LCR-ACTIVATED EXPRESSION OF CLONED K+ CHANNEL GENES IN MAMMALIAN CELLS

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Abstract

The human β-globin LCR confers high levels of expression on cis linked cDNAs and genes under control of the β-globin promoter, and certain genes under control of their native promoter elements. This heterologous expression is copy-number-dependent and position-independent in the nuclei of erythroid specific cells.

Mouse erythroleukemia (MEL) cells have been established as being electrophysiologically quiet with respect to K+ channels and suitable for patch-clamp analysis. Furthermore, the MEL-cell line has been utilized as an erythroid-specific host for the stable expression of mammalian delayed rectifier (RCK1), transient (A-type) (RatShal and hPCN2) and inward rectifier type (IRK1) voltage-activated K+ channel cDNAs under control of the β-globin promoter.

Parallel work has allowed the characterisation of a novel human delayed-rectifier type K+ channel (hCDR1) from human chromosomal DNA, and an analysis of the hCDR1 gene promoter region has been initiated. Furthermore, a 5.2kb BgIII-generated DNA fragment (hCDR15.2) containing the full hCDR1 coding-region and 2.4kb of upstream chromosomal sequence was isolated. hCDR15.2 has been linked to the human β-globin LCR and activation of native hCDR1 promoter elements in MEL-cell nuclei and subsequent detection of expressed hCDR1 homotetramers was achieved by patch-clamp analysis.

Finally, the establishment of a gene-activation library for the functional expression of novel and existing K+ channel genes has been attempted. A rabbit genomic DNA cosmid library was constructed with firstly a 1kb (nanolocus) LCR cassette shown to confer 30-40% full LCR activity and secondly a full LCR (6.5kb) based vector. 14 different nanolocus-based rabbit genomic cosmid library clones, shown to be homologues of voltage-activated K+ channel sequences, were isolated. No detectable expression was observed from any of these clones after stable integration into MEL-cell nuclei. Transcriptional silencing and insulation have been discussed as probable reasons for the lack of detectable expression from these 14 clones.
Acknowledgements

I would like to especially thank Prof. W. J. Brammar and Dr. Ed Conley for their support, guidance, enthusiasm and helpful input to my work. I would also like to thank the rest of the staff of the ICI Joint Laboratory for their help and moral support during my time at Leicester.

Many thanks also go to Dr. Noel Davies (Dept. Physiology, Leicester University) for his collaboration with all the electrophysiology that is presented in this thesis.
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenine 5'-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytosine 5'-triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxyguanine 5'-triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxythymine 5'-triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>IAA</td>
<td>Isoamyl alcohol</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-thiogalactoside</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propanesulphonic acid</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard sodium citrate</td>
</tr>
<tr>
<td>tk</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
</tbody>
</table>
## CONTENTS

### CHAPTER 1  INTRODUCTION

Part 1 Voltage-activated K⁺ channels

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.2. The diversity of K⁺ channels</td>
<td>1</td>
</tr>
<tr>
<td>1.3. Overview of voltage-activated K⁺ channels</td>
<td>2</td>
</tr>
<tr>
<td>1.4. Delayed rectifiers</td>
<td>2</td>
</tr>
<tr>
<td>1.5. Transient (A-type)</td>
<td>2</td>
</tr>
<tr>
<td>1.6. Inward rectifiers</td>
<td>3</td>
</tr>
<tr>
<td>1.7. <em>Shaker</em>: A route to the molecular study of voltage-activated K⁺ channels</td>
<td>3</td>
</tr>
<tr>
<td>1.8. Delayed rectifier and transient types</td>
<td>4</td>
</tr>
<tr>
<td>1.9. minK</td>
<td>5</td>
</tr>
<tr>
<td>1.10. Inward rectifiers</td>
<td>5</td>
</tr>
<tr>
<td><strong>Molecular structure of voltage-activated K⁺ channels</strong></td>
<td></td>
</tr>
<tr>
<td>1.11. Delayed rectifier and transient types</td>
<td>6</td>
</tr>
<tr>
<td>1.12. Gating</td>
<td>6</td>
</tr>
<tr>
<td>1.13. Identification of the pore</td>
<td>7</td>
</tr>
<tr>
<td>1.14. minK</td>
<td>8</td>
</tr>
<tr>
<td>1.15. Inward rectifiers</td>
<td>8</td>
</tr>
<tr>
<td>1.16. The oligomeric nature of voltage-activated K⁺ channels</td>
<td>8</td>
</tr>
</tbody>
</table>

Part 2  Eukaryotic expression systems employed for K⁺ channel gene expression

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.17. Introduction</td>
<td>9</td>
</tr>
<tr>
<td>1.18. Cell-free expression system</td>
<td>10</td>
</tr>
<tr>
<td><strong>Direct cytoplasmic microinjection of RNA</strong></td>
<td></td>
</tr>
<tr>
<td>1.19. Microinjection of <em>Xenopus</em> oocytes</td>
<td>11</td>
</tr>
<tr>
<td>1.20. Microinjection of clonal mammalian cells</td>
<td>12</td>
</tr>
</tbody>
</table>
## Contents

1.21. Vaccinia virus 12
1.22. Baculovirus 13
1.23. Promoter/enhancer integrative vectors 13

**Part 3 The Human β-globin Locus Control Region (LCR)**

1.24. Introduction 15
1.25. DNase I hypersensitive (HS) sites 15
1.26. The Locus Control Region (LCR) 16

**Domains within the LCR**

1.27. Overview 17
1.28. Activity of the individual SH sites 17
1.29. Globin gene switching 19
1.30. Factors influencing individual LCR-gene interactions 20
1.31. LCR involvement in developmental switching 21
1.32. MEL-cells 22

**CHAPTER 2 MATERIALS AND METHODS**

2.1. Chemicals 23
2.2. Enzymes and proteins 23
2.3. Bacterial strains and culture conditions 24
2.4. Cloning vectors 24
2.5. Methods of sterilisation 25

**Nucleic acid methods**

2.6. Solutions used during the handling of nucleic acids 25
2.7. Oligonucleotides used 30
2.8. Purification of high molecular weight genomic DNA from tissues 32
2.9. Purification of total RNA from tissues 32
2.10. Large-scale preparation of plasmid DNA 33
2.11. Small-scale purification of plasmid DNA 33
2.12. Gel electrophoresis 34
2.13. Restriction enzyme digestions and Southern (DNA) blotting 34
Contents

2.14. Northern (RNA) blotting 35
2.15. Hybridisation of membrane-immobilised nucleic acids 35
2.16. Removal of probes and re-use of DNA and RNA blots 36
2.17. Preparation of $^{32}$P radiolabelled probes 36
2.18. Purification of DNA fragments from agarose gels 37
2.19. Generation of DNA molecules with blunt ends 37
2.20. Ligation of DNA molecules and transformation of competent E. coli cells 37
2.21. Transformation of competent E. coli cells 38
2.22. Screening Bacterial Colonies using DNA Probes 40
2.23. Nucleic acid sequencing 40
2.24. PCR methods 40
2.25. Primer extension 41

In Vitro cell culture methods

2.26. Solutions used during the culture and transformation of animal cells 42
2.27. Cell lines cultured In Vitro 42
2.28. General cell culture 43
2.29. Subculturing and handling of cells 43
2.30. Cryogenic storage of cells 43
2.31. Transfection of MEL-cells 44
2.32. Induction of MEL-cells for expression studies 44

CHAPTER 3 ISOLATION AND CHARACTERISATION OF A HUMAN CARDIAC DELAYED RECTIFIER $K^+$ CHANNEL GENE (hCDR1g) FROM HUMAN GENOMIC DNA

3.1 Introduction 45

Identification of two hCDR1c genomic homologues by PCR, Southern blotting, and sequence analysis

3.2. PCR analysis 45
3.3. Southern blotting analysis 46
3.4. Sequence analysis 46
3.5. Confirmation of the identity of hCDR1 to the hFCN1 and HK2 published 47 sequences.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6. Mapping of the upstream region of hCDR1 from genomic sequence in</td>
<td>47</td>
</tr>
<tr>
<td>cosmids 5 and 11</td>
<td></td>
</tr>
<tr>
<td>3.7. Subcloning of a 5.2kb BglII fragment (hCDR15.2) from cosm id 11</td>
<td>48</td>
</tr>
<tr>
<td>containing the hCDR1 coding region and 2.4kb of upstream region</td>
<td></td>
</tr>
<tr>
<td>3.8. Primer-extension analysis on total human heart and pancreatic RNA</td>
<td>48</td>
</tr>
<tr>
<td>to determine the transcriptional start sites for hCDR1 in these two tissues.</td>
<td></td>
</tr>
<tr>
<td>3.9. Sequence analysis of the hCDR1 upstream region from hCDR15.2</td>
<td>49</td>
</tr>
<tr>
<td>Discussion</td>
<td></td>
</tr>
<tr>
<td>3.10. Isolation and characterisation of hCDR1g</td>
<td>50</td>
</tr>
<tr>
<td>3.11. Primer extension analysis identifies a putative TATA-less promoter 2</td>
<td>50</td>
</tr>
<tr>
<td>and 3bp upstream of the published hPCN1 cDNA sequence</td>
<td></td>
</tr>
<tr>
<td>3.12. Two putative TATA-containing promoter sites</td>
<td>53</td>
</tr>
<tr>
<td>3.13. The glucocorticoid response element</td>
<td>53</td>
</tr>
<tr>
<td>3.14. Conclusions</td>
<td>53</td>
</tr>
<tr>
<td>CHAPTER 4  REGULATED AND FUNCTIONAL EXPRESSION OF K⁺ CHANNEL cDNAs AND GENES UNDER CONTROL OF THE HUMAN β-GLOBIN LCR</td>
<td></td>
</tr>
<tr>
<td>4.1. Introduction</td>
<td>55</td>
</tr>
<tr>
<td>4.2. Analysis of ion-channels in native MEL-cells</td>
<td>55</td>
</tr>
<tr>
<td>Functional expression of a cDNA encoding a subunit of the voltage-activated K⁺ channel RCK1</td>
<td>56</td>
</tr>
<tr>
<td>4.3. Approach</td>
<td>56</td>
</tr>
<tr>
<td>4.4. Construction of the RCK1 expression unit</td>
<td>57</td>
</tr>
<tr>
<td>4.5. Expression of RCK1</td>
<td>58</td>
</tr>
<tr>
<td>Expression of two voltage-activated A-type K⁺ channel subunits</td>
<td>58</td>
</tr>
<tr>
<td>4.6. Approach</td>
<td>58</td>
</tr>
</tbody>
</table>
Contents

Construction of the hPCN2 and Rat Shal -pEV3 expression constructs

4.7. hPCN2 59
4.8. Rat Shal -pEV3 60
4.9. Expression of hPCN2 and Rat Shal 61

Functional expression of the intronless mouse inwardly rectifying K⁺ channel IRK1

4.10. Approach 61
4.11. Establishment of an intronless IRK1 coding-region and construction of RK1-pEV3 62
4.12. Expression of IRK1 63

Activation of hCDR15.2 under native promoter control

4.13. Approach 63
4.14. Construction of the hCDR15.2-pGSE1417 expression construct 63
4.15. Expression of hCDR1 from hCDR15.2 64

Attempted expression of IK8 and K13: two novel K⁺ channel subfamily members

4.16. Approach 65

Construction of the K13-pNV1 and IK8-pNV1 expression units

4.17. K13-pNV1 66
4.18. IK8-pNV1 67
4.19. Expression analysis on K13-pNV1 and IK8-pNV1 67

Discussion

4.20. The first application of LCR/MEL gene-activation to the functional expression of cDNAs and genes encoding mammalian K⁺ channels 67
4.21. MEL-cells 68
4.22. Characteristics of the MEL/LCR system 69
4.23. Future prospects for K⁺ channel expression in the MEL/LCR expression system 69
4.24. Expression analysis on IK3-pNV1 and K13-pNV1 71

CHAPTER 5 CONSTRUCTION OF LCR-BASED GENOMIC GENE-ACTIVATION LIBRARIES FOR EXPRESSION-CLONING OF EXISTING AND NOVEL TYPES OF K⁺ CHANNELS

5.1. Introduction 73

The nanolocus LCR cosmid system

5.2. Approach 75
5.3. Construction of pNANCOSS 76

Experiment to demonstrate the functionality of the LCR nanolocus cassette in pNANCOSS

5.4. Approach 78
5.5. Construction of the two hCDR15.2-pNANCOSS constructs containing hCDR15.2 in both orientations 78
5.6. Activation of hCDR15.2 in both orientations with respect to the LCR-nanolocus in pNANCOSS 79

Construction of a rabbit genomic library in the vector pNANCOSS

5.7. Approach 79
5.8. Construction 79

Retrieval of rabbit K⁺ channel sequences from the rabbit genomic-pNANCOSS library for expression analysis

5.9. Screening the rabbit genomic-pNANCOSS library with six PCR amplified K⁺ channel probes 81
5.10. Isolation and expression analysis on 10 positively hybridising clones 81
5.11. Further screening of the pNANCOSS-rabbit genomic library with three 83
full length voltage activated K⁺ channel gene probes

The microlocus LCR cosmids system

5.12. Approach 84
5.13. Construction of the pUCOS1 microlocus LCR-based cosmid expression vector 85

Experiment to demonstrate the functionality of the microlocus LCR in pUCOS1

5.14. Approach 86
5.15. Construction of pUCOS1-hCDR15.2 86
5.16. Expression of hCDR1 from a 4.7kb genomic fragment in pUCOS1 87
5.17. Construction of a rabbit genomic library in the vector pUCOS1 87

Discussion

5.18. Properties of the LCR nanolocus in pNANCOS8 87
   No detectable expression from 15 voltage activated rabbit K⁺ channel homologues after linkage to the LCR nanolocus in pNANCOS8
5.19. Putative reasons for no detectable expression 88
5.20. Probable reasons for no detectable expression 89

The pUCOS1 microlocus LCR based library strategy

5.21. The nature of the micolocus LCR in pUCOS1 91
5.22. Screening the pUCOS1-rabbit genomic library 91

CHAPTER 6 DISCUSSION

6.1. Introduction 92
6.2. hCDR1 and K⁺ channel promoter analysis 92
6.3. LCR activation of K⁺ channel cDNAs and genes 93
6.4. LCR gene activation libraries 94
6.5. The LCR nanolocus 94
CHAPTER 1

Introduction
1.1. Introduction

Ionic channels are macromolecular pores in cell membranes, and are most obvious as the fundamental excitable elements in the membranes of excitable cells. These channels bear the same relationship to electrical signalling in nerve, muscle and synapse as enzymes bear to metabolism. They act as molecular amplifiers, opening and closing to shape the signals and responses of the nervous system. Excitation and electrical signalling involves the movement of ions through ionic channels, with Na⁺, K⁺, Ca⁺, and Cl⁻ ions appearing to be responsible for almost all of this action. Each channel may be regarded as an excitable molecule, being specifically responsive to some form of stimulus. This stimulus may be a membrane potential change, a neurotransmitter or other chemical stimulus or a mechanical deformation. The channel's response, called gating, is apparently a simple opening or closing of the pore. The open pore has the important property of selective permeability, allowing a restricted class of ions to flow passively down their electrochemical activity gradients at a rate that is very high (>10⁶ ions per second). Ionic channels are undoubtedly found in the membranes of all cells, and their known functions include establishing a resting membrane potential, shaping electrical signals, controlling cell volume and regulating the net flow of ions across epithelial cells of secretory and resorptive tissues.

1.2 The diversity of K⁺ channels

The diversity of ionic channels that are selective for K⁺ ions is great and this group has been established as being more diverse than any other group of ion channels. The different K⁺ channel types are defined and distinguished more by their gating characteristics than by their ionic selectivity or pharmacology. Currents carried by voltage-gated potassium channels can be categorised into delayed rectifier, inward (anomalous) rectifier and transient (A-) types. In the other category of ligand-gated potassium channels are those gated by calcium, ATP, neurotransmitters, intracellular kinases and GTP-binding proteins. For a review of these channel types see (Rudy, 1988).
1.3. Overview of voltage-activated K\(^+\) channels

Voltage-activated K\(^+\) channels form the largest and most varied class of ionic channels and are broadly diversified to help set the resting potential, repolarize the cell, hyperpolarize the cell and shape voltage movements for action potentials. First described in membranes of skeletal muscle and nerve axons, voltage-activated K\(^+\) conductances are ubiquitous in electrically excitable as well as non-excitable cells. Voltage-activated K\(^+\) conductances are classified into three major families, distinguished mainly by their response to changes in membrane voltage (Table 1.1). Figure 1.1 provides examples of whole cell currents from each of the 3 major classes.

**Table 1.1. Classification of voltage-activated K\(^+\) channels**

<table>
<thead>
<tr>
<th>Family</th>
<th>Activated by</th>
<th>Fast inactivation</th>
</tr>
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<tbody>
<tr>
<td>Delayed rectifiers</td>
<td>Depolarisation</td>
<td>No</td>
</tr>
<tr>
<td>Transient channels</td>
<td>Depolarisation</td>
<td>Yes</td>
</tr>
<tr>
<td>Inward rectifiers</td>
<td>Hyperpolarisation</td>
<td>-</td>
</tr>
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</table>

1.4. Delayed rectifiers

Current through delayed rectifier channels (Figure 1.1 A) turns on with a brief delay following the onset of a membrane depolarisation, and persists while the depolarisation is maintained. Under physiological conditions, open channel currents are larger in the outward than the inward direction, reflecting the different extracellular and intracellular concentrations of K\(^+\) ion. A good example of a delayed rectifier is the K\(^+\) conductance of the squid giant axon (Hodgkin and Huxley, 1952), which causes the quick repolarisation that terminates the action potential.

1.5. Transient (A-type)

The permeability of transient (A-type) K\(^+\) channels (Figure 1.1 B) is activated by membrane depolarisation, then decays spontaneously and rapidly while the depolarisation is maintained. This causes a reduction of the time taken for an action potential to be initiated as well as a marked increase in the number of
Figure 1.1. Examples of the three major classes of voltage-gated K⁺ channels

A. Delayed rectifier. Example of whole cell currents from the cloned voltage-gated K⁺ channel RCK1 (Baumann et al., 1988) expressed in mouse erythroleukemia (MEL) cells. Currents were induced by steps ranging from -30 to +40 mV. Each step was followed by a repolarisation to -40 mV to obtain outward tail currents; the holding potential was -80 mV and the interpulse interval was 2.5s. RCK1 the holding potential was -80 mV and the voltage protocol was the same as that described in Figure 4.9A.

B. Transient (A-type). Example of whole cell currents from the cloned voltage-gated K⁺ channel hPCN2 (Philipson et al., 1990). The holding potential was -100 mV and a series of 150 ms pulses from -30 to +40 mV were applied, with an interpulse interval of 20 s. C. Inward rectifier. Example of whole cell currents from the cloned voltage-gated K⁺ channel KAT1 (Schachtman et al., 1992) expressed in oocytes. Depolarizing pulses from a holding potential of -60mV were applied 40 hours after injection with KAT1 mRNA.
action potentials generated by a constant depolarising pulse. Transient K⁺
currents were first described in mollusc neurons (Hagiwara et al., 1961).

1.6. Inward rectifiers

Inward rectifiers (Figure 1.1 C) mirror the characteristics of delayed rectifiers,
and possess the property of passing larger K⁺ currents in the inward, as
opposed to the outward direction. This so called 'anomalous' rectification is
due to a time-dependent gating process and/or to a time-independent
rectification inherent to the permeability of the open pore. The channels are
maximally opened by a large hyperpolarisation and pass sustained currents.
Their time-independent rectification is in the direction opposite to that
expected from the asymmetry of the physiological K⁺ ion distribution.
Anomalous rectification was first discovered in frog skeletal muscle (Katz,
1949), and is typically found in membranes that generate long-lasting
depolarisations, such as cardiac muscle.

1.7. Shaker: A route to the molecular study of voltage activated K⁺
channels

Because K⁺ channel proteins are very low in abundance, Drosophila genetics
proved the best approach to the molecular study of K⁺ channels. The X-linked
Shaker mutations were among the earliest behavioural variants identified in
Drosophila. It became apparent that the characteristic spontaneous excitability
and poor motor control resulted from defects in transient, voltage-activated
potassium channels encoded by this Shaker locus (Jan and Jan, 1976). The
subsequent cloning of 120kb of Drosophila chromosomal DNA encoding the
Shaker locus, by chromosome walking and the physical mapping of mutations,
proved successful in revealing the structure of the voltage-gated Shaker K⁺
channel (Papazian et al., 1987; Kamb et al., 1988; Fongs et al., 1988). When
expressed in the Xenopus oocyte, cDNAs hybridising to the Shaker locus gave
rise to distinct, transient K⁺ currents (Timpe et al., 1988), similar to those seen in
Drosophila muscle (Salkoff, 1983).

The Shaker gene was shown to exhibit extensive alternate splicing (Schwarz et
al., 1988). By screening Drosophila cDNA libraries at low stringency, three
genes, from three separate loci were identified (Butler et al., 1989; Wel et al.,
The proteins encoded by these three Shaker homologue genes were termed Shal, Shab and Shaw. When expressed in the Xenopus oocyte system it was shown that these four K⁺ channel types display a diversity of biophysical properties. Shal, like Shaker, encodes channels that carry an A-type K⁺ current, while Shab and Shaw encode channels that carry a delayed rectifier-type K⁺ current. Shab encoded channels that inactivate very slowly, while Shaw encoded channels that have no discernible inactivation state. These channels also differ in their voltage-sensitive properties and relative ion-selectivity (Wei et al., 1990).

The molecular diversity of mammalian voltage-activated K⁺ channels

1.6. Delayed rectifier and transient types

The close relation between Shaker, Shal, Shab and Shaw was evident, due to an approximately 40% identity in the protein coding S1-S6 regions between any two of these Drosophila sequences.

Strong homology with the Drosophila ShAl cDNA resulted in the cloning of the first mammalian voltage-activated K⁺ channel, the mouse brain MBK-1 cDNA (Tempel et al., 1988). This clone showed a 70% identity to the Shaker gene, but only approximately 40% to Shal, Shab and Shaw. This suggested that MBK-1 was a specific Shaker homologue, and that Shal, Shab and Shaw might similarly be expected to have mammalian homologues.

A number of reports has now established that each Drosophila gene does have one or more mammalian homologues (Frech et al., 1989; Yokoyama, 1989; Wei et al., 1990; Pak, 1991; Pak et al., 1991; Roberds and Tamkun, 1991). In contrast to Drosophila, which has only one member of each of the Shaker, Shal, Shab and Shaw subfamily members, at least four Shaw genes, two Shal genes and as many as twelve Shaker subfamily genes may be present in the mammalian genome (Jan and Jan, 1990; Rudy, 1991). Only one Shab homologue isolated from mouse brain (Wei et al., 1990; Pak, 1991) and an allelic homologue from rat brain have so far been cloned (Frech et al., 1989).

The various mammalian Shaker homologues so far reported are intronless and appear to have arisen by gene-duplication (Chandy, 1990). This contrasts with the single Drosophila Shaker gene, where diversity of channel activity is
generated by alternate splicing (Baumann et al., 1987; Schwarz et al., 1988). Mammalian K⁺ channels encoded by the Shaw subfamily have two levels of producing diversity. These are alternative splicing as well as gene-duplication (Yokoyama, 1989; Ghanshani et al., 1992).

Further diversity within this group was seen with the isolation of two DRK1 homologues, IK8 and K13 (Drewe et al., 1992). These two clones have so far been inexpressible in oocytes. Sequence comparison to published K⁺ channel genes has predicted IK8 to be a distant member of the Shab family, whereas K13 appears to define a new family of K⁺ channel.

1.9. minK

A new K⁺ channel type was reported when a rat kidney cDNA called minK was isolated by expression cloning in oocytes (Takumi et al., 1988). The minK channel produces a slow outward current and is unusual in that it has a very small DNA coding region of only 390bp and a predicted molecular weight of 15 kilodaltons. The human minK gene has also been cloned and is very similar to the rat gene (Murai et al., 1989). The arrangement of the minK gene has been shown to be complex (Iwai et al., 1990), with the minK mRNA containing multiple transcriptional start sites.

1.10. Inward rectifiers

Kubo et al. have reported the first example of cloning an inward rectifier-type K⁺ channel (Kubo et al., 1993). Here, expression cloning in Xenopus oocytes was employed to isolate a 5.5kb message encoding the inward rectifier K⁺ channel IRK1. The cloned coding sequence was shown to be short, with a predicted DNA coding sequence of 1284bp, and expression in unknown homomultimers was possible from a single subunit. The coding region of this gene has been shown to be intronless (see Section 4.11).
Molecular structure of voltage-activated \( \text{K}^+ \) channels

1.11. Delayed rectifier and transient types

Analysis of \( \text{K}^+ \) channel primary sequences has provided an insight into both the structure-function relationship of these channels and also their topographical location in biological membranes. Hydropathy profiles of membrane proteins indicate which sections of the protein are located in the membrane, and which sections project either into the cytoplasm or the extracellular fluid. There are six potential transmembrane domains in all studied \( \text{K}^+ \) channel sequences. The absence of an amino terminal leader sequence in voltage-dependent \( \text{K}^+ \) channels suggests a cytoplasmic location for their amino and carboxy termini. Figure 1.2A shows a current model for the structure of a voltage-dependent \( \text{K}^+ \) channel.

1.12. Gating

During an action potential, electrically excitable membranes undergo voltage swings in the order of 0.1 volts. Electric fields this high exert large forces on charged residues within membranes, and membrane potential is the signal used to drive voltage-activated channels between open and closed conformations.

The fourth transmembrane domains of all mammalian delayed rectifier sequences display identity to the \( S4 \) region of the \textit{Electrophorus electricus} (Electric eel) voltage-dependent sodium channel, a sequence believed to function as the voltage-sensor of this channel (Catterall, 1986). It consists of a repeat Arg/Lys-\( \text{X} \)-\( \text{X} \) motif (where \( \text{X} \) represents a hydrophobic amino acid). This arrangement forms a string of positively-charged amino acids across the membrane, and has been seen in no protein except voltage-gated \( \text{Na}^+ \), \( \text{Ca}^{2+} \) and \( \text{K}^+ \) channels. The voltage-sensing function of \( S4 \) however remains unproven, because experiments in which the \( S4 \) charges are systematically altered have not yet yielded interpretable alterations of voltage-dependent gating for \( \text{K}^+ \) channels (Papazian et al., 1991).
Evidence for the mechanism of inactivation has been obtained. Two reports have shown that inactivation is related to the NH2-terminal region in Shaker (Hoshi, 1990; Zagotta et al., 1990). Upon expression in the Xenopus oocyte, a Shaker variant with 41 amino acids deleted from the NH2-terminus was shown to behave identically to the wild-type channel, but failed to inactivate. Point mutations in this region, and trypsin-treatment of the intracellular surface membrane of Xenopus oocytes expressing Shaker, were shown to have the same result. Inactivation was shown to be reconstituted upon addition of a separate 20-residue peptide mimicking the deleted sequence (Zagotta et al., 1990).

1.3. Identification of the pore

Somewhere in the K+ channel polypeptide there must be one or more transmembrane stretches of sequence that provide the lining of the ion-permeation pathway. Amino acid groups presented to the interior of the aqueous environment of the pore provide the structures which allow K+, but not Na+, to permeate freely. The pore is as narrow as 3Å in places (Hille, 1973) and probably formed at the central interface of four subunits juxtaposed in the cell membrane.

Site-directed mutagenesis in conjunction with altered toxin blockade of Shaker channels with charybdotoxin, the neurotoxin from scorpion venom, has located the pore within the S5-S6 linker (MacKinnon and Miller, 1989; MacKinnon and Yellen, 1990). Experiments using the much smaller K+ channel blocker, tetraethylammonium (TEA), confirmed these reports (MacKinnon and Yellen, 1990).

A confirmatory experiment showed that the K+ conduction pore is contained wholly within the S5-S6 linker. This involved constructing chimeras of two mammalian K+ channels. One of these, NGK2, has high single-channel conductance, high-affinity block by external TEA, and low-affinity block by internal TEA. The other, DRK1, has threefold lower conductance, ten-fold lower sensitivity to external TEA, and about 100-fold higher sensitivity to internal TEA. Substitution of a 24-residue stretch from the S5-S6 linker of NGK2 into the equivalent region of DRK1 produced a channel with NGK2-like open pore behaviour (Hartmann, 1991).
Figure 1.2. Molecular structure of delayed rectifier and transient (A-type) voltage-gated K⁺ channels

A. Current view of functional domains in voltage-gated K⁺ channels. The important structural regions include the N-terminal ball, the intracellular chain region and the conduction pore that lies within the S5-S6 linker region. B. Through protein-protein interactions, the functional voltage activated K⁺ channel consists of four monomeric subunits in a symmetrical pore-forming arrangement. Depicted is an ariel view of the predicted tetrameric arrangement.
1.14. minK

Sequence of the minK gene in conjunction with hydropathy analysis of the encoded protein has predicted a single transmembrane domain (Takumi et al., 1988). (See Figure 1.3B).

1.15. Inward rectifiers

Upon hydropathy analysis, IRK1 appears to have only two putative transmembrane domains (see Figure 1.3A). The comparison of the H5 region of this channel with the H5 regions of other voltage-activated K⁺ channels gives evidence for conservation of the K⁺ selective pore region, predicted to be located between the M1-M2 region of IRK1. In conclusion, IRK1 appears to form a new class of K⁺ channel structure, completing cloned examples of all the major types of voltage activated K⁺ channel.

1.16. The oligomeric nature of voltage-activated K⁺ channel

When Shaker was sequenced, the motif of six putative transmembrane helices with a distinct S4-region was postulated to be homologous to the four subunit structure of voltage-gated Na⁺ and Ca2⁺ channels (Noda et al., 1984). The argument was circumstantial, but recently MacKinnon proved quantitatively that the functional channel is a tetramer. Mixing mRNA coding for toxin-sensitive and toxin-insensitive channels at known ratios, and injecting into Xenopus oocytes, the fraction of toxin-sensitive channels could be calculated assuming that random mixing of the different mRNA species was occurring, and the subsequent statistical analysis gave evidence for a tetrameric structure (MacKinnon, 1991). A more recent report has provided further evidence for these observations. Here, 2-5 K⁺ channel subunits were linker in a single open reading frame with individual constructs being tagged by a K⁺ channel subunit mutant altering sensitivity to TEA. Altered TEA sensitivity was only seen when the altered TEA sensitive mutant was coexpressed with a dimer or trimer, but not a tetramer (Liman et al., 1992). Figure 1.2B shows the proposed tetrameric assembly of a typical voltage activated K⁺ channel. The oligomeric nature of the IRK1 and minK functional channels has yet been undetermined.
Figure 1.3. Molecular structure of the minK and IRK1 voltage-gated K+ channels

A. Putative transmembrane topology of the cloned inward rectifier IRK1 subunit (Kubo et al., 1993) depicting the H5 putative K+ selective pore region within membrane spanning domains M1 and M2. B. Putative transmembrane topology of the cloned minK subunit (Takumi et al., 1988) depicting the single transmembrane domain.
Part 2. Eukaryotic expression systems employed for $K^+$ channel gene expression

1.37. Introduction

The following section reviews eukaryotic expression systems that have been applied to the functional expression of $K^+$ channel proteins. Table 1.3. shows a summary of eukaryotic gene expression systems which have been employed for the expression of $K^+$ channel genes, and Table 1.2. summarises the relative merits of oocytes versus mammalian cells for heterologous expression of $K^+$ channels, as the expression systems described in this section employ one of these two cell environments for expression.
Table 1.2. Summary of the relative merits of oocytes versus mammalian cells for heterologous expression of $K^+$ channels

<table>
<thead>
<tr>
<th><strong>OOCYTES</strong></th>
<th><strong>DISADVANTAGES</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ADVANTAGES</strong></td>
<td><strong>DISADVANTAGES</strong></td>
</tr>
<tr>
<td>Oocytes are very large, allowing simple microinjection of mRNA and 2-electrode voltage clamp</td>
<td>Large capacitance leads to poor temporal resolution in voltage-clamp experiments</td>
</tr>
<tr>
<td>Individual cells can easily be identified, manipulated, and a high percentage of cells express the protein</td>
<td>There are seasonal variations in expression</td>
</tr>
<tr>
<td>Recordings are stable for long periods and extracellular solutions can be simply exchanged</td>
<td>There are variations between oocytes from different frogs and between oocytes from a single frog</td>
</tr>
<tr>
<td>Rapid screening is possible for expression cloning</td>
<td>This is a transient expression system: cells die in a few days</td>
</tr>
<tr>
<td></td>
<td>The several endogenous conductances may mask expressed channels</td>
</tr>
<tr>
<td></td>
<td>Perfusion for precise control of internal solutions is tedious</td>
</tr>
<tr>
<td><strong>MAMMALIAN CELLS</strong></td>
<td><strong>DISADVANTAGES</strong></td>
</tr>
<tr>
<td><strong>ADVANTAGES</strong></td>
<td><strong>DISADVANTAGES</strong></td>
</tr>
<tr>
<td>Are well suited for high-resolution patch-clamp recording</td>
<td>Recordings are stable for tens of minutes at the most</td>
</tr>
<tr>
<td>Ability to produce clonal cell lines stably expressing foreign genes</td>
<td>Intracellular solutions cannot easily be changed</td>
</tr>
<tr>
<td>Large variety of cell lines allows complementation and selection of endogenous channel repertoire</td>
<td>Each recording requires a new patch pipette, so that screening is inefficient</td>
</tr>
<tr>
<td>High-level expression can be attempted in large-scale cultures</td>
<td></td>
</tr>
<tr>
<td>Posttranslational events (e.g., glycosylation) may be appropriate for expressed mammalian proteins</td>
<td></td>
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</table>

1.18. Cell-free expression system

The use of a cell-free expression system has been reported for the functional expression of *Shaker* potassium channels (Rosenberg and East, 1992). Cell-free translation into protein, microsomal membrane processing of nascent channel proteins, and reconstitution of newly synthesized ion-channels into planar lipid bilayers was employed to synthesize, glycosylate, process into membranes and record *in vitro* the activity of functional ShIR $K^+$ channels. Channel
<table>
<thead>
<tr>
<th>SYSTEM</th>
<th>PROMOTER</th>
<th>EXPRESSED K+ CHANNELS</th>
<th>REPORTED HOST CELL RANGE</th>
<th>TIME NEEDED TO ESTABLISH EXPRESSION</th>
<th>STABLE OR TRANSIENT EXPRESSION</th>
<th>LEVEL OF EXPRESSION</th>
<th>EXPRESSED CHANNEL CHARACTERISTICS</th>
<th>EASE OF EXPERIMENTAL REPRODUCTION</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>CELL-FREE</td>
<td>T7 RNA polymerase promoter</td>
<td>ShlR</td>
<td>Cell-free (Planar lipid bilayer)</td>
<td>4-5 hours</td>
<td>Transient in vitro transcription and translation</td>
<td>Mean open time was 96ms greater</td>
<td>Full procedure must be repeated</td>
<td>(Rosenberg and East, 1992)</td>
<td></td>
</tr>
<tr>
<td>CYTOPLASMIC MICROINJECTION OF RNA INTO XENOPUS OOCYTES</td>
<td>T7 RNA polymerase promoter</td>
<td>First examples were ShAl and ShB1</td>
<td>Xenopus oocyte</td>
<td>6 hours to 5 days</td>
<td>Transient</td>
<td>4μA whole-cell currents for ShB1 2 days post injection</td>
<td>Full procedure must be repeated Seasonal and donor Xenopus variation hinder reproduction</td>
<td>First reported (Timpe et al., 1988)</td>
<td></td>
</tr>
<tr>
<td>CYTOPLASMIC MICROINJECTION OF RNA INTO MAMMALIAN CELLS</td>
<td>T7 RNA polymerase promoter</td>
<td>Kv2.1</td>
<td>Clonal mouse fibroblasts (L-cells) and rat basophilic leukemia cells (RBL-1)</td>
<td>4 hours to 2 days</td>
<td>Transient</td>
<td>2nA whole-cell currents in 6 hour post injected RBL-1 cells</td>
<td>Similar</td>
<td>Full procedure must be repeated</td>
<td>(Ikeda et al., 1992)</td>
</tr>
<tr>
<td>VACCINIA VIRUS (VV)</td>
<td>Vaccinia virus 7.5KDa early promoter</td>
<td>Shaker H4</td>
<td>Rat primary cell-cultures Xenopus oocytes Clonal mouse fibroblasts (L-cells) and rat basophilic leukemia cells (RBL-1) NIH mouse 3-3 fibroblasts Monkey kidney (CV-1) cells</td>
<td>1-3 days</td>
<td>Transient</td>
<td>Whole-cell currents of 1-10μA in Xenopus oocytes 1-2nA for other cell types</td>
<td>Similar</td>
<td>Repeat transfection of host cells necessary</td>
<td>(Leonard et al., 1989; Yang, 1991; Karnchin et al., 1991)</td>
</tr>
<tr>
<td>BACULOVIRUS</td>
<td>Autographa california (AcMNPV) polyhedrin promoter</td>
<td>Shaker H4</td>
<td>Army-worm caterpillar Spodoptera frugiperda (SF) cells</td>
<td>15 hours - 3 days</td>
<td>Transient</td>
<td>Whole cell currents of 3nA 3 days post-transfection</td>
<td>Activation curves are shifted 15mV to more depolarised potentials</td>
<td>Repeat transfection of host cells necessary</td>
<td>(Klaiber et al., 1990)</td>
</tr>
<tr>
<td>PROMOTER/ENHANCER INTEGRATIVE VECTORS</td>
<td>hSPTD and Mouse metallothionein promoter</td>
<td>RCK1 BAK4</td>
<td>Mammalian muscle cell-line (Sol-8) Neuroblastoma (2a) cell line</td>
<td>Stable expression</td>
<td>Stable</td>
<td>Variable</td>
<td>Similar</td>
<td>Can store transfected cells as frozen pellets</td>
<td>(Koren et al., 1990, Garcia-Guzman, 1992)</td>
</tr>
</tbody>
</table>

Table 1.3. Summary of eukaryotic gene expression systems which have been employed for the expression of K+ channel genes
conductance and inactivation were close to those recorded in *Xenopus* oocytes, but activation and deactivation kinetics were faster than the 10-20ms seen previously (Hoshi, 1990).

The use of the system was exemplified in the analysis of ShIR K⁺-channels. Differences in activation and deactivation kinetics were observed after comparison of expression data in oocytes. This may be due to effects of bilayer lipids, incomplete carbohydrate processing, or other differences between oviduct microsomes and *Xenopus* oocytes.

**Direct cytoplasmic microinjection of RNA**

1.19. Microinjection of *Xenopus* oocytes

Direct cytoplasmic microinjection of RNA in amphibian eggs has been an important approach to the expression of K⁺ channel genes and was first reported by Timpe *et al.* in 1988 (Timpe *et al.*, 1988). Here, the injection of complementary RNA, transcribed *in vitro* from a coding sequence placed downstream of a powerful heterologous promoter, into a *Xenopus* oocyte was performed.

Advantages of this method are that oocytes are large, allowing simple microinjection of RNA and simple 2-electrode voltage clamp analysis. Individual cells can easily be identified and manipulated, and a high percentage of injected cells will express exogenous channel protein. Also, recordings are stable for long periods and extracellular solutions can be simply exchanged.

While this approach has been valuable in characterising the products of cloned and manipulated coding sequences, it has several limitations. Oocytes show seasonal variations in their capacity for expression, and there are variations between oocytes from different frogs and between oocytes from a single frog. Oocytes must be defolliculated before any recordings can be taken from the processed oocyte. In the process of defolliculation, all outer layers of the oocyte are enzymatically or mechanically removed, leaving the oocyte surrounded by the vitelline membrane. This treatment leads to damage of the vitelline membrane, generating holes which serve as "nonspecific ion channels",
allowing passive influx of Na\(^+\) and Ca\(^+\) and passive efflux of K\(^+\). During oocyte development, the expression of endogenous ion-channels (Dascal, 1987; Bourinet et al., 1992), neurotransmitter receptors and receptor-channel subunits (Fluharty et al., 1991) occurs, which may mask expressed channels. Post-translational modifications of exogenous channel proteins will be different and endogenous proteins that can specifically influence the activity of ion channels in mammalian cells will be absent or altered. This system is also limited in that it is transient, with cells dying after a few days post-injection.

1.20. Microinjection of clonal mammalian cells

Heterologous expression of a human K\(^+\) channel by direct cytoplasmic microinjection of cRNA in clonal mammalian cells has also been reported (Ikeda et al., 1992). The cloned human delayed rectifying K\(^+\) channel Kv2.1 was expressed in clonal mouse fibroblasts (L-cells) and rat basophilic leukemia cells (RBL-1). Both gave similar activation and inactivation parameters six hours after microinjection of cRNA. This approach provides a useful alternative to transient expression in the oocyte, giving rise to the availability of different cell backgrounds for the introduction of useful clones. However, equipment for the convenient injection of complementary RNA into mammalian cells can be expensive, and in general, recordings were reported to be not as stable in mammalian cells when compared to the Xenopus oocyte.

1.21. Vaccinia virus

A number of studies have explored the use of vaccinia virus vectors to express ion channel genes, both in Xenopus oocytes (Yang, 1991) and in mammalian cells (Leonard, 1989; Karschin, 1991). This large DNA virus was first shown to be a useful vector following the expression of a voltage-gated Drosophila K\(^+\) channel in a variety of cell-lines (Leonard et al., 1989). Expression in this system involves cloning of cDNA or genomic coding regions downstream of the vaccinia 7.5kDa promoter in a vaccinia recombination plasmid. Transfection of the vaccinia recombination plasmid with wild type vaccinia DNA onto host cells is then undertaken prior to identification and purification of a recombinant virus. This recombinant virus can then be used for transient transfection of host cells prior to recording.
All three reported applications of the system in various cell backgrounds gave similar channel characteristics as the identical genes expressed in *Xenopus* oocytes. Vaccinia-based vectors have the advantage of being functional in a wide range of mammalian cell types, but require complex isolation of a recombinant virus prior to functional expression.

1.22. Baculovirus

The expression of K⁺ channel sequences in a Baculovirus-infected insect cell-line has been described (Klaiber *et al.*, 1990). This system provides an electrophysiologically-quiet background in the form of the army-worm caterpillar *Spodoptera frugiperda* (Sf9) cell-line. These cells are convenient for patch and whole cell recording, though there was a reported systematic shift of 15mV to more depolarised potentials in the activation curves of expressed channels compared to expression in the *Xenopus* oocyte. Manipulation of the cloned sequence into the viral genome is a time-consuming procedure, involving cloning into a plasmid vector downstream of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) polyhedrin promoter, followed by recombination with wild-type AcMNPV virus in recipient cells. Labour-intensive identification of recombinant virus particles, also a requisite for the vaccinia system, make this approach unsuitable for processing large numbers of candidate clones. Also, post-translational modifications of exogenous channel proteins will be different and endogenous proteins that can specifically influence the activity of ion channels in mammalian cells will be absent or altered (Miller, 1988).

The Baculovirus system is however suited to the production of the high yields required for structural studies of channel proteins.

1.23. Promoter/enhancer integrative vectors

The stable expression of a K⁺ channel under the control of the inducible metallothionein promoter has been reported (Koren *et al.*, 1990). Here, the coding sequences for the delayed rectifier potassium channel, RCK1, was linked in *cis*, downstream of the metallothionein promoter, prior to transfection of a mammalian myoblast cell line (Sol-8). Transient expression assays were
undertaken 72 hours post-transfection, and stable transfectants were isolated after selection in the eukaryotic antibiotic G418. Two out of seven clones isolated expressed the channel, and one of the clones continued to express the channel at least six-months after isolation. Expressed current characteristics were identical to those where the same clone was expressed in *Xenopus* oocytes, also undertaken in this study.

A further report described the expression of a fast inactivating K⁺ channel (BAK4) in a neuroblastoma (Neuro-2a) cell line (Garcia-Guzman *et al.*, 1992). This expression was under the control of the hsp70, inducible heat-shock promoter, and currents were similar to those described after expression of the same channel in oocytes (Stühmer *et al.*, 1989).

These type of systems are affected greatly by the expression construct’s chromosomal integration site, and many clones may have to be screened to find integrants that have established in areas of active chromatin.

Advantages with this approach include the ease of experimental reproduction using sub-cultured stocks from expressing clones, and the stable nature of expression allows no constraints on time available for analysis.
1-Introduction

Part 3 The Human β-globin Locus Control Region (LCR)

1.24. Introduction

The human β-globin gene cluster is situated on the short arm of chromosome 11 and consists of five genes (ε, Gγ, Aγ, δ, and β), spanning approximately 60kb, which are successively expressed during human development. The site of erythropoiesis shifts along the gene cluster during development in a manner characteristic of the relative chromosomal position of the five β-globin cluster genes. The site of erythropoiesis begins in the embryonic yolk sac with expression of the ε gene, followed by that of the two γ genes in the fetal liver and finally by the expression of the minor (δ) and major (β) β-like genes in the adult bone marrow.

1.25. DΝαse I hypersensitive (HS) sites

The presence of DΝαse I hypersensitivity in the chromatin around genes is often associated with the transcriptional activity of these same genes, and this linkage between the phenomena of DΝαse I hypersensitivity and transcriptional activity is a general one, for a review see (Gross and Garrard, 1988). The discovery of DΝαse I hypersensitivity in the region of the active β-globin gene (Kimura et al., 1983) and the observation of super-hypersensitive (SH) sites located far upstream of the human β-globin gene cluster (Tuan et al., 1985; Forrester et al., 1986; Forrester et al., 1989), has led to a greater understanding of the developmental regulatory mechanisms of this group of genes. These far upstream SH sites were shown to be distinct from local hypersensitive sites associated with transcriptionally active nucleosome-free regions in the near vicinity of the individual β-globin cluster genes (Stalder et al., 1980), by their earlier appearance in the course of DΝαse I digestion, and have been termed SH sites because of this.

Five SH sites in the region 6-25kb 5‘ of the human ε gene, and a single site approximately 20kb 3‘ of the β gene were identified (Tuan et al., 1985). These sites, along with the five β-globin cluster genes encompassed a domain of about 90kb (Figure 1.4), and most of these SH sites are erythroid cell-specific.
Figure 1.4. Chromosomal organisation of the human θ-globin gene cluster

The cluster map depicts the position of the genes (boxes) and the positions of the super hypersensitive sites, depicted as arrows and denoted 1-6.
The location of the SH sites with respect to the globin genes, taken together with their developmental programme of appearance, led to the suggestion that these sites could define the boundaries of a large domain of active chromatin (Tuan et al., 1985). Such suggestions were presented due to the existence of such domains in the chicken β (Stalder et al., 1980) and ovalbumin (Lawson et al., 1982) gene families.

1.26. The Locus Control Region (LCR)

Evidence that the SH sites might serve as LCRs came from experiments where human non-erythroid cells were fused with mouse erythroleukaemia (MEL) cells with the production of a transcriptionally active human β-globin cluster, exhibiting all the HS sites characteristic of a transcriptionally active domain (Forrester et al., 1987). These results suggested that the human β-globin SH sites might function as regulatory elements, which through their effect on chromatin structure, act on all the genes in the β cluster. The characterisation of the genetic nature of a γ β-thalassemic patient also provided evidence for an LCR (Kioussis et al., 1983). In this patient an extensive upstream human β cluster deletion was characterised. It was shown that the β gene itself had no mutations and was transcriptionally active in HeLa cells, β gene transcripts being indistinguishable from those found in normal reticulocytes. Further evidence came from the identification of a 30kb deletion removing the 5' β-globin gene activation-region hypersensitive sites 5' of the ε gene in a patient with γβ-thalassemia. This disorder is characterised by decreased neonatal γ- and β-globin chain synthesis and a postnatal β-thalassemic trait (Driscoll et al., 1989).

To ascertain the function of these HS sites, a "minimal" was constructed with 21kb of upstream DNA containing all the SH sites linked to the human β-globin gene with all its known local regulatory elements and finally a 12kb region containing the downstream HS site (Grosveld et al., 1987). All transgenic mice carrying this construct in an unrearranged form expressed the human β gene. Furthermore, high levels of human β mRNA and protein were produced in an integration site independent manner. Moreover, the expression was quantal with respect to the copy number of the integrated transgene, as compared to the endogenous mouse β mRNA and protein levels.
In experiments where only the local regulatory sites accompany the introduced human β gene, most of the transgenic mice did not express detectable amounts of human β mRNA and protein. In cases where expression was detectable, a typical level of <1% of the mouse β mRNA and protein was detected, with no relation between expression level and transgene copy number being observed (Ryan et al., 1989). Failure to express was probably due to integration into "silent" heterochromatin. It was clear that β gene linkage to the regions surrounding the SH sites liberates them from random chromatin position effects, and assured high-levels of erythroid-specific expression. These regions have been termed locus control regions (Orkin, 1990).

1.27. Overview

Upstream of the human β-globin gene domain, five SH sites are found at approximately 6 (SH site 1), 11 (SH site 2), 14.5 (SH site 3), 17.5 (SH site 4) and 21kb (SH site 5) from the γ gene (Tuan et al., 1985) (see Figure 1.4). SH sites 1, 2 and 4 are erythroid specific, due to their presence in MEL and K562 erythroid specific cells, and absence in non erythroid specific cells (Tuan et al., 1985). SH site 3 is present in human erythroleukemia (HEL) cells, but not in K562 cells (a human leukemia cell line in which the embryonic γ-globin gene is predominantly expressed) (Tuan et al., 1987). SH site 5 is present in both erythroid and non-erythroid cells, and it may not be associated with the β-globin cluster, due bipartly to its non erythroid specificity, and distal position with respect to the γ-globin gene (Tuan et al., 1985). In addition, another erythroid-specific HS site (SH site 6) is found 20kb downstream of the β gene (Tuan et al., 1985). Figure 1.4 shows the relative positions of these SH sites in the β-globin gene domain.

1.28. Activity of the individual SH sites

Much investigation has been undertaken on the individual SH sites, observing their individual, and combined activities.

Early work on the individual SH sites (described as sites 4-1 proceeding upstream from the γ gene in the dominant control region-LCR equivalent),
showed sites 1 and 4 conferred little transcriptional activation. Sites 2 and 3 give rise to independent, high-level, β-gene-linked expression with each site accounting for approximately 50% of the activity of the full β-globin LCR sites 1-4 after assay in MEL cells. However, sites 2 and 3 together did not give rise to full inducibility in the absence of sites 1 and 4, suggesting the latter's necessity for full, regulated expression (Collis et al., 1990).

Little work has been reported on site 1. Suggestions are that SH site 1, necessary for full LCR activity, may be important in opening up the chromatin around the β-gene domain, and has been shown to independently attribute little LCR activity on linked genes (Fraser et al., 1990; Fraser et al., 1993).

The major activity of SH site 2 has been located on a 300bp core fragment, and independently on a 373bp fragment (Caterina et al., 1991) containing a triple repeat of a combination of GATA1 binding sites and G-rich sequences that are spaced approximately 30bp apart (Talbot et al., 1990), overlapping the major SH site in vivo. Within the fragments functional erythroid-specific GATA1, a dimer NF-E2, H-BP, J-BF and AP-1 protein binding sites were also identified (Caterina et al., 1991; Talbot and Grosveld, 1991; Ellis et al., 1993). Furthermore, experiments in transgenic mice showed that SH site 2 alone was sufficient for developmental regulation in Gy to Ay and γ to β switches, similar to those observed in normal human development (Morley, 1992). Recently, it has been shown that the LCR activity of SH site 2 is not dependent on any one factor-binding site for position-independent expression, but SH site 2 core concatamers of 2 or more copies leads to a partially active LCR, the copies possibly interacting with each other to form an area of open chromatin, a possible basis for LCR function (Ellis et al., 1993).

SH site 3 has been shown to account for the highest level of independent LCR activity, showing levels of 70% in MEL cells and transgenic mice when compared to the full LCR (Fraser et al., 1990). SH site 3 initially delineated on a 1.9kb fragment (Collis et al., 1990), was later shown to direct high levels of expression on a human β-gene from a 255bp core fragment (Phillipsen et al., 1990). Many transcription factor-binding sites have been observed in this region, including a functional erythroid-specific NF-E1 site, multiple Sp1, CAAC box and a single AP-1/NF-E2 binding site (Talbot and Grosveld, 1991; Strauss and Orkin, 1992). Later experiments have shown that a specific
I-Introduction

combination of a G-rich sequence, flanked on each side by one binding site for
the transcription factor GATA1, was essential for position-independent
expression of a linked β-globin gene in erythroid cells (Philipsen et al., 1993).
SH site 3 can drive expression of both the human γ- and β-globin genes and is
the most active site of the LCR in the embryonic yolk sac and foetal liver of
transgenic mice (Fraser et al., 1990; Fraser et al., 1993).

SH site 4 was initially shown to independently confer approximately 30% full
LCR activity, in a copy number-dependent, position-independent manner
(Collis et al., 1990; Fraser et al., 1990). Further work isolated SH site 4 to a 280bp
fragment that is functional in providing position-independent expression in
both MEL cells and transgenic mice (Fraser et al., 1990). Bandshift analysis has
shown the presence of binding sites for the erythroid-specific proteins GATA1
and NF-E2, along with a number of ubiquitous proteins, including jun/fos, Sp1
and TEf2 (Pruzina et al., 1991). Furthermore, a GACTGGC protected motif, a
putative CCAAT binding site, outside the localized 280bp fragment has been
shown to be critical for site 4 functioning when linked to adjacent SH sites
(Walters et al., 1991).

SH site 6, 20kb 3' of the β-globin gene, has been shown to have little effect on
the production of β mRNA in transgenic mice, when compared to a
construct containing all of the sites (Ryan et al., 1989; Talbot et al., 1989).

1.29. Globin gene switching

Correct stage-specific gene switching in the β-globin gene domain must depend
on specific interactions of the LCR with the regulatory elements of the
individual globin genes. An early report accounting for ϵ to β switching in the
chicken provided data for ϵ to β promoter competition of a shared enhancer.
Embryonically, the enhancer favoured interaction with the ϵ promoter, whereas
in the adult, the stage-specific DNA binding protein NF-E4 switched
association to the β promoter (Choi and Engel, 1988). A further report showing
mutations in the NF-E4 site conferring reciprocal changes in ϵ to β expression,
support this model (Foley and Engel, 1992).

The simplest model of globin gene switching involves the LCR interacting
successively, and in a mutually exclusive manner, with the ϵ-, γ-, and β-globin
1-Introduction

Evidence has shown that the choice of specific LCR-globin gene promoter interaction has several parameters.

Stage-specificity may be linked to a particular array of expressed transcription factors at a defined developmental stage. This may involve the presence of unique stage-specific factors, may operate through changes in abundance or involve structural modification of erythroid or ubiquitous factors (Minie et al., 1992). Recently, a nuclear factor called 50γ from the K562 foetal stage erythroleukemia cell line has been described as possibly controlling stage-selection between γ and β expression. The factor was present at high concentrations in the K562 cell line, at low level in MEL cells, and was shown to bind to a region in the γ promoter. This interaction is a possible determinant for preferential γ stage-selection, and therefore LCR interaction (Jane et al., 1992).

Evidence to support that proximity of a gene to the LCR has important consequences on stage-selection has been reported. Transgenic mice transfected with an LCR-γβ construct exhibit silencing of the distal β gene during early embryogenesis. When the order was reversed (LCR-βγ), the β gene was expressed prematurely in the yolk sac. Competition of the promoter proximal to the LCR for early stage expression seems apparent from this work (Hanscombe et al., 1991). Similar results were reported when LCR-γ and LCR-β constructs were shown to express their linked genes throughout development, whereas an LCR-γβ construct restored normal fetal to adult expression (Enver et al., 1990). Further evidence comes from a report where homologous recombination was used to insert a hygromycin B-resistance cassette into the globin cluster just downstream of SH site 2. Interaction of the LCR with the inserted proximal gene inactivated the distal β gene, and a new HS site was present in the enhancer/promoter of the hygromycin B-resistance cassette (Kim et al., 1992). Figure 1.5 shows a current model for LCR involvement in globin gene switching.

1.30. Factors influencing individual LCR-gene interactions

It has been shown that β-gene silencing during early embryonic development is mainly achieved through competition with the γ-globin gene for the influence of the LCR. In contrast, when linked singly to an LCR, the embryonic ε and foetal γ genes are appropriately expressed at their early stages of development.
and then shut off (Shih et al., 1990; Dillon and Grosveld, 1991; Lloyd et al., 1992).

These results lead to the proposition of negatively acting factors, or silencers, acting on the regulatory regions of these genes. Reported has been a mutation, possibly disrupting a GATA1 binding site in the γ promoter, which led to persistent expression of that gene into adult life. GATA1 may thus play a role in binding to the γ promoter and silencing expression late in development (Berry et al., 1992). A silencer region in the ε-promoter has also been identified between -177 and -392bp relative to the mRNA initiation site (Cao et al., 1989).

Furthermore, two erythroid-specific nuclear proteins have been shown to bind to this region (Gutman et al., 1992). Gumucio et al. have provided further information by showing that the protein CSFB-1, previously shown to act as a repressor in other systems, binds to silencer sequences in both the ε and γ gene promoters (Gumucio et al., 1992).

1.3.1. LCR involvement in developmental switching

Two conclusions regarding LCR function can be drawn. Firstly, the LCR has a chromatin opening function. Within the LCR sequences are a large number of transcription factor binding sites, and the LCR may operate by binding these factors to form a DNA protein complex. This complex may then function to displace histones, keep chromatin free from these DNA compacting proteins, and establish loops with the β genes now available for transcription (Felsenfeld, 1992).

Secondly, this same LCR seems to selectively interact with the β gene promoters contributing to stage-specific expression. Evidence for this developmental specificity of the SH sites within the LCR has been reported. Here, site 3 was shown to be most active during the embryonic period. Site 4 showed the highest activity during the adult stage, and expressed the γ genes at low level during the embryonic period. Site 2 ascribed equivalent levels of γ or β transgene expression throughout development and site 1 showed a similar pattern to site 2, although the activity was much lower (Fraser et al., 1993).

Two possible models for LCR developmental involvement might be considered. Firstly, the SH sites may form a large complex with which the β genes would successively interact. Secondly, the SH sites might be dedicated for interaction with a particular gene or genes in the cluster, present data being
unable to discriminate between either model. Figure 1.5 provides a current model for developmental gene-switching.

1.32. MEL-cells

Murine erythroleukaemia (MEL) cells (Friend, 1971) are the most commonly used erythroid tissue-culture model system, and have been employed extensively in the work described in this thesis. These erythroid progenitor cells are transformed by the Friend virus complex and are arrested at the pro-erythroid stage of development. However, upon treatment with various chemical agents, including dimethyl sulfoxide (DMSO), MEL-cells are induced to undergo a pattern of terminal erythroid differentiation which closely mimics the analogous process *in vivo*, including the high level synthesis of globin proteins (Kabat *et al.*, 1975). Previous studies have shown that the human β-globin LCR sequences are fully functional in MEL-cells (Blom van Assendelft *et al.*, 1989). This activity gives rise to equal amounts of human β-globin message and endogenous (mouse) β-major globin mRNA on a gene copy-number basis. This phenomenon overcomes the problem of "position effects" encountered in several other mammalian systems, characterised by highly variable expression levels dependant upon the position of integration of the transfected genes within the host genome (Palmiter and Brinster, 1986; Nandi *et al.*, 1988).
Figure 1.5. Model for globin gene regulation during development (Hanscombe et al., 1991) and relationship to heterologous expression of genes proximal to the LCR.

A. During developmental expression, the relative order of genes to the LCR is an important parameter. The LCR has a preference for acting with a different δ cluster gene moving away from the LCR during development. The polarity of gene expression can be modelled in terms of factors influencing contact between the LCR and activatable gene promoters. In adult stages of development, promoter-mediated 'silencing' for the proximal genes has been observed for the ε and γ-globin genes (above). B. During heterologous expression in MEL cells or transgenic animals, the LCR and heterologous gene can be juxtaposed and activated as an independent transcriptional unit within the chromatin of erythroid specific cells. Furthermore, the relative proximity of the LCR and promoter will maximize the chance of direct physical interaction and subsequent transcriptional initiation.
CHAPTER 2

Materials and Methods
2 - Materials and Methods

2.1. Chemicals

General laboratory chemicals were supplied by Fisons (Loughborough), BDH (Poole) or Sigma (Poole) unless otherwise stated and were of analytical grade or equivalent. Bacterial culture media were obtained from Difco (East Molesley) and animal cell culture media from Gibco BRL (Paisley). Radiolabelled nucleotides were supplied by Amersham International (Amersham). Millipore Super-Q® system (Millipore Corporation, Bedford) treated water was used for all solutions.

2.2. Enzymes and proteins

Restriction endonucleases were purchased from Gibco BRL or New England Biolabs (via CP Laboratories, Bishop's Stortford). T4 polynucleotide kinase was supplied by Gibco BRL. DNA polymerase I (Klenow fragment) was supplied by either Amersham International or Gibco BRL. T4 DNA polymerase was obtained from New England Biolabs. T7 DNA polymerase and Sequenase™ (modified T7 DNA polymerase) were obtained from Pharmacia P-L Biochemicals (Milwaukee). Taq DNA polymerase was obtained from Amersham or Northumbria Biologicals Limited (Northumbria). T4 DNA ligase was purchased from Gibco BRL and New England Biolabs. Avian Myeloblastosis Virus reverse transcriptase was obtained from Life Sciences Inc. (St. Petersberg). Proteinase K was purchased from Boehringer Mannheim (Lewes). RNase A (pancreatic RNase) and DNase I were obtained from Sigma. BSA (enzyme grade) was obtained from Gibco BRL. DNase-free RNase A and RNase-free DNase I were prepared by the methods described (Sambrook et al., 1989).

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2.3. Bacterial strains and culture conditions

Strains of *Escherichia coli* K12 used are given in Table 2.1. Bacteria were grown using the solid and liquid media given below.

**Table 2.1. Bacterial Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SURE</td>
<td>(e^{14}\text{(mcrA)}, \Delta(\text{mcrCB-hsdSMR-mrr})171,) (\text{sbcC, recB, recJ, umuC::Tn5(kan^R)}, \text{uwrC, supE44, lac, gyrA96, relA1, thi-1, endA1}) [F (\text{proAB, lacI9ZAM15, Tn10, (tetR)})]</td>
<td>(Greener, 1990)</td>
</tr>
<tr>
<td>NM554</td>
<td>(\text{recA13, araD139, } \Delta(\text{ara-leu})7696,) (\Delta(\text{lac})7A, \text{galU, galK, hsdR, rpsL (str}^R), mcrA, mcrB</td>
<td>(Raleigh and Murray, 1988)</td>
</tr>
</tbody>
</table>

**Solid media:**

Luria agar (LA): tryptone, 1% (w/v); yeast extract, 0.5% (w/v); NaCl, 0.5% (w/v); agar, 1.5% (w/v).

**Liquid media:**

Luria broth (LB): tryptone, 1% (w/v); yeast extract, 0.5% (w/v); NaCl, 0.5% (w/v).

2xYT broth (2xYT): tryptone, 1.6% (w/v); yeast extract, 1% (w/v); NaCl, 0.5% (w/v).

2.4. Cloning vectors

Routine subcloning was performed in the vector pBluescript SK+ which was purchased from Stratagene (San Diego).
2.5. Methods of sterilisation

Autoclaving: Autoclaves used to sterilise media and disposable plastic-ware were as follows: a Cabburn 8cu.ft. capacity autoclave (Cabburn Sterilisers, Shoeburyness) set to attain a temperature of 121°C for 30 minutes; a model ST19 portable electric autoclave (Dixon's surgical instruments, Wickford) set to attain a temperature of 121°C for 20 minutes.

Dry sterilisation: A B&T "Unitemp" sterilising cabinet (Laboratory thermal equipment, Oldham) was used to sterilise glass-ware. The cabinet was set to attain a temperature of 160°C for 6 hours.

Filter sterilisation: Small volumes (up to 50ml) were filter sterilised by passing through Acrodiscs (Gelman Sciences, Ann Arbor) with a pore size of 0.2mm. Larger volumes (50-500ml) were filter sterilised using Nalgene 0.2mm vacuum filter sterilising units (Nalgene, Rochester, New York).

Nucleic acid methods

2.6. Solutions used during the handling of nucleic acids

Acrylamide solution (40%): 380g of acrylamide (Serva, Heidelberg or National Diagnostics, Aylesbury) and 2g of N,N'-methylene bisacrylamide (Serva) were dissolved in 1lt of Q water. The solution was deionised using Amberlite® MB-1 ion exchange resin (Sigma), filtered through Whatman™ No. 1 filter paper (Whatman International, Maidstone) and stored in the dark at 4°C.

Ammonium acetate (5M): 38.5g of ammonium acetate was dissolved in 100ml of Q water; following filter sterilisation, the solution was stored at room temperature.

Caesium chloride (5.7M): 96g of caesium chloride (BRL, optical grade) was dissolved in 100ml of 0.1M EDTA (pH 8), sterilised by autoclaving and stored at room temperature.
Caesium chloride (1.3, 1.5 and 1.7g/ml): Caesium chloride (BRL, optical grade) was dissolved at the specified densities in 1 buffer. After checking the densities using a refractometer (Bellingham+Stanley, Tunbridge Wells), solutions were sterilised by autoclaving and stored at room temperature.

Calcium chloride (1M): 21.9g of calcium chloride·6H2O (BDH) was dissolved in 100ml of Q water, sterilised by autoclaving and frozen in 10ml aliquots at -20°C.

Chloroform/iso-amyl alcohol: 98% (v/v) chloroform (Fisons), 2% (v/v) iso-Amyl alcohol (Fisons) was prepared and stored at room temperature.

Citric acid (1mM): 4.2g of citric acid (BDH) was dissolved in 20ml of Q water, sterilised by filtration and stored at room temperature.

Colony neutralising solutions: 0.5M Trizma base, 1.5M NaCl; pH adjusted to 7.4 with concentrated HCl. Stored at room temperature.

ddNTP solutions (10mM): Powdered ddNTPs (Sigma) were dissolved in the specified volume of TE and stored at -20°C: 10mg ddTTP, 1639ml; 5mg ddCTP, 862ml; 5mg ddGTP, 806ml; 2mg ddATP, 323ml.

DEPC treated Q water: 0.1% (v/v) DEPC was added to Q water. After shaking vigorously, the bottles of Q water were left overnight in a fume cupboard with loosened caps. The DEPC-treated Q water was then autoclaved and stored at room temperature.

dNTP solutions (0.1mM): Powdered dNTPs (Sigma) were dissolved in the specified volume of TE and stored at -20°C: 10mg of dATP, 186.9ml; 5mg of dTTP, 103.7ml; 5mg of dGTP, 98.6ml.

dNTP solutions (50mM): Powdered dNTPs (Sigma) were dissolved in the specified volume of TE and stored at -20°C: 5mg of dTTP, 155.8ml; 10mg of dCTP, 338ml; 10mg of dGTP, 316ml; 10mg of dATP, 339ml.
Materials and Methods

DTT (1m): 1.55g of DDT (Sigma) was dissolved in a final volume of 10mL of 10mM sodium acetate (pH 5.6) and sterilised by filtration. 1mL aliquots were stored at -20°C.

EDTA (0.5m, pH 8): 186.1g of EDTA (Fisons) was dissolved in 1L of Q water adjusted to pH to 8.0 with NaOH pellets and 10M NaOH. Following sterilisation by autoclaving, the solution was stored at room temperature.

Ethidium bromide (10mg and 5mg/mL): Ethidium Bromide (Serva) was dissolved at the specified concentration in Q water and stored in opaque plastic bottles at room temperature.

Ethanol (100%, 80%, 70%): Ethanol (Fisons) was diluted as required with Q water and stored at -20°C.

Formamide, deionised (100%): Formamide (Fisons) was deionised using Amberlite MB-3 ion exchange resin (Sigma), filtered through Whatman No.1 filter paper and stored in aliquots at -20°C.

GFM buffer: 804mL of 6.9M glyoxal (deionised); 3.89mL of 100% formamide (deionised) and 306mL of MOPS (10x) were mixed together and frozen at -70°C in 250mL aliquots.

Glycerol (45%): Glycerol (Fisons) was dissolved at a concentration of 45% (w/v) in Q water and filter sterilised. The solution was stored at room temperature.

Glyoxal, deionised (6.9M): Glyoxal was supplied as a 40% (w/v) solution (equivalent to 6.9M) by BDH. After deionising with AG® 50W-X8(D) 20-50 mesh ion exchange resin (Bio-Rad, Richmond) and filtering through Whatman No.1 filter paper, the solution was stored in 1mL aliquots at -70°C.

HEPES (2m, pH 6.6): 9.53g of HEPES (Sigma) was dissolved in a final volume of 20mL Q water after adjusting the pH to 6.6 with 5M NaOH. After filter sterilisation, the solution was stored at 4°C.
Materials and Methods

HCl (0.25M): 21.55ml of concentrated HCl (Fisons) was added to 978.45ml of Q water. The solution was stored at room temperature.

IPTG (100mM): 238mg of IPTG (Sigma) was dissolved in 10ml of Q water and stored at -20°C.

λ Buffer: 6mM Tris-HCl (pH 8); 10mM MgCl₂ (Fisons), 100mM NaCl (Fisons); 0.05% (w/v) gelatin (Fisons). Sterilised by autoclaving and stored at room temperature.

Magnesium chloride (1M): 20.3g of MgCl₂.6H₂O was dissolved in 100ml of Q water, sterilised by autoclaving and stored at room temperature.

Magnesium sulphate (1M): 24.6g of MgSO₄.7H₂O (Fisons) was dissolved in 100ml of Q water, sterilised by autoclaving and stored at room temperature.

Maltose (20%): 4g of maltose (Sigma) was dissolved in 20ml of Q water and filter sterilised. The solution was stored at 4°C.

MOPS buffer (10x): 0.2M MOPS, 50mM sodium acetate.3H₂O, 1mM EDTA (pH 8). The pH was adjusted to 8 with NaOH prior to autoclaving. The solution was stored at room temperature.

PBS: Dulbecco's modified PBS (without Mg²⁺ or Ca²⁺) was prepared by dissolving ten tablets (ICN Flow) in 1000ml of Q water. The PBS was dispensed (250ml aliquots) into 500ml tissue culture bottles (Gibco BRL). Following sterilisation by autoclaving, the solution was stored at room temperature.

PCI: 50% (v/v) phenol (Fisons), 48% (v/v) chloroform, 2% (v/v) iso-Amyl alcohol; equilibrated against 10mM Tris-HCl (pH 8) and kept in the dark at 4°C.

Phenol (liquified): Liquified phenol containing 0.1% (w/v) 8-hydroxy-quinoline and equilibrated against 100mM Tris (pH 7.6) was obtained from Fisons.
Materials and Methods

Sodium acetate, (3M, pH 5.6): 40.8g of sodium acetate·3H2O (Fisons) was dissolved in 100ml of Q water, after adjusting the pH to 5.6 with glacial acetic acid. The solution was sterilised by autoclaving and stored at room temperature.

Sodium acetate, (1.1M, pH 7): 14.96g of sodium acetate·3H2O was dissolved in a final volume of 100ml of Q water after the pH had been adjusted to 7. Following sterilisation by autoclaving, the solution was stored at room temperature.

Sodium chloride (4M): 23.4g of sodium chloride (Fisons) was dissolved in 100ml of Q water and sterilised by autoclaving. The solution was then stored at room temperature.

SDS (10%): 50g of SDS (Fisons) was dissolved in 500ml of Q water and stored at room temperature.

Sodium hydrogen phosphate (0.5M): 89g of Na2HPO4·2H2O (Fisons) was dissolved in 11 of Q water. After sterilising by autoclaving, the solution was stored at room temperature.

Sodium hydroxide (10M): 200g of sodium hydroxide (Fisons) was added slowly to 400ml of Q water. After adjusting the volume to 500ml, the solution was stored at room temperature.

Southern denaturation solution: 0.5M NaOH, 1.5M NaCl; autoclaved and stored at room temperature.

Southern neutralisation solution: 0.5M Tris-HCl (pH 7.4), 3M NaCl; autoclaved and stored at room temperature.

SSC (20x): 3M NaCl, 0.3M tri-sodium citrate (Fisons); pH adjusted to 7 with NaOH, autoclaved and stored at room temperature.

TAE (10x): 48.4g of Trizma base (Sigma) and 20ml of 0.5M EDTA (pH 8) were dissolved in 11 of Q water after the pH had been adjusted to 7.5 with glacial acetic acid (Fisons).
2 - Materials and Methods

TBE (10x): 108g of Trizma base, 55g of boric acid (Fisons) and 9.3g of EDTA were dissolved in 1lt of Q water.

TBE (0.5x) Acrylamide (6%) Urea: 50ml of 10 x TBE, 430g of urea (Serva) and 150ml of 40% acrylamide were dissolved in 1lt of Q water and stored in the dark at 4°C.

TBE (2.5x) Acrylamide (6%) Urea: 250ml of 10 x TBE, 430g of urea, 150ml of 40% acrylamide, 50g of sucrose (BRL) and 50mg of bromophenol blue (Sigma) were dissolved in 1lt of Q water and stored in the dark at 4°C.

TE: 10mM Tris-HCl (pH 7.2), 1mM EDTA (pH 8); autoclaved and stored at room temperature.

Tris-HCl (1M): 121.1g of Trizma base was dissolved in 1lt of Q water after adjusting the pH as required with concentrated HCl. Following sterilisation by autoclaving, the solution was stored at room temperature.

X-gal (20mg/ml): 200mg of X-gal (5-Bromo-4-Chloro-3-indoly1-β-D-galactoside) obtained from Novabiochem (Nottingham) was dissolved in 10ml of dimethylformamide (Fisons) and stored in the dark at -20°C.

2.7. Oligonucleotides used

Synthetic oligonucleotides used during the course of the work are shown in Table 2.2.
Table 2.2. Synthetic oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide Number</th>
<th>5'-3' sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CAATAAATTATCACAGAAGT</td>
</tr>
<tr>
<td>2</td>
<td>AACCAGGGAACCCATTTC</td>
</tr>
<tr>
<td>3</td>
<td>TCTCCTGCTCTCTCTCTGCTGAGC</td>
</tr>
<tr>
<td>4</td>
<td>CTGCTAGGGGCCCGAG</td>
</tr>
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<td>5</td>
<td>AACCAGATGAGAATAGC</td>
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<tr>
<td>6</td>
<td>GAAATCCAGAGCTATTC</td>
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<td>7</td>
<td>CATGTTATTTCAAGCTCC</td>
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<tr>
<td>T7</td>
<td>AATACGACTCACTATAAG</td>
</tr>
</tbody>
</table>
2.8. Purification of high molecular weight genomic DNA from tissues

Human tissue was powdered in a Mikro Dismembrator II (FT Scientific Instruments, Tewksbury) at liquid nitrogen temperature; other tissues were powdered under liquid nitrogen in a mortar and pestle. The powder was then resuspended in 20 ml sterile PBS, mixed gently, and spun at 3,000 rpm for 10 minutes. The supernatant was discarded, and the separated cells resuspended in 5 ml of SE buffer (150 mM NaCl; 100 mM EDTA, pH 8) plus 1/10 volume of 10% SDS and 1/40 volume of proteinase K (20 mg/ml) in Corex (DuPont Scientific Instruments, Delaware) tubes, mixed gently and incubated overnight at 37°C. 1/2 volume of PCI was added and after mixing, the emulsion was spun at 10,000 rpm in a HB-4 rotor (DuPont) within a Sorvall RC-5B centrifuge for 2 minutes. The aqueous phase was transferred to a fresh tube and the phenol was re-extracted with 1/4 volume of SE. The supernatant was then dialysed against 2 litres of TE buffer overnight at 4°C. The sample was then carefully removed from the dialysis tubing before observation of the DNA quality on agarose gels.

2.9. Purification of total RNA from tissues

Total RNA was purified from whole tissues by either cesium chloride centrifugation or by acid guanidinium thiocyanate-phenol-chloroform extraction. All solutions were prepared using DEPC-treated Q water and all vessels containing RNA were soaked in DEPC-treated Q water prior to use. The two methods are described below:

1. Cesium chloride centrifugation: Powdered tissues were prepared as described in section 2.8 above. Without thawing, the powder was transferred to 5 ml of TLE and phenol-extracted twice. Both aqueous phases were pooled and subjected to cesium chloride pad (5 M CsCl) centrifugation at 25,000 rpm in a TST41:14 swing-out rotor (Sorvall®) in a Sorvall® OTD 50B ultracentrifuge (DuPont) for 18 hr at 25°C. The purified RNA was ethanol precipitated, resuspended in DEPC-treated Q water and stored at -70°C. The quantity and quality of the RNA was checked by spectrophotometric analysis and agarose gel electrophoresis respectively.
2. Guanidinium thiocyanate extraction: Tissue was homogenised on ice in 1ml of extraction buffer (4M guanidium thiocyanate, 25mM sodium citrate, pH7, 0.5% sarcosyl, 0.1M 2-mercaptoethanol) then extracted with phenol. The aqueous phase was precipitated twice with isopropanol (Fisons), washed with 70% ethanol, dried and resuspended in 0.5% SDS or DEPC-treated Q water. The quantity and quality of the RNA was checked by spectrophotometric analysis and agarose gel electrophoresis respectively.

2.10. Large-scale preparation of plasmid DNA

Two methods were used for large-scale plasmid DNA purification. Subsequent to purification by both methods, the quantity and quality of the DNA was checked by spectrophotometric analysis and agarose gel electrophoresis respectively.

1. Detergent lysis followed by CsCl/EtBr equilibrium density centrifugation was essentially as described in (Sambrook et al., 1989). Briefly, bacterial cultures were lysed with Triton-X100 and supercoiled plasmid DNA separated from other nucleic acids by centrifugation in a solution of CsCl/EtBr at 39,000rpm for 48hr in a T1270 rotor (DuPont Scientific Instruments). After isolation of the supercoiled plasmid DNA, isopropanol extraction followed by dialysis removed EtBr and CsCl from the sample respectively. After ethanol precipitation, the DNA was resuspended in Q water.

2. Alkaline lysis and purification of DNA by the use of Qiagen columns (Hybaid, Teddington) was performed according to the manufacturer's instructions.

2.11. Small-scale purification of plasmid DNA

A 5ml overnight culture was grown from a single colony or a glycerol stock in 2xYT under appropriate antibiotic selection. A 1.6ml aliquot was spun down in a microcentrifuge tube, the pellet resuspended in 100ml sterile water and incubated on ice for 5-minutes. 200µl of alkaline SDS (1% SDS, 0.2M NaOH) were added, mixed and the cells incubated on ice a further 5minutes. Next, 150µl of potassium acetate solution (3M acetate, 3M
potassium) was added, the solution gently mixed and incubated on ice for a final 5-minutes. Precipitated matter was collected by centrifugation and discarded, the supernatant was retained. RNase A was added to 100μg/ml and the solution incubated at 37°C for 1 hour. Precipitated protein was removed by phenol extraction and the plasmid DNA was recovered by ethanol precipitation.

2.12. Gel electrophoresis

1. Agarose. Nucleic acids were separated by electrophoresis through horizontal agarose slab gels (Seakem HGT or NuSieve GTG; both purchased from Flowgen, Sittingbourne), which varied between 0.8 and 5% (w/v). Gels were made and run in either 0.5 x TAE (DNA) or 1 x MOPS (RNA) buffers. Ethidium bromide was added to the gel and buffer at 0.5mg/ml for DNA gels to allow visualisation of the DNA when illuminated by ultraviolet light (254nm wavelength). When a permanent record of a gel was required, the gel was photographed using a Polaroid MP4 land camera loaded with Polaroid type 667 black and white positive film (Polaroid, St. Albans).

2. Polyacrylamide. Nucleic acids were separated by electrophoresis through vertical polyacrylamide gels of various percentages and types. 6% denaturing gels were used for separating the products of DNA sequencing reactions. Denaturing gels were of two types: non-gradient (purely 0.5 x TBE) or gradient (0.5 x TBE at the top and 2.5 x TBE at the bottom). The products of primer-extension analysis were separated on a 7.5% denaturing gel containing 42% (w/v) urea and 0.5 x TBE. All gels were run in TBE buffer of the same strength as that in the gel except gradient gels which contained 0.5 x TBE in the upper chamber and 1 x TBE in the lower chamber.

2.13. Restriction enzyme digestions and Southern (DNA) blotting

Restriction endonucleases were used according to the manufacturers' instructions and incubated for sufficient time to attain complete digestion. 1/10 volume of loading buffer (20mM EDTA, 50% (v/v) glycerol, 0.05% (w/v) bromophenol blue) was added and the sample loaded on an agarose
Materials and Methods

gel together with a suitable size-marker. After electrophoresis and photography, capillary transfer of the DNA to a nylon membrane (Hybond-N, Amersham) was performed essentially as described by (Sambrook et al., 1989) using 20 x SSC as a transfer buffer. The filter was dried and the DNA cross-linked to the membrane by irradiation with ultraviolet light (254nm).

2.14. Northern (RNA) blotting

RNA was glyoxalated by incubation in 33-50% GFM buffer (depending on sample volume) at 55°C for 15 minutes. 1/10 volume of loading buffer [40% (v/v) formamide, 50% (v/v) glycerol, 1 x MOPS, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF (BDH)] was added and the samples loaded on a 0.8 or 1% neutral agarose gel in 1 x MOPS buffer. Marker tracks containing aliquots of an RNA ladder (BRL) were treated in an identical manner and loaded onto the gel. After electrophoresis, the RNA was transferred (without any pretreatment) to a Nylon membrane as described for Southern blotting and cross-linked by ultraviolet irradiation. To reverse the glyoxalation, the filter was baked at 80°C for 1hr. To visualise the marker RNAs, these tracks were removed from the filter and stained in 0.04% (w/v) methylene blue, 500mM sodium acetate, pH5.6 until blue and destained in water.

2.15. Hybridisation of membrane-immobilised nucleic acids

1. DNA. Prehybridisation was performed for a minimum of 10 minutes at 65°C in Church and Gilbert buffer, 7% (w/v) SDS, 1% (w/v) BSA, 0.5M NaPO4 pH7 and 1mM EDTA as described. Hybridisation was performed in this solution with 0.5ng/ml of radiolabelled probe for 16hr at 65°C. Prehybridisation and hybridisation were performed in a volume of 20ml in sealed Hybaid oven hybridisation bottles.

After hybridisation the filters were washed at 2 x SSC, 0.1% (w/v) SDS at 65°C for heterologous probes, and 0.1 x SSC, 0.1% (w/v) SDS for homologous probes, for 3 x 30 minute periods. Filters were blotted dry and autoradiographed at -70°C with intensifying screens or at room temperature, depending on the strength of signal. Three types of X-ray film were routinely used: Kodac X-OMAT™ (Eastman Kodak Company, New York),


2. Materials and Methods

Fuji RX™ (Fuji Photo Film Company, Japan) and Hyperfilm™ MP (Amersham).

2. RNA. Filters were prehybridised and hybridised in an identical manner to DNA filters. Filters were blotted dry and autoradiographed in an identical manner to Southern blot filters.

2.16. Removal of probes and re-use of DNA and RNA blots

1. DNA blots. Probe sequences were removed by incubating the filter in 0.4M NaOH at 45°C for 30 minutes followed by incubation in 0.1 x SSC, 0.1% (w/v) SDS, 0.2M Tris-HCl (pH 7.5) at 45°C for 30 minutes. After probe removal the filter was prehybridised and hybridised as normal.

2. RNA blots. Probe sequences were removed by incubating for 1-2hr at 65°C in 5mM Tris-HCl (pH 8), 2mM EDTA, 0.1 x Denhardt’s solution. After probe removal the filter was prehybridised and hybridised as normal.

2.17. Preparation of 32P radiolabelled probes

32P radiolabelled probes were generated by three different methods:

1. Random priming method. The method used was that of Feinberg and Vogelstein (1983). Labelling reactions were performed at either 37°C for 30 minutes or 20°C for 4-5hrs with 2u of Klenow polymerase (Amersham). New preparations of DNA were checked for efficiency of radionucleotide incorporation as follows: 1μl (approximately 1/100) of the probe mix was pipetted onto a 2cm circle of DE81 paper (Whatman), dried and Cerenkov counted in a Tri-Carb®, Minaxi-b 4000 series liquid scintillation counter (Packard Instruments, Downers Grove, Illinois). After washing off the unincorporated nucleotide with 0.5M Na2HPO4, the filter was dried and counted again. Typically 70-80% incorporation was seen, and probes with over 60% incorporation were used without further purification.

2. End-labelling. Oligonucleotides and short DNA fragments were labelled using T4 polynucleotide Kinase-catalysed transfer of 32P from [γ32P] ATP to the free 5’ hydroxyl group of the nucleic acid. The efficiency of transfer was
estimated by chromatographic analysis of a small aliquot (approximately 1\%) of the reaction mixture on DE81 paper. The $[^{32}\text{P}]$ ATP migrates at the solvent (0.3M ammonium formate) front, whereas the radiolabelled DNA remains at the origin. Relative proportions of radioactivity in each fraction were estimated by autoradiography of the DE81 paper. Well over 90\% incorporation of $^{32}\text{P}$ into the DNA was regularly seen.

2.18. Purification of DNA fragments from agarose gels

The desired fragment was excised from an agarose gel, placed inside dialysis tubing containing a small amount of 0.5 x TAE and electrophoresed at 10 V/cm for 15 minutes. The eluted DNA was then ethanol precipitated, dried and resuspended in a suitable volume of Q water.

2.19. Generation of DNA molecules with blunt ends

Blunt ends were generated by removing protruding 3' termini and filling in protruding 5' termini. Protruding 5' termini were filled essentially according to the method of Wartell and Reznikoff (1980). Briefly, DNA was included in a nick-translation reaction catalysed by Klenow polymerase (Amersham). After heat inactivation (70\°C for 5 minutes) of the enzyme the DNA could be included directly in ligation reactions. Protruding 3' termini were removed using the 3'5' exonuclease activity of T4 DNA polymerase. Briefly, following restriction endonuclease digestion, dNTPs were added to a concentration of 0.1mM. 1-2 units of T4 polymerase was then added and incubated at 12\°C for 15 minutes followed by heat inactivating the enzyme (75\°C for 10 minutes). The DNA could then be included directly in ligation reactions.

2.20. Ligation of DNA molecules and transformation of competent E. coli cells

Ligations were performed using T4 DNA ligase at 15\°C. Standard reactions were performed in a 10\mu l volume with 10-100ng of vector and an appropriate amount of insert to give a 1:1 molar ratio of vector:insert. Occasionally, greater amounts of insert were used (up to a 10-fold excess) without problem. For ligation of molecules containing cohesive ends, T4
2. Materials and Methods

DNA ligase from Gibco BRL at 0.1 u/μl final concentration was used; incubations being for 4-24 hr. For ligation of molecules containing blunt ends, T4 DNA ligase from New England Biolabs at 200 u/μl (equivalent to 3 BRL u/μl) final concentration was used and incubations were for a minimum of 24 hr.

2.2. Transformation of competent E. coli cells

Two methods were routinely used for DNA uptake and transformation of E. coli cells. Method 1 is a chemical transformation procedure, whereas method 2 is one based on electroporation.

Method 1

Preparation of competent cells. A single colony was picked from an agar plate and grown overnight in 10 ml LB. A 1 ml aliquot was diluted 1:100 in LB prewarmed to 37°C and the culture grown to an optical density at 550 nm of 0.5. It was chilled on ice for 15 min and the cells collected as a pellet by centrifugation at 4°C, 3,500 rpm for 5 min. The pellet was carefully drained of yB, the cells resuspended in 40 ml of TbfI (TbfI is: 30 mM Potassium acetate, 100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl₂ and 15% (v/v) glycerol at pH 5.8 and filter sterile) and incubated on ice for 5 min. The cells were collected by a second centrifugation as above and the pellet resuspended in 4 ml TbfII (TbfII is: 10 mM MOPS pH 6.5, 75 mM CaCl₂, 10 mM RbCl and 15% (v/v) glycerol, filter sterile). Following a 15 min incubation on ice the cells were flash frozen in 200 ml aliquots in microfuge tubes using liquid nitrogen. They could be stored in this state at -70°C until required.

Introduction of DNA. An aliquot of competent cells was thawed on ice for 15 min. 50-100 ng of DNA from a ligation, or 5-10 ng of closed circular DNA, was chilled on ice while the cells were thawing. The DNA was added to the cells, mixed by gentle agitation and incubated on ice for 1 hour. The DNA-cell mix was incubated at 42°C for 1 minute and finally returned to ice for 5 minutes.
Selection of transformed cells. 1ml of 2xYT was added and the cells incubated at 37°C with shaking for 1 hour. The cells were collected by centrifugation at full speed for 30 secs in a micro centrifuge the 2xYT discarded and the pellet resuspended in 100ml fresh 2xYT. Dilutions were prepared at 1:10, 1:10² and 1:10³ and the cells plated on LA, including appropriate antibiotics and chromogenic selective agents if required. Transformed cells could be seen as discrete colonies on one or more of the plates following an overnight incubation at 37°C.

Method 2

Preparation of cells. 100mls of LB was inoculated with 200µl of an overnight culture of an appropriate host E.coli strain, and the cells grown to an optical-density at 600nm of 0.7. The cells were spun down at 4000rpm for 5 minutes, the supernatant discarded and the cells resuspended in 100mls of Q water. The cells were again spun at 4000rpm for 5 minutes, the supernatant discarded and the cells resuspended in 50mls of Q water. Another spin at 4000rpm for 5 minutes was followed by discarding the supernatant and resuspending the cell pellet in 2mls of 10% glycerol. A final spin at 4000rpm was followed by discarding the supernatant and the final cell pellet resuspended in 200µl of 10% glycerol. After separation into 40µl aliquots, one aliquot was used per electroporation on fresh cells or cells stored at -70°C.

Electroporation procedure. A Bio-Rad (Hemel Hempstead) gene pulsar electroporator was set to a capacitance of 25µF, a voltage of 1.5kv and a resistance of 1kOhm. Transforming DNA was added in a volume of 1-4 ul to a 40ul aliquot of cells. The cell-DNA mixture was added to a precooled biorad electroporation cuvette with a 2mm electrode gap. A pulse was delivered and a time constant of 18-24 ms was routinely observed. The transformed cells were then removed from the cuvette and placed in 1ml of 2xYT and incubated at 37°C for 1 hour before plating out.

Selection of transformed cells. Selection of transformed cells was achieved in an identical manner to Method 1.
2.2. Screening Bacterial Colonies using DNA Probes

A gridded nylon filter (orientated) was placed on an agar plate and individual colonies picked onto the membrane using sterile tooth-picks (the same colonies were also replicated onto a master plate). The plate was then inverted and grown at 37°C until the colonies became visible. At this time the filter was peeled off the plate and transferred to 3MM paper soaked in Southern denaturing solution. After 5 minutes, the filter was transferred to 3MM paper soaked in 1M Tris-HCl (pH 7.2) for 1 minute prior to transfer to 3MM paper soaked in colony neutralising solution for 5 minutes. The filter was then washed quickly in 1 x SSC to remove bacterial debris before drying and cross-linking the DNA to the filter by irradiation with ultraviolet light. The filter was then probed as described for Southern blots.

2.23. Nucleic acid sequencing

A T7Sequencing™ kit was obtained from Pharmacia and used according to their instructions. Plasmid DNA from large-scale and small-scale preparations was used with equal success.

2.24. PCR methods

PCR was used for amplifying sequences from DNA/cDNA in several protocols. Amplifications were performed using a Perkin Elmer Cetus DNA thermal cycler (model No. N8010177, Perkin Elmer Corporation, Norwalk). Buffers used were either those supplied with the Taq DNA polymerase or the buffer specified by Anglian Biotech Ltd. [167μl of 2M Tris-HCl (pH 8.8), 83μl of 1M ammonium sulphate, 33.5μl of 1M MgCl₂, 3.6μl of 2-mercaptoethanol (Sigma), 3.4μl of 10mM EDTA (pH 8), 75μl of each 100mM dNTP (Pharmacia, ultra pure), 85μl of 10mg/ml BSA (BRL)]. Reaction volumes were 50 or 100μl containing 2.5μl of Taq polymerase and overlayed with 50μl of mineral oil (Fisons). Primers were present at 1mM and template DNA at 10-300ng.

For amplification of DNA sequences using two known primers, the reactions were set up according to the protocol supplied with the DNA
thermal cycler and amplification performed using 30 cycles of: 94°C melt for 1 minute, anneal for 1 minute and extension at 72°C for 3 minutes. The final extension was for 10 minutes, followed by a 4°C soak. In addition, an initial 94°C melt for 4 minutes was employed for amplification from genomic DNA. Annealing temperatures were usually 5°C below the calculated melting temperature of the oligonucleotide primers. PCR experiments which generated background amplification products under the above conditions were repeated using a modified "touchdown" PCR programme. In this programme the annealing temperature dropped 2°C every second cycle from 70°C to 52°C, with a final 20 cycles at 50°C annealing temperature. All other parameters were identical to non-touchdown programmes.

2.25. Primer extension

Primer extension analysis of total human heart and human pancreatic RNA was performed essentially as described by (Sambrook et al., 1989). Briefly, the radiolabelled single-stranded primer sequence was mixed with the RNA and ethanol precipitated. The pellet was resuspended in hybridisation buffer, heated to 85°C for 10 minutes, then incubated at 45°C overnight. After recovering the nucleic acid by ethanol precipitation, the primer was extended using AMV RVT at 42°C for 40 minutes. The RNA was then removed by treatment with RNase and the cDNA recovered by ethanol precipitation. After addition of loading dye and boiling, the cDNA was loaded onto a 7.5% denaturing polyacrylamide gel using M13 sequencing reactions as size markers. After electrophoresis, the gel was dried down and autoradiographed overnight at room temperature.
2. Materials and Methods

In Vitro cell culture methods

2.2.6. Solutions used during the culture and transformation of animal cells

DMEM: DMEM (without sodium pyruvate, with 4500mg/l glucose) was purchased from Gibco BRL and stored at 4°C. The medium was supplemented with 10% (v/v) FCS (Gibco BRL), 2mM glutamine (Gibco BRL) and antibiotic (100u/ml penicillin and 100mg/ml streptomycin, Gibco BRL).

PBS (tissue culture): Dulbecco's modified PBS (without Mg^{2+} or Ca^{2+}) was prepared by dissolving ten tablets (ICN Flow) in 1000ml of Q water. The PBS was dispensed (250ml aliquots) into 500ml tissue culture bottles (Gibco BRL). Following sterilisation by autoclaving, the solution was stored at room temperature.

Electroshock buffer for MEL-cells: Electroshock buffer for the electroporation of MEL-cells contained 140mM NaCl, 25mM HEPES pH 7.5 (correct pH obtained using 4M NaOH) and 0.75mM Na2HPO4. 500ml aliquots of the buffer were filter sterilised. The final pH of the buffer was approximately pH 7.

Gene pulser cuvettes: Gene pulser cuvettes with a 0.4cm electrode gap, for the electroporation of MEL cells, were obtained from Biorad.

Geneticin (G418): Geneticin (G418) powder was dissolved in Q water to a final concentration of 200mg/ml (w/v) and stored as 1ml aliquots at -20°C.

2.27. Cell lines cultured In Vitro

MEL-C88 murine erythroleukaemia cells (Deisseroth and Hendrick, 1978) were employed.
2.28. General cell culture

Cell manipulations were performed in a class II microbiological safety cabinet (Medical Air Technology Ltd., Manchester). Cells were routinely cultured in 10ml of medium in 9cm Nuncion® (Gibco BRL) dishes. All mammalian cells were incubated at 37°C (5% CO2 atmosphere) in a humidified incubator.

2.29. Subculturing and handling of cells

To concentrate MEL-cell lines (non-adherent cells), the medium containing cells was centrifuged in a sterile 10ml cone base plastic tube (Northern Media Supply Ltd., North humberside) for 5 minutes at 2000rpm in a Heraeus DigiFugeGL (Heraeus Equipment Ltd., Brentwood). The medium was aspirated and the cells resuspended in a suitable volume of fresh medium. To subculture these cell-types, log-phase cells were diluted as required (up to one hundred-fold) into fresh medium in 9cm dishes (10ml per dish).

2.30. Cryogenic storage of cells

Cells from one 9cm dish were pelleted as above and resuspended in 2ml of medium containing 10% DMSO (Sigma, cell culture grade). 100ul aliquots were dispensed into 1.0ml ampules and cooled slowly to -196°C before storing in liquid nitrogen in a model LR40 cryostorage refrigerator (Jencons [Scientific] Ltd., Leighton Buzzard).

Growth of cells from frozen samples was performed as follows: a vial of frozen cells was placed in a water bath at the normal culture temperature of the cells until thawed. The cells were then transferred to 9ml of culture medium in a 9cm dish and incubated overnight. The following day, the medium was changed for fresh medium and incubation continued.
2.31. Transfection of MEL-cells

Expression constructs were linearized with a suitable restriction enzyme prior to electroporation. $1 \times 10^7$ MEL cells in logarithmic growth phase were washed twice with PBS and resuspended in 0.9ml of electroshock buffer before being mixed with 25 μg quantities of expression constructs. The cell-DNA mixture was placed in a genepluser cuvette and electroporated in a Bio-Rad Gene Pulsar™ at 960 mF and 250 V in sterile electroshock buffer. Following electroporation, cells were diluted in fresh culture medium to $10^4$ - $10^5$ cells/ml; 24-hours later, G418 was added (final concentration 1 mg/ml) to select for transfectants. Semi-adherent colonies (at a size visible to the naked eye) appeared 10-14 days post-transfection, and were either pooled or grown in separate wells under G418 selection. Independently-propagated cell-lines were numbered and aliquots taken for storage in liquid nitrogen. Cells were pelleted in a bench-top centrifuge (~1500 RPM, 10 min) and gently resuspended in 1/10th volume complete DMEM medium supplemented with 10 % DMSO before slow freezing (200 ml aliquots) in liquid nitrogen vapour.

2.32. Induction of MEL-cells for expression studies

For expression studies, cells were thawed at 37°C, and 50 ml diluted into 10 ml complete DMEM medium. Exponential growth was maintained for a minimum of four days prior to addition of DMSO (2 % v/v) to induce differentiation and expression. Cells were suitable for electrophysiological analysis and RNA expression assay between 0.5 to 5 days post-induction.
CHAPTER 3

Isolation and characterisation of a human cardiac delayed rectifier K+ channel gene (hCDR1g) from human genomic DNA
3.1 Introduction

A search was initiated for cDNA clones derived from mRNAs encoding voltage-sensitive K⁺ channels expressed in the human heart by screening an adult male heart cDNA library with a PCR-generated probe corresponding to the S4 segment of MBK-1 (Tempel et al., 1988). A cDNA insert of 1657 bp (hCDRlac) was sequenced and predicted to encode a voltage activated K⁺ channel by its strong homology to published K⁺ channel sequences. Strong homology was observed between this sequence and the cloned mouse K⁺ channel kvl (Swanson et al., 1990). This cloned cDNA could not be used to express functional channels since it lacked sequences encoding the N-terminal region as far as the S2 transmembrane domain. It was therefore used as a probe to screen at high stringency for the presence of the corresponding genomic clones in a human genomic library in the cosmid vector pcos2EMBL (Poustka et al., 1984). A number of genomic clones were isolated which showed strong homology to hCDRlac.

Identification of two hCDRlac genomic homologues by PCR, Southern blotting, and sequence analysis

3.2 PCR analysis

Using the 20 strongest positives, identified by their relative binding signal to 32P labelled hCDRlac, an initial experiment was undertaken to see whether a diagnostic PCR band could be generated. Two primers, oligonucleotides 1 and 2 (see materials and methods), which produced a diagnostic 1200 base pair band using hCDRlac as template, were used to see if they could produce a similar band with any of the 20 isolated cosmids. Five of the cosmid isolates, visualised following electrophoresis on a 0.8% agarose gel, produced a diagnostic 1200 base pair PCR band. Two isolates, cosmids 5 and 11 were propagated and taken forward for further characterisation (see Figure 3.1).
Figure 3.1.  PCR analysis on 20 hCDR1c putative homologues

PCR analysis using oligonucleotides 1 and 2 on 20 hCDR1c putative homologues. Lanes 5 and 11 correspond to positive isolates 5 and 11, taken for further analysis. Lane C is a positive control lane of hCDR1c DNA as template. The black arrows indicate the desired 1200bp diagnostic band.
3.3. Southern blotting analysis

Figure 3.2 shows the results from a Southern blotting experiment to identify bands which hybridized to hCDR1c as a \( ^{32} \)P labelled probe, and to observe any identity between cosmids 5 and 11.

Some differences between cosmids 5 and 11 were observed. Examples of this were seen after digestion with \( \text{EcoRV} \). Cosmid 11 showed positively hybridizing bands at 1.2kb and one at >8.4kb, whereas cosmid 5 produced a band at 5kb and one >8.4kb which is smaller than the larger band produced with cosmid 11. Lane 6 shows bands of size 4kb and 7kb with cosmid 5, whereas cosmid 11 shows only a band of >8.4kb after cutting with \( \text{SstI} \).

However, examples of band sets that look identical between cosmids 5 and 11 can be observed. These are seen in lane 8, where 2 small bands of 0.8 and 0.6kb can be seen on both blots after digestion with \( \text{SstII} \), and lane 2 where a band of 1.4kb is observed on both blots after digestion with \( \text{PstI} \).

After an appropriate deduction of 6.1kb for the pcos2EMBL vector sequences, addition of the separate band sizes from each cosmid sample digest before Southern blotting gave an estimated insert size of 32.5kb for cosmid 5 and 30.5kb for cosmid 11. After observation and comparison of the bands on each Southern blot, the results from this experiment indicate that the DNA cloned into the two cosmids are from the same chromosomal region, with cosmid 5 having 2.0kb of extra genomic sequence.

3.4. Sequence analysis

The 1.2kb \( \text{EcoRV} \) fragment from cosmid 11 which was observed to hybridize to hCDR1c was isolated and cloned into the \( \text{pSK}^+ \) \( \text{pBluescript} \) vector for sequence analysis. Sequence data employing \( \text{T3} \) and \( \text{T7} \) primers (see materials and methods for their sequence), was generated from both termini of this fragment. Sequence generated from the \( \text{T7} \) primer (Figure 3.5) confirmed the fragment's identity to hCDR1c. Due to the results reported in section 3.5, it was subsequently shown that sequence generated from the \( \text{T7} \) primer corresponded to a position heading downstream from the \( \text{EcoRV} \) site, at position 2120 of the
Figure 3.2. Southern blotting analysis on cosmids 5 and 11.

1µg samples of cosmids 5 and 11 were cut with a variety of restriction enzymes and separated through 0.8% agarose gels prior to Southern blotting. 32P labelled hCDR1c was used as the probe. Figure 3.1a shows aliquots of cosmid 5 and Figure 3.1b aliquots of cosmid 11 after Southern blotting and probing. The following scheme applies to both blots. Lane 1 HindIII, lane 2 PstI, lane 3 SalI, lane 4XbaI, lane 5 EcoRV, lane 6 SstI, lane 7 SmaI, lane 8 SstII, lane 9 EcoRI and lane 10 a 20ng positive control of 1657bp hCDR1c. Size markers in kb are indicated down the left-hand side of the Figure.
3 - Isolation and characterization of hCDR1g

human insulinoma $K^+$ channel, hPCN1 (Philipson et al., 1991), a homologue of hCDR1 (see Figure 3.6). Sequence generated from the T3 primer showed no homology to the full hPCN1 cDNA sequence. This was predicted as the 1.2kb EcoRV fragment would extend beyond the 3' end of the published hPCN1 sequence, therefore corresponding to flanking genomic sequence. The predicted position of this 1.2kb EcoRV fragment is shown in Figure 3.8.

3.5 Confirmation of the identity of hCDR1 to the hPCN1 and HK2 published sequences.

During the course of our work it became apparent that the hCDR1 sequence was identical to a 2.8kb $K^+$ channel cDNA sequence isolated in two independent studies, designated hPCN1 (Philipson et al., 1991) and HK2 (Tamkun, 1991), isolated from the human pancreas and heart respectively.

3.6 Mapping of the upstream region of hCDR1 from genomic sequence in cosmids 5 and 11

To map the upstream region of the hCDR1 sequence from cosmids 5 and 11, restriction enzyme sites that cut only once at the 3' end of hCDR1 beyond the coding region in the downstream untranslated region (dUTR) were determined. These unique sites were then used in an attempt to identify, by Southern blotting analysis, fragments larger than the predicted cDNA sequence alone would generate. Figure 3.3 shows the results of this analysis. A 1013 base-pair EcoRI-BamHI fragment from hCDR1c was used for probing the Southern blot. The position of this probe with respect to hCDR1 is shown in Figure 3.4. From sequence analysis of hPCN1, six enzymes, BglII, NdeI, BamHI, EcoRI, DraI and SspI were chosen for single 3' cut sites in the hCDR1 dUTR within cosmids 5 and 11. All these sites were predicted to be downstream of the 1013 bp probe sequence, therefore insuring only sequence upstream of these sites to be identified by the probe.

Positively hybridising bands from cosmid 11 generating a band size extending beyond the published cDNA sequence for hPCN1 were BglII, EcoRI, NdeI, SspI and DraI. These were accurately sized and interpreted in the form of a restriction map shown in Figure 3.4.
Figure 3.3. Southern blotting analysis on cosmids 5 and 11 to map upstream of the hCDR1 coding sequence from hCDR152.

1μg samples of cosmids 5 and 11 were digested to completion with 6 different restriction enzymes, and the products separated on a single 0.8% agarose gel. A 1013 base pair EcoR1-BamH1 fragment from hCDR1c was then used to probe a Southern blot produced from this gel. Lane 1 cosmid 11 BglII, lane 2 cosmid 11 Ndel, lane 3 cosmid 11 BamHI, lane 4 cosmid 11 EcoRI, lane 5 cosmid 11 Dral, lane 6 cosmid 11 SspI, lane 7 cosmid 5 BglII, lane 8 cosmid 5 Ndel, lane 9 cosmid 5 BamHI, lane 10 cosmid 5 EcoRI, lane 11 cosmid 5 Dral and lane 12 cosmid 5 SspI. Size markers in kb are indicated down the left-hand side of the Figure.
Figure 3.4.  Restriction map of the upstream region of hCDR1g.

The map shows the 5 restriction enzyme sites, BglII, EcoRI, NdeI, DraI and SspI upstream of the hCDRI coding region as determined by Southern blotting. Their relative mapping positions from the 3' region of hCDRI (region of 3' cut sites) are indicated upstream of the polyadenylation consensus site (polyA). The position of the 1013bp probe from hCDRIc used to map these 5 restriction enzyme sites is also indicated.
Figure 3.5. Sequence analysis from the 5' region of the 1.2kb EcoRV fragment from cosmid 11 using the T3 primer

Sequence ladder from the T3 generated 5' region of the 1.2kb EcoRV fragment from cosmid 11. The arrow shows the first G residue of recorded sequence, and each base specific lane is labelled at the top of the ladder.
A. Sequence comparison of the T7 generated sequence from the EcoRV cosm id 11 fragment (labelled 11) to the hPCN1 published sequence (labelled H). Only 5 sequence differences were recorded. hPCN1 was used as the reference sequence. A dash refers to a nucleotide loss, a lower case letter to a sequence change and a nucleotide letter symbol above a sequence position to an extra nucleotide present following the indicated sequence position. B. Sequence comparison of the 5' region of the 1.2kb EcoRV cosm id 11 fragment to published K+ channel sequences. hPCN1 is shown to have the strongest homology. Black areas represent sequence homology, and white areas sequence differences. C. Relative position of the confirmatory 5' region of the 1.2kb EcoRV cosm id 11 fragment sequence to hPCN1. The sequence is indicated extending from nucleotide position 2120 and downstream.
3.7. Subcloning of a 5.2kb BgII fragment (hCDR15.2) from cosmid11 containing the hCDR1 coding region and 2.4kb of upstream region

From the previous experiment, five enzymes, BgIII, EcoRI Ndel and DraI were predicted to produce a subclone of cosmid 11 including the full hCDR1 coding region and varying amounts of upstream sequence. To confirm this, a 5.2kb BgII fragment (hCDR15.2), which from Figure 3.2 was shown to hybridise strongly to hCDR1c with an estimated 2.4kb of upstream region, was isolated and cloned into the vector pBluescript SK+.

Sequence analysis from both termini using T7 and T3 primers confirmed that hCDR15.2 was the predicted subclone. 3' sequence using the T3 primer confirmed the clone's position in the dUTR, just upstream of the polyadenylation consensus sequence - AATAAA, and 5' sequence showed no homology to the full hCDR1 cDNA sequence or with pCOS2EMBL vector sequences, the only other possible source of this sequence apart from upstream human genomic hCDR1 sequence.

It was thus concluded that hCDR15.2 did contain the full hCDR1 coding region and 2.4kb of upstream sequence.

3.8. Primer-extension analysis on total human heart and pancreatic RNA to determine the transcriptional start sites for hCDR1 in these two tissues.

Primer-extension analysis on total human heart and pancreatic RNA, using a 24bp oligonucleotide (oligo. 3), complementary to a position -206 to -185 base pairs with respect to the translational start point was performed to determine the transcriptional start sites for hCDR1 in these two tissues. This was undertaken to investigate the possibility of alternate transcriptional start-sites for hCDR1 in heart and pancreas due to reported differences in detected RNA band sizes of 4kb from pancreas (Philipson et al., 1991) and 2.5 and 1.5 from heart (Tamkun, 1991), determined by Northern blot analysis.

Two apparently identical labelled bands of equal intensity were identified after the primer-extension products from both human heart and pancreas were separated on a 7.5% denaturing polyacrylamide gel. Figure 3.7 shows this result, with the arrow indicating the major species at 47 and 48 base pairs. The
Figure 3.7. Primer extension analysis on total human heart and total human pancreatic RNA.

Primer extension analysis on 25μg total human heart RNA and 100μg of total human pancreatic RNA using an end labelled 24bp oligonucleotide (oligo. 40) The oligonucleotide was complementary to the 5' end of the published hPCN1 cDNA sequence. Products were separated on a 7.5% denaturing polyacrylamide gel. Lanes 1-4 are M13 mp18 sequence markers corresponding to A, C, G and T respectively. Lane H was extension on total human heart RNA and lane P total human pancreatic RNA. The major primer-extension products are indicated by the arrow.
Figure 3.8. Restriction map of the upstream region of hCDR15.2.

Restriction map of the upstream region of hCDR15.2, indicating the relative positions of the oligonucleotides (3-7) used to sequence both strands of the 468 bp upstream sequence, and the position of the 1200 bp EcoRV fragment from cosmid 11 employed to produce confirmatory sequence to identity with hPCN1. The outer limits of the BgIII fragment, the published hPCN1 cDNA 5' terminus, translational start point (+1), and polyadenylation consensus sequence are also shown.
sizes were determined by their relative position to the sequence ladder in the centre of the gel, generated from a known M13 mp18 sequence template, using a 16bp oligonucleotide (oligo. 8) to prime the generation of this sequence. The positions of the transcriptional start-sites were deduced from the known position of hybridisation of the 24bp primer (oligo. 3) and the precise size of the extension-products. The major species at 47 and 48 base pairs in size would indicate a start site of 2 and 3 base pairs upstream of the start of the published cDNA sequence for hPCN1, equivalent to sequence positions -230 and -231 with respect to the translational start point.

3.9. Sequence analysis of the hCDR1 upstream region from hCDR15.2

In an attempt to elucidate the sequence and regulatory regions of hCDR1, 468bp of sequence data from hCDR15.2, upstream of the hCDR1 coding region was generated in an attempt to find sequence elements that might be important in the control of this gene. During the process of sequencing the upstream region of hCDR1 from hCDR15.2, sequence data corresponding to 1324bp of upstream sequence from the hCDR1 homologue hPCN1 was made available (unpublished data). The following section reports the consensus of these two sequences, and relative sequence domains that may be important in the regulation of this gene.

468bp of upstream hCDR1g sequence was generated using the five 18bp oligonucleotides (oligos. 3-7) on hCDR15.2 as template. Sequence data were obtained on both positive and negative strands, with the positions of each oligonucleotide shown in Figure 3.8. Figure 3.9 shows the comparison of this sequence to the 1324bp obtained from upstream of hPCN1. The sequences are clearly homologous with only 11 observed sequence differences when comparison of the hCDR1 upstream sequence to the first 468bp of the hPCN1 upstream sequence is undertaken.

Figure 3.10 shows the hPCN1 upstream sequence alone with putative regulatory sequence elements, and putative transcription start-sites as determined by the primer-extension analysis undertaken in section 3.8.

Putative sequence motif elements which may be part of the hCDR1 promoter structure are shown. These are two spl binding site consensus sequences,
Figure 3.9. Comparison of the 468bp of upstream sequence from hCDR15.2 compared to the equivalent sequence from upstream of hPCN1

Comparison of the 468bp of upstream sequence from hCDR15.2 compared to the equivalent sequence from upstream of hPCN1. Stars indicate identical sequence using hPCN1 upstream sequence as the comparative sequence. A letter in place of a star indicates a difference in sequence corresponding to that letter. A dash indicates a gap in the hCDR1 sequence comparing to hPCN1 and a letter under two dashes indicates extra base-pairs of sequence, before the sequence position indicated by the dashes, in the hCDR1 sequence which are not present in the hPCN1 equivalent sequence. The 468bp of hCDR1 sequence are shown extending upstream from the published hPCN1 sequence.
Figure 3.10. Sequence analysis of the hPCN1 (hCDR15.2) upstream region.

Sequence of the hPCN1 (hCDR15.2) upstream region. M at position 1 indicates the translational start site. The large arrow beneath position -228 indicates the 5'-end of the published hPCN1 sequence. The two arrows extending upwards and downstream from positions -230 and -231 indicate the two transcriptional start sites as determined by primer extension. The two boxed motifs at positions -264 and -311 show spl binding site consensus sequences. The consensus at position -480 indicates a glucocorticoid response element binding consensus. The triangular markings above positions -986 and -1152 indicate the start of two TATA box consensus sequences. The underlined motif at position -1513 indicates the start of a CAAT box consensus sequence. All sequence positions are with respect to the translational start site.
Isolation and characterisation of hCDR1g

Starting at positions -264 and -311, 33 and 80bp upstream of the first putative transcription start site. Two TATA box motifs starting at positions -986 and -1152 are also present. These are accompanied by a single CAAT box motif starting at position -1513, which could be associated with one of the TATA box motifs if there was a transcription start site in this region.

Discussion

3.10. Isolation and characterisation of hCDR1g

The experiments in this chapter have established that 2 cosmid clones, cosmids 5 and 11, which have commonality between them, encompass the hCDR1 locus. It has also been established that a subclone of cosmid 11, a 5.2kb BglII fragment, called hCDR15.2, contains the full coding region of the hPCN1 equivalent sequence, and 2.4kb of upstream genomic sequence, determined by sequence analysis from both termini of this subclone. HCDR1 was also shown to be truncated upstream of the polyadenylation sequence, as determined from sequence comparison of the 3' terminus of hCDR15.2 to hPCN1. Cloning of genomic regions encompassing K+ channel sequences will provide a direct route to analysis of the upstream and downstream elements controlling these genes in their native cell-types. The following discussion looks at characterisation of the upstream elements of hCDR1, and possibilities for transcriptional regulation of this gene.

3.11. Primer extension analysis identifies a putative TATA-less promoter 2 and 3bp upstream of the published hPCN1 cDNA sequence

Primer extension analysis on total human heart and pancreatic RNA (Figure 3.7) detected two putative transcriptional start sites at positions -230 and -231, 2 and 3bp upstream of the published hPCN1 cDNA sequence. The two primer extension product bands were of equal intensity for both tissues, indicating, in the event of these two sites being authentic, an equivalent usage of these sites for transcriptional initiation. No TATA or CAAT binding motifs were observed in the vicinity of these two putative transcriptional start sites, but two Sp1 binding sites (Kadonaga et al., 1986) were observed in the near upstream vicinity of the two sites (see Figure 3.10). The region was also observed to have a high GC content.
A number of eukaryotic genes that have promoters high in a G+C content lack the characteristic TATA and CAAT boxes. These include the hypoxanthine guanine phosphoribosyltransferase gene (Melton et al., 1984), the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase gene (Reynolds et al., 1984) and the adenosine deaminase gene (Valerio et al., 1985). These encode enzymes with housekeeping functions with expression at a low constitutive level in a wide number of different tissues. Also the promoters of several cellular growth control genes such as c-Harvey ras (Ishii et al., 1985), c-Kirsten ras (Hoffman et al., 1987), the epidermal growth factor receptor (Ishii et al., 1985), and the nerve growth factor (NGF) receptor gene (Sehgal et al., 1988) do not contain the typical TATA and CAAT transcriptional consensus sequences, but have multiple GC box motifs.

A comparison of these promoters to hCDRI showed a distinct similarity to the NGF receptor gene promoter. Both show a common structural feature among this class of promoters in the possession of multiple GGGCGG sequences in the 5' upstream region. These sequences are similar to the GC boxes in the 21-bp repeat region of the simian virus 40 promoter and are recognised by transcription factor Sp1 (Kadonaga et al., 1986). The NGF receptor gene has 4 such consensus sequences upstream of its transcriptional start sites, whereas hCDRI has 2.

The most striking similarity between these two promoter regions is that there appears to be directed transcription primarily from two adjacent nucleotides instead of using multiple sites of initiation. Most constitutively expressed genes contain multiple initiation sites distributed over a 15 to 20-base region that are all used with the same frequency (Reynolds et al., 1985). In this case, specificity of transcription initiation must be controlled by regulatory elements other than the consensus TATA sequence. An element termed the initiator, has been implicated in transcription initiation to specific nucleotide positions in TATA-less and TATA-containing promoters (Smale et al., 1989). The initiator, which has a loose consensus sequence of 5'-PyPyCAPyPyPyPyPyPyPy-3' was shown to independently, at low level, direct transcription from a single nucleotide position, the A nucleotide within the initiator consensus from the lymphocyte-specific terminal deoxynucleotidyl transferase gene. This was augmented by the presence of a TATA box, and augmented in the absence of a TATA box, but
in the presence of the SV40 early promoter elements, containing Sp1 binding sites. These initiator sites have also been observed in several mammalian TATA-containing genes (Corden et al., 1980).

Another TATA-less promoter, the SV40 major late promoter, has been shown to contain an important sequence element, with no sequence similarity to a TATA box, 30bp upstream of its transcription initiation site (Wiley et al., 1991). This sequence was shown to bind the cloned human TATA box binding protein (TBP), hTFIID*, and lead to transcription initiation. It was also observed that this element has genetic properties similar to those of a TATA box in that it directed RNA polymerase II to initiate transcription approximately 30bp downstream of its location, and inactivation of the element resulted in increased heterogeneity in the sites of transcription initiation. The report concluded that many, if not all TATA-less promoters differ from TATA-box containing promoters simply in the affinity of their -30 regions for binding of TFIID, with other nearby sequence elements contributing to functional transcription initiation.

Pugh et al. have shown a requirement for a TFIID complex for TATA-less transcription, this TFIID complex consisting of many regulatory factors including the TBP (Pugh et al., 1991). They suggested that the preinitiation complex remains essentially unaltered at both TATA-containing and TATA-less promoters but the promoter-binding specificity of the TFIID complex is achieved through protein-protein interactions with 'tethering' factors, (proteins shown to be present only in the transcription complex of a TATA-less promoter, distinct from Sp1 and TBP and promoter bound Sp1), instead of direct protein-DNA interactions between the TBP subunit and the TATA-box.

The hCDR1 putative TATA-less promoter showed no consensus with the initiator sequence from the NGF-receptor gene or the -30 region from the SV40 major late promoter. Sequences within the vicinity of the two transcriptional start-sites may however be important for accurate transcription initiation, but as yet remain undefined. The two Sp1 binding sites upstream of the transcriptional start-sites may be crucial in the regulation of this putative promoter, being crucial for the transcription initiation complex to form (Pugh and Tijan, 1991).
3.12. Two putative TATA-containing promoter sites

Further sequence analysis of the upstream region identified two TATA-box motifs shown in Figure 3.10. It has been established that the simplest promoter is one containing a TATA-element, with or without a transcriptional start-site or initiator (Breathnach and Chambon, 1981). A consensus CCAAT box is also present >350bp upstream from the two TATA elements. Many eukaryotic promoters possess a regulatory element with this consensus between 60 and 80bp upstream of the initiation site (Benoist et al., 1980; McNight and Tijan, 1986). The significance of this consensus is however questionable due to its large distance from the nearest TATA element.

3.13. The glucocorticoid response element

Downstream from the two TATA box motifs is a glucocorticoid response element (GRE) consensus sequence (Moore et al., 1984). Glucocorticoid hormones are released as part of the stress response and regulate secretion by the pituitary. Studies on the rat hCDRI homologue, the Kv1 gene, showed that treatment of rats, and a clonal rat pituitary cell-line, with the glucocorticoid agonist dexamethasone effected increased transcriptional levels of this Kv1 message in rat hypothalamus and anterior pituitary, and from the clonal rat pituitary cell-line (Levitan et al., 1991). This would indicate that this element would have a similar role in the human, and a similar experimental regime may confirm this prediction.

3.14. Conclusions

From Northern blot analysis, message sizes of 4kb from human pancreas (Philipson et al., 1991) and 2.5 and 1.5kb from human heart (Tamkun, 1991) were reported. Allowing for a polyadenylation addition of approximately 20-250 adenosine residues (Wickens, 1990), a 4kb message would give a transcriptional start site that could be accomodated by both the TATA box elements shown in Figure 3.10, making both these consensus TATA elements strong candidates for the promoter site employed in the pancreas. Message sizes of 2.5 and 1.5kb, observed from heart would fall well within the predicted coding region of the gene, and as it has been established in this work that the gene is intronless, it is unlikely that some alternatively spliced variants are
Involved in the production of these message sizes. Therefore the presence of these messenger species must have some other explanation.

Transcript mapping in this study has shown two putative transcript start sites for human heart and pancreas, which would be predicted to give a message size of approximately 2.9-3.1kb, taking into consideration the addition of a polyadenosine tract to the mature message. This message size was not observed on blots from Northern analysis from human heart and pancreatic RNA. Primer extension in this vicinity may have been hindered by a high G+C content leading to premature termination, giving rise to the false appearance of these two transcriptional start sites. In the case that a transcriptional start site is present further upstream, it may explain the reason why the published cDNA has only extended to this position upstream of the translational start site.

SI analysis would be a good candidate to confirm these two transcriptional start sites, or confirm that termination of the products was due to secondary RNA structure, or a high G+C content in the region surrounding these putative transcriptional start sites.
CHAPTER 4

Regulated and functional expression of K+ channel cDNAs and genes under control of the human β-globin LCR
4.1. Introduction

Functional expression of $K^+$ channel sequences is a fundamentally important requirement for the study of these membrane-associated proteins at a molecular level. Without functional expression, classification of the different cloned types of $K^+$ channel genes would be difficult. Comparison at the nucleotide and amino-acid level has shown close homology between different $K^+$ channel sequences, yet the expression of these same sequences has shown a diverse variety of expressed $K^+$ channel types. Furthermore, the wealth of knowledge that has accumulated on the functional definition of specific sequence elements of $K^+$ channel gene sequences, by sequence manipulation, would not be available without functional expression.

Sections 1.17-1.23 have reviewed the status of available systems for the expression of $K^+$ channel cDNAs and genes. At the time of this work, a novel expression strategy was under development at ICI Pharmaceuticals (Alderley Edge, U.K.) for the high-level expression of proteins, and was kindly made available for the study of the system's potential use for expression of $K^+$ channel cDNAs and genes. This system employs sequence elements from the human $\beta$-globin LCR gene activation region, incorporated into various vectors designed for the expression of cDNA and gene sequences. The vectors are used in conjunction with an erythroid-specific, mouse erythroleukaemia (MEL) cell-line (Friend et al., 1971), and the system has been termed the LCR/MEL expression system. The system has recently been published, providing a full description and data for human growth hormone expression under the control of the $\beta$-globin promoter (Needham et al., 1992). The following section reports the establishment of this novel expression strategy for the regulated and functional expression of $K^+$ channel cDNAs and for the first example of activation of a $K^+$ channel gene under the control of its own promoter.

4.2. Analysis of ion-channels in native MEL-cells

The heterologous $K^+$ channels described in this chapter could be studied in isolation since the cell differentiation protocol, essential for LCR gene activation, did not produce any voltage-dependent currents in native MEL-C88 cells (Deisseroth and Hendrick, 1978). The existence of three types of ion channels in these cells, a $\text{Ca}^{2+}$-activated $K^+$ channel, a stretch-activated channel, and a $\text{Cl}^-$
Figure 4.1. Membranes of non-transfected MEL-C88 cells do not express any voltage-activated K⁺ currents following differentiation.

A family of leak-subtracted whole-cell currents was measured in two non-transfected, DMSO-induced MEL cells, four days post-induction. One recording was made with less than 2 nM Ca²⁺ in the pipette and the other with 1 μM Ca²⁺ in the pipette as indicated. The holding potential was -80 mV and the voltage protocol is shown above. Note the absence of any voltage-dependent currents in both cells.
channel, the latter two being labile, has been reported (Arcangeli et al., 1987). With the very low free [Ca²⁺] in our normal pipette solution, any contamination from Ca²⁺-activated K⁺ currents were negligible, with non-leak corrected currents at test potentials up to +60 mV being no larger than 10 to 20 pA. When free [Ca²⁺] was raised to 1 μM, a significant current could be detected before leak subtraction. In the example shown in Figure 4.1, the non-leak corrected current at +40 mV was 81 pA. Following leak subtraction, however, no residual voltage-activated current was unmasked. Thus under the conventional recording conditions employed, endogenous currents did not interfere with measurements on heterologous K⁺ channels.

Functional expression of a cDNA encoding a subunit of the voltage-activated K⁺ channel RCK1

4.3. Approach

To ascertain if functional expression of K⁺ channel gene sequences could be achieved in the LCR/MEL system, an initial experiment was undertaken to establish whether a previously characterised K⁺ channel cDNA sequence, the RCK1 cDNA subunit (Baumann et al., 1988), could be functionally expressed into subunit multimers under the control of the human β-globin LCR. The RCK1 clone was provided as a gift from Dr. O. Pongs (Ruhr-Universität Bochum, Bochum, FRG), and functional expression of this cDNA subunit had already been established in Xenopus oocytes (Stühmer et al., 1989). These features made this cDNA sequence a good candidate for the characterisation of this proposed new approach to K⁺ channel expression.

The 3.74kb RCK1 cDNA subunit was provided as a plasmid in the vector pBluescriptKS+ (Stratagene Inc.). The cDNA had been cloned into the EcoRI site of this vector, with an orientation 5' to the M13 primer site in the polylinker of pBluescriptKS+.

At the time of this experiment, the only approach to the production of a cDNA expression construct for use in the LCR/MEL system was a two-step cloning procedure. This procedure entailed cloning of the cDNA of interest downstream of the human β-globin promoter and upstream of the exonII-exonIII-polyadenylation sequences from the human β-globin gene in the expression vector pEC3 (see Figure 4.2). The exonII-exonIII-polyadenylation sequences
from the human β-globin gene were shown to provide mRNA processing and maturation signals and to give stability to the final mRNA in induced MEL-cells prior to translation (Collis et al., 1990; Needham et al., 1992). This promoter-cDNA-exonII-exonIII-polyadenylation sequence cassette was then transplaced downstream of the β-globin microlocus LCR in the vector pGSE1417 shown in Figure 4.3, and this final construct linearized prior to transfection into MEL-cells.

4.4. Construction of the RCK1 expression unit

A large scale preparation of RCK1 cDNA was undertaken by Quiagen plasmid purification after transformation of 10ng of the RCK1/pSK+ plasmid into SURE E. coli competent cells, and growth of a single ampicillin resistant isolate in 500ml liquid culture. The RCK1 cDNA was then removed from the pKS+ vector by digesting 50μg of the Quiagen purified RCK1/pSK+ plasmid with EcoRI, followed by purification of the 3.74kb cDNA band from the 2.9kb pSK+ vector band by agarose gel electrophoresis, and electroelution.

200ng of the EcoRI fragment was then ligated with 50ng of pEC3 previously cut to completion with EcoRI. To obtain a clone with the RCK1 cDNA in the correct orientation, a diagnostic ClaI cut was performed on 6 isolates identified as containing an RCK1 cDNA insert by colony hybridisation using a 32p labelled RCK1 cDNA insert. From sequence analysis of the published RCK1 cDNA sequence, ClaI was shown to cut once at a position 869bp from the 5' end. Taking into consideration the ClaI sites in pEC3, it was predicted that in the correct orientation, with the cDNA 3' to the β-globin promoter, band sizes of 5.77, 2 and 1.47kb should be expected. In the wrong orientation band sizes of 3.77, 3.47 and 2kb were expected. One of the six isolated clones, isolate 5, gave the correct diagnostic set of bands.

The second step involved removal of the promoter - RCK1 cDNA - exonII-exonIII - polyadenylation sequence cassette (RCK1 expression cassette) from pMEG3. Sequence analysis on RCK1 and observation of the available cloning sites in pGSE1417 indicated that this 7.24kb cassette could be removed intact by an Asp718 digestion. The RCK1 expression cassette was then transplaced directly into the Asp718 site of pGSE1417 by conventional ligation. The 7.24kb RCK1 expression cassette was removed by digestion of a 50μg Quiagen-purified quantity of the correct orientation RCK1-pEC3 construct (isolate 5) with
Figure 4.2. Plasmid diagram of the cDNA 'expression cassette' vector pEC3.

The pEC3 vector backbone is based on pBluescript (Stratagene Inc.) with a new polylinker inserted between the ClaI and SstI sites. The polylinker comprises the sites ClaI, HindIII, EcoRI, XhoI, BglII, SalI, and NotI. The human β-globin promoter was cloned between the 5' ClaI and HindIII sites of this new polylinker. The 3' region of the human β-globin gene (base pairs 2037-4845) which encodes part of the β-globin 2nd exon, the 2nd intron, 3rd exon, poly-adenylation recognition site and approximately 2kb of 3' β-globin flanking DNA was cloned between the BamHI and PstI sites in the polylinker (Needham et al., 1992). cDNAs were cloned into the unique sites between the promoter and 3' β-globin gene sequences.
Figure 4.3. Plasmid diagram of the human β-globin LCR containing expression vector pGSE1417

pGSE1417 contains a 6.5kb (microlocus) NotI fragment containing the four 5′ hypersensitive sites from the human β-globin gene. This fragment was cloned into a specially designed polylinker in a pUC18 vector containing the 2.0kb partial NarI-tk-neo² gene fragment conferring G418-resistance upon transformation into MEL-cells (Talbot et al., 1989). cDNA expression cassettes and genomic fragments were cloned into one or more of the unique sites - ClaI, Asp718 or SstII.
Asp718, and subsequent separation of this 7.24kb cassette from the 4.9kb remaining pEC3 vector sequences by agarose gel electrophoresis and electroelution.

This cassette was then ligated into pGSE1417 prepared by digestion with Asp718. Colony hybridisation was used to identify recombinants using an aliquot of 32P labelled RCK1 cDNA as the probe. Twelve recombinants were identified by their binding to the RCK1 cDNA probe, and ClaI digestion was used to ascertain the orientation the RCK1 expression cassette with respect to the LCR sequences. Band sizes of 16.47 and 1.27kb were predicted for the cassette 5' to the LCR sequences, and band sizes of 10.5, 5.97 and 1.27kb for the cassette 3' to the LCR sequences. From 12 clones, 4 were shown to have the RCK1 expression cassette 5' to the LCR, and 8 3' to the LCR sequences. One clone with the RCK1 expression cassette 3' to the LCR was amplified by Quiagen plasmid purification and taken forward for expression analysis. This expression construct termed pEC3: cRCK1 is shown in Figure 4.4a.

4.5: Expression of RCK1

50μg of the RCK1 expression construct pEC3:cRCK1 was prepared for expression studies by digestion with PvuI to linearize the DNA before electroporation into MEL-cells. 1x10^7 MEL-cells, in log phase culture, were electroporated with the 50μg aliquot of linearized pEC3:cRCK1 and stable, G418-resistant colonies established, isolated and induced with 2% DMSO prior to electrophysiology. Following conventional selection and induction, individual G418-resistant cells from pooled populations were subjected to electrophysiological analysis (Figure 4.5a). The characteristics of the RCK1 currents were similar to those expressed in Xenopus oocytes (Stühmer et al., 1989), with half-maximal activation occurring at -23.4 ± 1.1 mV (n=3).

Expression of two voltage-activated A-type K+ channel subunits

4.6: Approach

The next step was to establish whether the MEL/LCR system could be used for the expression of A-type (fast-inactivating) voltage-activated K+ channel subunits as well as the previously characterised delayed rectifier type, RCK1. Two suitable cDNA candidates were made available. One was the previously
Figure 4.4. Expression constructs for (a) non-inactivating (RCK1) and (b) inactivating (hPCN2) voltage-sensitive K⁺ channel cDNAs.

The K⁺ channel subregion nomenclature is described in the legend to Figure 4.9. The MEL cell expression cassette pEC3 (see Figure 4.2) contains a multiple cloning segment (MCS) for insertion of cDNAs or intact genomic fragments lacking a functional promoter. In order to confer integration-position-independent, copy-number-dependent expression levels, construct (a), pEC3: c-RCK1 was cleaved with Asp718 and construct (b), pEC3: c-hPCN2 with ClaI and Asp718 and the resulting fragments inserted into the Asp718 site (= KpnI site; Asp718 and KpnI are isoschizomeric) and directionally into the ClaI and Asp718 sites of pGSE1417 respectively, downstream of the four hypersensitive sites. The expression cassettes were cloned into pGSE1417 with the promoter 5' to the
Figure 4.5. Examples of whole-cell RCK1, hPCN2 and RatShaII currents stably expressed in MEL cells following transfection of LCR-cDNA constructs driven by the β-globin promoter.

For RCK1 the holding potential was -80 mV and the voltage protocol was the same as that described in Figure 4.9A. For hPCN2 and RatShaII the holding potential was -100 mV and a series of 150 ms pulses from -30 to +40 mV were applied, with an interpulse interval of 20 s.
characterised Rat Shal cDNA, isolated from a rat hippocampus cDNA library, and kindly supplied by Dr. Timothy Baldwin (University of California, San Francisco, U.S.A.). Digestion with HindIII would yield a 2.7kb RatShal cDNA insert and the remainder Rcf/CMV vector sequences, as described (Baldwin et al., 1991). The second clone obtained, hPCN2, was isolated from a human fetal skeletal muscle cDNA library and was a gift from Dr. L. Phillipson (University of Chicago, Chicago, U.S.A.). This clone is an RCK4 homologue and was provided as a 2300bp cDNA insert in the vector pSP64T(Melton). The hPCN2 cDNA has been fully sequenced (Phillipson et al., 1990), and functional expression of this clone has been reported (Lee et al., 1991).

Construction of the hPCN2 and Rat Shal -pEV3 expression constructs

4.7. hPCN2

The hPCN2 plasmid sample provided was transformed into SURE E.coli competent cells and a single transformant grown in 500ml liquid culture and plasmid DNA prepared by Quiagen purification. The hPCN2 cDNA was removed by digestion of a 50μg sample of this DNA with BglII, separation on a 0.8% agarose gel and electroelution of the 2.3kb hPCN2 cDNA band. 400ng of this band was ligated to 50ng of the pEC3 expression vector DNA previously cut to completion with BglII. SURE E.coli competent cells were transformed with the products of the ligation and 100 ampicillin-resistant colonies were picked and subjected to colony hybridisation using the full 2.3kb hPCN2 cDNA as a 32P labelled probe. Figure 4.6 shows 7 hPCN2 positives on a plate with a total of 50 isolates. A mini preparation of plasmid DNA was produced from 10 hPCN2 positive clones, as shown from colony hybridisation. A sample of these isolated plasmids were then digested to completion with BglII to look for recombinants. All 10 clones were recombinant producing a 2.3kb cDNA band along with a 5.5kb pEC3 band.

To find a recombinant with the hPCN2 cDNA in the correct orientation, a diagnostic EcoRI and XbaI cut was undertaken on the four recombinants. An XbaI site 400bp from the 3' end of hPCN2 and a unique EcoRI site in the polylinker cloning site of pEC3 upstream of the BglII cloning position was predicted to produce a diagnostic 400bp band if the clone was in the wrong orientation. Two clones produced this 400bp band after double digestion with the two enzymes, and two did not. One of the clones that did not, clone 21, was
4. Regulated and functional expression of $\beta$-chain cDNAs and genes under control of the human $\beta$-globin LCR

grown in large-scale culture and hPCN2-pEC3 plasmid DNA prepared by Quiagen purification.

To place the hPCN2 expression cassette (promoter-cDNA-exonII-exonIII-polyadenylation sequences) from hPCN2-pEC3 downstream of the LCR sequences in the vector pGSE1417, 50$\mu$g of hPCN2-pEC3 DNA was cut to completion with $\text{Cleal}$ and $\text{Asp718}$ and the hPCN2 expression cassette separated from the remainder pEC3 vector sequences by agarose gel electrophoresis and electrolution. 100ng of this DNA was then ligated to 100ng of pGSE1417 cut to completion with $\text{Cleal}$ and $\text{Asp718}$. The products of the ligation were transformed into E.coli SURE competent cells, and 10 out of 12 isolated ampicillin-resistant colonies were shown to contain the required recombinant hPCN2 expression cassette-pGSE1417 plasmid, shown after a diagnostic $\text{Cleal}$ and $\text{Asp718}$ digestion. This final hPCN2 expression construct is shown in Figure 4.4b. The SURE E.coli clone harbouring the construct was grown in large scale liquid culture, and hPCN2 expression cassette-pGSE1417 plasmid DNA isolated by Quiagen purification.

4.8. Rat Shal-pEV3

A Quiagen preparation of the Rc/CMV-RShal plasmid was undertaken and a 50$\mu$g aliquot digested with $\text{HindII}$. The 2.7kb RShal cDNA insert was then separated from the remainder 3.6kb Rc/CMV vector sequences by separation on a 0.8% agarose gel and electrolution.

At this time, a new single-step cDNA expression vector was made available by Dr. M.Needham (ICI pharmaceuticals, Alderley Edge, U.K.), in the form of the 13.7kb pEV3 vector, shown in Figure 4.7. A 5$\mu$g aliquot of pEV3 was digested to completion with $\text{EcoRI}$ and $\text{SaiI}$, two single-site enzymes in the polylinker of pEV3, and the vector band isolated from the small polylinker fragment by agarose gel electrophoresis, and subsequent electrolution.

A 100ng amount of the $\text{HindII}$-cut Rat Shal cDNA was mixed with the pEV3 vector, cut with $\text{EcoRI}$ and $\text{SaiI}$, and the S'-overhangs of these fragments filled in using Klenow polymerase. These blunt-ended fragments were then ligated together, and clones containing the Rat Shal insert identified by colony hybridisation using a $^{32}$P labelled $\text{HindII}$ Rat Shal cDNA probe. Twelve positive clones were isolated.
Figure 4.6. Colony hybridisation to identify hPCN2-pEC3 recombinants

Seven putative hPCN2-pEC3 recombinants as determined by colony hybridisation using the hPCN2 cDNA sequence as a $^{32}$P labelled probe.
Recombinants with the Rat Shal cDNA in the correct orientation to the β-globin promoter were identified by a diagnostic BamHI digest. BamHI cuts once, 1kb from the 3' end of the Rat Shal cDNA. Therefore in the correct orientation band sizes of 6, 3.6, 3, 2.3 and 1kb were expected and in the wrong orientation sizes of 4.2, 3.6, 3 and 2.3kb expected. One clone showing the predicted fragments for the correct orientation was isolated and scaled up for expression analysis. This final Rat Shal-pEV3 construct is shown in Figure 4.8.

50μg samples of both Rat Shal-pEV3 and hPCN2-pEV3 were digested to completion with PvuI before electroporation into MEL-cells, and selection of stable G418-resistant derivatives.

4.9. Expression of hPCN2 and Rat Shal

Both cDNA clones produced cells showing transient type K+ channel currents as predicted. All recordings were taken between 1 and 4 days post induction.

The hPCN2 currents were transient, having an inactivation time-course of the order 20 ms at room temperature. Half-maximal activation of the example shown in Figure 4.5b occurred at -9 mV (as obtained by calculating the chord conductance from the peak current). The cDNA encoding the inactivating mammalian K+ channel, Rat Shal, produced transient type K+ channel currents similar to those described (Baldwin et al., 1991). An example of whole cell currents is shown in Figure 4.5c.

Functional expression of the intronless mouse inwardly rectifying K+ channel IRK1

4.10. Approach

In March 1993 the first example of the cloning of an inwardly rectifying K+ channel was reported in Nature (Kubo et al., 1993). The channel structure is reviewed in Chapter 1 of this thesis. It therefore seemed a logical approach to attempt to isolate the IRK1 coding region consisting of 1284bp of DNA and attempt to express the clone in the LCR/MEL system. PCR was firstly employed to establish the intronless identity of the IRK1 coding region and
Figure 4.7. Plasmid diagram of the human β-globin LCR-single step cloning expression vector pEV3

pEV3 was constructed by transferring a ClaI-Asp718 cassette from pEC3 (Figure 4.2) into pGSE1417 (Figure 4.3) prepared by digestion to completion with ClaI and Asp718. All restriction enzyme sites indicated are unique, and cDNAs were cloned into one of the 4 unique sites just downstream of the β-globin promoter.
subsequently to isolate the IRK1 coding region from mouse genomic DNA. This IRK1 coding region was then ligated into pBluescript SK+, and then removed intact from the pSK+ multiple cloning site prior to incorporation into the LCR expression vector pEV3.

4.11 Establishment of an intronless IRK1 coding-region and construction of IRK1-pEV3

Two 18bp oligonucleotides (oligos. 25 and 26) were used to PCR amplify the IRK1 coding region. Oligonucleotide 25 was designed to encompass the methionine ATG and endogenous Kozak sequence of IRK1. Oligonucleotide 26 was designed to encompass the final TGA stop codon and 12bp of DNA downstream in the IRK1 3' untranslated region. A touchdown PCR program was used to amplify the predicted 1304bp IRK1 coding region using 100ng of mouse genomic DNA as template. Upon separation on a 2% agarose gel, a 1304bp band was observed indicating that IRK1 is intronless in the coding-region. An identical procedure was performed using 100ng of mouse genomic DNA as template to produce a 1034bp band for isolation.

The resultant 1304bp amplified band was removed from a 2% agarose gel, isolated by electrodialysis and 100ng of the resultant band, after undergoing a fill-in reaction with Klenow DNA polymerase was ligated into 50ng of pBluescript SK+ previously cut to completion with EcoRV. After transformation of E.coli SURE competent cells, blue/white selection was employed to isolate 12 white pSK+-IRK1 recombinants. A diagnostic KpnI digest was then used to identify the relative orientation of the IRK1 coding region in pSK+. With a KpnI site close to the 5' end of the IRK1 coding region, either a 2.9 and 1.3kb band set was predicted for an IRK1 orientation 3' to the pSK+ KpnI site, and a 4.2kb band with the coding region 5' to the pSK+ KpnI site.

One clone with an IRK1 orientation 3' to the pSK+ KpnI site was isolated and the coding region of IRK1 removed on an EcoRI-XhoI fragment. 100ng of this 1304bp gel purified fragment was then ligated into 50ng of pEV3, previously cut to completion with EcoRI and SalI. After transformation of E.coli SURE competent cells, 12 ampicillin-resistant clones were propogated and a miniprep of DNA isolated from each. 10 of the 12 clones were shown to contain the 1304bp IRK1 coding region after a diagnostic EcoRI and NotI digestion releasing the 1304bp IRK1 band and the 13.7kb pEV3 vector band.
Figure 4.8. Expression construct for the inactivating RatShall cDNA.

Schematic diagram of the RatShall cDNA-pEV3 expression construct prior to electroporation into MEL-cells. The RatShall cDNA coding region is shown cloned in-frame downstream of the β-globin promoter in pEV3.
Figure 4.9. Examples of whole-cell IRK1 currents stably expressed in MEL cells following transfection of LCR-cDNA constructs driven by the β-globin promoter

IRK1 currents recorded from a MEL cell with 140mM K⁺ and 10mM Mg²⁺ in the pipette and 70mM K⁺ and 70mM Na⁺ outside. The holding potential was -30mV and 100ms pulses were applied to potentials between +50 and -190mV as indicated.
One isolate (No.4) was propagated and a large scale of pEV3-IRK1 plasmid DNA isolated by Quiagen purification.

4.12. Expression of IRK1

A 50µg sample of pEV3-IRK1 plasmid DNA was digested to completion with PvuI before electroporation into MEL-cells, and selection of stable G418-resistant derivatives.

Figure 4.9 shows an example of IRK1 currents recorded from a MEL cell with 140mM K⁺ and 10mM Mg²⁺ in the pipette and 70mM K⁺ and 70mM Na⁺ outside. The holding potential was -50mV and 100ms pulses were applied to potentials between +50 and -190mV as indicated.

Activation of hCDR1S.2 under native promoter control

4.13. Approach

The established expression of the RCK1 cDNA into functional K⁺ channels in MEL cells, under the control of the β-globin promoter, gave reason to investigate the possibility of activation of K⁺ channel genes with their native promoter sequences. A number of reports justifying such an investigation have been published and these are represented fully in the discussion. From Section 3.7, hCDR1S.2 was predicted to contain a full transcription unit for the voltage-activated K⁺ channel equivalent hPCN1 with the promoter elements partially characterised. This 5.2kb fragment was cloned into the microlocus LCR expression vector pGSE1417 shown in Figure 4.3, and taken forward for expression analysis in MEL cells to investigate the possibility of the activation of hCDR1 from native promoter elements by the human β-globin LCR.

4.14. Construction of the hCDR1S.2-pGSE1417 expression construct

200ng of hCDR1S.2 and 50ng of pGSE1417, previously cut to completion with Asp718, were mixed and the 5’ overhang ends of the fragments filled in using Klenow polymerase. The filled-in fragments were then ligated together. SURE E.coli competent cells were transformed with the products of the ligation, and 100 colonies isolated on L-ampicillin plates subjected to colony hybridisation
using $^{32}$P labelled hCDR15.2 as the probe. Twelve clones were observed to contain the hCDR15.2 insert. Five of these clones were shown to contain the hCDR15.2 promoter region in an orientation 5’ to the LCR sequences by a diagnostic BglII/Ndel digestion. This produced a diagnostic 5.3kb band due to the presence of an Ndel site 1kb from the 5’ end of hCDR15.2 shown from the mapping experiment in section 3.4, and the presence of a BglII site in the LCR-microlocus 4.3kb from the Asp718 cloning site. One of these pGSE1417/hCDR15.2 clones was grown in large scale liquid culture and plasmid DNA prepared by Quiagen purification. This construct is shown in Figure 4.10.

4.15. Expression of hCDR1 from hCDR15.2

The K⁺ currents of MEL cells containing the activated hCDR1 gene were characterised using both whole-cell and isolated patch recordings obtained between 1 and 3 days post-induction. Activation of hCDR1 occurred around -20 to -25 mV, which is in agreement with data published for hPCN1 currents expressed in Xenopus oocytes (Philipson et al., 1991). Substantial whole-cell currents could be measured 24 hours after induction of the cells. In two such cells, peak currents elicited by commands to +30 mV were 388 and 339 pA respectively, and in another cell a current of 109 pA was measured 7 hours following induction. Examples of whole-cell currents elicited by steps to between -30 and +40 mV from a holding potential of -80 mV, together with the current-voltage relationship, are shown in Figure 4.11 A and B. Mean peak current measured at +30 mV in 5 cells after 3 days induction was 441 ± 45 pA. Activation characteristics of the hPCN1 current were investigated using tail current analysis. The outward tail current at the end of each test pulse was obtained by repolarising the membrane to -40 mV for 100 ms before returning to the holding potential. Normalised conductance values were obtained from extrapolated exponential fits to the tail currents, calculated as $G/G_{max} = I_{tail}/I_{tail\ max}$ and plotted against test pulse potential (Figure 4.11C). The data were fitted to the Boltzmann relation: $G/G_{max} = 1/(1+\exp((V-V_1/2)/k))$ where $V_1/2$ is the voltage where the open probability of the channels is half the maximal open probability and $k$ is the steepness of the voltage-dependence. Mean values (± s.e.m.) of 6 cells were -3.94 ± 2.18 mV for $V_1/2$ and -8.45 ± 0.66 for $k$. Thus hCDR1 currents were fully activated at +20 to +30 mV.
The plasmid construct pGSE1417 (Talbot et al., 1989) carrying the four hypersensitive sites of the globin LCR (HS1-HS4) is shown juxtaposed to a 5.2 Kb fragment encompassing the hCDR1 K+ channel gene. Note the absence of any promoter element other than that provided by the genomic fragment. The K+ channel gene is subdivided into functional subregions according to the following prefix nomenclature: *utl-* upstream untranslated region 5' to the start codon; *int-* encoding an intracellular N-terminus; *mS1-* (mS2-, mS3-, mS4- ... etc.) encoding the membrane domain S1; *e12-* encoding an extracellular loop between membrane domains 1 and 2; *i23-* encoding an intracellular loop between membrane domains 2 and 3; *ict-* encoding an intracellular C-terminus; *dut-* downstream untranslated region, 3' to the stop codon.
Figure 4.11. Electrophysiological analyses following specific activation of the human K+ channel gene hCDR1.

A, examples of whole-cell hCDR1 currents induced by steps ranging from -30 to +40 mV. Each step is followed by a repolarisation to -40 mV to obtain outward tail currents; the holding potential was -80 mV and the interpulse interval was 2.5s. B, current-voltage relationship of this cell. C, activation curve obtained as described in the text. The line shows the fit of the data to the Boltzmann equation (see text) giving a $V_{1/2}$ of -4.48 mV and a $k$ value of -7.89. D, single hCDR1 K+ channels recorded from an inside-out patch at a test potential of +30 mV; the dashed line is the closed level. Filter cut-off (-3dB) 800 Hz. E, ensemble average of 328 records from the same patch (noisy trace); the smooth trace is a scaled whole-cell current from another cell at the same potential. F, all point amplitude histogram of selected records, the data have a bin width of 10 points for display purposes. The parameters in the Gaussian fit to the two peaks were 0.0 ± 0.129 and 0.456 ± 0.150 pA.
Outside-out patches of MEL cells expressing hCDR1 usually contained many active hCDR1 channels, and since their amplitude is small it was difficult to resolve individual events. We therefore used inside-out patches because this configuration usually yields fewer active channels in addition to having less background noise. Examples of single hCDR1 channels recorded from an inside-out patch at a test potential of +30 mV are shown in Figure 4.11D. The flickery nature of the openings suggests the presence of a very short-lived closed state. The time course of the ensemble average of 328 such traces coincides closely with the scaled whole-cell current (at the same test potential) of another cell expressing hCDR1 currents (Figure 4.11E). This indicates that the observed unitary events are those of hCDR1 channels. An amplitude histogram comprised of selected portions of the records is shown in Figure 4.11F. The Gaussian fit of the data gives a single channel amplitude of 0.456 ± 0.150 pA at +30 mV. Dividing the plateaux current of the ensemble average (0.210 pA) by the single channel amplitude gave a value for open state probability ($P_{open}$) of 0.46. Total current ($I$) is given by: $I = N \cdot i \cdot P_{open}$ where $N$ is total number of channels, and $i$ is single channel amplitude. Substituting values into this equation suggests that the number of hPCN1 channels expressed following activation of its own promoter ranged from about 1400 to 2700 per cell (3 days post-induction). Interestingly, clone populations which carried the hCDR15.2 genomic fragment inserted into the LCR expression vector pNANCOS8 (Figure 5.2) in the opposite orientation (i.e. 3' to the LCR sequences) expressed the hCDR1-characteristic current at comparable levels (see Section 5.6). This establishes that the promoter being utilised lies within the cloned BglII fragment and illustrates the ability of the globin LCR to activate promoters independently of their relative orientation.

Parallel experiments where LCR-TK-neo and hCDR15.2-TK-neo constructs were transfected into MEL-cells showed no detectable hCDR1 expression.

**Attempted expression of IKβ and K13: two novel K+ channel subfamily members**

4.16. Approach

Based upon similarities throughout the core region, voltage activated K+ channels belong to one of four subfamilies originally defined as Shaker, Shal, Shab and Shao. The only exception is a very slowly activating K+ channel (Isk),
which is completely unrelated (Takumi et al., 1988). The isolation of two putative K+ channel cDNAs, IK8 and K13, defining two new subfamilies based upon similarities of the amino-acid sequence to existing K+ channel sequences has been reported (Drewe et al., 1992). So far, attempts to express cRNA from these two cDNAs in Xenopus oocytes into functional channels has been unsuccessful. These two cDNA clones were obtained from Dr Roger Zuhlke (Dept. Cell Biology, University of Texas, U.S.A.) as cDNAs in the vector pBluescript SK-.

The 2761bp cDNA-K13 was derived from a directional library and cloned into the EcoRI and NotI sites of pBluescript SK-. The 2461bp cDNA-IK8 was cloned non directionally into the EcoRI site of pBluescript SK-. Both clones were provided in sense orientation.

At this time, a smaller version of the 6.5kb microlocus LCR region was made available in the form of a 950bp nanolocus LCR element. This consisted of the core elements around the DNaseI-hypersensitive sites 2, 3, and 4 of the human β-globin LCR (a kind gift from Dr. M. Antoniou, NIMR, Mill Hill, London.) and shown in Figure 5.1. This element was first shown to have LCR activity after incorporation of this 950bp nanolocus element in the vector pNANCOSS (see Figure 5.2), and subsequent activation of hCDR1.2 in either orientation with respect to this LCR element. This element had also been incorporated into the cDNA expression vector pNV1 (Figure 4.12), and was shown to have approximately 30% activity when expression levels from the human growth hormone cDNA were compared to expression from the human β-globin microlocus LCR (Dr. M. Needham personal communication). pNV1 is the vector which has been used in the following section.

Construction of the K13-pNV1 and IK8-pNV1 expression units

4.17. K13-pNV1

K13/pSK- was transformed into SURE E.coli competent cells and a 500ml culture Quiagen plasmid purification undertaken from a single transformant. A 50µg sample of this DNA was then cut to completion with EcoRI and NotI, and this 2.76kb band isolated from the 2.9kb pSK- vector sequences by agarose gel electrophoresis and electroelution of the isolated 2.76kb cDNA band. 100ng of this K13 cDNA band was then ligated into 50ng pNV1 expression vector previously cut to completion with EcoRI and NotI. This directional cloning event ensured all recombinants to be in the sense orientation with respect to the β-
Figure 4.12. Plasmid diagram of the single step cDNA nanolocus LCR expression vector pNV1.

pNV1 was created by replacing the 6.5kb microlocus LCR cassette from pEV3 (Figure 4.7) with the 950bp nanolocus cassette (Figure 5.1). All restriction enzyme sites indicated are unique, and cDNAs were cloned into one of the 6 unique sites just downstream of the β-globin promoter.
4. Regulated and functional expression of \( \text{K}^+ \) channel cDNAs and genes under control of the human \( \beta \)-globin LCR globin promoter. The products of the ligation were transformed into SURE E.coli competent cells and transformants plated out on L-ampicillin. 10 out of 12 transformants were shown to contain the cDNA after a diagnostic EcoRI and NotI cleavage.

4.18. IK8-pNV1

IK8-pNV1 was prepared in an identical manner to K13-pNV1 except in this case HindIII was used as the unique 5' bracketing enzyme, with NotI again being used as the 3' bracketing enzyme. After the transformants were plated out on L-ampicillin, 11 out of 12 transformants were shown to contain the cDNA after a diagnostic HindIII and NotI cleavage.

4.19. Expression analysis on K13-pNV1 and IK8-pNV1

50\( \mu \)g aliquots of the expression units K13-pNV1 and IK8-pNV1 were linearized with PvuI and resuspended in 50\( \mu \)l TE-buffer. MEL cells were electroporated with the constructs and G418 resistant clones established according to materials and methods.

Twelve clones from each experiment were taken forward for electrophysiological analysis 3 days post induction, along with a clone of hCDR15.2-pGSE1417 already established as being able to express voltage-activated currents. From 6 clones of K13-pNV1 and 6 clones of IK8-pNV1, no voltage-activated currents were observed after whole-cell patch clamp analysis. Control currents were observed from the hCDR15.2-pGSE1417 clone.

Discussion

4.20. The first application of LCR/MEL gene-activation to the functional expression of cDNAs and genes encoding mammalian \( \text{K}^+ \) channels

This chapter reports the first application of LCR/MEL gene-activation to expression of genes encoding mammalian \( \text{K}^+ \) channels. The system satisfies important experimental criteria for such applications, including a low endogenous current background, control of \( \text{K}^+ \) channels density, high cellular resilience under patch-clamp, simple culture/storage and high reliability. All clones which contain one or more un-rearranged independent transcriptional
Regulated and functional expression of $\text{K}^+$ channel cDNAs and genes under control of the human $\beta$-globin LCR

units (LCR + activatable promoter + full coding sequence) following transfection are stable expressors. In all the cases reported here, post-translational $\text{K}^+$ channel assembly into homo-tetramers takes place efficiently within this mammalian cell background. Moreover, the inactivation characteristics of the hPCN2 and Rat Shal channels observed following cRNA injection in *Xenopus* oocytes (Baldwin et al., 1991; Lee et al., 1991) are maintained. A further desirable feature of the LCR/MEL expression system is its simplicity. For the $\text{K}^+$ channels described here, neither the genomic nor cDNA $\text{K}^+$ channel constructs required extensive 'trimming' of the regions up- or downstream to the coding sequence to elicit expression. Expression does not depend upon gene-amplification or the use of high copy-numbers. Characteristically, $\text{K}^+$ channel expression derives from a low average number (<10) of LCR-gene copies.

4.21. MEL-cells

The MEL-C88 cells used in these studies have favorable characteristics for heterologous expression of ion channels. The endogenous electrophysiology of MEL cells has been investigated (Arcangeli et al., 1987) and the main current was found to be $\text{K}_{\text{Ca}}$. This current was absent in whole-cell recordings where free $[\text{Ca}^{2+}]$ in the pipette was strongly buffered by EGTA to about 2 nM. Under these conditions, control MEL cells can be considered silent and interference with measurements on heterologous channels were negligible. MEL cells are robust, and are cultured as semi-adherent 'floaters'. They can be patch-clamped without enzymatic treatment and achievement of high-resistance seals (>2 GΩ) was relatively straightforward. MEL cells have a rapid growth rate (doubling time ~ 10-16 hrs). Since MEL cells are erythrocytes in development, they are also likely to express outer membrane proteins such as the $\text{Na}^+$/Cl$^-$ symport, the $\text{Ca}^{2+}$/ $\text{Na}^+$/ $\text{K}^+$ ATPases and the exchangers for $\text{Cl}^-$ /$\text{HCO}_3^-$ ions (band 3) and $\text{Na}^+$/ $\text{H}^+$ ions.

For faster re-growth following selection, most of the experiments reported here have simply pooled recombinant (G418-resistant) colonies prior to electrophysiology. In the case of the hPCN2 and Rat Shal $\text{K}^+$ channel, however, multiple lines of stable transfectants were separately propagated. Each cell-line exhibits a characteristic level of expression which we routinely estimate by whole-cell current measurements and/or quantitative RNA hybridization. In the examples reported, MEL cells typically express at a level.
of $10^3$ - $10^4$ functional channels per cell at 4 days post-induction. In some cases, currents were observed 7 hours post-induction.

4.22. Characteristics of the MEL/LCR system

The establishment of stable, mammalian cell lines expressing heterologous $K^+$ channel genes is clearly of value, but can be labour-intensive with conventional cloning systems. The experimental difficulties arise largely from the dominant effect of the genomic context at the random sites of integration into the host cell genome. Thus many selected clones fail to express the gene of interest, while the rates of expression of others tend to be low and unpredictable.

LCR-based heterologous expression, however, is characterised by its independence of genomic context, guaranteeing expression levels which are strictly dependent on the copy-number of the transgene. For electrophysiological applications, it was found the separate propagation of clonal cell lines had two significant advantages. Firstly, it enabled cells with a range of 'quantal' ion channel expression levels to be isolated, based on the gene-dosage inherent to each propagated cell line. Secondly, incorporation of quantitative RNA assays prior to patch-clamp analysis can obviate the need for primary screening by detection of current. The time-course of $K^+$ channel mRNA expression following induction conforms to the pattern established for other heterologous genes (Needham et al., 1992). Within pooled populations of cells, RCK1-specific mRNA was shown to be present at approximately 0.4% total RNA at four-days post-induction. These features would permit experiments to determine the relationship between the gene copy number, specific mRNA levels and the number of expressed protein molecules following heterologous gene-activation. This study takes advantage of the fact that the stoichiometry of functional $K^+$ channel protein can be directly deduced for the entire cell by reference to unitary channel and whole-cell conductance measurements.

4.23. Future prospects for $K^+$ channel expression in the MEL/LCR expression system.

In addition to conferring position-independent gene-expression, the LCR possesses other attributes that can be exploited in the study of $K^+$ channels. It has been established that the LCR activates transcription through general,
proximal promoter elements (Antoniou and Grosveld, 1990). Thus it is not surprising to find that the LCR can activate heterologous promoters with diverse structures see (Blom van Assendelft et al., 1989; Talbot et al., 1990; Needham et al., 1992), including that of the hCDR1 gene described here. This property of the LCR should allow the direct characterization of K⁺ channel protein genes isolated from genomic libraries. A cosmid vector containing β-globin LCR elements and a selectable marker for mammalian cells has been constructed with this in mind and is reported in Chapter 5. There may also be circumstances when high-level gene-expression is undesirable: for example, in obtaining optimal membrane-protein densities for single-channel recording or in controlling the relative expression levels of K⁺ channel and effector molecules to minimize promiscuous cross-talk. In such cases lower levels of transcription can be obtained by introducing mutations into the promoter (Antoniou and Grosveld, 1990) or LCR elements (Collis et al., 1990; Fraser et al., 1990) or by simply modifying the cell culture and induction regimes (M. Needham, unpublished).

It has also been shown that the LCR can activate more than one gene linked in cis on the same DNA construct (Hanscombe et al., 1989; Talbot et al., 1990). This offers the scope for simultaneous activation of genes encoding channels and possible channel-modulatory molecules in the same cell and will be an important, direct route for stable reconstitution of cell-lines capable of producing specified proteins involved in complex electrophysiological phenotypes. In practice, the desired effect can be achieved by co-transfection of MEL cells with two independent constructs or by introducing more than one gene onto a single DNA construct.

The facility of direct gene-activation by the globin LCR offers powerful and timely solutions to several general problems associated with the stable expression or reconstitution of electrophysiological proteins from higher eukaryotes. Locus-activation could be a preferred route for expression of K⁺ channel coding sequences interrupted by introns and / or genes encoding large ion channel proteins. The LCR system is highly versatile in creating stable cell lines from native (or modified) cDNA or genomic K⁺ channel gene constructs, and should be seen as complementary to transient expression systems. In the uninduced state, cells carrying recombinant K⁺ channel genes will express them at low or undetectable levels. The induction system can be used in showing the electrophysiological consequences following de novo expression of heterologous
4. Regulated and functional expression of $K^+$ channel cDNAs and genes under control of the human $\beta$-globin LCR

genes and in studying the kinetics of ion channel assembly in vivo. These features, together with the wealth of information on gene-expression mechanisms within these cells, suggest that LCR/MEL gene activation methods will find many powerful applications within molecular physiology.

4.24. Expression analysis on IK8-pNV1 and K13-pNV1

From electrophysiological analysis on MEL cells transformed with the IK8-pNV1 and K13-pNV1 constructs no detectable currents were observed under conditions for voltage activated $K^+$ channels and a number of conclusions can be drawn.

It has been established that voltage activated $K^+$ channels form functional homo- and heterotetramers in eukaryotic membranes. The formation of tetramers was established by studying the interaction of a scorpion toxin with co-expressed wild-type and toxin insensitive mutant Shaker $K^+$ channels in Xenopus oocytes (MacKinnon, 1991). Evidence for the formation of homotetramers is due to the fact that to date, all published voltage-activated $K^+$ channels have been expressed into functional $K^+$ channels from a single cDNA subunit species. Other reports showing intermediate electrophysiological characteristics from the expression of mixed cRNA species support the evidence for the formation of heterotetramers. (Christie et al., 1990; Isacoff et al., 1990; McCormack, 1990; Ruppersberg et al., 1990). IK8 and K13 may be unable to form homotetramers as in the case of existing voltage activated $K^+$ channels, and may need to coassemble with existing $K^+$ channel subunits, or hereto undefined subunits to form functional channels.

Protein products of the approximate molecular weights from the cDNA sequences of these two $K^+$ channels was reported from a reticulocyte lysate cell-free expression system (Drewe et al., 1992), indicating that the IK8 and K13 cDNAs can be appropriately translated. Suggestions that the oocyte may be unable to process the primary translation products from IK8 and K13 in a correct manner so as to allow transportation and incorporation into the cell membrane now seem more unlikely due to non-functional expression in mammalian MEL cells. A further possibility may be that these clones need some other non channel protein subunit to functionally express. Furthermore, the possibility that these channels may not be voltage-activated and the binding of
some other, unknown ligand may be essential for expression, has to be considered.
CHAPTER 5

Construction of LCR-based genomic gene-activation libraries for expression-cloning of existing and novel types of K+ Channels
5.1. Introduction

K⁺ channels have been established as being encoded by the most diverse group of ion-channel genes. The following chapter investigates an approach to establish an LCR-based genomic gene-activation library for expression-cloning of existing and novel types of K⁺ channel genes. The investigation follows from a number of unique features that the human β-globin LCR conveys in the MEL/LCR gene activation system.

In Chapter 3, it was established that the human β-globin LCR activated expression of the human K⁺ channel hCDR1 from a 5.2kb genomic fragment (hCDR15.2) containing 2.4kb of upstream sequence, from native promoter sequences. Furthermore, this LCR-effect has been observed on a variety of heterologous promoters, reviewed in Section 4.2.3.

The activation of heterologous promoters by the human β-globin LCR is not governed by distance between the LCR and activatable promoter. This is seen with expression of cDNAs in vectors where the LCR is proximal to the β-globin promoter, and in expression of hCDR15.2, where the LCR-promoter distance was > 3kb. Both these observations are reported in Chapter 4. Furthermore, in vivo, the LCR interacts with the β-globin gene promoter with a distance of >50kb between the two interacting elements (Orkin, 1990).

Orientation of an activatable gene with respect to the LCR sequences has been shown to have no effect on the activatable expression of that gene. This has been shown in Section 5.6 of this chapter, and has been reported (see Section 4.2.3).

The MEL-cell membrane appears to be an excellent environment for K⁺ channel gene-expression. Analysis of expressed genes in MEL cells is achieved by patch-clamp recording. This is a sensitive technique that can detect a minimum expressed level of 20 K⁺ channels in the whole cell recording configuration (Dr. Noel Davies, personal communication), allowing a sensitive assay for any expressed channel. Experimentally, this may be important if a reduction in the activation of expression of novel K⁺ channel genes occurred. This may be due to a weak LCR interaction with certain promoter types, or if
competing promoter motifs are present between the LCR sequences and the promoter of interest.

Due to a low rate of expression of genes encoding many K⁺ channels, the cDNA route for isolation is often a difficult one, with large cDNA screening programs needed to isolate the most abundant K⁺ channel types. Isolation of rare channel species and developmentally expressed channel types from a single species would be difficult following a cDNA route. However, genomic DNA from a particular species would contain a representative number of every gene encoding a K⁺ channel. Furthermore, K⁺ channels have been established as being grouped in families of related genes. A vast majority of these genes are intronless, except the mammalian Shaw-related channels which have alternatively spliced 3' variants (see Section 1.8).

These facts together led to the idea of constructing an LCR-based gene-activation library for expression-cloning of existing and novel types of K⁺ channels. The library vector required a number of features, which are shown in Table 5.1.

Table 5.1. Desired features for an LCR library vector

<table>
<thead>
<tr>
<th>Feature number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Active LCR sequences in a form that could be easily inserted into the vector</td>
</tr>
<tr>
<td>2</td>
<td>A prokaryotic selectable gene</td>
</tr>
<tr>
<td>3</td>
<td>A eukaryotic selectable gene</td>
</tr>
<tr>
<td>4</td>
<td>Unique cloning sites for insertion of size-selected genomic DNA</td>
</tr>
<tr>
<td>5</td>
<td>Unique vector restriction enzyme sites to linearize isolated clones of interest prior to electroporation into MEL cells</td>
</tr>
<tr>
<td>6</td>
<td>Large insert capacity</td>
</tr>
</tbody>
</table>

A cosmid-based library was seen as the most logical approach to fulfill the criteria in Table 5.1.
5.2. Approach

The cosmid vector pTM (Grosveld et al., 1982), shown in Figure 5.2, was kindly provided by Dr. M. Antoniou (NIMR, Mill Hill, London) for construction of an LCR-based cosmid vector. The vector contains an ampicillin-resistance gene, a TK-neomycin resistance gene and unique BamHI and HindIII sites for insertion of size-fractionated genomic DNA.

LCR sequences in current use employed the 6.5kb micro-locus (Talbot et al., 1989) taken from the human β-globin gene domain. These sequences were shown to be functional in cDNA and genomic expression vectors for K+ channel gene expression (see Chapter 4), but were deemed too large for insertion in a vector of this type. A vector incorporating such a large cassette would be difficult to construct, and would reduce the size of the final genomic DNA insert.

A construct termed the nano-locus (see Figure 5.1), a 900bp cassette consisting of the core-sequences from the human β-globin LCR DNaseI hypersensitive sites 2-4 in the vector pPolyIII, was made available at this time by Dr. M. Antoniou in an untested form, and was employed in the construction of the vector. The 900bp nanolocus cassette was inserted as a blunt ended BamHI-HindIII fragment into the blunted ClaI site of pTM.

Finally, a number of unique restriction enzyme sites situated at a maximal distance from any functionally important parts of the vector were desired. These were desired to facilitate linearisation of library clones of interest prior to electroporation. For this purpose, four 8bp cutter sites, PacI, NotI, Pmel and Ascl were introduced into the SalI site of pTM on two self-annealing oligonucleotides (oligos. 9 and 10). Upon annealing, these oligonucleotides produced SalI-complementary 5' overhangs, compatible with the SalI site of pTM.

The SalI recognition-sites at the termini of the complementary oligonucleotides were lost due to a disruption of the SalI consensus site. This enabled the
Figure 5.1. The human LCR nano-locus

The human LCR nano-locus consisting of the core regions of the DNaseI hypersensitive sites (HSS) 2-4 from the human β-globin LCR. The building vector for the construct was pPolyIII. HSS2 (Talbot et al., 1990) was an EcoRI linkerEd 424bp fragment cloned into the EcoRI site of pPolyIII. HSS3 (Philipsen et al., 1990) was a Blunted HphI-Fnu4HI fragment cloned into the pPolyIII blunted BamHI site. HSS4 (Pruzina et al., 1991) was a Blunted SacI-AvaI fragment cloned into the HincII site of pPolyIII.
Figure 5.2. The expression vector pNANCOS8

The expression vector pNANCOS8 and its precursor vector pTM. The figure shows the introduction of the nano-locus into the Clal site of pTM and the introduction of the four 8bp cutter sites into the SalI site of pTM.
ligation mixture of SalI-cut vector and annealed complementary oligonucleotides to be cut with SalI, eliminating religated vector molecules when a complementary oligonucleotide cassette had not been inserted.

The two complementary oligonucleotides 9 and 10 and the positions of the 8bp cutter sites are shown in Figure 5.3.

Figure 5.3. Oligos. 9 and 10 showing their predicted annealing configuration with the 8bp restriction enzyme recognition sites PacI, NotI, Pmel, Ascl and the SalI compatible termini indicated.

\[
\begin{align*}
\text{PacI} & \quad \text{NotI} & \quad \text{Pmel} & \quad \text{Ascl} \\
5' & \text{TCGAGTTAATTAGCCCGCCCGGCGTITAAACGGCGGCCGCCC} \text{GGCC} & \quad 3' \\
& \text{CAATTAATTCCCGCGCCGCAAAATT} & \text{TTGCCCGCGGGGGCCGAGCT} & \quad 5' \\
\text{SalI} & \quad \text{SalI}
\end{align*}
\]

5.3. Construction of pNANCOS8

Two aliquots of SURE E.coli competent cells were separately transformed with 10ng of pTM cosmid DNA and 10ng of the pPolyIII-LCR nanolocus construct. An individual ampicillin-resistant colony from both transformations was propagated and large-scale plasmid isolation undertaken by Quiagen purification.

10μg of pTM cosmid DNA was then cut to completion with Clal, and 10μg of the pPolyIII-LCR nanolocus DNA sample cut to completion with HindIII and BamHI and the 900bp LCR nanolocus fragment isolated by agarose gel electrophoresis and subsequent electroelution.

50ng of pTM cut to completion with Clal and 200ng of the 900bp LCR nanolocus fragment were mixed together in a final volume of 25μl and the 5' overhang ends of the molecules filled-in according to materials and methods. In the same reaction, 1μl of 10mM ATP and 400units of T4 DNA ligase were added, and after incubation overnight at 4°C, an aliquot of SURE E.coli competent cells transformed with the products of this ligation.
After plating onto L-ampicillin, 100 colonies were subjected to colony hybridisation using an aliquot of $^{32}$P labelled 900bp LCR nanolocus fragment as the probe. 8 of the 100 colonies were shown to contain the 900bp LCR nanolocus fragment following digestion to completion of a plasmid DNA miniprep isolated from each with HindIII and BamHI. After separation of the products on a 0.8% agarose gel, band sizes of 8.5 and 0.9kb were visible for each of the 8 clones, confirming their recombinant identity.

1µg from one of the pTM-nanolocus LCR recombinants, Isolate 4, was then cut to completion with SflI. 10ng of oligos 9 and 10 were mixed in a final volume of 10µl, heated to 70°C, and allowed to anneal by slowly cooling to room temperature. 50ng of pTM-nanolocus LCR isolate 4 was then mixed with the annealed 10ng of oligos. 9 and 10 and ligated in a final volume of 20µl. An aliquot of SURE E.coli competent cells was transformed with the products of the ligation. 100 colonies, after plating the products of the ligation on L-ampicillin, were subjected to colony hybridisation using an aliquot of $^{32}$P-end labelled oligo. 9 as the probe, and 6 colonies were shown to contain the annealed oligos. 9 and 10 in the SflI site of pTM by a strong binding to the probe. This result was confirmed after digestion to completion of plasmid miniprep DNA isolated from 4 of the 6 oligo. 9 and 10 positives with NotI and PacI gave linear 9.5kb bands on a 0.8% agarose gel. Control pTM-nanolocus LCR recombinant isolate 4 DNA was shown not to cut with these two enzymes.

Isolate 2 was propagated and a maxiprep of plasmid DNA isolated by Quiagen purification. A diagnostic EcoRI digestion showed the nanolocus fragment in the pTM-nanolocus LCR recombinant to be in the orientation shown in Figure 5.2 after band sizes of 7, 1.5, 0.6 and 0.3kb were identified on a 0.8% agarose gel.

These features were built into the basic pTM cosmid to yield the vector pNANCOS8, shown in Figure 5.2.
Experiment to demonstrate the functionality of the LCR nano-locus cassette in pNANCOSS

5.4. Approach

Prior to construction of a genomic library in the vector pNANCOSS, a test genomic fragment, with its native promoter intact, was inserted into the vector. hCDR15.2 (Figure 3.4) was employed for this purpose, a genomic fragment shown to be activatable by the micro-locus LCR cassette (see Section 4.15). The BglII ends of hCDR15.2 were compatible with BamHI, and the fragment was ligated into the BamHI site of pNANCOSS, in both orientations with respect to the LCR sequences.

5.5. Construction of the two hCDR15.2-pNANCOSS constructs containing hCDR15.2 in both orientations

A 5μg aliquot of pNANCOSS DNA was cut to completion with BamHI. 50ng of this sample was mixed with 200ng of hCDR15.2 (isolated previously) and ligated together. After plating onto ampicillin, 100 colonies were subjected to colony hybridisation using an aliquot of 32P labelled hCDR15.2 fragment as the probe. 12 of the 100 colonies were shown to contain the insert by hybridising to the probe.

To find the relative orientation of hCDR15.2 in the 12 positively hybridising isolates, miniprep DNA from each was subjected to digestion with BamHI and HindIII. Due to the presence of a BamHI site 400bp from the 3' end of hCDR15.2, band sizes of 14 and 1.4kb were expected for hCDR15.2 in an orientation with the 5' end of hCDR15.2 proximal to the LCR sequences, and 9.2 and 5.8kb for hCDR15.2 orientated with its 3' end proximal to the LCR sequences. 8 clones were shown to be 5' proximal and 4 clones 3' proximal to the LCR sequences.

One clone orientated 5' and one clone 3' to the LCR sequences in pNANCOSS were propagated and a maxiprep of plasmid DNA isolated by Quiagen purification from each. 50μg of each was cut to completion with PvuI prior to electroporation into MEL cells. These constructs are shown in Figure 5.4.
Figure 5.4. The pNANCOS8-hCDR15.2 expression constructs

The pNANCOS8-hCDR15.2 expression constructs. Shown is the BgII hCDR15.2 fragment (described in Section 3.5) cloned into the BamHI site of pNANCOS8. Here, the hCDR15.2 fragment is cloned in an orientation with the 5' end of the coding sequence proximal to the LCR nano-locus. The fragment was also isolated in the opposite orientation with respect to the LCR nano-locus.
5.6. Activation of hCDR15.2 in both orientations with respect to the LCR-nanolocus in pNANCOSS

Following linearisation with Poul and electroporation into MEL cells, aliquots of cells from 6 G418-resistant colonies from both hCDR15.2 5' and 3' with respect to the nanolocus, were induced with 2% DMSO prior to electrophysiology. Large delayed-rectifier currents were observed from hCDR15.2 in both orientations in pNANCOSS. Whole-cell patch clamp recordings from an example of each 5' and 3' hCDR15.2 orientated clones are shown in Figure 5.5.

Construction of a rabbit genomic library in the vector pNANCOSS

5.7. Approach

Construction of a rabbit genomic library in the vector pNANCOSS was undertaken. This involved size-selection of Sau3AI partially digested high molecular-weight rabbit genomic DNA on sucrose gradients, and subsequent ligation of 37-45kb fractions into BamHI-digested and phosphatased pNANCOSS vector. This concatameric mixture was then packaged, and the cosmid library transfected into the host bacterium NM554 prior to titering and final amplification.

5.8. Construction

15μg of pNANCOSS DNA was cut to completion with BamHI. 3μg of this sample was then treated with calf intestinal alkaline phosphatase (CIAP) to remove the terminal phosphate groups from the linear pNANCOSS molecules.

High molecular weight rabbit genomic DNA was prepared and partial Sau3AI digests of this DNA, to give sizes of 33-45kb were performed. The partial digest giving an enriched fraction of 33-45kb sized fragments was scaled up to accommodate 100μg of high molecular weight rabbit genomic DNA. This sample, after analysis on a 0.3% agarose gel using KpI digested (30kb), XhoI digested (33kb) and wild type λ DNA (48kb) as markers on the gel, was layered onto a continuous 10-40% sucrose gradient and spun overnight at 28K.
Figure 5.5. Electrophysiological analyses following specific activation of the human $K^+$ channel gene hCDR1 from the pNANCOS8-hCDR1 genomic expression constructs.

Examples of whole-cell hCDR1 currents induced by steps ranging from -30 to +40 mV. Each step is followed by a repolarisation to -40 mV to obtain outward tail currents; the holding potential was -80 mV and the interpulse interval was 2.5s. A: Currents from hCDR15.2 in the 5' orientation to the nanolocus LCR. B: Currents from hCDR15.2 in the 3' orientation to the nanolocus LCR.
Thirty 0.8ml fractions were collected from the gradient and 20μl of each run on a 0.3% agarose gel with size-markers to find those fractions with an average genomic DNA size of 33-45kb. These fractions were pooled, diluted to 15ml with water, and the DNA precipitated by addition of 15ml isopropanol at room temperature. The DNA was collected by centrifugation at 10,000 rpm for 15 minutes. The final washed pellet was resuspended in 50μl of Q-filtered water.

3μg of pNANCOSS cut to completion with BamHI and treated with calf intestinal alkaline phosphatase was mixed with 2μg of the 33-45kb size fractionated rabbit genomic DNA, and the mixture ligated overnight in a final volume of 25μl.

2μl of this mixture was packaged using Stratagene (San Diego, California) Gold packaging extracts according to the manufacturer’s instructions. Titrating the library by infection of NM554 λ-sensitive cells and subsequent plating onto L-ampicillin at a concentration of 50μg/ml showed a titer of 1.83x10^6 colony forming units per 2μl of ligation mix. To check the representation of the library and the average insert size, 9 individual colonies were propagated and a miniprep of cosmid DNA isolated from each. After digesting aliquots with EcoRI and BamHI and separation on a 0.8% agarose gel, the library clones looked random, with an average insert size of >30kb (see Figure 5.6).

A further four 2μl aliquots of ligation mix were packaged to give a total library titer of 1.01x10^6. A library of 3.53x10^5 was predicted to have a 0.99 probability of being representative if an average insert size of 40kb was estimated. A total library titer of 1.01x10^6 was predicted to be approximately 3 times this probability.

The complete library was amplified according to the manufacturer’s instructions, and the amplified library stored at -70°C in 25% sterile glycerol at a titer of 3.5x10^5 colonies/μl.
Figure 3.6. Restriction enzyme analysis on 9 random clones from the pNANCOS8-rabbit genomic library

Lanes 1-9 show miniprep DNA from isolates 1-9 cut with EcoRI. Lanes 10-18 show miniprep DNA from isolates 1-9 cut with BamHI. Either side of these lanes are size markers of λ-HindIII digested DNA (23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6kb). All 9 clones showed an average insert size >30kb, and the clones were all different from each other, indicating the production of a random library.
5. Construction of LCR-based genomic gene-activation libraries for expression-screening of existing and novel types of $K^+$ channels

Retrieval of rabbit $K^+$ channel sequences from the rabbit genomic-pNANCOS8 library for expression analysis

5.9. Screening the rabbit genomic-pNANCOS8 library with six PCR amplified $K^+$ channel probes

5μl (1.7x10^6 clones) of the amplified library were plated onto 10 x 132mm L-ampicillin plates by vacuum blotting through a 137mm Buchner funnel onto 132mm Hybond-N discs (Amersham), and subsequent placement of the filters onto the preset 132mm L-ampicillin plates. These were allowed to grow overnight to pin-prick size, and the 132mm Hybond-N discs with approximately 1.7x10^5 colonies on each sandwiched together with another 132mm Hybond-N disc to make a single perfect replica. This was repeated for each of the 10 filters. Each replica filter was orientated to its original by 5 pinprick marks around the edge of the filters. The replica filters were then processed according to the colony hybridisation protocol prior to screening.

The library was then screened with a mixture of six PCR-amplified $K^+$ channel probes. This involved using 6 oligonucleotide pairs that would amplify specific 3'-regions of the voltage gated $K^+$ channels RCK1-5 (Roberds and Tamkun, 1991) and kV1 (Swanson et al., 1990) (see Figure 5.7). These probes, ranging in size from 278bp to 833bp, were pooled, an aliquot labelled, and finally employed to screen the 10 prepared filters.

After hybridisation overnight, four 30 minute washes in 2xSSC/0.1% SDS were performed before autoradiography.

5.10. Isolation and expression analysis on 10 positively hybridising clones

50 areas of 3mm square, corresponding to putative positive clones were isolated using a sterile scalpel from the 10 original filters, unprocessed for screening and regrown on L-ampicillin plates. These 50 areas were put through a second round of screening by vortexing the 3mm square filter pieces in 1ml of LB and plating 10μl of this onto 1x82mm L-ampicillin plate. A conventional colony-lift was then undertaken for each of these putative positive clones. 21 of
Figure 5.7. The 6 PCR generated 3' region K+ channel bands employed for screening the pNANCOS8-rabbit genomic library

Lane 1 shows a 388bp band to RCK1 generated using oligonucleotides 13 and 14; lane 2 a 500bp band to RCK2 generated using oligonucleotides 15 and 16; lane 3 an 833bp band to RCK3 generated using oligonucleotides 17 and 18. Lane 4 shows a 387bp band to RCK4 generated using oligonucleotides 19 and 20; lane 5 a 320bp band to RCK5 generated using oligonucleotides 21 and 22 and lane K shows a 278bp band to Kv1 generated using oligonucleotides 23 and 24. Lane S shows molecular weight markers of φ X174 DNA cut with HaeIII. From the top of the lane band sizes of 1.35, 1.08, 0.87, 0.60, 0.31, 0.28 and 0.27 are present.
these clones showed isolated hybridising signals after screening with an aliquot of the $^{32}$P labelled K$^+$ channel PCR band mixture.

The clones were graded according to their relative binding signal to the probes, as some were more strongly hybridising than others. Figure 5.8 shows a first and second round screen for one of the strongly hybridising positive clones.

6 strongly hybridising clones were isolated as single colonies, propagated and a maxiprep of cosmid DNA isolated by Quiagen purification. A 100ug aliquot of each cosmid DNA sample was then linearized with one of the unique 8bp enzyme sites present in the pNANCOS8 vector. The 6 clones were observed as having insert sizes >30kb and being different from each other by restriction enzyme digestion and separation of these products by agarose gel electrophoresis (see Figure 5.11). The six linearized clones were then electroporated into $2 \times 10^7$ MEL cells.

Upon selection of G418-resistant clones and induction by addition of 2% DMSO, electrophysiological recordings were taken from three isolated G418 positive clones from each of the original six clones that showed strong homology to the pool of six rat K$^+$ channel probes. After three days induction no K$^+$ channel currents were detected. Under identical conditions, control currents were observed from a pNANCOS8-hCDR15.2 clone already established as being able to express functional hCDR1 channels under control of the 900bp LCR nanolocus.

Four further clones, observed as being less strongly homologous to the 6 PCR generated probes (see Figure 5.11) were taken forward to expression as described for the 6 strongly hybridising clones. After three days induction no K$^+$ channel currents were detected. Under identical conditions, control currents were observed from a pNANCOS8-hCDR15.2 clone already established as being able to express functional hCDR1 channels under control of the nanolocus.
Figure 5.8. Example of a pNANCOS8-rabbit genomic library screen

(A) A first and (B) a second round screen for one of the strongly hybridising positive clones, homologous to the 6 PCR-amplified K+ channel probes RCK1-5 and Kv1. The isolate shown (clone 2) was a strongly hybridising positive homologue. The example of a strong positively hybridising clone 2 is indicated by an arrow in parts (A) and (B). This clone is also represented as lane 7 in Figure 5.11.
5.11. Further screening of the pNANCOS8-rabbit genomic library with three full length voltage activated $K^+$ channel gene probes

5x10^6 clones of the amplified library was plated out onto 10 x 132mm Hybond-N filters on L-ampicillin plates by vacuum blotting through a 137mm Buchner funnel, and filters processed as described in section 5.10.

Three probes, hCDR15.2 (see Figure 3.3) Rat Shal1 (Baldwin et al., 1991) and RCK1 (Baumann et al., 1988) were chosen to screen the library. Screening was undertaken and 18 strongly hybridising clones isolated and cosmid DNA purified and processed as described in section 5.10.

Upon restriction digestion with EcoRI and BamHI and separation through a 0.8% agarose gel, only five different clones were observed (see Figure 5.9). Upon Southern blotting analysis, these 5 different clones were shown to be most strongly homologous to the hCDR15.2 probe. This was observed after Southern blotting analysis on 3 identical gels as shown in Figure 5.9 with hCDR15.2, Rat Shal1 (Baldwin et al., 1991) and RCK1 (Baumann et al., 1988) as $^{32}$P labelled probes. Only strong hybridisation was observed with the hCDR15.2 probe (see Figure 5.10). These 5 clones were also shown to be different to the 10 clones isolated with the $K^+$ channel FCR generated probes by their different HindIII restriction enzyme patterns, after separation on a 0.8% agarose gel (see Figure 5.11).

100µg of the five different hCDR1 homologue cosmid clones were linearized with NotI. Upon selection of G418-resistant clones and induction by addition of 2% DMSO, electrophysiological recordings were taken from three isolated G418 positive clones from each of the four different clones that showed strong homology to hCDR15.2. After three days induction no $K^+$ channel currents were detected. Under identical conditions, control currents were observed from a pNANCOS8-hCDR15.2 clone already established as being able to express functional hCDR1 channels under control of the nanolocus-LCR.
Figure 5.9. Gel showing 5 different hCDR15.2, Rat *Shall* and RCX1 cDNA homologues

From left to right (1-18) 18 different hCDR15.2, Rat *Shall* and RCX1 cDNA homologues cut with (A) *EcoRI* and (B) *BamHI*. The five different library clones are indicated 1-5 cut *EcoRI* in (A), and their corresponding restriction enzyme patterns after digestion with *BamHI* (below (B)). At the right hand side of these lanes are size markers of λ-*HindIII* digested DNA (23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6kb)
Figure 5.10. Southern blotting analysis on the gel shown in Figure 5.9, using $^{32}$P labelled hCDR15.2 as probe.

Southern blotting analysis on the gel shown in Figure 5.9 using $^{32}$P labelled hCDR15.2 as probe. (A) A common 4.1kb hybridising band after digestion with EcoRI, a possible common region of rabbit chromosomal DNA with hCDR1-equivalent sequences present. (B) Different hybridising bands after digestion with BamHI and probing. The five different clones as observed from their restriction enzyme digestion patterns are indicated as 1-5. Down the left hand side of the margin are size-markers in kb.
Figure 5.11. The 15 K+ channel homologue pNANCOS8-rabbit genomic library clones used for expression in MEL-cells

The 5 different hCDR15.2, Rat ShalI and RCK1 cDNA homologues (1-5), alongside the 6 strongly hybridising (6-11) and 4 less strongly hybridising (12-14) RCK1-5 and Kv1 PCR band probed homologues. All samples were cut with HindIII. At the right hand side of these lanes are size markers of λ-HindIII digested DNA (23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6kb)
5.12. Approach

Due to the negative results observed with the nanolocus LCR cosmid system in Section 5.10 and 5.11, further investigation was pursued to establish a working system for LCR gene activation from a cosmid library.

The following section reports the establishment of a microlocus LCR cosmid system. Here, pEV3 (Figure 4.7) was employed as the starting point for the construction of the microlocus LCR-based cosmid expression vector. In construction, the 400bp β-globin promoter cassette was removed from pEV3 by digestion with Clal and EcoRI and replaced with a 1kb XbaI-EcoRI fragment containing the λ-cos site from the Triple Helix cosmid vector (THV) (Stratagene Inc.).

To assemble the vector, two oligonucleotides (oligos. 11 and 12) were employed to provide a linker sequence between the 13.3kb pEV3 Clal and 1kb THV XbaI fragment sites. After allowing to anneal, these oligonucleotides were designed to produce a short double stranded DNA molecule containing an internal 8bp recognition AscI site and XbaI and Clal compatible termini. These two oligonucleotides are shown in Figure 5.12.

**Figure 5.12.** Oligos. 11 and 12 showing their predicted annealing configuration with the 8bp recognition AscI site and XbaI and Clal compatible termini indicated.

```
5'   CTAGACGGCGCGCCGAT   3'
3'   TGCCGCGCGGCTAGC   5'
XbaI    AscI    Clal
```
In this version of the library, the 6.5kb microlocus LCR sequences from pEV3 were employed, and a rabbit library was subsequently constructed in this vector according to section 5.2.4.

5.13. Construction of the pUCOS1 microlocus LCR-based cosmid expression vector

pEV3 (Figure 4.7) was employed as the starting point for the construction of a microlocus LCR-based cosmid expression vector. 10μg of pEV3 was cut to completion with ClaI and EcoRI, and the 13.3kb vector sequences isolated from the 400bp cassette containing the β-globin promoter by separation through a 0.8% agarose gel and subsequent electroelution.

THV was then cut with XbaI and EcoRI to remove a 1kb fragment containing the λ-COS site. This fragment was isolated from the remainder 8kb vector sequences by separation through a 0.8% agarose gel and subsequent electroelution.

200ng in total of oligos. 11 and 12 were mixed together in Q-water in a volume of 20μl. The mixture was then heated to 70°C and allowed to cool slowly to room temperature to allow the oligos. to anneal.

Finally, a three way ligation containing 50ng of the pEV3 ClaI-EcoRI fragment, 50ng of the 1kb THV XbaI-EcoRI and 10ng of the annealed oligos. 11 and 12 was performed in a final volume of 20μl.

An aliquot of SURE E.coli competent cells were transformed with the products of the ligation. 24 colonies, after plating on L-ampicillin were propagated and a miniprep of plasmid DNA isolated from each. A diagnostic XbaI and EcoRI digestion indicated that 8 of the 24 colonies contained the 1kb THV fragment, after observation of this 1kb XbaI-EcoRI THV fragment, not present in control pEV3 cut with these two enzymes. This was further confirmed upon linearisation with the 8bp recognition site restriction enzyme Ascl, engineered into the complementary oligonucleotide linker.

One isolate, clone 6 was propagated, and a maxiprep of plasmid DNA isolated by Quiagen purification. This 14.2kb vector pUCOS1 is shown in Figure 5.13.
Figure 5.13. The LCR microlocus cosmid vector pUCOS1

The LCR microlocus cosmid vector pUCOS1 derived from pEV3 (Figure 4.7). Indicated is the l-cos site and the 8bp recognition Ascl site introduced in the complementary oligonucleotides 11 and 12. Other features are as described for pEV3 (Figure 4.6).
Experiment to demonstrate the functionality of the microlocus LCR in pUCOS1

5.14. Approach

To demonstrate the functionality of the microlocus LCR in pUCOS1, hCDR15.2 (see Figure 3.3) was employed as a test genomic fragment for expression. To facilitate the cloning of hCDR15.2 into pUCOS1, the previously constructed pSK+-hCDR15.2 clone (described in Section 3.5) was employed. This clone was established as being 5' to the EcoRI polylinker site of pSK+. It was established that digestion of pSK+-hCDR15.2 with EcoRI would remove 500bp of the upstream sequence, due to no EcoRI sites in the coding region of hCDR1 and the detection of a 500bp and a 7.6kb band after digestion of pSK+-hCDR15.2 with EcoRI (see Figure 5.14). A unique NotI site in the 3' region of the pSK+ vector sequence of pSK+-hCDR15.2 was used as the unique 3' bracketing enzyme site for removal of hCDR1. Finally, hCDR15.2, less 500bp of upstream sequence was removed by digestion of pSK+-hCDR15.2 with EcoRI and NotI, and this cassette was finally ligated into pUCOS1 previously cut with EcoRI and NotI.

5.15. Construction of pUCOS1-hCDR15.2

10μg of pUCOS1 was cut to completion with EcoRI and NotI, and the 14.2kb linear vector band isolated. Secondly, pSK+-hCDR15.2 was cut to completion with EcoRI and NotI, and the 4.7kb hCDR1 fragment (including 1.9kb of upstream sequence) isolated by separation of this band from the 500bp hCDR1 upstream, and 2.9kb pSK+ vector fragments. Both fragments were isolated by separation through a 0.8% agarose gel and subsequent electroelution.

50ng of the pUCOS1 and 4.7kb hCDR1 fragment were mixed and ligated together. An aliquot of SURE E. coli competent cells was transformed with the products of the ligation, followed by plating on L-ampicillin.

24 colonies were picked, and a miniprep of plasmid DNA isolated from each. 20 of the clones were shown to contain the intact 4.7kb hCDR1 fragment when digestion with EcoRI and NotI yielded a pUCOS1 vector band of 14.2kb and a
Figure 5.14. Agarose gel separation of pBluescript SK--nCDR15.2 cut to completion with EcoRI.

Agarose gel separation of pBluescript SK--nCDR15.2 cut to completion with EcoRI. Indicated are the 0.5 and 7.5 kb bands generated from this EcoRI digestion.
5. Construction of LCR-based genomic gene-activation libraries for expression-cloning of existing and novel types of K^+ channels

4.7kb hCDR1 fragment band. All clones were predicted to be in an orientation with the 5' end proximal to the LCR sequences in pUCOS1.

One clone, isolate 4, was propagated and a maxiprep of plasmid DNA isolated by Quiagen purification. 50µg of this construct was linearized with PvuI prior to electroporation into 1x10^7 MEL cells. This final hCDR1-pUCOS1 construct is illustrated in Figure 5.15.

5.16. Expression of hCDR1 from a 4.7kb genomic fragment in pUCOS1

Following induction, large delayed rectifier currents were observed from 3 clonal G418-resistant hCDR1-pUCOS1 MEL cell-lines. An example of these currents is shown in Figure 5.16.

5.17. Construction of a rabbit genomic library in the vector pUCOS1

Construction of a rabbit genomic library in pUCOS1 was performed in an identical manner to the method described in Section 5.8. Figure 5.17 shows 8 random clones after digesting an aliquot of each with EcoRI and BamHI. The different restriction digest patterns of these 8 clones indicated the library to be random and therefore representative of the rabbit genome. All 8 clones were predicted to have inserts of >30kb.

Discussion

5.18. Properties of the LCR nanolocus in pNANCOS6

Section 5.6 reports the first functional data on the properties of the LCR nanolocus. This condensed LCR element, consisting of core fragments from DNase I hypersensitive sites 2-4 of the human β-globin LCR (see Figure 5.1), appears to show partial LCR activity. Large delayed rectifier hPCN1 currents were observed from twelve random G418-resistant MEL clones, six of each with hCDR15.2 cloned in either orientation with respect to the nanolocus in pNANCOS6. This heterologous promoter activation, orientation-independent and high-level expression from the 12 analysed clones gave good reason to
Figure 5.15. Schematic diagram of the pUCOS1-hCDR1 genomic expression construct

Schematic diagram of the pUCOS1-hCDR1 genomic expression construct. The full hCDR15.2 fragment is shown at the top of Figure 5.15., with the EcoRI (500bp downstream of the genomic fragments BgIII site) and NotI site (from the multiple cloning site of pBluescript SK+) employed for subcloning this fragment indicated. The orientation of the EcoRI-NotI hCDR1 subclone and the cloning sites used in pUCOS1 are indicated by the dotted lines.
Figure 5.16. Electrophysiological analyses following specific activation of the human K+ channel gene hCDR1 from the pUCOS1-hCDR1 genomic expression construct.

Examples of whole-cell hCDR1 currents induced by steps ranging from -30 to +40 mV, 3 days post-induction. Each step is followed by a repolarisation to -40 mV to obtain outward tail currents; the holding potential was -80 mV and the interpulse interval was 2.5s.
Figure 5.17. Restriction enzyme analysis on 3 random clones from the pUCOS1-rabbit genomic library

Lanes 1-3 show miniprep DNA from 3 random clones from the pUCOS1-rabbit genomic library cut with EcoRI. Lanes 9-16 show miniprep DNA from isolates 1-3 cut with BamHI. Either side of these lanes are size markers of λ-HindIII digested DNA (23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6kb). All 9 clones showed an average insert size >30kb, and the clones were all different from each other, indicating the production of a random library.
Construction of LCR-based genomic gene-activation libraries for expression-cloning of existing and novel types of \( K^+ \) channels

proceed further with the production of a genomic library containing the LCR nanolocus in the library vector pNANCOSS.

Recently, more analysis on the LCR nanolocus has been undertaken. It has now been shown to confer approximately 30-40% of the full LCR activity and appears to exhibit some position-dependence with regards to the integration site in MEL cell chromatin. (M. Needham, and M. Antoniou, unpublished data).

No detectable expression from 15 voltage activated rabbit \( K^+ \) channel homologues after linkage to the LCR nanolocus in pNANCOSS

5.19. Putative reasons for no detectable expression

An encouraging result was seen when delayed rectifier currents, characteristic of hPCN1 were observed upon transfection of the pNANCOSS-hCDR15.2 construct into MEL cells. Large currents were observed from twelve MEL-cell G418-resistant clones with the hCDR15.2 fragment in both orientations with respect to the LCR-sequences.

These results showed that a \( K^+ \) channel gene could be driven from its own promoter on a genomic DNA fragment, and the resulting current detected by direct electrophysiological analysis from a single cell. Furthermore, pNANCOSS was functional with the LCR-sequences in the form of the nanolocus active in this vector.

A number of reasons why the isolated clones from the pNANCOSS-rabbit genomic library did not express are available for discussion.

One possibility is that the probes may have picked up a \( K^+ \) channel gene, but the channel coding sequence may have been truncated at one end of the cosmid insert, rendering it inexpressible. Alternatively the pool of probes may have retrieved inexpressible pseudogenes. The possibility that the clones did not contain \( K^+ \) channel sequences is also present, and must be considered.

Distance effects on activatable expression between the LCR and heterologous promoter have still to be investigated, and the nature of intervening DNA between the LCR and promoter may have hitherto unknown effects on
expression. Furthermore, gene-activation from heterologous promoters from different species in the MEL cell is still an area to be addressed. Gene-activation of a K⁺ channel has been successfully achieved from a human K⁺ channel gene promoter (from hCDR15.2), but promoter types from other organisms have yet to be investigated.

3.20. Probable reasons for no detectable expression

It seems unlikely that K⁺ channel pseudogenes are present in mammalian genomes, as to date no such sequences have been reported. Further, the possibility that all 15 K⁺ channel homologues were truncated seems unlikely due to the small transcriptional unit size of intronless genes for mammalian voltage-activated K⁺ channels in comparison to the large inserts retrieved in the case of the 15 K⁺ channel pNANCOS8-rabbit genomic homologues.

Therefore, the most likely reasons for no detectable expression from the 15 cosmid isolates would seem to be connected with one of three possibilities: the structure of the K⁺ channel gene promoters present in the rabbit genomic DNA inserts, the distance between the LCR sequences and K⁺ channel gene promoters and the nature of the DNA sequences surrounding the K⁺ channel genes.

Transcriptional silencing of genes by trans acting factors at promoters is well established. Several forms of transcriptional repression have been proposed (Levine and Manley, 1989). These include competition, quenching, direct repression and squelching modes of repression. Several proteins that bind to negative elements have also been described. For example, GCF, whose cDNA has been cloned, binds to GC-rich sequences and acts as a negative regulator (Kageyama and Pastan, 1989). Some silencers elements appear to consist of several cis-elements. The silencer fragment from the glutathione transferase P (GST-P) gene is one such example. Here, deletion or in vivo competition of each cis-element resulted in a decrease in activity, and no single element was shown to be important for full silencing activity (Imagawa et al., 1991). These findings indicate a cooperative function, including a spacing effect, between the silencer elements in the repression of promoter activity as has been observed for elements including TRE (Okuda et al., 1990) and the serum responsive element (Shaw et al., 1989). More recently, a common silencer element has been
identified in the SCG10 and type II Na\(^+\) channel genes at -1500 and -1000bp respectively from their promoter regions, binding a factor present in nonneuronal cells but not in neuronal cells (Mori et al., 1992).

Furthermore, silencing of the \(\beta\)-globin cluster \(\epsilon\) and \(\gamma\) genes during the adult stage of development gives a clear examples of the strength of silencing. Here, in the presence of the upstream LCR, correct developmental expression of the \(\epsilon\) and \(\gamma\) genes during the embryonic and foetal stages of developmental is observed. However, expression of these same genes is tightly shut off during adult development to allow correct expression of the \(\beta\) globin gene at this stage (see Section 1.30 for a full discussion and references).

Another important consideration concerns the identification and characterisation of an insulating element far upstream in the chicken \(\beta\)-globin locus (Dr. G.Felsenfeld, personal communication). This DNA sequence element has been isolated on a 1kb fragment and has been shown to confer reduced levels of expression on a \(neo\) gene when placed between mouse locus control region HS2 and the human \(\gamma\)-globin promoter coupled to the \(neo\) gene. Upon transfection of K562 cells approximately one-fold reduction in G418 resistant colonies is seen when the insulator is present as a single copy. However, approximately 30 fold reduction in G418 resistant colonies is observed when the element is present as a tandem dimer between the LCR and human \(\gamma\)-globin promoter coupled to the \(neo\) gene. Placing the insulator sequence outside the space between HS2 and the human \(\gamma\)-globin promoter has no effect on expression. Further, insulation appears to involve suppression of transcription and inactivation of chromatin structure over the promoter. Elements such as these may be common in chromosomal DNA to prevent 'leaky' expression of genes which could be lethal to certain cell-types.

Finally, it is clearly established that the LCR can activate the \(\beta\)-globin gene at a distance of approximately 50kb between the two elements (see Section 1.29). This provides evidence that the LCR could potentially activate a heterologous gene promoter at a large distance between the two elements from a retrieved cosmid library clone. The nature of activatable promoters has already been addressed in Section 4.20. The possibility that certain promoter types have very weak interactions or no interaction at all with the LCR remains an undetermined possibility.
5. Construction of LCR-based genomic gene-activation libraries for expression-cloning of existing and novel types of K⁺ channels

5.21. The nature of the microlocus LCR in pUCOS1

It was possible that the reasons for the failure of the nanolocus library strategy were simply a result of the nanolocus LCR element's inability to activate the retrieved rabbit K⁺ channel genes. For this reason, it seemed logical to attempt construction of a microlocus LCR cosmid vector. Section 5.13 describes this construction and Section 5.16 reports resultant activatable hCDR1 currents from a genomic fragment containing 500bp less DNA from the 5' end of hCDR15.2. This construction and genomic hCDR1 activation were good reasons for production of a rabbit genomic DNA library in this new vector.

5.22. Screening the pUCOS1-rabbit genomic library

After establishing that the pUCOS1-rabbit genomic library was representative, the library was screened with a number of voltage activated K⁺ channels gene probes. Three probes, hCDR15.2 (see Figure 3.3) Rat Shal (Baldwin et al., 1991) and RCK1 (Baumann et al., 1988) were chosen to screen the library. After 12 attempts at screening, achieved in an identical manner to Section 5.9, no K⁺ channels gene positives were able to be retrieved beyond the first round of screening. Positive (E. coli SURE cells harbouring pSK⁻K⁺ channel cDNA) and negative controls (E. coli SURE cells harbouring pSK⁺) were run at each stage, and were shown to be respectively positive and negatively hybridising after probing and washing. Reasons for non retrieval of clones remains unclear.
6. Discussion

6.1. Introduction

A number of clearly defined results have been obtained from this project which are important for the future study of $K^+$ channel molecular biology. Characterisation of a novel human $K^+$ channel gene and its promoter region have been achieved. Secondly, a novel strategy for the expression of $K^+$ channel cDNAs and genes based upon gene-activation by the human $\beta$-globin LCR has been established. These findings are finally discussed here in Sections 6.2 - 6.5.

6.2. hCDR1 and $K^+$ channel promoter analysis

Firstly, Chapter 3 describes the novel isolation of hCDR1, a human delayed-rectifier type $K^+$ channel and an analysis of this $K^+$ channel's promoter region. The isolation of hCDR1 from human genomic DNA was subsequently supported by the isolation of the same gene from human heart and pancreas cDNA (Philipson et al., 1991; Tamkun, 1991). The isolation of the hCDR1 gene on cosmid 11 gave the opportunity to study the promoter region of this human $K^+$ channel. A number of conclusions were drawn as to the possible regulatory elements involved in the promoter region of this gene and these are discussed in Chapter 3. To summarize, a putative TATAless promoter region was identified just upstream of the published hPCN1 cDNA sequence and two TATA box motifs defining putative promoter regions further upstream were observed.

Further support for these observations was provided from the LCR gene-activation data on hCDR15.2 in Chapters 4 and 5. Here, LCR activation of hCDR15.2 (see Section 4.15) and a derivative with 500bp less of upstream hCDR1 sequence (see Section 5.16) was seen. These results defined functional promoter elements, activatable by the human $\beta$-globin LCR, in a region 1900bp upstream of the published hPCN1 sequence.

These data provide the first investigation into the nature of a $K^+$ channel gene promoter. Furthermore, the approach of promoter analysis in combination with LCR-based gene-activation may provide a powerful approach to the study of $K^+$ channel promoter function. Recently, two rat genomic DNA regions encompassing two voltage activated $K^+$ channel genes were provided from Dr.
G. Chandy (Dept. Medicine, University of California), encompassing the intronless MK1 and MK2 genes (Chandy et al., 1990). Human β-globin LCR linkage to these rat genomic regions is proposed to help define the promoter regulatory elements of these two genes. Due to very low transcription levels of these genes in native tissues, LCR activation is proposed to provide a large pool of MK1 and MK2 mRNA from their respective native promoters for various analyses. Approaches such as these may prove valuable for K⁺ channel gene promoter studies.

6.3. LCR activation of K⁺ channel cDNAs and genes

Chapter 4 of this thesis describes the establishment of a novel expression system for the expression of K⁺ channel cDNAs and genes. Here, we see the first application of human β-globin LCR-based gene activation to the functional expression of the three major classes of voltage activated K⁺ channels. Also described is the first example of activation of a K⁺ channel gene, whose isolation was described in Chapter 3, from native promoter elements in MEL-cells. MEL-cells have been established as providing a suitably quiet electrophysiological background for the expression of K⁺ channel cDNAs and genes, and further application of this system to the stable expression of other K⁺ channels would be logical.

Of particular interest is the observation of high levels of expression of hCDR1 from the hCDR15.2 construct in MEL-cells in the absence of a polyadenylation signal (Section 4.15). Nearly all mRNAs in eukaryotic cells end in a homopolymer of 20-250 adenosine nucleotides. This poly(A) tail, which is added in the nucleus following transcription, both stimulates translation and stabilizes the message (Wiclenz, 1990). It is possible that the LCR is stimulating such high levels of hCDR1 transcription that the absence of a poly(A) tail on hCDR1 mRNA has little effect on expressed K⁺ channel subunits forming functional, membrane-associated channels.

Stable expression of the K⁺ channels described in this thesis has allowed collaboration with the ion-channel physiology group at Leicester University, who are proceeding with detailed kinetic studies on the nature of these expressed channels in MEL-cells. The activation and inactivation properties of these currents are being studied using both whole-cell and single channel
recording. Additionally it is envisaged that the permeation properties of several expressed K channels will be investigated and future work will involve site-directed mutagenesis in an attempt to correlate structure with function.

6.4. LCR gene activation libraries

Chapter 5 describes the attempts at the establishment of a gene-activation library for the expression of existing and novel K+ channel genes. Reasons for the non-expression of the 14 rabbit K+ channel gene homologues retrieved as pNANCOSS-rabbit genomic clones have been fully discussed in Chapter 5. The presence of upstream promoter regulatory elements which silence promoter activation seem a strong possibility for the non-detectable expression from the 14 rabbit K+ channel gene homologues.

Maue et al. (1990) conducted an investigation into the nature of the upstream elements involved in cell-specific expression of the type II sodium channel gene. Transient expression assays showed that deletion of upstream sequences resulted in an 80-fold increase in reporter gene activity in skeletal muscle cells, suggesting the presence of negative elements in this region (Maue et al., 1990). Reports such as these, and the observation of other silencers (see Section 5.20) require the upstream regions of voltage-activated K+ channel genes to be investigated for the presence of such elements, possible determinants for the non-expression of the 14 rabbit K+ channel gene homologues.

6.5. The LCR nanolocus

Finally, data reported in this thesis has shown the functional potential of smaller versions of the human β-globin LCR. Due to the fine analysis of the functional domains of the β-globin LCR, and identification of core hypersensitive domains within the full LCR (see Section 1.28), constructs such as the nanolocus may provide a powerful approach to high-level erythroid-specific expression where there are constraints on the cloning capacity which a full 6.5kb β-globin LCR would confer. Furthermore, for the expression of K+ channel cDNAs and genes, the nanolocus, shown to confer approximately 30-40% full LCR activity, has been shown to be a useful element. Data in Chapter 5 has shown that the nanolocus can activate a heterologous promoter (Section
5.6), and activate in an orientation independent manner, characteristic of LCR function. Furthermore, the nanolocus has been shown to be useful in the expression of $K^+$ channel cDNAs, of which LCR-nanolocus-activated expression of the mouse inward rectifier, IRK1, has been achieved (data not shown).

It is anticipated that the results in this thesis should find wide application for the molecular study of the biology of $K^+$ channel genes.
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