be considered.

1.1.1 Myeloid cell development

Haemopoietic stem cells, located in the bone marrow, liver and spleen of developing embryos and in the bone marrow of adults, give rise to a variety of lineages including cells which are the precursors of erythroid, megakaryocytoid, lymphoid and myeloid forms. The lineage towards which a stem cell will differentiate is determined by clonal proliferation, induced by glycoproteins such as the interleukins (IL) and colony stimulating factors (CSF). Myelopoiesis is mediated initially by granulocyte-erythrocyte-monocyte/macrophage CSF (GEMM-CSF), granulocyte/monocyte (GM)-CSF, monocyte (M)-CSF and multi-CSF. In addition, both IL-3 and IL-6 play a role in signalling differentiation toward mature phenotypes. The various CSFs involved act on developing cells in combination, rather than exclusively, where the relative combinations will influence the lineage-specific differentiation (reviewed by Metcalf, 1988).

Stem cell differentiation to a granulocyte/macrophage-colony forming unit (CFU-GM) is followed by differentiation to a monoblast, promonocyte and monocyte (Figure 1.1.1).
Figure 1.1.1. Schematic representation of myeloid development and the relative positions of differentiative block in U937 and HL60 cells.
This process occurs within the adult bone marrow over approximately six days after which the monocyte enters the blood stream (reviewed by van Furth, 1992). From the blood stream, monocytes may then enter tissue following adhesion to vascular endothelium, either randomly or in response to inflammatory stimuli (see section 1.1.2), where they differentiate to tissue macrophages, signalled by cytokines such as tumour necrosis factor-α (TNFα) or IL-1. Macrophages can still undergo a further stage of differentiation whereby they form multinucleated giant cells, signalled by interferon-γ (IFNγ) (reviewed by Johnston, 1988).

1.1.2 Monocyte/macrophage function

Human monocytes have a half-life of about 3 days (18 hours in mice) in the blood stream. The number of cells adhering to endothelial cells, that is the marginating pool, is not known in humans, but 60% of all murine monocytes are marginated. Interaction with vascular endothelium is mediated by a variety of surface receptors on the monocyte that bind to extracellular matrix proteins and integrin molecules (reviewed by Springer, 1990). Upon entering tissue, the monocyte differentiates into a tissue-type macrophage. For example, in the liver the cell is observed as a Kupffer cell, in lung tissue as an interstitial or alveolar macrophage, in bone as an osteoclast, and in brain tissue as a microglial cell.

A significant step in macrophage function is its activation in the tissue from a resting state. The precise function of this activation differs between tissues and experimental systems. For example, peritoneal macrophages have been used for murine studies where agents have been injected in vivo to induced activation while others have injected these agents to study mechanisms of recruitment rather than activation (see below).

One key property of monocytes and macrophages is their relative facility to adhere to other cells such as lymphatic or vascular endothelium, and to extracellular proteins such as laminin and fibronectin. This property was defined as a feature of the monocyte/macrophage in vitro as well as in vivo by Pawlowski et al. (1985) and (1988). It is now known that this is achieved via a series of adhesion receptors expressed on the
surface of monocyte/macrophages. Adhesion is also required for many monocyte/macrophage functional properties including phagocytosis of large particles, cytotoxicity, chemotaxis, tissue recruitment and production of inflammatory mediators. There are three families of cell surface proteins that mediate adherence: the integrins, the immunoglobulin gene superfamily, and the selectins (reviewed by Springer, 1992). The integrins are large heterodimeric glycoproteins responsible for cell-cell and cell-matrix interactions. They are classified into subfamilies by a common β subunit. β1 comprises of a β-chain (CD29) and a series of possible α-chains (CD49a to CD49f) which represent the receptors VLA-1 to 6. β2 comprises CD18 with either CD11a (LFA-1), CD11b (Mac-1) or CD11c (p150/95). There are also β3 (cytoadhesins) and β7 subfamilies. β1 and β3 integrins preferentially bind collagen, laminin, fibronectin and vitronectin, whereas β2 integrins mainly mediate cell-cell interactions. CD11a/CD18 recognise immunoglobulin family receptor molecules such as ICAM-1 (CD54), ICAM-2 (CD102) and ICAM-3. The selectins, comprising L-selectin (CD62L), P-selectin (CD62P) and E-selectin (CD62E), are involved in leukocyte-endothelium interactions including rolling of granulocytes along vascular endothelium. Selectins recognise oligosaccharides such as Le^X (CD15) and sialyl Le^X (CD15s) (Prieto et al., 1994 and references therein).

Prieto et al. (1994) performed extensive analysis of adhesion receptor expression by peripheral blood monocytes, tissue macrophages and the myeloid cell lines U937, THP-1, KG-1 and HL60. Their results showed that monocytes abundantly express adhesion molecules, in particular, the integrins β1 and β2. Intracellular pools of β2 integrin (CD11a/CD18) are also activated following stimulation of these cells with the chemotactant formyl-Met-Leu-Phe (fMLP)(Spertini et al., 1992). Comparison of blood monocytes with in vitro differentiated macrophages and alveolar macrophages showed there to be down regulation of two of the three forms of CD11 (CD11a and CD11b) and CD18 following differentiation from monocyte to macrophage (Spertini et al., 1992). Similar down regulation from monocyte to alveolar macrophage was seen for the β1 integrins, as well as ICAM-2, L-selectin, CD15 and CD15s. It would seem likely that
heterogeneous expression of adhesion molecules by macrophages is associated with the tissue in which they are expressed. For example, alveolar macrophages express less CD11b than peritoneal macrophages, which do not express CD11a (Strassmann et al., 1985).

Of the four cell lines, differentiated by phorbol ester (PMA) treatment, studied by Prieto et al. (1994), none displayed all the changes to adhesion molecule expression observed when monocytes develop into macrophages. Untreated U937 cells, the most mature of the cell lines studied, bore the closest resemblance to blood monocytes. It was suggested that phorbol ester-induced differentiation of these cell lines is more relevant to monopoiesis than to the monocyte to macrophage differentiation phase.

The importance of β2 integrins is demonstrated in patients with β2 deficiency whose monocytes show defective migration in response to fMLP and fail to enter sites of infection. They also have scarring of ulcerated cutaneous infections, supporting the role of monocyte/macrophages in tissue repair (Wahl and Wahl, 1992).

1.1.3 The U937 cell line

The U937 cell line an example of a monoblastic cell line that is committed to monocyte differentiation and serves as a useful model to study aspects of this differentiation as well as monocyte/macrophage function (Figure 1.1.1). The enormous quantity of literature concerning the U937 cells reflects the value of this cell line. Discussion of U937 cells here, and where relevant the acute myelomonocytic cell line HL60, will be restricted to details concerning treatment of these cells with phorbol esters, cytokines, retinoic acid (RA) and 1α,25-dihydroxycholecalciferol (D3).

1.1.3.1 General characteristics of U937 cells

Originally isolated from a patient with diffuse histiocytic lymphoma (Sunderström and Nilsson, 1976), U937 cells proliferate in suspension culture in the presence of growth
factors supplied by foetal calf serum. Figure 1.1.1 shows the myeloid cell lineage and the point of maturation arrest associated with the U937 cell line. U937 cells, when growing normally, have a doubling time of between 20 and 48 hours, and express few Fc, C3 or chemotactic peptide receptors (Sunderström and Nilsson, 1976). Induction of differentiation can be achieved by incubating cells with a wide range of agents such as phorbol ester 12-myristate 13-acetate (PMA), cytokines, RA and D3 (Forseck et al., 1985; Ralph et al., 1983; Olsson and Breitman, 1982; Amento et al., 1983).

1.1.3.2 The switch from the proliferative to the non-proliferative state of U937 cells during differentiation

Following treatment of U937 cells with differentiating agents, changes in protein levels occur that are associated with the shift from the proliferative to the non-proliferative state. Such proteins include Myc, Max, Mad, GTA, Myb, Fos and Jun (discussed in section 1.1.4.1). Myc, the protein product of the c-myc gene, has been shown to dimerise in vitro with the protein Max and binds DNA as a heterodimer at specific binding sites to activate transcription of target genes. Max may also heterodimerise with Mad, and as such, will bind DNA and function as a repressor of transcription (reviewed by Marcu et al., 1992).

After PMA treatment of U937 cells, levels of Myc are seen to decline whilst Mad levels increase. These changes broadly correlate with cessation of cell proliferation. More specifically, the ratio of Myc-Max heterodimers to Mad-Max heterodimers is seen to switch from the former to the latter as differentiation progresses to the extent that no Mad-Max dimers are detected in untreated U937 cells and no Myc-Max dimers are detected 48 hours after PMA treatment (Ayer and Eisenman, 1993).

The tyrosine kinase GTA has been associated with cell cycle control, having a 46% sequence homology with the human cell cycle control gene, cdc2. PMA treatment of U937 cells results in increased GTA protein levels as well as its tyrosine kinase activity. GTA is thought to influence cell cycle control through phosphorylation of the golgi
enzyme β-1,4-galactosyltransferase whose expression also fluctuates throughout cell cycle (Kraft et al., 1992).

Nguyen et al. (1993) studied the role of the transcription factor Egr-1 in monoblastic differentiation. Egr-1 is expressed in U937 and HL60 cells treated with PMA, although not expressed in HL60 cells differentiated towards a granulocyte. The importance of this apparent macrophage-specific transcription factor was shown by the observation that antisense Egr-1-specific oligodeoxynucleotides blocked monocytic differentiation. Furthermore, enforced expression of Egr-1 in HL60 cells blocked their ability to undergo granulocytic differentiation. It was noted that Egr-1 transfected HL60 cells expressed 50-fold more c-myc than normal HL60 cells. Nguyen et al. (1993) suggested that since Myb can transactivate c-myc, constitutive expression of Egr-1 blocks granulocytic differentiation through activation of c-myc. Normal c-myc expression in HL60 cells differentiated towards granulocytes rapidly decreases to nothing within 3 to 4 hours, unlike that seen in HL60 cells differentiated towards monocytes where expression is still observed after 6 hours (also observed in Egr-1 transfected cells). These results show that Egr-1 plays an important role in restricting a multipotent stem cell to a specific monocytic lineage.

1.1.3.3 U937 cells as a model for monoblastic differentiation

The expression of adhesion receptors by U937 cells resembles expression in blood monocytes but does not match changes in expression following differentiation into tissue macrophages (Prieto et al., 1994). When cells are induced to differentiate with PMA they acquire a macrophage morphology, adherent properties, expression of Fc receptors, antibody-dependent cellular cytotoxic activity (ADCC), the ability to phagocytose, and produce lysozyme and alkaline phosphatase (Harris and Ralph, 1985). Despite the length of time that has elapsed since isolation of this cell line, the properties have remained sufficiently stable with respect to differentiation potential, and thus U937 cells are still
used in experiments which can be extrapolated to devise basic models of normal monocyte/macrophage function *in vivo*.

### 1.1.4 Differentiation of U937 cells

#### 1.1.4.1 PMA-induced differentiation of U937 cells

PMA activates protein kinase C (PKC) which in turn leads to further downstream signal transduction pathways. One pathway leads to activation of the transcription factor NFκB. In its inactive state, NFκB is bound to its inhibitor protein, IκB. Phosphorylation of IκB leads to their dissociation allowing NFκB to translocate to the nucleus to activate transcription of target genes (Baeuerle and Baltimore, 1988). PKC activation also stimulates the activity of the AP-1 binding proteins Fos and Jun. Pulverer *et al.* (1993) observed increased AP-1 binding with 15 minutes of PMA treatment of U937 cells. Jun was also phosphorylated at specific regulatory serine residues at the N-terminus DNA binding domain. This phosphorylation was attributed to the activity of mitogen activated protein kinase (MAPK), itself activated as a result of serine/threonine phosphorylation events that begin with PKC phosphorylation of Raf-1. Furthermore, it was also demonstrated that GSK-3b, which represses Jun activity by C-terminal phosphorylation of Jun, was itself inhibited by PKC. In addition to PKC stimulation, other pathways are also activated. For instance, PMA down regulates phospholipase Cγ-1 (PLCγ-1) expression, correlating with growth arrest. Moreover, PLCγ-2 levels remain constant suggesting that this isoform functions in differentiated U937 cells (Lee *et al.*, 1995).

Other significant effects of PMA treatment of U937 cells include the synthesis of cytokines such as IL-1α, TNFα and CSF-1 (Knudsen *et al.*, 1986; Liu and Wu, 1992); adherence to plastic; increased expression of cyclo-oxygenase (required for prostanoid production), increased expression of CD18 and ICAM-1; production of lysozyme and oxygen radicals (reviewed by Harris and Ralph, 1985). Pretreatment with glucocorticoids prior to PMA induction results in cells that, although growth arrested, no longer display the above characteristics (Hoff *et al.*, 1992).
1.1.4.2 Retinoic acid- and 1α, 25-dihydroxycholecalciferol-induced differentiation of U937 cells.

RA, either as all-trans RA or 9-cis RA (see section 1.3), will induce partial growth arrest and differentiation of U937 cells (Bhalla et al., 1989). RA is required for LPS-stimulated induction of IL-1β and TNFα synthesis (Taimi et al., 1993), and has also been shown to down regulate expression of the TNF receptor isoforms p60 and p80 in U937 cells (Totpal et al., 1995). For these reasons it is thought that RA will induce cells to differentiate to an intermediate stage of development, distinct from a monocyte that requires further co-stimulatory signals to achieve differentiation. Treatment of U937 cells with the active metabolite of D3, 1α,25-dihydroxy-D3, has a growth arresting effect, more profound than RA, and induces the accumulation of IL-1β and IL-6 mRNA. However, protein levels of these cytokines do not increase unless co-stimulated with LPS. The high affinity receptor for D3, VDR (see section 1.3), is upregulated in U937 cells by D3 and PMA (Hewison et al., 1989a). Also, D3 has an autocrine effect on its own activity whereby D3 increases the activity of 24-hydroxylase, the enzyme responsible for converting D3 from the active 1α,25-dihydroxy-D3 form to the inactive 24,25-dihydroxy-D3 form. Conversely, PMA will increase the activity of 1α-hydroxylase, responsible for producing the less active 1,24,25-D3 form (Hewison et al., 1989b). Taken together, these effects show a specific developmental control of D3 metabolism during monopoiesis.

The synergistic effects of D3 with other agents have been established in U937 cells in a number of instances including TGFβ and GM-CSF (Testa et al., 1993; Zuckerman et al., 1988). RA and D3 used in combination will induce levels of cytokine secretion comparable with those seen for D3 with LPS (Taimi et al., 1993). Co-operation between RA and D3 has also been observed in HL60 cells leading to monocytic differentiation (Brown et al., 1994). The differential effect of RA and D3 on U937 cells is exemplified by the expression of CD11b and CD14, where D3 induces expression of both antigens.
and RA only induces expression of CD11b (Sellmayer et al., 1994). Either of these treatments, in combination with IFNγ, induces CD11b expression, although RA with IFNγ still failed to induce CD14 expression. Zhang et al. (1994) determined that the CD14 gene promoter region -128 to -70, with respect to the transcription start site, contains a D3-responsive element. Although it did not include a consensus D3 response element (VDRE, see section 1.3) it did possess two Sp-1 binding sites required for activation of this promoter in response to D3. D3 and RA also have antagonistic effects on the expression of CD14 and the Fc receptor for IgE, CD23. PMA and RA will induce U937 cell expression of CD23, while PMA and D3 (but not RA) can induce CD14 expression. Öberg et al. (1993) demonstrated that D3 suppresses CD23 expression in RA- and PMA-induced U937 cells, while RA inhibited D3- and PMA-induced CD14 expression. Such antagonistic roles are reflected in the differential response of HL60 cells to D3 and RA leading to respective monocyte/macrophage and granulocyte differentiation lineages. These studies have parallels in animal models. Chick yolk sac macrophages, grown in primary culture, have the ability to differentiate towards osteoclasts by first becoming large multi-nucleated giant cells (MNGC). RA promotes proliferation of these macrophages while D3 promotes formation of MNGCs. Furthermore, RA strongly inhibits D3's ability to induce MNGC formation (Woods et al., 1995). These various data indicate the importance of D3 and RA in being able to signal different monocyte/macrophage activities, depending on tissue localisation and/or cell-cell interactions.

1.1.4.3 Cytokine-induced differentiation of U937 cells

Most attention has been paid to the effects of cytokines on U937 cells. As discussed above, these immunomodulatory proteins influence the lineage-specific development of haemopoietic cells as well as function in the activation of monocyte/macrophages. IFNγ was identified as having differentiating effects on U937 cells leading to several macrophage-like characteristics. In contrast, CSF-1 had no differentiating effect but was growth stimulatory (Harris et al., 1985). Treatment of U937 cells with GM-CSF resulted
inhibition of proliferation and induction of TNFα production and secretion (Cannistra et al., 1987). The presence in the medium of secreted TNFα does not account for the growth inhibitory effect of GM-CSF since addition of anti-TNF antibodies does not prevent this effect. TNF treatment of U937 cells results in 50% growth inhibition at 1pM concentrations (Peetre et al., 1986), and signals its effect via two possible isoforms of TNF receptor, p60 and p80, which mediate distinct cellular responses through different intracellular domains. It has recently been shown that the p60 isoform engages neutral sphingomyelinase (SMase) activating the serine/threonine kinases, Raf-1, and the mitogen-activated protein kinase (MAPK) signalling pathway (Belka et al., 1995; Winston et al., 1995).

As described above, the synergistic effect of cytokines with D3 has a more profound effect on U937 differentiation than with cytokine alone. The combination of D3 and TGFβ leads to monocytic differentiation in U937 and HL60 cells. Pretreatment of cells with TGFβ followed by D3 treatment has a similar effect, whereas D3 pretreatment does not (Testa et al., 1993). A similar effect was observed for both GM-CSF and IFNγ in combination with D3, with enhanced anti-proliferative effects and increased expression of differentiation markers (Zuckerman et al., 1988; Kelsey et al., 1993). It should be noted that in combinational experiments, concentrations of D3 are at low levels (10^{-8}M or less) rather than at high levels (100nM) (Zuckerman et al., 1988; Testa et al., 1993). At low concentrations, D3 has little effect on U937 or HL60 cell proliferation or differentiation but is sufficient to elicit a strong response in the presence of co-stimulatory factors.

To summarise, the derivation of heterogeneous populations of monocytes and macrophages in the body appears to be a result of numerous co-stimulatory events that involve D3, RA, LPS, and cytokines. The control of these agent's production and activity, mediated by cell-cell interactions and/or tissue micro-environment, appears to guide
monocyte/macrophage development to the appropriate mature phenotype via adhesion receptors (for tissue-specific recruitment) and functional activation.

The studies described above have used the U937 cell line as a model for studying the biology of monocyte/macrophage development and activity. The large quantity of research that has utilised this cell line is a testament to the usefulness of U937 cells. For this reason the U937 cell line was used in this thesis.
1.2 The c-fgr proto-oncogene

This section gives an overview of the c-src family of protein tyrosine kinases, of which c-fgr is a member, before discussing specific features of c-fgr expression and function. The intention is to illustrate the structural and functional importance of this family within mammalian systems in general and thus provide a background against which the function of c-fgr in monocytes and macrophages can be more clearly understood.

1.2.1 The c-src family of non-receptor protein tyrosine kinases

In 1911 Peyton Rous reported that a cell-free extract of a fibrosarcoma could transmit the tumour between chickens. The transmittable agent later proved to be a virus, the Rous Sarcoma Virus (RSV), which was shown to encode a viral oncogene v-src. The cellular homologue of v-src is the proto-oncogene c-src, and this became the first of a number of structurally related proto-oncogenes to be isolated to make up a family currently consisting of nine members: c-src, c-fgr, c-yes, fyn, lyn, lck, hck, blk and yrk. The chromosomal location of these genes and their predicted protein product are shown in Table 1.2.1. The cDNAs of this gene family have been isolated through homologous sequence identity of their structural regions. The importance that Src family members play in cell signalling has become apparent through the wide range of tissues where they are expressed, as well as the number of other proteins with which they can interact. This is a consequence of the nature of their structure which facilitates multiple varieties of protein-protein interactions.

1.2.1.1 Structural features

The amino acid sequences of the proteins encoded by the c-src gene family have been predicted from the nucleotide sequence of the corresponding cDNA clones. The basic structure of the Src protein is shown in Figure 1.2.1. Each protein possesses a unique region of 60-90 amino acids from the N-terminal, and sharing 60 to 70% homology over
Table 1.2.1. Predicted protein product and chromosomal location of c-src gene family members in humans. References: Drebin et al., 1995; Dracopoli, et al., 1988; Popescu et al., 1987; Quintrell et al., 1987; Perlmutter et al., 1988a; Yamanashi et al., 1987.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein product</th>
<th>Chromosomal localisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>blk</td>
<td>p55blk</td>
<td>8 p22-p23</td>
</tr>
<tr>
<td>c-fgr</td>
<td>p55c-fgr</td>
<td>1 p31-pter</td>
</tr>
<tr>
<td>fyn</td>
<td>p59fyn(B), p59fyn(T)</td>
<td>6 q21</td>
</tr>
<tr>
<td>hck</td>
<td>p59hck</td>
<td>20 q11-q12</td>
</tr>
<tr>
<td>lck</td>
<td>p56lck</td>
<td>1 p32-p35</td>
</tr>
<tr>
<td>lyn</td>
<td>p53lyn, p56lyn</td>
<td>8 q13-pter</td>
</tr>
<tr>
<td>c-src</td>
<td>p60c-src (3 isoforms)</td>
<td>20 q13.3</td>
</tr>
<tr>
<td>c-yes</td>
<td>p62c-yes</td>
<td>18 q21.3</td>
</tr>
<tr>
<td>yrk</td>
<td>p60yrk</td>
<td>not determined.</td>
</tr>
</tbody>
</table>
Figure 1.2.1. General structure of members of the Src family of protein tyrosine kinases.
its remaining length. The conserved regions consist of three domains of which homologues are found in a range of other functionally related genes. The C-terminal Src homology 1 (SH1) domain is a catalytic domain that acts as a tyrosine kinase, capable of phosphorylating tyrosine residues on other proteins as well as auto-phosphorylating its own tyrosine residues (Y-416 in Src). The SH2 domain is capable of binding with other tyrosine-phosphorylated proteins as well as with a phosphotyrosine at its own C-terminal. The SH3 domain can facilitate interactions with proline-rich regions of other proteins.

Two conserved tyrosine residues (Y-416 and Y-527 in p60^{S src}) are the targets for phosphorylation. Y-416 lies within the catalytic domain and can be auto-phosphorylated. Y-527 lies at the C-terminus of the protein and is involved in the regulation of kinase activity.

Several of the src gene family members encode more than one protein product as a result of post-translational modification. Three variants of p60^{S src} (Src), differing in their SH3 domain, have been identified (Pyper and Bolen, 1990). Two variants of p59^{lyn} (Fyn), which differ in their catalytic domain, are found either in the brain (the B-isoform) or in T-cells (the T-isoform)(Cooke and Perlmutter, 1989). Variation in the unique region of the lyn protein product (Lyn) gives rise to two isoforms, p53^{lyn} and p56^{lyn} (Stanley et al., 1991).

**The unique N-terminal region**

The N-terminal regions of Src family members are generally highly diverged with the exception of a conserved glycine at position 2. This glycine residue is responsible for the translocation of newly translated protein to the inner surface of the cell membrane. This is achieved by the post-translational addition of a 14 carbon saturated fatty acid, myristate, to G-2 (reviewed by Resh, 1994). Myristate is covalently bound to G-2 following the removal of the N-terminal initiator methionine. Mutation of G-2 to alanine or glutamate residues in Src prevents myristylation and the mutated protein is not translocated (Kamps
et al., 1986), but the kinase activity of the mutated protein is not affected (Buss et al., 1986). G-2 forms part of an N-terminal consensus motif: M-G-X-X-X-S/T (Resh, 1993). This motif has been found also in the il-, o- and z- forms of the α-subunit of G protein, itself associated with the inner surface of the cell membrane. Myristate alone is not sufficient to bind the associated molecule effectively to the cell membrane (Peitzsch and McLaughlin, 1993). However, as 30% of the total phospholipids of the membrane possess negatively charged head groups, there is an interaction with three arginine residues at 14 to 16 and three lysine residues at 5, 7 and 9 in Src (Zhou et al., 1994)(Figure 1.2.2).

Post-translational acetylation of p56\textsuperscript{Lck} (Lck) and Fyn on cysteine at residues 3 and/or 5/6 with a 16 carbon fatty acid, palmitate, has been observed (Paige et al., 1993; Shenoy-Scaria et al., 1993). A similar process is observed in Gα subunits, which is upregulated upon binding of agonist. Furthermore, mutation of G-2 within Gα subunits blocks both myristylation and palmitylation, suggesting that myristylation is a prerequisite for palmitylation, or that myristylation enhances accessibility to membrane-bound palmityl transferase. Shenoy-Scaria et al. (1993) demonstrated that palmitylation of Src proteins may enhance interactions with glycosyl phosphatidylinositol-linked proteins and further influence sub-cellular localisation.

A well documented N-terminal domain interaction is that of Lck with the cytoplasmic tail of the T-cell co-receptor CD4 (and CD8)(Shaw et al., 1989). This interaction involves CD4's N-terminal 32 amino acids, including two cysteine residues, with two cysteine residues of Lck (20 and 23): an interaction that is not dependent on myristylation (Turner et al., 1989).
**Figure 1.2.2.** The N-terminal region of Src family proteins and Gα subunits, showing myristate (myr) at G-2. Conserved residues in boxes, basic residues lysine (K) and arginine (R) (Resh, 1994).
The catalytic (SH1) domain.

The 256 amino acid catalytic domain of Src extends from 260 to 516 and is capable of phosphorylating its protein substrate on tyrosine residues. The domain is not uniformly conserved between other src family members but consists of spaced regions of homology which function in catalysis (Hanks et al., 1988)(Figure 1.2.3). These sub-domains, denoted I (at the N-terminal region of the SH1 domain) through to XI, possess consensus sequences that function as an ATP binding site (sub-domain I)(Sternburg and Taylor, 1984), phospho-transfer reaction via a conserved lysine residue (II)(Kamps and Sefton, 1986) and phosphate group interactions via two aspartate residues at 386 and 404 in Src and Mg\(^{2+}\) salt bridges (VI and VII)(Brenner, 1987). These same features are also found within the kinase domain of serine/threonine (S/T) kinases. However, sub-domains VI and VIII in tyrosine kinases differ from S/T kinases and suggests that they are involved in tyrosine specificity (Hanks et al., 1988).

Each member of the Src family possesses two highly conserved tyrosine residues. The C-terminal tyrosine (Y-527 of Src, Y-505 of Lck and Y-511 of Fgr) is a major target of phosphorylation in vivo and is involved in the regulation of kinase activity through interaction with the SH2 domain (see section 1.2.1.3). The viral oncogenes v-src, v-fgr, and v-yes all have mutations that result in the loss of this C-terminal tyrosine residue (Katamine et al., 1988; Takeya and Hanafusa, 1983; Sukegawa et al., 1987). Removal or replacement of this residue from Lck, Fyn, Hck or Src gives rise to proteins with elevated kinase levels and transforming activity (Amrein and Sefton, 1988; Kawakami et al., 1988; Perlmutter et al., 1988b; Kmiecik and Shalloway, 1987). Furthermore, the polyoma virus protein p58\(^{MT}\) has been shown to bind to the C-terminal of Src, Fyn or Yes immediately upstream of the tyrosine with the effect of preventing phosphorylation, a process that correlates with cell transformation (Courtneidge, 1985; Courtneidge and Smith, 1983; Cheng et al., 1988; Kornbluth et al., 1990). The crystal structure of the insulin receptor tyrosine kinase domain, which contains numerous highly conserved amino acid residues with other tyrosine kinases (including Src), revealed the importance...
Figure 1.2.3. (a) Structure of the catalytic domain of Src. Numbered black boxes represent subdomains. (b) Amino acid sequences of each domain, highly conserved residues are underlined (Hanks et al., 1988)
of the autophosphorylated tyrosines in the regulation of catalytic activity. Also indicated was the importance of arginine and lysine residues in the formation of salt bridges with phosphotyrosine (Hubbard et al., 1994). Phosphorylation of Y-527 is catalysed by a distinct family of non-receptor protein tyrosine kinases, of which p50cSk (Csk) was first identified by Nada et al. (1991) (see section 1.2.1.3).

Tyrosine 416 of Src (Y-400 of Fgr) is a target of autophosphorylation. The function of this is unclear since mutation of Y-416 results in only a small decrease in kinase activity of both v-Src (Cross and Hanafusa, 1983) and c-Src activated by Y-527 removal (Kmieciak and Shalloway, 1987; Piwnica-Worms, et al., 1987). A similar mutation of Lck, activated by Y-505 removal, does result in loss of kinase activity (Buss et al., 1986) and it is believed that this is due to loss of conformational changes that would normally allow access of substrates to the catalytic site (Hanks, et al., 1988). This suggests that Src and Lck differ by the level of regulation achieved by tyrosine phosphorylation at these two sites which may relate to their tissue-specific function.

The Src Homology 2 domain

The SH2 domain, like the SH3 domain (discussed below), has been found to be conserved in several proteins, particularly those involved in signal transduction (see section 1.2.1.4). These proteins include other tyrosine kinases such as ZAP-70, Csk, p150c-abl, p98c-fps, and proteins that lack tyrosine kinase domains such as the p85 subunit of phosphotidylinositol 3-kinase (PI 3-K), phospholipase Cγ-1 (PLCγ-1), ras GTPase activating protein (ras GAP) and Grb-2 (Figure 1.2.4). The presence of SH2 domains in functionally related proteins suggested that the SH2 domain might play a central role in their interactions. Indeed, SH2 domains have been found to bind with high affinity to phosphotyrosine-containing proteins. In fact, the SH2 domain will bind specifically to phosphotyrosine, although surrounding amino acids will confer a binding specificity. For example, the consensus binding site for the SH2 domain of the p85 subunit of PI 3-K is Y-X-X-M, whereas for the SH2 domain in Grb-2 it is Y-X-N-X.
Figure 1.2.4. Proteins that contain SH2 (white boxes), SH3 (grey boxes), or SH1 (tyrosine kinase) domains (hatched boxes).
An extensive analysis of SH2 recognition motifs was performed by Songyang et al. (1993). Using a phosphopeptide library they determined that the SH2 domains of Src, Fyn, Lck, Fgr, Abl, Crk and Nck preferred the motif Y-hydrophobic-hydrophilic-I/P whilst the SH2 domain of PI 3-K, PLCγ and SHPTP2 favoured Y-hydrophobic-X-hydrophobic. Src proteins all selected the sequence Y-E-E-I. As a result of the interactions of SH2 domains in protein tyrosine kinase mediated signalling pathways, it has become clear that they are involved in controlling the assembly of multimeric complexes (see section 1.2.1.4).

The crystal structure of the SH2 domains of Src, PI 3-K and Grb2 was determined by Waksman et al. (1992). Although the SH2 domains of these proteins have only 25 to 40% homology, there exist structural similarities. Specifically, the SH2 domain is a modular hemi-spherical structure where the flat surface contains a deep pocket. This will bind phosphotyrosine of an interacting protein, facilitating an induced fit, probably by rearrangement of arginines (R-155 and R-175 in Src) which opens the binding site (Waksman et al., 1992).

**The Src Homology 3 domain**

The SH3 domain, like the SH2 domain, is found in other signalling proteins (Figure 1.2.4). Smaller than the SH2 domain, the SH3 domain (residues 86 to 136 in Src) has been shown to interact with proline rich peptides and allow protein-protein interactions. Crystal structures of the SH3 domain of α-spectrin and solution structures of SH3 from Src, PI 3-K and PLCγ have shown conserved aromatic residues and variable loops (Musacchio et al., 1992; Noble et al., 1993; Yu et al., 1992; Booker et al., 1993; Koyama et al., 1993; Kohda et al., 1993). The Src and PI 3-K SH3 domains differ in structure from each other, with PI 3-K possessing a 15 amino acid insert not present in Src SH3. However both have ligand binding sites localised to the conserved aromatic residues that form a hydrophobic patch on the surface of the protein. The loops surrounding this area may confer ligand specificity. Yu et al. (1994) have found that
binding of proline-rich motifs does not result in conformational change. Rather, the SH3 domain represents a preformed template. Motifs found to bind the SH3 domain of v-Fgr and PI 3-K were R-P-R-P-L-P-P-P-T and P-P-R-P-L-P-V-A-P-G-S respectively (the highly conserved residues in bold type) deriving a consensus binding sequence X-P-Φ-P-P-X-P (where Φ is a hydrophobic residue). The proline residues appear to function as scaffolding or binding whereas non-proline residues such as arginine or leucine confer specificity (Yu et al., 1994).

Work by Abrams and Zhao (1995) using chimeric proteins to delete or interchange SH3 domains between Lyn, Blk and Src, revealed that Lyn, lacking its SH3 domain, had reduced kinase activity but was capable of transforming NIH 3T3 cells. This is in contrast to SH3-deleted Src, which had increased kinase activity, but could still transform 3T3 cells, suggesting that there is functional variation between the SH3 domains of Src and Lyn. Substitution of Lyn SH3 domains with either Src or Fyn SH3 showed increased in vitro kinase activity, whereas substitution with Blk SH3 had no effect. This was attributed to an activating arginine residue in the Fyn and Src SH3 domains that replaces an isoleucine of Lyn. The Blk SH3 domain, when placed into Lyn, resulted in a relatively conservative substitution of isoleucine to valine.

1.2.1.2 Regulation of kinase activity

For Src proteins to be an effective part of a cellular signalling mechanism it is crucial that their kinase activity be switched on or off at the appropriate time; constitutively active Src has been shown to be able to transform cells. The catalytic activity of Src proteins is controlled by their phosphorylation and dephosphorylation. This is most likely achieved by interactions with other proteins that possess kinase or phosphatase activity which would act on the regulatory C-terminal tyrosine residue. Phosphorylation of Src at Y-527 has the effect of repressing Src kinase activity by promoting an intramolecular interaction with the SH2 domain which physically masks the kinase domain. There is evidence, as described above, that the SH3 domain is also involved in stabilising this conformation.
The phosphorylation of Y-416 is associated with the activated state of the protein and would appear to be involved in stabilising the active conformation (Figure 1.2.5). In terms of free energy states the repressed protein can "breathe" between active and repressed states due to a small energy barrier. When neither Y-527 or Y-416 are phosphorylated there is assumed to be a semi-active state which is stabilised by Y-416 phosphorylation (reviewed by Cooper and Howell, 1993).

The importance of the specific regulation of Src proteins is underlined by the discovery of a family of protein tyrosine kinases that specifically phosphorylate Src proteins. Nada et al (1991) isolated a protein tyrosine kinase, Csk, which contains SH2, SH3 and tyrosine kinase domains and is capable of specifically phosphorylating Src at Y-527. Following this, it was shown that Lck and Fgr were also phosphorylated by Csk (Bergman et al. 1992; Ruzzene et al., 1994). The in vivo role of Csk has been clearly demonstrated in T-cells where over-expression of Csk in a murine T-cell line led to the down regulation of T-cell receptor (TCR)-induced tyrosine phosphorylation and the down regulation of lymphokine production, a process associated in part with Lck and Fyn (see section 1.2.1.4)(Chow et al., 1994). The directed mutation of Csk expression by production of a Csk-/- mouse (a so-called "knock out") (see Table 1.2.2) showed that there was a complete block of T-cell and B-cell differentiation (Gross et al., 1995). Csk-/- mouse embryos died after 10 days of gestation showing defects of the neural tube (associated with actin filament), dysfunction of cell matrix adhesion protein and cell-cell adhesion protein (Imamoto and Soriano, 1993; Nada et al., 1993). Kinase activity of Src and Fyn was greatly enhanced in cells derived from these mutant mice, although phosphorylation of the C-terminal Y-527 of Src was reduced but not eliminated (Imamoto and Soriano, 1993). Creation of Csk-/-Src-/- and Csk-/-Fyn-/- double knockouts by Thomas et al. (1995) showed the relative importance of unregulated Src and Fyn proteins in producing the lethal phenotype of Csk-/- mice. Csk-/-Src-/- mice were rescued from the lethal phenotype of the Csk-/- single mutant whereas Csk-/-Fyn-/- mice still died after 10 to 11 days of gestation.
Figure 1.2.5. Conformation changes of repressed and activated Src protein stabilised by phosphorylation of either tyrosine-527 (pY-527) or tyrosine-416 (pY-416), and energy profiles of the conformational states (Cooper and Howell, 1993).
Analysis of the tyrosine phosphorylation of specific proteins associated with cytoskeletal development showed that Csk regulated-Src was responsible for phosphorylation of cortactin, tensin, paxillin and Fak, while Fyn acted only on paxillin and Fak. Csk expression was not noted as being widespread through all tissues. This would suggest that only certain Src family members require a high level of precise regulation (supplied by Csk) to effect their correct function, such as Fyn and Lck in TCR signalling. Alternatively, other related Src-regulating tyrosine kinases exist which have yet to be cloned. In support of the latter argument, a homologue of the csk gene was isolated by McVicar et al. (1994) called lsk (expressing p57lsk). This is expressed in human brain, resting and activated NK cells, and activated T-cells. Also, Klages et al. (1994) reported the isolation of a brain-specific Src kinase gene in mice called ctk which is 53% homologous with murine csk and encodes a 52kDa protein product. Other Csk-like kinases include MATK, the human homologue of ctk, the human haemopoietically restricted HYL gene, and a murine brain and T cell-specific gene ntk. (Bennett et al., 1994; Sakano et al., 1994; Chow et al., 1994). Evidently there is now a growing family of Csk proteins which act in a tissue and/or target protein-specific manner.

In contrast to the phosphorylation of Src proteins, little is known concerning the dephosphorylation. The best known example of specific protein tyrosine phosphatase (PTPase) activity is the leukocyte common antigen co-receptor CD45. A partial amino acid sequence (PTP 1B) was isolated by Tonks et al. (1988) which had no homology to known serine/threonine (S/T) phosphatases. PTP 1B was then found to be homologous to CD45, which at that time had no known function. Although an intrinsic PTPase domain had previously been noted in functional assays, it became clear that cross-linking of CD45 to CD4/CD8 by MHC class II interaction (see section 1.2.1.4) physically brings CD4/CD8-associated Lck into proximity with the PTPase domains of CD45. This allows dephosphorylation of Lck at Y-505, which in turn activates Lck and allows interaction with the TCR (Mustelin and Altman, 1990; Ledbetter et al., 1988). A similar interaction between CD45 and CD5- or CD3- associated Fyn also occurs (Shiroo et al., 1992;
Burgess et al., 1992). Recently, it was shown that Csk can also phosphorylate CD45, activating its PTPase activity and generating a phosphotyrosine residue that can bind to the SH2 domain of Lck (Autero et al., 1994). This suggests that the regulatory circuit involving Csk, CD45 and Lck is more complicated than was previously thought.

The mechanism of phosphorylation and dephosphorylation of Y-416 remains unclear. Clearly it would be counter-productive if Csk were to phosphorylate this tyrosine, thereby favouring an active conformational state (see section 1.2.1.2). Likewise, PTPase mediated dephosphorylation of Y-416 would favour an inactive conformation.

1.2.1.3 Expression and function of Src family proteins

The pattern of src gene family expression can be divided in two groups: those that are ubiquitously expressed (c-src, c-yes, yrk and fyn) and those expressed solely in the haemopoietic cell lineages (c-fgr, blk, lck, hck and lyn). In a number of studies, the targeted disruption of specific src family genes in transgenic mice has been employed to help elucidate their function. A summary of these is shown in Table 1.2.2.

Ubiquitously expressed Src family proteins

Although Src is expressed in most cell types, attention has focused on a few cell types where expression is particularly high. Src is expressed at a high level in platelets, where it is localised to the plasma membrane and the surface connected canalicular system, although its function is unclear (Ferrell et al., 1990). The c-src gene is seen to be expressed in many tissues but is particularly abundant in the developing chick embryo and in the adult central nervous system (CNS). Src is localised to nerve growth cones where it has been suggested that it is involved in regulating neurite extension during contact-mediated guidance through neural development towards its target (Maness et al., 1988). Src mediated phosphorylation of α- and β- tubulin, proteins associated with growth cone membranes, supports this hypothesis (Maness and Matten, 1990).
Table 1.2.2. Transgenic mice with targeted disruption of *src* family genes (and *csk*), and summary of effects.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Effect on mouse</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-4 weeks postnatally; osteoclast dysfunction.</td>
<td></td>
</tr>
<tr>
<td>c-<em>yes</em></td>
<td>None</td>
<td>Grant <em>et al.</em>, 1992.</td>
</tr>
<tr>
<td>c-<em>fgr</em></td>
<td>None</td>
<td>Lowell <em>et al.</em>, 1994.</td>
</tr>
<tr>
<td>c-<em>fgr</em> and <em>hck</em></td>
<td>Susceptibility to <em>Listeria monocytogenes</em> infection.</td>
<td>Lowell <em>et al.</em>, 1994.</td>
</tr>
<tr>
<td><em>csk</em> and c-<em>src</em></td>
<td>No apparent effects.</td>
<td>Nada <em>et al.</em>, 1993.</td>
</tr>
<tr>
<td><em>csk</em> and <em>fyn</em></td>
<td>Similar phenotype to <em>csk</em>/*- mice.</td>
<td>Thomas <em>et al.</em>, 1995.</td>
</tr>
</tbody>
</table>
In adult CNS tissue Src appears to have a role in exocytosis, by which neurotransmitters were released from nerve synapses, and in neuro-endocrine secretion (Barkenow et al., 1990). Src^-/- transgenic mice develop normally, with no apparent defects of its platelets or in the brain. They did however die 3 to 4 weeks after birth from severe osteopetrosis (Soriano et al., 1991). It was later revealed that Src is normally expressed in osteoclasts (Horne et al., 1992). Subsequently, Boyce et al. (1992) showed that osteoclasts from Src^-/- mice failed to form ruffled borders, a characteristic of actively resorbing osteoclasts, and failed to form normal resorption pits on dentine slices in culture. These data would suggest that Src may be involved in regulating cytoskeletal structure in osteoclasts, perhaps by phosphorylating cytoskeletal proteins (Boyce et al. 1992).

The apparent normality of platelets and neural development in Src^-/- mice may be explained by the fact that a number of other Src family members, including Fyn and Yes, are also expressed in these tissues. It is possible that these proteins can substitute functionally for the loss of Src (functional redundancy). In contrast, Fyn, Lyn and Yes appear to be unable to substitute for the loss of Src in osteoclasts, even though they are expressed in these cells (Horne et al., 1992). Apparent functional redundancy is observed in other Src family knock-outs, indicating the requirement of multiple knock-out mice to be bred if Src family protein function is to be elucidated. It was proposed by Erickson (1993) that, in addition to functional redundancy, there may also be superfluous expression of certain proteins (including Src family members) which arise from the energy cost of switching off a gene being greater than the cost of producing unused proteins.

The Fyn protein has been shown to be expressed abundantly in human fibroblasts, umbilical vein endothelium, lymphocytes, monocytes, platelets and the brain (Kawakami et al., 1986; Kawakami et al., 1989). Fyn is involved in the signal transduction mechanism associated with the platelet derived growth factor (PDGF) receptor, T-cell receptor, B-cell receptor (see section 1.2.1.4), interleukin-2 (IL-2) receptor, IL-7 receptor,
low affinity IgE receptor (FceRII), platelet glycoprotein IV (CD36), and the prolactin receptor (Twamley et al., 1992; Samelson et al., 1990; Flaswinkel and Reth, 1994; Kobayashi et al., 1993; Venkitaraman and Cowling, 1992; Sugie et al., 1991; Huang et al., 1991; Clevenger and Medaglia, 1994).

The use of Fyn−/− mice has revealed many details concerning the function and expression of Fyn. Fyn is expressed by neurons in specific regions of the CNS, including the hippocampus, although no obvious abnormalities were apparent in mutant mice (Stein et al., 1992). Further investigation revealed three subtle defects (Grant et al., 1992). First, mice displayed impaired long term potentiation (LTP). This is a process by which the strength of synaptic activity can be improved by a particular pattern of activity. Second, the mice had impaired spatial learning, determined by their inability to learn the solution to a water maze. Third, they had subtle defects in the structure of the hippocampus, which is involved in the long-term storage of memory. Disruption of c-src and c-yes, also expressed in the hippocampus, did not result in the above effects. Grant et al. (1992) proposed that Fyn is important in the development of the hippocampus and also has a role in the mature synapse, related to the induction of LTP. Another strain of Fyn knock-out mice, deficient in both Fyn-T and Fyn-B isoforms, was produced by Yagi et al. (1993). Homozygous offspring from heterozygous parents survived to adulthood whilst homozygous offspring from homozygous parents died from being unable to suckle. It is of note that homozygous mothers were able to nurse homozygous pups if lactation was first activated by the suckling of heterozygous pups from another litter. It was suggested that Fyn−/− mice had an abnormality of the modified glomerular complex in the olfactory bulb, which acts in transmission of suckling stimulus to the hippocampus (Yagi et al., 1993). Umemori et al. (1994) observed that Fyn−/− mice had reduced amounts of myelin in their brains compared to normal mice, although the number of neurons was normal. Fyn-B was found to be physically associated with the large myelin-associated glycoprotein which is thought to be involved in signal transduction during the initial stages of myelination. The reason for the differing phenotypes of the Fyn−/− mice
produced by Grant et al. (1992) and those by Yagi et al. (1993) is unclear, although it should be noted that they used different strategies to disrupt \( fyn \) expression.

Both \( c\text{-yes} \) and \( yrk \) are expressed in a broad range of tissues including brain, spleen, liver, muscle, intestine, kidney, placenta and skin (Zhao et al., 1990; Sudol et al., 1993). In the cerebellum, the \( c\text{-yes} \) gene is expressed in some neurons that also express \( c\text{-src} \), although at different stages of development (Sudol et al., 1988). In the kidney, Yes is found in the epithelium of the proximal tubules, while in the skin it is expressed in keratinocytes within the basal layer of the epidermis, becoming less abundant following differentiation (Sukegawa et al., 1990; Krueger et al., 1991). Yes has been shown to be activated following engagement of the high affinity IgE receptor (FceRI) in a mouse mast cell line PT-18, although in RBL-2H3 cells (a rat basophilic leukaemia cell line) Src and Lyn were activated (Eiseman and Bolen, 1992). Yes, along with Src and Fyn is believed to be involved in signalling from the PDGF receptor (see section 1.2.1.4).

**Haemopoietic cell-restricted Src family proteins**

The cells of the haemopoietic lineage have been extensively studied, due largely to the number of established cell lines and the ease with which normal cells can be isolated from animals and studied \textit{in vitro}. For this reason, the Src proteins have been more comprehensively characterised than would have been in less accessible tissues. In particular, Lck and Fyn have been subject to considerable analysis. The site of Src family expression in haemopoietic cells is shown in Table 1.2.3.

Through the use of \( \text{Lck}^{-/-} \) and \( \text{Fyn}^{-/-} \) mice, it was established that Lck and Fyn expression is developmentally regulated in T-cells. Lck is involved in transducing the signal from ligand-bound TCR and the IL-2\( \beta \) receptor (Xu and Littman, 1993; Hatakeyama et al., 1991). Molina et al. (1992) observed that there was a severe reduction in CD4\(^+\)/CD8\(^+\) immature thymocytes, no mature CD4 or CD8 single positive thymocytes
Table 1.2.3. Members of the c-src gene family whose expression (●) is restricted to haemopoietic lineages.

<table>
<thead>
<tr>
<th></th>
<th>blk</th>
<th>hck</th>
<th>lck</th>
<th>lyn</th>
<th>c-fgr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocyte/macrophage</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Granulocyte</td>
<td>●</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet</td>
<td></td>
<td></td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>T-lymphocyte</td>
<td></td>
<td>●</td>
<td></td>
<td>●</td>
<td></td>
</tr>
<tr>
<td>B-lymphocyte</td>
<td>●</td>
<td>●</td>
<td></td>
<td>●</td>
<td>(●)</td>
</tr>
</tbody>
</table>
and very few peripheral T-cells in Lck\(^{-/-}\) mice. This block in T-cell development was pinpointed to a specific stage of thymopoiesis, prior to the appearance of CD4 and CD8, during the rearrangement of the V\(\beta\) and V\(\alpha\) genes (Levin et al., 1993). These rearrangements are required for the expression of the TCR. It is of note that the Lck\(^{-/-}\) thymocytes showed rearrangement of \(\beta\)-chain genes but not \(\alpha\)-chain genes and it was proposed by Levin et al. (1993) that Lck might ordinarily serve to signal the opening of the \(\alpha\) locus for access of recombinase. A similar maturational block was observed by Mombaerts et al. (1992) in thymocytes with targeted disruption of the \(\beta\) chain gene. Less striking results were obtained from the targeted disruption of Fyn. Although ubiquitously expressed throughout the body, there was no obvious effect on the phenotype of homozygous mice, as discussed above (Stein et al., 1992). Analysis of T-cell populations revealed that signalling from the TCR was abrogated in CD4/8 single positive thymocytes yet normal TCR signalling was observed in mature peripheral T-cells. Despite this signalling abnormality, T-cell development was unhindered (Appleby et al., 1992). With regard to the negative selection of self class I and II presenting antigens, a process that involves TCR signalling, Stein et al. (1992) suggested that Fyn may contribute to clonal deletion late in thymic differentiation, and Fyn\(^{-/-}\) mice could potentially have impaired deletion of self-reactive thymocytes leading to auto-immune responses. However, no evidence of auto-immune disorder was observed.

Blk is expressed exclusively in B-cells and appears to be involved in the signalling mechanism of the B-cell antigen receptor (BCR) along with Fyn and Lck, as seen by the activation of these proteins upon engagement of the receptor (Dymecki et al., 1990; Burkhardt et al., 1994)(see section 1.2.1.4). Blk expression is developmentally regulated, detected in pro-B-cells prior to assembly and functional expression of immunoglobulin heavy chain genes, and is maintained in pre-B-cells and B-cells before diminishing following differentiation of B-cells to plasma cells (Dymecki et al., 1992).
Lyn protein is found in B-lymphocytes, monocytes, macrophages and platelets (Yamanashi et al., 1989). In B-cells, Lyn is physically associated with the BCR complex, its kinase domain being activated following engagement of the receptor (Yamanashi et al., 1991). This is followed by association of PI 3-K with Lyn, phosphorylating the p85 subunit and inducing PI 3-K activity (Yamanashi et al., 1992). The modes of interaction between the two Lyn isoforms, p53\(^{lyn}\) and p56\(^{lyn}\), differ with respect to their association and levels of kinase activity following engagement of the BCR. This is probably as a result of differences in the N-terminal domain of p53\(^{lyn}\) and p56\(^{lyn}\) where 21 amino acid residues (P-23 to R-43 or V-24 to P-44) are missing from p53\(^{lyn}\) (Yamanashi et al., 1991b). Interaction between Lyn and the lipopolysaccharide (LPS) receptor, CD14, in monocytes induced the activation of Lyn. Fgr and Hck were also activated although they were not found to be physically associated with CD14 (Stefanova et al., 1993). Activation of monocytes with LPS results in the production and secretion of cytokines. However, pretreatment of cells with the tyrosine kinase inhibitor, herbimycin A, results in a complete block of cytokine production showing the importance of tyrosine kinase activity in CD14 signalling pathways (Stefanova et al., 1993).

Human Hck and Fgr are both expressed in monocytes, macrophages, granulocytes and B-cells. B-cell expression of \(c\)-fgr is observed in a small subpopulation of the bone marrow mantle zone (Link and Zutter, 1995), and Epstein Barr virus (EBV) transformed B-cells (see section 1.2.2)(Ziegler et al., 1987; Ziegler et al., 1988; Cheah et al., 1986). LPS treatment of monocytes, as discussed above, upregulates \(hck\) mRNA levels while \(c\)-fgr mRNA levels remain unchanged (Ziegler et al., 1988). In contrast, granulocytic differentiation of the HL60 cell line induced by treatment with D3 results in the upregulation of \(c\)-fgr expression but has no effect on \(hck\). (Katagiri et al., 1991). These differences would imply that they play different roles in myeloid cells (see section 1.2.2.4).
1.2.1.4 Signal transduction mechanisms involving Src family proteins

The specific mechanisms by which Src proteins interact with surface-bound receptors in order to contribute to signal transduction have been elucidated for a few examples of receptor types. These include the (i) PDGF receptor, (ii) T-cell receptor, (iii) B-cell receptor and (iv) the Fc receptors, and are described below.

Following binding of a ligand to its surface receptor the signal is transmitted to the nucleus via a number of mechanisms involving increased tyrosine phosphorylation and/or S/T phosphorylation, metabolism of phosphotidyl inositides, upregulation of diacylglycerol (DAG) and mobilisation of intracellular calcium. A number of proteins are involved in achieving these responses: PLCγ-1, one of several isoforms, is responsible for the cleavage of the phospholipid phosphotidylinositol 4,5 bisphosphate (PIP$_2$) to the second messengers DAG (which acts through protein kinase C) and inositol triphosphate (which activates calcium release). An alternative fate of PIP$_2$ is to be phosphorylated by the p130 subunit of PI 3-K to produce phosphotidylinositol 3,4,5 triphosphate second messenger. Other mechanisms allow the activation of ras GTPase activating protein (GAP) to produce GTP. Each of these second messengers then activate further down stream proteins, ultimately leading to a nuclear response. This can also be achieved by SH3 domain-containing adapter molecules such as Grb2, SOS and Vav interacting with receptor-associated tyrosine kinases. Further signal transduction may involve phosphorylation of Raf (a S/T kinase) which subsequently activates the mitogen activated kinase (MAP) series of kinases. This topic has been reviewed by Fry et al., 1993.

The PDGF receptor

The Src, Yes and Fyn proteins are co-expressed in fibroblasts where it has been suggested that they have a role in regulating fibroblast proliferation by means of a functional interaction with the PDGFβ receptor (PDGF-R). Binding of PDGF to PDGF-R results in dimerisation of the receptor, activation of its cytoplasmic tyrosine kinase domain and
autophosphorylation of a number of tyrosine residues within the cytoplasmic tail. These events are accompanied by a small increase in the kinase activity of the cellular pool of
Figure 1.2.6. Model for the interaction of Src protein with the PDGF-R
Fyn, Src and Yes, and by the physical association with a small proportion of these proteins with the activated PDGF-R (Kypta et al., 1990)(Figure 1.2.6). This association results from binding of the SH2 domain of Fyn, Src or Yes to phosphorylated Y-579 or Y-581 of the PDGF-R (Mori et al., 1990). The association is transient, possibly because the complexes are rapidly internalised and degraded. There is evidence that Fyn, Src and Yes are activated by their association with the PDGF-R and possibly phosphorylated at novel sites (Twamley et al., 1992). The intermolecular binding of the Src SH2 domain to phosphorylated Y-579 and Y-581 of the PDGF-R would disrupt intramolecular binding of Src's SH2 domain to T-527 (Mori et al., 1993). This would result in the unfolding and subsequent activation of the tyrosine kinase (see section 1.2.1.2). Hence, it would seem that the activation of Src would not require the services of a PTPase in order to dephosphorylate Y-527. Indeed, it appears that the Src SH2 domain has a higher affinity for the phosphotyrosine-containing binding sites on the PDGF-R than for its own phosphorylated Y-527 (Roussel et al., 1991; Liu et al., 1993). The binding of Src, Fyn or Yes to the activated PDGF-R is thought to allow the recruitment of their substrates to this site. In support of this, a number of other signalling molecules are recruited to the activated PDGF-R by SH2-phosphotyrosine interactions. These include PI 3-K, PLCγ-1 and rasGAP (reviewed by Pawson and Schlessinger, 1993). The formation of a signal transduction complex at the inner surface of the plasma membrane is thought to extend the range of substrates that are modified by PDGF-R stimulation beyond that which could be modified by the receptor alone.

The T-cell antigen receptor (TCR)

Unlike the PDGF-R, the TCR lacks an intrinsic tyrosine kinase domain, hence the requirement to recruit cytosolic tyrosine kinases to the receptor (reviewed by Weiss and Littman, 1994). As discussed above, the Src proteins Lck and Fyn represent two such tyrosine kinases, along with the ZAP-70 protein tyrosine kinase. The TCR is a multi-subunit complex consisting of an α-β heterodimer and ζ-ζ homodimer subunits, of which α and β (Ti subunits) are largely extracellular, whereas ζ is largely cytoplasmic and
responsible for cytosolic interactions in response to ligand binding by α and β. With these
is associated CD3, consisting of a γ-ε dimer and an ε-δ dimer (Figure 1.2.7). A feature of
the cytoplasmic region of ζ and all CD3 subunits is the motif Y-X-X-L-X(7)-Y-X-X-L/I,
called the tyrosine activation motif (TAM), also known as the antigen recognition
activation motif (ARAM)(Reth, 1989; Flaswinkel and Reth, 1994). These TAMs have
also been observed in the cytoplasmic domains of the BCR and most Fc receptors (see
below). It was determined that TAMs are the binding sites of SH2 domains following the
phosphorylation of the tyrosine residues. This was supported by the observation that the
Y-X-X-L of the TAM is similar to the phosphotyrosine binding sequences found by
Songyang et al. (1993) that bind to the phosphotyrosine binding pocket and to a second
site for Y+3 (the lysine residue) of the Src SH2 domain (Flaswinkel and Reth, 1994).
Upon engagement of antigen-bearing major histocompatibility complex (MHC) to the
TCR a number of sequential events are seen to occur. The rapid association of ZAP-70 to
the ζ subunit, via SH2 and TAM interaction, first requires tyrosine phosphorylation of the
ζ subunit's TAM. This phosphorylation is possibly catalysed by Fyn since it is seen to be
associated with either CD3 ε or with ζ itself (Gauen et al., 1992). Iwashima et al. (1994)
used a CD8-ζ fusion protein to demonstrate that, following stimulation of the chimeric
receptor (using an anti-CD8 antibody), Lck was required to phosphorylate the ζ portion
prior to binding of ZAP-70. Whether Lck or Fyn performs this function in vivo is unclear.
However, as discussed in section 1.2.1.3, expression of these PTKs is developmentally
regulated and may reflect different signalling requirements between immature
thymocytes and mature T-cells. Lck, associated with CD4/CD8, contributes to the TCR
complex by the binding of CD4/CD8 to the same MHC molecule that is bound to the
TCR. It is noteworthy that this allows an interaction of the TCR with activated Lck, not
by contributing its kinase domain to the TCR complex, but by contributing its SH2
domain (Xu and Littman, 1993). The function of this is unknown, but it is thought that for
efficient signalling from the TCR, it is required that a stable ternary complex must be
formed, for which CD4/CD8-Lck association is necessary (Xu and Littman, 1993).
Figure 1.2.7. General structure of T-cell antigen receptor, B-cell antigen receptor, FcεRI and FcγRIII. Tyrosine activation motif: (TAMs), black boxes.
Once the signalling complex is formed, downstream signalling molecules are then activated. These include activation of ZAP-70 by phosphorylation of Y-493 by Lck (Chan et al., 1995), association of p120/130, a putative substrate of Fyn (da Silva et al., 1993), binding of PI 3-K to the Lck SH3 domain, and activation of PI 3-K by Lck (Vogel and Fujita, 1993), and activation of PLCγ-1 by ZAP-70 and/or Lck (Weber et al., 1992). The interactions between Lck, Fyn and the TCR are illustrated in Figure 1.2.8. The complexity of the mechanism by which the TCR binds to MHC and transmits its signal from the extracellular domain of the TCR to cytosolic signalling molecules may well be an indication of the diversity of the possible T-cell responses.

The B-cell antigen receptor complex

The B-cell antigen receptor complex (BCR) has a number of similarities with the TCR in terms of the mechanism by which binding of ligand (denatured protein or carbohydrate in a soluble, particulate or cell-bound form) leads to activation of signalling molecules. Different tyrosine kinase proteins are observed: Src proteins involved are thought to be Blk, Fyn and Lyn, whilst p70^Syk (Syk) plays a role like that of ZAP-70. Functionally homologous features of BCR subunits include two Igα-Igβ heterodimers which form the active cytoplasmic portion of the BCR in the same way that ζ does in the TCR. The involvement of co-receptors also contributes to signalling, these being CD21 associated with CD19 and the protein TAPA-1 (reviewed by Weiss and Littman, 1994). Binding of antigen to the BCR leads to recruitment of Syk to TAMs on the Igα-Igβ subunits following tyrosine phosphorylation by Lyn or Fyn. It is unclear whether this phosphorylation is catalysed by Lyn or Fyn associated with CD19/CD21 or not, although it has been proposed that the CD19/CD21 complex delivers Lyn to the BCR upon cross-linking (van Noesel et al., 1993). It has been demonstrated that BCR stimulation subsequently leads to activation of PI 3-K as well as GAP and Vav (Gold et al., 1992; Downward et al., 1992; Gold et al., 1993).
Figure 1.2.8. Putative pathways of T-cell receptor signalling involving protein tyrosine kinases Lck, Fyn, Csk and ZAP-70.
1.2.1.5 C-src gene family transcription

The genomic structure of chicken c-src, murine hck, human and murine lck and human c-fgr genes have been determined (Takeya and Hanafusa, 1983; Ziegler et al., 1988; Rouer et al., 1989; Garvin et al., 1988; Patel et al., 1990b; Gutkind et al., 1991). Each of these genes contain ten downstream coding exons, conserved in both nucleotide sequence and positioning of the splice sites. These exons encode the conserved regions of each protein, for example, exon 3 encodes the SH3 domain, exons 4 to 7 encode the SH2 domain, and exons 7 to 12 encode the tyrosine kinase domain. The upstream exons of src family genes are highly variable between genes including the number of upstream untranslated exons.

Examination of the human c-src promoter by Bonham and Fujita (1993) revealed a GC rich sequence which lacked both TATA and CAAT boxes. At least 14 transcription start sites were identified between approximately -350 and -630bp upstream of exon 1a. The promoter region also contained six Sp-1 and seven AP-2 binding sites (see section 3.2). Finally, analysis of promoter sequences using a reporter gene indicated that the promoter region up to -635bp was required to direct reporter gene transcription and longer fragments of the promoter, upstream of approximately -1500bp, had a repressive effect. The region between -635 and -462 contained several Sp-1 and AP-2 binding sites.

The promoter region of c-yes contains many of the features observed in the c-src promoter, namely, being GC rich with no TATA box, although there was a CAAT box. There were also Sp-1, AP-1 and Myb binding sites. GC box-like sequences, which can bind Sp-1, were found to be important for activating transcription of a reporter gene, as demonstrated by DNase I footprinting and site directed mutagenesis (Matsuzawa et al., 1991).

For human c-src and c-yes promoter analysis 10T1/2 (mouse fibroblasts) and CV-1 (African green monkey cells) cell lines have been used respectively. In each case, the cell system in which each promoter was studied does not necessarily represent the ideal
transcription mechanism that would control these promoters \textit{in vivo}. Nonetheless, since c-
\textit{src} and c-\textit{yes} are ubiquitously expressed throughout human tissues, the likelihood of
developmental- and tissue-specific factors differing from 10T1/2 and CV-1 cells is reduced.

The human and murine \textit{lck} genes have two promoters which give rise to two mRNAs
differing in their 5' untranslated region. The upstream promoter (type II) in the human \textit{lck}
gene, directs transcription from a cluster of at least five transcription start sites separated
by 35kb from the downstream type I promoter (Takadera \textit{et al.}, 1989). Unlike the type II
promoter, use of the type I promoter is developmentally restricted to immature
thymocytes (Reynolds \textit{et al.}, 1990). Ets-1 binding elements between -91 and -119 (with
respect to the transcription start site) and a repressive element between -512 and -570
appear to be important in regulating this promoter (Leung \textit{et al.}, 1993). These data were
derived by transiently transfecting promoter fragments, linked to a reporter gene, into a
T-lymphoma cell line, Jurkat. PMA treatment of Jurkat cells resulted in a reduction in
type I transcripts and an increase in type II transcripts even though there are also two
Ets binding elements within the type II promoter (Leung and Miyamoto, 1991). It has been
suggested that the differential expression of type I and type II expression is governed by
different members of the Ets-1 family of transcription factors (Leung \textit{et al.}, 1993).

The \textit{lyn} gene promoter was found to be GC rich and lacked TATA and CAAT boxes but
did contain OTF, PEA3 and Myb binding sites. The region of the promoter to -54bp,
containing the OTF binding site, was required to direct transcription of a reporter gene in
the B-cell line Raji (Uchiumi \textit{et al.}, 1992).

Zwollo and Desiderio (1994) identified the activity of a transcription factor that was
specific to B-cell expression of \textit{blk}. The promoter sequence, again lacking TATA and
CAAT boxes, contained a binding site for this B-cell specific transcription factor, BSAP,
between -68 and -48bp.
Lichtenberg et al. (1992) reported that the \textit{hck} promoter, as with all \textit{src} genes studied, lacked TATA and CAAT boxes, and contained Sp-1 and AP-2 binding sites. They were unable to show, using reporter plasmid constructs, regions of the promoter responsible for myeloid-specific expression of \textit{hck}.

The \textit{c-fgr} promoter will be discussed in section 1.2.3.

1.2.1.6 Post-transcriptional modification of \textit{c-src} family genes

Splice variants of \textit{c-src}, \textit{fyn} and \textit{lyn} give rise to variant protein products. \textit{C-src} mRNA gives rise to p60\textsuperscript{c-src} protein. However, in specific regions of the CNS an alternative transcript is expressed which contains an additional 18 nucleotides originating from an exon NI, which lies between exons 3 and 4, encoding p60\textsuperscript{c-src+} (Black 1991). A further splice variant includes an exon inclusion between NI and exon 4, called NII (Piper and Bolen, 1990). These Src isoforms appear to differ in their phosphorylation patterns even although they differ in their SH3 domain (Brugge \textit{et al.}, 1987), which would suggest alternative protein-protein interactions via the SH3 domain.

The two isoforms of Fyn (B and T) arise from two alternative exon 7 transcripts. The brain form, Fyn-B, is derived from exon 7A containing-mRNA whereas the thymic form, Fyn T, is derived from exon 7B-containing mRNA (Cooke \textit{et al.}, 1989). These isoforms appear to differ in their ATP binding site in the kinase domain, with exon 7A (Fyn-B specific) encoding G-X-G-X-X-G, and 7B (Fyn-T) encoding G-X-G-X-X-A (a motif not seen in other protein tyrosine kinases [Hanks \textit{et al.}, 1988]) (Cooke \textit{et al.}, 1989). Although Fyn-T and Fyn-B activities have not been compared, Cooke \textit{et al.} (1991) showed both isoforms equally capable of enhancing thymocyte responses.
1.2.1.7 Conclusions

The Src family of non-receptor protein tyrosine kinases represent a highly versatile group of molecules, evidenced both by the broad range of tissues where they are expressed and by the variety of protein-protein interactions. This could be considered a consequence of the structure of Src proteins facilitating multiple types of interactions in a tissue and substrate specific manner. In addition to playing a central role in receptor signalling mechanisms, the Src proteins have also been shown to function in signalling other cellular activities, such as guidance of neural development by Src.

1.2.2 Structure, expression and function of the c-fgr proto-oncogene

1.2.2.1 Isolation of the c-fgr gene

A strain of a feline sarcoma virus was isolated from a spontaneous sarcoma of an eight-year-old domestic cat by Rasheed et al. (1982). This virus, the Gardner-Rasheed feline sarcoma virus (GR-FeSV), encoded a 70kDa phosphoprotein which possessed tyrosine kinase activity (Naharro et al., 1983b). Molecular analysis of the viral genome revealed a gag-actin-onc mRNA transcript encoded by GR-FeSV where the onc sequence (subsequently designated v-fgr) was found to have homologues in the DNAs of diverse vertebrate species (Naharro et al., 1984; Naharro et al., 1983b). It was suggested that v-fgr had originally been transduced from a cellular equivalent (Naharro et al., 1983a). Tronick et al (1985) identified the human c-fgr gene (the homologue of v-fgr), although the primary structure of the c-fgr protein product p55c-fgr (Fgr), derived from cDNA clones, was not determined until 1988 (Katamine et al., 1988). In the same year, c-fgr was mapped to chromosome 1 p31-pter (Dracopoli et al., 1988). Latterly, King and Cole (1990) have isolated the murine c-fgr gene.

1.2.2.2 Structure of p55c-fgr

The basic structure of Fgr is the same as the other members of the Src family of protein tyrosine kinases (discussed in section 1.2.1). Comparison of the amino acid sequence of Fgr with other Src proteins is summarised in Table 1.2.4. Drebin et al. (1995) produced
phylogenetic trees using data based on amino acid homologies for SH2, SH3 and kinase domains between Src proteins (Figure 1.2.9). The Src family is divided into two groups on a phylogenetic basis, representing the ubiquitously expressed members (Src, Yes and Fyn) and the haemopoietically expressed members (Blk, Lck, Hck and Lyn). It is of note that Fgr shows a greater relatedness to the ubiquitous group than to the haemopoietic group. The significance of this is not known but one might speculate that during the evolutionary development of the Src family there was a divergence into two groups, ubiquitous and haemopoietic. It is conceivable that Fgr arose from the ubiquitous group and later acquired a haemopoietic function and restriction of expression, analogous to the haemopoietic group.

1.2.2.3 Expression of c-fgr mRNA and its protein product, p55\(^{c-fgr}\)

The c-fgr proto-oncogene has been found to be expressed specifically in myeloid cells such as monocytes, macrophages, granulocytes and neutrophils. There is also expression of c-fgr in bone marrow mantle zone B-lymphocytes, and in B-lymphomas resulting from Epstein-Barr virus infection. Recently, Wechsler and Monroe (1995a) have determined the expression of murine Fgr in mature splenic B-cells. Furthermore, they have shown murine Fgr to be activated in response to BCR cross-linking (see below)(Wechsler and Monroe, 1995b)

*B-lymphoid expression*

Initially, c-fgr mRNA was found to be expressed in B-lymphocytes infected with the Epstein-Barr virus (EBV)(Inoue \textit{et al.}, 1987). Study of Burkitt's lymphoma and non-Burkitt's lymphoma cell lines, and cell lines derived from EBV-transformed umbilical cord and peripheral blood cells indicated that c-fgr expression was associated with the presence of EBV (Cheah \textit{et al.}, 1986). Normal human B-cells, other than those of the bone marrow mantle zone, were found not to express c-fgr mRNA. However, following immortalisation of primary B-cells with the EBV strain B95-8, a ten-fold increase in c-fgr expression is observed (Knutson, 1990; Patel \textit{et al.}, 1990a).
Table 1.2.4. Homology between predicted human Fgr amino acid sequence and other Src family members. (Brickell and Patel, 1988)

<table>
<thead>
<tr>
<th></th>
<th>Human Fgr (1 to 75)</th>
<th>Human Fgr (76 to 529)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N-terminal unique region</td>
<td></td>
</tr>
<tr>
<td>Human Lyn</td>
<td>19%</td>
<td>60%</td>
</tr>
<tr>
<td>Human Hck</td>
<td>23%</td>
<td>62%</td>
</tr>
<tr>
<td>Human Fyn</td>
<td>29%</td>
<td>76%</td>
</tr>
<tr>
<td>Human Src</td>
<td>15%</td>
<td>77%</td>
</tr>
<tr>
<td>Human Yes</td>
<td>17%</td>
<td>76%</td>
</tr>
</tbody>
</table>
Figure 1.2.9. Phylogenetic trees of the e-src gene family of tyrosine kinases (excluding yrk). The trees schematically represent the structural relationships based on comparison of the catalytic domain (a), SH2 domain (b), and SH3 domain (c).
The expression of c-fgr would appear to be associated with the EBV viral protein EBNA-2. This is based on the observation that EBV conversion of EBV-negative B-lymphoma cell lines with the EBV strain HR-1 does not result in induction of c-fgr expression (Patel et al., 1990a; Knutson et al., 1990). HR-1 lacks both EBNA-2 and EBNA-LMP (Latent Membrane Protein). Infection of EBV-negative B-cell lines with recombinant EBV which carry EBNA-2 alone increased c-fgr mRNA accumulation (Knutson, 1990). It seems likely that EBNA-2, although on its own unable to immortalise B-cells, is involved in promoting c-fgr expression (see section 1.2.3). However, it is also likely that c-fgr is not involved in the immortalisation of B-cells, where EBNA-1 has been implicated, but is expressed as an indirect consequence of EBNA-2 expression in the same cell. Recently, Link and Zutter (1995) used single cell immunohistochemical and immunofluorescent techniques to identify apparent expression of Fgr protein in normal bone marrow mantle zone B-cells, a small population of resting B-cells which secrete immunoglobulin in response to pokeweed mitogen (Gadol et al., 1988). This observation would support the argument that in itself expression of c-fgr is not sufficient to transform normal B-cells.

IFNγ treatment of EBV-positive cell lines such as Daudi and Raji results in a 56% reduction in c-fgr expression, correlating with down regulation of c-myc expression (Sharp et al., 1989). This would imply that c-fgr expression is not necessarily controlled by EBV proteins and that Fgr is involved in growth regulation by interferons, perhaps in mantle zone B-cells. It would seem equally plausible that IFNγ treatment of these cells could down regulate the mechanism by which EBV proteins promote c-fgr expression or even down regulate EBV proteins themselves. The demonstration that Fgr is expressed in a developmentally restricted manner in murine splenic B-cells, and that it is associated with BCR signalling challenges the widely held belief that Fgr expression is confined to myeloid lineages, at least in mice (Wechsler and Monroe, 1995a and 1995b).

Myeloid expression

C-fgr is expressed in the myeloid lineage of cells. In particular, c-fgr mRNA accumulates in more differentiated cells such as peripheral blood monocytes, tissue macrophages,
granulocytes, neutrophils and natural killer cells (Ley et al., 1989; Notario et al., 1989; Inoue et al., 1990). Much of the understanding of the specific expression of c-fgr has been determined using the HL60 and U937 cell lines (see section 1.1). As previously discussed, treatment of these cell lines with differentiating agents such as PMA, RA, D3 and TNFα will give rise to a monocyte/macrophage (for U937) or a granulocyte (for some treatments of HL60). In each case, accumulation of c-fgr mRNA and Fgr protein occurs as a function of granulocytic or monocytic differentiation (Notario et al. 1989). Treatment of U937 and HL60 cells with PMA induces c-fgr expression by 6-8 hours after treatment, reaching maximal levels by 24 hours (Faulkner et al., 1992; Miyazaki et al., 1993). The kinetics of c-fgr mRNA expression in U937 cells differs between various treatments. PMA and D3 treatment leads to a gradual accumulation of c-fgr transcripts with maximal levels being achieved by 48 to 72 hours, with PMA inducing the highest levels of expression. TNFα provokes a rapid induction of expression, with maximal levels being achieved by 8 hours, before subsequently declining. D3 and TNFα in combination induce levels of c-fgr mRNA accumulation greater than that seen for either treatment on their own. Also, the expression occurs within 4 hours and increases to this higher level where it is maintained by 48 to 72 hours (Faulkner et al., 1992). These data suggest that PMA, TNFα and D3 signal different modes of c-fgr expression, relating to alternate signalling mechanisms and possibly different mechanisms of transcription regulation.

1.2.2.4 Function of Fgr protein in myeloid cells

Expression of Fgr in myeloid cells (as described above) would indicate that Fgr plays a role in cellular functions associated with a mature cell. The fact that maximal c-fgr mRNA accumulation is not reached until 12-24 hours following induction of differentiation would argue against Fgr playing a role in signalling differentiation. Although Willman et al. (1987) showed that the growth-stimulatory cytokine CSF-1 will down regulate c-fgr mRNA in adherent bone marrow monocytic cells, suggesting a role in cell cycle control, there has been no direct evidence to support this notion. In support of a function in mature cells, Fgr has been shown to be myristylated and subsequently
translocated to the plasma membrane as well as be identified in secretory (secondary) granules (Gutkind and Robbins, 1989). The association of catalytically active Fgr with these membranes increases following neutrophil activation with fMLP. This suggests a role in the regulation of exocytosis in neutrophils. A clearer indication of the role of c-fgr was determined by Hamada et al. (1993) who demonstrated there to be a physical association between Fgr and the low affinity IgG receptor, FcγRII, in human neutrophils. The FcγRII (of which there are three homologous but distinct genes, -A, -B and -C) is responsible for signalling phagocytosis, generation of reactive oxygen intermediates, antibody-dependent cellular cytotoxicity, and release of lysosomal enzymes. These functions are effected by the binding of the Fc portion of IgG, particularly within the context of opsonised target cells. The kinase activity of Fgr was increased by the presence of both goat anti-mouse IgG and cross-linking of FcγRII. This suggested that physical cross-linking of Fgr was required for activation. Co-immunoprecipitation experiments, using an anti-FcγRII antibody and goat anti-mouse IgG showed associated Fgr levels to decrease following receptor cross-linking and this was consistent with the notion that, following ligand binding, the receptor is internalised. The nature of the Fgr-FcγRII interaction is not known, but as discussed earlier (section 1.2.1.4), Fc receptors possess a TAM motif that would allow for a Fgr SH2-FcγRII association. This is supported by the fact that a truncated form of an FcγRII isoform (FcγRIIA), which lacks part of its TAM, is unable to initiate calcium mobilisation and phagocytosis of large particles (Odin et al. 1991).

In mouse monocyte/macrophages, Fgr has been associated with the membrane proteins Ly6C and p70, proteins of unknown function but thought to be receptors for extracellular signals (Hatakeyama et al., 1994). This study showed Fgr was not associated with FcγRII. Hatakeyama et al. (1994) point to large differences between the N-terminal domains of murine and human Fgr, in particular residues 12-62, which may account for differences in Fgr-receptor association between the two species, but argue against the involvement of
TAM-Fgr interaction as suggested above. They suggest that p70 may represent a functional murine equivalent of human FcγRII.

An association between the C3 complement receptor LFA-1 (CD11b/CD18) with activation of Fgr kinase activity in TNF-induced activation of human neutrophils was studied by Berton et al. (1994). LFA-1 stimulation leads to the activation of neutrophils and monocytes to engulf particles. Treatment of neutrophils with PMA, TNF or fMLP all lead to increase Fgr kinase activity, whilst blocking LFA-1 with an anti-CD18 antibody prevented Fgr activation (Berton et al., 1994).

These data taken together strongly implicate Fgr as functioning principally in signalling mechanisms associated with phagocytosis via Fc receptors and possibly via LFA-1, and is consistent with the idea that Fgr mediates functions in mature cells rather than regulating myeloid development. Generation of Fgr"/- and Hck"/- single knock out mice and Fgr"/-Hck"/- double knock out mice indicated that the expression of Fgr and Hck in murine cells are not critical for haemopoiesis, which was unimpaired, although Hck"/- macrophages displayed reduced phagocytosis (Lowell et al., 1994). Double mutant mice had a susceptibility to infection by the bacterium Listeria monocytogenes. The reason for this specific immunodeficiency is unclear. Disruption of IFN-γ receptor also results in susceptibility to Listeria (Huang et al., 1993). However, double mutant mice macrophages still responded normally to heat-killed Listeria and to IFN-γ, as seen by production of reactive nitrogen free radicals (Lowell et al., 1994). These studies would suggest that Fgr function, in the murine monocyte/macrophage at least, can be performed by Hck, and probably vice versa.

1.2.3 Regulation of c-fgr gene expression

The first analysis of the regulation of c-fgr transcription was in B-lymphoid cell lines that had been converted by EBV strain B95-8. c-fgr transcription in these cells give rise to two mRNA transcripts of 2.9 and 3.5kb (Brickell and Patel, 1988). Similar transcripts had
been found in EBV converted normal human peripheral B-cells (Inoue et al., 1987). The 3.5kb transcript represents an incompletely processed mRNA that still contains intron 2. Different polyadenylation signals resulted in varying lengths of cDNA clones derived from c-fgr transcripts. The function of alternative polyadenylation is unclear but may signal some form of post-transcriptional regulation of c-fgr expression. The c-fgr cDNA clones showed there to be untranslated regions at both ends of the mature transcript (Brickell and Patel, 1988; Inoue et al., 1987).

Isolation of 5' genomic sequences of the human c-fgr gene by Patel et al. (1990b) permitted characterisation of its gene structure and promoter activity. Gene structure had previously been determined for exons 4 to 12 indicating, by homology with the avian c-src gene, the likely ancestral duplication of c-src to derive c-fgr (Parker et al., 1985; Nishizawa et al., 1986). A cosmid genomic clone, cF2.3, was isolated containing a 36.5kb insert. Sequence analysis and comparison with cDNA clones isolated from B-lymphoid cells revealed two untranslated exons (1a and 1b) and a translation initiation codon in the third exon (exon 2). S1 nuclease mapping showed there to be a cluster of twelve transcription start sites between 14 and 56bp upstream of exon 1a (Patel et al., 1990b). Within the region upstream of these start sites, a number of other structural features included: three Alu repeat sequences, (TG)21 and (TG)14 tracts (known to have transcription enhancer activity in certain circumstances), AP-2 and Sp-1 consensus binding site sequences, and the absence of TATA and CAAT elements.

Although c-fgr expression in EBV+ B-lymphocytes is directed through a promoter upstream of exon 1a, it became apparent that this promoter is not functional in myeloid cells. Isolation of c-fgr cDNA clones from normal human peripheral blood monocytes revealed that exon 1a was not expressed in myeloid c-fgr mRNA transcripts. Instead, c-fgr cDNAs possessed untranslated exons not seen in B-cell transcripts (Gutkind et al., 1991). These exons (M1 and M2), later to be shown to give rise to rare splice variants, were mapped by Gutkind et al. (1991) approximately 11kb and 2kb upstream of exon 1a.
(see section 3.2). Following this, Link et al. (1992) and Patel and Brickell (1992) isolated further c-fgr cDNA clones possessing a more commonly expressed untranslated exon (M4, of unknown genomic location), derived from IFNγ-treated U937 cells. These various c-fgr mRNAs from B-cells and myeloid cells were a product of alternative splicing, with exon M4 representing the major c-fgr 5' exon specific to myeloid expression (see section 3.2). This is summarised in Figure 1.2.10. Consequently, it was presumed that sequences upstream of exon M4 would possess myeloid-specific promoter activity. The nature of the myeloid-specific promoter of c-fgr is examined in this thesis (see section 1.4).
Figure 1.2.10. Differential splicing of c-fgr RNA in myeloid cells and EBV transformed B-lymphocytes.
1.3 The retinoid X receptor

The retinoid X receptors (RXRs) belong to a superfamily of nuclear receptors that function as ligand-dependent trans-acting transcription factors, capable of inducing or repressing transcription of a target gene through its promoter. Members of this superfamily so far identified include two classes of receptors: (i) retinoic acid receptors (RAR), retinoid X receptors (RXR), ultraspircle (usp) in Drosophila, farnesoid X-activated receptor (FXR), vitamin D receptor (VDR), peroxisome proliferator activated factor receptor (PPAR), thyroid hormone receptor (TR), and (ii) oestrogen receptor (ER), and the glucocorticoid receptor (GR). ER and GR are steroid receptors that differ from the first class of receptor by their mechanism of DNA binding and activation (Evans, 1988; Forman et al., 1995). There are also approximately 150 orphan receptors currently identified, and this number is certainly destined to increase. A feature of all these receptors is their requirement to dimerise in order to bind DNA and activate transcription (in the presence of bound ligand) usually as heterodimers for the first class of receptors, and as homodimers in the second class of receptors. The first class most commonly form heterodimers with RXRs, which function as accessory proteins. RXRs also form homodimers in the presence of its high affinity ligand, 9-cis RA. This section will describe the structure and function of the RXRs and relate these to possible roles in the myeloid cell lineage which led to the investigation of the RXRs during monoblastic differentiation.

1.3.1 Isolation of the retinoid X receptors

The importance of vitamin A (retinol), from which other retinoids are derived, has long been known to be associated with photoreceptors in rods and cones in the human eye. Deficiency of retinol leads to 'night blindness'. However it was also noted that retinol deficient mouse embryos developed congenital malformations of the foetal eyes and heart (VAD syndrome)(reviewed by Blomhoff, 1994). This suggested that retinol, or its derivatives, function in foetal development. Other effects of retinoids, specifically
retinoic acid (RA), on differentiation, haemopoiesis and development, led to the hypothesis that, as a small lipid soluble molecule, RA might function in the same way as steroid and thyroid hormones. Namely, a specific protein receptor for RA might exist as there is for steroid and thyroid hormones.

Initially, Chytil and Ong (1978) discovered a cellular RA binding protein (CRABP) which was believed to be the functional receptor for RA. Study of CRABP and related proteins showed them not to be the cognate receptor for RA. However, in 1987 Petkovich et al. and Giguère et al. reported the isolation of retinoic acid receptor -α (RARα). Subsequent isolation of RARβ and RARγ defined a family of receptors which possessed ligand binding and DNA binding domains, features which indicated membership of a nuclear receptor superfamily. Up to this time it had been assumed that the active form of RA was all-trans RA until the significance of 9-cis RA was determined (discussed below). The structure of 9-cis RA and all-trans RA are shown in Figure 1.3.1.

The human retinoid X receptor -α (RXRα) was isolated from a liver and kidney cDNA library using a low stringency hybridisation with a RARα DNA probe (Mangelsdorf et al., 1990). RXRβ and RXRγ isoforms have since been isolated from human, rodent and chicken tissues (Mangelsdorf et al., 1992; Yu et al., 1991; Rowe et al., 1991; Leid et al., 1992). It became apparent that the RXRs represented a distinct gene family from the RARs (Table 1.3.1). It was noted that even though there is only 27% sequence similarity between the ligand binding domains of RXRα and RARα, both were activated by retinoic acid (Mangelsdorf et al., 1990). The high affinity ligand for the RXRs was later found to be a specific isomer of retinoic acid, 9-cis retinoic acid (9-cis RA) (Heyman et al., 1992). In contrast, both 9-cis RA and all-trans RA bind the RARs with high affinity (Levin et al., 1992). Recently, Willy et al. (1995) isolated another high affinity receptor for 9-cis RA called LXR which represents a third, distinct retinoid receptor.
Figure 1.3.1. Chemical structures of all-trans retinoic acid and 9-cis retinoic acid.
Table 1.3.1. DNA binding domain and ligand binding domain sequence homologies of human RXRα with other nuclear receptors (Mangelsdorf *et al.*, 1994).

<table>
<thead>
<tr>
<th></th>
<th>DNA binding domain</th>
<th>Ligand binding domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>human RARα</td>
<td>61%</td>
<td>27%</td>
</tr>
<tr>
<td>human TRβ</td>
<td>53%</td>
<td>24%</td>
</tr>
<tr>
<td>Drosophila USP</td>
<td>86%</td>
<td>44%</td>
</tr>
<tr>
<td>human GR</td>
<td>52%</td>
<td>22%</td>
</tr>
</tbody>
</table>
1.3.2 Retinoid metabolism

Retinol, acquired through the diet from vegetables and fish in the form of retinol or β-carotene, is stored in the liver as retinol. Retinol binds to retinol binding protein and is delivered to tissues where it enters the cell and binds to cellular retinol binding protein (CRBP). The retinol is then oxidised by cytochrome P-450 oxidases to all-trans RA, which in turn binds cellular retinoic acid binding protein (CRABP). The action of isomerases will then produce 9-cis RA. RA isomers (all-trans, 13-cis and 9-cis) are also found in plasma at a concentration of approximately 2nM (3.5-5.4ng/ml) and may be protein bound (reviewed by Blomhoff, 1994). CRABP-I and -II are expressed throughout embryonic and adult tissues and is thought to regulate the cellular concentration of RA, although targeted disruption of CRABP-II has virtually no effect on mouse embryogenesis (except for a single digit duplication on one forepaw)(Fawcett et al., 1995).

1.3.3 Structure/function relationships of the nuclear receptor superfamily

The structure of the RXRs (summarised in Figure 1.3.2) closely resembles the RARs and other members of the nuclear receptor superfamily, and gives an understanding as to the method by which these proteins function. Structural regions, labelled A to E, include a ligand binding domain, DNA binding domain, dimerisation interface and regions associated with activation of transcription (AF-1 and AF-2 regions).

1.3.3.1 DNA binding

RXR dimerisation, either as a homodimer or as a heterodimer with other nuclear receptors, binds to the DNA of specific gene promoter sequences. Subsequent transactivation of transcription is then facilitated by the presence of bound ligand. The DNA binding domain (DBD) of RXR consists of 94 amino acids and is the most highly conserved region between the nuclear receptors. The DBD contains two cysteine-zinc fingers which form two perpendicular helices in a single structural domain. Unlike other nuclear receptors, the RXR DBD contains a third helix (helix 3) found immediately after
helix 2 which is thought to mediate protein-protein and protein-DNA interactions (Figure 1.3.3)(Lee et al., 1993). It is likely that the direct interaction of RXR with DNA involves helix 1 lying across the major groove and helix 3 (also known as the T-box) projecting towards the minor groove. This has the effect of bringing basic amino acids into close proximity with the DNA, facilitating a strong interaction with the DNA. As part of a binding homodimer, the T-box of the upstream-bound RXR DBD can also interact with the second zinc finger of the downstream-bound RXR DBD, hence serving as a dimerisation interface (see section 1.3.3.3)(Lee et al., 1993). Recently, Rastinejad et al., (1995) have determined the crystal structure of the RXR-RAR-DNA complex, confirming the nature of protein-DNA interactions (described above) that establish sequence-specificity of the various RXR heterodimer interactions.

The 5bp consensus sequence response element, to which nuclear receptors bind, comprises of two so-called half sites. The consensus core sequence of the half site is 5'-A/G-G-T/G-T-C'-3'. These half sites are arranged either as direct repeats, inverted repeats or palindromic repeats, all of which are spaced by between 1 to 6bp. The spacing between the half sites confers receptor specificity and transcription activity. Steroid receptors such as ER or GR, which usually homodimerise, tend to bind palindromic repeats. Receptors that involve dimerisation with RXR commonly bind direct repeat response elements. Generally the RAR response element (RARE) has a direct repeat, spaced by 5bp (a DR-5 element), VDRE has a DR-3 element, and TRE and LXRE have a DR-4 element. RXR homodimers will bind to DR-1 elements in the presence of 9-cis RA. It is noteworthy that these interactions are not absolute. RXR homodimers can also bind to DR-2 and DR-5 elements(Zhang et al., 1992), and RXR-RAR heterodimers can bind to DR-2 elements (Leid et al., 1992; Durand et al., 1992)(Figure 1.3.4). The DR spacing restricts the possible heterodimer-DNA interactions by steric hindrance.
Figure 1.3.2. General structure of RXRα.
Figure 1.3.3. Schematic representation of the RXRα DNA binding domain. Helix regions in grey boxes; Zinc finger cystein residues circled in black; D-box residues in individual squares; vertical numbers above sequence, amino acid number (Lee et al., 1993).
Figure 1.3.4. Variously spaced response element/dimer interactions involving RXR, RAR, VDR, TR and ligands required to activate DNA-bound receptor complexes.
For example, the RXR-TR dimer is unable to bind a response element spaced less than 4bp due to a helix structure in the TR DBD that forms an interface with RXR (see section 1.3.3.3) (Rastinejad et al., 1995). The extent of dimer-DNA association can vary, depending on the specific sequence of the response element. For example, the RARE of the ApoAI promoter, a DR-2 element, can bind RXR-RAR dimers and activate transcription, whereas the CRBP-I promoter RARE, also a DR-2, can neither bind nor be activated by RXR-RAR dimers (Zhang et al., 1992). Evidence that receptor binding and activation of transcription may be governed by the sequence of the spacer nucleotides was supplied by Katz et al. (1995) and Vennström (1995) who showed that alterations to the DR-4 spacer of a TRE could in some cases abolish transactivation, and in other cases enhance transactivation.

### 1.3.3.2. Ligand binding.

The ligand binding E domains of RXR and RAR have a molecular weight of approximately 25,000 and contain large numbers of hydrophobic amino acids which are thought to supply pockets into which 9-cis RA fits (Evans, 1988; Green and Chambon 1988). RXRx, RXRβ and RXRγ bind 9-cis RA with dissociation constants (Kd) of 15.7, 18.3 and 14.1 nM, respectively (Allenby et al., 1993). There is relatively little sequence homology between the LED of RXR and RAR even though both will bind 9-cis RA (RAR's Kd between 0.2 and 0.7 nM) (Allenby et al., 1993). However, RAR will also bind all-trans RA with a high affinity (Kd also between 0.2 and 0.7 nM) indicating a structurally diverse method of ligand binding that permits both RA isomers. Crystal structure analysis of the RXRx LED by Bourguet et al. (1995) revealed two binding pockets for 9-cis RA called A and B. The LDB consists of 11 α-helices (H1 to H11) which are folded such that pocket A, formed from H1, H3, H5 and H11, and pocket B, formed from H5 and H7, can both bind 9-cis RA. Evidence from this and other studies would suggest that pocket B is the actual binding site for 9-cis RA (Forman and Samuels, 1990). It is thought that binding of 9-cis RA to RXR induces a conformational change that facilitates the formation of homodimers and exposes the AF-2 (which is located in
the E domain) to interact with other proteins involved in transcription initiation (see section 1.3.3.4)(Leid, 1994). RXR is capable of forming homodimers and binding to DNA in the absence of 9-cis RA, although without the ligand the dimer cannot activate transcription. Exposure of AF-2 also allows interaction with the DNA binding domain and may contribute to sequence specificity of the receptor.

1.3.3.3 Receptor dimerisation

Nuclear receptors contain two dimerisation interfaces that permit either homo- or heterodimerisation. One lies in the LDB, consisting of approximately 20 amino acids. Mutation of these residues results in the loss of dimerising ability of RAR, VDR and TR with RXR. However, mutation of the equivalent 20 amino acids in RXR does not affect its ability to form dimers with unmutated RAR, VDR or TR, although RXR homodimerisation was not studied (Rosen et al., 1993). From these data one might speculate that since both partners of the dimer are bound to two direct repeat half sites, and hence orientated in the same direction, the dimerisation interface of the downstream receptor faces upstream to interact with a downstream facing region of the other receptor (RXR)(Figure 1.3.5). The LDB dimerisation interface does not mediate dimer specificity but acts to stabilise the dimer complex (Mader et al., 1993).

The second dimerisation interface is located in the DBD, and is associated with the T-box (as described above) of the RXR DBD during homodimerisation on a DR-1 element (Lee et al., 1993). Zechel et al. (1994b) showed that the dimerisation interface is provided by the T-box region of the downstream RAR and the CII finger (excluding the D-box) of RXR for heterodimerisation of RXR-RAR on a DR-2 element. A similar dimerisation mechanism is employed during formation of RXR-RXR homodimers on DR-1 elements. Analysis of DBD-mediated dimerisation on DR-4 (RXR-TR) and DR-5 (RXR-RAR) revealed that the D-box component of the RXR CII finger is specifically required to interact with the tip of the RAR CI finger (Figure 1.3.3).
Figure 1.3.5. Model of receptor dimerisation (solid line arrows) via DNA binding domain, and transactivation via AF-1, AF-2 and non-receptor proteins (dashed line arrows). Functional domains in squares.
RXR dimerisation with TR, however, involves the same RXR region interacting with a 7 amino acid prefinger, N-terminal of TR's CI (Zechel et al., 1994a). In summary, the DBD dimerisation interface defines the specificity of the homo- or heterodimer DR binding repertoire (Zechel et al., 1994a). Studies of dimerisation and receptor-DNA interaction have also shown that the polarity of heterodimer binding is significant in transactivation. RXR will bind to the 5' upstream DR half site, and RAR, TR or VDR will bind to the 3' downstream DR half site (Predki et al., 1994). As described above, downstream regions of the RXR DBD interact with upstream regions of the dimer partner's DBD (Figure 1.3.5). The signal delivered by nuclear receptors depends on both the ligand available and the response element to which the receptor will bind. For example, all-trans RA will bind RAR and activate transcription through a DR-2 or DR-5 element as a heterodimer with RXR. However, 9-cis RA will also achieve the same result, but in addition, 9-cis RA will promote the formation of RXR homodimers which can more strongly activate transcription through a DR-1, DR-2 or DR-5 element (Zhang et al., 1992). RAR-RXR heterodimers bound to a DR-1 element have been shown to repress transactivation in the presence of ligand (Kurokawa et al., 1994). In this case, RAR is bound to the upstream half-site which allosterically prevents 9-cis RA binding to RXR. Further varieties of nuclear receptor signalling were elucidated by Carlberg et al. (1993) who showed that, in addition to RXR-VDR signalling via DR-3 VDRE, VDR can homodimerise and activate transcription via a DR-6 element in the presence of its ligand, D3. Similarly, TR can activate transcription via a DR-4 element in the presence of its ligand, thyroid hormone (T3) (Brent et al., 1992). An interaction between VDR and TR was observed by Schröder et al. (1994) whereby VDR and TR heterodimerise. In the presence of D3, VDR-TR dimers form and associate with a DR-4 element with the 5'-VDR polarity being significant. In the presence of T3, TR-VDR (5'-TR polarity) will associate with a DR-3 element. Of interest is the fact that unliganded VDR or TR homodimers, bound to their appropriate response element, have a repressive effect on transcription. Addition of ligand will induce dissociation of the homodimer from some, but not all, response elements and form heterodimers with TR/VDR in the absence of RXR (Yen et al., 1994;
Schräder et al., 1994). The presence of RXR will induce the conversion of VDR homodimers to RXR-VDR heterodimers following binding of D3 to the VDR homodimer (Cheskis and Freedman, 1994).

1.3.3.4 The autonomous transactivation functions AF-1 and AF-2

The modulation of transactivation by steroid receptors such as ER has been shown to be as a result of two regions in the protein, the autonomous transactivation functions (AF-1 and AF-2)(reviewed by Gronemeyer, 1991). It was hypothesised that similar regions would exist in the RXR and RAR proteins. Such regions would allow for interaction with other proteins bound to the promoter to form activated transcription initiation complexes. Nagpal et al. (1992) demonstrated the presence of an AF-2 region in the E domain of RXR and RAR. An AF-1 region in the A/B domain was shown to modulate the activity of the ligand-inducable AF-2 in a promoter context-specific manner (Nagpal et al., 1993). For example, a chimeric protein RARα1(regions A, B and C)-RARγ1(regions D, E and F) activated RAREs normally activated by RARα1, and vice versa (Nagpal et al., 1992). To conclude, AF-2 transactivation through co-operation with other cellular proteins on the promoter, is modulated by AF-1 interaction which governs promoter specificity (Figure 1.3.5). Innumerable combinations of RXR and RAR isoforms that may dimerise, each defining promoter specificity, demonstrates the vast array of possible responses that may be derived from activation by a single ligand, RA.

1.3.3.5 Interactions with non-nuclear receptor proteins

Little is known concerning protein interactions between DNA bound RXR homo- and heterodimers and the downstream transcription initiation complex., although LeDouarin et al. (1995) have recently shown an interaction between the murine RXR AF-2 and a protein TIF1. Transactivation by RXR-RAR heterodimers can initiate transcription via interaction with the TATA box-associated transcription factor protein TFIIID. This interaction, observed in transfected embryonal carcinoma cells, required additional activity from a protein that can bridge TFIIID with RAR. This protein was described as
having activity similar to the adenovirus protein E1A (Berkenstam et al., 1992). Study of VDR transactivation implicated a direct interaction between SP-1, AP-1 and Oct-1 transcription factors as well as there being, in some instances, a further adapter protein (akin to the E1A-like protein described above) (Liu and Freedman, 1994). A direct interaction between VDR and TFIIB had been reported by MacDonald et al. (1995) that was ligand independent. The same study revealed that the interaction between RXR-VDR and TFIIB did not involve contact between the RXR portion of the heterodimer.

The interaction of RXR which is not bound to DNA with non-nuclear receptor proteins provides a further level of transcriptional control of certain gene promoters. RARα and RARβ have been shown to interact with and repress promoter activation by the Fos/Jun dimer that comprises the AP-1 transcription factor. This repression is achieved by preventing DNA binding of AP-1 (Schüle et al., 1991). More recently, Salbert et al. (1993) reported the ability of RXRα to interact with AP-1 in repressing transcription of the TGFβ1 promoter. As with RAR inhibition of AP-1 activity, RXRα is not bound to a DNA response element. It is noteworthy that RXRα appears to interact with AP-1 as a homodimer, whereas RAR is monomeric during AP-1 interaction (Salbert et al., 1993; Schüle et al., 1991). Additionally, 9-cis RA greatly enhances RXRα-AP-1 association. Specific mutations of RXRα indicated that anti-AP-1 activity was situated in the C-terminal portion of the LBD associated with the C-terminal heptad repeat, a probable leucine zipper. In particular, a point mutation of L-422 to Q (glutamine) resulted in 80% loss of RXRα anti-AP-1 activity. Other point mutations within this region resulted in greater than 50% loss of anti-AP-1 activity (Salbert et al., 1993).

1.3.4 Expression of the retinoid X receptor in foetal and adult tissues

Retinoic acid has been known to be important in controlling embryonic development in mammals. Roles for RA include formation of the nervous system, limb morphogenesis and craniofacial differentiation. The homologue of RXR in Drosophila, usp, has been shown to dimerise with the ecdysone receptor (EcR), which is involved in signalling the
stages of metamorphosis (Yao et al., 1993; Karim et al., 1993). The close sequence homology of *usp* with RXR and EcR with FXR demonstrates the capacity of this mechanism to signal a precise sequence of developmental events that has allowed for its evolutionary conservation between insects and mammals. The expression of RAR and RXR have been extensively studied in chick and mouse embryos (for a review of RAR expression see Rowe et al., 1992). Excess levels of RA lead to abnormalities in embryonic development such as craniofacial malformation and digit deletion in the limbs (Kochhar, 1973). Local application of RA to the anterior region of the developing chick limb bud results in mirror image duplication of the wing which can also be produced by transplanting the posterior region of the limb bud to the anterior region (Tickle et al., 1982; Saunders and Gasseling, 1968). Study of embryonal murine RXR expression showed RXRα and RXRβ to be expressed in a wide range of tissues including liver, skin, intestine and the face, whereas RXRγ was more restricted to discrete areas of the central nervous system and muscle (Mangelsdorf et al., 1992; Dollé et al., 1994). Mice with targeted disruption of RXR and RAR isoforms displayed lethal abnormalities of the eye and heart. RXRα−/− mice had thinned myocardium and a defect of the ventricular septum arising from early differentiation of myocytes (Kastner et al., 1994; Sucov et al., 1994). Extensive eye defects included shortened ventral retina, thickened cornea and ventral rotation of the lens (Kastner et al., 1994). Double knockouts of RXRα, RARα and RARγ extended the eye and heart abnormalities (Kastner et al., 1994). Similar defects are observed in vitamin A deficient embryos, establishing genetic evidence that dietary retinol is the source of RXR and RAR ligand.

In adult murine tissues expression of RXRβ is widespread, while RXRα expression is restricted to liver, skin and kidney tissues. RXRγ expression is restricted to muscle and heart tissues (Mangelsdorf et al., 1992; reviewed by Mangelsdorf et al., 1994). Recently, Saltou et al. (1995) observed that targeted expression of a dominant negative RAR in mice inhibited the development of skin, blocking the switch of keratin expression from
immature K1 and K14 to mature K10 and K5. The effect of this was to prevent the full formation of outer skin layers like the horny, granular, spinous and granular layers.

1.3.5 Expression of retinoid X receptor in haemopoietic cells

Treatment of myeloid leukaemias with retinoic acid, and its subsequent complete remission, has strongly implicated the involvement of retinoid receptors in overcoming the differentiative block in these cells. Indeed, treatment of the HL60 cell line with RA will lead to granulocytic differentiation (see section 1.1). RA does not, however, represent a miracle cure since recurrence of the leukaemia will occur up to six months following RA treatment of the patient (reviewed by Warrell et al., 1993). Li et al. (1994) have shown that RA-resistant HL60 cells, derived from chronic treatment of cells with RA, contain mutated RARα. It was established that in the majority of acute promyelocytic leukaemia (APL) patients there existed a chromosomal translocation of chromosome 15 to chromosome 17, t(15;17) (Rowley et al., 1977; reviewed by Grignani et al., 1994). This translocation was shown to result in the expression of a fusion protein arising from RARα and PML encoding genes located at the chromosomal breakpoint loci (de Thé et al., 1990). It was originally believed that the fusion protein, PML-RAR, acted as a dominant negative RAR. However, recent understanding of the function of PML has suggested that it is a combination of PML and RAR disruption that leads to leukaemogenesis. The PML protein contains structural features that implicate its role in control of transcription, including a DNA-binding cysteine rich region (RING finger motif), two zinc fingers and a coiled coil region (for dimerisation)(Kakizuka et al., 1991). In normal myeloid cells PML is localised in the nucleus as part of a subnuclear domain, known as a PML oncogenic domain (POD), which also contains RNA and other proteins associated with transcriptional control (Dyck et al., 1994; Weis et al., 1994; Koken et al., 1994). Within these PODs, PML is homodimerised. In APL cells, PML staining is dispersed throughout the nucleus and cytoplasm, heterodimerised with PML-RAR. Following incubation of these cells with RA, PODs are reformed and cells differentiate (Dyck et al., 1994; Weis et al., 1994). Earlier work by Perez et al. (1993) had shown that
RXR could heterodimerise with PML-RAR in vitro and in transfected Cos cells, suggesting RXR is sequestered in the cytoplasm away from normal function. They also demonstrated that D3-dependent activation of a VDRE was inhibited. In support of PML-RAR as a dominant negative RAR, Weis et al. (1994) described the co-localisation of RXR with PML-RAR in APL cells but could not be detected with PML structures following RA treatment. Hence, not only does PML-RAR act as a dominant negative RAR protein, it also acts as a dominant negative PML protein, preventing formation of PML homodimerisation and POD formation. Other, less common APLs have arisen from translocations involving RARα with other transcription factor-like proteins such as PLZF (t[11;17]; Chen et al., 1993).

Granulocytic differentiation of HL60 cells, following RA treatment, is signalled via RARα (Collins et al., 1990). Expression of a dominant negative RARα in HL60 cells results in the loss of their ability to differentiate. This same mutated RARα, when transfected into a murine multipotent haemopoietic progenitor cell line, FDCP mix A4, alters these cells' normal (predominantly) neutrophilic differentiation to mast cell/basophilic differentiation (Tsai et al., 1992). Hence, the importance of RARα in controlling the mode of haemopoietic differentiation would seem clear. The use of synthetic retinoids have shown that differentiation is signalled specifically by RXR-RAR heterodimers. Dawson et al. (1994) used a RAR-specific retinoid analogue TTNPB and a RXR-specific retinoid analogue SR11217 to demonstrate that only TTNPB was capable of inducing differentiation of HL60 cells.

Incubation of HL60 cells with 9-cis RA for greater than six days leads to induction of apoptosis of the differentiated cells. With the use of receptor specific retinoids it was demonstrated the neither TTNPB (RAR-specific) or LGD1069 (RXR specific) could induce apoptosis on their own. However, sequential treatment with TTNPB, to promote differentiation, followed by LGD1069 to promote apoptosis, indicated that differentiation is signalled by RXR-RAR heterodimers and apoptosis is signalled by RXR-RXR
homodimers (Nagy et al., 1995). It would seem likely that differentiation is induced by activation of genes containing a DR-5 or DR-2 RARE in their promoter, and apoptosis is induced by activation of genes containing a DR-1 RARE in their promoter. Further to this, two proteins seen to be associated with apoptosis have been studied. BCL-2 is a membrane associated protein which has been implicated in the suppression of apoptosis (Benito et al., 1994). Davies (1995) has shown that RARγ has a suppressive effect on the expression of this protein. Furthermore, tissue transglutaminase (TGase) is seen to accumulate in HL60 cells prior to apoptosis (Chiocca et al., 1989). Study of the murine TGase homologue has revealed a putative DR-5 RARE while RXR-specific agonists upregulate TGase expression in HL60 cells, indicating that RXR activates TGase expression through a bifunctional DR-5 RARE (Davies, 1995). The importance of receptor-specific retinoid analogues, not only as a tool in dissecting the activities of the pan-agonist 9-cis RA, also has implications for their use in a clinical context. Problems of toxicity and leukaemic relapse with apparent RA resistance make receptor-specific retinoid analogues an attractive option in anti-cancer therapies. Recently, the RXR-2c specific retinoid has begun clinical trials for leukaemia treatment. The same principal of using receptor-specific analogues has also been applied to produce D3 analogues, so-called deltanoloids, which overcome the highly undesirable side effects of D3 administration.

A role for RXR in T-cell function was demonstrated by Yang et al. (1993) whereby activation-induced T-cell apoptosis, the mechanism of thymic clonal deletion (negative selection), can be inhibited in vitro by treatment with 9-cis RA. Activation-induced apoptosis requires the accumulation of the protein Fas (CD95) bound to its agonist Fas-ligand (Brunner et al., 1995). The effect of 9-cis RA, in contrast to the induction of apoptosis in granulocytes, has been shown to be due to the repressive effect of RXR on the Fas-ligand promoter, which in turn will not be available for Fas association and subsequent signalling of apoptosis.
Since their discovery in 1990, the retinoid X receptors have been shown to play a central role in mediating development and differentiation of foetal and adult tissues. The small number of ligands that can interact with RXR, either directly in the case of 9-cis RA, or indirectly via other nuclear receptors, signal a vast number of possible cellular responses. This is due to the variety of response element interactions and cell-specific non-receptor protein interactions. The importance of RXR in the mediation of haemopoietic cell development has become clear through studies of APL cells and the HL60 cell line, particularly with respect to granulocytic differentiation and apoptosis. Little attention has been paid to the role of retinoid receptors in monocytic differentiation. This issue, however, is addressed in this thesis (see section 1.4).
1.4 Aims of this thesis

The aims of this thesis were to study the regulation of transcription of certain specific genes during monoblastic differentiation and peripheral monocyte/macrophage function. Two genes associated with this process have been characterised using U937 cells as a model for monoblastic differentiation. Firstly the c-fgr proto-oncogene, which serves as a useful marker of successful differentiation. The myeloid-specific promoter of c-fgr was analysed in an attempt to delineate the transcription regulatory mechanisms that are activated following induction of U937 differentiation. Specifically, the aim of this work was to identify sequences of the c-fgr promoter that are required for transcriptional activation of this gene.

Secondly, the retinoid X receptors have been shown to be important in regulating gene transcription in many cell and tissue types. In particular, their role in myeloid differentiation is evident from the ability of RA and D3 to differentiate normal and leukaemic myeloid cell lineages. The intention was to modulate the endogenous expression of RXRα and RXRβ in U937 cells by establishing stably transfected cell lines. By studying the effects of altered expression of RXRα and RXRβ on the transfected cell it was hoped to determine the role played by these genes in regulating gene transcription in the monocyte/macrophage lineage.
CHAPTER 2

MATERIALS AND METHODS
Chapter 2 Materials And Methods

Appendix 2 describes the constituents of solutions referred to in this section.

2.1 Screening of a Genomic Library

2.1.1 Primary Screen

A human genomic library (a gift from D. Kioussis, National Institute for Medical Research, London)(Kioussis et al., 1987), consisting of cosmid vector (cos202) containing 30-40kb Sau3A partial digest fragments of human genomic DNA, transformed into E. coli (ED8767), was plated at a density of 200 000 cells per plate on to three 250x250mm square plates containing LB+amp agar with Hybond N (Amersham International, Amersham, Bucks, UK) nylon membrane placed on its surface. Using a glass spreader the cells were evenly spread over the surface of the membrane and the plates incubated overnight at 37°C. These three plates were the master plates.

The membrane of each master plate was lifted from the plate and placed on 3MM paper (Whatman International Ltd., Maidstone, Kent) and a fresh dampened membrane placed on top, sandwiching the colonies and thus transferring cells to the fresh membrane. Using a syringe needle and Indian ink a series of marks were made by piercing holes through both in order to allow alignment at a later stage. The duplicate membrane was placed on to a fresh LB+amp agar plate (cells upward) as above and incubated overnight. This procedure was repeated for a second fresh membrane. The master membranes were placed on to fresh plates containing 20% glycerol (v/v) and stored at 4°C. The following day, duplicate membranes were individually removed from the plates, floated onto a dish containing Denaturation Solution for 7mins, lifted out and floated on three changes of Neutralising Solution for 3mins each. When this was complete, the cells on the membrane were then gently wiped off using a damp tissue. The filters were allowed to air dry and the DNA was UV-cross linked to the membrane according to the manufacturer's instructions (UV Stratalink 2400, Stratech).
The membranes were prehybridised in Southern prehybridisation mix at 65°C for 4 hours before adding the exon M4-specific probe, Probe 1 (see section 2.3), and the membranes hybridised overnight at 65°C. The membranes were washed at 65°C as follows: (i) Twice in 6xSSC, 0.5% (w/v) SDS for 30mins, (ii) Twice in 2xSSC, 0.5% SDS for 30mins and (iii) Twice in 0.1xSSC, 0.5% SDS for 60mins. All hybridisations were performed in an oven using cylindrical bottles on a rotisserie (Hybaid) turning at approximately 6rpm. Membranes were then exposed to X-ray film (X-OMAT, Eastman Kodak Company, Rochester, NY.) overnight at -70°C. After developing, the film was examined for duplicate signals.

Positive clones were isolated by placing the master membrane on a light box, and then, using a sterile tooth-pick, scrape off cells from the region on the master plate where the positive cosmid clone lay. The tooth-pick was then transferred to an Eppendorf tube containing 200μl LB+amp broth. It was assumed that approximately 10^6 cells were transferred to the broth. A secondary screen would be required in order to be able to isolate a single colony with the appropriate cosmid.

2.1.2 Secondary Screen

From the 200μl LB+amp-broth containing the picked clone, three dilutions were plated on to 145mm diameter petri dishes containing Hybond N membrane on LB+amp agar. These were incubated overnight. The same procedure as for the primary screen (using duplicate plates for each dilution) was performed except that membranes were hybridised with a probe specific to exons M4, 1b, 2, 3 and 4. (see section 2.3). Single positive colonies were picked from the secondary screen and grown to allow cosmid DNA extraction (see section 2.9).
2.2 Restriction mapping of cosmid clones.

5-10μg of DNA was single and double digested, for example with XhoI, BglII, XhoI+BglII, and incubated overnight at 37°C. All restriction enzymes used were manufactured by Gibco-BRL (Paisley, UK). After incubation, 4μl Loading Buffer was added to each reaction and mixed well. Reactions were electrophoresed on large (250x190mm) 0.7% (w/v) agarose gels (made with TBE) for 3-4 hours at 4V.cm⁻¹ until bands were well separated. The gel was photographed under UV light. A λ1kb ladder (λ1kb)(Gibco) or HindIII digested λ (λHindIII)(Gibco) were used as size markers and were run alongside the digested DNA. The gel was soaked in 0.1M HCl for 3mins to partially depurinate the DNA. The gel was placed in 0.5M NaOH for 30mins to denature DNA within the gel prior to blotting.

Five Hybond N membranes were cut to size and labelled 1 to 5. A capillary blot system was set up. A series of blots were taken so as to give 5 identical membrane blots from the same gel as follows:

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Blotting time</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>15mins</td>
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<tr>
<td>2</td>
<td>7mins</td>
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<tr>
<td>3</td>
<td>3mins</td>
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<tr>
<td>4</td>
<td>3mins</td>
</tr>
<tr>
<td>5</td>
<td>overnight</td>
</tr>
</tbody>
</table>

The membranes were allowed to dry before UV cross-linking.

Membranes were hybridised using various probes (Probes 1 to 6, see section 2.3), washed (as described for cosmid screen hybridisation) and exposed to X-ray film at -70°C.

2.3 DNA probes

Probe 1 (Figure 2.1)

Plasmid pFM2 (Figure 2.1) contains the complete c-fgr cDNA insert derived from an IFNγ-treated HL60 cell cDNA library (Patel and Brickell, 1992). This plasmid was
digested with *SstII* and concentrated by precipitating with ethanol. A polymerase chain reaction (PCR) was set up containing primers specific to exon 1b (Primer 1b: 5'ACGTGCCTGTATGAGCG3') and the multiple cloning site (Primer SK 5'CTAGGTGATCAAGATCT3') proximal to the 5' end of the insert. The reaction was performed in the presence of 30μCi 32P-αdCTP at 3000Ci/mmol (NEN Dupont, Stevenage, Herts, UK) in order to radio-label the resulting exon M4-specific PCR product. The reaction mix was as follows: 50ng pFM2 (SstII digested); 360ng forward primer (SK, Figure 2.1); 360ng reverse primer (1b, Figure 2.1); 10μl 10 x Taq DNA polymerase buffer (Gibco); 1.3μl 10mM dNTPs; 150μCi 32P-αdCTP; ddH2O to 100μl; 1 unit Taq DNA polymerase (Hoffmann-LaRoche, Baslé, Switzerland). The reaction mix was overlaid with 100μl mineral oil. Reactions was performed as follows: 4mins at 94°C, followed by 35 cycles comprising 1min at 94°C, 1 min at Tm-X°C and Y seconds at 72°C (X is between 3 and 5°C below the melting temperature [Tm] of the oligonucleotide primer with lowest Tm, and Y is the extension time, which was approximately 20 seconds for the 100bp Probe 6 PCR product). The primer's Tm (in °C) was determined by the calculation: 4(number of G and C nucleotides) + 2(number of A and T nucleotides). When the reaction was completed, the mineral oil was removed by addition of 100μl chloroform, mixed and centrifuged and the upper aqueous phase removed. The PCR product was purified from unincorporated isotope and unused primer by spinning the solution through a G-50 Sephadex (Sigma, Poole, Dorset, UK) column. The specific activity of the probe was then estimated by trichloro-acetic acid (TCA) precipitation and scintillation counting (see section 2.4).

The PCR is liable to produce spurious products that may give rise to radio-labelled sequences that have the ability to hybridise to cosmid sequences, rather than for exon M4. To avoid DNA polymerase "over-running" primer SK, pFM2 was cut with *SstII*. Also, a dot blot (see section 2.14) was made using pSK (which contains similar sequences to cos202) as a negative control and pFM2 as a positive control.
Figure 2.1. Specificity of probes 1 to 6 (see text for details).
This dot blot was included in each hybridisation using Probe 1 to be assured of the probe’s exon M4-specificity.

Probe 2 (Figure 2.1)

pFM2 was double digested with *EcoRI* and *HinCII* and of the resulting 3kb, 2.4kb and 0.57kb fragments, the 0.57kb fragment was oligolabelled (see section 2.4). This fragment contained the 5’ region of pFM2 insert including exons M4, 1b, 2, 3 and part of 4.

Probe 3 (Figure 2.1)

The 2.4kb fragment of the pFM2 digest, as described for Probe 2, contains the 3’ region of the insert which was oligolabelled. This probe was specific for exons 4 to 12.

Probe 4 (Figure 2.1)

B2 is a *BamHI* fragment containing exon 1a, which was subcloned from the cosmid cF2.3 (Patel *et al.*, 1990b). It was digested with *BamHI* and *PstI* and the 450bp fragment of B2 (5’ end) was oligolabelled. This probe was specific for exon 1a and flanking genomic sequences.

Probe 5 (Figure 2.1)

The plasmid Bluescript SK+ (pSK)(Stratagene, San Diego, CA,) was digested with *TaqI* and the 1.5kb fragment containing sequences for the ampicillin resistance gene, was isolated and oligolabelled.

Probe 6 (Figure 2.1)

pB1-1 was digested with *PstI* and *HinCII* and the resulting 130bp fragment, from the 5’ region of the insert, was oligolabelled.
2.4 Oligolabelling of DNA with $^{32}$P-$\alpha$dCTP

DNA labelling was based on the method described by Feinberg and Vogelstein, (1989). The DNA to be labelled was digested with the appropriate restriction enzymes and run on a 0.7% (w/v) low melting point (LMP) gel made with TAE buffer. After running (at 4V/cm) until bands were clearly separated, the band of DNA to be labelled was excised with a scalpel blade and placed into an Eppendorf tube. ddH$_2$O was added at 3ml per gram of gel slice and boiled for 7min. This solution was be stored at -20°C until required. The fragment was thawed and boiled for 5min and then put into a 37°C water bath for 5min. From this the following reaction was then set up: 33µl melted gel slice, 10µl Oligolabelling Buffer, 3µl $^{32}$P-$\alpha$dCTP (NEN-Dupont) and 2 units of Klenow DNA polymerase (Gibco). This reaction was incubated overnight at room temperature. The probe was purified by adding 20µl 4M sodium acetate, 130µl TE and 500µl ethanol to precipitate the probe. This was placed at -20°C for 30mins and then pelleted in a microcentrifuge for 10mins. The supernatant was discarded and the pelleted probe was resuspended in 100µl ddH$_2$O. The activity of the probe was estimated by TCA precipitation. 5µl of 10% (w/v) bovine serum albumin (Sigma) was added to a fresh Eppendorf tube and 1µl resuspended probe was placed on the side of the tube. 1ml of 10% TCA was added, mixed and placed on ice for 10mins. A glass-fibre filter disk (Whatmann) was placed on a filtration apparatus and the TCA/probe/BSA slowly pipetted onto it. The filter was placed into 5ml of scintillation fluid (EcoScint O, National Diagnostics, Atlanta, GA.) and the activity measured in a liquid scintillation counter (LKB Pharmacia, Uppsala, Sweden). The probe was used in hybridisation solution at a specific activity of 10$^6$cpm/ml.

2.5 Procedure for sub-cloning of specific DNA fragments

Both the plasmid vector (2-10µg) (eg pGL-2basic) and the plasmid/cosmid (10-20µg), containing the fragment to be subcloned, were digested with the appropriate restriction enzymes. It was important that equimolar amounts of vector and insert DNAs were used. The DNAs were run on a 0.7% (w/v) LMP agarose gel in TAE buffer, from which the
fragment and vector were excised and placed into an Eppendorf tube. 3 ml of NaI (GeneClean kit, Bio101, Stratech Scientific, La Jolla, CA.) per gramme of gel slice were added to the Eppendorf and heated in a 45°C water bath for 5 mins until the gel had dissolved. The DNA was purified using the GeneClean kit (Bio 101) according to the manufacturer's instructions and eluted in 15 µl ddH2O.

The ligation reaction was set up as follows: 3.5 µl insert DNA; 3.5 µl plasmid DNA; 2 µl 5x T4 DNA ligase buffer (Gibco); 1 µl 5 mM ATP; 1 unit T4 DNA ligase (Gibco), and incubated overnight at 15°C. For ligations where two enzymes were used, for example a PstI site at one end and a Smal site at the other end, equimolar concentrations of vector and insert DNA were used. If there was only one enzyme used, recircularisation of the vector was very likely and so the insert concentration was increased to between 2 and 5-fold to the vector concentration. For blunt ended ligations, the concentration of ATP was reduced to less than 0.1 mM, and 5 µl of T4 DNA ligase were used. For pSK, recircularisation will result in the production of functional β-galactosidase in transformed cells. On an LB+amp agar plate containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)(Sigma) these colonies will appear blue whereas cells with pSK containing an insert will be white, since the insert interrupts the lacZ' gene.

Competent cells were transformed (see section 2.10) with 5-10 µl of ligation mix, or with 10 ng of a control plasmid in order to judge the ligation efficiency. The cells were incubated overnight on LB+amp agar plates (containing 0.5 µg/ml X-Gal). Two LB+amp agar plates were prepared, one of which had a Genescreen+ filter disk (NEN Dupont) placed on top (Grunstein and Hogness, 1975). This disk had a grid drawn on it in pencil, each square of the grid numbered. A similar grid was drawn on the underside of the other duplicate plate so that each colony (representing a single ligated clone) could be picked using a sterile tooth-pick onto both the filter plate and the duplicate plate. Also on the filter plate were included positive and negative control cells: 5 µl thawed glycerol stock of cells containing either the vector or the insert. Both plates were incubated overnight. The
following day, the filter was removed from the filter plate (the duplicate placed in 4°C) and floated on Denaturation Solution for 7mins followed by twice on Neutralising Solution for 3mins each. Cell debris was then gently wiped off using a tissue. This was dried and hybridised with a 32P-labelled probe specific for the inserted DNA fragment. Following hybridisation, the filter was exposed to X-ray film and developed. Colonies which gave a positive signal were picked from the corresponding grid on the duplicate plate, inoculated into 10ml of LB+amp broth and incubated overnight. The following day, plasmid was extracted from the cultures (see section 2.13) and digested with appropriate restriction enzymes to determine (i) that the correct insert was present, (ii) that only one insert was ligated and (iii) the orientation of the insert was correct.

To derive blunt ends from restriction digests that produce 5' protruding ends, 10mM dNTPs and 2 units of Klenow polymerase were included in the digestion reaction, "filling in" the ends. For 3' protruding ends to be blunted, 2 units of Klenow polymerase was incubated with purified digested DNA with restriction digest buffer in the absence of dNTPs. Under these conditions, Klenow polymerase acts as a 3'-exonuclease, removing single-stranded DNA and producing a blunt ended fragment.

2.6 Production of competent E. coli cells
A 500ml culture of the E. coli strain DH5α was grown in LB broth (no ampicillin) to a density of OD580 = 0.4. The cells were harvested by centrifugation at 3000rpm, 10min, at 4°C. As much medium as possible was removed before resuspending cells in 15ml of ice cold RFBI and placed on ice for 15min. The cells were spun down again as above and resuspended in 4ml of ice cold RFBII. 500μl aliquots were transferred to Eppendorf tubes and snap-frozen in liquid nitrogen before immediately transferring to -70°C for storage.

2.7 Transformation of plasmid DNA into competent E. coli strain DH5α
An aliquot of competent cells was thawed slowly and placed on ice immediately. To 250μl of the aliquot were added 50ng of plasmid DNA (or 5 to 10μl of ligation mix) and
placed on ice for 30-45min. This was placed in a 42°C water bath for 2min and returned to ice for 10min. 1ml of LB+amp broth was added and incubated for 30-45min at 37°C. The cells were then centrifuged for 5min, the supernatant removed and the cells resuspended in 200ul of fresh LB+amp broth. This was spread on an LB+amp agar plate using a glass spreader and incubated overnight, with transformed cells forming colonies which can then be isolated.

2.8 Sequencing of DNA

Plasmid DNA was sequenced by dideoxy-chain termination (Sanger et al., 1979) using the ‘Sequenase Version 2.0’ (USB, United States Biochemical Corp., Cleveland, Ohio) double stranded DNA sequencing system according to the manufacturer’s instructions. 10ug of plasmid DNA were made up to 20ul with ddH₂O and denatured with 2ul 2M NaOH for 10mins. The mixture was neutralised with 8.8ul 5M ammonium acetate and the single stranded DNA precipitated with 124ul of ethanol and centrifuged for 10min. The DNA pellet was washed once with 70% (v/v) ethanol resuspended in 7ul ddH₂O, 2ul Sequenase Buffer (USB) and 1ul 10pmol/ul oligonucleotide primer, heated to 65°C for 5mins and allowed to cool slowly to room temperature. To the annealed template-primer were added 1ul 0.1M DTT, 2ul dNTPs, 1ul Sequenase DNA polymerase (all USB) and 1ul 35S-αdATP. This labelling reaction was incubated at 37°C for 10mins. 4ul aliquots of the labelling reaction were transferred to 4 Eppendorfs containing 2.5ul 8uM ddATP termination mix, ddCTP termination mix, ddTTP termination mix or ddGTP termination mix (all USB). This termination reaction was incubated at 37°C for 5mins. The reaction was stopped using 4ul Stop Solution (USB). DNA from these reactions were resolved by polyacrylamide gel electrophoresis (described below).

Two glass sequencing gel plates were washed carefully, dried and assembled with spacer strips between them. The plates were taped together so that the acrylamide could be poured between the plates without any leakage. The gel mix was as follows: 3.6ml 10xTBE, 23.75ml Diluent (National Diagnostics), 8.65ml Sequagel Concentrate
(National Diagnostics), 115μl ammonium persulphate at 1.12g/5ml (made freshly or stored in frozen aliquots) and 15μl N, N, N', N'-tetramethylethylenediamine (TEMED) (Biorad, Hemel Hempstead, UK); Mixed and poured immediately. It was important that no air bubbles were caught in the gel. Finally the comb was placed upside-down so as to give a flat edge at the top of the gel, below the eared plate upper edge. Once the gel had polymerised (after about 30 min) the tape was removed from the bottom edge, the plates clamped into a gel tank, and TBE added to the upper and lower reservoirs. The comb was eased out of the gel, reversed and replaced into the gel with the teeth pointing down. The points of the teeth were pushed into the gel to about 1mm depth, forming the wells. Alternate lanes were loaded with 4μl Stop Solution to check for leaking wells before pre-running for 30mins at 30Watts to warm the gel up. Stopped termination reactions were placed in a 72°C water bath for 2mins and placed immediately on ice prior to loading. 4μl of sample were loaded onto the gel in the order A, C, G and T and run under the same conditions as the pre-run until the faster moving (pale blue) dye reached the bottom of the gel resolving the sequence between approximately 50 to 150bp downstream of the primer binding site. Run times were extended to obtain sequences further downstream. When the run was complete, the gel apparatus was dismantled, the tape removed and the eared plate carefully lifted off leaving the gel on the plate below. This was immersed in a tray containing fixative (10% acetic acid, 10% methanol [v/v]) for 15-20min. The plate was laid on the bench and a piece of 3MM paper lowered onto it and then slowly lifted again, bringing the gel (now stuck to the paper) with it. This was then dried on a vacuum gel-drier (Biorad) for 1 hour. After drying, the gel was exposed to X-ray film overnight and the sequence read from the film.

2.9 Small and large scale plasmid preparation

2.9.1 Small scale plasmid preparation

Bacterial cells were grown overnight in 10ml of LB+amp broth and harvested by centrifuging at 3000rpm, 10min at 4°C. The medium was removed and the cells resuspended in 200μl Solution I, transferred to an Eppendorf tube and placed on ice.
400μl of freshly made Solution II were added, mixed and left on ice for 5min. 200μl of Solution III were added, mixed and left on ice for 10min. The Eppendorf tube was centrifuged at 12 000g for 15min at 4°C and the supernatant transferred to a fresh Eppendorf and added to this was 1μl of DNase-free RNase (10mg/ml)(Boeringer Mannheim, Lewes, UK) for 1 hour. Plasmid DNA was then purified using the GeneClean kit (Bio101, Stratech) according to manufacturer’s instructions. The plasmid DNA was resuspended in 20μl ddH₂O and stored at -20°C.

2.9.2 Large scale plasmid preparation

Large scale plasmid preparation was made by the alkaline lysis and polyethylene glycol (PEG) precipitation method as described by Sambrook et al. (1989). Bacteria containing the desired plasmid were grown in 800ml of LB+amp broth overnight. Cells were harvested by centrifugation at 3000rpm for 15mins, the pellet washed in 20ml SET and respun. Cells were resuspended in 18ml Solution I to which was added 2ml fresh lysozyme (10mg/ml in 10mM Tris, pH 8) and 40ml Solution II. These were mixed and incubated at room temperature for 10mins. 20ml Solution III were added and the mixture stored on ice for 10mins before centrifuging at 3000rpm for 15mins at 4°C. Filtered supernatant was mixed with 2/3 volume propan-2-ol to precipitate nucleic acids, which were pelleted by centrifugation at 5000rpm for 15mins at 4°C. The pellet was dissolved in 3ml TE. This was transferred to a 15ml corex tube and 3ml ice-cold 5M LiCl added. This precipitated RNA, which was removed by centrifugation at 10 000rpm for 10mins. DNA was removed from the supernatant by ethanol precipitation, re-dissolved in 500μl TE containing 20μg/ml DNase-free RNase (Boeringer Mannheim) and incubated at 45°C for 1 hour. Plasmid DNA was precipitated from this solution by the addition of 500μl 1.6M NaCl containing 13% (w/v) PEG (BDH Merck, Poole, Dorset), incubated at 4°C for 5mins before centrifuging at 13 000rpm for 10mins. The pellet was resuspended in 200μl ddH₂O, phenol/chloroform extracted, ethanol precipitated and resuspended in ddH₂O at 2μg/μl.
2.10 Culture of the U937 cell line.

U937 cells (European Culture Collection of Animal Cell Cultures, Porton Down, Wilts, UK) were maintained in RPMI 1640 medium supplemented with 10% heat inactivated foetal calf serum, 50µM 2-mercaptoethanol, 100u/ml penicillin and 100µg/ml streptomycin, 10mM HEPES and 2mM L-glutamate (all Gibco). 10ml of cell culture at 5x10^5 cells/ml were split 1 into 9 twice weekly with fresh medium and incubated at 37°C, in 5% CO2. All cell culture was performed under aseptic conditions in a laminar flow hood.

Cells were treated with PMA (Sigma), at a concentration of 5-10ng/ml; human recombinant TNFα (Genentech, San Francisco, CA.) was used at 5-10ng/ml (30-100u/ml); D3 (a gift from M. Uskokovic, Hoffman-La Roche, Nutley, NJ.) at 100nM; all-trans RA (Sigma) and 9-cis RA (a gift from M. Klaus, Hoffman-La Roche, Basel, Switzerland) at 1µM (Faulkner et al, 1992). Since D3 and RA were diluted in ethanol, negative control experiments required the addition of no more than 0.1% ethanol.

2.11 Electroporation of U937 cells

U937 cells were grown to a density of 10^6 cells/ml in 100ml. These were then split 1 in 2 and incubated overnight. Cells were counted and pooled into a single 50ml falcon tube (Sterilin), washed once with PBS and then repelleted (1000rpm, 10min, 4°C). Finally, the pellet was resuspended in Hank’s Balanced Salt Solution (HBSS, Gibco) such that there were 250µl per electroporation containing 2.5x10^7 cells. Cells were kept on ice at all times. 250µl of cells and 35-50µg of DNA (in 20µl) were placed into an electroporation cuvette (Biorad) and put on ice for 5min before pulsing in a Genepulse (Biorad), set at 220V, 960µF. The time constance, K, was noted for each pulse. K was usually between 50 and 100 and should not have varied by more than 10 between samples in the same experiment. Cuvettes were then placed on ice for 10min. 1ml of medium was then added to the cuvette.
For transient transfections, the electroporated cells were transferred to 50ml flask containing 9ml of medium and incubated overnight. The following day any treatments were performed by removing 5ml of cell culture to a fresh 10ml flask and adding appropriate treatment with 5ml of fresh medium. After required time points cells were harvested for reporter gene expression assays. Since PMA induces adherence in U937 cells, PMA treatments were performed in 90mm petri dishes allowing adherent cells to be removed using a rubber policeman when harvesting cells. For stable transfections, the electroporated cells were transferred to a fresh 250ml flask containing 19ml fresh medium. The cells were incubated for 48hrs prior to the addition of selecting antibiotics.

2.12 Estimation of electroporation efficiency

To determine the efficiency of transfection for a single electroporation and hence be able to standardise samples from differing electroporations, the method described by Abken and Reifenrath (1992) was employed. 100μl of cell lysate were incubated with 2μl RNaseA (10mg/ml) (Boeringer Mannheim) for 30min and 2μl Proteinase K (10mg/ml, Sigma) (Boeringer Mannheim) for 1-2 hours at 45°C. 200μl of 20xSSC were added to the lysate and the whole solution applied on to Hybond N using a dot blot manifold (Bethesda Research Laboratories, Bethesda, MD.) attached to a vacuum pump. The dot blot was dried and an ampicillin gene- or luciferase gene-specific oligolabelled probe derived from pSK or pGL-2basic, respectively, were hybridised to the dots of extracted DNA. The dot blot was exposed to a phosphor-imager screen for 1 hour and analysed on a phosphor-analyser (BioRad GS-250 Molecular Imager) according to the manufacturer's instructions (BioRad).

2.13 Chloramphenicol acetyl transferase (CAT) assay

Transfected cells were harvested by drawing off the culture with a pipette into a universal and centrifuging at 1000rpm for 10min. The cells were washed once in PBS, resuspended in 100μl 0.25mM Tris-HCl (pH7.8) and then transferred to an Eppendorf tube. The cells were lysed by freeze/thaw lysis: 5min in liquid nitrogen followed by 5min in a 37°C
water bath, repeated three times. The cell lysate was centrifuged for 5min and the supernatant transferred to a fresh tube. The chloramphenicol acetyl transferase (CAT) reaction was performed as follows: 70μl 0.25M Tris-HCl (pH 7.8); 35μl ddH2O; 20μl cell lysate; 1μl ^14C-Chloramphenicol (NEN Dupont); 20μl 4mM Acetyl Coenzyme A (Boeringer Mannheim). Positive and negative control reactions consisting of 1 unit of CAT enzyme (Boeringer Mannheim) or 20μl 0.25mM Tris-HCl were included. Reactions were incubated at 37°C for 10-30min. The chloramphenicol and acetylated derivatives were extracted by addition of 1ml ethyl acetate, vortexing for 30sec and centrifuging for 5min. The top organic phase containing chloramphenicol was removed to a fresh Eppendorf and dried down in a freeze drier for 2 hours, the chloramphenicol precipitating about the edge of the tube. The chloramphenicol and acetylated derivatives were separated by ascending thin layer chromatography (TLC) by resuspending in 20μl ethyl acetate, spotting onto the TLC plate (Whatman) and placing in a chromatography jar containing 2cm 95:5 chloroform:methanol (v/v). When the solvent front reached the top of the plate it was lifted out of the jar and allowed to dry. The plate was then exposed to X-ray film for 1 to 2 weeks.

2.14 Luciferase assay

Cells were harvested as described for the CAT assay. After washing with PBS, the cells were then lysed with 250μl Lysis reagent (Promega Corp., Madison, WI), incubated at room temperature for 15min, the cell debris removed by 30sec centrifugation and the supernatant transferred to a fresh Eppendorf. 80μl of cell lysate was added to 400μl Luciferase assay reagent (Promega) and immediately placed into a luminometer (Turner Luminometer 20E, Sunnyvale, CA.) and the light emittance measured on a potentiometer over a period of 1min. Alternatively, samples were placed into a scintillation counter (LKB Pharmacia) programmed to detect bioluminescence over a period of 1min.
2.15 Isolation of mRNA from U937 cells

Extraction of total RNA was performed using the method described by Chomczynski and Sacchi (1987). U937 cells were seeded 1 in 10 with medium and incubated for 3 days. This culture was then split 1 in 2 and incubated overnight. 5 x 10^6 cells were harvested by centrifugation and resuspended in 1ml Solution D, 100μl 2M sodium acetate, 1ml phenol and 200μl chloroform:IAA before transferring to 2 Eppendorf tubes. After mixing by inversion the tubes were centrifuged at 10 000g for 20mins at 4°C. The upper aqueous layer was transferred to a fresh tube, mixed with 1ml propan-2-ol and stored for 1 hour at -20°C. The precipitated RNA was pelleted by centrifugation and resuspended in 300μl of Solution D and again precipitated with propan-2-ol. Finally the total RNA pellet was resuspended in 100μl ddH2O. Messenger RNA was isolated from the total RNA solution using the Promega Magnasphere PolyA+ Isolation System according to the manufacturer's instructions, and resuspended in 250μl ddH2O.

2.16 Northern blotting and hybridisation

RNA was blotted on to Hybond N membrane by electrophoresing the RNA at 4V/cm on a 1% agarose gel containing 2% formaldehyde (v/v) in a MOPS buffer and capillary blotted. The blot was prehybridised in Northern Prehybridisation Mix at 65°C for 4 hours prior to adding the riboprobe (see section 2.17) and hybridised overnight at 65°C. The following day the membrane was washed as follows: 6xSSC, 0.5% SDS; 3xSSC, 0.5% SDS; 1xSSC, 0.5% SDS; and 0.1xSSC, 0.5% SDS; all for 15mins at 65°C and then exposed to X-ray film for photography, and phosphor-imaging screens for quantitative analysis. To control for mRNA loading on the gel, the blot was re-hybridised with a probe specific to β-actin mRNA (Alonso et al., 1986).

2.17 Synthesis of RNA probes

The DNA which is to be transcribed must lie adjacent to either the viral promoters T7 or T3 DNA-dependant RNA polymerase binding sites. The plasmid pSK (into which all
RXR cDNAs had been inserted) had T7 and T3 promoters flanking the multiple cloning site. Prior to transcription, the plasmid was linearised using a suitable restriction enzyme such that the DNA was cut at the end distal from the transcription start site. For example, to produce an antisense RXRα riboprobe, the plasmid pSK(3-7)-RXRα was first linearised with a \textit{NotI} digestion and the transcription performed using T7 RNA polymerase. This DNA, cleaned by phenol extraction, was then referred to as the template.

Into an Eppendorf tube was placed 5μl 5x Transcription buffer (Promega), 0.5μl 1M DTT, 1.2μl ATP, 1.2μl GTP, 1.2μl UTP (each at 10mM), 1μl 50mM CTP, 2-3μg template DNA, 6μl \textsuperscript{32}P-αCTP, 10.9μl ddH\textsubscript{2}O, 0.5μl RNase inhibitor (RNasin, Promega), 1μl T3/T7 RNA polymerase (Promega). This was incubated for 40mins at 37°C. After this time a further 1μl RNA polymerase was added and the incubation continued for a further 40mins. The DNA template was then removed by the addition of 1μl 20mg/ml yeast tRNA, 0.5μl 1M DTT, 0.5μl RNasin and 0.5μl RNase free DNasel (Promega). This was the incubated for 10mins at 37°C. Finally, 5μl 4M NaCl, 4μl 1M DTT and 154μl ddH\textsubscript{2}O were added to the riboprobe and phenol extracted, precipitated with ethanol and used immediately in hybridisations.

\textbf{2.18 Establishment of stably transfected U937 cells}

U937 cells were electroporated with 50μg of plasmid DNA and incubated for 48 hours before adding selecting antibiotic (for pMEP-4 clones this would be 300μg/ml Hygromycin B [Boeringer Mannheim]). The antibiotic began to kill cells in an untransfected control plate after 5 days. After 7 to 10 days the cell suspension was layered on to Histopaque 1077 (Sigma) in a 20ml universal and centrifuged at 1500rpm for 20mins at room temperature. Healthy cells which remained at the interface between the medium and the histopaque were drawn off and pipetted into a fresh universal, washed in HBSS and resuspended in medium containing antibiotic. This was repeated between 3 and 5 days later depending on the extent of cell death. As soon as the cells
were clearly beginning to grow and had reached approximately $5 \times 10^6$ cells/ml, aliquots were frozen as stocks.

2.19 Cell proliferation assay
Aliquots of 100µl of a $2 \times 10^5$/ml culture of proliferating cells were placed into the wells of a 96-well plate (each treatment in quadruplicate) with 100µl medium containing the appropriate treatment. Plates were incubated for 66 hours before adding 36µl of $^{125}$I-deoxyuridine (dUR)(in HBSS) and incubated for a further 6 hours. Alternatively, after 48 hours, 10µl $^3$H-thymidine was added and followed by 24 hour incubation. Cells were harvested using a Titertek cell harvester (Skatron, Lier, Norway) on to paper disks which were then placed into a gamma counter (Packard Cobra II Autogamma, Pangbourne, Berks.) or a scintillation counter (LKB Pharmacia), and the CPM noted.

Percent inhibition of proliferation (% inhibition) in Figure 4.1.21 was calculated as follows:

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100 - \left[ \frac{\text{CPM treated}}{\text{CPM untreated cells}} \right] \times 100 \] = % inhibition

2.20 Receptor binding assay
2.20.1 Displacement assay
Proliferating cells were centrifuged at 1000 rpm and resuspended in RPMI at a final concentration of $2 \times 10^7$ cells/ml. 50µl of this suspension were aliquoted in to an Eppendorf containing 100fmol of $^3$H-9-cis RA (Amersham) and a range of concentrations of unlabelled-9-cis RA (0, 0.01, 0.03, 0.1, 0.3, 1, 3 or 10 µM) in RPMI. The cell suspensions were incubated for 1 hour at 37°C and then immediately placed on ice, 200µl ice cold HBSS added, mixed and centrifuged at 7000 rpm for 5mins. The supernatant was removed and the pellet resuspended in 200µl of HBSS and centrifuged as before. The pellet was resuspended in 200µl Binding assay lysis buffer and stored on ice for 5 minutes before repeating this step. At all stages the tubes were stored on ice where possible. Finally, the pellet was resuspended in 150µl ethanol and the radioactivity measured in a scintillation counter (LKB Pharmacia).
Results (in CPM) were converted to DPM, based on the efficiency of the counter, and plotted against unlabelled 9-cis RA concentration. Displacement of bound $^3$H-9-cis RA forms a sigmoid curve where 50% displacement occurs at a unlabelled concentration equivalent to the receptor binding affinity, $K_d$.

2.20.2 Saturation assay
Proliferating cells were centrifuged at 1000 rpm and resuspended in RPMI at a final concentration of $2 \times 10^7$ cells/ml. 50μl of this suspension were aliquoted into an Eppendorf containing a linear range of concentrations $^3$H-9-cis RA (3-100fmol) and 50μl RPMI. This set of tubes represented total binding. An identical set of Eppendorf tubes were set up (excluding 50μl RPMI) containing 50μl 20μM unlabelled 9-cis RA in RPMI. This set of tubes represented non-specific binding. A set of Eppendorfs containing 100μl RPMI and the appropriate quantity of $^3$H-9-cis RA as were used for each concentration to determine the precise amount used when plotting saturation curve. The tubes were incubated and processed as described in section 2.19.1. A saturation curve was plotted $^3$H-9 cis RA (fmol) bound against $^3$H-9-cis RA (fmol) added.

2.21 Staining of U937 cells for flow cytometry
Cells ready for staining were washed twice 10% normal rabbit serum (Gibco) in HBSS (NRS/HBSS)(w/v) and 0.1% sodium azide (w/v) before incubating in 10% NRS/HBSS on ice for 10mins. Cell concentration was adjusted to $2 \times 10^6$ cells/ml and 100μl aliquots placed into a ‘V’ bottomed 96-well plate (Falcon). The plate was centrifuged at 1500rpm for 5mins at 4°C, supernatant removed and into each well added 50μl the appropriate primary antibody ($1/100$ dilution). The plate was then incubated on ice for 5mins. The cells were washed twice with 100μl 10% NRS/HBSS before adding 50μl secondary antibody (fluorescent-labelled rabbit anti-mouse IgG [Dako Ltd, Bucks, UK], $1/40$ dilution). The plate was incubated on ice for 30mins before washing twice with 100μl 10% NRS/HBSS. Cell-antibody complexes were fixed with 4% paraformaldehyde/PBS...
and stored at 4°C. For flow cytometric analysis, cells were resuspended in 500µl HBSS and analysed on a Becton Dickinson FACscan according to manufacturer's instructions.
CHAPTER 3

REGULATION OF C-\textit{FGR} EXPRESSION IN U937 CELLS DURING DIFFERENTIATION
3.1 Results

Analysis of the promoter region of \textit{c-fgr} during differentiation of monoblastic cells would lead to a greater understanding of the transcriptional mechanisms that are active during this process. Hence, it was necessary to isolate the promoter region that functions in this system, using U937 cells as a model for monoblastic differentiation.

3.1.1 Isolation of cDNA clone containing exon M4

Comparison of cDNA clones derived from B-lymphoid cell lines with a cDNA clone derived from IFN\(\gamma\)-treated U937 cells (pFM2) showed there to be an alternative 5' exon: exon 1a in the B-lymphoid clone and exon M4 in the myeloid cDNA (Link \textit{et al.}, 1992; Patel and Brickell, 1992). Previous work by M. Patel had shown that the myeloid-specific exon M4 was not present in the genomic clone cF2.3 isolated during study of the B-lymphoid promoter (see section 1.2.2.3). Hence, it was necessary to screen a cosmid library of human genomic fragments for one containing exon M4.

3.1.2 Isolation of genomic clones containing exon M4

Using a 100bp probe specific for exon M4, derived from the plasmid pFM2 by PCR using primers immediately flanking exon M4 (see section 2.3), a cosmid library was screened for clones containing exon M4. This probe (rather than one containing other \textit{c-fgr} exons) was used in the primary screening in order to avoid re-isolating cF2.3 through downstream sequences. Three positive signals were identified (Figure 3.1.1). These were isolated and rescreened (the secondary screen) using a probe specific to exons M4 through to exon 4 (Figure 3.1.2)(see section 2.3). Six clones were isolated and digested with \textit{EcoRI}, \textit{BamHI}, or \textit{HindIII} (Figure 3.1.3). From these digests it was possible to determine that clones 1I, 1II and 2II were identical. Also, clone 3I had similar bands as 2II, indicating that they overlap. Clones 1I (renamed c1) and 3I (renamed c3) were then selected for further examination.
Figure 3.1.1. Primary screen autoradiograph showing hybridisation of positive clones with an exon M4-specific probe, indicated by arrows, that align with signals on an autoradiograph of a duplicate membrane (not shown).
Figure 3.1.2. Representative autoradiographs from the secondary screen. Arrows indicate the hybridisation of an exon M4-specific probe (a) that was reproduced on a duplicate membrane (b).
Figure 3.1.3. Restriction digestions of cosmid clone isolates II (c1), III, 2I, 2II, 2III and 3I (c3). *BamHI*: lanes 1, 6, 11, 15, 20 and 25. *EcoRI*: lanes 2, 7, 12, 16, 21 and 26. *EcoRI+BamHI*: lanes 3, 8, 13, 17 and 27. *HindIII*: lanes 4, 9, 14, 18 and 28.
3.1.3 Restriction digest mapping of genomic clones c1 and c3

By restriction digest mapping and hybridising with various $^{32}$P-labelled DNA probes (section 2.3 and Figure 2.1) a series of Southern blots were derived. From these data and partial restriction maps published by Patel *et al.* (1990b) and Gutkind *et al.* (1991), a map of c3 was generated (Figure 3.1.7). Cosmid clone c3 was found to completely overlap c1, extending to a *Sau3AI* restriction site immediately downstream of exon 12, whereas the 3' end of c1 lay immediately upstream of exon 12. Representative blots showing the mapping of exon M4 to a specific *XhoI* fragment and mapping of an *EcoRI* site 4.6kb upstream of exon M4 are shown in Figure 3.1.4 and 3.1.5 respectively. Subcloning of the 4.4kb *EcoRI* fragment, indicated in Figure 3.1.5, into pSK (by P. Kefalas) allowed for further mapping of this region. As shown in Figure 3.1.6, only *AccI* and *AflII* cut within the inserted fragment at positions 2.6kb and 0.8kb upstream of M4, respectively. Exon M4 was mapped to a position 11.1kb upstream of exon 1b. Both clones extended approximately 8kb upstream of exon M4. Cosmid cF2.3 overlapped c1 and c3 from immediately upstream of exon 1a and extending 30kb downstream (Figure 3.1.7). Having determined the position of exon M4 within a 1.6kb*BamHI* fragment (indicated in Figure 3.1.8) it was possible to further study the *c-fgr* myeloid-specific promoter region immediately upstream of this exon.

3.1.4 Sequencing of exon M4

Using flanking *BamHI* restriction sites, exon M4 and surrounding sequences were subcloned in the plasmid pSK to derive the construct pB1-1. Southern blot analysis of pB1-1, digested with *EcoRI, XhoI, Stul* and *Smal*, using an M4-specific $^{32}$P-labelled DNA probe confirmed the presence of M4 in pB1-1 (Figure 3.1.9). The pB1-1 insert was fully sequenced using primers synthesised in the laboratory.
Figure 3.1.4 Restriction digestion of cosmid c1 and hybridisation to an exon M4-specific probe. Lane 1, XhoI; 2, BglII; 3, XhoI+BglII; 4, XhoI+HindIII; 5 Lambda HindIII size marker; 6, XbaI; 7, PstI; 8, EcoRI; 9, SacI; 10, Lambda size marker. The arrow indicates the M4-containing XhoI fragment.
Figure 3.1.5. Restriction digestion of cosmid c1 and hybridisation to a probe specific to the 5' region of the pBl-1 insert. Lane 1, Xhol; 2, BglII; 3, Xhol+BglII; 4, Xhol+HindIII; 5, LambdaHindIII size marker; 6, XbaI; 7, PstI; 8, EcoRI; 9, SacI; 10, Lambda5 kb size marker. The arrow indicates the 4.4 kb EcoRI fragment cloned into pSK-Eco4.5.
Figure 3.1.6. (a) Restriction digest of pSK-Eco4.5. Lane 1, HindIII; 2, Sall; 3, AccI; 4, AflII; 5, RsaI; 6, KpnI; 7, Clal; 8, EcoRV; 9, EcoRI; 10, undigested; 11, Lambda1kb size marker. (b) Hybridisation of the AccI digest to a probe specific to the 5' region of pB1-1 (Probe 6).
Figure 3.1.7 The relative overlaps of cosmid clones c3, cF2.3 and c1 (a), and a restriction map of c3 (b).
Figure 3.1.8. Restriction digest of cosmid clones c1 (Lanes 1, 5, 9 and 13), cF2.3 (lanes 2, 6, 10 and 14) and c3 (lanes 3, 7, 11 and 15). LambdaHindIII size marker, lanes 4 and 12; pGEM size marker, lane 8. BamHI fragment subcloned into pB1-1 (lane 5) is indicated with an arrow.
Figure 3.1.9. Restriction digestion and hybridisation of pB1-1 to an exon M4 specific probe. Lane 1, BamHI; 2, Smal; 3, BamHI+Smal; 4, Lambda_HindIII size marker; 5, XhoI; 6, XhoI+BamHI; 7, XhoI+Smal; 8, Lambda_kb size marker; 9, Stul; 10, Stul+BamHI.
The primers used were (i) KS \((5'-\text{CGAGGTCGACGGTCTCG}-3')\) at 5' end the pSK poly linker, (ii) SK \((5'-\text{CTAGGTGATCAAGATCT}-3')\) at the 3' end of the pSK poly linker, (iii) M1-1 \((5'-\text{CAGTGATGGAGAAAAGC}-3')\) within exon M1 (Gutkind et al., 1991) at -347 to -330, with respect to the major myeloid start site, directing the sequence downstream, (iv) M3-2 \((5'-\text{ACTCGCGGCTGCTGGAG}-3')\) within exon M4 at -62 to -44 directing the sequence downstream, and (v) M3-3 \((5'-\text{CTGGAGCCCAATTCTGG}-3')\) within exon M4 at +120 to +103 directing the sequence upstream (Figure 3.1.10). From the derived sequence of the pBl-1 insert, the splice donor site of exon M4 was identified. In addition, a full restriction map was derived using the computer programme Microgenie (Beckman, Palo Alto, CA.). Link et al. (1992) noted two transcription start sites 99 and 98bp upstream of the Smal restriction site within M4. The sequence (Figure 3.1.11) was noted as having a GC content of 57.5% (from 0 to -772). Also, 30bp upstream of the transcription start site was the sequence AATAAA, a putative TATA-box (Link et al, 1992). Computer analysis using the PCgene programme (IntelliGenetics Inc., Mountain View, CA.) was used to search for consensus transcription factor binding sequences (Faisst and Meyer, 1992). This showed there to be 7 different putative transcription factor consensus binding sequences at thirteen locations (Table 3.1.1 and Figure 3.1.11). Computer sequence homology analysis with the EMBL sequence database revealed there to be two putative Alu repeat sequences which are also observed in the B-lymphoid promoter (Patel et al, 1990b).
Figure 3.1.10. Diagram of the insert in pH1-1, showing the location of primer binding sites used for sequencing.
Figure 3.1.11. Sequence of c-fgr promoter region (-1688 to 0bp) and exon M4 (+1 to +125bp). Exon M4 in stippled box; bold type, restriction sites used for sub-cloning; bold type in italics, consensus transcription factor binding sites; arrows show transcription start sites (Link et al., 1992); AATAA box in grey square; Alu like repeat sequences bracketed.
Table 3.1.1. Table of putative transcription factor consensus binding sequences found in the c-fgr myeloid promoter. N, any nucleotide (Faisst and Meyer, 1992).

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Concensus binding sequence*</th>
<th>Number of mismatches</th>
<th>Position</th>
<th>Inducer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-2</td>
<td>CCC(T)AACGGGX</td>
<td>1</td>
<td>-497 to -489</td>
<td>PMA, retinoic acid</td>
</tr>
<tr>
<td></td>
<td>CCCAAGGG/C(T)</td>
<td>1</td>
<td>-833 to -836</td>
<td></td>
</tr>
<tr>
<td>IRBP</td>
<td>AGTGCAC(G)T</td>
<td>1</td>
<td>-823 to -815</td>
<td>PMA</td>
</tr>
<tr>
<td></td>
<td>AGTGCAC(G)T</td>
<td>1</td>
<td>-1177 to -1171</td>
<td></td>
</tr>
<tr>
<td>ISGF2</td>
<td>CTTTCTC(T)TTT</td>
<td>1</td>
<td>-925 to -916</td>
<td>IFNα, IFNγ, prolactin</td>
</tr>
<tr>
<td></td>
<td>C(G)TTTCTCTTT</td>
<td>1</td>
<td>-1297 to -1287</td>
<td></td>
</tr>
<tr>
<td>Myb</td>
<td>TAACG/T(A)G</td>
<td>1</td>
<td>-462 to -456</td>
<td>[myeloid differentiation]</td>
</tr>
<tr>
<td></td>
<td>TAACG(G)GG</td>
<td>1</td>
<td>-494 to -489</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TAACG/T(A)G</td>
<td>1</td>
<td>-1391 to -1386</td>
<td></td>
</tr>
<tr>
<td>NFκB</td>
<td>GGG(C)AA/C(G)TTTCC</td>
<td>2</td>
<td>-504 to -495</td>
<td>PMA, LPS, TNFα</td>
</tr>
<tr>
<td></td>
<td>GGGAA/C(T)TT(T)CC</td>
<td>2</td>
<td>-1424 to -1415</td>
<td></td>
</tr>
<tr>
<td>PEA3 (also binds Ets-1)</td>
<td>AGGAAG</td>
<td>0</td>
<td>-150 to -144</td>
<td>PMA, EGF, v-src, serum</td>
</tr>
<tr>
<td>Sp-1</td>
<td>GGGCGGG</td>
<td>0</td>
<td>-140 to -134</td>
<td>[Developmentally regulated]</td>
</tr>
<tr>
<td></td>
<td>GGGCTGG</td>
<td>0</td>
<td>-206 to -198</td>
<td></td>
</tr>
</tbody>
</table>

*Nucleotide in parenthesis denotes mismatched nucleotide of consensus nucleotide immediately preceding it.
3.1.5 Promoter analysis using a chloramphenicol acetyl transferase reporter gene

The ability of the c-fgr myeloid promoter region to direct expression in U937 cells was studied using a chloramphenicol acetyl transferase (CAT) reporter gene. Three overlapping fragments of the promoter of varying sizes were cloned (by M. Patel) into the plasmid pGCAT-A (Frebourg and Brison, 1988) extending from a Smal site at +99 to -116 (an Acyl site; pGCAT-116), -359 (a HincII site; pGCAT-359) or -772 (a BamHI site; pGCAT-772) with respect to the major myeloid start site (Figure 3.1.12) (see Appendix C). U937 cells were transiently transfected with each of the CAT constructs and, after 24 hours incubation, were split into dishes, one of which was untreated, the other treated for 48 hours with 10ng/ml PMA. Also included in these experiments were pIE-CAT (Rous sarcoma virus immediate early promoter) as a positive control and pGCAT-A as a negative control. From this it was hoped to deduce the regions of the promoter required to direct transcription of the CAT gene (and therefore by inference the c-fgr gene) upon differentiation of U937 cells. CAT activity was determined by reacting 20μl of cell lysate with 14C-chloramphenicol and separating acetylated chloramphenicol products by thin film chromatography, which were subsequently autoradiographed (Figure 3.1.13). A signal was observed in pGCAT-116, pGCAT-359 and pGCAT-772, all three of which were less than that observed for pGCAT-A. In addition, a signal was detected for pGCAT-MR, where the -359 fragment was inserted in an antisense orientation as a further negative control. It became clear that the reporter plasmid pGCAT-A was itself capable of directing CAT expression in U937 cell in the absence of any inserted promoter fragment. This was apparent by the obvious CAT activity detected when pGCAT-A was transfected as a negative control. Increasing lengths of inserted DNA had the effect of reducing this effect in a size-dependent manner.
Figure 3.1.12. Plasmid CAT constructs, showing region of c3 from which fragments were derived and cloned into pGCAT-A. B, BamHI; S, SmaI.
Figure 3.1.13. Representative autoradiograph showing CAT activity in transfected U937 cells following 48 hour treatment with 10ng/ml PMA.
3.1.6 Promoter analysis using a luciferase reporter gene

Two problems were encountered using CAT as a reporter gene. Firstly, the vector appeared to be active in U937 cells, and secondly the CAT reporter signal was not very strong. The luciferase gene has a number of advantages as a reporter gene over the CAT gene. The assay is quicker and less variable, there is no requirement to use radio-isotope, and the luciferase reporter system has been reported to be up to 100 times more sensitive than CAT (Alam and Cook, 1990). To overcome the problem of low sensitivity using c-fgr-CAT constructs, we decided to use a luciferase reporter plasmid. We first determined whether the luciferase reporter plasmid, pGL-2basic, had intrinsic promoter activity in U937 cells. Figure 3.1.14 shows that it does not.

To construct plasmids containing the varying lengths of promoter sequence, equivalent to those used for pGCAT constructs, it was necessary to first clone fragments into pGL-2basic via a pSK intermediary. This was because the pGL-2basic vector had a limited multiple cloning site that did not possess suitable restriction sites. The effect of using an intermediate vector was to introduce sequences of the pSK multiple cloning site into pGL constructs. Hence, to have a faithful negative control it was necessary to clone the multiple cloning site of pSK into pGL-2basic using flanking *SacI* and *KpnI* sites (Figure 3.1.15)(see Appendix 3). This improved the multiple cloning site of pGL-2basic by producing more available restriction sites which could be exploited for cloning other fragments. This new construct was called pGL-BSK. Transfection of pGL-2basic or pGL-BSK into U937 cells demonstrated that these vectors did not possess intrinsic promoter activity in the presence of 10ng/ml PMA (Figure 3.1.14).
Figure 3.1.14. Luciferase activity in transfected U937 cells following 48 hour treatment with 10ng/ml PMA. Mean of 3 experiments ± SEM.
Eight overlapping c-fgr promoter constructs were produced, extending from *SmaI* at +99 to -128 (at *AvrII*; pGL-128), -343 (at *XhoI*; pGL-343), -752 (at *PstI*; pGL-752), -911 (at *DraI*; pGL-911), -1137 (at *SmaI*; pGL-1137), -1211 (at *Esp3I*; pGL-1211), -1688 (at *RsaI*; pGL-1688), and approximately -3200 (at *AccI*; pGL-3200) with respect to the major myeloid start site (Figure 3.1.16)(see Appendix 3). Briefly, this was achieved as follows:

1) A 4.5kb *EcoRI* fragment of cosmid c1, extending from -649 to approximately -4600 and overlapping the 5' end of the pBI-1 insert, was subcloned into pSK to derive pSK-Eco4.5. Analysis of this plasmid revealed an *AccI* site at -3200 with respect to the myeloid start site (see section 3.1.3). Digestion of pSK-Eco4.5 with *EcoRI* and *AccI* produced a 2.6kb fragment which was inserted into the 5' region of pBI-752 (lacking -649 to -752 through similar digestion). This gave rise to pBI-3200. Flanking *KpnI* and *SacI* sites were used to clone the pBI-3200 insert into pGL-2basic to give rise to pGL-3200 (Figure 6.15).

2) Plasmid pGL-1688 was produced by inserting a 1kb fragment, produced by *RsaI* and *AflIII* digestion of pSK-Eco4.5, into pGL-752 that had been digested with *KpnI* (and the ends filled using klenow DNA polymerase to give blunt ends) and then *AflIII*. The blunt cutting *RsaI* enzyme would allow ligation with the blunted *KpnI* site (Figure 6.14).

3) To produce pGL-1211, pGL-1688 was first digested with *Esp3I*, which cuts only once to linearise the plasmid. The 5' overhang at each end of the linearised plasmid resulting from this cut was blunted using Klenow DNA polymerase. This blunt-ended, linearised plasmid was then digested with *DraIII*, which removed the 5' region of the insert up to -1211, leaving a 3' overhang at one end of the remaining plasmid, which was then blunted using Klenow DNA polymerase in the absence of deoxynucleotides. The resulting blunt-ended plasmid was recircularised with DNA ligase to give rise to pGL-1211 (Figure 6.13).
4) Digestion of pGL-1688 with *SmaI* produced a 1.2kb fragment (-1137 to +99). This was cloned into *SmaI* digested pGL-2basic to give rise to pGL-1137 (Figure 6.12). The orientation of the insert was checked by digestion with *EcoRI* which produced an expected 1445bp fragment (not shown).

5) A *SmaI* digestion of pGL-1688 produces a 1.2kb fragment (as described above). Further digestion of this fragment with *DraI* produced a 1.1kb fragment that was cloned into the *SmaI* site of pGL-2basic to give rise to pGL-911 (Figure 6.10). *EcoRI* digestion also produced a 1445bp indicating that fragment was inserted in the correct orientation (not shown).

6) Plasmid pGL-752 was produced by digesting pGCAT-772 with *PstI* and *SmaI*, deriving a 752bp fragment which was cloned into pSK to give rise to the construct pBI-752. Flanking *KpnI* and *SacI* sites (of pSK) were used to excise the 872bp insert of pBI-752 which was cloned into these sites of pGL-2basic (Figure 6.9).

7) Plasmid pGL-343 was produced by *XhoI* digestion of pBI-752, removing the 400bp 5' region, and religating the cohesive ends to produce the construct pBI-343. The 440bp fragment derived from *KpnI* and *SacI* digestion of pBI-343 was then cloned into pGL-2basic (Figure 6.8).

8) Plasmid pGL-128 was produced by digestion of pGCAT-MR with *AvrII* deriving a 212bp fragment which was blunted with klenow DNA polymerase. This blunted fragment was subsequently digested with *HindIII* and the resulting 158bp fragment cloned into *HindIII* and *SmaI* of pGL-2basic (Figure 6.7).

Plasmids pGL-1688, pGL-752, pGL-343 and pGL-128 were constructed by myself, and plasmids pGL-3200, pGL-1211, pGL-1137 and pGL-911 were constructed by P. Kefalas.
Figure 3.1.15. (a) Restriction digestion of positive isolates with *XhoI* (during cloning of pGL-BSK) with correct construct in lane 2. The expected 120bp fragment, containing the insert, is indicated by an arrow. Lambda10kb size marker, lane 9. (b) Restriction map of the multiple cloning site of pGL-BSK.
Figure 3.1.16. Plasmid luciferase constructs, showing region of c3 from which fragments were derived and cloned into pGL-2basic. B, BamHI; S, Smal.
Plasmid pGL-2control (containing the SV40 promoter) was used as a positive control and pGL-BSK and/or pGL-2basic as a negative control. Plasmids were introduced into U937 cells by electroporation and incubated for 24 hours. The transfected cells were divided into two cultures, one of which remained untreated whilst the other was treated with 10ng/ml PMA or 100nM D3 + 100u/ml TNFα and incubated for a further 48 hours. Cells were harvested, lysed, and luciferase activity determined using either a luminometer or scintillation counter.

Efficiency of transfection was monitored by dot blot hybridisation and phosphor-imagery. 100μl of RNase- and Proteinase K-treated cell lysate were dot blotted, hybridised with a 32P-labelled DNA probe specific for the luciferase gene and analysed by phosphor-imagery. For each sample, luciferase activity was corrected for variations in transfection efficiency by dividing the value obtained from luminometry by the value obtained from dot blot hybridisation. Results from a representative experiment are shown in Table 3.1.2.

Figure 3.1.17a shows luciferase activity directed by promoter fragments in undifferentiated U937 cells, while Figure 3.1.17b shows luciferase activity in PMA treated cells. The data represent three separate experiments, expressed in arbitrary light units ± SEM. The c-fgr gene fragment extending from -128 to +99 (pGL-128) did not direct any luciferase expression above the background level seen with pGL-2basic, but the longer c-fgr gene fragments each directed luciferase expression approximately 10-12 times background. As shown in Figure 3.1.18, PMA treatment had no effect on the luciferase expression directed by pGL-128, pGL-343 or pGL-752. However, PMA induced an approximately 4-fold increase in the luciferase expression directed by pGL-1137, pGL-1211, pGL-1688 and pGL-3200. These data demonstrate that the 385bp region between positions -1137 and -752 contains one or more PMA-response elements. The plasmid pGL-911 was capable of directing an approximately 2-fold increase in luciferase activity. This would suggest that the fragment -911 to +99 may overlap the PMA-response element.
Table 3.1.2. Representative c-fgr promoter analysis experiment showing luciferase activity (measured on a scintillation counter as *luc* CPM), the signal from the dot blot (measured on phosphor-analyser as dot CPM), and *luc* CPM+dot CPM which corrects for transfection efficiency. "-ve", untreated; "+PMA", treated for 48 hours with 10ng/ml PMA.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Treatment</th>
<th><em>luc</em> CPM</th>
<th>dot CPM</th>
<th>[<em>luc</em> CPM+dot CPM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGL-128</td>
<td>-ve</td>
<td>61296</td>
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<tr>
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<td>+PMA</td>
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<td>17238</td>
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</tr>
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<tr>
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<td>17030</td>
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</tr>
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</table>
Figure 3.1.17. Basal luciferase activity in transfected U937 cells (a) and induction of luciferase activity following 48 hour PMA treatment (b). Mean of 3 experiments ± SEM.
Figure 3.1.18. Fold induction of luciferase activity in transfected U937 cells following 48 hour treatment with 10ng/ml PMA. Mean of 3 experiments ± SEM.
Link et al. (1992) have described a PMA response element contained within a -772 to +99 fragment. The possibility that a PMA-response element might exist between -772 and -752 was explored by constructing a plasmid (pGL-772) that contained the fragment -772 to +99. Figure 3.1.19 shows that this fragment did not direct increased luciferase activity in PMA-differentiated U937 cells.

Plasmids pGL-3200 and pGL-1688 were both unable to induce luciferase activity when transfected U937 cells were treated with 100nM D3 in combination with 10ng/ml TNFα (Figure 3.1.20).
Figure 3.1.19. Representative experiment showing fold induction of luciferase in transfected U937 cells following 48 hour treatment with 10ng/ml PMA.
Figure 3.1.20 Luciferase activity in transfected U937 cells in the presence or absence of 10ng/ml TNFα in combination with 100nM D3. Mean of 3 experiments ± SEM.
3.2 Discussion

Differentiation of U937 cells has been shown to lead to the accumulation of c-fgr mRNA. The protein product of this gene, p55c-fgr, has been associated with signalling from the Fc receptor, FcγRII, which ultimately signals monocyte/macrophage phagocytosis. Consequently, c-fgr may be considered a useful marker of a differentiated monocyte/macrophage. How the expression of this gene is controlled at a transcriptional level is of much interest for further understanding the mechanisms of monoblastic differentiation.

3.2.1 Isolation and mapping of exon M4-containing cosmid clones

Comparison of c-fgr cDNA clones derived from EBV transformed B-lymphoma cell lines and IFNγ-treated U937 cells revealed that myeloid-specific expression of c-fgr was directed through a promoter not previously identified. Exon M4, unique to myeloid c-fgr mRNA transcripts, is a non-coding exon that had been found to be absent in the genomic cosmid clone cF2.3, which extends from approximately 4kb upstream of the coding region to approximately 20kb downstream of exon 12 (Patel et al., 1990b). Consequently, a library of human genomic cosmids was screened using an exon M4-specific probe to isolate a clone containing exon M4. Two such clones, c1 and c3, were isolated that almost completely overlapped, with c3 containing exon 12 at its 3' end. An extensive restriction map of c3 was determined showing exon M4 to be located approximately 11.1kb upstream of the c-fgr coding exons (Figure 3.1.7). This was confirmed by Link et al (1992) who published an equivalent map having isolated a lambda genomic clone (λ-fgr2). For the purposes of promoter analysis using sequences upstream of exon M4, c3 was superior to λ-fgr2 in so far as c3 extends 6kb further upstream of exon M4 than λ-fgr2.
3.2.2 The sequence of exon M4 and the c-fgr myeloid promoter

Exon M4 was further studied by sub-cloning the 1.6kb BamHI fragment of c3 that contains exon M4 into pSK to derive pBl-1. The complete insert of pBl-1 was sequenced. Exon M4 was found to be 122bp in length and had a high GC content of 76%. It is possible that the high GC content plays a role in post-transcriptional regulation since the 5' untranslated region of c-sis has been shown to inhibit translation of c-sis mRNA by 40-fold (Rao et al., 1988).

Computer analysis of the c-fgr promoter was performed on sequences extending from +99 upstream to -1688 with respect to the major myeloid transcription start site (determined by Link et al., 1992)(Figure 3.1.11). The sequence AATAA, situated 27bp upstream of the start site may function as a TATA box. This is of interest since no TATA boxes have yet been observed in c-src family gene promoters. Sub-sequence homology searches were performed using consensus transcription factor binding sites allowing for 0, 1 or 2 mismatches, the results of which are shown in Table 3.1.1 and Figure 3.1.11. Specifically, analysis was limited to transcription factors that are known to be activated by PMA or upon myeloid differentiation. Binding sites for seven transcription factors (all PMA-inducable except for ISGF-2 and Sp-1) were found at multiple positions. Two binding sites for each of PEA3, AP-2, IRBP, Sp-1, and NFκB were identified, with one mismatch for each AP-2 and IRBP site, two mismatches for each NFκB, and no mismatches for each PEA3 and Sp-1 site. Two binding sites (with one mismatch) were observed for the IFN-inducible ISGF2 protein and three binding sites (with one mismatch) for Myb (Faisst and Meyer, 1992, and references therein). As in the hck, c-yes and c-src gene promoters, there were Sp-1 binding sites, as well as Myb binding sites that are found in c-yes and lyn promoters. AP-2 sites are found in the c-src and hck promoters, and PEA3 is found in the lyn promoter. In addition, the lck promoter has been shown to be activated by the Ets-1 transcription factor which can also bind to the PEA3 binding site. The presence of the Myb binding sites is of interest since it has been implicated in the regulation of haemopoietic growth and differentiation, and is itself developmentally
regulated in HL60 cells according to the lineage of differentiation (Boise et al., 1992). It seems unlikely that any of the above transcription factors are involved in PMA activation of c-fgr since all their binding sites do not lie within the later defined PMA-responsive region (see section 3.2.3).

A further feature of the c-fgr promoter sequence is the presence of three Alu-like repeat sequences (Rogers, 1985) at -1218 to -1061, -879 to -518, and -409 to -220. Alu-like repeats are also observed in the B-lymphoid c-fgr promoter, although the significance of their presence in promoter sequences is not understood (Patel et al., 1990b).

3.2.3 The identification of a PMA-responsive region in the c-fgr promoter

The accumulation of c-fgr mRNA transcripts following differentiation of U937 cells occurs within 8 hours, reaching a maximal level by 48 hours (Faulkner et al., 1992). To determine the region(s) of the c-fgr promoter responsible for binding factors that promote transcription in response to PMA, a series of overlapping promoter constructs of varying length, linked to a reporter gene, were produced. Initially, the bacterial CAT gene was used as a reporter in transient transfection studies. However, in addition to a low level of CAT activity seen for the positive control plasmid pIE-CAT (attributable to poor transfection efficiency) the negative control plasmid pGCAT-A (the vector alone) was capable of producing CAT activity in the presence of PMA. Furthermore, it was apparent that the inserted c-fgr promoter fragment decreased CAT activity with increased size of insert. It is conceivable that cryptic promoting activity observed in pGCAT-A is activated from sequences upstream of the multiple cloning site, which is down regulated as a function of the distance between this region and the CAT gene. Problems arising from cryptic promotion of CAT reporter plasmids in U937 cells have been previously observed and have resulted in attempts to counteract this effect using mechanisms such as strings of terminating codons upstream of the multiple cloning site (Boshart et al., 1992). Attempts to use CAT reporter plasmids to determine myeloid promoter activity of hck in
U937 cells and the c-fgr B-lymphoid promoter in EBV+ B-cell lines have both proved unsuccessful (Lichtenberg et al., 1992; Patel and Brickell, personal communication).

The subsequent availability of firefly luciferase reporter plasmids represented an attractive alternative to CAT. Having the advantage of up to 100-fold increased sensitivity over CAT (Alam and Cook, 1990), it became clear that the luciferase vector, pGL-2basic, when transiently transfected into U937 cells and treated with PMA, did not possess the cryptic promoting properties seen for pGCAT-A. One drawback with pGL-2basic was the limited multiple cloning site which did not contain suitable restriction sites for producing promoter constructs. In order to produce promoter constructs it was necessary to clone fragments into pSK and then, using flanking Kpnl and Sacl restriction sites at each end of the pSK multiple cloning site, insert the fragment into the Kpnl and Sacl restriction sites of pGL-2basic. In doing this, sequences from the pSK multiple cloning site were now also being included into the pGL construct. Wary of introducing possible cryptic promoting activity in to the plasmid, it was also necessary to clone the pSK multiple cloning site itself in to pGL-2basic, deriving pGL-BSK, which could be used as a more faithful negative control. pGL-BSK was shown to have virtually no luciferase activity, equivalent to that observed for pGL-2basic. A benefit of creating pGL-BSK was that it represents a more versatile reporter plasmid, by virtue of its improved multiple cloning site, allowing for future pGL constructs to be produced more easily.

Eight pGL constructs of varying sized overlapping promoter fragments were produced ranging from the shortest, pGL-128, to the largest, pGL-3200. Transient transfection of these plasmids into U937 cells was followed by their incubation in the presence or absence of PMA. Analysis of luciferase activity in untreated cells determined that sequences up to -343 were required to promote basal levels of transcription (Figure 3.1.17a), consistent with the low level of c-fgr mRNA expression observed in untreated U937 cells (Faulkner et al., 1992). The basal level of luciferase activity does not significantly increase for larger fragments suggesting that constitutively active expression
is promoted from sequences between -343 and -128. Within this region there is a putative PEA3 transcription binding site and two Sp-1 binding sites. Either of these two transcription factors may be involved in the basal level of promoter activity.

PMA treatment of U937 cells, following their transient transfection with pGL promoter constructs, showed that sequences upstream of -1137 contained a PMA responsive region capable of inducing an approximately 4-fold increase in luciferase activity which did not significantly change with increasing fragment length (Figure 3.1.17b and 3.1.18a). The plasmid pGL-911 induced luciferase activity 2-fold by PMA treatment suggesting that there may be a partial overlap with a PMA responsive element that results in a 50% reduction in activity. These data indicate that the PMA-responsive region of the c-fgr myeloid promoter lies within, and possibly overlaps the 3' end of the 226bp region between Smal and Drai restriction sites. This sequence only contains the putative binding site for ISGF2, which is IFN-induced rather than PMA-induced (Imam et al., 1990), and would seem unlikely to be involved in the PMA-induced expression of luciferase.

Link et al. (1992) reported a PMA-responsive region between -772 and -433. While pGL-752 did not show PMA inducibility, the possibility of responsive sequences between -772 and -752 was investigated by the production of pGL-772. This construct did not promote luciferase activity above basal levels observed for pGL-752. The discrepancy between the results described here and those of Link et al. (1992) are possibly due to different experimental methods. Link and co-workers cloned a c-fgr promoter fragment (the same -772 to +99 BamHI/Smal fragment as pGL-772) in front of the neomycin resistance gene, neo. This construct was then stably transfected into U937 cells, where transfected clones were selected using neomycin. Activation of the c-fgr promoter was then determined by northern blot detection of c-fgr-neo mRNA transcripts in transfected cells, with or without PMA treatment. It would seem possible that the procedure of neomycin selection could select for transfected clones that express neo more efficiently, perhaps by some minor recombination event. Alternatively, since luciferase activity is
dependent on expression of a functional protein, there exists the possibility of post-transcriptional regulation occurring which might not necessarily affect c-fgr-neo mRNA accumulation.

3.2.4 D3 and TNFα do not induce c-fgr promoter-luciferase activity

Although D3, used in combination with TNFα, induces a rapid accumulation of c-fgr mRNA in U937 cells within 4 hours (Faulkner et al., 1992), none of the pGL c-fgr promoter constructs displayed inducible luciferase activity (Figure 3.1.20). This result would indicate that there is not a D3+TNFα response element within the promoter that results in production of exon M4-containing mRNA. It may be that D3+TNFα activates a different promoter. Gutkind et al. (1991) and Link et al. (1992) have described rare PMA-induced mRNA transcripts that contain exons M1 (located approximately 250bp upstream of M4), M2, M3 and 1.5 (each located proximal to exons 1a and 1b). In addition to the major myeloid M4-1b-[2 to 12] c-fgr transcripts, there are also M1-M2-1b-[2 to 12] and M4 splicing into either M3, M2 and M3-1.5 before joining exon 1b (Figure 3.2.1). If, for example, D3+TNFα promotes endogenous c-fgr expression via a promoter upstream of exon M1 to produce M1-M2-1b-[2 to 12] transcripts, the pGL constructs used here could not promote luciferase activity. This is because the splice donor of exon M1 has its next available splice acceptor within the SV40 sequences of pGL that are downstream of the luciferase gene. c-fgr mRNA transcripts in D3+TNFα-differentiated U937 cells have not been studied to determine what 5' exons are present. If different untranslated exons are utilised following D3+TNFα-induced differentiation it may account for the different kinetics of c-fgr mRNA accumulation observed during PMA-induction.

Conclusions drawn from this work, and future experimentation that requires to be performed, are found in the section 5.1.
Figure 3.2.1. Rare myeloid c-fgr mRNA transcripts (Link et al., 1992).
CHAPTER 4

THE ROLE OF THE RETINOID X RECEPTORS IN U937 CELLS DURING DIFFERENTIATION
Chapter 4.1 Results

The role retinoic acid and D3 plays in the induction of monoblastic differentiation implicates the retinoid X receptors as mediators in this process. Therefore, the second aim of this project was to characterise RXR expression in U937 cells, and to alter endogenous expression in transfected cell lines and clones, thus elucidating their role in this model system.

4.1.1 Analysis of RXRα and RXRβ expression during differentiation of U937 cells
RXRα and RXRβ expression was investigated by extracting mRNA from proliferating U937 cells, northern blotting and hybridising with RXRα, RXRβ and β-actin specific 32P-labelled RNA probes. The signal from the β-actin hybridisation was as a control for mRNA loading. A low level of RXRα and RXRβ mRNA transcripts were present in these cells (Figure 4.1.1a). The expression of these genes was upregulated following treatment with 10ng/ml PMA in a time-dependent manner (Figure 4.1.1b). Densitometry, using a BioRad GS-670 Imaging Desitometer, showed that both RXRα and RXRβ mRNAs were up-regulated 8-fold and 3-fold, respectively, from the undifferentiated cell when corrected for β-actin hybridisation (Figure 4.1.2).

4.1.2 Construction of RXRα and RXRβ expression plasmids pMEP-RXRαA, pMEP-RXRαS and pMEP-RXRβA
Since RXR mRNAs were upregulated during differentiation, implying that these genes play a role during this process, we were encouraged to elucidate further the function of RXRα and RXRβ. Transfected cell lines were established in which RXRα mRNA was over-expressed or in which endogenous RXRα and RXRβ expression was inhibited.
Figure 4.1.1. Hybridisation of RXRα-, RXRβ- and β-actin-specific riboprobes to mRNA from untreated U937 cells (a), and following incubation with 10ng/ml PMA for 24, 48 and 72 hours (b). Expected sizes of RXRα, RXRβ and β-actin mRNAs were 4.4kb, 3.8kb and 2.1kb, respectively. Size marker, in the form of rRNA was not used due to the problem of extensive cross-hybridisation. Hybridised membrane was exposed to X-ray film for 1 to 3 days. RNA loading was controlled by determining β-actin mRNA expression (see Figure 4.1.2).
Figure 4.1.2. The time-dependent expression of RXRa and RXRβ mRNA in PMA treated U937 cells, as determined by densitometry of autoradiographs shown in Figure 4.1.1. RNA loading controlled for using β-actin signal.
To achieve this an EBV-based mammalian expression vector, called pMEP-4, was used (Groger et al., 1989; reviewed by Brickell and Patel, 1991)(Figure 4.1.3). The plasmid pMEP-4 has a number of features which are necessary for expressing the gene inserted into its multiple cloning site. Firstly, the cloning site is immediately downstream of the human metallothionein promoter, hMTIIa. This promoter is induced by the presence of heavy metal ions such as Cd$^{2+}$ or Zn$^{2+}$ (in the form of CdCl$_2$ or ZnSO$_4$), and hence facilitates induction of expression of the inserted gene. Downstream of the multiple cloning site lies the SV40 intron and polyadenylation and termination signal sequence, SVpA/T. To maintain this plasmid in the nucleus as an episomal DNA element, and so prevent chromosomal integration (into potentially detrimental sites), the EBNA-1 and OriP sequences from EBV are included. The EBNA-1 protein maintains episomal plasmids by binding and activating the OriP replication origin. The hygromycin B resistance gene, hph, allows selection of transfected cells. Hygromycin B is an antibiotic which inhibits the growth of a broad range of cells including bacterial, plant, fungal and mammalian cells. Specifically, hygromycin B inhibits protein synthesis at the level of translation at the ribosome (Gonzalez et al., 1978). The resistance gene hph encodes a phosphotransferase which inactivates hygromycin B by phosphorylating it (Malpartida et al., 1983). Finally, two bacterial plasmid components, the ampicillin resistance gene, amp, and the bacterial origin of replication, ColEl, allow for manipulation of the plasmid in bacteria.

The complete coding region of human RXRa gene (in the plasmid vector pSG-5, a gift from D. J. Mangelsdorf, UT Southwestern Medical Centre, Dallas, TX.)(Mangelsdorf et al., 1990) was excised using EcoRI sites at the 5' and 3' ends to give a fragment of 1866bp. This was ligated into pSK at the EcoRI site of its multiple cloning site (see Figures 4.1.4 and 6.16). Two clones were derived with inserts in opposite orientations. These were called pSK(3-7)-RXRa and pSK(7-3)-RXRa. The orientation of the inserts was verified by digesting these constructs with SacI which is shown in Figure 4.1.5.
Flanking *HindIII* and *NotI* sites were used to clone the RXRα insert into pMEP-4, yielding pMEP-RXRαS and pMEP-RXRαA,
Figure 4.1.3. Diagram of pMEP-4. \textit{hMTII}a, metallothionein CdCl2-inducible promoter; \textit{hph}, Hygromycin B resistance gene; MCS, multiple cloning site; \textit{SVpA/T}, SV40 polyadenylation signal and termination signal; \textit{Ori P}, EBV eukaryotic origin of replication; \textit{EBNA-1}, EBV transactivator of \textit{Ori P}; \textit{ColE1}, \textit{E. Coli} origin of replication (Groger \textit{et al.}, 1989).
Figure 4.1.4. The gene maps of the plasmids pSK(7-3)-RXRα, pSK(3-7)-RXRα and pSK-RXRβ. Dashed arrow indicates orientation of the gene insert with respect to the T7 and T3 regions of the pSK vector.
Figure 4.1.5. Confirmation of the orientations of the RXRα cDNA inserts in pSK(7-3)-RXRα and pSK(3-7)-RXRα by digestion with SacI. Expected 3197bp and 1629bp fragments from pSK(7-3)-RXRα and 4333bp and 291bp fragments from pSK(3-7)-RXRα labelled a to d, respectively.
with the inserts lying respectively in the sense and antisense orientations, with respect to hMTIIa. The orientations were checked by SacI digestion and hybridisation with a $^{32}$P-labelled DNA probe specific to RXR$\alpha$ (Figure 4.1.6).

The complete human RXR$\beta$ coding region (in pSK, a gift from D. J. Mangelsdorf and R. M. Evans)(Figure 4.1.4) was digested with KpnI and NotI and the 2.8kb fragment (representing the complete RXR$\beta$ coding sequences) was ligated into KpnI/NotI cut pMEP-4. This gave rise to the construct pMEP-RXR$\beta$A, which contained the RXR$\beta$ insert in the antisense orientation with respect to hMTIIa (Figure 6.17). The orientation was checked by performing an XhoI digest, shown in Figure 4.1.7.

A number of unsuccessful attempts were made to ligate RXR$\beta$ into pMEP-4 in the sense orientation.
Figure 4.1.6. (a) Confirmation of the orientations of the RXRα inserts in pMEP-RXRαS and pMEP-RXRαA by digestion with SacI. Expected 8700bp and 3600bp fragments from pMEP-RXRαS and 7300bp and 5000bp fragments from pMEP-RXRαA labelled a to d respectively. (b) Southern hybridisation of the same gel with a^{32}P-labelled probe specific to RXRα. Hybridisation to 8700bp and 5000bp fragments labelled E and F respectively.
Figure 4.1.7. Confirmation of the orientation of the RXRβ insert in pMEP-RXRβ by digestion with XhoI. Expected 10.4kb and 2.8kb fragments from pMEP-RXRβ labelled a and b respectively.
4.1.3 Production of pooled transfectant U937 cells and isolation of clones

The constructs pMEP-RXRαS, pMEP-RXRαA and pMEP-RXRβA were transfected into U937 cells by electroporation. This was performed on two occasions for pMEP-RXRα-S and pMEP-RXRα-A giving rise to transfected cell pools UαS(1), UαA(1), UαS (2) and UαA(2). UαS(1) and UαA(1) were used for subsequent experiments. A single pMEP-RXRβ-A transfected cell pool, UβA, was established. These lines and related clones are summarised in Table 4.1.1. Transfected cell pools were grown to large numbers (without passage) for 2 weeks before freezing down several aliquots in liquid nitrogen for storage until required. This process was necessary as such pools contain a heterogeneous population of cells in which, although all are resistant to hygromycin B, would not all necessarily express the transgene due to mutation events. Non-expressing cells might have a selective advantage over expressing cells and hence supplant these after successive passages. For subsequent experiments frozen aliquots were thawed and grown for 1 week prior to experimentation.

A negative control cell line, MEP, was used throughout experiments. In this line, pMEP-4, with no inserted gene, had been stably transfected into U937 cells and subsequently cloned (by M. Dabrowski; see Hewison et al, 1994).

As transfectant pools represent a heterogeneous population of cells (see above), it was necessary to isolate individual clones that could be studied further. To achieve this, cell pools were plated at limiting dilution, and the resulting clones grown. The cloning procedure generated 15 clones from UαA(1), 15 clones from UαS(1) and 12 from UβA (listed in Appendix 4). From these clones, suitable candidates for further study were selected on the basis of their response to 9-cis RA, a response that had been indicated by preliminary cell proliferation assays on transfectant pools (see section 4.1.4).
Table 4.1.1. Transfected cell pools and related clones used in experiments (see also Appendix 4).

<table>
<thead>
<tr>
<th>Transfectant cell pools</th>
<th>Inserted gene</th>
<th>Orientation</th>
<th>Derived clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>UαA(1)</td>
<td>RXRα</td>
<td>Antisense</td>
<td>αB5A</td>
</tr>
<tr>
<td>UαS(1)</td>
<td>RXRα</td>
<td>Sense</td>
<td>αG2S</td>
</tr>
<tr>
<td>UβA</td>
<td>RXRβ</td>
<td>Antisense</td>
<td>βF9A</td>
</tr>
<tr>
<td>MEP</td>
<td>-</td>
<td>-</td>
<td>MEP</td>
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</tbody>
</table>
4.1.4 Preliminary cell proliferation assays

Initial experiments were performed to determine what effect the transfected plasmids had on differentiation and whether or not this effect was dependent on induction of inserted gene expression through the hMTIIa promoter by CdCl2. Proliferation was assayed by uptake of 125I-dUR by transfected cells in the presence or absence of differentiating agents and 10μM CdCl2. All cell proliferation assays were performed in quadruplicate wells of a 96-well plate. Inhibition of proliferation was taken as a rough indication of differentiation of transfected cells.

By comparing the extent of growth inhibition of treated cells with untreated cells it was clear that UαS cells showed greater sensitivity to 9-cis RA, all-trans RA and D3 than MEP control cells (Figure 4.1.8a). This sensitivity became significantly more marked in the presence of CdCl2 (using paired Student's t-test: 9-cis RA, p<0.005; all-trans RA, p<0.01; D3, p<0.05 versus UαS cells without CdCl2)(Figure 4.1.8b). By contrast, treatment of UαA cells with 9-cis RA, all-trans RA and D3, when compared with untreated UαA cells, were less inhibited by these agents than were MEP cells (Figure 4.1.8a). The presence of CdCl2 did not alter the response of UαA cells to 9-cis RA or all-trans RA, although D3 appeared to now have a growth inhibitory effect on UαA cells (p<0.05 versus D3-treated cells without CdCl2)(Figure 4.1.8b).

TNFα and PMA inhibited growth of UαS, UαA and MEP cells to the same extent, both in the presence and absence of CdCl2 (Figure 4.1.9).
Figure 4.1.8. Proliferation of MEP, \( \alpha \)S and \( \alpha \)A cells following treatment with 1\( \mu \)M 9-\textit{cis} retinoic acid, 1\( \mu \)M all-\textit{trans} retinoic acid or 100nM D3 (a) and with 10\( \mu \)M CdCl\(_2\) (b). Determined by uptake of 125I-dUR ± SEM.
Figure 4.1.9. Proliferation of MEP, UαS and UαA cells following treatment with 10ng/ml PMA or 100u/ml TNFα in the absence (a) or presence of 10μM CdCl₂ (b). Determined by the uptake of¹²⁵I-dUR ± SEM.
Proliferation assays performed on transfectant pools defined growth response characteristics that were exploited as a means for selecting suitable cloned cell lines for further experiments.

An RXRα antisense clone was isolated by performing proliferation assays, in the presence or absence of 9-cis RA or D3, on a random selection of clones derived from UαA(1). The clone αB5A was completely resistant to the anti-proliferative effect of these treatments whereas other clones, such as αD9A and αH6A, responded in the same way as MEP control cells (Figure 4.1.10). In the presence of CdCl₂, αB5A cells were resistant to D3, but slightly inhibited by 9-cis RA (a feature that did not re-occur in subsequent experiments; see section 4.1.6). Although the proliferation of untreated αB5A cells was less than other cell lines in this experiment, later experiments showed αB5A cells to divide at a rate comparable to MEP cells (see section 4.1.6).

Using the same procedure as described above, a suitable RXRα sense clone was selected from clones derived from UαS(1). Proliferation of the clone αG2S was very strongly inhibited in the presence of 9-cis RA (approximately 95%) whereas other clones, such as αF7S, responded in the same way as MEP cells (Figure 4.1.11). D3 treatment of αG2S cells did not exert a response that was greater than that of αF7S or MEP in the absence or presence of CdCl₂.

In both cases, the response of αB5A and αG2S was more profound compared to their respective cell pools UαA and UαS. This could be explained by the presence in the pools of MEP-like clones, such as αH6A and αF7S, which would have the effect of attenuating the overall proliferative response in transfected cell pools.

The proliferative response of the transfected cell pool UβA to any of the above treatments did not differ from MEP cells (Figure 4.1.12). Analysis of UβA-derived clones, such as βF9A, did not show a response that differed from MEP when treated with 9-cis RA or
D3, in the presence or absence of CdCl₂ (Figure 4.1.13). Therefore, no further experiments were performed on RXRβ-transfected U937 cells.
Figure 4.1.10. Proliferation of MEP, and UαA clones αB5A, αD9A and αH6A upon treatment with 1μM 9-cis retinoic acid or 100nM D3 in the absence (a) or presence (b) of 10μM CdCl₂. Determined by uptake of ¹²⁵I-dUR ± SEM.
Figure 4.1.11. Proliferation of MEP, and UαS clones αG2S and αF7S upon treatment with 1μM 9-cis retinoic acid or 100nM D3 in the absence (a) or presence (b) of 10μM CdCl₂. Determined by uptake of ¹²⁵I-dUR ± SEM.
Figure 4.1.12. Proliferation of MEP and UβA cells in the presence or absence of 9-cis RA, 100nM D3 or 10ng/ml PMA in the presence (a) or absence (b) of 10μM CdCl₂. Determined by uptake of^{125}I-dUR ± SEM.
Figure 4.1.13. Proliferation of MEP, and UβA clone βF9A upon treatment with 1μM 9-cis retinoic acid or 100nM D3 in the absence (a) and presence of 10μM CdCl₂(b). Determined by uptake of ¹²⁵I-dUR ± SEM.
4.1.5 Characterisation of RXRα expression in U937 clones expressing sense (αG2S) or antisense (αB5A) RNA

It was important to determine whether RXRα expression in the transfected cell lines αG2S and αB5A was indeed altered and thereby eliciting the observed responses. RXRα mRNA levels were examined in these cells by northern hybridisation, and receptor binding assays were performed to examine protein levels. Levels of functional protein were determined using a RARE-linked reporter gene. Western blotting could not be used since a suitable anti-RXRα antibody was not available.

4.1.5.1 Northern hybridisation analysis of transfectant clones

αG2S, αB5A and MEP cells were incubated for 24 hours in the presence or absence of 10μM CdCl2. mRNA was then extracted from these cells, northern blotted and hybridised with a 32P-labelled dsDNA probe, produced by oligo-labelling. A dsDNA probe was used instead of a riboprobe as, for unknown reasons, numerous attempts to use the latter probe type proved unsuccessful. As shown in Figure 4.1.14, αG2S cells expressed high levels of RXRα that increased slightly in the presence of CdCl2. Untreated αB5A cells did not show any apparent hybridisation. MEP cells also showed no apparent expression of RXRα, although low levels of expression would have been expected (as observed in U937 cells). Following treatment of αB5A cells with CdCl2 a signal was observed that possibly arises from hybridisation of the dsDNA probe with antisense RXRα RNA derived from pMEP-RXRαA. The cross hybridisation of the RXRα probe with a small amount of contaminating ribosomal RNA (rRNA) acted as a control of RNA loading onto the gel.

4.1.5.2 Receptor binding assays.

Two types of binding assays were employed in an attempt to determine the ability of the transfectant cell lines αG2S, αB5A and MEP to bind 3H-9-cis RA through RXRα. Saturation assays were performed, in which cells were incubated with a range of 3H-9-cis RA concentrations allowing for the determination of binding affinity and the number of
receptors per cell. Non-specific binding of $^3$H-9-cis RA was determined by incubating cells in an identical range of $^3$H-9-cis RA concentrations with a 2000-fold excess of unlabelled 9-cis RA. The presence of $^3$H-9-cis RA in this second range of samples represents non-specific binding which, when subtracted from the first range of samples (representing total binding), determines specific binding.

A problem encountered when performing these experiments was that concentrations of $^3$H-9-cis RA above approximately 100-200fmol led to a high degree of non-specific binding. Consequently, at lower concentrations binding was observed but not necessarily at saturating levels. Although maximal binding ($B_{\text{max}}$) observed for αB5A cells appeared to be less than that for MEP cells ($2.5 \times 10^{-5}$ fmol/cell and $9 \times 10^{-6}$ fmol/cell respectively), $B_{\text{max}}$ for αG2S cells ($1.4 \times 10^{-6}$ fmol/cell) was less than both αB5A and MEP cells. From data derived from saturation binding assays it was not possible to establish the number of available retinoid receptors for each cell line.

Displacement binding assays were used as an alternative to saturation assays. Displacement assays would not enable the number of retinoid receptors for each cell line to be determined but would be able to detect if there were greatly reduced or increased levels of specifically bound RA. For these experiments, a set concentration of 100fmol $^3$H-9-cis RA (based on data obtained in saturation assays) was incubated with cells, in the presence of a range of concentrations of unlabelled 9-cis RA, up to a concentration capable of completely displacing receptor bound $^3$H-9-cis RA (ie. 10μM). The data represented in Figure 4.1.15 demonstrated limited displacement of $^3$H-9-cis RA in each cell line, with 10μM unlabelled 9-cis RA not displacing more than $2/3$ of total $^3$H-9-cis RA. This result again indicated a high level of non-specific binding of 9-cis RA in these cell lines. Since these cell lines contain a number of proteins that will bind 9-cis RA with various binding affinities, binding assays would also reflect this alternative binding too. Hence, binding assays proved to be unsuitable for determining RXRα levels in transfectant cells.
Figure 4.1.14. Hybridisation of mRNA extracted from αG2S, αB5A and MEP cells with a $^{32}$P-labelled RXRα-specific dsDNA probe. Untreated: lane 1, 3 and 5. Treated with 10μM CdCl₂: lanes 2, 4 and 6. Expected size of RXRα RNA was 4.8kb. Size marker, in the form of rRNA was not used due to the problem of extensive cross-hybridisation. Hybridised membrane was exposed to X-ray film for 7 days. No apparent endogenous RXRα mRNA was visible, possibly due to low sensitivity of dsDNA probes compared to that of ribo-probes observed in Figure 4.1.1.
Figure 4.1.15. Representative displacement plots showing the inhibition of $^3$H-9-cis RA binding to cell lines MEP (a) αB5A (b) and αG2S (c).
4.1.5.3 Transient transfection of a luciferase-retinoic acid response element reporter construct

Differences in levels of functionally active RXRα protein between the three cell lines αG2S, αB5A and MEP were examined by studying the ability of each transfectant to activate a retinoic acid response element (RARE). This was done by transiently transfecting cells with a plasmid construct pR140-luc (a gift from D. Cash, Ludwig Institute for Cancer Research, Riding House Street, London). pR140-luc consists of nucleotides -124 to +14 of the RARβ2 promoter, which includes a DR-5 RARE, cloned immediately upstream of a luciferase reporter gene (Vivanco Ruiz et al., 1991)(Figure 4.1.16). pR140-luc was introduced into MEP, αB5A and αG2S cells by electroporation, incubated overnight in 20ml of medium and divided into two 10ml cultures, one of which was treated with 10ng/ml PMA. The undifferentiated and PMA-differentiated cells were incubated for a further 48 hours in the presence or absence of 1μM 9-cis RA and/or 10μM CdCl2. Cells were harvested and assayed for luciferase activity, which was normalised for transfection efficiency by dot-blot hybridisation (see section 3.1). Figure 4.1.17a shows the luciferase activity of undifferentiated cells. Both MEP and αB5A showed a very small response to 9-cis RA which was unaffected by the presence or absence of CdCl2. However, 9-cis RA-treated αG2S cells showed an approximately 25-fold induction of luciferase activity which was increased to 80-fold induction by CdCl2 treatment. This result clearly shows the presence of high levels of functionally active RXRα protein that is increased by induction of the hMTIIa promoter of pMEP-RXRαS in αG2S cells. Induction of luciferase activity in PMA-differentiated cells (Figure 4.1.17b) was attenuated compared to undifferentiated cells, with the exception of MEP+9-cis RA+CdCl2 and αB5A+9-cis RA.
pR140-luc

5' -- CGGGTAGGGTTCACCGAAAGTTCACTCGC - [luc] 3'

Figure 4.1.16. Diagram showing the DR-5 RARE (bold type) of the human RARβ2 gene promoter in pR140-luc.
Figure 4.1.17. Luciferase activity in MEP, αG2S and αB5A cells transfected with pR140-luc and treated with 1μM 9-cis RA and 10μM CdCl₂ as shown, in the absence (a) and presence (b) of 10ng/ml PMA. Mean of 3 experiments ± SEM.
4.1.6 Phenotypic characterisation of transfected U937 clones expressing sense (aG2S) or antisense (aB5A) RXRα RNA

4.1.6.1 Cell proliferation assays.

The growth of untreated cells over 72 hours was compared between αG2S, αB5A and MEP by counting cell number at 0, 24, 48 and 72 hour time points (in triplicate) using a haemocytometer. Growth of both αG2S and αB5A transfectants was slower than MEP cells, with doubling times of approximately 24 hours, 20 hours and 19 hours, respectively (Figure 4.1.18). Trypan blue staining showed no significant cell death over this time (less than 5%, data not shown) with no difference between the cell lines.

Preliminary experiments indicated that transfected cells did not require CdCl₂ to elicit a significant response (or lack of response) to RA, D3, PMA or TNFα. For this reason, CdCl₂ treatment was omitted from further experiments for the purposes of clarity.

The proliferative response of αG2S and αB5A to retinoic acid, D3, PMA and TNFα was examined as a guide to these cells' ability to differentiate. The effect of RA on αG2S and αB5A cells was examined. Incubation of cell lines with a range of 9-cis RA (30nM to 3μM) for 72 hours showed αB5A to be completely resistant to the antiproliferative effect of 9-cis RA compared to approximately 35-40% inhibition observed for MEP in 3μM 9-cis RA (Figure 4.1.19). In contrast, αG2S displayed up to 95% inhibition in 3μM 9-cis RA (Figure 4.1.20) and 70% for 3μM all-trans RA (Figure 4.1.21). All-trans RA also failed to inhibit αB5A proliferation at a 1μM concentration (Figure 4.1.22a). The dose response of αG2S to 9-cis RA (Figure 4.1.20) and all-trans RA (Figure 4.1.21) showed these cells to be strongly inhibited, achieving MEP cells' maximal level of growth inhibition by 40nM and 400nM concentrations respectively. Comparison of 9-cis RA and all-trans RA treatment revealed that the 50% inhibitory concentrations of αG2S were 60nM and 500nM, respectively. These results show that nearly a 10-fold larger concentration of all-trans RA was required to give an equivalent degree of growth inhibition as 9-cis RA.
Incubation of αB5A with 100nM D3 for 72 hours failed to inhibit proliferation (Figure 4.1.22a) whereas αG2S was inhibited by D3 to the same extent as MEP (Figure 4.1.22b). For clarity, Figure 4.1.22b is expressed as a percentage inhibition of proliferation induced by D3 against untreated cells (for method of calculation see section 2.19).

The effect of 9-cis RA and D3, used in combination, on RXRα transfectant clones was examined. Figure 4.1.23 shows that 100nM 9-cis RA+100nM D3 had a greater growth inhibitory effect on MEP and αG2S cells than with either of these treatments alone. Surprisingly, αB5A cells, although unaffected by 9-cis RA or D3 alone, were significantly inhibited by their combined treatment (p<0.05 versus untreated αB5A cells). It should be noted that this experiment was performed using $^3$H-thymidine rather than $^{125}$I-dUR.

Treatment with 30u/ml TNFα had a similar effect on αG2S, αB5A and MEP (Figure 4.1.24a). PMA treatment also showed a similar response in MEP and αG2S cells (approximately 90% growth inhibition), whereas αB5A cell growth, although still approximately 80% inhibited, was significantly less than MEP and αG2S cells (p<0.005)(Figure 4.1.24b).
Figure 4.1.18. Time-dependent growth of MEP, αG2S and αB5A cells expressed as the number of cells/ml ± SEM.
Figure 4.1.19. Proliferation of MEP and αB5A cells treated with 9-cis RA, determined by uptake of $^{125}$I-dUR ± SEM.

*p<0.001 compared to equivalently treated MEP cells. Representative experiment performed on at least 3 separate occasions.
Figure 4.1.20. Proliferation of MEP and αG2S cells treated with 9-cis RA, determined by uptake of $^{125}$I-dUR ± SEM. *p<0.001 compared to equivalently treated MEP cells. Representative experiment performed on at least 3 separate occasions.
Figure 4.1.21. Proliferation of MEP and αG2S cells treated with all-trans RA, determined by uptake of $^{125}$I-dUR ± SEM. *p<0.001 compared to equivalently treated MEP cells. Representative experiment performed on at least 3 separate occasions.
Figure 4.1.22. Proliferation of MEP and αB5A cells treated with 1μM all-trans RA or 100nM D3, determined by the uptake of $^{125}$I-dUR ± SEM. *p<0.001 compared to equivalently treated MEP cells. Representative experiment which was performed on at least three separate occasions (a). Effect of D3 on MEP and αG2S cell proliferation, determined by uptake of $^{125}$I-dUR, and expressed as percent inhibition compared to untreated cells (b).
Figure 4.1.23. Proliferation of MEP, αG2S and αB5A cells treated with 0.1μM 9-cis RA and 100nM D3 alone and in combination. Determined by uptake of ³H-thymidine ± SEM. *p<0.01, **p<0.05 v's untreated cells.
Figure 4.1.24. Proliferation of MEP, αG2S and αB5A cells treated with 30u/ml TNFα (a) and 10ng/ml PMA (b). Determined by uptake of $^{125}$I-dUR ± SEM. *p<0.05 compared to treated MEP cells.
4.1.6.2 Analysis of expression of the β2-integrins CD11a, CD11b, CD11c and CD18

The cell surface expression of the β2-integrins CD11a, CD11b, CD11c and CD18 was examined by flow cytometry. The cell lines αG2S, αB5A and MEP were incubated for 48 hours in the presence or absence of 1μM 9-cis RA before staining with antibodies against CD11a, CD11b, CD11c, CD18 (all from the Fifth Leucocyte Differentiation Antigen workshop). CD45, the leucocyte common antigen, was also stained for as a positive control. Secondary staining was performed using a fluorescence-labelled rabbit anti-mouse IgG antibody. The cells were then analysed by flow cytometry (operated by M. George). The results are shown in Table 4.1.2, Figure 4.1.25 and Figure 4.1.26.

Both CD11a and CD18 were expressed by 100% of the population of all three cell lines, before and after 9-cis RA treatment. CD11b and CD11c were expressed in approximately 60% of untreated αB5A and MEP cells, and approximately 80% of untreated αG2S cells. Following 9-cis RA treatment, CD11b and CD11c was expressed in approximately 90% of all three cell lines.

The most striking result was the change in mean fluorescence intensity (ΔMFI) of CD11a, CD11b, CD11c and CD18 surface expression on αG2S cells following 9-cis RA treatment. For each antigen the ΔMFI for αG2S cells ranged from 67 to 180, whereas for MEP and αB5A cells, ΔMFI ranged from -16 to 62. The induced expression of these antigens on αG2S cells can be seen on the histograms (in Figure 4.1.25) as emerging second peaks. The actual MFI (Table 4.1.2) for CD11a, CD11c and CD18 was lower in untreated αG2S cells than in untreated MEP cells. Hence, 9-cis RA induces greater upregulation of these three antigens but does not necessarily hyper-express them under these conditions. Only CD11b appears to be hyper-expressed in response to 9-cis RA compared to MEP cells.
Figure 4.1.25. Flow cytometry of MEP, αG2S and αB5A cells following 48 hour incubation with 1μM 9-cis RA (+RA) or with medium alone (-ve). Stained with (from front to back) secondary antibody alone [dark blue], CD11a [pale blue], CD11b [green], CD11c [yellow], and CD18 [red]. Y-axis is the number of cells and the X-axis the log fluorescence.
Table 4.1.2. Mean fluorescence intensity and percent positive expression of the β2-integrins CD11a, CD11b, CD11c and CD18 in MEP, αG2S and αB5A cells in the absence (-ve) or presence of 1μM 9-cis RA (+RA), determined by flow cytometry. (See also Figure 4.1.24 and 4.1.25). Data determined from a single experiment.

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Figure 4.1.26. Expression of CD11a, CD11b, CD11c and CD18 of MEP, αG2S and αB5A cells determined by flow cytometry. Change in mean fluorescence intensity (ΔMFI) following 48 hour treatment with 1μM 9-cis RA (RA), and MFI of CD45 positive control in panel (a). Percent positive transfectant clones untreated (b) or 9-cis RA-treated (C). Data determined from a single experiment.
Chapter 4.2 Discussion

The role of retinoid receptors in the control of myelopoiesis has been extensively studied, particularly with respect to granulocytic differentiation. Although the effects of RA on U937 cells have been characterised, the role of the retinoid receptors in monopoiesis has not been determined. In most cases D3 and RA both require the presence of RXR to facilitate activation of their relevant response elements through dimerisation with their cognate receptors VDR, RAR or RXR. Understanding the control of gene expression during differentiation of U937 cells by RXR would help to delineate the mechanisms by which these cells alter their phenotype towards a monocyte/macrophage.

4.2.1 RXRα and RXRβ mRNAs are expressed in U937 cells

Northern blot analysis of RNA isolated from proliferating U937 cells using probes specific for RXRα and RXRβ showed there to be low levels of expression (Figure 4.1.1a). Defacque et al. (1994) were unable to detect RXRα protein in U937 cells using an anti-RXRα antibody in immunoblots. Since the presence of RXR would be required to initiate a cascade of responses to RA or D3, they did not rule out the possibility that there may be very low protein levels that fall below the limit of detection by immunoblotting. Alternatively, they suggested that RXRβ-RAR and RXRβ-VDR heterodimers could signal RA and D3 responses. The northern blotting data presented here support either of these possibilities.

PMA-induced differentiation of U937 cells showed a time dependent increase of RXRα and RXRβ mRNA levels (Figure 4.1.1b). This suggests that RXRα and RXRβ play a role in differentiated U937 cells. Such a role might include the retinoid-signalled down-regulation of TNF-receptor and VDR-mediated protection of apoptosis (Totpal et al., 1995; Hewison et al., 1995)(see section 4.2.3).
4.2.2 Production of stably transfected U937 cells

To study the roles that RXRa and RXRβ play during differentiation, U937 cells were transfected with RXRa or RXRβ cDNA-containing expression vectors that would alter the expression levels of endogenous RXRa and RXRβ. To do this, the expression vector pMEP-4 was used. The complete RXRa coding region was cloned into pMEP-4 in either sense or antisense orientations (with respect to the hMTIIa promoter) to derive the plasmids pMEP-RXRαS and pMEP-RXRαA, respectively. The complete RXRβ coding region was cloned into pMEP-4 in an antisense orientation to derive pMEP-RXRβA. These plasmids were introduced into U937 cells by electroporation and transfected cells were selected and maintained in medium containing hygromycin B. Pooled transfectants derived from pMEP-RXRαS, pMEP-RXRαA and pMEP-RXRβA (UαS, UαA and UβA respectively) were established (Table 4.1.1).

Transfected U937 cells that express antisense RXRa RNA were expected to have reduced (or complete loss of) endogenous RXRa protein levels. Expression of antisense RNAs as a mechanism of gene regulation has been observed in biological systems involving proteins such as transwindase and helicase which facilitate the action of dsRNA-specific nuclease (reviewed by Nellen and Lichtenstein, 1993). This method of gene regulation is thought to account for one mechanism by which c-myb expression is controlled in HL60 cells (Boise et al., 1992). dsRNA molecules formed between endogenous RXRa or RXRβ mRNAs and antisense transcripts from pMEP-RXRαA or pMEP-RXRβA would be digested by dsRNA nuclease resulting in loss of the cells' ability to produce RXRa or RXRβ protein. This method of targeted gene knockout differs from the use of antisense oligodeoxynucleotides (ODNs). It is thought that ODNs bind target RNAs and physically block translation of the molecule at the ribosome (reviewed by Stein and Cheng, 1993). Bergman et al. (1993) used ODNs to target the expression of c-myc in U937 cells, successfully down-regulating Myc protein levels. The use of ODNs has certain disadvantages including: possible non-specific toxic effects of ODNs on the cells; unsuitable design of ODN sequence, as some sequences more effectively inhibit target...
gene expression than others relating to which region of the target mRNA they bind; and unsuitable design of negative control ODN sequences (random sequences may still bind target sequences or other important cellular gene mRNAs). There also exists the possibility that certain ODNs may also target sequence-related homologues (discussed by Wagner, 1994). Problems of target specificity, such as antisense RXRα also targeting RXRβ, are overcome since the pMEP constructs express the complete sequence of the target gene.

Preliminary experiments were performed on the pooled transfectants using cell proliferation assays. The transfected U937 clone, MEP, which contains the pMEP-4 plasmid without any insert (Hewison et al., 1994), was included in these and future experiments as a negative control. MEP cells have been shown to behave in a very similar manner to the parental U937 cell line in both morphology, growth kinetics and cell surface antigen expression (Hewison et al., 1994; L. Faulkner, University College London, personal communication). Hence, MEP provided a faithful negative control cell line, showing that the presence of hygromycin B does not significantly alter the normal phenotype of transfected cells. Cell proliferation was determined by incorporation of 125I-dUTP into DNA during S phase of cell division. Results indicated that all-trans RA, 9-cis RA, D3, TNFα and PMA all inhibited proliferation of MEP cells. 9-cis RA, all-trans RA and D3 inhibited proliferation of UαS to a greater extent than MEP whereas UαA showed no significant inhibition (p>0.1) with these treatments (Figure 4.1.8). The presence of CdCl2, although expected to induce transcription of the gene insert, did not alter the response of these cells markedly. Assuming these results are due to altered RXRα expression, the fact UαS and UαA responded to RA and D3 in the absence of CdCl2 suggests that the transfected gene in the expression vector is being transcribed at a sufficient level to elicit an effect on the cell. The hMTIIa promoter is known to be "leaky" in some conditions, possibly due to heavy metal ions in the growth medium which can activate it (Hewison et al., 1995). In addition to the metal response element in hMTIIa there are also two AP-1 binding sites (Lee et al., 1987) which may be active in these cells.
As described in section 4.1.3, the population of cells within pooled transfectants is unstable. Hence, clones from each transfected pooled were isolated by limiting dilution for further experimentation. This procedure yielded between 12 and 15 clones per pooled transfectant (see Appendix 4) from which clones were selected for further study on the basis of their growth response to 9-cis RA and D3. The UαA-derived clone, αB5A, displayed a complete resistance to the growth inhibitory effects of 9-cis RA. By contrast, the UαS-derived clone, αG2S, was profoundly inhibited by the same treatment by up to 95%. For both UαA and UαS there were clones which behaved as MEP cells which would explain the slightly attenuated effects of RA and D3 on these cells. Again, CdCl2 did not have a significant effect on the proliferative response of αG2S or αB5A following RA or D3 treatment.

Neither the transfectant pool UβA, nor any of the UβA-derived clones studied, showed any response that differed from MEP, with or without CdCl2. For this reason, further work was restricted to RXRa transfected cells rather than RXRβ transfected cells.

It was important to characterise the expression of RXRa mRNA and protein in transfectant clones if changes in U937 cell phenotype were to be attributed to the overexpression or ablation of RXRa expression in αG2S and αB5A, respectively. Northern hybridisation analysis using a dsDNA probe indicated that high levels of RXRa mRNA are expressed in αG2S cells that increased slightly following treatment with CdCl2 (Figure 4.1.14). There appeared to be little or no detectable expression of RXRa mRNA, comparable to that of MEP cells. However, treatment of αB5A cells with CdCl2 lead to a signal that might represent hybridisation of the dsDNA probe with antisense RXRa RNA derived from the transfected plasmid, pMEP-RXRαA.
Two methods were employed to study RXRα protein expression: receptor binding assays and transient transfection of a RARE-luciferase reporter construct. Immunoblotting would have been a third method but no suitable anti-RXRα antibody was available.

Two types of receptor binding assays were performed using $^3$H-9-cis RA. To begin with, saturation assays were performed, the results of which could have been used to determine the number of retinoid receptors per cell. This method involves the incubation of transfectant clones with increasing concentrations of $^3$H-9-cis RA until all receptor binding sites are saturated. In plots of free $^3$H-9-cis RA concentration against bound ligand (data not shown) the subsequent curve should flatten at the point of saturation, termed $B_{\text{max}}$. The binding affinity of the receptor, defined as the dissociation constant ($K_d$), can be determined by extrapolating the point on the Y-axis at $1/2 \ B_{\text{max}}$ through the curve to the X-axis. Specific binding of $^3$H-9-cis RA decreased above approximately 1nM free $^3$H-9-cis RA. Also, the approximate $B_{\text{max}}$ values for MEP and αG2S (respectively 2.8 x 10^{-5} and 1.4 x 10^{-5} fmol bound ligand per cell) were higher than that for αB5A (9 x 10^{-6} fmol bound ligand per cell) indicating fewer retinoid receptors in αB5A. However, calculation of the $K_d$ from each of these cell lines (100-400fmol) was lower than the expected $K_d$ of the RXRs in vitro (between 14 and 19nM; Allenby et al., 1993). This implies that the $B_{\text{max}}$ determined from these experiments is not the true $B_{\text{max}}$. Hence, as an alternative to saturation binding assays, displacement assays were performed, where a set concentration of $^3$H-9-cis RA was incubated with a range of concentrations of unlabelled 9-cis RA. The results were plotted as DPM of bound $^3$H-9-cis RA versus the concentration of unlabelled 9-cis RA. The resultant curve can be used to determine $K_d$ by extrapolation at the point of 50% displacement (on the Y-axis), but does not give an indication of the number of receptors per cell. Figure 4.1.15 shows displacement curves from representative experiments. More receptors should give a higher DPM, equivalent to total binding at 100fmol of $^3$H-9-cis RA. This was not observed. MEP cells showed the greatest level of binding of the three cell lines. There appeared to be least displaceable binding for αB5A, consistent with the notion that there
are reduced levels of RXRα protein, but since αG2S also showed less displaceable binding than MEP, it is not possible to conclude whether this assay represents true differences in RXRα protein levels. The inability to obtain meaningful data from receptor binding assays may be accounted for by high levels of non-specific binding of ^H-9-cis RA as well as the presence of several other endogenous proteins, such as the RARs and CRABPs, that can bind 9-cis RA (with differing affinities) within the transfected cell lines.

The activity of a RARE-luciferase reporter construct (pR140-luc) was examined in undifferentiated and differentiated cells, in the presence or absence of CdCl2 (Figure 4.1.17). Transient transfection of pR140-luc into MEP and αB5A cells showed that 9-cis RA induced a low level of luciferase activity in these cells. 9-cis RA treated pR140-luc-transfected αG2S cells induced a 25-fold increase in luciferase activity. This effect was increased to 80-fold induction of luciferase activity in the presence of CdCl2. This experiment clearly demonstrates the presence of functional RXRα protein that is constitutively expressed in αG2S cells. CdCl2 upregulation of the luciferase activity in αG2S cells proved that the RXRα protein in these cells is derived from pMEP-RXRαS and is directed by the hMTIIa promoter. The ability of αB5A cells to activate the RARE of pR140-luc would suggest that endogenous RXRα expression in these cells is only partially attenuated. It is also likely that endogenous RXRβ and/or other RARs can also bind and activate pR140-luc. Indeed, the RARβ2 promoter of pR140-luc has been shown to be activated by all isoforms of RXR and RAR (Underhill et al., 1994). Following PMA-differentiation, luciferase activity in αG2S cells was reduced, compared to undifferentiated αG2S cells. In contrast, MEP and αB5A cells appeared to have slightly increased luciferase activity. The reasons for these observations are not known. However, it should be noted that the data for MEP and αB5A are within a range close to the sensitivity of the assay and as such may not be wholly reliable.
Each of the above experiments have been employed to determine the nature of RXRα expression in αG2S and αB5A cells at the protein level by receptor binding assays and RARE-linked luciferase assays. Northern analysis indicated αG2S cells expressed high levels of RXRα mRNA, both in the absence and presence of CdCl₂. In the absence of CdCl₂, RXRα mRNA was not detected in αB5A cells, while it seems feasible that the signal observed in CdCl₂ treated cells represents antisense RXRα RNA. While receptor binding assays failed to demonstrate the RXRα content of αB5A and αG2S cells, the use of pR-140-luc clearly showed that αG2S cells express elevated levels of RXRα that are capable of binding and activating a DR-5 RARE in the presence of 9-cis RA. Furthermore, CdCl₂ further increased the levels of RXRα expression, proving that this RXRα protein originates from pMEP-RXRαS in αG2S cells.

4.2.3 RXRα is involved in the proliferative response of U937 cells to RA and D3

The effect of RA treatment on the transfectant clones was assessed using cell proliferation assays. αB5A cells displayed characteristic resistance to the antiproliferative effects of a range of concentrations of 9-cis RA up to 3μM (Figure 4.1.19). Similarly, all-trans RA did not inhibit the growth of αB5A cells (Figure 4.1.21a). The dose-dependent inhibition of αG2S cell growth in response to 9-cis RA and all-trans RA was clearly much more profound than that of MEP cells (Figure 4.1.20 and 4.1.21). These data not only indicate that RXRα specifically modulates the antiproliferative effect of RA, but that RAR is likely to be involved. All-trans RA activates gene transcription via RXR-RAR dimers on DR-2 or DR-5 RAREs. 9-cis RA produces stronger gene activation through 9-cis RA binding of RXR-RAR dimers and by promoting RXR-RXR homodimer formation that can also transactivate from DR-2 and DR-5 RAREs (Zhang et al., 1992). This is supported by the 10-fold greater growth inhibition of αG2S cell growth by 9-cis RA over all-trans RA. The greater potency of 9-cis RA to differentiate myeloid leukaemia cell isolates and established cell lines has been demonstrated (Sakashita et al., 1993). Receptor-specific retinoid analogues have shown that granulocytic differentiation of HL60 cells is signalled via RXR-RAR dimers and not RXR-RXR homodimers (Dawson
et al., 1994; Heyman et al., 1995). This does not rule out RXR-RXR homodimer involvement in monocytic differentiation, although the issue could be clarified by investigating whether or not an RXR-specific retinoid analogue, such as LG1069, is capable of inducing a response similar to 9-cis RA in αG2S cells. As discussed in section 4.2.1, Defacque et al. (1994) suggested that very low levels of RXRα are present in untreated U937 cells and are required to initiate the cascade of events that lead to differentiation following RA treatment. If this were the case it would explain why pMEP-RXRαA, in αB5A cells, does not require CdCl₂ induction of antisense RXRα RNA since the existing low levels of expression arising from this plasmid are sufficient to knockout endogenous RXRα protein levels. Likewise, over-expression of RXRα protein in αG2S would result in higher levels of activated, dimerised RXRα protein, amplifying the RA response.

The antiproliferative response to D3 was completely blocked in αB5A cells (Figure 4.1.22a). This suggests that the receptor of D3, VDR, exclusively heterodimerises with RXRα in order to signal cessation of proliferation. It was therefore surprising that αG2S cells were not hyper-responsive to D3 treatment, and behaved in the same way as MEP (Figure 4.1.22b). It was noted that the transfectant pool UαS did have a stronger response to D3 than MEP cells. The reason for this is not known. Nonetheless, in the case of αG2S cells, the question arises: why can attenuated levels of RXRα block D3-signalled differentiation through reduced numbers of RXRα-VDR dimers, whereas over-expression of RXRα does not lead to increased D3-mediated responses through higher numbers of RXRα-VDR dimers? It is possible that the response of αG2S cells to D3 is limited by a restricted number of non-receptor proteins that bridge the VDRE-bound RXRα-VDR dimer to the transcription initiation complex. Alternatively, D3-responsive genes that signal differentiation may have a slow rate of transcription, the effect of which would be to limit the number of available VDRE sequences that can be bound by RXRα-VDR dimers. A further hypothesis is that there are a limited number of VDR molecules with which RXRα can dimerise, thereby restricting the cellular response to D3. In support of
this hypothesis, Hewison et al. (1994) have produced stably transfected U937 cells that over-express VDR (called DH39) using the pMEP-4 expression vector. DH39 cells were shown to have an increased antiproliferative response to D3 treatment, reminiscent of the RA response of αG2S cells. It may be possible that the increased sensitivity to D3 in DH39 cells is in part facilitated by normally saturating levels of RXRα being able to form more heterodimer complexes with VDR. Hewison et al. (1995) have recently generated antisense VDR RNA-expressing transfected U937 cells, called DH42. These cells were resistant to D3 treatment in the presence of low levels of antisense VDR RNAs (arising from a leaky hMTIIa promoter). The behaviour of DH42 cells, whereby low levels of antisense VDR expression can inhibit the differentiating effect of D3, is also reminiscent of αB5A cell responses to D3 and RA. However, CdCl2 treatment of DH42 cells reduced VDR levels still further, resulting in apoptosis; a process that is ligand-independent. Hence, VDR signals two different cellular responses, differentiation and resistance to apoptosis. Whether the latter response is dependent on dimerisation with RXRα is not known, although a brief study of apoptosis in MEP, αG2S and αB5A cells (induced by cyclohexamide) by R. Cairns for a BSc project (in our laboratory) did not reveal any apparent differences between these three cell lines. This does not necessarily rule out the possibility that RXRβ may be involved.

The combined effect of RA and D3 being able to overcome the resistant phenotype of αB5A cells and induce growth arrest (Figure 4.1.23) is difficult to explain. 9-cis RA and D3 treatment induces upregulation of RAR and VDR, respectively. Upregulation of both of these receptors by their respective ligands would probably require RXR; however, it should be recalled that RXRβ (and possibly low levels of RXRα) are available in αB5A cells. It is conceivable that RAR-VDR dimers may be able to signal the antiproliferative response in αB5A cells. RAR-VDR heterodimer activation of a DR-6 response element (from the human osteocalcin gene promoter) has been described in vitro, although the ligand required for this activity was all-trans RA (Schräder et al., 1993). If this is the case, then this result defines an additional signalling mechanism that is involved in
monoblastic differentiation. The combined treatment of RA and D3 has been observed as having a strong differentiative effect on U937 and HL60 cells, which is greater than that achieved by either of these treatments alone, and leads to a more mature phenotype (Taimi et al., 1993; Brown et al., 1994). Such enhanced effects may be as a result of this additional signalling mechanism.

As discussed in section 1.3, PML-RAR is thought to produce a block in myeloid differentiation by acting as a dominant negative RAR as well as a dominant negative PML protein. In APL cells that express PML-RAR, RXR is believed to be sequestered away from its normal function in signalling differentiation. PML-RAR has been stably transfected into U937 cells by Grignani et al. (1993). These cells were shown to be resistant to the differentiating effect of D3, consistent with the notion that RXRα is sequestered by PML-RAR. This work also recapitulates the behaviour of αB5A cells in response to D3. However, PML-RAR transfected U937 cells displayed heightened sensitivity to 1μM all-trans RA. This apparent contradiction to the response of αB5A cells to RA would rule out PML-RAR having a dominant-negative effect on normal RXRα activity during RA treatment of these cells.

Both PMA and TNFα, in the presence or absence of CdCl2, inhibited MEP, UoA, UoS and UβA cell growth to the same extent of approximately 95% and 50%, respectively (Figure 4.1.9 and 4.1.12). Similarly, αG2S cells responded in the same way as MEP cells to PMA and TNFα (Figure 4.1.24). However, αB5A cells showed a measure of resistance to PMA treatment. This implies that although RXRα and RXRβ do not play a role in the differentiative signal of TNFα, there remains the possibility that PMA induction might involve RXRα. Signalling mechanisms that are distinct from the RXRs, or act downstream of RXR-mediated signals, would account for the TNFα response as well as suggest a possible convergence of RXR activity with PMA-associated signalling. For example, RXRα may signal the production of a protein responsible for PKC activation, an action when performed directly by PMA (see section 1.1) would bypass a RXRα step.
Signalling from the TNF-receptor is thought to be transduced via the Raf-1, PKC, PLC, PLA2, and neutral SMase (Belka et al., 1995) (see section 1.1) and hence, unlikely to require the activity of RXRα.

### 4.2.4 RXRα influences the expression of the β2-integrins

RA treatment of U937 cells does not induce differentiation *per se* but induces differentiation commitment (see section 1.1.4). Nonetheless, β2-integrin expression was shown to be upregulated in all three cell lines following 48 hour treatment with 1μM 9-cis RA. A number of differences were observed between αG2S cells and MEP cells which are described in detail in section 4.1.6.2. The most significant of these were firstly, the increased proportion of cells expressing CD11b and CD11c (expressed as percent positive) prior to 9-cis RA treatment compared with MEP and αB5A cells. Secondly, 9-cis RA induced the greatest increase in the number of CD11a, CD11b, CD11c and CD18 antigens per cell (expressed as ΔMFI) on αG2S. Of these antigens, CD11b was expressed on αG2S cells at a higher level than MEP and αB5A cells prior to treatment, and this was greatly increased following 9-cis RA treatment. For each antigen, 9-cis RA had the effect of inducing a population of high expressing αG2S cells that are seen on the histograms of Figure 4.1.25 as a secondary peak. These data indicate that RXRα may be involved in mediating the expression of these β2-integrins during monoblastic differentiation, particularly with respect to CD11b. The lower density of CD11a, CD11c and CD18 antigens on untreated αG2S cells, compared with MEP cells, raises the possibility that over expression of RXRα may slightly down-regulate their expression, perhaps through suppression of Fos/Jun-DNA binding (a ligand-independent activity of the RXRs; see section 1.3.3.5).

αB5A cell expression of the β2-integrins was found to closely match that observed for MEP control cell expression. If αB5A cells were completely blocking the differentiating
effects of RA one might expect there to be no changes in cell surface antigen expression. As this was not the case, two possible explanations could account for the apparently normal response to RA. Firstly, the expression of the $\beta_2$-integrins may be under the control of certain RAREs, either directly or indirectly, which can also be activated by RXR$\beta$. It is not known whether this represents functional redundancy, whereby RXR$\beta$ is equally capable of performing the task normally done by RXR$\alpha$, or whether RXR$\beta$ normally signals $\beta_2$-integrin expression. In the latter case, increased RXR$\alpha$ expression in $\alpha$G2S cells may be inducing high levels of $\beta_2$-integrin expression by activating promiscuously the same RAREs. A second explanation for the normal behaviour of $\alpha$B5A may be that a block of RA-induced growth inhibition does not necessarily represent a block of differentiation. RXR$\alpha$ may be important in signalling the anti-proliferative effect of RA but not other differentiative characteristics. Finally, the levels of RXR$\alpha$ may be sufficiently reduced in $\alpha$B5A cells to block RA-signalled growth arrest but not $\beta_2$-integrin expression. This experiment has established that RXR$\alpha$ is capable of signalling $\beta_2$-integrin expression in response to RA, but it seems likely that other retinoid receptors are involved.

Conclusions drawn from this work, and future experiments to be performed, are found in the section 5.2.
CHAPTER 5

CONCLUSIONS AND FUTURE OBJECTIVES
Chapter 5 Conclusions and future objectives

5.1 Control of c-fgr gene expression during U937 cell differentiation

The activity of the myeloid-specific promoter of the c-fgr proto-oncogene has been characterised using the U937 cell line as a model for monoblastic differentiation. In this thesis it has been shown that in these cells, basal levels of c-fgr expression are directed by the region from -752 to -343bp, with respect to the transcription start site. The region from -1137 to -772bp contains sequences responsible for induction of c-fgr expression in response to PMA. This promoter is not functional in EBV-transformed B-cell lines, its activity being unique to myeloid cell lineages. Hence, transcription factors that activate the myeloid-specific c-fgr promoter must also be specifically expressed and activated during myeloid differentiation. In order to determine which transcription factors bind to the myeloid-specific promoter during differentiation, the precise region of binding will have to be determined. This will require fine mapping of the PMA response element (to approximately 100bp) by construction of further luciferase reporter plasmids in the region surrounding -911bp. Following this, band shift (gel retardation) assays and DNA footprinting assays will have to be performed to determine the precise binding sequences of PMA-induced transcription factors, and to allow for the identification and isolation of the bound transcription factors.

Combined treatment with D3 and TNFα did not induce promoter activity in any of the pGL constructs. A likely explanation for this could be that these agents induce c-fgr expression via a different promoter. To determine where this promoter is, it would first be necessary to identify the major c-fgr mRNA transcript that is induced by this treatment since it has not been shown whether or not such transcripts would contain exon M4. The kinetics of D3+TNFα-induced c-fgr mRNA accumulation differs from that induced by PMA. Therefore, it might be concluded that there are two c-fgr promoters defining alternative signalling pathways that relate to differing functional properties of a mature monocyte/macrophage.
5.2 Regulation of gene expression by RXRα during U937 cell differentiation

In this thesis it has also been established that RXRα and RXRβ mRNAs are expressed in undifferentiated U937 cells. Furthermore, differentiation of U937 cells, by PMA treatment, leads to upregulation of RXRα and RXRβ mRNA levels. Stable transfection of U937 cells with sense or antisense RXRα RNA expressing plasmids has generated two novel cell lines, αG2S and αB5A, which allow for elucidation of the function of RXRα in this lineage.

The inability of αB5A, the antisense expressing cell line, to growth arrest in response to RA treatment indicates a pivotal role for RXRα. Whether this results from complete loss of RXRα expression, or reduction of RXRα to levels below a required threshold, remains unclear. The level of RXRα mRNA expression in αB5A cells was studied by northern hybridisation with a dsDNA probe specific to RXRα. Untreated αB5A cells did not appear to express detectable RXRα, while the treatment with CdCl2 resulted in a signal that may represent hybridisation with antisense RXRα RNA. CdCl2 induction of the transfected gene in αB5A cells did not appear to be required to achieve a RA-resistant phenotype, suggesting that low levels of antisense RXRα RNA in CdCl2-untreated cells may be sufficient to elicit this change in response. Defacque et al. (1994) were unable to detect RXRα protein in undifferentiated U937 cells. It is possible that differentiation commitment may require de novo expression of RXRα protein from low levels of mRNA prior to growth arrest. Such a hypothesis might be tested by RA treatment of U937 cells which have been incubated in cyclohexamide (inhibiting translation of RXRα mRNA). Over expression of RXRα mRNA in αG2S cells resulted in hyper-responsiveness to RA, characterised by almost complete growth arrest of these cells. This effect, complementary to that of αB5A, further demonstrates the ability of RXRα to signal growth arrest. The difference in the levels of response of αG2S to 9-cis RA and all-trans RA suggests that RXRα could signal differentiation through target gene promoters via the bi-functional DR-2 and/or DR-5 RAREs, binding as RXRα-RAR heterodimers in the presence of all-trans RA, and binding predominantly as RXRα-RXR homodimers in the presence of 9-
cis RA. This issue could be clarified by the use of receptor-specific retinoids such as TTNPB and LDG1069 in cell proliferation assays.

The importance of RXRα in D3-signalled differentiation was demonstrated by the inability of D3 to inhibit αB5A proliferation. Interestingly, αG2S behaved in the same way as control cells in response to D3 treatment, suggesting a limiting factor in D3-mediated growth arrest. The combination of 9-cis RA and D3 on αB5A inhibited proliferation. This raises the possibility that under these circumstances, growth arrest may be signalled by RAR-VDR dimers (see section 4.2.3).

TNFα inhibits proliferation equally in all cell lines tested, indicating a signalling pathway that is RXRα-independent. PMA also inhibits proliferation of all cell lines, although the effect on αB5A is less marked. Again, this indicates an alternative signalling pathway, but does not rule out the possibility that this pathway converges at some point with that involving RXRα.

The analysis of β2-integrins, as markers of differentiation, showed that αB5A had a normal response to 9-cis RA. Conversely, αG2S showed increased expression of each antigen studied, especially CD11b. It was concluded that, while RXRα is capable of inducing CD11a, CD11b, CD11c and CD18 expression, RXRβ may also be able to do so. This result was similar to that observed when RARE activity in αG2S and αB5A cells was studied using pR140-luc. High luciferase activity was observed in 9-cis RA treated αG2S cells whereas luciferase activity in αB5A cells resembled MEP control cells. This supports the notion that other endogenous retinoid receptors, such as RXRβ, are active in αB5A cells. Alternatively, the normal response of αB5A cells could be attributable to insufficient abrogation of RXRα in these cells such that β2-integrin expression, mediated by endogenous RXRα, is unaffected.
The implication from these experiments is that RXRα may not be responsible for signalling monoblastic differentiation per se, but is an absolute requirement for initiating the cascade of events that lead to growth arrest following RA or D3 treatment.

Downstream targets for RA and/or D3 in signalling cessation of proliferation need to be identified. Candidate genes include those encoding Myc, Mad, Egr-1, and Myb, all of which have been shown to control the proliferative state of U937 cells. For example, RXRα may act to down-regulate (either directly or indirectly) Myc expression, or alternatively act to upregulate Mad expression (see section 1.1). Such questions could be explored by comparing the expression of these candidate genes (by northern analysis) during RA and D3-induced differentiation of αB5A and αG2S cells.

Features of differentiated U937 cells warrant further analysis to determine the role of RXRα in monocyte/macrophage function. For example, expression of surface antigens, such as CD14, CD23, and CD38 (Drach et al., 1994), could be investigated, as could the role of the RXRs in protection from apoptosis (associated with VDR), and the RA-mediated down regulation of TNF receptors.

U937 cells transfected with RXRβ antisense expressing plasmids (UβA and derived clones) behaved in the same way as control cells in cell proliferation assays. Since RXRβ expression in these cells has not been studied, it cannot be concluded that RXRβ is not involved in the control of growth arrest. However, if RXRβ is involved in this process it might be expected that RXRα antisense transfected cells would remain sensitive to RA and D3 treatment (via endogenous RXRβ), which is not the case. Nonetheless, RXRβ is upregulated during monoblastic differentiation and so would be very likely to play a role in the mature cell. For this reason it would be desirable to produce RXRβ sense expressing transfected U937 cells. The possibility of producing RXRα/RXRβ double antisense transfected cells is also attractive. Finally, the expression of RXRγ has not been...
examined in U937 cells, although RXRγ expression has been found in HL60 cells (Brown
et al., 1994), suggesting a further line of investigation.

Changes in gene expression during monoblastic differentiation have been studied from
the perspective of the regulators (the RXRs) and of the regulated (c-fgr). This thesis has
contributed to our understanding of the means by which monoblastic cells differentiate
towards a monocyte/macrophage by identifying a role for RXRα in growth arrest, and
characterising the promoter of a lineage-specific gene, c-fgr, during this process.
APPENDICES
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>EBNA</td>
<td>EBV-determined nuclear antigen</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EMBL</td>
<td>European Molecular Biology Laboratories</td>
</tr>
<tr>
<td>ER</td>
<td>oestrogen receptor</td>
</tr>
<tr>
<td>fMLP</td>
<td>formyl-methionine-leucine-phenylalanine</td>
</tr>
<tr>
<td>FXR</td>
<td>farnesoid X-activated receptor</td>
</tr>
<tr>
<td>G</td>
<td>glycine</td>
</tr>
<tr>
<td>GAP</td>
<td>Ras GTPase activating protein</td>
</tr>
<tr>
<td>GEMM-CSF</td>
<td>granulocyte-erythrocyte-monocyte/macrophage CSF</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte/macrophage CSF</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>GR-FeSV</td>
<td>Gardner-Rasheed feline sarcoma virus</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid</td>
</tr>
<tr>
<td>I</td>
<td>isoleucine</td>
</tr>
<tr>
<td>IFNγ</td>
<td>interferon-γ</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin 6</td>
</tr>
<tr>
<td>Kd</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>L</td>
<td>leucine</td>
</tr>
<tr>
<td>LB + amp</td>
<td>Lauria-Bertoni with ampicillin</td>
</tr>
<tr>
<td>LBD</td>
<td>ligand binding domain</td>
</tr>
<tr>
<td>LMP</td>
<td>low melting point</td>
</tr>
<tr>
<td>LTP</td>
<td>long term potentiation</td>
</tr>
<tr>
<td>M</td>
<td>methionine</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage CSF</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NRS</td>
<td>normal rabbit serum</td>
</tr>
<tr>
<td>ODN</td>
<td>oligodeoxynucleotides</td>
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</table>
P  proline
PDGF  platelet derived growth factor
PDGF-R  PDGF receptor
PEG  polyethylene glycol
PI 3-K  phosphotidylinositol 3-kinase
PKC  protein kinase C
PLCγ  phospholipase C-γ
PMA  phorbol 12-myristate 13-acetate
PPAR  peroxisome proliferator-activated receptor
PTPase  protein tyrosine phosphatase
R  arginine
RA  retinoic acid
RAR  retinoic acid receptor
RARE  retinoic acid response element
RSV  Rous sarcoma virus
RXR  retinoid X receptor
S  serine
sd  standard deviation
SDS  sodium dodecyl sulphate
SEM  standard error mean
SH2  Src homology 2
Smase  sphingomyelinase
SSC  sodium saline citrate
T  threonine
T3  thyroid hormone
TAE  tris-acetate EDTA
TAM  tyrosine activation motif
TBE  tris-borate EDTA
TCA  trichloroacetic acid
TCR  T-cell antigen receptor
TEMED  N, N, N', N'-tetramethylethylediamine
TGFβ  transforming growth factor-β
Tm  melting temperature
TNFα  tumour necrosis factor-α
TR  thyroid hormone receptor
TRE  thyroid hormone response element
VAD  vitamin A deficiency
VDR  vitamin D receptor
VDRE  vitamin D response element
X-Gal  5-bromo 4-chloro 3-indolyl β-D-galactopyranoside
Y  tyrosine
ZAP-70  p70 zeta-associated protein
Appendix 2  Solutions

Binding assay lysis buffer
10mM Tris-HCl, 250mM sucrose, 1% Triton X-100 (v/v), pH 7.4

Chloroform:IAA
Chloroform:Iso-amyl alcohol at 49:1, stored under water

ddH₂O
Reverse osmosis purified water, further purified by a resin filters (Milli-Q plus 185, Millipore, Watford, Beds.) sterilised by autoclaving at 120°C for 30mins. For working with RNA, water was autoclaved with 10μl diethylpyrocarbonate (DEPC) per 500ml of water in glassware that had been baked for 3 hours at 180°C.

Denaturation Solution
0.5M NaOH, 1.5M NaCl

100 x Denhardt’s Solution
10g Ficoll, 10g Polyvinylpyrrolidone, 10g BSA in 500ml ddH₂O.

L. uria-Bertoni with ampicillin (LB + amp) broth/ agar
1% tryptone (Bacto), 0.5% yeast extract (Bacto), 1% NaCl, (2% w/v agar [Difco]).
Ampicillin (amp)(Pharmacia) was made to a stock of 100mg/ml and added to autoclaved LB broth or agar (prior to setting) at 1μl stock per millilitre of LB.

Oligo-labelling Buffer
Solution O = 1.25M Tris-HCl pH 8.0, 0.125M MgCl₂
Solution A= Solution O 1μl
2-mercaptoethanol 18μl
100mM dATP 5μl
100mM dGTP 5μl
100mM dTTP 5μl

Solution B = 2M HEPES pH6.6 (pH with 4M NaOH)
Solution C = Random 6mer hexanucleotide at 90ODu/ml (Boeringer Mannheim)

Mix A,B and C as A:B:C = 2:5:3
Stored at -20°C.

10xMOPS buffer (pH 5.5-7.0)
0.2M MOPS (3-[N-morpholino] propanesulphonate)
50mM Sodium acetate
10mM EDTA

Neutralisation Solution
0.5M Tris-HCl, 3M NaCl, pH7.0

Northern Prehybridisation Mix
50% (v/v) formamide
5xDenhardt’s solution (filtered)
5xSSC
0.1% (w/v) SDS
100mg/ml herring sperm DNA
200mg/ml yeast total RNA

10 x PBS
1.3M NaCl, 70mM Na2HPO4, 30mM NaH2PO4
RFBI (100ml):
Potassium acetate 0.3g
MnCl$_2$.2.H$_2$O 0.99g
1mM CaCl$_2$ 1ml
RbCl(0.5g/ml) 2.4ml
200mM acetic acid 1ml
ddH$_2$O up to 100ml and filter sterilised

RFBII (50ml):
100mM MOPS (pH6.8) 5ml
1M CaCl$_2$ 3.75ml
RbCl (0.5g/ml) 120µl
Glycerol 7.5ml
ddH$_2$O up to 50ml and filter sterilised

SET
100mM NaCl, 10mM Tris-HCl, 1mM EDTA, pH 8.0

Solution D
4M Guanidium thiocyanate
250mM Sodium citrate (pH7.0)
0.5% (w/v) sarcosyl
100mM 2-mercaptethanol

Solution I
50mM glucose, 25mM Tris-HCl, 1mM EDTA pH8.0

Solution II
200mM NaOH, 1% (w/v) SDS
Solution III
60ml 5M Potassium acetate, 11.5ml acetic acid, 28.5ml ddH2O

Southern Prehybridisation Mix
6xSSC
5xDenhardt’s solution
0.5% (w/v) SDS.

20 x SSC
300mM Sodium citrate, 3M NaCl

50 x TAE
2M TrisHCl, 1M Acetic acid, 100mM EDTA

10 x TBE
900mM Tris-HCl, 900mM Boric acid, 12.5mM EDTA, pH8.4

TE
10mM Tris-HCl, 1mM EDTA, pH8.0

All basic chemicals supplied by Merck BDH.
Appendix 3  Cloning strategies.
Figure 6.1. Cloning strategy for the construction of pB1-1.
Figure 6.2. Cloning strategy for the construction of pGCAT-113.
Figure 6.3. Cloning strategy for the construction of pGCAT-359 and pGCAT-MR.
**Figure 6.4.** Cloning strategy for the construction of pGCAT-772
Figure 6.5. Cloning strategy for the construction of pGL-BSK.
Figure 6.6. Cloning strategy for the construction of pSK-Eco4.5.
**Figure 6.7.** Cloning strategy for the construction of pGL-128.
**Figure 6.8.** Cloning strategy for the construction of pGL-343.
**Figure 6.9.** Cloning strategy for the construction of pGL-752.
Figure 6.10. Cloning strategy for the construction of pGL-772.
pGL-1688 digested with Smal to produce a 1236bp fragment

1236bp fragment digested with Dral (blunt cutter) to produce 1010bp fragment

pGL-2basic linearised with Smal

Figure 6.11. Cloning strategy for the construction of pGL-911.
Figure 6.12. Cloning strategy for the construction of pGL-1137.
pGL-1688 digested with $Esp3I$ to linearise plasmid, and blunted with klenow polymerase

Linearised plasmid digested with $DraIII$, removing 5' sequences, and blunted with klenow polymerase

blunted ends religated

pGL-1211

Figure 6.13. Cloning strategy for the construction of pGL-1211.
Figure 6.14. Cloning strategy for the construction of pGL-1688.

- pGL-L digested with KpnI
- Fill ends with KpnI by incubation with Klenow polymerase to give blunt ends.
- Digested with AflIII
- Linearised pGL-752
- pSK-Eco4.5 digested with Rsal & AflIII
- 1kb fragment
- ligate
- pGL-1688
Figure 6.15. Cloning strategy for the construction of pGL-3200.
Figure 6.16. Cloning strategy for the construction of pMEP-RXRαS and pMEP-RXRαA.
Figure 6.17. Cloning strategy for the construction of pMEP-RXRβA.
Appendix 4  List of transfectant clones.

UαA(1) clones.

<table>
<thead>
<tr>
<th>αB5A</th>
<th>αB8A</th>
<th>αC1A</th>
<th>αC3A</th>
<th>αD1A</th>
<th>αD5A</th>
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</thead>
<tbody>
<tr>
<td>αD9A</td>
<td>αD11A</td>
<td>αE4A</td>
<td>αF1A</td>
<td>αF5A</td>
<td>αF10A</td>
</tr>
<tr>
<td>αG10A</td>
<td>αH6A</td>
<td>αH12A</td>
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UαS(1) clones.

<table>
<thead>
<tr>
<th>αB3S</th>
<th>αB4S</th>
<th>αB5S</th>
<th>αC1S</th>
<th>αE1S</th>
<th>αE9S</th>
</tr>
</thead>
<tbody>
<tr>
<td>αF7S</td>
<td>αG1S</td>
<td>αG2S</td>
<td>αG3S</td>
<td>αG6S</td>
<td>αG8S</td>
</tr>
<tr>
<td>αG9S</td>
<td>αH7S</td>
<td>αH9S</td>
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UβA clones.

<table>
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<tr>
<th>βB8A</th>
<th>βB9A</th>
<th>βB10A</th>
<th>βC6A</th>
<th>βC11A</th>
<th>βD12A</th>
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<tbody>
<tr>
<td>βE8A</td>
<td>βE10A</td>
<td>βE11A</td>
<td>βF2A</td>
<td>βF9A</td>
<td>βF11A</td>
</tr>
<tr>
<td>βG4A</td>
<td>βG5A</td>
<td>βG6A</td>
<td>βG9A</td>
<td></td>
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Appendix 5     Publications arising from this thesis

REFERENCES


