Investigation into the role of plakoglobin in *Xenopus* development.

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Desmosomes are rivet-like intercellular junctions which link intermediate filament networks of adjacent cells, dissipating mechanical stress over the tissue as a whole. They are composed of a number of different proteins and glycoproteins including the transmembrane adhesive molecules (the desmosomal cadherins) and the plaque-localised armadillo family protein, plakoglobin (PG).

Following preliminary experiments showing a desmosomal cadherin was expressed during early *Xenopus* development, before fully formed desmosomes had been observed, it was decided to investigate the potential morphogenetic role of desmosomal cadherins during early development. Initially it was decided to characterise the expression pattern of desmosomal components through early *Xenopus* development using a panel of antibodies, which were screened for cross-reactivity by Western blotting. Sections were prepared using a variety of techniques, and conditions optimised for immunostaining. These optimal conditions, however, did not maintain tissue integrity at early stages of development, and the proposed time course study was abandoned. In parallel an attempt was made to clone a *Xenopus* desmosomal cadherin using a variety of methods, for use in complementary *in situ* analysis and intervention studies, unfortunately without success, and so after two years this project was abandoned.

Following published experiments which illustrated the role of β-catenin (a molecule closely related to the desmosomal protein PG) in the Wnt signalling pathway, it was decided to further elucidate the potential signalling role of PG in early pattern formation. GST-fusion proteins of the unique N- and C-terminal domains of *Xenopus* PG and of the N-terminal two fifths of human PG were made, and antibodies generated against them. These were used to study PG through early *Xenopus* development by Western blotting, and revealed an interesting non-uniform expression pattern. When Fab fragments were made and microinjected into embryos, they caused non-specific toxic effects that were not significantly more severe than those generated by Fab fragments from control antisera. No double axis embryos were generated above background levels, which would have indicated a specific activation of the Wnt pathway.
signalling pathway, although these were produced when positive control anti-β-catenin F\textsubscript{ab} fragments were injected.

Little is known about the function of the terminal regions of PG, or indeed whether any other molecules bind to them. PG becomes tyrosine phosphorylated after certain stimuli and it has been postulated that the phosphorylation site lies in one of the terminal regions. The fusion proteins were therefore used for \textit{in vitro} binding and kinase studies in an attempt to address these questions. Of the molecules tested, only α-catenin was found to associate with the N-terminal domain of PG, in a phosphorylation-independent manner, and the site of tyrosine phosphorylation could not be identified.
Abstract ................................................................................................................................. 1

Table of Contents .................................................................................................................. 3

List of Figures ........................................................................................................................ 8

Abbreviations .......................................................................................................................... 10

Acknowledgements ............................................................................................................... 13

Chapter 1: General introduction ...................................................................................... 14

1.1 Adhesion molecules ........................................................................................................ 16

1.1.1 Immunoglobulin superfamily ................................................................................... 16

1.1.2 Selectins ................................................................................................................... 21

1.1.3 Integrins ................................................................................................................... 23

1.1.4 Cadherins ............................................................................................................... 25

1.1.4.1 Classical cadherins ........................................................................................... 26

1.1.4.2 The catenins ...................................................................................................... 27

1.1.4.3 Non-classical cadherins ................................................................................... 29

1.1.4.3.1 Unconventional cadherins ........................................................................ 29

1.1.4.3.2 Desmosomal cadherins ............................................................................. 29

1.1.4.3.3 Cadherin-like molecules ........................................................................... 31

1.1.4.4 Cadherins in development ............................................................................... 32

1.2 Adhesive junctions .......................................................................................................... 34

1.2.1 Cadherin adhesive junctions ................................................................................... 34

1.2.1.1 Adherens Junctions .......................................................................................... 34

1.2.1.2 Desmosomes .................................................................................................... 39

1.2.2 Other cell junctions ................................................................................................. 41

1.2.2.1 Focal adhesions/focal contacts ........................................................................ 41

1.2.2.2 Hemidesmosomes ............................................................................................ 42

1.2.2.3 Tight junctions. .................................................................................................. 42

1.2.2.3.1 Dlg-R or MAGUKs (Membrane Associated GUanylate Kinase) family of proteins ................................................................................................................. 43

1.2.2.4 Gap junctions ................................................................................................... 44

1.3 Armadillo superfamily of proteins ................................................................................. 46

1.3.1 Similarity between PG, β-catenin and armadillo....................................................... 50

1.3.1.1 Armadillo (arm) .............................................................................................. 50

1.3.1.1.1 Armadillo in the Drosophila wingless signalling pathway. ......................... 51

1.3.1.2 β-catenin ........................................................................................................... 55

1.3.1.2.1 The Wnts in early Xenopus pattern formation ........................................... 58

1.3.1.2.2 β-catenin in early Xenopus Wnt pathway ................................................... 60

1.3.1.3 Plakoglobin ....................................................................................................... 60

1.3.1.3.1 PG in Xenopus development ..................................................................... 62

1.4 Early Xenopus pattern formation ............................................................................ 63

1.4.1 Maternal information ............................................................................................... 63

1.4.2 Fertilisation and setting up of Nieuwkoop centre ................................................. 63

1.4.3 Blastula stage ........................................................................................................... 64

1.4.3.1 Mesoderm induction and Spemann’s organiser ............................................ 64
Chapter 2: Materials and Methods

2.1 Buffers

2.2 Bacterial Strains, plasmids, and cell lines used in this study

2.3 Library Screening

2.3.1 Making a radiolabelled nucleic acid probe

2.3.2 Screening a λZap2 library with cDNA

2.3.2.1 Preparation of a plating culture

2.3.2.2 Plating of library

2.3.2.3 Lifting of library

2.3.2.4 Probing filters

2.3.2.5 Identifying putative clones

2.3.3 Screening a λZap2 expression library with antibodies

2.3.4 In vivo excision protocol for subcloning from λZap2 vector

2.3.5 Isolation of λ DNA

2.3.6 Touchdown PCR

2.4 Other molecular Biology

2.4.1 PCR (Polymerase Chain Reaction)

2.4.2 Agarose gel electrophoresis

2.4.3 Southern blot

2.4.4 Probing a Southern

2.4.5 Normal GENECLEAN®

2.4.6 Double GENECLEAN®

2.4.7 Digestion with restriction enzymes

2.4.8 Ligation into pGEX

2.4.9 Preparation of competent DH5α and transformation with plasmid DNA

2.4.10 Maintenance of stocks of transformed DH5α

2.4.11 Mini-prep DNA preparations

2.4.12 Maxi-prep DNA preparations

2.4.13 Sequencing of DNA

2.5 Protein biochemistry

2.5.1 Production and isolation of trpE-DP or trpE-Dsg1 fusion proteins

2.5.2 Production and purification of GST-fusion Proteins

2.5.3 Expression and purification of His6-Dsg1 fusion protein

2.5.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

2.5.4.1 Glycine-buffered

2.5.4.2 Tricine-buffered

2.5.5 Staining of SDS Polyacrylamide gels

2.5.5.1 Coomassie Blue staining

2.5.5.2 Silver staining

2.5.6 Fluorography

2.5.7 Western blot transfer

2.5.7.1 Semi-dry blot

2.5.7.2 Wet blot

2.5.8 Staining Western blots

2.5.8.1 Staining proteins on Western blots with Ponceau S
2.5.8.2 Staining proteins on Western blots with amido black ..............................................102
2.5.9 Probing a Western blot ...............................................................................................102
2.5.10 In vitro kinase assay .................................................................................................103
2.5.11 Binding assays ........................................................................................................103
  2.5.11.1 Fusion proteins ....................................................................................................103
  2.5.11.2 Immunoprecipitations ........................................................................................104
  2.5.11.3 Overlays ............................................................................................................104
2.5.12 Protein concentration estimation using Bio-Rad Protein Assay .........................104
2.6 Histology/sectioning methods ......................................................................................106
  2.6.1 Slide subbing ...........................................................................................................106
  2.6.1.1 TESPA subbing of slides ....................................................................................106
  2.6.1.2 Gelatin subbing of slides ...................................................................................106
  2.6.2 Embryo fixation protocols .......................................................................................106
    2.6.2.1 Paraformaldehyde fixation ...............................................................................106
    2.6.2.2 TCA fixation ...................................................................................................106
    2.6.2.3 Methanol fixation ............................................................................................107
  2.6.3 Embedding and sectioning protocols ......................................................................107
    2.6.3.1 Acrylamide embedding and sectioning ............................................................107
    2.6.3.2 'OCT' embedding and sectioning ....................................................................107
    2.6.3.3 Wax embedding and sectioning ........................................................................108
  2.6.4 Staining of sections ................................................................................................108
    2.6.4.1 Dewaxing of wax sections before immunostaining ......................................108
    2.6.4.2 Immunostaining ..............................................................................................108
    2.6.4.3 Giemsa counterstaining ................................................................................109
2.7 Antibody methods .........................................................................................................110
  2.7.1 Antibody production and sera preparation .............................................................110
  2.7.2 Antibody purification ...............................................................................................110
    2.7.2.1 Preparation of an Affi-10 gel affinity column ...............................................110
    2.7.2.2 Antibody purification by affinity chromatography ........................................110
  2.7.3 Fab fragment generation ..........................................................................................111
  2.7.4 Preparing a cross-linked Protein A-bead-antibody affinity column .....................112
2.8 Xenopus laevis techniques ............................................................................................113
  2.8.1 Xenopus NP40 lysate preparation for original Western blot ..............................113
  2.8.2 Routine preparation of NP40 embryo extracts for SDS-PAGE .........................113
  2.8.3 Preparation of organ extracts for SDS-PAGE ......................................................114
  2.8.4 Embryo subcellular fractionation protocol ...........................................................114
  2.8.5 Microinjections into 4 cell stage Xenopus ............................................................115
  2.8.6 Whole mount antibody detection to track injection ..........................................115
  2.8.7 Whole mount antibody muscle counter-stain .......................................................116
2.9 Tissue Culture .............................................................................................................117
  2.9.1 Growth ....................................................................................................................117
  2.9.2 Passaging ..............................................................................................................118
  2.9.3 NP40 lysis .............................................................................................................118
  2.9.4 NIF fraction extract ..............................................................................................118
  2.9.5 Whole cell lysis (Laemmli buffer) .......................................................................118
  2.9.6 Radioactive labelling ............................................................................................119
  2.9.7 Freezing cells .......................................................................................................119
  2.9.8 Infection of SF9 cell line ......................................................................................119
  2.9.9 Purification of recombinant EGF receptor ............................................................120
  2.9.10 Membrane preparation of A431 cells .................................................................120
Chapter 3: Initial antibody studies on *Xenopus* and attempted cloning of a *Xenopus* desmoglein

3.1 Introduction ................................................................................................................... 121
3.2 Screening of antisera for cross-reactivity with *Xenopus* ........................................... 123
3.3 Fluorescence immunohistology. .................................................................................... 123
3.4 Attempted cloning of a *Xenopus* desmoglein ............................................................. 128
    3.4.1 Southern blot analysis of *Xenopus* genomic DNA .............................................. 134
    3.4.2 Screening of a λZap2 *Xenopus* liver library using a cDNA probe ..................... 134
        3.4.2.1 Initial screening process ................................................................................ 134
        3.4.2.2 Attempts to isolate the insert DNA by alternative methods. ....................... 137
    3.4.3 Attempted isolation of DSG clone from other libraries using cDNA ................ 149
    3.4.4 Screening of a λZap2 DSG clone library (stage 52 tail) using antibodies .......... 149
    3.4.5 Touchdown PCR. ................................................................................................ 155
3.5 Summary and discussion ............................................................................................... 157
    3.5.1 Antibody studies .................................................................................................... 157
    3.5.2 Attempted desmoglein cloning ............................................................................. 159
        3.5.2.1 Southern blot .................................................................................................. 160
        3.5.2.2 DNA screening ............................................................................................... 161
        3.5.2.3 Antibody screening ......................................................................................... 163
        3.5.2.4 PCR ............................................................................................................... 163
    3.5.3 Current state of knowledge about desmosomal cadherins during early vertebrate
devlopment ..................................................................................................................... 164

Chapter 4: Production of GST-fusion proteins XPGN, XPGC, HPGN and
generation of antibodies ................................................................................................. 167
4.1 Introduction ................................................................................................................... 167
4.2 Production of fusion proteins ....................................................................................... 167
4.3 Specificity of antibodies by Western blot .................................................................... 168
4.4 Zooblot of antibodies .................................................................................................... 173
4.5 Conclusion and discussion ............................................................................................ 173

Chapter 5: Examination of the role of PG in early *Xenopus* development. .............. 179
5.1 Introduction ................................................................................................................... 179
5.2 PG expression in early *Xenopus* development .......................................................... 179
5.3 Generation and purification of Fabs fragments ............................................................. 185
5.4 Microinjections of anti-XPGN, anti-XPGC, pre-immune and anti-β–catenin Fab
    fragments into *Xenopus* embryos .............................................................................. 188
5.5 Conclusion and discussion. ........................................................................................... 199
    5.5.1 PG expression pattern ........................................................................................... 199
        5.5.1.1 Comparison with other PG and β-catenin expression data .............................. 199
        5.5.1.2 Possible interpretations of data ...................................................................... 201
        5.5.1.3 Possible functional significance of data ......................................................... 203
    5.5.2 Microinjection results ............................................................................................ 204

Chapter 6: In vitro assays ................................................................................................. 213
6.1 Introduction ................................................................................................................... 213
    6.1.1 PG tyrosine phosphorylation ................................................................................ 213
    6.1.2 Binding studies. ...................................................................................................... 214
6.2 Examination of tyrosine phosphorylation of fusions .................................................... 214
6.3 Fusion protein binding studies. .................................................................................... 217
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Schematic of adhesion molecules.</td>
<td>18</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Schematic representation showing primary structure of adhesion molecules.</td>
<td>20</td>
</tr>
<tr>
<td>Figure 3</td>
<td>The junctions of the cell.</td>
<td>36</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Connections to the cytoskeleton at the adherens junction and desmosome.</td>
<td>38</td>
</tr>
<tr>
<td>Figure 5</td>
<td>The armadillo family of molecules.</td>
<td>48</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Schematic of wingless signalling pathway during segmentation of <em>Drosophila</em>.</td>
<td>54</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Gastrulation and neurulation in <em>Xenopus laevis</em>.</td>
<td>66</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Inductive interactions in early <em>Xenopus</em> development.</td>
<td>71</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Outline of <em>in vivo</em> excision process.</td>
<td>85</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Southern blot apparatus.</td>
<td>90</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Screening of antisera for cross reaction with <em>Xenopus</em>.</td>
<td>125</td>
</tr>
<tr>
<td>Figure 12</td>
<td>List of antisera screened to test for cross reaction with <em>Xenopus</em> samples.</td>
<td>127</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Immunostaining of SVK14 cells.</td>
<td>130</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Immunostaining of <em>Xenopus</em> sections.</td>
<td>132</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Southern blot of <em>Xenopus</em> genomic DNA using the three human desmoglein isoforms as probes.</td>
<td>136</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Example autoradiographs from primary round of screening using radiolabelled DNA probes.</td>
<td>139</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Example autoradiographs from the secondary round of screening.</td>
<td>141</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Example autoradiographs from the tertiary round of screening.</td>
<td>143</td>
</tr>
<tr>
<td>Figure 19</td>
<td>λ DNA preparations and Southerns from putative clones.</td>
<td>146</td>
</tr>
<tr>
<td>Figure 20</td>
<td>Results of mini-preps of putative clones.</td>
<td>148</td>
</tr>
<tr>
<td>Figure 21</td>
<td>Table of libraries tested for screening.</td>
<td>150</td>
</tr>
<tr>
<td>Figure 22</td>
<td>Southern blots of λ preparations from different libraries.</td>
<td>152</td>
</tr>
<tr>
<td>Figure 23</td>
<td>Results using alternative methods in an attempt to isolate a Dsg clone.</td>
<td>154</td>
</tr>
<tr>
<td>Figure 24</td>
<td>Degenerate primer sequences.</td>
<td>156</td>
</tr>
<tr>
<td>Figure 25</td>
<td>Schematic outline of cloning strategy for production of PG fusion proteins.</td>
<td>170</td>
</tr>
<tr>
<td>Figure 26</td>
<td>Purification of fusion proteins.</td>
<td>172</td>
</tr>
<tr>
<td>Figure 27</td>
<td>Specificity of anti-fusion protein sera.</td>
<td>175</td>
</tr>
<tr>
<td>Figure 28</td>
<td>Ability of antisera to recognise PG across species: zooblot results.</td>
<td>177</td>
</tr>
<tr>
<td>Figure 29</td>
<td>Expression of PG and β-catenin in early <em>Xenopus</em> development (Example 1).</td>
<td>182</td>
</tr>
<tr>
<td>Figure 30</td>
<td>Expression of PG and β-catenin in early <em>Xenopus</em> development (Example 2).</td>
<td>184</td>
</tr>
<tr>
<td>Figure 31</td>
<td>Example of antibody purification and F\textsubscript{ab} production.</td>
<td>187</td>
</tr>
<tr>
<td>Figure 32</td>
<td>Examples of secondary axis phenotypes produced by microinjection of anti-β-catenin Fab fragments.</td>
<td>190</td>
</tr>
<tr>
<td>Figure 33</td>
<td>Examples of phenotypes produced after microinjection of anti-fusion protein F\textsubscript{ab} fragments.</td>
<td>192</td>
</tr>
</tbody>
</table>
Figure 34  Bar charts illustrating results of microinjection experiments using anti-XPGN F\textsubscript{ab} fragments.  194
Figure 35  Bar charts illustrating results of microinjection experiments using anti-XPGC F\textsubscript{ab} fragments.  196
Figure 36  Table showing example of data obtained from one microinjection experiment (anti-XPGN 2006 F\textsubscript{ab} fragments).  198
Figure 37  Amino acid sequence comparison of PG (human and *Xenopus*) and β-catenin (*Xenopus*).  208
Figure 38  Schematic indicating reported binding domains of PG.  210
Figure 39  Human desmoglein 1 cytoplasmic domain amino acid sequence.  216
Figure 40  *In vitro* kinase assays.  219
Figure 41  Fusion protein precipitations of 35S-labelled A431 cells.  222
Figure 42  Western blot analysis of fusion protein precipitations.  224
Figure 43  Amino acid sequences of PG and β-catenin with potential tyrosine phosphorylation sites marked.  227
<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>arm</td>
<td>Armadillo</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CIP</td>
<td>Calf intestinal phosphatase</td>
</tr>
<tr>
<td>D</td>
<td>Aspartate</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>^{35}S-dATP</td>
<td>Deoxyadenosine 5'-triphosphate (α-^{35}S)</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytidine 5'-triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxyguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleoside 5'-triphosphate</td>
</tr>
<tr>
<td>ddNTP</td>
<td>Dideoxyribonucleoside 5'-triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxythymidine 5'-triphosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DP</td>
<td>Desmoplakin</td>
</tr>
<tr>
<td>Dsc</td>
<td>Desmocollin</td>
</tr>
<tr>
<td>Dsg</td>
<td>Desmoglein</td>
</tr>
<tr>
<td>DTIT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>dH_2O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Animal Cell Cultures</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethyleneglycol-bis(β-aminophylether)N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>F</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>F_{ab}</td>
<td>Univalent fragment of immunoglobulin G</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>G</td>
<td>Glycine</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosyl phosphatidylinositol</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase-3β</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>H</td>
<td>Histidine</td>
</tr>
<tr>
<td>HGF/SF</td>
<td>Hepatocyte growth factor/scatter factor</td>
</tr>
</tbody>
</table>
XPGN N-terminal region of *Xenopus* plakoglobin (see figure 25)
Y tyrosine
Acknowledgements.

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Chapter 1: General introduction.

This thesis is concerned with the roles of certain desmosomal molecules during early vertebrate development. This section provides a brief outline of the introduction, which presents the molecules relevant to this study in the wider context of other adhesive and cell junctional molecules, and also introduces the reader to the early development of Xenopus, the model organism used during the study.

A large number of different adhesive molecules are expressed during the life of a multicellular organism and can be classified into four main groups: the immunoglobulin superfamily, the selectin family, the integrin family and the cadherin superfamily, all of which are discussed in section 1.1 (Figure 1).

Some of these adhesive molecules assemble into larger structures, cell junctions, mediating more stable forms of adhesion and aiding tissue strength and integrity by linking the cytoskeletons of different cells together. Members of the cadherin superfamily are involved in anchoring cells to each other at adherens junctions, which link into the actin cytoskeleton, and at desmosomes, which link into the intermediate filament cytoskeleton. The integrins are involved in anchoring cells to the extracellular matrix at focal adhesions, which link into the actin cytoskeleton, and at hemidesmosomes, which link into the intermediate filament network. Other cell junctions also exist to aid tissue integrity and cell communication; these are tight junctions (which form a seal around the cell differentiating the apical and basolateral extracellular environments), and gap junctions (which allow the transfer of small molecules and electrical impulses directly between cells). These are all discussed in further detail in section 1.2.

A large part of this thesis is concerned with plakoglobin (PG), a member of the armadillo superfamily of molecules. These are a diverse group of proteins that act intracellularly, a subgroup of which are localised at desmosomes and adherens junctions and are involved in signalling and cell fate determination. This superfamily is discussed in section 1.3.
Aspects of this thesis are concerned with the roles of cadherins and PG during early *Xenopus* development. An overview of early pattern formation in *Xenopus* is given in section 1.4.

The specific aims of this doctorate are outlined in section 1.5.
1.1 Adhesion molecules.

See Figures 1 and 2.

1.1.1 Immunoglobulin superfamily.

The immunoglobulin superfamily (Ig SF) of proteins is a large family of mainly membrane-associated molecules that mediate Ca\(^{2+}\)-independent adhesion and signal transduction (Doherty et al., 1991; Schuch et al., 1989) in developmentally-regulated patterns. They are particularly abundant in the immune and nervous systems where they mediate complex interactions between diverse cell types, playing important roles in regulating the immune response (Roitt, 1994; Springer, 1990), and in developmental processes such as axonal guidance and fasciculation (Rutishauser and Landmesser, 1991; Sonderegger and Rathjen, 1992). For reviews see Buck (1992); Hunkapiller and Hood (1989); Williams (1987).

Most members are single-spanning transmembrane proteins although some link to the membrane using a GPI-anchor, for example one splice variant of N-CAM (Owens et al., 1987), and a few others exist only as secreted proteins, for example the proteoglycan perlecan (Noonan et al., 1988). The molecules contain a variable number of extracellular immunoglobulin-like domains, which consist of between 70 and 110 amino acids arranged in two parallel \(\beta\) sheets that are stabilised by disulphide bonds and define the family (Figure 1; see Vaughn and Bjorkman, 1996 for review of domain structure), resulting in a conserved tertiary structure thought to act as a scaffold on which different unique determinants can be displayed. Other than these Ig repeats, there is enormous variety in the primary structure of the members of this family, with different members having different structural motifs (e.g. N-CAM has fibronectin type III repeats localised more proximally to the membrane; Figure 2).

Members of the family can bind homotypically (for example N-CAM binds to itself in the vertebrate nervous system; see Walsh and Docherty, 1991 for review of N-CAM) and heterotypically, both to other members of the family (for example CD2/LFA-2 binds CD58/LFA-3; Dustin and Springer, 1990) and to non-related molecules (for example ICAM-1 binds the leukointegrins \(\alpha_4\beta_2\); Marlin and Springer,
FIGURE 1: Schematic of adhesion molecules.

Adapted from Hynes (1994).

- Lectin-like domain of selectin
- EGF-like domain of selectin
- Complement regulatory-like domain of selectin
- Globular head region of integrin subunits containing the adhesion binding site; this domain of the α subunit contains some of the repeat regions, and includes the cation binding site.
- Cysteine rich repeat domain of β integrin subunit
- Immunoglobulin-like repeat
- Mucin like ligand
- Fibronectin type III domain
- Extracellular cadherin repeat
- Extracellular cadherin anchor repeat
- Cell adhesion recognition site (includes HAV motif for classical cadherins)
FIGURE 2: Schematic representation showing primary structure of adhesion molecules.

Key:

- Fibronectin type III domain
- Immunoglobulin-like repeat
- Plasma membrane
- Complement regulatory-like domain
- Lectin-like domain
- EGF-like domain
- Integrin α subunit repeats
- Cysteine-rich repeat domains of integrin β subunit
- Extracellular anchor domain
- Cadherin repeat domain
- Cadherin propeptide domain

- Site of cleavage for heavy and light chains of integrin α subunit
- Site of divalent cation binding
- Alternate splice site
1987 and \( \alpha_M^3 \beta_2 \); Diamond et al., 1991; Staunton et al., 1988, and N-CAM recognises the extracellular matrix molecule heparan sulphate; Cole and Akeson, 1989). Generally, homotypic binding appears to be mediated by the "internal" domains requiring anti-parallel alignment of interdigitating molecules, and heterotypic binding by the N-terminal Ig domains (for review see Holness and Simmons, 1994). As well as functioning alone family members can associate together to form receptors, as in the case of CD8 and CD4 with the T cell receptor in antigen recognition; Parnes et al., 1989.

The adhesive strength of some of the family members can be modulated (probably by steric hindrance effects) by the binding of polysialic acid to the membrane proximal Ig-like domains; when large amounts of polysialic acid are attached to N-CAM (such as in embryonic tissues) adhesiveness is reduced, and when less is bound (such as in adult tissues) adhesiveness is increased (Rutishauser, 1992). It is thought that the intracellular domain of some family members may also regulate adhesive function (DeLisser et al., 1994; Doherty et al., 1992), and recently it has been suggested that members of this family are also involved in signal transduction into the cell through second messenger systems such as intracellular calcium, pH and cAMP level modulation (Rosales et al., 1995 and references therein). Indeed a number of the newly discovered receptor protein tyrosine phosphatases (RPTPs) belong to the immunoglobulin superfamily, implying a direct involvement of family members in intracellular signalling (Gebbink et al., 1993; Walton and Dixon, 1993).

1.1.2 Selectins.

The selectins (previously known as the LEC-CAMs) are a family of transmembrane glycoproteins that bind specific carbohydrate groups to mediate adhesion in a \( \text{Ca}^{2+} \)-dependent manner. They mediate heterotypic interactions between leukocytes and platelets or vascular endothelial cells, and are involved in leukocyte trafficking and early events in extravasation. The so called "rolling" of leukocytes that precedes more stable adhesive interactions before extravasation is mediated by selectin adhesion (Lasky, 1992; see Welply et al., 1994, and Rosen and Bertozzi, 1994 for reviews).
At present the family contains three known members: E-selectin (expressed on activated endothelial cells), L-selectin (expressed constitutively on lymphocytes) and P-selectin (expressed on activated platelets and endothelial cells). The molecules consist of, from the N-terminus, a C-type lectin domain of approximately 120 amino acids (which contains the region involved in calcium dependency and ligand binding; Drickamer, 1993; Kansas et al., 1991; Pigott et al., 1991), an EGF-like repeat of approximately 34-40 amino acids (which may be involved in recognising a protein component of the ligand; Kansas et al., 1991; Siegelman et al., 1990), a domain of variable size which contains repeats of approximately 62 amino acids that are related to complement binding proteins, a transmembrane domain, and a short cytoplasmic domain (Figure 2).

It is thought that the selectins can bind different ligands provided they contain a recognisable structural epitope based on the sialyl-Lewis x motif, which contains sialic acid and fucose residues in specific linkages, within the carbohydrate moiety of the ligand (Cummings and Smith, 1992; Rosen and Bertozzi, 1994; Varki, 1992). Indeed L-selectin has been shown to recognise at least three biological ligands: GlyCAM-1/Sgp50 (Dowbenko et al., 1993; Imai et al., 1991; Lasky et al., 1992), CD34/Sgp90 (Baumhueter et al., 1993; Imai et al., 1991), and MAdCAM-1 (Berg et al., 1993). All of these ligands, as well as the one biological P-selectin ligand so far identified, PSGL-1 (Norgard et al., 1993; Sako et al., 1993), contain extracellular mucin domains consisting of a highly extended rigid serine/threonine-rich polypeptide backbone densely decorated with O-linked carbohydrate chains (Figure 1). This has led to the hypothesis that most if not all selectin ligands in vivo are mucin-like molecules, although this remains to be confirmed as more biologically active ligands are found for the different selectins. As well as selectins binding multiple ligands, the affinity a selectin has for a specific ligand can also be modulated by intracellular signals (Gimbrone et al., 1989; Spertini et al., 1991).
1.1.3 Integrins.

The integrins are a family of cell-adhesion molecules that play important roles in cell migration during embryogenesis, wound healing, thrombosis, immune defence mechanisms and oncogenic transformation (Albelda and Buck, 1990). They are transmembrane heterodimeric glycoproteins consisting of an $\alpha$ subunit (120-180kD) non-covalently linked to a $\beta$ subunit (90-110kD) in a 1:1 complex (Figure 1). The subunits associate via their extracellular domains producing a structure with a globular head region on two stalks which traverse the membrane, and the ligand binding site is thought to be at or near to the interface of the two subunits in the head region (Hynes, 1992a; Loftus et al., 1994). The majority of integrin-mediated adhesion has been shown to be highly dependent on the presence of divalent cations (usually $\text{Ca}^{2+}$), although recent work (Bergelson et al., 1993; Masumoto and Hemler, 1993; Miles et al., 1986) has identified a different ligand binding mechanism by some of the members of the family that is independent of cation binding. Ligand binding mechanisms are reviewed in Haas and Plow (1994); general integrin reviews can be found in Hynes (1987; 1992a).

The $\alpha$ subunits, of which 16 vertebrate isoforms are currently known, have a large extracellular domain containing seven 24-45 amino acid repeats spaced 20-30 amino acids apart, the most membrane proximal 3 or 4 of which are thought to contribute to the divalent cation binding sites needed for receptor function. This extracellular domain is followed by a transmembrane domain and small cytoplasmic domain of approximately 20-75 amino acids (Figure 2). The $\alpha$ subunits can be divided into two subgroups, with the exception of $\alpha_4$, depending on their post-translational modifications. The first group are proteolytically cleaved near the C-terminus, producing an extracellular heavy chain that is disulphide bonded to a membrane-spanning light chain of approximately 25-30kD (for example $\alpha_6$). The second group, which includes $\alpha_1$, $\alpha_2$, $\alpha_L$, $\alpha_M$ and $\alpha_X$, have an insert of 180-200 amino acids called the I domain spliced in between the second and third most N-terminal repeats which replaces the first of the four cation-binding sequences of the $\alpha$ subunit. The I domain has homology with the collagen binding domain of von Willebrand factor, cartilage
matrix protein and complement proteins, and has been implicated in ligand- and cation-binding functions of the integrin (Bilsland et al., 1994; Michishita et al., 1993; Randi and Hogg, 1994). The α₄ subunit fits neither of these groupings as it lacks an I domain, can be expressed in an intact state, or cleaved at a different site to the first group of α subunits and expressed as two non-covalently joined segments of 85 and 75kD. In addition to these extracellular cleavage sites, certain α subunits have been reported to be alternatively spliced in the cytoplasmic domain; for example α₃ (Tamura et al., 1991) and α₆ (Hogervorst et al., 1991).

The β subunits, of which 8 vertebrate isoforms are currently known, consist of a large extracellular domain containing four 40 amino acid cysteine-rich repeats, a transmembrane region and a cytoplasmic domain of approximately 50 amino acids with small clusters of amino acids conserved between subunits (except β₄ which has 1018 amino acids in its cytoplasmic domain)(Figure 2). At least some of the β subunits can be alternatively spliced in the cytoplasmic domain; for example β₁ (Altruda et al., 1990), β₃ (van Kuppevelt et al., 1989), and β₄ (Hogervorst et al., 1990; Suzuki and Naitoh, 1990; Tamura et al., 1990). The cytoplasmic portion of the β subunit anchors the integrin dimer to the cytoskeleton by linking into the actin filament network of the cell via talin, α-actinin and a complex of proteins often organised into focal adhesions (section 1.2.2.1), except for β₄ which links to the intermediate filament network instead, often at hemidesmosomes (section 1.2.2.2).

Different combinations of α and β subunits produce receptors for different ligands including extracellular matrix proteins (for example fibronectin and laminin), complement components (for example C3b) and other cells via members of the IgSF (for example VCAM 1). Often a given integrin will bind more than one ligand (for example α₃β₁ binds fibronectin, laminin and collagens; Hynes, 1992b), and similarly a given ligand may have more than one integrin receptor (for example fibronectin binds α₃β₁, α₄β₁, α₅β₁, α₆β₁, αIβ₃, αvβ₃, αvβ₆ and α4β7; Hynes, 1992b), with receptors for the same ligand often producing different cellular responses (for example both αvβ₃ and αvβ₅ interact with the RGD sequence of vitronectin but αvβ₃ localises to focal contacts whereas αvβ₅ does not; Wayner et al., 1991). Most cells have more than one
integrin present on their surface, and most integrins are present on a variety of cells, but a given integrin may have a different ligand affinity on different cells (Kirchhofer et al., 1990b), or even on the same cell depending on the divalent cation present or its particular conformational state (Gailit and Ruoslahti, 1988; Kirchhofer et al., 1990a, 1991).

The affinity of a particular integrin may be increased or decreased by a conformational switch induced either by an extracellular stimulus, so called "outside-in" signalling, or by intracellular changes, so called "inside-out" signalling (reviewed in Damsky and Werb, 1992; Ginsberg et al., 1992; Hynes, 1992a; Juliano and Haskill, 1993; Kornberg and Juliano, 1992; Sastry and Horwitz, 1993; Schwartz, 1992; Smyth et al., 1993; Williams et al., 1994b). This variability of adhesion is particularly important in developmental processes when cells must remain plastic and capable of changing responses if necessary (for example α6β1 is present in a deactivated form on chick embryonic day 11 retinal neurones but can be reactivated to bind laminin - Neugebauer and Reichardt, 1991), and in immunological processes when cells must be able to quickly change their adhesive state (for example αIIbβ3 is present in an inactive form on resting platelets, but once platelets are activated by agonists such as thrombin, the integrin is "switched on" and binds soluble fibrinogen leading ultimately to clot formation - Phillips et al., 1991; Shattil et al., 1994).

1.1.4 Cadherins.

The cadherins are a multigene superfamily of predominantly homophilically binding Ca^{2+}-dependent cell-cell adhesion molecules. They are single pass/type 1 transmembrane glycoproteins (with the exception of T-cadherin described below) that contain in their extracellular domains an 110 amino acid motif repeated a number of times, the so-called cadherin repeat, which defines the superfamily. Each repeat includes the two putative Ca^{2+}-binding sequences, DXNDN and DXD. Family members play important roles in vertebrate development and morphogenesis (for reviews see Kemler et al., 1989; Marrs and Nelson, 1996; Takeichi, 1991, 1995), and
can be split into 4 main categories, the first of which, the classical cadherins, is described below.

1.1.4.1 Classical cadherins.

The classical cadherins include B-cadherin, E-cadherin, EP-cadherin (also called CLP for cadherin-like protein), N-cadherin, P-cadherin, R-cadherin, U-cadherin, and XB-cadherin. They are highly related at the amino acid level consisting of a large extracellular domain, a transmembrane domain and a highly conserved cytoplasmic domain of approximately 150 amino acids (Figure 2).

The extracellular domain consists of five related cadherin repeat regions, named EC1-5 from the N-terminus. The fifth, most membrane proximal, domain has low homology with the other four, but plays an important role in adhesion; Ozawa et al. (1991) showed that a monoclonal antibody directed against this domain (DECMAl) is sufficient to block cell-cell binding in adhesion assays. The N-terminal 113 amino acids, which includes the EC-1 domain, have also been shown to be essential for adhesion, as this region contains a site of adhesive interaction, and is the most conserved extracellular region between species and across subclasses. The site of adhesive interaction or cell adhesion recognition site (CAR) has been shown by Nose et al. (1990) to be centred around the HAV motif within this 113 amino acid segment, and alterations to the HAV sequence, or to the few amino acids on either side, drastically affect adhesive specificity.

Recently X-ray crystallography work (Nagar et al., 1996; Shapiro et al., 1995) and NMR studies (Overduin et al., 1995) have started to give some indications about how the cadherins might bind cells together at the molecular level. It is thought that the molecules on the cell surface align in pairs, each member of the pair then interacting with one of another pair on the opposing cell via the most N-terminal repeat regions, interdigitating in a zipper like mechanism (Figure 1).

Homophilic adhesion generally occurs between members of the same cadherin subclass, i.e. E-cadherin binds E-cadherin, although some evidence for heterotypic adhesion exists. Inuzuka et al. (1991) showed that R- and N-cadherins could bind
together when transfected into L-cells, although they showed a preference for homophilic binding; Murphy-Erdosh et al. (1994 and 1995) showed B- and E-cadherins bind heterophilically and are often co-expressed in the same tissue during chick development suggesting this heterophilic interaction may occur in vivo; Karecla et al. (1995) and Cepek et al. (1994) have shown that E-cadherin can bind the integrin $\alpha_6\beta_7$; and Williams et al. (1994a) have suggested that N-cadherin interacts with FGF receptor-1 (although biochemical evidence to support this remains to be shown) indicating that the cadherins could be involved in more complex interactions than previously thought.

The cytoplasmic tail of the classical cadherins links with the actin microfilament network via a group of molecules called the catenins, often at adherens junctions (see section 1.2.1.1 below), which are essential for cadherin-mediated adhesion. The integrity of the cytoplasmic region is fundamental to cadherin-mediated adhesion, and disruption of the cadherin-catenin complex induces loss of cytoskeletal binding and adhesiveness. It is thought that the cell modulates cadherin adhesion via this cytoplasmic domain, indeed Stappert and Kemler (1994) have shown that a cluster of serine residues within the C-terminal region of E-cadherin are needed for complex formation with the catenins, and that phosphorylation of these residues has an important role to play in controlling the adhesion complex integrity. Regulation of the cadherin-catenin complex is particularly important in morphogenesis when specific cells co-express certain cadherin types (see section 1.1.4.4).

**1.1.4.2 The catenins.**

$\alpha$-Catenin, a protein of 102 kD, is a vinculin-like molecule which is thought to connect directly to the actin microfilament network (Nagafuchi et al., 1991; Rimm et al., 1995) via its carboxyl terminus (Nagafuchi et al., 1994). It is essential for cadherin-mediated adhesion as illustrated by Watabe et al. (1994) when cell lines expressing cadherin but lacking $\alpha$-catenin were unable to adhere until transfected with $\alpha$-catenin, and by Nagafuchi et al. (1994) when C-terminal truncated non-functional E-cadherin was fused with $\alpha$-catenin and endowed transfected L-cells with adhesive properties. It does
not bind cadherin directly (Aberle et al., 1994), but is linked into the cadherin adhesion complex via β-catenin which it binds through its N-terminal domain (Jou et al., 1995).

β-catenin, a molecule of 92kD with homology to plakoglobin (PG) and the Drosophila segment polarity gene product armadillo, interacts tightly and directly with the cadherin cytoplasmic domain via its central armadillo repeat region (Hülsken et al., 1994), and binds α-catenin via its N-terminal domain (between amino acids 120 and 137; Aberle et al., 1994). It acts as a regulatable molecular bridge controlling cadherin-mediated adhesion, and when truncated disrupts adhesion (Oyama et al., 1994; discussed further in section 1.3).

γ-catenin also co-precipitates with cadherins. It has a molecular weight of approximately 83kD, but two dimensional gel electrophoresis has shown that it is composed of more than one protein (Piepenhagen and Nelson, 1993). One of its constituent proteins is PG, a member of the armadillo family of proteins with high levels of homology to β-catenin (see section 1.3), which binds α-catenin via its N-terminal domain (between amino acids 109 and 137; Aberle et al., 1996), and binds cadherin directly through its armadillo repeats (Sacco et al., 1995; Wahl et al., 1996) competing for the same site as β-catenin (Stappert and Kemler, 1994). The other γ-catenin protein(s) remains to be identified.

p120cas, another member of the armadillo family of proteins (Reynolds et al., 1992) initially identified as a src family tyrosine kinase substrate, has also recently been identified as a catenin (Aghib and McCrea, 1995; Reynolds et al., 1994; Shibamoto et al., 1995). It binds the cytoplasmic domain of cadherins directly (Jou et al., 1995; Shibamoto et al., 1995; Staddon et al., 1995), but it is not clear if it binds α-catenin in vivo (Jou et al., 1995; Shibamoto et al., 1995) and its role within the complex is not understood.

The catenins have also been shown to complex with other proteins, for example b-catenin has been shown to link the EGF receptor to the cadherin complex (Hoschuetzky et al., 1994), suggesting that EGF-mediated signalling has a role to play in modulating cadherin-mediated adhesion; and APC (a tumour suppressor protein of the armadillo family of molecules) directly competes with cadherins to bind catenin
complexes via β-catenin or PG (Hülsken et al., 1994; Rubinfeld et al., 1993; Su, 1993; discussed further in section 1.3).

1.1.4.3 Non-classical cadherins.
The non-classical cadherins can be considered in three groups discussed below.

1.1.4.3.1 Unconventional cadherins.
The second class in the cadherin superfamily is the unconventional cadherins. These include: M-cadherin, in which FAL replaces HAV at the adhesion interaction site (Donalies et al., 1991); LI-cadherin which has an extra repeat domain, AAL at the adhesion interaction site and a short cytoplasmic domain (Berndorff et al., 1994); cadherins-5 (also known as VE-cadherin) through to -12, discovered by PCR and present in the brain, which also differ at the adhesion interaction site but are similar in structure to the classical cadherins (Suzuki et al., 1991; Tanihara et al., 1994); T-cadherin which has a cadherin extracellular domain attached to the outside of the cell via a GPI-anchor (Ranscht and Dours-Zimmerman, 1991); and DE-cadherin (the Drosophila E-cadherin homologue) which has a much longer extracellular domain than the vertebrate molecules, due to the presence of additional repeats, the insertion of a cysteine-rich sequence and a sequence with similarity to the laminin-A globular domain, although the cytoplasmic domain is highly conserved (Oda et al., 1994).

1.1.4.3.2 Desmosomal cadherins.
The third class in the superfamily is the desmosomal cadherins, a group of molecules closely related to conventional cadherins with 5 extracellular cadherin-like repeats, but an alternative motif at the CAR site and considerably different cytoplasmic domains.

The desmosomal cadherins consist of two subclasses, the desmocollins (Dsc) and desmogleins (Dsg) which exist as a number of different isoforms; at least one Dsc and one Dsg are required to form desmosomes (see section 1.2.1.2). The desmosomal cadherin genes are clustered on chromosome 18 (Simrak et al., 1994; and personal communication Dr R. S. Buxton), and it seems likely that they arose from a primordial
gene which duplicated and diverged, initially giving rise to an ancestral desmocollin and desmoglein, each of which were then copied again at least twice giving rise to the three different isoforms known today. The desmocollins Dsc1, Dsc2, and Dsc3 each exist in two splice variant forms, a and b, which differ only at their C-termini (Figure 2). Their homologies at the amino acid level are 53% for Dsc1 and 2, 67% for Dsc2 and 3, and 52% for Dsc1 and 3 (King et al., 1995), with the cell-adhesion recognition sites being YAT, FAT and YAS for Dsc1, 2 and 3 respectively. The desmogleins Dsg1, Dsg2, and Dsg3 have an extra unique C-terminal repeat domain and RAL (Dsg1 and Dsg 3) or YAL (Dsg2) as the motif at the site of cell adhesion. Their homologies at the amino acid level are 37% for Dsg1 and 2, 40% for Dsg2 and 3, and 47% for Dsg1 and 3 (Schäfer et al., 1994). Dsg1 (Pemphigus foliaceous antigen) and Dsg3 (Pemphigus vulgaris antigen, PVA) are the autoantigens in the autoimmune blistering skin diseases of the same names (Allen et al., 1993; Amagai et al., 1992; Stanley, 1993).

The different isoforms of both the desmocollins and desmogleins are expressed in tissue-specific and developmentally-regulated patterns, although a single cell can express constitutively more than one type of Dsg and Dsc. Dsg2 and Dsc 2 are the most widely expressed isoforms of the desmosomal cadherins, being found in all desmosome-containing tissues including both simple and stratified epithelia, non-epithelial tissues such as heart, carcinomas, and many cultured epithelial cells, often being the only isoforms represented (Schäfer et al., 1994; Nuber et al., 1995; Koch and Franke, 1994). As such these desmosomal cadherins are proposed to be the fundamental isoforms synthesised during development, and are thought to be the most primordial/ancestral versions. The other desmosomal cadherins all have much more restricted expression patterns and are thought to be associated with differentiation of specific stratified epithelia: Dsc1 is detected only in epidermis and, unexpectedly, the lymph nodes (Nuber et al., 1995); Dsc3 is found in all stratified epithelia examined (Nuber et al., 1995); Dsg1 and Dsg3 are both restricted to certain specialised epithelia, mainly stratified squamous epithelia (Schäfer et al., 1994). The various isoforms also have specific expression patterns throughout the layers of the epidermis, reflecting
progressive differentiation states: Dsg1 is restricted to the uppermost layers of the epidermis (stratum comeum and granular layers); Dsg2 is found in the basal areas of the epidermis; Dsg3 is expressed only in the basal and lower spinous layers of the epidermis; Dsc1 is found in the upper spinous and granular layers of the epidermis; Dsc2 localises mainly to the lower cells of the epidermis; and Dsc3 has apparently the same localisation as Dsc2 (Amemann et al., 1993; Schäfer et al., 1994; King et al., 1995; Nuber et al., 1995; also see Buxton and Magee, 1992; Koch and Franke, 1994; Magee and Buxton, 1991, for further information).

Troyanovsky et al. (1993) have shown the cytoplasmic domains of the desmosomal cadherins to be important in assembling the plaque region of the desmosome (see section 1.2.1.2), with the long splice variant of desmocollin capable of recruiting the molecules necessary to form a desmosomal plaque. They have also shown that desmocollin 1a binds desmoplakin at a membrane-proximal 12 amino acid domain (Troyanovsky et al., 1994a). The desmogleins interact directly with PG via a centrally located 19 amino acid motif (Mathur et al., 1994; Troyanovsky et al., 1994b). This motif is present at the C-terminus of the desmocollins, which also interact directly with PG (Kowalczyk et al., 1994), and has significant homology to the 30 amino acid motif located at the C-terminus of the classical cadherins which interacts with β-catenin and PG (Stappert and Kemler, 1994).

1.1.4.3.1 Cadherin-like molecules.

The final class in the superfamily is the cadherin-like molecules. This is a diverse class of molecules that share motifs with the cadherins to varying degrees, usually in the extracellular domain, but that can vary in overall structure. The protocadherins are a group of molecules that are highly expressed in the brain of a variety of organisms and consist of 6 or 7 extracellular cadherin-like repeats, a transmembrane domain and a cytoplasmic domain that differs greatly from the conventional cadherins (Sano et al., 1993). Other molecules in this subclass include dachsous, a Drosophila gene product involved in imaginal disc morphogenesis, containing 27 cadherin repeats in its extracellular domain (Clark et al., 1995); and the
fat tumour suppressor gene that was initially discovered in *Drosophila* (Mahoney *et al.*, 1991), but has recently been found to have vertebrate homologues (Dunne *et al.*, 1995). The latter codes for a huge transmembrane protein (5147 amino acids) that includes 34 cadherin repeats in its extracellular domain and, like *dachsous*, has sequence similarity in its cytoplasmic domain to the β-catenin-binding domain of the classical cadherins.

1.1.4.4 Cadherins in development.

Cadherins have been shown to have important roles during development (for reviews see Marrs and Nelson, 1996; Takeichi, 1995; Takeichi *et al.*, 1990; Takeichi, 1988; and Takeichi, 1987). Cells expressing particular subclasses of cadherins specifically adhere and segregate together (Nose *et al.*, 1988; Nose and Takeichi, 1986) giving rise to the hypothesis that cadherins are involved in sorting cell types, particularly during morphogenesis, histogenesis and regeneration. One model to explain how cadherins might mediate differential segregation *in vivo* proposes that cells preparing to leave a parental group begin expressing a new cadherin, causing a change in adhesive specificity; the cells would then have lower affinity for some of their original neighbours and increased affinity for other cells which also express the new type of cadherin, leading to regrouping. Indeed spatiotemporal expression patterns of specific cadherins *in vivo* correlate well with morphogenetic events such as segregation of cells into layers. An example of this occurs during mouse neural development; undifferentiated ectoderm expresses E-cadherin only, but as the neural plate invaginates the levels of E-cadherin decrease and N-cadherin increase on the invaginating cells, until at neural tube closure N-cadherin only is present, with the overlying ectoderm still expressing E-cadherin. Similarly, neural crest cells down-regulate N-cadherin, express different cadherin types during migration (cadherin-11 has been suggested in the mouse; Hoffmann and Balling, 1995; Kimura *et al.*, 1995; Nakagawa and Takeichi, 1995), and finally express the cadherins of the target tissue when they reach their final destination (Crossin *et al.*, 1985; Duband *et al.*, 1988; Hatta *et al.*, 1987; Hatta and Takeichi, 1986; Ranscht and Bonner-Fraser, 1991; Thiery *et al.*, 1984). It has also been shown that disruption of E-cadherin-mediated adhesion causes an epithelial-
mesenchymal transition in epiblast cell explants from the mouse primitive streak (Burdsal et al., 1993).

As well as the expression pattern of cadherins changing with developmental stages, the adhesive intensity of cadherins can be strengthened or weakened by intracellular signals. Briecher and Gumbiner (1994) showed that C-cadherin-mediated cell adhesion activity of blastomeres in animal cap explants is down-regulated when treated with activin, although the actual levels of the cadherin remained constant. During compaction in the early mouse embryo stronger adhesion between blastomeres is induced by activation of E-cadherin already present on the cell surface (Fleming and Johnson, 1988), probably via protein kinase C and increased serine/threonine phosphorylation of E-cadherin (Bloom, 1989).

Further evidence of the importance of cadherins in development has been supplied by experiments using synthetic peptides containing the HAV motif which have been found to inhibit compaction in mouse embryos (Shirayoshi et al., 1983) and neurite outgrowth of astrocytes (Neugebauer et al., 1988; Tomaselli et al., 1988). E-cadherin knockout experiments in mice (Laure et al., 1994) have shown how essential the cadherins are in the early stages of development as death occurred before implantation. Heasman et al. (1994) showed that over-expression of cadherins in Xenopus embryos causes reduced dorsal axial structure formation. It has been suggested that a decrease in cadherin expression on the surface of tumour cells may be involved in invasion and metastasis (Behrens et al., 1989; Hashimoto et al., 1989; Shimoyama et al., 1989; Takeichi, 1993) leading to the suggestion that these molecules may also be tumour suppressor proteins.
1.2 Adhesive junctions.

See Figures 3 and 4, and Gumbiner (1996) and Rosales et al. (1995) for reviews.

1.2.1 Cadherin adhesive junctions.

Cadherin family members not only mediate cell adhesion transiently as described above, but also form part of the "adherens" type junctions to mediate more permanent adhesive interactions between cells (see Tsukita et al., 1992; Garrod, 1993, and below).

1.2.1.1 Adherens Junctions.

Adherens junctions (also known as zonula adherens) are calcium-dependent intercellular junctions, located beneath tight junctions but above desmosomes on the lateral surface of epithelial cells (Figure 3), which link into the actin cytoskeleton, and are essential for the establishment and maintenance of cell polarity; they must be present before other cell-cell junctions, such as tight and gap junctions, can be formed (Gumbiner et al., 1988; Jongen et al., 1991; Marrs and Nelson, 1996 and references therein). Electron microscopy reveals them to have an intercellular space of approximately 20nm filled with homogeneous amorphous material and an intracellular "loosely woven mat"-like plaque region. The transmembrane region of the junction is formed by classical cadherins which link via the catenins to the plaque structure where proteins such as tenuin, radixin, zyxin, vinculin and α-actinin link to the actin cytoskeleton. ZO-1 has also been localised to the plaque region of the adherens junction (Itoh et al., 1993; see section 1.2.2.3 below) although its role here remains to be determined.

Adherens junctions not only hold cells together but act as a signalling point from which the cell receives instructions and information about its environment to enable it to behave appropriately; the down-regulation of adherens junctions often precedes metastasis (Takeichi, 1993). Adherens junctions are a major site of protein phosphorylation, with protein kinase C (Tsukita et al., 1990), many proto-oncogenes including src family kinases (Tsukita et al., 1991), and the receptor type protein
FIGURE 3: The junctions of the cell.

Schematic diagram of a simple epithelial cell showing the various types of adhesive mechanisms used by the cell. Taken from Garrod (1986).
Apical Surface

Microvilli

Tight Junction

Zonula Adhaerens

Desmosome

Gap Junction

Non-junctional Membrane Adhesion

Focal Contact

Basal Lamina

Hemidesmosome
FIGURE 4: Connections to the cytoskeleton at the adherens junction and desmosome.

Schematic illustrating the major connections to the cytoskeleton at adherens junctions (A), and desmosomes (B). Adapted from Cowin and Burke (1996).

- Cadherin repeat domain with cell adhesion recognition site.
- C domain

β-cat  β-catenin
Pg    plakoglobin
α-cat α-catenin
αA    α-actinin
V     vinculin
IF    intermediate filament network
N     N terminus
C     C terminus
A

E-cadherin

E-cadherin

β-cat/Pg

αA

V

F-actin

B

Desmoglein

Desmocollin b form

Desmocollin a form

Desmoglein

Plakophilin 1
tyrosine phosphatase PTPμ (Brady-Kalnay et al., 1995) being associated with the junction. These together with other signalling pathways (including the Wnt pathway; see section 1.3.1.2.1) regulate junction stability, and cadherin function and signal into the cell, communicating its state within the higher order of the organism. Assembly of adherens junctions can be regulated by src-mediated tyrosine phosphorylation (Behrens et al., 1993; Hamaguchi et al., 1993; Matsuyoshi et al., 1992; Watabe et al., 1993), and protein kinase C-mediated signalling has been shown to regulate adherens junction formation and adhesiveness (Lewis et al., 1995; Williams et al., 1993; Winckel et al., 1990). The EGF receptor also co-localises with adherens junctions and can modulate junction protein phosphorylation levels; EGF-induced tyrosine phosphorylation of β-catenin or PG at the adherens junction induces disassembly of the cadherin-catenin complex and breakdown of the junctions (Hoschuetzky et al., 1994; Shibamoto et al., 1994). See Volberg (1992) and section 1.3.1.2 for further information about control of cadherin-mediated adhesion.

1.2.1.2 Desmosomes.

Desmosomes, also known as maculae adherentes, are rivet-like cell-cell junctions (0.1-0.5μm in diameter), found in most epithelia and the intercalated discs of the heart (as well as a few other tissues), where tissues are subject to mechanical stress. The intermediate filament network of cells links with the cytoplasmic side of desmosomes, such that shearing forces exerted upon a cell are dissipated through the tissue as a whole and thus play an important role in maintaining tissue integrity.

Cross-section electron micrographs through desmosomes reveal a highly organised, almost crystalline, intercellular gap region of about 20-30nm with an electron dense midline, and a dense plaque structure running parallel to (but slightly separated from) the inside of the plasma membrane, into which intermediate filaments loop (Figure 3).

Desmosomes are composed of a number of different proteins and glycoproteins (for reviews see Schwarz et al., 1990 and Garrod et al., 1990) which are sufficiently conserved across species that cells from animals as diverse as human and frog can
make desmosomes between them (Mattey and Garrod, 1985). The main components are the non-glycosylated proteins plakoglobin (see section 1.3.1.3) and the desmoplakins, which locate to the cytoplasmic plaque structure, and the transmembrane glycoproteins the desmosomal cadherins (see section 1.1.4.3.2).

Desmoplakin 1 (Green et al., 1990) is a molecule of 250kD, that probably aggregates into higher order structures, consisting of an N-terminal domain, a central domain containing a heptad repeat sequence characteristic of many α-fibrous proteins (leading to a two chained coiled-coil structure upon dimerisation), and a C-terminus composed of three regions (each of which includes approximately five repeats of a 38-residue motif) which fold to form a globular structure stabilised by intrachain ionic interactions. Desmoplakin 2, 215kD, lacks the central chain region as a result of alternative splicing (Green et al., 1990). Desmoplakins are found in the outer plaque of the desmosome where the intermediate filaments interact, and the globular C-terminal domain has been shown by deletion analysis to be involved in intermediate filament linkage (Stappenbeck et al., 1993; Stappenbeck and Green, 1992). The desmoplakins are part of a larger family including BPAG1 (Bullous pemphigoid antigen 1, a 240kD protein found in hemidesmosomes; Tanaka et al., 1991), IFAP-300 (intermediate filament associated protein of molecular weight 300kD, found in desmosomes and hemidesmosomes; Skalli et al., 1994), plectin (a 470kD protein found in the subplasmalemmal network as well as desmosomes in a number of cells; Wiche et al., 1991), and envoplakin (a 210kD component of desmosomes and the cornified envelope in epidermis involved in tylosis oesophageal cancer; C. Ruhrberg and F.M. Watt, personal communication).

Desmosomes are not simply static structures maintaining tissue integrity and strength in adults, they also have a more dynamic role to play during development. For example, studies of early mouse development show that they play the traditional strengthening role at blastocoel formation, helping the cell sheet withstand the build up of hydrostatic pressure, but later (for example in the detachment of neural crest cells from the dorsal lip and in hair formation) they are present then disappear selectively as cells segregate to form subpopulations, defining which of the original population will
form a given tissue (see Larsen and Wert, 1988). The role of desmosomal components in human and murine kidney formation has also been examined (Garrod and Fleming, 1990), and the results suggest they play a role here in early development. Data from I. King (personal communication) also support the notion that they are dynamic in early mouse development; different desmosomal cadherin isoforms are expressed in different tissues at different times presumably helping differentiate cell populations into separate groups.

1.2.2 Other cell junctions.

1.2.2.1 Focal adhesions/focal contacts.

These are cell-matrix contacts (Figure 3) where integrins cluster together and link into the actin cytoskeleton of the cell via the cytoplasmic domain of the β subunit (Sastry and Horwitz, 1993; Pavalko and Otey, 1994, and references therein). They are electron-dense structures which share many component proteins with adherens junctions. Proteins that localise to focal adhesions include the structural molecules: talin, which binds vinculin (Gilmore et al., 1993) and the integrin β subunit directly (Horwitz et al., 1986); α-actinin, which binds actin (Kuhlman et al., 1992), vinculin (McGregor et al., 1994), zyxin (Crawford et al., 1992), and also binds β subunits directly (Lewis and Schwartz, 1995; Otey et al., 1990); vinculin, which also binds actin (Kuhlman et al., 1992); vinculin, which also binds paxillin (Wood et al., 1994) and tensin (Lo et al., 1994); paxillin, which also binds pp125FAK (Turner and Miller, 1994); tensin; fimbrin, which binds and bundles actin (Pavalko and Otey, 1994); and zyxin. The calcium-dependent protease calpain II (Beckerle et al., 1987), various kinases including focal adhesion kinase pp125FAK (Schaller et al., 1992), protein kinase C (Jaken et al., 1989; Woods and Couchman, 1992), src kinases (Rohrschneider, 1980), and small G proteins (Ridley and Hall, 1992; Symons and Mitchison, 1992) also associate with focal adhesions indicating that as well as an adhesive role this complex has a signalling role. Indeed integrin ligation-induced phosphorylation of pp125FAK has been observed in a number of different cells including fibroblasts and platelets (Freedman et al., 1993; Guan et al., 1991; Hanks et al., 1992; Kornberg et al., 1992; Kornberg et al., 1991; Lipfert et al., 1992), and may
be involved in cytoplasmic signalling to the nucleus (Juliano and Haskill, 1993). Ligand binding also leads to other signal transduction events such as elevation of intracellular pH and Ca^{2+} levels (Richardson and Parsons, 1995).

It has been shown that tyrosine phosphorylation is probably involved in complex assembly and disassembly; phosphorylation of paxillin and pp125^{FAK} is concomitant with focal adhesion formation (Burridge \textit{et al.}, 1992; Romer \textit{et al.}, 1992). For further reviews see Clark and Brugge (1995), Gumbiner (1993b), Gumbiner (1996), Huttenlocher \textit{et al.} (1995), Richardson and Parsons (1995), and Rosales \textit{et al.} (1995).

1.2.2.2 Hemidesmosomes.

These are punctate junctions that mediate adhesion of basal cells to the basement membrane in stratified and transitional epithelia (Figure 3). Ultrastructurally they resemble half desmosomes with a dense plaque underlying the plasma membrane with cytokeratin filaments in close association, but at a molecular level they are very different. The main adhesive molecule of hemidesmosomes is the integrin α_6β_4, which binds laminin in the basement membrane and links back to a plaque structure within the cell. It is thought that BPAg2, the 180kD bullous pemphigoid antigen which has regions of homology to the triple helical domain of collagen, may also play a role in adhering to the matrix. The plaque structure includes: the 230kD bullous pemphigoid antigen (also known as BPAg1), which has repeat units homologous to some found in desmoplakin suggesting it may play a similar role to that of desmoplakin in desmosomes; a 200kD protein possibly providing the linkage to the intermediate filament network; a 500kD protein called HD-1 about which little is known that may be involved in intermediate filament attachment.

1.2.2.3 Tight junctions.

The tight junction, also known as the zonula occludens, is an area where two cells become so closely apposed that under the electron microscope the outer leaflets of adjoining cell membranes apparently converge. By freeze-fracture analysis they appear
as long parallel, ribbons of intramembranous particles that surround the cell, with shorter ribbons interconnecting the main array (see Gumbiner, 1993a and Citi, 1993 for mini-reviews). They form apically in epithelial cells lying above both the adherens junctions and desmosomes (Figure 3) and are involved in establishing and maintaining epithelial and endothelial cell polarity. They form a seal with regulatable permeability to small ions around the cell, essentially segregating the extracellular environment of the cell physically into apical and basolateral areas that cannot mix. There appears to be a diffuse band of cytoplasmic material just inside the junction where it is thought actin filaments link into the tight junction.

Occludin, a protein of 65kD that spans the membrane four times (as do the connexins in gap junctions), is the tight junction integral membrane protein (Furuse et al., 1993) and binds ZO-1 and ZO-2. ZO-1 (a 225kD protein also found at adherens junctions of non-epithelial cells; Itoh et al., 1993), ZO-2 (a 160kD protein which has been shown to immunoprecipitate with ZO-1; Gumbiner et al., 1991), cingulin (a molecule of 140kD which is structurally similar to the rod portions of myosin; Citi et al., 1988), and 7H6 (a 155kD protein; Zhong et al., 1993) all localise to this cytoplasmic plaque region of tight junctions. It is thought that spectrin may also have a role in the plaque region as tetramers can bind ZO-1 (Itoh et al., 1991).

1.2.2.3.1 Dlg-R or MAGUKs (Membrane Associated GUanylate Kinase) family of proteins.

ZO-1 and ZO-2 are part of the expanding Dlg-R family, the first family in which the 80-90 amino acid DHR repeat was discovered. The Dlg-R family includes the Drosophila tumour suppressor protein Discs large (dlg, involved in the formation of septate junctions), ZO-1, ZO-2, SAP90/PSD-95, and p55. The members are thought to play roles in signal transduction at tight, septate and synaptic junctions (reviewed in Kim, 1995 and Anderson, 1996), and interestingly the human homologue of dlg has recently been shown to bind the armadillo repeat-containing tumour suppressor protein APC (see section 1.3 and Matsumine et al., 1996). The family members contain between 1 and 3 DHR repeats, an SH3 motif and a modified guanylate kinase domain.
The DHR repeat is thought to act like SH2 or SH3 domains in that it appears to bind motifs on other molecules, in particular the N-terminal domain of band 4.1 family protein molecules (which includes ezrin, moesin and radixin), and may act to localise multidomain proteins to cell-cell junctions thus enabling specific signalling functions. DHR repeats are more widespread than previously thought being present in molecules without the SH3 and guanylate kinase domains such as the *Drosophila* wingless signalling pathway protein dishevelled, the *lin-2* gene product involved in *C.elegans* vulval development, AF-6 protein, five syntrophins, neuronal nitric acid synthase, and a number of protein tyrosine phosphatases including PTP-BAS, PTPH1, and PTP-MEG (Ponting and Phillips, 1995).

1.2.2.4 Gap junctions.
These are intercellular communicating junctions where two cells become closely apposed and allow the electrical and chemical coupling of the cells (Figure 3). The intercellular gap of 2-4nm is bridged by a lattice of subunits which allow small hydrophilic molecules (of 1kD or smaller) to pass freely between the two cells. A junction is formed when two "connexons" from opposing cells associate head to head. A connexon consists of a hexamer of connexin molecules arranged within the membrane such that a central pore region is formed; the connexons associate such that the pores line up to form a regulatable channel. For a review see Beyer (1993).

The connexins are a multigene family of non-glycosylated proteins of 26-43kD that span the membrane 4 times with both the N- and C-termini lying within the cell and which are expressed in a cell specific pattern (Willecke *et al.*, 1991). The transmembrane and extracellular domains of the connexins are highly conserved; the cytoplasmic domain is more structurally diverse, possibly being important for allowing specific control of the junctions in certain cell types.

Communication by gap junctions can be controlled by intracellular Ca\(^{2+}\); when levels are raised the connexins tilt and the channel closes, when levels fall the connexins become more upright and the channel opens and allows the movement of molecules between cells (Unwin and Ennis, 1984). Calmodulin has been shown to bind
sequences on the intracellular side of the junction and it is thought that it may have a role in modulating gap junction function (Beyer, 1993; Zimmer et al., 1987). Cadherin-mediated adhesion is a prerequisite for gap junction formation (Mège et al., 1988; Meyer et al., 1992), and E-cadherin has also been shown to modulate gap junction communication by post-translationally regulating connexin 43 (Jongen et al., 1991). It has been shown that in cancerous cells gap junction communication is much reduced (Klann et al., 1989, and cell phenotype can be normalised by transfection with connexin cDNA inducing more gap junction communication (Eghbali et al., 1990; Evans, 1992). When Wnt-1 RNA is injected into fertilised Xenopus eggs there is an increase in gap junction communication in the ventral blastomeres before axis duplication (Olson et al., 1991) indicating that gap junction communication has important roles to play in pattern formation.
1.3 Armadillo superfamily of proteins.

This is a family of intracellular proteins characterised by the presence of a so-called armadillo (arm) repeat, a hydrophobic motif of approximately 42 amino acids, found imperfectly repeated a number of times within the molecules (Figure 5). Members of this superfamily include PG, β-catenin, armadillo itself (a Drosophila protein shown to have functions in the wingless signalling pathway and in the Drosophila equivalent of adherens junctions), p120cas src kinase substrate (which localises to the adherens junction as well as existing in a cytoplasmic pool), plakophilin 1 (also known as band 6 protein, which is associated with certain types of desmosomes, binds keratins and which has more similarity to p120 than to other members of the family; Hatzfeld et al., 1994; Heid et al., 1994), p0071 (a protein found in adhesion plaques; Hatzfeld and Nachtsheim in press), APC (the tumour suppressor adenomatous polyposis coli gene product; Birchill, 1994), smgGDS (an exchange factor for ras-related small G proteins which gets phosphorylated on tyrosine residues; Kikuchi et al., 1992), regulatory subunits of protein phosphatase 2A from yeast and human (van Zyl et al., 1992), EF3 (a yeast translation elongation factor; Qin et al., 1990), α- and β-adaptin (conserved adaptor molecules from coated pits; Ponnambalan et al., 1990), srp1 (a Saccharomyces cerevisiae suppressor of RNA polymerase 1 mutations involved in nuclear protein transport; Yano et al., 1992), and related proteins RCH1 (a protein that interacts with the V(D)J recombination inducing protein RAG-1; Cuomo et al., 1994), importin (a protein that promotes nuclear import of karyophilic proteins; Görlich et al., 1994), and pendulin (a tumour suppressor molecule in haematopoietic cells that shuttles between the cytoplasm and nucleus in a cell-cycle dependent manner; Küssel and Frasch, 1995).

The role of the repeat region is still unknown but given that it is present in a variety of proteins, which perform a range of functions, it has been postulated that it might act as an adapter region bringing proteins together into specific complexes (Peifer et al., 1994a). Indeed this idea is supported by studies of β-catenin and PG which show that different molecules bind to various domains on the proteins; cadherin or APC bind in a mutually exclusive fashion to the armadillo repeat region of PG or β-
FIGURE 5: The armadillo family of molecules.

Schematic representation of the main members of the armadillo superfamily.

- approximately 40 amino acids
- inserts found in alternative splice variants of p120 and p0071
- Armadillo repeat based on the imperfect repeat consensus (Peifer et al., 1994a):

\[
\begin{align*}
\text{LKNLS}^* & \quad \text{K}^* \quad \text{NK}^* \quad \text{ALL}^* \quad \text{GGLPALV} \quad \text{KLL}^* \quad \text{S}^* \quad \text{KE}^* \quad \text{L}^* \quad \text{AA}^* \quad \text{A} \\
\text{R} & \quad \text{R} & \quad \text{R} & \quad \text{I} & \quad \text{I} & \quad \text{R} & \quad \text{R} & \quad \text{I} & \quad \text{H} & \quad \text{H} & \quad \text{H} & \quad \text{VV} & \quad \text{V} & \quad \text{H} & \quad \text{H} & \quad \text{V}
\end{align*}
\]

Subfamilies homology:

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Armadillo</td>
<td>60-70% identity</td>
</tr>
<tr>
<td>Plakoglobin</td>
<td></td>
</tr>
<tr>
<td>β-catenin</td>
<td></td>
</tr>
<tr>
<td>p120</td>
<td></td>
</tr>
<tr>
<td>Plakophilin 1</td>
<td>33% identity</td>
</tr>
<tr>
<td>p0071</td>
<td></td>
</tr>
<tr>
<td>Pendulin</td>
<td></td>
</tr>
<tr>
<td>Srp-1</td>
<td>48-62% identity</td>
</tr>
<tr>
<td>Rch-1</td>
<td></td>
</tr>
<tr>
<td>Importin</td>
<td></td>
</tr>
<tr>
<td>APC</td>
<td></td>
</tr>
<tr>
<td>smgGDS</td>
<td></td>
</tr>
<tr>
<td>Protein Phosphatase 2a</td>
<td>&lt;25% identity</td>
</tr>
<tr>
<td>Elongation Factor 3</td>
<td></td>
</tr>
<tr>
<td>β-Adaptin</td>
<td></td>
</tr>
</tbody>
</table>

Armadillo
Plakoglobin
β-catenin
p120
Plakophilin 1
p0071
Pendulin
Srp-1
Rch-1
Importin
APC
smgGDS
PP2a
EF3
β-Adaptin
catenin (Hülsken et al., 1994), α-catenin binds the N-terminal armadillo repeat region of PG (Aberle et al., 1996; Sacco et al., 1995) or β-catenin (Aberle et al., 1994), and the EGF receptor binds the central core region of β-catenin whilst the latter remains in a cadherin complex (Hoschuetzky et al., 1994). The arm repeats do not appear to bind homophilically, different motifs are involved in recognising these sites.

The proteins in this family have a variety of functions within the cell. Some members are involved in cell adhesions, for example PG at both the adherens and desmosomal junctions, β-catenin and p120 at the adherens junction and plakophilin at the desmosome. Others are involved in signalling functions, for example β-catenin (Funayama et al., 1995; Guger and Gumbiner, 1995), smgGDS (Hiraoka et al., 1992), p120 (Downing and Reynolds, 1991; Reynolds et al., 1989) and APC (Peifer, 1996). A number of proteins in this family bind to each other; APC binds to the armadillo repeat region of PG and β-catenin (Hülsken et al., 1994; Rubinfeld et al., 1995; Shibata et al., 1994), while PG forms homodimers in the cytoplasm of the cell (Cowin et al., 1986; Kapprell et al., 1987).

The armadillo repeats might also be involved in nuclear localisation. Importin is involved in nuclear protein transport; it allows the binding of karyophillic proteins to the nuclear pore complex, although whether importin acts by binding to the nuclear localisation signal (NLS) of the protein and carrying it to the pore complex, or by exposing a site for the NLS on the pore complex itself, is not known (see Adam, 1995 and references therein for further information). Also, when PG, β-catenin or deletions of β-catenin essentially containing just the armadillo repeat region are microinjected into Xenopus embryos the molecules localise to the nucleus (see section 1.3.1.2.1 below, Karnovsky and Klymkowsky, 1995 and Funayama et al., 1995), and this appears to be an endogenous mechanism as recent reports (W.W. Franke, personal communication, UCL seminar 1995) indicate that when isolated under mild conditions, plakophilin can be found in the nucleus of a number of different cell types. This possible nuclear localisation effect and the fact that a number of members of this family are involved in signalling makes it interesting to speculate that at least some of the armadillo repeat-containing proteins might act in a manner analogous to the
JAK/STAT system of signal transducers (see Briscoe et al., 1994 for mini-review on JAK/STAT signalling pathway). In this scenario family members would be modified by an initial signal, released from a specific cellular site and go on to bind specific targets via the armadillo repeat domains. Instead of the signal being passed on by other molecules in a cascades (for example as is the case in the MAP kinase cascade), the protein would move from the original subcellular location (the membrane or cytoplasm) to the nucleus, transmitting the signal directly itself.

1.3.1 Similarity between PG, β-catenin and armadillo.

β-catenin, PG and armadillo are the most related molecules of the armadillo family. β-catenin is considered the vertebrate orthologue of the Drosophila protein armadillo, the two proteins sharing 71% identity over their entire length (Xenopus β-catenin and Drosophila armadillo; Peifer et al., 1992). PG and β-catenin are 65% identical over their entire length (Xenopus sequences; Fouquet et al., 1992), whereas PG and armadillo share 63% identity over their whole length (Drosophila armadillo and human PG; Peifer and Wieschaus, 1990). The homology between these molecules increases significantly when the armadillo repeat regions alone are considered. It is interesting to note that the homology between the same repeat in these different proteins is higher (approximately 75%) than between different repeats in the same protein (approximately 20-30%) despite the evolutionary distance. This suggests that individual repeats may have specific functions modulating different protein-protein interactions, the molecule acting in a modular fashion, perhaps like the different EGF-repeats in the Drosophila notch protein mediating interactions with specific ligands (Davis, 1990; Kelley et al., 1987; Rebay et al., 1991).

1.3.1.1 Armadillo (arm).

The Drosophila protein armadillo exists as two isoforms, a predominant isoform of 105-110kD, and a nervous system-specific isoform of 82kD produced by alternative splicing (Peifer et al., 1993a). The smaller isoform lacks the C-terminal domain of the
protein entirely and it is thought that this truncation may determine to some extent the role of armadillo in this environment.

Armadillo exists mainly (approximately 75%) as a membrane-bound pool where it plays an adhesive role in the Drosophila version of adherens junctions (Peifer, 1993), presumably modulating cell adhesion in a fashion similar to that of β-catenin in vertebrate adherens junctions (see section 1.3.1.2 below). The signalling molecules notch (Fehon et al., 1991), sevenless (Tomlinson et al., 1987) and abl (Bennett and Hoffman, 1992) all localise to the Drosophila adherens junction and may play a role in junction regulation. Studies of truncated armadillo proteins in oogenesis have shown that proteins lacking the C-terminal domain of armadillo, similar to the truncated nervous system isoform, are sufficient for cell adhesion and integrity during oogenesis, whilst removing further repeat regions disrupts oogenesis (Peifer et al., 1993b). This indicates that the N-terminal and repeat regions of armadillo are sufficient for the proteins adhesive function.

Despite the fact that most armadillo in the cell is involved in adhesions, its cytoplasmic form, which plays a role in the wingless signalling pathway, has been better studied (see below and Peifer et al., 1993a and references therein for review). The C-terminal portion of armadillo is necessary for this signalling function, and it is thought that the truncation of the nervous system isoform prevents it acting in this manner in that location.

1.3.1.1 Armadillo in the Drosophila wingless signalling pathway.

During development of a multicellular organism a single cell has somehow to become a mass of cells with different functions and fates in specific positions. This setting up of a body plan, or pattern formation, has been studied in a number of different organisms (Gilbert, 1994). During Drosophila development an early stage of body plan development involves the segmentation of the larva, with each cell in a segment then being assigned a position (more anterior or posterior) by the so called "segment polarity genes". The segmentation and polarity is reflected in a distinctive denticle pattern on
the ventral side of the embryo, the larval cuticle pattern, which acts as a convenient marker for the underlying cells' fate.

Wingless is one of the segment polarity genes (reviewed in Klingensmith and Nusse, 1994; Perrimon, 1994), which also has important roles to play at later stages of development but for the purposes of this section it will only be considered at this early stage of development (see section 1.3.1.2.1 for references of other roles of wingless). The wingless gene product is a secreted morphogenetic glycoprotein, one of whose functions is to instruct posterior cells in the segment to secrete naked cuticle; when wingless signal is interfered with, the cuticle develops with denticles all over indicating that the cells within the segment have not been assigned their proper fate; all cells have adopted an anterior fate.

Armadillo is one of the proteins involved in the wingless signal transduction cascade, and the accumulation of a stripe of armadillo protein within the segment, mimicking wingless localisation, is one of the first cellular responses in this pathway. The regulation of armadillo is fundamental to the control of wingless signalling and is performed post-translationally. Absolute levels of armadillo do not seem critical for wingless signalling, rather the relative levels of membrane-bound versus cytoplasmic forms seem to be important (Peifer et al., 1994c; Peifer and Wieschaus, 1990), although the levels of armadillo present at the membrane appear to be relatively constant whether in wild type, wingless or shaggy (see below) mutants. This supports the notion that the membrane-associated pool is not involved in wingless signal transduction and that the molecule has at least two different roles, one for adhesion and one for signalling (Peifer et al., 1994c).

The gene product immediately upstream of armadillo in the wingless signalling cascade, as identified by genetic epistasis studies (Martinez Arias, 1994; Peifer et al., 1994c), is shaggy (also known as zeste white-3), a serine/threonine kinase (Figure 6). Glycogen synthase kinase-3 (GSK-3β) is the vertebrate orthologue of shaggy, with 76% identity, and is regulated by tyrosine phosphorylation; unusually, it is normally phosphorylated on tyrosine in resting cells (Hughes et al., 1993). In the absence of wingless signal shaggy is active and, either directly or indirectly, phosphorylates
FIGURE 6: Schematic of wingless signalling pathway during segmentation of *Drosophila*.

Taken from Magee (1995). Recently Dfz 2 (a novel member of the *frizzled* gene family) has been shown to act as a wingless receptor (Bhanot *et al.*, 1996).

**Gene Products:**
- Wg: wingless
- Porc: porcupine
- Dsh: dishevelled
- Zw3: Zeste-white 3
- Arm: armadillo
- Dcad: *Drosophila* cadherin
- PTK: Protein tyrosine kinase
- rrp: ras-related protein
- En: engrailed
- Hh: hedgehog
- Ptc: patched
- Fus: fused
- Cos2: costal-2
- Ci^D: cubitus interruptus
- Gsb: gooseberry

**Genes:**
- en: engrailed
- ptc: patched
- ci-D: cubitus interruptus
- wg: wingless
armadillo which results in very little of the protein being present in the cytoplasm, probably due to an increase in turnover rate, so inhibiting armadillos signalling function. However, when wingless is present, shaggy is inhibited through dishevelled (one of the DHR domain-containing proteins mentioned in section 1.2.2.3.1), allowing the dephosphorylation of armadillo (Magee, 1995; Noordermeer et al., 1994; Siegfried et al., 1994; Yanagawa et al., 1995). This results in an accumulation of the cytoplasmic pool of armadillo allowing the protein to act as a signal transducer possibly by activating an inactive cytoplasmic kinase, or by regulating nuclear import of some factor leading to gene transcription and cell fate determination.

Mutations of armadillo that lack the C-terminal domain are less sensitive to phosphorylation induced by shaggy, which supports the theory that this area of the molecule is involved in wingless signalling whilst the truncated molecule has been seen to still be sufficient for adhesion (Peifer et al., 1994b).

Tyrosine phosphorylation of armadillo has also been noted (Peifer et al., 1994b) and may be involved in a different regulatory pathway modulating armadillo function, perhaps regulating junctional assembly in a manner similar to that of β-catenin in vertebrate adherens junctions. Peifer et al. (1994b) have shown that the membrane-bound form of armadillo, which is most serine/threonine phosphorylated, is also the most tyrosine phosphorylated (this appears to be unlike β-catenin where tyrosine phosphorylation seems to destabilise adherens junctions - see below).

1.3.1.2 β-catenin.

β-catenin is a 92kD intracellular protein found both in a soluble pool and complexed with cadherins and α-catenin at adherens junctions. It is necessary for cadherin-mediated cell-cell adhesion functioning as a regulatable molecular bridge between the adhesive molecule (cadherin) and the cytoskeleton linker protein (α-catenin). Cadherins bind to the central armadillo repeat domain of β-catenin (Hülsken et al., 1994), and α-catenin binds the N-terminal region (Aberle et al., 1994). It plays a central regulatory role in cell adhesion modulating the strength of cadherin-mediated adhesion, during development controlling cadherin-mediated morphogenetic
movements, and in the adult preventing or allowing tumour progression. It also has an important adhesion-independent signalling role involved in determining cell fate.

It has become increasingly clear over recent months that β-catenin is controlled in a variety of ways and the final regulatory picture is not clear or simple. One of the ways that β-catenin is regulated is by tyrosine phosphorylation. Volberg (1992) showed that there is a strong correlation between loss of adhesion and the state of tyrosine phosphorylation of adherens junction proteins, especially β-catenin, and Sommers et al. (1994) showed that the tyrosine phosphorylation state of β-catenin is important for the level of adhesiveness in E-cadherin transfected cells. However, recent work by Takeda et al. (1995) with cells that express a cadherin-α-catenin fusion protein has shown that tyrosine phosphorylation of other adherens junction proteins may be more involved than previously thought in regulating adhesion. Tyrosine phosphorylation of β-catenin can be induced by a number of stimuli: Shibamoto et al. (1994) showed tyrosine phosphorylation of β-catenin stimulated by EGF or HGF/SF causes loss of adhesiveness, and Matsuyoshi et al. (1992) showed v-src transformation of cells results in tyrosine phosphorylation of some adherens junction proteins that are normally serine/threonine phosphorylated including β-catenin. Kinch et al. (1995) showed that tyrosine phosphorylation of β-catenin induced by ras-transformation results in reduced adhesion. The site of tyrosine phosphorylation on β-catenin remains to be mapped precisely although Hoschuetzky et al. (1994) obtained tyrosine phosphorylation with recombinant EGF receptor of β-catenin but not of a the core region alone indicating that the phosphorylation sites are in the terminal domains of the protein.

The EGF receptor has been shown to co-localise with cadherins in the basolateral membrane of epithelial cells (Fukuyama and Shimizu, 1991), and β-catenin has been shown to associate with the EGF receptor via the armadillo repeat region (Hoschuetzky et al., 1994) probably acting as a link to the cadherin complex. The autophosphorylated EGF receptor is thought to associate with the cadherin-catenin complex, and the tyrosine phosphorylation of β-catenin induced by receptor activation is thought to cause the release of the cadherin-catenin complex from the actin
microfilament network resulting in the loss of cell adhesion seen by Shibamoto et al. (1994) mentioned above. β-catenin has also been shown to bind the core region of the c-erbB-2 gene product (a region with extensive homology to the EGF receptor), and it is possible that phosphorylation of β-catenin induced by this oncogenic tyrosine kinase destabilises cadherin-mediated adhesion helping lead to metastasis (Kanai et al., 1995).

β-catenin has also been reported to be serine/threonine phosphorylated and given the proteins homology with armadillo, this phosphorylation may be involved in regulating the signalling of β-catenin in the Wnt pathway (see section 1.3.1.2.1 below); indeed recent work by Yost et al. (1996) has shown that Xgsk3 (the *Xenopus* homologue of shaggy) directly phosphorylates β-catenin *in vitro* and *in vivo*. Reports by Lechleider et al. (1994) and Byers et al. (1994) at the 1994 ASCB meeting show that in certain breast cancer cell lines β-catenin associates with a serine/threonine kinase, and that serine phosphorylation modulates both the levels and subcellular localisation of β-catenin; okadaic acid treatment leads to increased β-catenin levels, and serine phosphorylated β-catenin is targeted to the membrane, as is the case with serine/threonine phosphorylated armadillo in *Drosophila*.

An additional regulatory mechanism of β-catenin involves APC, another member of the armadillo family of molecules. APC is a cytoskeleton-associated tumour suppressor protein of 2843 amino acids implicated in familial adenomatous polyposis coli, an inherited autosomal disorder characterised by the early onset of multiple adenomatous polyps of the colon and a high likelihood of colon carcinoma (see Burchill, 1994; Peifer, 1996; Polakis, 1995 for further information about APC). Its role within the cell is still not fully understood but it has been shown to act by modulating the intracellular levels, and so function, of β-catenin within the cell. Munemitsu et al. (1995) showed that APC regulates β-catenin levels by down-regulating the amount of "free" (i.e. uncomplexed) β–catenin found in the cell and targeting it for degradation. Recently, Rubinfeld et al. (1996) showed that APC has a key role to play in the Wnt signalling pathway (see below) by physically linking GSK-3β and β-catenin in a GSK-3β-phosphorylation-dependent manner, regulating intracellular levels of β-catenin. Work by Papkoff et al. (1996) confirms the role of
APC in the Wnt signalling pathway by showing that Wnt-1 signal transduction stabilises the APC-catenin complex. APC binds β-catenin at multiple independent sites (Rubinfeld et al., 1995) and directly competes with E-cadherin for the central armadillo repeat region of β-catenin (Hülsken et al., 1994). The fact that APC binds β-catenin in the armadillo repeat region means that α-catenin can remain in the complex possibly linking APC to the actin cytoskeleton. APC has been shown to bind to microtubules and it is possible that it acts to link the catenins to this network allowing a different and as yet unidentified form of regulation (Munemitsu et al., 1994; Smith et al., 1994). Some of the most common mutations in APC compromise its tumour suppressor activity by preventing its ability to regulate β-catenin levels (Munemitsu et al., 1995), again indicating the central importance of β-catenin in the cell.

1.3.1.2.1 The Wnts in early Xenopus pattern formation.

The Wnts are a family of secreted glycoproteins that encode the vertebrate homologues of wingless, and have been shown to have spectacular roles to play in early vertebrate pattern formation. For example McMahon and Bradley (1990) and Thomas and Capecchi (1990) have shown that homozygous Wnt-1 null mutants lead to embryonic death in mice due to the complete lack of cerebellum development. Recently the Wnt genes have been grouped into two functional classes, the Wnt-1 and Wnt-5A classes, based on assays performed in Xenopus embryos and mammalian cells lines (Du et al., 1995; Moon et al., 1993; see section 1.4 for outline of early Xenopus development). Members of the Wnt-1 class (including XWnt-1, -3A, -8, and -8b), when ectopically expressed in Xenopus blastomeres, induce secondary axis formation and rescue Nieuwkoop centre activity in UV-ventralised embryos suggesting that Wnt signalling is part of the organising activity in Xenopus (Du et al., 1995; McMahon and Moon, 1989; Smith and Harland, 1991 and reviewed in Moon et al., 1993), whereas members of the Wnt-5A class (including XWnt-5A, -4, and -11) inhibit morphogenetic movements without overtly altering cell fate (Du et al., 1995; Moon et al., 1993). Similarly the mouse Wnt genes have been grouped according to their transforming ability of C57MG
cells; Wnt-1 family members (Wnt-1, -3A, and -7A) transform at high frequency, whereas Wnt-5A members (Wnt-4, and -5A) do not (Wong et al., 1994).

In early *Xenopus* development XWnt 11 and XWnt 5A are both expressed from egg onwards, and XWnt 8 expression begins at the mid-blastula transition. The other Wnts come on at later stages (see Moon et al., 1993, for summary). Wnts of the class 1 family also cause an increase in gap junction communication (Olson et al., 1991), and given that gap junction communication has been implicated in pattern formation (Fraser et al., 1987; Warner and Lawrence, 1982), this supports the potential role of Wnts in early pattern formation in vertebrates. Xwnt 11 is found in the expected expression pattern for a molecule involved in setting up the initial organising centre (see section 1.4.6 below and Slack, 1994 and Ku and Melton, 1992).

Other members of the Wnt signalling pathway have also been shown to produce similar disruptions to normal pattern formation in *Xenopus*: Sokol et al. (1995) have shown that dishevelled can induce secondary axes when over-expressed in prospective ventral mesoderm blastomeres, and He et al. (1995), and Pierce and Kimelman (1995) have produced similar effects with dominant negative injections of GSK-3β mRNA. Also Bradley et al. (1993) and Hinck et al. (1994) have shown that levels of β-catenin and PG accumulate upon increased expression of Wnt-1 (by post-translationally increasing their metabolic stability) showing the pathway is conserved between *Drosophila* and vertebrates.

Wnt family molecules are also implicated in other stages of vertebrate and invertebrate development including CNS development (vertebrates: Shimamura et al., 1994; Smolich and Papkoff, 1994; Wolda et al., 1993; invertebrates: Fradkin et al., 1995; Kaphingst and Kunes, 1994), limb polarity specification (vertebrates: Parr and McMahon, 1995; invertebrates: Wilder and Perrimon, 1995), dermal development (Tanda et al., 1995), and their abnormal expression can give rise to cancers (Huguet et al., 1995). For general reviews see Klingensmith and Nusse (1994) and Parr and McMahon (1994).
1.3.1.2.2 β-catenin in early *Xenopus* Wnt pathway.

The role of β-catenin within the *Xenopus* Wnt pathway has been confirmed recently. McCrea *et al.* (1993) showed that when Fab fragments generated against β-catenin are microinjected into blastomeres, secondary axis embryos are generated. Heasman *et al.* (1994) showed that depletion of β-catenin by injection of antisense RNA reduces dorsal mesodermal structures, and Funayama *et al.* (1995) showed that over-expression of β-catenin or the armadillo repeat region alone is sufficient to induce secondary axes. β-catenin can also increase gap junction communication (Guger and Gumbiner, 1995) showing that it mimics all of the activities of members of the Wnt family studied so far.

Work by Guger and Gumbiner (1995) suggest that β-catenin may act as part of the *Xenopus* Nieuwkoop centre (see section 1.4 for explanation of early *Xenopus* development and definition of Nieuwkoop centre). When it is over-expressed by microinjection of RNA into the vegetal ventral region in 4-32 cell embryos, secondary axis embryos are produced but the cells over-expressing β-catenin do not themselves contribute to axial structures directly; β-catenin does not alter cell fate autonomously but acts to create an inductive centre for axis specification. They also showed that β-catenin can rescue UV-irradiated embryos indicating that it can contribute to *de novo* axis formation, but that β-catenin alone is insufficient to induce mesodermal markers or make animal caps elongate (unlike activin or noggin). Fagotto and Gumbiner (1994) have also showed that β-catenin expression is concentrated at the dorsal blastopore lip during gastrulation, suggesting that it may have a role to play in involution or dorsalisation as well as a role in the Nieuwkoop centre, although it could well be acting more as an adhesion modulating protein here, less as a signalling molecule.

1.3.1.3 Plakoglobin.

Plakoglobin (PG) is a molecule of 83kD that is highly conserved throughout evolution. It is unique because it is expressed in the plaque regions of both desmosomes (where it has traditionally been studied) and adherens junctions. Like armadillo and β-catenin, it also exists in a soluble cytoplasmic pool and has been postulated to have a signalling role as well as its better known role in adhesive junctions. PG binds α-catenin at its N-
terminal region (Aberle et al., 1996; Sacco et al., 1995), has been shown to bind APC in a manner similar to β-catenin (Rubinfeld et al., 1995), and when tyrosine-phosphorylated it associates with classical cadherins but not with APC (Shibata et al., 1994). Again like β-catenin it binds to the core region of the c-erbB-2 gene product suggesting it has a role in to play in signalling as well as simply in adhesion (Kanai et al., 1995), and phosphorylation on tyrosine residues induced by EGF or HGF/SF correlates with loss of classical cadherin-mediated adhesiveness (Shibamoto et al., 1994).

PG and β-catenin differ in that PG associates with desmosomes whereas β-catenin normally does not. At the desmosome PG binds a region of the cytoplasmic domain of the desmosomal cadherins called the C-domain (centrally located on the desmogleins and terminally located on the desmocollins), which corresponds to the conserved carboxyl-terminus of the classical cadherins, and is essential for formation of the desmosomal plaque and attachment of cytokeratin filaments (Mathur et al., 1994). The exact domains of PG involved in binding both the classical and desmosomal cadherins remain to be precisely defined, with recent papers indicating a variety of sites on PG that are involved in the interactions (Chitaev et al., 1996; Troyanovsky et al., 1993; Troyanovsky et al., 1994b; Wahl et al., 1996; Witcher et al., 1996; Figure 38) possibly, as suggested by Birchmeier, Behrens and co-workers (Hülsken et al., 1994; Wahl et al., 1996), because the protein wraps around the cytoplasmic domain of the cadherins or desmosomal cadherins using multiple sites for interaction. However, recent data (P. Cowin and S. Troyanovsky personal communications from the 1996 Gordon Conference) suggest that the desmoglein and α-catenin binding sites on PG overlap, explaining why α-catenin does not localise at desmosomes and why desmosomes do not link to the actin microfilament network, and that the predominant classical cadherin binding domain is localised towards the middle armadillo repeats of the molecule. The desmogleins and desmocollins post-translationally regulate PG levels by decreasing the rate of its degradation (Kowalczyk et al., 1994). PG has different binding strengths for the different molecules associating most tightly with the C-domain of desmogleins, to a lesser degree with that of the desmocollins, and most
weakly with that of E-cadherin (Chitaev et al., 1996; Kowalczyk et al., 1994). PG binds classical cadherins in a manner similar to that of β-catenin but with lower affinity, being washed away from a cadherin immunoprecipitation whilst β-catenin remains firmly bound to the complex (Aberle et al., 1994; Peifer et al., 1992). Sommers et al. (1994) showed that in an E-cadherin-transfected cell line where adhesiveness is low, PG levels are reduced whereas β-catenin levels are normal but the protein is highly tyrosine phosphorylated. Lampugnani et al. (1995) showed that in VE-cadherin/cadherin 5-mediated adhesions β-catenin associates quickly at adhesions whereas PG only associates with junctions in tightly confluent cells, is readily lost from them when the monolayer is wounded, and that its level of expression at the junctions is highly variable.

1.3.1.3.1 PG in Xenopus development.

PG has been less studied than β-catenin in early development therefore the amount of data available is scant. However, Fouquet et al. (1992) have shown that PG mRNA is produced and stored in oocytes and eggs and it is possible that this maternal pool contributes to junctional structures connecting the oocyte to the follicle epithelium during oocyte maturation. DeMarias and Moon (1992) examined expression following fertilisation and showed that PG RNA is present in fertilised eggs through to tadpole stage, and that the proteins are present throughout early development. This maternal pool could allow the rapid formation of desmosomes, which have been reported from stage 7 onwards (Franz et al., 1983) and other plaque structures in the pre-gastrulation embryo. This expression pattern contrasts with that seen in the mouse (J. Collins personal communication - abstract submitted for ASCB conference 1996) which shows that PG is not expressed until late cleavage stages. Karnovsky and Klymkowsky (1995) showed that, like β-catenin, over-expression of PG during early Xenopus development results in secondary axis formation, with nuclear localisation of the armadillo repeat protein, and that this phenotype could be rescued by co-injection of the cytoplasmic domain of human Dsg1.
1.4 Early Xenopus pattern formation.

For many years Xenopus has been used as a model system in which to study pattern formation and address fundamental developmental questions such as how a fertilised cell becomes a multilayered multicellular organism with the correct body layout, and how cells are assigned certain specific fates within the organism so that they develop appropriately. This section will attempt to give a brief overview of early pattern formation in Xenopus (see Slack, 1994, Kessler and Melton, 1994 and Gilbert, 1994 for further details).

1.4.1 Maternal information.

When the Xenopus egg is laid it is radially symmetric around the animal-vegetal axis formed during oogenesis. This axis is easy to identify as the animal hemisphere is pigmented and the vegetal hemisphere, which contains the yolk platelets stored in the cell, is lighter in colour. The egg contains various proteins and mRNAs, localised to different regions along the axis of the egg, which the embryo uses in the early stages of development; the zygote's own genes are dormant until the mid-blastula transition which actually occurs late in the blastula stage.

1.4.2 Fertilisation and setting up of Nieuwkoop centre.

Upon fertilisation the radial symmetry of the egg is broken by sperm entry; the point opposite the site of sperm entry will ultimately become the dorsal side of the embryo. The formation of the dorso-ventral axis occurs by a process called cortical rotation, which entails the plasma membrane and a small shell of underlying cytoplasm (the cortex) moving about 30° with respect to the internal cytoplasm, leading to the formation of a dorso-vegetal signalling centre known as the Nieuwkoop centre or organiser-inducing centre (Gerhart et al., 1989). At the end of rotation the future dorsal side is where vegetal cortex adjoins animal cytoplasm, and the future ventral side is where animal cortex adjoins vegetal cytoplasm.
Cortical rotation occurs before the first cell division and is essential for further normal development; if it is prevented for example by UV irradiation of the vegetal pole, the embryo develops radially ventral. This is known as a UVO phenotype and is caused solely by the inhibition of rotation (due to the UV preventing the polymerisation of microtubules) which in turn prevents the formation of the Nieuwkoop centre, and can be completely rescued by artificial rotation of the embryo (Gerhart et al., 1989; Scharf and Gerhart, 1980).

1.4.3 Blastula stage.

The embryo divides forming a blastula, essentially a ball of cells with a central cavity called the blastocoel (Figure 7). Division continues until there are approximately 10-20,000 cells when the next stage of development, gastrulation, begins.

1.4.3.1 Mesoderm induction and Spemann's organiser.

When cells from the animal region or the vegetal region of a blastula stage embryo are taken and cultured separately different types of tissue are generated; animal pole cells differentiate into atypical epidermis and vegetal pole cells form an unstructured cell mass. Mesodermal derivatives are never produced unless vegetal and animal cells are cultured together (Nieuwkoop, 1969a; Sudarwati and Nieuwkoop, 1971). Co-culture experiments have shown that signals produced by vegetal cells are needed by overlying animal cells to cause them to develop into mesoderm (Gurdon et al., 1985; Nieuwkoop and Ubbels, 1972), and that the type of mesoderm produced is determined by the vegetal cells (Dale et al., 1985; Nieuwkoop, 1969b). This process, when a signal is produced by one set of cells so that another responsive set of cells change their fate, is called induction.

During normal development vegetal cells at the blastula stage release signals which induce the overlying animal cells to become mesoderm, so making the third body layer of the developing organism. It is thought that there are two types of signal that emanate from the vegetal cells to induce the overlying animal cells to develop into mesoderm: a general one acting all around the circumference of the embryo that causes
FIGURE 7: Gastrulation and neurulation in *Xenopus laevis*.

Taken from Gilbert (1994).

(i) Schematic of invagination movements that occur during gastrulation.

(ii) Schematic of sagittal section through gastrulating embryo:
   - A: late blastula stage showing position of blasocoel
   - B: initiation of gastrulation with cells beginning convergent extension at the animal cap and invaginating at the blastopore lip.
   - C: early-mid gastrulation with invagination beginning at ventral blastopore lip and blastocoel being displaced as cells continue invaginating at dorsal blastopore lip.
   - D: late gastrulation with the blastopore closing and the majority of cells invaginated and the archenteron formed.
   - E: end of gastrulation with the three body layers now defined and in the correct position ready for neurulation to begin.

(iii) Dorsal surface view of neurulation:
   Neurulation begins at the future posterior of the embryo where the blastopore closes and gradually moves up the embryo in an anterior direction, the neural folds lifting up and closing to form the neural tube.

(iv) Schematic of transverse section through neurulating embryo:
   The neural folds lift up at either edge of the neural plate eventually fusing at the top to form the neural tube.
the mesoderm to be ventro-lateral in character and develop into structures such as blood cells and mesothelium, and a more specialised signal generated in the Nieuwkoop centre (in the dorsal area alone) that causes the mesoderm to become axial (dorsal) in type and eventually develop into structures such as the notochord and segmented muscle (Boterenbrood and Nieuwkoop, 1973; Dale et al., 1985; Gimlich and Gerhart, 1984; Slack et al., 1984). The region in which the prospective axial mesoderm lies is known as the Spemann's organiser or simply the organiser, and is the area that will become the dorsal blastopore lip at the beginning of gastrulation.

1.4.4 Gastrulation.

Gastrulation is a process of complex cell movements which transforms the embryo from a ball of cells where the future body layers are lined up one on top of the other along the animal-vegetal axis (presumptive ectoderm at the animal pole, mesoderm at the marginal zone and endoderm at the vegetal pole), into a structure where the future body layers are in the correct position i.e. the ectoderm lies on the surface of the embryo, the endoderm lines the inside of the embryo, and the mesoderm lies in between (Figure 7).

The cells on the outside of the embryo elongate, flatten and migrate inside the embryo at a small invagination on the surface called the dorsal blastopore lip, in a manner similar to that produced if one pushes a finger inwards on the surface of an inflated balloon. The cavity formed by the invagination of the cells, the archenteron, is the presumptive gut. As gastrulation proceeds the blastopore lip expands laterally until it encircles the whole of the vegetal pole forming the blastopore; the remaining exposed vegetal cells are called the yolk plug. Eventually the blastopore shrinks in size and moves down the embryo until all the yolk plug cells are internalised (Figure 7).

The anterior-posterior axis of the embryo is also set during gastrulation: the mesoderm that migrates first through the dorsal blastopore lip gives rise to the future anterior of the embryo, and the mesoderm where the yolk plug closes in the ventral margin forms the future posterior.
As well as the physical movements, a complex set of inductive processes occurs during gastrulation; neural induction occurs at this stage and involves the ectoderm of the animal region being induced to form neural plate which later becomes the neural tube and nervous system. The animal pole ectoderm is believed to be induced synergistically by a tangential signal from the underlying invaginating mesodermal cells and a planar signal emitted from the organiser (Dixon and Kintner, 1989). Another inductive process, dorsalisation, also occurs during gastrulation; this is when cells that would otherwise develop into ventral mesoderm migrate into the embryo via the dorsal blastopore lip and are induced, by dorsal mesoderm in that area (the organiser), to form dorsal type mesoderm e.g. muscle (Slack et al., 1984). Mesoderm that invaginates via the ventral or lateral blastopore lips develops into the mesodermal mantle that generates ventral type derivatives e.g. heart, kidney, blood, bones.

1.4.5 Neurulation.

This is the stage when morphogenetic movements produce the neural tube (which goes on to form the brain and spinal cord), and generate neural crest cells (which go on to form, amongst other cell types, the peripheral neurones and glia and the pigment cells of the skin). The neural plate lifts up at the edges forming neural folds, the central part sinks into the embryo, and the folds move inwards until they fuse together. The process begins at the future posterior end of the embryo (the site of the closed yolk plug) and progresses along the anterior-posterior axis until the whole neural tube is formed (Figure 7).

The end of neurulation is the first stage at which one can observe, non-intrusively, the prospective final pattern of the embryo; it has distinct markings that allow one to identify the future antero-posterior and dorso-ventral axes of the animal. For further details of development see Nieuwkoop and Faber (1967; gives the original Xenopus stages and descriptions of internal morphology), Hausen and Riebesell (1991; contains plates of sections at different stages and helps visualise what is happening at different stages of development as well as good descriptions of morphogenetic
movements) and Gilbert (1994; good general book dealing with all aspects of
development).

1.4.6 Nature of Nieuwkoop centre.

It is thought that the Nieuwkoop centre is set up by cytoplasmically localised
determinants, probably mRNA, laid down during oogenesis (see Slack, 1994 and
Kessler and Melton, 1994, for reviews). Cortical rotation somehow activates the
factor(s), probably by translation or post-translational modifications, at a particular site
setting up the signalling centre and starting body plan formation. A number of
molecules are potential candidates that may constitute the Nieuwkoop centre as defined
by the experiments below.

Studies to identify the candidate inducer molecules of the Nieuwkoop centre
have made use of the fact that explants from the animal cap will give rise to only
ectodermal derivatives if left to develop alone. For the animal cap assay, candidate
proteins are placed in the culture mix with the explant and the types of tissue generated
analysed to see if mesoderm has been formed, and if it is ventral or axial. A similar
assay is the autoinduction assay in which embryos are injected with candidate RNA (or
less commonly cDNA), caps cut at the blastula stage, cultured and examined to see if
mesoderm is generated and if so what type it is. These types of experiments led to the
discovery that two classes of proteins can induce mesoderm production: the first class
are the fibroblast growth factor (FGF) family, and the second the transforming growth
factor β (TGFβ) family, a group of molecules that to be active must first be processed
from a longer precursor protein then dimerise. Among the FGF family of mesoderm
inducers identified by these assays are bFGF (Kimelman and Kirschner, 1987;
Kimelman and Maas, 1992; Thompson and Slack, 1992) and kFGF (Thompson and
Slack, 1992). Members of the TGFβ family that are active include: activins (Smith et
al., 1990; Smith et al., 1993; Thomsen et al., 1990), Vg-1 chimeras which allow the
molecule to be processed generating the mature protein in vivo (Dale et al., 1993;
Thomsen and Melton, 1993), nodal and *Xenopus* nodal-related genes (Jones et al.,
1995; Smith et al., 1995), and bone morphogenetic protein-4, also known as BMP4 or
FIGURE 8: Inductive interactions in early *Xenopus* development.

Adapted from Slack (1994).

**Regions:**
- light green: animal
- light pink: vegetal
- dark green: dorsal animal
- dark pink: dorsal vegetal
- orange: organiser
- light yellow: mesoderm
- dark yellow: somitic mesoderm
- blue: neural plate

**Genes:**
- bra: brachyury
- Epi-1: an epidermal marker
- fkh: forkhead
- gcd: goosecoid
- lim: Xlim-1
- N-CAM: neural cell adhesion molecule
- not: Xnot
- twi: twist
- sna: snail
Stage | State | Signal required for induction of next state
--- | --- | ---
Unfertilized egg | Animal | sperm entry point
 | Vegetal | Dorsal Cortical rotation
Fertilized egg | IP$_3$ | Wnts active?
 | Vg-1 active? | Kinase cascade FGF activin? Vg-1? BMPs?
Late blastula | brachyury | Vg-1 active?
 | noggin follistatin | noggin
DVR-4 (Dale et al., 1992; Jones et al., 1992). bFGF and BMP4 induce ventral type mesoderm, whereas Vg-1 chimera, nodal and activin induce dorsal type mesoderm and so may be candidate molecules for the Nieuwkoop centre.

As well as inducing mesoderm in animal cap explants, any candidate molecule must also be able to rescue an axis-deficient embryo; it must be able to induce a new Nieuwkoop centre when injected into a UV-irradiated embryo. If an active molecule is injected into the ventral side of a normal embryo, a double axis embryo is generated that at its most extreme leads to a tadpole with two heads leading into one trunk and tail. Using this assay members of the TGFβ family give positive results: e.g. Xenopus nodal-related genes (Jones et al., 1995), Vg-1 chimera (Dale et al., 1993; Thomsen and Melton, 1993), activin (Thomsen et al., 1990) and its receptor (Kondo et al., 1991). Members of the Wnt family, and members of the Wnt signalling pathway, also work in this assay: e.g. Wnt-1, Xwnt-3A, Xwnt-8, and to a lesser extent Xwnt-11 (Ku and Melton, 1992; McMahon and Moon, 1989; Smith and Harland, 1991; Sokol et al., 1991; Wolda et al., 1993), β-catenin (Funayama et al., 1995; Guger and Gumbiner, 1995), PG (Karnovsky and Klymkowsky, 1995), and dominant negative mutants of glycogen synthase kinase-3 (He et al., 1995; Pierce and Kimelman, 1995). Other molecules have also been identified by this assay including the secreted polypeptide noggin (Smith and Harland, 1992), and the homeobox gene product goosecoid (Cho et al., 1991). Li+ injection can also cause double axis formation or rescue UV-irradiated embryos and is thought to perturb the inositol phosphate cycle, implying a role for these second messengers during axis development (Busa and Gimlich, 1989).

This last assay is not specific for the Nieuwkoop centre, but will also give a positive result if the molecule is active in the Spemann's organiser. To help determine if a candidate molecule is active in the Nieuwkoop centre or in the Spemann's organiser the expression pattern at gastrulation can be examined; whilst cells that form the Nieuwkoop centre eventually become endoderm, organiser cells involute during gastrulation and contribute to axial mesoderm. Using this criterion goosecoid is involved in the organiser (Cho et al., 1991), whereas for example β-catenin is involved in the Nieuwkoop centre (Guger and Gumbiner, 1995).
The final criterion that any candidate inducer molecule has to fulfil is that it is present at the appropriate time and place during development at biologically active levels. Recently it has been proposed (Slack, 1994) that actually only two of the above mentioned molecules fulfil all the criteria to be involved in the Nieuwkoop centre: Xwnt-11 and Vg-1, if somehow processed *in vivo* to allow it to become active, both of which are encoded by vegetally-localised mRNAs (Ku and Melton, 1992; Weeks and Melton, 1987). It has been suggested that Vg-1 is the initiator of the Nieuwkoop centre activity, with activin, noggin and Wnts acting downstream within the centre, initiating other signalling cascades and specifying the next stages of development (Slack, 1994; Figure 8).

1.4.7 Nature of Spemann's organiser.

The Spemann's organiser has to fulfil four functions: it initiates gastrulation by the formation of the blastopore lip, it makes head mesoderm and notochord, it causes the dorsalisation of mesoderm, and it induces overlying ectoderm to become the neural plate at gastrulation. Given that it has all these functions, it is obvious that it is a complex area that will have many different genes acting in concert to cause different groups of cells to follow different fates. Little is known about the nature of the organiser and it is an area of intense study; some candidates have been identified that probably function here, but the whole story is still far from clear.

A number of genes have been shown to be expressed in a pattern resembling that of the organiser including: goosecoid (Cho *et al.*, 1991), forkhead (Dirksen and Jamrich, 1992), lim (Taira *et al.*, 1992), noggin (Smith and Harland, 1992) and, after an initial phase of universal expression, Xnot (von Dassow *et al.*, 1993). It has been suggested that goosecoid may be responsible for the involution function of the organiser (Niehrs *et al.*, 1993) and that noggin acts as the neural plate inducer and the mesodermal dorsaliser (Slack, 1994).
1.5 Aim of projects undertaken.

Desmosomes have a well characterised structural role to play, but the fact that they contain proteins belonging to a family with proven morphoregulatory roles, the cadherins, prompted the question whether these desmosomal cadherins could also act independently from the desmosome and have a morphoregulatory role to play during development. Preliminary studies by Dr Al Magee showed that a desmoglein was present in early *Xenopus* development at a stage before desmosomes as fully formed structures had been described (described from stage 7 onwards [Franz et al., 1983]), supporting this possibility. It was decided to investigate this potential alternate role using *Xenopus* as a model system due to its easy accessibility at all stages of development for possible intervention experiments. The first aim was to map and compare the expression pattern of certain desmosomal components through early *Xenopus* development by immunohistology and RNA *in situ* analysis, determining at what stage the desmoglein assembled into desmosomes and in which subcellular compartments the protein was present beforehand. However, before the *in situ* analysis could be performed, a *Xenopus* desmoglein clone was required. A further aim was therefore to clone a *Xenopus* homologue of a desmoglein from a cDNA library. This clone could then also be used for later overexpression studies, and used to generate antisense oligonucleotides/sequences and deletion constructs for dominant negative studies to determine what effects disruption of normal desmoglein levels had upon development. This work is described in chapter 3. Unfortunately these aims were not fulfilled for a variety of reasons and a new project was pursued.

Due to the time spent on the first project (2 years), the aims for the second project were more limited and focused than those described above, although if successful could be extended at a later stage by other members of the laboratory. A desmosomal cytoplasmic plaque protein, PG, is a member of the armadillo family of molecules. Following work performed by McCrea *et al.* (1993) which showed that a closely related member of this family, β-catenin, plays a role in the *Xenopus* Wnt signalling pathway, it was decided to investigate whether PG also had signalling
functions using similar methodology. The aims of this project were therefore to generate antibodies that recognised PG specifically, and use F_{ab} derivatives of these for microinjection experiments in *Xenopus* to determine if they altered the Wnt signalling pathway. This work is described in chapters 4 and 5.

It had also been shown that certain stimuli, including EGF, cause both β-catenin and PG to become tyrosine phosphorylated, but the location of the phosphorylation site was unknown. A further aim was therefore to use the reagents generated during the antibody production protocol, to determine if the site of phosphorylation was located in one of the unique end regions of PG. This work is described in chapter 6.

Also to make full use of the reagents generated, a further aim was to determine if the unique domains of PG bound any other proteins from a panel of possible candidates *in vitro*, and whether these associations were dependent on tyrosine phosphorylation induced by EGF signalling. This work is also described in chapter 6.
2.1 Buffers.

Unless stated otherwise, chemicals were supplied by BDH-Merck Ltd, Leics.

20x Denharts: 4g Ficoll, 4g BSA, 4g PVP in 1L 20x SSC.

L-Agar  
L-broth + 1% agar

L-Broth  
10g bacto-tryptone, 5g yeast Extract, 10g NaCl, 1 litre dH2O, pH 7.5

LB Amp  
L-Broth with 50µg/ml ampicillin

1x Laemmli loading buffer  
2% SDS, 10% glycerol, 100mM Tris pH 6.8, 0.05% w/v bromophenol blue (with DTT 50-100mM for reduced samples)

M9 minimal medium  
50mM Na2HPO4, 20mM KH2PO4, 10mM NaCl, 20mM NH4Cl, 0.4% [w/v] glucose, 0.1% [w/v] casamino acids, 2mM MgSO4, 0.2mM CaCl2, 10µg/ml thiamine

MTPBS  
16mM Na2HPO4, 4mM NaH2PO4, 150mM NaCl

1x NAM  
110mM NaCl, 2mM KCl, 1mM Ca(NO3)2, 1mM MgSO4, 0.1mM Na2EDTA, 2mM sodium phosphate pH 7.0, 1mM NaHCO3, 50mg/ml gentamycin

NP40 Lysis buffer  
1% NP40, 150mM NaCl, 1mM DTT, 50mM Tris pH 8.0, 1mM PMSF, 10µl/ml aprotinin (2.2mg/ml stock)
NP40 Lysis buffer + Phosphatase inhibitors
NP40 lysis buffer plus 1mM sodium orthovanadate, 5mM NaF, 10mM sodium pyrophosphate

NZY Broth:
5g NaCl, 2g MgSO₄·7H₂O, 5g Yeast extract, 10g NZ Amine (casein hydrolysate)

NZY agar:
as NZY broth but with 15g Difco Agar

NZY top agarose:
as NZY broth but with 0.7% agarose

PBS-A
170mM NaCl, 10mM Na₂HPO₄, 3.5mM KCl, 1.8mM KH₂PO₄

PBS-T
PBS-A + 0.1% Tween 20

10x PCR Buffer
200mM Tris pH 8.75, 100mM KCl, 100mM (NH₄)₂SO₄, 20mM MgCl₂, 1%

Triton X-100, 1mg/ml BSA

SM buffer:
100mM NaCl, 10mM MgSO₄, 50mM Tris HCl pH 7.5, 0.01% Difco gelatin

20x SSC:
3M NaCl, 0.3M Na₃Citrate

XIB
88mM NaCl, 1mM KCl, 15mM Tris pH 7.5
2.2 Bacterial Strains, plasmids, and cell lines used in this study.

**Bacteria.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>supE44 $,\Delta$ lacU169 (φ80 lacZΔM15)</td>
</tr>
<tr>
<td></td>
<td>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
</tr>
<tr>
<td></td>
<td>(Hanahan, 1983; Bethesda Research Laboratories, 1986)</td>
</tr>
<tr>
<td>TG1</td>
<td>$F,\prime traD36$ proAB+ lacI9 lacZΔE15 $\Delta$(lac-pro) supE thi hsdD5</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, ac, [F' proAB, lacI9ZΔE15, Tn10 (tet')].</td>
</tr>
</tbody>
</table>

**Plasmids.**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pATH2</td>
<td>Expression vector. (Dieckmann and Tzagoloff, 1985)</td>
</tr>
<tr>
<td>pET 21a</td>
<td>Expression vector from Novagen R&amp;D systems, Oxon.</td>
</tr>
<tr>
<td>pGEX 2T</td>
<td>Expression vector from Pharmacia Biotech, Herts.</td>
</tr>
<tr>
<td>pGEX 3X</td>
<td>Expression vector from Pharmacia Biotech, Herts.</td>
</tr>
<tr>
<td>Bluescript KS+</td>
<td>Cloning vector from Stratagene, San Diego.</td>
</tr>
</tbody>
</table>

**Cell Lines.**

<table>
<thead>
<tr>
<th>Line</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>Human squamous carcinoma (ECACC No: 85090402)</td>
</tr>
<tr>
<td>SW480</td>
<td>Human Colon adenocarcinoma (ECACC No: 87092801)</td>
</tr>
<tr>
<td>Sf9</td>
<td><em>Spodoptera frugiperda</em> (ECACC No: 89070101)</td>
</tr>
</tbody>
</table>
XL  

*Xenopus laevis* liver-derived cell line

(Anizet *et al.*, 1981)
2.3 Library Screening.

Good microbiological practice was used for all techniques.

2.3.1 Making a radiolabelled nucleic acid probe.

Probes were made from human cDNA plasmids in the laboratory using the Megaprime kit and \(^{32}\)P-dATP from Amersham following the protocol set out in the handbook accompanying the kit. This was essentially as follows:

3\(\mu\)l (25ng) DNA, 5\(\mu\)l random nonamer primers, and 23\(\mu\)l dH\(_2\)O were placed in a 0.5ml microfuge tube and boiled for 5 minutes, before 5\(\mu\)l reaction buffer ("a concentrated buffer containing Tris-HCl, pH 7.5, MgCl\(_2\) and 2-mercaptoethanol"), and 3\(\mu\)l each of dCTP, dGTP and dTTP ("nucleotides in concentrated buffer solutions containing Tris-HCl, pH 8.0, 0.5mM EDTA") were added. The tube was spun briefly in a microfuge and 2\(\mu\)l enzyme solution ("1 unit per \(\mu\)l DNA polymerase 1 'Klenow' fragment [cloned] in 50mM potassium phosphate pH 6.5, 10mM 2-mercaptoethanol and 50% glycerol"), and 3\(\mu\)l \(\alpha^{32}\)P-dATP (Amersham) added, the tube spun again and incubated at 37°C for 10 minutes. The labelled DNA was then purified in one of two ways, either by precipitation or by passing through a Sephadex\textsuperscript{Tm} G50 column. For the precipitation method 50\(\mu\)l dH\(_2\)O, 20\(\mu\)l tRNA (at 1\(\mu\)g/\(\mu\)l), 1\(\mu\)l glycogen, 15\(\mu\)l 3M NaAc, and 300\(\mu\)l ice-cold 100% ethanol were added, the mixture left for 15 minutes on ice then spun for 10 minutes in a microfuge, the supernatant discarded and the DNA resuspended in 200\(\mu\)l dH\(_2\)O. For the spin column method 0.9ml Sephadex\textsuperscript{Tm} G50 (Pharmacia) equilibrated in STE (10mM Tris-HCl pH 8.0, 1mM EDTA, 0.1 M NaCl) was packed in a 1ml syringe barrel, placed in a 10ml centrifuge tube with a decapped 1.5 ml microfuge tube at the bottom, and pre-washed with 0.1ml STE by centrifugation at 1600g for 5 minutes. The probe DNA was then applied to the top in a final volume of 0.1ml (made up with STE), and spun as before. The DNA was collected in the 1.5ml tube with the unincorporated nucleotides being retained in the beads. Before use 130\(\mu\)l denatured salmon sperm DNA (10mg/ml) were added to the probe and boiled for 5 minutes.
2.3.2 Screening a λZap2 library with cDNA

The library was a *Xenopus* adult male liver library kindly supplied by Dr J.R. Tata. XL1-Blue *E.coli* were used as the host bacteria for screening. Stocks were maintained on LB tetracycline plates (tetracycline at 12.5mg/ml).

2.3.2.1 Preparation of a plating culture.

A 250ml flask with 50ml LB broth, containing 0.2% maltose and 10mM MgSO₄, was inoculated with a bacterial colony from a stock plate. The mixture was grown overnight at 32°C with shaking, the bacteria spun down for 10 minutes at 2500rpm in a Mistral 3000i centrifuge at room temperature and the supernatant discarded. The bacteria were washed once with 10mM MgSO₄, then resuspended in 0.4 volumes of 10mM MgSO₄ (i.e. 20ml) and could be stored for up to 3 weeks at 4°C.

2.3.2.2 Plating of library.

Primary screening was performed using 22cm square plates which had been allowed to dry for 2 days at room temperature. In a 50ml conical tube 100μl phage library (diluted such that 2-5x10⁵ pfu were added per plate) were added to 0.6ml of plating culture bacteria and incubated for 15 minutes at 37°C. 40ml NZY top agarose containing 10mM MgSO₄, held at 42°C, was then added to the bacterial culture and gently mixed before being poured onto the dried NZY plates. The plates were allowed to harden at room temperature (approximately 45 minutes) before being upturned and incubated for 8 hours or overnight at 37°C.

Further rounds of screening were carried out using 150mm diameter dishes with 0.2ml plating culture incubated at 37°C for 15 minutes with phage stock diluted to give 5x10⁴ pfu/plate. 6.5ml NZY top agarose with 10mM MgSO₄ were mixed with each culture before plating out.
2.3.2.3 Lifting of library.
The plates were then placed at 4°C for at least 2 hours (to ensure that the top layer of agarose did not lift from the base layer of agar) before replica lifting using Hybond™-N+ filters (Amersham Life Science, Bucks.). The first filter was laid on the plate for 1 minute, and the duplicate for 3 minutes, before being placed onto 2 pieces of Whatman 3MM paper thoroughly soaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 7 minutes colony side up. The filters were then neutralised for 3.5 minutes in a similar manner using neutralising solution (1.5M NaCl, 1mM EDTA, 0.5M Tris-HCl pH 7.2) before being washed in 2x SSC and any attached agarose removed. The filters were then dried between two pieces of Whatman paper and baked at 80°C for 2 hours to fix the DNA, and the plates stored at 4°C until required.

2.3.2.4 Probing filters.
Probes were made as set out in section 2.3.1, and the filters probed as for a Southern blot as set out in section 2.4.4 except that the washing conditions for the filters were milder than those described, with the primary filter wash being in 0.4x SSC and 0.1% SDS for 15 minutes at 50°C, and the second wash for 15 minutes at 50°C in 0.4x SSC.

2.3.2.5 Identifying putative clones.
When the films were developed, any plaques corresponding to spots that were identified on both the replica filters were picked and placed in 1ml SM buffer and placed at 4°C at least overnight. Dilutions of the supernatant were used for the next round of screening, and the whole screening process was repeated until all plaques on a dish were positive when probed.

2.3.3 Screening a λZap2 expression library with antibodies.
A λZap2 stage 51-52 Xenopus tail cDNA expression library (a kind gift from Mrs B. Bennett) was screened using XL1-Blue as the host bacteria. The library was plated out as described in section 2.3.2.2 using 150mm diameter plates; however, instead of overnight incubation at 37°C, the plates were incubated for 2 hours at 42°C, IPTG
impregnated filters (supported nitrocellulose from Sartorius) placed over the plate surface, and the plates incubated at 37°C for 6 hours. The plates were then placed at 4°C for 1 hour to overnight, the filters lifted off and the plates stored at 4°C until required. The filters were rinsed in PBS-A, blocked and developed as for an alkaline phosphatase Western blot (see section 2.5.9) using affinity-purified pan-desmoglein 920 antibody as the primary antibody (for 3 hours at 37°C). Putative positive plaques were rescreened by removing an agar plug from the region of the plate which corresponded to the signal on the filter, and placing it in 0.5ml SM buffer with 20μl chloroform. The plugs were then stored at 4°C where they were stable for up to a year. The whole screening process was repeated until all the plaques on the plate gave positive signals.

2.3.4 In vivo excision protocol for subcloning from λZap2 vector.

One of the advantages of using the λZap2 vector (Stratagene) is its ability effectively to subclone itself into the Bluescript phagemid, the so called in vivo excision process. (See Figure 9 for explanation of this process).

In a 50ml conical flask 200μl XL1-Blue cells (OD_{600}=1.0), 200μl of the λZap2 phage stock (derived from the cored plug) and 1μl R408 or ExAssist® helper phage were incubated for 15 minutes at 37°C. 5ml of 2x YT media were then added and the flask set shaking at 37°C for 3 hours, before heating the tube at 70°C for 20 minutes and spinning the tube for 5 minutes at 4000g. The supernatant (containing any phagemid packaged as filamentous phage particles) was decanted into a sterile tube. Dilutions of this were incubated with 200μl XL1-Blue cells (OD_{600}=1.0) for 15 minutes at 37°C then plated out on freshly prepared LB/Ampicillin plates and incubated overnight at 37°C. Any colonies appearing should contain the pBluescript SK- double stranded plasmid including the cloned DNA insert, which can then be amplified by normal mini-prep or maxi-prep methods (see sections 2.4.11 and 2.4.12).
FIGURE 9: Outline of in vivo excision process.

Adapted from information accompanying λZap2 library (Stratagene).

Built into the vector are λ bacteriophage origin of replication initiation and termination sites. Thus when host bacteria are infected with both the vector and an λ type "helper phage" (R408 or ExAssist available from Stratagene), the initiation site is recognized by λ derived proteins which nick one of the two vector DNA strands before beginning replication downstream until the λ termination site is encountered. The single stranded DNA produced contains all the sequences of the pBluescript SK(−) phagemid, including any insert clone. The DNA is then circularised, packaged as an λ type bacteriophage, and secreted from the host, all under control of the λ "helper phage" proteins.

Once the phagemid is secreted the mixture is heated to 70°C which kills the host bacteria, but allows the secreted bacteriophage to survive. The single stranded phagemid is then "rescued", and isolated from the "helper phage" which have also survived the heat treatment, by mixing it with a fresh population of host cells and spreading on LB Ampicillin plates (1L L-Broth with 15g Difco agar and ampicillin at 50mg/ml). Colonies which grow contain the double stranded phagemid; bacteria containing the "helper phage" die because they are not ampicillin resistant. Colonies can then be treated as normal for DNA isolation.

KEY:
A-J lambda structural proteins
att lambda attachment site
T3/T7RNA polymerase promoters
1. Coinfect *E. coli* with lambda ZAP clone and F1 helper phage.
2. Grow culture 1-6 hours at 37°C for automatic excision.
3. Heat culture 20 min at 70°C to inactivate *E. coli* & lambda, but not packaged phagemid.
4. Infect *E. coli* with packaged plasmid and plate on ampicillin plates to recover excised phagemid.
2.3.5 Isolation of \( \lambda \) DNA.

10\( \mu l \) of plating culture (see section 2.3.2.1) were added to 50ml L-Broth and infected with 330\( \mu l \) phage solution then incubated overnight at 37\(^\circ\)C with shaking. 500\( \mu l \) chloroform were added, and the culture incubated for a further 20 minutes before being spun at 10,000 rpm for 10 minutes at 4\(^\circ\)C in a Beckman JA20 rotor. The supernatant was then respun at 16,000 rpm for 2 hours at 4\(^\circ\)C, again in a Beckman JA20 rotor, the resultant pellet resuspended in 700\( \mu l \) SM buffer, 35\( \mu l \) 20\% SDS and 7\( \mu l \) 0.5M EDTA, and the suspension heated to 68\(^\circ\)C for 15 minutes to break open the phage protein coat. 700\( \mu l \) of phenol/chloroform (1:1) were then added and mixed to remove protein. The aqueous layer was removed to a fresh tube and the DNA ethanol precipitated, dried under vacuum and resuspended in 50\( \mu l \) water.

2.3.6 Touchdown PCR.

Using this method, sequences that hybridise under more stringent conditions are selectively amplified over those with a worse match, and it can be a useful method of cloning across species, using degenerate primers, when there are areas of highly conserved sequence in the molecule one is attempting to clone.

The PCR reaction was set up as described in section 2.4.1, except that degenerate primers were used (designed by Dr G. Wheeler based on the bovine and human sequences) and a non-proofreading type polymerase was used (Amplitaq\textsuperscript{®}, Perkin Elmer, Cheshire). The conditions for the PCR were the same as set out in section 2.4.1 except that the annealing temperature varied during the early rounds of amplification; the first round of amplification was carried out at a hybridisation temperature 10\(^\circ\)C higher than the optimal annealing temperature, with the temperature reducing by 2\(^\circ\)C in each of the subsequent rounds of amplification until the correct annealing temperature was reached. 20 rounds of amplification were then carried out at the optimal annealing temperature before the final cycle.
2.4 Other molecular Biology.

Good microbiological practice was used for all techniques.

2.4.1 PCR (Polymerase Chain Reaction).

Reactions were carried out using cloned Pfu DNA polymerase (that has proof reading capability) and PCR buffer obtained from Stratagene (La Jolla, California). For one reaction 5 μl 10 x PCR buffer were added to 5 μl dNTP mix (2 mM of each dATP, dCTP, dGTP, dTTP), with 1 μl each primer (stock concentration of 50 μM), 0.35 μl enzyme (1.25 units) and 36.65 μl distilled sterile water in a sterile 0.5 ml microfuge tube. To this 1 μl of DNA (approximately 20 ng) was added, the mixture vortexed briefly and a drop of mineral oil added to the top. The PCR was carried out in a Hybaid Omni-Gene apparatus according to the following conditions:

<table>
<thead>
<tr>
<th>step</th>
<th>cycle 1</th>
<th>cycle 2-32</th>
<th>cycle 33</th>
</tr>
</thead>
<tbody>
<tr>
<td>melting</td>
<td>94°C 3 min 50 sec</td>
<td>94°C 1 min 30 sec</td>
<td>94°C 1 min 30 sec</td>
</tr>
<tr>
<td>annealing</td>
<td>xx°C 2 min 00 sec</td>
<td>xx°C 1 min 30 sec</td>
<td>xx°C 1 min 20 sec</td>
</tr>
<tr>
<td>elongation</td>
<td>72°C 2 min 00 sec</td>
<td>72°C 1 min 45 sec</td>
<td>72°C 15 min 00 sec</td>
</tr>
</tbody>
</table>

The annealing temperature, xx, depended on the composition of the primers and was calculated according to the following equation:

\[ 4(A+T) + 2(G+C) = T_m \]

The annealing temperature was T_m-5°C. The lower of the two annealing temperatures for a particular set of primers was used. When the reaction was finished the sample was frozen on dry ice, the oil removed and a sample then run out on an agarose gel to check the product length.

2.4.2 Agarose gel electrophoresis.

1% agarose gels were prepared using Sigma type 1: low EEO agarose melted in 1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.3), poured into a gel mould with a comb inserted and allowed to cool at room temperature. Once set the gels were placed in a
BRL Horizon 58 apparatus for minigels (6x8cm), a BRL Horizon 11.14 for midigels (11x14cm), or a BRL Horizon 20.25 for maxigels (20x25cm) with enough 1x TAE to cover the gel for running buffer. DNA samples were loaded with 1/10 volume of loading buffer (15% Ficoll 400, 0.25% xylene cyanol, 0.25% bromophenol blue in dH₂O). Minigels were run for approximately 1 hour at 80V, midi gels overnight at 20V and maxigels overnight at 30V. Gels were then placed to stain in a solution of ethidium bromide (1mg/ml) for at least 10 min, then viewed on a ultraviolet lightbox.

2.4.3 Southern blot.
Agarose gels prepared as set out in section 2.4.2 were taken after ethidium bromide staining and photography, and placed in a large volume of 0.5M NaOH, 1.5M NaCl for 30 minutes at room temperature to denature the DNA. The gels were then neutralised by soaking in a large volume of 1.5M NaCl, 1M Tris-HCl pH 8.0 for 1 hour at room temperature before transfer. The blotting system was set up as shown in Figure 10, using 20x SSC as the reservoir solution and Hybond-N or Hybond-N+ filters (Amersham). The system was left to transfer overnight, and the filters baked at 80°C for 2 hours to fix the DNA.

2.4.4 Probing a Southern.
The filters were layered with nylon spacers in 2x SSC, carefully rolled so that no air bubbles were introduced and placed in Hybaid tubes along with 30ml prehybridisation solution (7.5ml 20x Denhart's solution, 1.5ml 20% SDS, 21ml dH₂O). The tubes were then placed in a rolling Hybaid oven at the hybridisation temperature (60°C unless otherwise stated) for a period of two to four hours, after which the prehybridisation solution was replaced by pre-warmed hybridisation solution (30ml per tube: 7.5ml 20x Denhart's stock, 1.5ml 20% SDS, 7.5ml 20%w/v dextran sulphate, 13.5ml H₂O) and the radiolabelled probe prepared as described in section 2.3.1 added. The filters were left to hybridise overnight at 60°C in the rolling Hybaid oven, washed once in 1x SSC, 0.1% SDS for 15 minutes at 50°C, and once in 1x SSC for 15 minutes at 50°C. The
FIGURE 10: Setting up of Southern blot apparatus

Method as described in section 2.4.3.
weight

paper towels

4 pieces of 3MM paper

gel

filter

3MM wick

piece of glass as support

transfer buffer
filters were then covered in Saranwrap and placed against film with enhancer screens at -70°C overnight.

2.4.5 Normal GENECLEAN®.
Identified DNA products on an agarose gel were purified using the GENECLEAN® kit and protocol from Bio 101 Inc. (La Jolla, California). Essentially this involved cutting the band out of the gel, adding three volumes of the NaI stock solution and incubating at 56°C for 5 min to melt the agarose. GLASSMILK was added (5μl per initial 5μg or less DNA, then a further 1μl per 0.5μg DNA above 5μg), the suspension mixed and incubated for 5-30 min on ice. The DNA-GLASSMILK complex was then pelleted and washed 4 times with NEW WASH. 25μl distilled water was then added to the beads-DNA complex, the sample heated to 56°C for 3 min before pelleting the beads again and removing the supernatant which contained the DNA. This was performed twice so the DNA was in a final volume of 50μl.

2.4.6 Double GENECLEAN®.
Products of PCR reactions were prepared for ligation using the GENECLEAN® kit and protocol from Bio 101 Inc. (La Jolla, California), performing a double GENECLEAN®. Essentially this involved adding 3 volumes of NaI and 15μl GLASSMILK® to the final PCR reaction mixture (leaving behind the oil), the mixture being left on ice for 30 min, then the mixture washed and the DNA isolated as set out under section 2.4.5. This constituted the first GENECLEAN.

Half of the DNA was then digested with appropriate enzymes to produce "sticky ends" as set out in section 2.4.7, and the reaction stopped by the addition of 1μl 0.5M EDTA pH 8.0.

The DNA was then "GENECLEANed" as before using 10μl GLASSMILK® and was eluted into 25μl distilled water. The DNA was then ready for ligation into a vector.
2.4.7 Digestion with restriction enzymes.

These were carried out using appropriate buffers and enzymes following manufacturers
protocols (Boehringer Mannheim). 5 units of enzyme were allowed per microgram of
DNA, and the incubation carried out in sterile 1.5ml microfuge tubes (not more than
1/10 final volume was enzyme).

For HPGN, the BclI digest of the PCR product was performed at 50°C for 3
hours with buffer M, the pGEX 3X vector BamHI digest for 3 hours at 37°C with
buffer B, and the orientation after ligation checked by digestion with SphI and EcoRI at
37°C for 3 hours in buffer H.

For XPGN and XPGC, the PCR products were digested for 2 hours with BclI at
50°C then EcoRI added and the mixture placed at 37°C for a further 2 hours with
buffer H, and the pGEX 2T vector was digested with BamHI and EcoRI for 2 hours at
37°C with buffer B.

2.4.8 Ligation into pGEX.

Vector preparation:
1-2 µg vector DNA was digested with appropriate enzymes in a final volume of 25µl as
set out in section 2.4.7. After 1.5 hours 2µl (1 unit/µl) of calf intestinal phosphatase
(CIP, from Boehringer Mannheim) were added and the mixture incubated for another
hour. The digest reaction was then run out on a 1% TAE agarose gel as set out in
section 2.4.2, the linearised vector excised and purified using GENECLEAN® as set
out in section 2.4.3.

Insert preparation:
The PCR product was prepared as set out in section 2.4.4.

Ligation Reaction:
8.5µl insert DNA and 1µl vector DNA, or vector DNA alone (as a negative control)
were added to 2µl 10x Boehringer Mannheim T4 ligation buffer (660 mM Tris-HCl,
50mM MgCl₂, 10mM dithiothreitol, 10mM ATP, pH 7.5) and 2µl Boehringer
Mannheim T4 DNA ligase (10^3 units/ml) and water added to a volume of 20µl. The reaction was incubated at 15°C overnight, then 5µl of this mix added to 20µl water and used to transform competent cells.

2.4.9 Preparation of competent DH5α and transformation with plasmid DNA.

Preparation of Competent Cells:
Competent cells were prepared according to the CaCl2 method. 15ml of LB were placed in a 50 ml tube, inoculated with bacteria from a growing culture, and incubated overnight with shaking at 37°C. The culture was then diluted 1/100 (1ml into 100ml) with fresh LB and incubated in a 1 litre flask with shaking for a further 2.5 hours to ensure the cells were in log phase. The cells were then collected into 2 x 50ml polypropylene Falcon centrifuge tubes, spun down at 3,000rpm for 5 min in a Mistral 3000i benchtop centrifuge and resuspended in 50ml ice-cold 0.1M CaCl2, spun down again at 4°C, and resuspended in 10ml CaCl2. They were then left on ice for 45 min and could be stored for up to 4 days at 4°C.

Transformation:
100µl competent cells were taken and 5µl ligation mix with 20µl water added in a 1.5ml microfuge tube, gently mixed and left on ice for 35 min. The cells were then heat shocked in a water bath at 42°C for 3 min, replaced on ice for 5 min before 700µl LB were added and the cells incubated for a further 30 min in a waterbath at 37°C. The cells were then spun down for 3 min at 13,000rpm in a microfuge, before being plated out in a minimal volume of LB on L-agar plates containing 50µg/ml ampicillin. The plates were allowed to dry at room temperature, then incubated overnight at 37°C.

2.4.10 Maintenance of stocks of transformed DH5α.
Colonies were picked from plates using a sterile toothpick, re-streaked on L-agar plates with ampicillin at 50µg/ml to form master plates and grown overnight at 37°C.

Frozen stocks of transformed cells were made by inoculating 50ml LB, containing ampicillin at 50µg, with part of a colony from the master plate, growing the
culture overnight at 37°C with agitation, pelleting the cells and resuspending them in 1/10 the original volume of LB with ampicillin, adding an equal volume of sterile glycerol and mixing the solution thoroughly, allowing it to settle for 30 min then storing aliquots in 1.5ml microfuge tubes at -70°C until required. When used, samples were removed from the still frozen tubes using a sterile toothpick, streaked out on L-agar plates with ampicillin and grown overnight at 37°C before use as normal.

2.4.11 Mini-prep DNA preparations.
These were performed using StrataClean™ (from Stratagene Ltd., Cambridge) resin following an adaptation of the alkaline lysis protocol. 5ml of LB with ampicillin were inoculated with a single bacterial colony and grown overnight at 37°C with shaking. The bacteria were pelleted, resuspended in a 1.5ml microfuge tube in 100μl ice-cold solution 1 (50mM glucose, 52mM Tris pH 8.0, 10mM EDTA), 200μl freshly prepared solution 2 added (200mM NaOH, 1% SDS) and mixed by rapid inversion before incubating the mixture on ice for 5 min. 150μl ice-cold solution 3 were added (3M KAc, 11.5% glacial acetic acid) and mixed in by rapid inversion of the tube for 10 sec before a 10 min incubation on ice. The sample was spun at 13,000 rpm for 15 min at 4°C in a microfuge and the supernatant transferred to a clean 1.5ml microfuge tube. 20μl StrataClean™ resin were added to the supernatant and vortexed for 5 sec, incubated for 1 min at room temperature and spun again for 2 min in a microfuge. The supernatant was removed, 2 volumes of absolute ethanol added, vortexed and incubated for 30 min on ice. The sample was spun for 15 min at 4°C, the supernatant removed and the pellet allowed to air dry. 1ml ice-cold 70% ethanol were added, vortexed for 5 sec and re-centrifuged for 5 min at 4°C. The supernatant was again removed and the pellet dried for 20 min in a Heto "speed-vac" before being resuspended in 50μl dH2O.

2.4.12 Maxi-prep DNA preparations.
These were performed using QIAGEN columns (from QIAGEN Inc., Chatsworth, California) following the manufacturers protocol. 100-500 ml LB with ampicillin were inoculated and grown overnight at 37°C with shaking. The bacteria were pelleted in a
Beckman JA10 rotor at 6,000rpm for 15 min at 4°C resuspended in 10ml buffer P1 (100μg/ml RNase A, 50mM Tris, 10mM EDTA, pH 8.0), 10ml buffer P2 added (200mM NaOH, 1% SDS), mixed by gently inverting the tube and incubated at room temperature for 5 min. 10ml ice-cold buffer P3 (3M KAc, pH 5.5) were added and incubated on ice for 20 min before centrifugation at 4°C for 30 min at 15,000rpm in a Beckman JA20 rotor. If the supernatant was not clear when removed, it was re-centrifuged for 15 min under the same conditions and poured onto an already equilibrated QIAGEN-tip 500 (equilibrated with 10ml QBT buffer: 750mM NaCl, 50mM MOPS, 15% ethanol, 0.15% Triton X-100, pH 7.0). Once the supernatant had gone through, the tip was washed with 2x 30ml buffer QC (1M NaCl, 50mM MOPS, 15% ethanol, pH 7.0) and the bound DNA eluted with 15ml buffer QF (1.25M NaCl, 50mM Tris, 15% ethanol, pH 8.5). The DNA was then precipitated with 0.7 volumes isopropanol and pelleted at 11,250rpm in a Beckman JA20 rotor at 4°C for 30 min, washed with 15ml ice-cold 70% ethanol, re-centrifuged and allowed to air dry before being resuspended in 500μl dH2O.

2.4.13 Sequencing of DNA.

All sequencing was carried out using the Sequenase® Version 2.0 kit (United States Biochemical Corp.) which utilises the dideoxy chain termination method. 20μl DNA from a QIAGEN maxi-prep was denatured by the addition of 5μl fresh 1M NaOH, incubated at room temperature for 5 min, spun through a spin dialysis column of CL6B Sepharose (Pharmacia Biotech, Herts) for 1 min at 750rpm in a Mistral 3000i centrifuge and the resultant solution put on ice. 2μl Sequenase 5x Reaction buffer (200mM Tris pH 7.5, 100mM MgCl2, 250mM NaCl), 1μl oligonucleotide primer (0.5 pmole/μl) and 7μl of the freshly denatured DNA were mixed in a fresh 1.5ml tube and incubated for 2 min at 65°C, allowed to cool gradually to <35°C over a period of 20 min, to allow the primers to anneal to the DNA, then put on ice. This annealed template was then added to 2μl 1x labelling mix (5x mix: 7.5μM dGTP, 7.5μM dCTP, 7.5μM dTTP), 1μl 0.1mM DTT, 0.5μl 35S-dATP (Amersham International plc, Bucks.), 1μl Mn2+ buffer (0.15M sodium isocitrate, 0.1M MnCl2) and 2μl diluted
Sequenase® polymerase (1μl Sequenase® DNA polymerase with 8μl ice-cold enzyme
dilution buffer: 10mM Tris pH 7.5, 5mM DTT, 0.5mg/ml BSA), mixed thoroughly and
incubated at room temperature for 5 min. 3.5μl of the labelling mix was then added to
2.5μl each of four Termination mixtures, A,T,G, or C (80μM each dNTP and 8μM one
ddNTP in 50mM NaCl) preheated to 37°C, and the reactions allowed to continue for 5
min at 37°C before 4μl stop solution were added (95% formamide, 20mM EDTA,
0.05% bromophenol blue, 0.05% xylene cyanol FF). Samples were either frozen at
-20°C or used immediately by heating them to 75°C before loading them onto a
sequencing gel.

The vertical BRL model S2 electrophoresis system was used, and the
polyacrylamide gels were produced using Sequagel reagents (National Diagnostics) to
give a 6% gel. After electrophoresis the gel was fixed using 2 litres fixing solution
(10% acetic acid, 10% methanol), dried on a slab gel drier and exposed to X-OMAT™
AR Kodak film overnight at room temperature.
2.5 Protein biochemistry

2.5.1 Production and isolation of trpE-DP or trpE-Dsg1 fusion proteins.

A culture of TG1 cells transformed with pATH2 vector containing sequences of DP or Dsg1 (previously prepared by others in the laboratory, see Figure 12 for details of sequence used) was grown with shaking overnight at 37°C in 5ml M9 minimal medium supplemented with 20µg/ml tryptophan and 50µg/ml ampicillin. The culture was then diluted into 50ml of prewarmed M9 medium with ampicillin but without tryptophan and incubated for 1 hour at 30-32°C in a shaking waterbath. 250µl indole acrylic acid (1mg/ml, an artificial inducer of the trp operon) was then added and the incubation continued for a further 3 hours. The cells were pelleted for 15 min at room temperature at 2500rpm in a Mistral 3000i centrifuge, then resuspended in 0.5-1.0ml cracking buffer (6M urea, 1% [w/v] SDS, 1% [v/v] β-mercaptoethanol, 10mM Na2HPO4, pH 7.2) and incubated for 3 hours at 37°C. The lysates were passed repeatedly through a 21 gauge hypodermic needle to shear genomic DNA, and were centrifuged at 13,000rpm in a benchtop microfuge at 4°C to pellet insoluble debris. The lysates were then run on large scale preparative SDS-PAGE, the fusion protein band identified, by Coomassie blue staining, excited and electroeluted at 50V overnight in an Atto electroelution chamber.

2.5.2 Production and purification of GST-fusion Proteins.

An overnight culture of DH5α cells transformed with the pGEX vector with insert was grown with shaking in LB with ampicillin (final concentration 50µg/ml) at 37°C. This was then diluted 1/10 in pre-warmed medium and grown for 1 hour with shaking at 37°C before inducing fusion protein production with IPTG (final concentration 0.1mM) and growing for a further 5.5 hours. The cells were spun for 5 min at 10,000rpm in a Beckman JA10 rotor at 4°C and stored at -20°C overnight. They were then resuspended on ice in 1/100th volume of MTPBS including protease inhibitors (50µg/ml aprotinin, 50µg/ml leupeptin, 2mM EDTA, 2mM PMSF), sonicated twice on ice for 1 min using a wide bore probe and kept on ice for at least a further 15 min.
Triton X-100 was added to a final concentration of 1% and a further 1/50th volume of PMSF (100mM stock) added before spinning out the cell debris at 15,000 rpm for 10 min at 4°C in a Beckman JA20 rotor. The supernatant was taken and added to 3ml 50% glutathione-agarose beads (Sigma Chemical, Dorset) in MTPBS, and placed on a blood mixer at 4°C for at least one hour. The beads were pelleted for 5 min at 1,000 rpm in a Mistral 3000i centrifuge, washed three times in ice-cold MTPBS containing 1% Triton X-100, and a further two times in ice-cold MTPBS alone. The fusion protein was then batch eluted twice for 30 min at 4°C with one bead volume of freshly prepared 10mM reduced glutathione (Sigma Chemical, Dorset) in 50mM Tris-HCl pH 8.0 and the eluates pooled.

The fusion proteins were either dialysed overnight at 4°C against 1/10th XIB then lyophilised to 1/10th the original volume ready for microinjection, or dialysed against MTPBS alone and concentrated either by ultra-filtration or lyophilisation to 200μg/ml ready for injection for antibody production.

2.5.3 Expression and purification of His6-Dsg1 fusion protein.

The host bacteria (BL21(DE3)pLysS) with the vector (pET21a from Novagen) containing the Dsg1 cytoplasmic region were grown to an OD₅₉₀ of 1.5 in 100ml LBAm at 37°C. IPTG was added to a final concentration of 0.1mM and the cells grown for a further 25 min at 37°C with agitation, before 100μg/ml rifampicin were added and the culture grown for a further 2.5 hours. The cells were spun down at 3000rpm for 20 min in a Mistral 3000i centrifuge then resuspended in 0.5ml 10mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 100μg/ml lysozyme, rapidly frozen on a dry ice/isopropanol bath, left for 1 hour then thawed. 10mM MgCl₂ was added to titrate away the EDTA, and 50μg/ml aprotinin, 50μg/ml leupeptin, 2mM PMSF were added before adding 10μl of 50mg/ml DNase 1 and syringing the lysate. Imidazole was added to 10 mM final concentration, and the lysate loaded onto a column of Ni²⁺-NTA Resin (from Qiagen) previously packed and equilibrated with 10mM Tris pH 7.5, 150mM NaCl, 10mM imidazole. The column was washed with four column volumes
of 10mM Tris pH 7.5, 150mM NaCl, and the fusion protein specifically eluted with 250mM imidazole pH 7.0.

2.5.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5.4.1 Glycine-buffered.
These gels were used to separate proteins and prepare them for Western blotting. Separating gels were made using a mixture of 15% acrylamide stock solution (15% acrylamide, 0.4% bisacrylamide [purchased as stock solution from Anachem, Luton], 0.1% SDS, 375mM Tris pH 8.9) and 0% acrylamide stock solution (0.1% SDS, 375mM Tris pH 8.9) to obtain gels from 7.5% to 15% (to resolve according to the molecular weight of the examined proteins), polymerised with 0.05% TEMED and 0.05% APS and allowed to set at room temperature with a thin layer of isopropanol on the top. The isopropanol was then removed and the stacking gel poured (4.5% acrylamide, 0.12% bisacrylamide, 0.1% SDS, 125mM Tris pH 6.7 polymerised with 0.1% TEMED and 0.1% APS), a comb inserted and allowed to set at room temperature. Samples were loaded in Laemmli loading buffer with or without DTT (50-100mM) depending on whether the proteins of interest were to be examined reduced or non-reduced, and run in an Atto Mini-slab apparatus (Genetic Research Instrumentation Ltd., Essex) with 25mM Tris, 200mM glycine, 0.1% SDS as anode and cathode buffer.

2.5.4.2 Tricine-buffered.
Tricine-buffered SDS-PAGE gels were used to look at fusion proteins and digested fusion proteins for better resolution at lower molecular weights. The separating gels were made using 10ml acrylamide stock (48% w/v acrylamide, 1.5% w/v bisacrylamide), 10ml gel buffer (3M Tris pH 8.45, 0.3% SDS), 10ml dH2O, 150µl 10% APS, 15µl TEMED, poured, covered with a thin layer of isopropanol and allowed to set at room temperature. The isopropanol was removed and the stacking gel poured (1ml acrylamide stock, 3.1 ml gel buffer, 8.4ml water, 125µl 10% APS, 12.5 µl TEMED), a comb inserted and allowed to set. Samples were loaded in normal Laemmli sample
loading buffer with 50-100mM DTT, the gel was then run in an Atto Mini-slab apparatus using 200mM Tris pH 8.9 as the anode buffer and 100mM Tris pH 8.9, 100mM tricine, 0.1% SDS as the cathode buffer at 100-200V for approximately 2 hours (until the dye front reached the bottom of the gel).

2.5.5 Staining of SDS Polyacrylamide gels.

2.5.5.1 Coomassie Blue staining.

This was carried out to visualise proteins on an SDS-PAGE gel. Gels were fixed and stained in stain solution (2% Coomassie blue R250, 45% methanol) for 30-60 min at room temperature with agitation, then placed in number of changes of destain solution (20% methanol, 7% glacial acetic acid) with agitation at room temperature, or with slight heating, until the required amount of destaining had occurred (usually overnight if not heated). The amount of protein present on the gel could estimated when compared to a dilution series of standard protein (e.g. BSA) run at the same time.

2.5.5.2 Silver staining.

This was carried out to visualise protein that could not be detected by Coomassie blue staining after SDS-PAGE. All procedures were carried out at room temperature wearing gloves to ensure no further protein attached to the gel. Gels were fixed in 50% methanol, 10% acetic acid for 30 min, transferred to a solution of 5% methanol, 7% acetic acid for a further 30 min, fixed for a further 30 min in 10% glutaraldehyde, then soaked overnight in a large volume of distilled water. The gel was then soaked in a fresh solution of DTT (5μg/ml) for 30 min, placed into 0.1% silver nitrate for 30-60 min, rinsed twice with distilled water, rinsed twice with fresh developer solution (3% sodium carbonate, 0.0185% formaldehyde), then soaked in developer solution for 5-30 min (usually approximately 10 min) until the bands were sufficiently visible. Development was stopped by the addition of 5ml 2.3M citric acid per 100ml developer solution, and the gel stored in distilled water or 0.03% sodium carbonate.
2.5.6 Fluorography.

SDS-PAGE of radioactive samples was run as described in section 2.5.4. The gel was then removed and placed in 1M sodium salicylate for 15-20 min at room temperature before being dried onto Whatman® 3MM as backing paper at 60°C for 1-2 hours. The gel was then exposed to pre-flashed X-OMAT™ AR film (Kodak) overnight, or for longer if required.

2.5.7 Western blot transfer.

2.5.7.1 Semi-dry blot.

Four pieces of gel sized Whatman® 3MM paper soaked in transfer buffer (25mM Tris, 192mM glycine, 20% methanol, 0.05% SDS, pH 8.3) were placed on the lower (positive) electrode, a wetted piece of supported nitrocellulose (Hybond™-C extra, Bio-Rad Laboratories Ltd., Herts.) cut to the same size placed on top, the gel, pre-equilibrated in transfer buffer, placed above and four more pieces of soaked Whatman paper placed above again. Any excess buffer was removed to prevent a short circuit between the upper and lower electrodes. Gels were transferred using a semi-dry blot apparatus (Biometra Fast blot apparatus) running at 1mA/cm² of gel for 2-3 hours.

2.5.7.2 Wet blot.

The "sandwich" was constructed essentially the same as for a semi-dry blot and was placed in a wet-blot apparatus (Bio-Rad Mini Trans-blot Cell apparatus, Bio-Rad Laboratories Ltd., Herts.) such that the blot was completely immersed in transfer buffer with the nitrocellulose membrane towards the anode. The transfer was left running at 25V overnight at 4°C. If the proteins to be blotted were large or difficult to transfer, "strong" transfer buffer was used instead (100mM Tris, 760mM glycine, 0.1% SDS, 20% methanol pH 8.3).

Particularly large proteins, such as APC, were transferred using a different blot buffer (25mM Tris; 192mM glycine; final pH 8.2), a cooled Hoeffer system and extreme transfer conditions: 400mA for the first hour followed by 1.5A for the next 18 hours.
2.5.8 Staining Western blots.

2.5.8.1 Staining proteins on Western blots with Ponceau S.
This was used temporarily to visualise proteins on the Western blot before probing took place to check transfer. Blots were stained for 5 min in Ponceau S solution (0.5% w/v in 5% w/v trichloroacetic acid) then rinsed with water until bands were visualised as desired.

2.5.8.2 Staining proteins on Western blots with amido black.
This was used as a more permanent stain to visualise proteins on a blot. Blots were placed in stain (0.1% amido black, 45% methanol, 10% acetic acid) for 1-5 min, then rinsed a few times rapidly with destain (45% methanol, 1% acetic acid) and allowed to destain in a final wash until bands were visualised as desired. The blot could then be kept for a few days in water for photography.

2.5.9 Probing a Western blot.
After Ponceau staining, as set out in 2.4.6.1, blots were blocked for 1 hour at 37°C or overnight at 4°C in blocking solution (5% Marvel in PBS-A with 0.1% Tween 20, 0.02% sodium azide), washed twice for 5 min in PBS-A, and twice for 10 min in PBS-T, and incubated with an appropriate concentration of antibody in blocking solution for 1-3 hour at room temperature. After this time, the blot was washed as before and incubated for 1 hour at room temperature with secondary antibody (goat-anti-rabbit or goat-anti-mouse antibody conjugated with horseradish peroxidase purchased from Bio-Rad Laboratories Ltd., Herts. or goat-anti-rabbit conjugated with alkaline phosphatase from Sigma Chemical, Dorset) at 1/1000 in blocking solution without azide. The blot was then washed as before and developed using ECL™ reagents (Amersham International plc, Bucks.) for the blots incubated with horseradish peroxidase conjugate, exposing the blot to X-OGRAPH BLUE X-ray film (X-ograph Imaging systems, Wilts.) for varying periods of time until the desired exposure was obtained, or in AP Buffer (100mM Tris pH 9.5, 100mM NaCl, 5mM MgCl₂) with NBT.
(33μl 50mg/ml 70% dimethylformamide stock per 5ml AP Buffer) and BCIP (16.5μl 50mg/ml in dimethylformamide stock per 5ml AP Buffer) for 5-20 min until bands developed as required.

2.5.10 In vitro kinase assay.

A431 membranes or EGF receptor bound to Protein A-beads via the R1 antibody (beads 1:1 in NP40 lysis buffer) were obtained as set out in section 2.9.10 and 2.9.9 respectively. 10μl of these membranes or 10μl 1:1 slurry of beads were added to 5μg fusion protein, 24μl 2.5x kinase buffer (250mM NaCl, 125mM Hepes pH 7.5, 12.5mM MgCl₂, 12.5mM MnCl₂, 25μM ATP) with or without 1μl EGF (10μg/ml stock) and water to 60μl, incubated for 30 min at 30°C, then 15μl 5x Laemmli loading buffer with DTT added, and the samples heated to 99°C in a PCR machine for 3 min to stop the reaction.

2.5.11 Binding assays.

2.5.11.1 Fusion proteins.

NP40 lysates of radiolabelled or EGF-treated or untreated A431 cells were obtained as set out in section 2.9.3. The lysate from one confluent 90mm dish was pre-cleared with 160μl of a 1:1 slurry of glutathione-agarose beads (Sigma Chemical, Dorset) or 320μl of a 1:1 slurry of nickel-agarose beads (QIAGEN Inc., Chatsworth, California) in NP40 buffer for 1 hour at 4°C. The beads were spun down, 40μg of fusion protein added to the supernatant and mixed for 1 hour at 4°C. 40μl of a 1:1 slurry of fresh glutathione-agarose beads or 80μl of a 1:1 slurry of nickel-agarose beads were then added and mixed again for 1 hour at 4°C. The beads were pelleted and washed, as were the "pre-clear" beads, 4 times with ice-cold NP40 buffer and once with ice-cold 50mM Tris pH 6.8 before being taken up into 1x Laemmli loading buffer, heated and run on SDS-PAGE as set out under section 2.5.4.
2.5.11.2 Immunoprecipitations.

NP40 lysates of radiolabelled or EGF-treated or untreated A431 cells were obtained as set out in section 2.9.3. The lysate from one quarter of a confluent 90mm dish was pre-cleared overnight with 10μl of pre-immune serum. 40μl of a 1:1 slurry of Protein A-beads in NP40 buffer were added and mixed for 30 min at 4°C, the beads pelleted and 10μl of terminal bleed serum added to the supernatant and mixed for 1 hour at 4°C. 40μl of fresh 1:1 slurry of Protein A-beads were added for 1 hour then the beads pelleted again. The beads, as well as the "pre-clear" beads, were washed 4 times with NP40 buffer, once with 50mM Tris pH 6.8, then heated to 99°C in a PCR machine for 3 min in 1x Laemmlili loading buffer before being run out on SDS-PAGE as set out in section 2.5.4.

2.5.11.3 Overlays.

Whole A431 lysates were obtained as set out in section 2.9.5, run out on SDS-PAGE and Western blotted as set out in sections 2.5.4 and 2.5.7. The blots were blocked overnight at 4°C in TST (10mM Tris 7.5, 150mM NaCl, 0.1% Tween 20), washed 4x 5 min in TBS (50mM Tris pH 7.5, NaCl 150mM) then incubated with fusion proteins at 10μg/ml in TST for 2 hours at room temperature. The blots were washed 2x 5 min in TBS, 2x 10 min in TST, then probed with anti-GST antibody in TST for 1 hour 30 min at room temperature, washed as before, incubated with alkaline phosphatase-conjugated anti-rabbit antibody for a further hour at room temperature, and developed using NBT and BCIP as set out in section 2.5.9.

2.5.12 Protein concentration estimation using Bio-Rad Protein Assay.

This is an adaptation of the Bradford protein estimation method and manufacturers protocol was followed (Bio-Rad Laboratories Ltd., Herts.). A dilution series of BSA in buffer was made (diluted in XIB for F_ab fragment estimations) and 5μl of each dilution pipetted in duplicate into wells of a 96 well microtiter plate, as were 5μl of the sample of interest. The stock dye solution was diluted 1 in 5 and 250μl added to each well and mixed. After between 10 and 45 min incubation at room temperature, the plate was
placed in a microtiter plate reader and the absorbence at 620nm read. A standard curve was drawn and the concentration of sample protein estimated using it.
2.6 Histology/sectioning methods.

2.6.1 Slide subbing.

This is a process in which slides are thoroughly cleaned, then coated with a thin film of material (TESPA or gelatin) resulting in a surface that promotes maximal adherence of sections.

2.6.1.1 TESPA subbing of slides.

Slides were cleaned by dipping them in 10% HCl (12M), 70% ethanol, rinsed in dH2O, washed with 95% ethanol and dried at 150°C for 5 minutes and allowed to cool. They were then dipped in 2% TESPA in acetone for 10 seconds, before being rinsed twice in acetone and once in dH2O. The slides were then dried at 42°C in a clean atmosphere.

2.6.1.2 Gelatin subbing of slides.

Slides were cleaned by dipping them in 10% HCl (12M), 70% ethanol, rinsed in dH2O, washed with 95% ethanol and dried at 150°C for 5 minutes and allowed to cool. A solution of 2.5g gelatin, 0.25g chrome alum (CrK(SO₄)2·12H₂O) in 500ml water was prepared by heating, then filtered and cooled on ice. The slides were then dipped into the solution and allowed to dry in a dust-free atmosphere.

2.6.2 Embryo fixation protocols.

2.6.2.1 Paraformaldehyde fixation.

The embryos were demembranated (removing the vitelline membrane using two pairs of fine forceps), placed in an ice-cold solution of freshly made 4% paraformaldehyde (made up in 70% PBS-A) and placed at 4°C overnight. They were then washed briefly in 70% PBS-A before being embedded.

2.6.2.2 TCA fixation.

The embryos were demembranated, placed in ice-cold 2% TCA and placed at 4°C overnight. They were then washed briefly in 70% PBS-A before being embedded.
2.6.2.3 Methanol fixation.
The embryos were demembranated, placed in ice-cold 100% methanol (or 80% methanol, 20% DMSO) and placed at -20°C overnight.

2.6.3 Embedding and sectioning protocols

2.6.3.1 Acrylamide embedding and sectioning.
For acrylamide embedding the embryos were placed in ice-cold AEM solution (Acrylamide Embedding Medium stock solution: 8.4g acrylamide, 13.4mg bisacrylamide, 10ml 10x PBS-A and water to 100ml) with TEMED (1μl per ml of AEM) for 5-18 hours at 4°C. 50μl 10% APS and 10μl TEMED were added to 10 ml AEM, gently mixed and poured into a number of aluminium foil moulds to form solid acrylamide bases, and allowed to set under vacuum (in a dessicator). The embryos were then orientated on this base and more AEM with TEMED and APS added to fill the mould to the brim. The medium was allowed to set under vacuum overnight, or with a layer of Parafilm over the top to exclude air and the moulds placed at 4°C. The samples were then frozen in a dry ice-ethanol slurry before being mounted and cut (6-10μm sections) on a Shandon Cryotome 620 cryostat at -23°C and sections collected on gelatin-subbed slides.

2.6.3.2 'OCT' embedding and sectioning.
For 'OCT' embedding (Tissue-Tek® O.C.T. compound from Raymond A. Lamb, London) the embryos were placed in 2.5% sucrose, 0.1% sodium azide for 30 minutes at room temperature after fixation as a cryoprotection measure to prevent the embryos splitting too much when frozen. They were then placed in OCT medium for 30 minutes at room temperature before freezing to allow impregnation of the medium. To mount the samples, a daub of medium was first frozen in isopentane (2-methylbutane) previously cooled in liquid nitrogen for 3-5 minutes (if left in the liquid nitrogen too long, the isopentane becomes too cold, the medium freezes too quickly, and cracking occurs causing problems when sections are cut), a slight indentation made and the embryo placed and orientated in this, before being covered with more medium and
frozen. The samples were then allowed to equilibrate to -23°C before being cut on a Shandon Cryotome 620 cryostat (6-10μm sections) and collected on gelatin-subbed slides.

2.6.3.3 Wax embedding and sectioning.

The embryos were incubated for 30 minutes with agitation at 4°C, unless otherwise stated, in the following solutions: twice in 70% saline; once in 1:1 70% saline:100%ethanol; twice in 70% ethanol; twice in 85% ethanol; twice in 95% ethanol; twice in 100% ethanol; twice in toluene at room temperature; once in a 1:1 mix of toluene with fibrowax at 60°C; twice in fibrowax at 60°C; then transferred into plastic moulds and orientated in fresh fibrowax at 60°C. Once cooled, the blocks were trimmed and mounted on a wooden block and 6μm sections cut at room temperature using a Reichert-Jung Biocut microtome (Raymond A. Lamb, London). The ribbons of sections were floated on a 50°C waterbath, collected on TESPA-subbed slides and allowed to dry on an 'electrothermal slide drying bench' (Raymond A. Lamb, London).

2.6.4 Staining of sections.

Acrylamide- and OCT-embedded sections did not need any pre-treatment before immunostaining and so were used as retrieved from the cryostat.

2.6.4.1 Dewaxing of wax sections before immunostaining.

Slides were dipped for 10 minutes twice in Xylene, then gradually rehydrated by dipping for 2 minutes each as follows: twice in 100% ethanol, then once each in 90%, 70%, 50%, 30% ethanol and finally twice for 5 minutes in PBS-A.

2.6.4.2 Immunostaining.

Sections were kept in a moist atmosphere. The slides were blocked in 2% normal goat serum for at least 30 minutes, before being rinsed in PBS-A. The primary antibody was made up at 1/100 in PBS-A with 0.1% TritonX-100, 2% foetal calf serum and 0.1% sodium azide and placed on the slides in a humidified box and incubated at room
temperature overnight. The slides were rinsed twice for 10 minutes with slight agitation in PBS-A before the secondary antibody (biotinylated goat-anti-rabbit from Vector laboratories, Peterborough at 1/100 in PBS-A) was added and incubated in a humidified box, for 3 hours at room temperature. The slides were again rinsed in PBS-A, and the tertiary antibody (FITC conjugated streptavidin from Vector Laboratories, Peterborough at 1/500 in 10% foetal calf serum in PBS-A) added and left in the dark at room temperature in a humidified box for 2-3 hours. The slides were rinsed and a solution of DAPI (at 1μl/ml) and eriochrome black (at 0.005%w/v, used to quench autofluorescence of the yolk platelets) in PBS-A added for 10 minutes before being rinsed again. The slides were then mounted using Citifluor (City University, London) or Vectashield™ (from Vector Laboratories, Peterborough) and viewed under the fluorescence microscope. Glycerol had also been used as a mounting medium and found not to be as good for enhancing fluorescence.

2.6.4.3 Giemsa counterstaining.
Alternate sections were sometimes collected on separate slides for counterstaining to help identify features of the embryo. Giemsa solution (Sigma Chemical, Dorset) was diluted 1:1 with dH2O and placed on the slides for 1 minute at room temperature. The slides were then rinsed four or five times until most of the excess stain was removed and mounted in glycerol.
2.7 Antibody methods.

2.7.1 Antibody production and sera preparation.

Rabbits were injected subcutaneously at six sites on the back with 0.5ml fusion proteins (200μg/ml) homogenised with an equal volume of Freunds adjuvant (complete for the primary immunisation and incomplete for the boosts), then boosted at monthly intervals six times with bleeds a week later, before terminal bleeding/exsanguination. Sera were prepared by leaving the blood to clot at 37°C for 30 min, then placing it to contract on ice for 1 to 2 hours or overnight at 4°C. The blood was spun for 10 min at 1,000rpm at 4°C in a Mistral 3000i centrifuge, the supernatant re-spun for 10 min at 1,500rpm at 4°C to pellet any red blood cells, then the supernatant removed, aliquoted and stored at -20°C. Aliquots taken to 4°C had 0.02% azide added for preservation.

2.7.2 Antibody purification.

2.7.2.1 Preparation of an Affi-10 gel affinity column.

Recombinant protein, prepared either by electroelution from bacterial preparations for Dsg1 and DP fusion proteins (see section 2.5.1) or by glutathione-agarose affinity chromatography for GST-PG fusion proteins (see section 2.5.2), was dialysed against PBS-A, MTPBS or other non-Tris based buffers before being allowed to couple with a slurry of Affi-10 beads in buffer (Bio-Rad Laboratories Ltd., Herts.), in a ratio of at least 200μg protein per ml of settled beads, overnight at 4°C. The beads were rinsed three times with buffer, transferred to a column and pre-eluted with 0.1M glycine pH 3.0, rinsed with buffer then pre-eluted with 0.1M triethylamine pH 11.5 and rinsed again. A sample of beads was taken and tested by SDS-PAGE for protein binding.

2.7.2.2 Antibody purification by affinity chromatography.

1ml of serum was added to 2ml buffer (as in section 2.7.2.1) and agitated overnight at 4°C with 2ml Affi-10-trpE-DP or -trpE-Dsg1 beads, then the beads were packed into a disposable minicolumn (Amicon) and rinsed with buffer (5 column volumes). The reactive antibodies were eluted using 10x 1ml aliquots of 0.1M glycine pH 3.0, or with 10x 1ml aliquots of 0.1M triethylamine pH 11.5, collected into tubes containing 50μl
2M Tris-HCl pH 8.0 to neutralise the solution as it was collected, and the column rinsed and re-equilibrated with buffer.

In the case of the antibodies to be used for microinjection, the antisera were run initially over an Affi-10-GST column (to remove GST-reactive antibodies) and the supernatant and first wash (i.e. the GST-non-reactive fractions of the antiserum) were then recycled over a Protein A column five times. This column was then eluted using 10x 1ml aliquots of 0.1M glycine pH 3.0 collected into tubes containing 100μl 1M Tris-HCl pH 8.0.

Samples from each fraction were run out by SDS-PAGE (non-reducing), and fractions that contained antibodies were pooled. If antibodies were be used for F\textsubscript{ab} fragment generation they were dialysed overnight against 100mM sodium acetate pH 5.5, or if they were to be used for microinjection experiments they were dialysed against 1/5x XIB to be further concentrated to 1x XIB by lyophilisation.

2.7.3 F\textsubscript{ab} fragment generation.
The anti-GST-depleted serum was concentrated to approximately 5mg/ml in 100mM sodium acetate pH 5.5. Cysteine and EDTA were added to a final concentration of 50mM and 1mM respectively. 1μg papain was added per 500μg of antibody, the solution mixed well and incubated at 37°C for 2 hours. Iodoacetamide was then added to a final concentration of 75mM to quench the reaction and incubated at room temperature for 30 min (papain requires reducing conditions, provided by the cysteine, to work; when iodoacetamide is added, cysteine is alkylated and the reaction stops). The F\textsubscript{ab} fragments were then isolated by running the solution over a Protein A column to which they did not bind whereas the F\text{c} fragments and uncut antibodies bound. The F\textsubscript{ab} fragments were then dialysed against 1/5x XIB overnight and lyophilised to 1/5 volume to concentrate them to approximately 5mg/ml ready for microinjection. The concentration was confirmed by protein assay as set out in section 2.5.12 and running samples out on SDS-PAGE and Coomassie staining as set out in sections 2.5.4 and 2.5.5.
2.7.4 Preparing a cross-linked Protein A-bead-antibody affinity column.

Antibodies were allowed to bind to Protein A-beads for 2 hour or overnight at 4°C with agitation (approximately 2mg antibody per ml wet beads) in PBS-A. The beads were washed twice with 10 volumes 0.2M sodium borate pH 9.0, resuspended in 10 volumes sodium borate, and dimethylpimelidate (Sigma Chemical, Dorset) added to a final concentration of 20mM. The slurry was mixed for 30 min at room temperature and the cross-linking reaction stopped by washing with 0.2M ethanolamine pH 8.0 then incubating the beads in ethanolamine for 2 hours at room temperature or overnight at 4°C. The beads were then washed with PBS-A and stored in PBS-A with azide at 4°C until required.
2.8 *Xenopus laevis* techniques.

2.8.1 *Xenopus* NP40 lysate preparation for original Western blot.

200μl of ice-cold lysis buffer (50mM TrisHCl pH 7.5, 1% NP40, 100mM NaCl, 1mM EDTA, 5mg/ml Pepstatin, 5mg/ml Leupeptin, 5mg/ml Antipain, 1% Aprotinin and 1mM PMSF) were added to fifteen staged embryos in a 500μl microfuge tube which were then homogenised by pipetting up and down through a yellow Gilson tip (if the embryos were a late stage and much larger than early stages, fewer embryos were used, or more lysis buffer added). The mixture was vortexed for 45 seconds before being replaced onto ice for 15 minutes. An equal volume of ice-cold Freon (1,1,2-trichlorotrifluoroethane) was then added and the mixture vortexed again for 45 seconds. The microfuge tube was then spun at 6500 rpm in a refrigerated microfuge for 5 minutes. The lysate separated into three layers: the lower phase contained the Freon and the majority of the yolk platelets, the interface contained the NP40-insoluble components of the embryo, and the aqueous phase contained the NP40-soluble (cytosolic and membrane) components of the lysate. The upper phase was TCA precipitated overnight at 4°C, by addition of ice-cold TCA to a final concentration of 10%, then washed twice in cold 5% TCA and twice in ice-cold 100% ethanol. The pellet was dried in a Speedvac and resuspended in 100μl 1x Laemmli loading buffer with boiling. The interface fraction was washed in PBS-A, dried in a Speedvac and resuspended in loading buffer with boiling. The lower phase fraction, containing the yolk platelets, was discarded.

2.8.2 Routine preparation of NP40 embryo extracts for SDS-PAGE.

Aliquots of different stage embryos were routinely stored at -70°C in 0.5ml tubes. 0.1μl aprotinin (2.2mg/ml stock) and 0.1μl PMSF (100mM stock) per embryo were added to the aliquots as they defrosted on ice. 10μl/embryo lysis buffer (50mM Tris pH 7.5, 50mM KCl, 2.5mM MgCl2, 0.5mM DTT, 1% NP40, 5μg/ml leupeptin, 5μg/ml pepstatin) were added on ice and the embryos homogenised by pipetting up and down a yellow Gilson tip. The homogenate was spun at 13,000rpm in a microfuge at 4°C, to remove the yolk platelets, and the supernatant taken. This was spun again if necessary.
and the lipid layer removed from the surface. Laemmli loading buffer with DTT was
added to a final concentration of 1x and the samples heated in a PCR machine to 99°C
for 3-5 min.

2.8.3 Preparation of organ extracts for SDS-PAGE.
Two hearts, or approximately 4cm² skin from a freshly sacrificed adult male *Xenopus*
was rinsed briefly in ice-cold 70% PBS-A, diced into small pieces on ice and 4ml ice-
cold extraction solution added (50mM DTT, 3% SDS, 50mM Tris pH 6.7) in a 50ml
tube. The tissue was homogenised on ice using a polytron (15 sec bursts at half speed).
The samples were heated at 70°C for 5 min, spun at 4°C for 15 min at 2,000rpm in a
Mistral 3000i centrifuge, the supernatant aliquoted and stored at -70°C until needed.

2.8.4 Embryo subcellular fractionation protocol.
10 embryos of a particular stage were homogenised in 1ml ice-cold SF buffer (250mM
sucrose, 10mM HEPES pH 7.5, 2mM MgCl₂, 1mM EGTA, 0.5mM EDTA, 1mM
PMSF, 10μl/ml aprotinin, 5μg/ml leupeptin, 5μg/ml pepstatin), and spun at 750g for 5
min at 4°C to pellet the yolk platelets and large portions of membrane. This pellet was
resuspended in 50μl SY buffer (0.5% NP40, 150mM NaCl, 10mM HEPES pH 7.5,
1.5mM EDTA, 0.5mM EDTA, 1mM PMSF, 10μl/ml aprotinin (2.2mg/ml stock),
5μg/ml leupeptin, 5μg/ml pepstatin) and spun at 16,000g for 10 min at 4°C giving a
pellet of yolk and NP40-insoluble proteins and a supernatant of membrane proteins.
Both these samples were resuspended in Laemmli buffer in preparation for SDS-PAGE
analysis. The supernatant from the original spin was layered above 100μl 2M sucrose
and spun at 150,000g for 30 min at 4°C using a Beckman SW55Ti rotor in a Beckman
L8-70 ultracentrifuge. The supernatant represented soluble proteins to which TCA was
added to a final concentration of 10%. The proteins precipitated on ice for 30 min,
then were washed twice with 5% TCA, and twice with 100% ethanol, air dried and
taken up into Laemmli buffer for SDS-PAGE analysis. The pellet from the sucrose
spin represented the majority of the embryos membranes. 1ml cold 10% TCA was
added to this fraction and the proteins precipitated on ice for 30 min then washed and prepared as set out above.

2.8.5 Microinjections into 4 cell stage Xenopus.
Female frogs were primed with 800 units of human chorionic gonadotrophin (HCG) 12 hours before eggs were required. The eggs were then "squeezed" (gentle peristalsis of the mothers ventro-lateral surface) and fertilised by brushing fresh testis from a sacrificed male over them. The embryos were flooded with 3/4 Normal Amphibian Medium (1x NAM: 110mM NaCl, 2mM KCl, 1mM Ca(NO₃)₂, 1mM MgSO₄, 0.1mM Na₂EDTA, 2mM sodium phosphate pH 7.0, 1mM NaHCO₃, 50mg/ml gentamycin) and allowed to rotate which usually takes about 20 min at room temperature. Once rotation had occurred the embryos were de-jellied in 2% cysteine hydrochloride pH 8.0 and cultured in 3/4 NAM until injection.

Injections were performed using an air-driven injector (Inject+matic apparatus) with the embryos in a solution of 3/4 NAM containing 4% Ficoll to reduce any blebbing that may occur due to breaking the embryo membrane; sample size was 30-45 embryos per set of injections. Varying amounts of protein or Fₐb fragment solutions (from 0.1-0.5ng total) were injected into a ventral blastomere at the four cell stage (identified by being one of the two darker pigmented cells at that stage) usually in a volume of 10nl, and the embryos allowed to develop in the 3/4 NAM with Ficoll for a few hours, then transferred to 1/10 NAM before gastrulation to prevent exogastrulation. The embryos were allowed to develop until approximately stage 34 and were scored for defects/abnormal development.

2.8.6 Whole mount antibody detection to track injection.
Microinjected embryos were fixed for 5 min at room temperature in methanol, bleached at least overnight in 70% methanol-10% H₂O₂, re-hydrated in PBS-A, blocked for a few hours at room temperature in 1% Marvel-PBS-T and incubated with alkaline phosphatase-conjugated anti-rabbit antibody overnight at 4°C. The embryos
were then extensively washed (5x 1 hour) and developed using NBT and BCIP (reagents as set out in section 2.5.9).

2.8.7 Whole mount antibody muscle counter-stain.
Microinjected embryos were fixed in 4% paraformaldehyde overnight at 4°C, washed twice for 30 min in PBS-A before being dehydrated in sequential methanol dilutions: 50%, 70% and two times 100% for 30 min each at room temperature. The embryos were then bleached extensively at room temperature (2 or more days) in 70% MeOH, 10%H₂O₂.

The embryos were washed 2x 5 min and 2x 30 min in PBS-BT (0.2% BSA, 0.1% Triton X-100 in PBS-A), incubated in PBS-BTN (PBS-BT with 5% normal goat serum) for 30 min then incubated overnight at 4°C with 12/101 (mouse monoclonal antibody muscle specific marker (a kind gift from the Smith laboratory at the NIMR) at 1/750 in PBS-BTN. The embryos were washed 3x 5 min and 4x 30 min in PBS-BT, once for 30 min in PBS-BTN and incubated overnight at 4°C with horseradish peroxidase-conjugated secondary antibody (Sigma Chemical, Dorset) diluted 1/100 in PBS-BTN. They were washed again in PBS-BT 3x 5 min and 4x 30 min before development, then incubated for 10 min in DAB solution (0.3mg/ml NiCl₂, 0.3mg/ml DAB [Sigma Chemical, Dorset] in PBS-BT), transferred to glass staining wells with fresh DAB solution with 0.03% H₂O₂ and the reaction allowed to continue for 5-10 min. The reaction was stopped by adding PBS-A and 2mM EDTA. The embryos were then sequentially dehydrated in 30 min each 25%, 50%, 75% methanol/PBS-A and 100% methanol, then were cleared for 10 min at room temperature in 2:1 benzyl alcohol: benzyl benzoate and photographs taken at different magnifications on a Leica Wild M8 binocular microscope, with Leica Wild MPS 52 camera attached, and exposure times controlled by a Leica MPS 48 control panel (Kodak Ektachrome 64T professional film used).
2.9 Tissue Culture.

All tissue culture was carried out in a laminar flow class II tissue culture hood.

2.9.1 Growth.

A431 cells (an adherent cell line derived from a human epidermal carcinoma expressing large quantities of EGF receptor) were routinely grown at 37°C with 5% CO₂ in complete DMEM medium (Gibco BRL/Life Technologies) supplemented with 10% foetal calf serum (Sigma Chemical, Dorset), 0.006% penicillin, 0.01% streptomycin and 0.0292% glutamine in 75cm² flasks.

SW480 cells (an adherent cell line derived from a grade 3-4 colon adenocarcinoma carrying a truncated APC product) were routinely grown at 37°C in the absence of CO₂ in Liebovitz L-15 medium (Gibco BRL/Life Technologies) supplemented with 10% foetal calf serum, 0.006% penicillin, 0.01% streptomycin and 0.0292% glutamine in 75cm² flasks.

XL-cells (a *Xenopus laevis* liver-derived cell line) were routinely grown at room temperature and atmospheric CO₂ in the dark, in 70% (v/v) diluted Liebovitz L-15 medium (Gibco BRL/Life Technologies) supplemented with 10% foetal calf serum, 0.006% penicillin, 0.01% streptomycin and 0.0292% glutamine in 75cm² flasks.

Sf9 cells (a non-adherent/loosely adherent insect cell line derived from pupal ovarian tissue of *Spodoptera frugiperda*) were routinely cultured at room temperature (25°C) in TC100 insect medium (Gibco BRL/Life Technologies) supplemented with 10% foetal calf serum without added antibiotics. They were usually grown in suspension with stirring, and plated out onto tissue culture plates when preparing for infection with baculovirus.

Media were stored at 4°C, and cells re-fed with fresh medium every 2-3 days. If the cells were transferred to tissue culture dishes, they were incubated in a humidified box (containing paper towels dampened with distilled water containing 1% CuSO₄) to reduce evaporation.
2.9.2 Passaging.
Sf9 cells were grown in suspension, by Mr C. Young of Biological Services at the NIMR, and were diluted routinely by him. A431, SW480, and XL cells were passaged by trypsinisation when confluent. The medium was poured off, and the cells rinsed two times with PBS-A, or with 70% PBS-A in the case of XL cells. A thin layer (approximately 1ml) of 0.25% trypsin in 140mM NaCl, 0.3mM EDTA, pH 8.0 was added to the cells and any excess poured off. The cells were left in the hood until they detached, the flask hit hard once on the side to loosen any remaining rounded up attached cells, and fresh medium added. A 1/10 or 1/20 dilution was made into fresh flasks.

2.9.3 NP40 lysis.
This was performed when wishing to keep complexes of proteins intact. A 90mm dish was plated out, and when 80-90% confluent the medium was aspirated off, the cells rinsed two times with PBS-A, and 1ml of ice-cold NP40 buffer added (1% NP40, 150mM NaCl, 1mM DTT, 50mM Tris pH 8.0, 1mM PMSF, 10μl/ml aprotinin [2.2mg/ml stock]) with or without phosphatase inhibitors (1mM sodium orthovanadate, 5mM NaF, 10mM sodium pyrophosphate). The cells were then scraped using the blunt end of a 1ml Gilson tip, the lysate collected into a 1.5ml microfuge tube, cell debris spun down by centrifugation (5-10min at 13,000rpm in a benchtop microfuge), and the supernatant stored at -70°C until required.

2.9.4 NIF fraction extract.
This was the NP40 insoluble fraction obtained from section 2.9.3 which was resuspended in 1x Laemmli sample buffer containing 50mM DTT, and contains the nuclei, intermediate filaments and desmosomes from the cells.

2.9.5 Whole cell lysis (Laemmli buffer).
This was performed when wishing to solubilise the maximal amount of protein from the cell. A 90mm dish was plated out, and when 80-90% confluent the medium was
aspirated off, the cells rinsed two times with PBS-A (or with 70% PBS-A if for XL-cells), and 0.5-1ml 1x Laemmli sample buffer containing 50mM DTT added if the samples were to be run directly on a gel, or 0.5-1ml 1% SDS, 25mM Tris pH 6.8 added otherwise. The cells were then scraped using the blunt end of a 1ml Gilson tip and the lysate taken up and down a narrow gauge syringe needle to shear the DNA, before being collected into a 1.5ml microfuge tube and stored at -70°C until required.

2.9.6 Radioactive labelling.
An 80% confluent 90mm dish was labelled overnight, the medium being replaced with 5ml labelling medium (95% methionine-free medium with 10% dialysed [against PBS-A] foetal calf serum, glutamine and antibiotics, plus 5% complete medium, 0.5mCi 35S-TRAN label [ICN Biomedicals Ltd., Oxfordshire]). The cells were then lysed in either NP40 Buffer and frozen or scraped in PBS-A, the cells spun down, the PBS-A removed and the pellet frozen at -70°C until required.

2.9.7 Freezing cells.
The cells were trypsinised as usual and taken up in fresh medium into centrifuge tubes. These were spun at 500rpm for 5 min in a Mistral 3000i centrifuge at room temperature, the medium removed and the cells taken up into 1.5ml of ice-cold freezing medium (complete medium with 10% DMSO). The suspension was then placed in an ice-cold cryotube and frozen in liquid nitrogen vapour over a period of 5 hours or more, followed by transfer to liquid nitrogen for long term storage.

2.9.8 Infection of Sf9 cell line.
Cells were obtained in suspension and the density calculated using a haemocytometer. Dilutions were made if necessary so that 3x10^6 cells were plated out into 60mm dishes or 5ml flasks, the cells allowed to settle at room temperature for 30-60 min, the medium replaced with 5ml fresh medium with an appropriate amount of EGF receptor recombinant baculovirus (kindly supplied by Dr George Panayotou, Ludwig Institute e.g. 50μl). The cells were grown and monitored for 2-3 days, the medium (a virus
stock) removed and stored at 4°C, the cells harvested and spun down for 7 min at 750 rpm in a Mistral 3000i centrifuge, washed with PBS-A and frozen at -70°C as a pellet until required.

2.9.9 Purification of recombinant EGF receptor.
A pellet of infected Sf9 cells was taken from -70°C, resuspended on ice in 200µl NP40 buffer with phosphatase inhibitors, taken up and down a narrow gauge syringe to shear the DNA and the cell debris spun out in a microfuge at 13,000rpm for 5 minutes at 4°C. 10µg R1 anti-EGF receptor antibody were added to the supernatant, and the mixture agitated for 1 hour at 4°C. 25µl of a 1:1 slurry of Protein A-beads were added for 1 hour at 4°C with agitation, the pellet spun down and the beads washed twice in ice-cold NP40 buffer with protease inhibitors then used immediately for kinase assay.

2.9.10 Membrane preparation of A431 cells.
Cell membranes were obtained by taking the p100 fraction from 2 confluent 150mm tissue culture dishes of A431 cells. Cells were washed twice in cold PBS-A then once in cold hypotonic lysis buffer (5mM Tris pH 7.5, 1mM EGTA, 1mM MgCl2) and scraped into 5 ml of lysis buffer. Cells were left on ice for 10 min to swell, broken by passage through a narrow gauge syringe needle 20 times and pelleted for 10 min at 2,000rpm at 4°C. The supernatant was pelleted for 30 min at 50,000 rpm in a Beckman SW55 rotor to generate the p100 pellet. This was dissolved in 1ml NP40 lysis buffer with phosphatase inhibitors, followed by spinning for 30 min at 4°C in an SW55 rotor at 50,000rpm and removing the supernatant. This was aliquoted out and stored at -70°C until required.
Chapter 3: Initial antibody studies on *Xenopus* and attempted cloning of a *Xenopus* desmoglein.

3.1 Introduction.

At the time this project was begun (Autumn 1991), little was known about the role of desmosomes and desmosomal proteins during vertebrate development, and an ongoing aim of the laboratory was to investigate this area. *Xenopus* was chosen as a model system in which to begin addressing this question, due to the ease of accessibility of the developing embryo throughout all stages of development, and also due to the recent investigations that had been conducted characterising the expression pattern of various classical cadherins in *Xenopus* development giving a background with which any results could be compared (for example Angres *et al.*, 1991; Choi & Gumbiner, 1989; Choi *et al.*, 1990; Detrick *et al.*, 1990; Ginsberg *et al.*, 1991; Herzberg *et al.*, 1991; Levi *et al.*, 1991a & 1991b; Müller *et al.*, 1992; Winklbauer *et al.*, 1992). Other studies had confirmed the importance of cadherins during *Xenopus* development, and illustrated ways in which the questions concerning the function of the desmosomal cadherins could be addressed using this system, reinforcing the decision to use *Xenopus* as a model organism (for example Detrick *et al.*, 1990, and Fujimori *et al.*, 1990, both examined mutant phenotypes when N-cadherin expression was perturbed). Interestingly, these studies also indicated that although cadherins were as important in early *Xenopus* development as in other vertebrates, there were also differences in the precise molecules used, particularly in the very earliest stages of development. For example in the mouse, E-cadherin appears to be the only cadherin expressed during the earliest stages of development (it is present from the egg stage onwards; Vestweber *et al.*, 1987), whereas in *Xenopus* E-cadherin is not expressed until the beginning of zygotic transcription at the midblastula transition (Choi & Gumbiner; 1989). A surprising variety of different cadherins were shown to be present at the very earliest stages of development by a number of different laboratories and the situation did not appear at all clear (C-cadherin [Choi *et al.*, 1990], EP-cadherin [Ginsberg *et al.*, 1991], U-cadherin [Angres *et al.*, 1991; Müller *et al.*, 1992 ], and XB-cadherin [Herzberg *et
It has since been shown, however, that some of these cadherins are identical (e.g. XB- and U-cadherins), and some are allelic variations (e.g. EP- and C-cadherin) thus helping to clarify matters greatly. The excellent review by Kühl and Wedlich (1996) explains the current understanding of classical cadherins' expression patterns and roles during early *Xenopus* development.

Preliminary Western blot experiments performed by Dr Al Magee showed that a desmoglein is present in a NP40-soluble fraction in early *Xenopus* development (at stages before fully formed desmosomes had been observed; described from stage 7 onwards by Franz et al., 1983). This allowed for the possibility that the desmosomal cadherins might have roles during development other than simply as components of desmosomes, and confirmed *Xenopus* as a reasonable choice of model organism in which to conduct investigations. It was therefore decided to characterise the expression pattern of certain desmosomal proteins at these early stages of development, to determine at what stage the desmoglein assembled into desmosomes, and into which subcellular compartments the protein localised beforehand. If the desmoglein was expressed at the cell surface throughout all stages of development, this would tend to indicate an adhesive role independent of its association at desmosomes. These expression studies were initially undertaken using conventional fluorescence immunohistology, described in sections 3.2 and 3.3, and were to be enhanced, once the basic expression pattern was understood, with confocal studies to determine subcellular localisation's at specific developmental points. These antibody-based studies were to be complemented later with RNA *in situ* analysis once a *Xenopus* desmoglein clone had been identified. The search for a desmoglein clone is described in section 3.4. Once the clone was isolated intervention experiments were planned (full length overexpression studies, as well as dominant negative studies using deletion constructs and antisense oligonucleotides/sequences) to determine if the desmoglein had a morphoregulatory role to play during development. Unfortunately, as described in section 3.4, no desmoglein homologue was isolated, and so these studies were not feasible.
3.2 Screening of antisera for cross-reactivity with Xenopus.

To begin the expression studies a panel of 17 polyclonal antibodies generated in the laboratory were screened by Western blot for cross-reaction with extracts prepared from either XL cells (a liver-derived desmosome bearing cell line; Anizet et al., 1981) or from unfertilised Xenopus eggs prepared as set out in section 2.8.1. Of these antisera four polyclonals gave positive reactions (Figure 11A). Two were directed against a desmoglein (920 and 921 both generated against the cytoplasmic domain of human Dsg1; Wheeler et al., 1991) and two against desmoplakin (121 and 145, both generated against the C-terminal domain of human desmoplakin; Arnemann et al., 1993). A variety of the antisera were also used for immunoprecipitations using radiolabelled XL cell lysates, with similar results; 920, 121 and 145 all gave positive bands at the correct molecular weight (Figure 11B). A summary of the antisera tested and results obtained is given in Figure 12.

Specific antibodies from the antisera 920 and 121 were then purified by affinity chromatography as set out in section 2.7.2, using recombinant fusion-protein from the pATH vector obtained as set out in section 2.5.1, for use in immunohistology and for screening described later in section 3.4.4.

3.3 Fluorescence immunohistology.

A variety of fixation, embedding and mounting techniques (see section 2.6) were tested over a period of approximately 12 months using the cross-reactive antibodies (both the antisera and purified antibodies) on SVK14 cells (a human keratinocyte cell line) and 6-10μm sections of Xenopus embryos (stages 29 onwards) to determine the optimal conditions for antibody reactivity. Fixations included 100% methanol, 4% TCA or 4% paraformaldehyde; embedding materials included acrylamide, historesin (Reichert-Jung, Cambridge Instruments; sections kindly prepared by Mr J. Asante, Histology section NIMR), wax or 'OCT', and mounting media included Citifluor, glycerol or Vectashield™ as set out in materials and methods. Each pair of antisera directed against the same epitope (i.e. 920 and 921, or 121 and 145) reacted identically under the same conditions.
FIGURE 11: Screening of antisera for cross reaction with *Xenopus*.

A  Examples of Western blots showing cross reaction of antisera 121 (anti-desmoplakin) and 920 (anti-pan desmoglein) with *Xenopus* samples. Arrows indicate position of proteins, markers indicated on right.

- DP  desmoplakin
- Dsg  desmoglein
- I  *Xenopus* egg extract
- II  *Xenopus* XL cell whole cell extract
- III  *Xenopus* XL cells NIF extract

The Western blots pre-date routine use of ECL reagents, so the BCIP/NBT colour system was used and unfortunately the results have faded considerably since the experiments were performed hence only two blots are shown.

B  Results of immunoprecipitation experiments using various antisera and XL cell lysates.

The numbers at the top indicate the antiserum used for immunoprecipitation in that lane. Arrows indicate positions of positive band. Dsg, desmoglein; DP, desmoplakin.
**FIGURE 12: List of antisera screened to test for cross reaction with Xenopus samples.**

In general the antisera were generated in pairs of rabbits against the same immunogen.

<table>
<thead>
<tr>
<th>Name</th>
<th>Generated Against</th>
<th>Blot Result</th>
<th>IP Result</th>
</tr>
</thead>
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<tr>
<td>37</td>
<td>Human Dsg-1 extracellular domain</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>38</td>
<td>as 37</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>918</td>
<td>human Dsg-1 cytoplasmic domain: bps 2218-3422 in pATH2 including the DG specific repeats</td>
<td>-/+</td>
<td>ND</td>
</tr>
<tr>
<td>920</td>
<td>as 918</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>921</td>
<td>as 918</td>
<td>+</td>
<td>ND</td>
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<tr>
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<td>human Dsg-1 cytoplasmic repeat</td>
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<td>-</td>
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<tr>
<td>971</td>
<td>as 970</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>human Dsc2a C terminal peptide: KLH conjugated last 12 amino acids</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>as 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>human Dsc2 fusion</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>629</td>
<td>as 3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>144</td>
<td>human Dsc2 extracellular domain: bps 1-2084 extracellular fragment in pATH2 vector</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>154</td>
<td>as 144</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>human Dsc b form C-terminal peptide: KLH conjugated last 11 amino acids of human Dsc2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>as 11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>name</td>
<td>generated against</td>
<td>blot result</td>
<td>IP result</td>
</tr>
<tr>
<td>------</td>
<td>------------------</td>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>121</td>
<td>human DP C-terminal domain: bp 4247-5228 of human DP in pATH2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>145</td>
<td>as 121</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
For SVK14 cells on slides, methanol fixation gave good results for both the anti-desmoglein and anti-desmoplakin antisera; paraformaldehyde treatment was satisfactory for the anti-desmoplakin antisera but not for the anti-desmoglein sera; treatment with TCA quenched the epitopes for both sets of antisera (Figure 13). Treatment of the slides following the protocol used for wax embedding, or exposure to acrylamide, also quenched the epitopes.

For *Xenopus* sections, repeated trials indicated that methanol, TCA, historesin and wax treatments were unsuitable as they apparently destroyed/altered the epitopes the antibodies recognised. Best results were obtained using paraformaldehyde fixation followed by 'OCT' embedding and Vectashield™ mounting for both sets of antisera (Figure 14), although the anti-desmoglein antisera were much poorer reagents than the anti-desmoplakin antisera, and they gave much more variability in their response. When these fixation and embedding techniques were used to prepare sections from the early stages of development, tissue integrity was severely compromised and so were unsuitable for use in attempting to map the expression pattern of the molecules throughout development. The yolk which made up the bulk of the embryo at these early stages repeatedly fell off the slides taking large areas of the cells with it, making identifying any areas of staining impossible (this was probably due to lack of deep medium penetration).

If time had not been limiting, the investigation could have been continued by attempting whole mount staining followed by sectioning, but as this technique had not been used before in the laboratory, the amount of time needed to optimise the technique before getting useful results would have been too long and was not attempted. This work was carried out in parallel with negative cloning experiments described in section 3.4, and so due to time limitations it was abandoned at this point.

### 3.4 Attempted cloning of a *Xenopus* desmoglein.

The original Western blot data from Dr AI Magee, as well as the data from Figure 11, showed that *Xenopus* has at least one desmoglein homologue and it was decided to
**FIGURE 13:** Immunostaining of MDCK cells.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH</td>
<td>Methanol fixed cells</td>
</tr>
<tr>
<td>Para</td>
<td>Paraformaldehyde fixed cells</td>
</tr>
<tr>
<td>DP</td>
<td>Cells stained for desmoplakin</td>
</tr>
<tr>
<td>DG</td>
<td>Cells stained for desmoglein</td>
</tr>
<tr>
<td>NRS</td>
<td>Cells stained with normal rabbit serum</td>
</tr>
</tbody>
</table>
FIGURE 14: Immunostaining of *Xenopus* sections.

NRS  Section stained with Normal rabbit serum
DG  Section stained with anti-desmoglein antibodies
DP  Section stained with anti-desmoplakin antibodies

Note intercellular staining in epidermis with both anti-desmoglein and anti-desmoplakin antisera.
attempt it's cloning for use in *in situ* analysis and for intervention experiments. Although all materials present in the laboratory were generated using human desmoglein sequences, the fact that frog and human cells can form desmosomes between them (Mattey and Garrod, 1985) suggested the human and frog homologues are sufficiently similar that the human-derived reagents would detect a *Xenopus* homologue during the screening process.

There are two traditional methods for screening a cDNA library, both with their advantages and disadvantages when attempting to bridge the gap between two species as divergent as humans and *Xenopus*. The first method of screening uses radiolabelled nucleic acid probes (DNA screening). We had the human sequences for three isoforms of desmoglein in the laboratory at the time, so this method of screening was a possibility. An attractive feature of screening this way is that probes will hybridise with appropriate sequences regardless of reading frame, so the whole complexity of the library is screened. The disadvantages however are that the DNA sequence is likely to be very divergent over such a large evolutionary gap and so low stringency hybridisation and washing conditions have to be used to ensure any similar sequences are detected. Unfortunately, this will also lead to the detection of a larger number of false positives. In the particular case of desmogleins there is also the problem of humans having multiple isoforms with *Xenopus* possibly having a single more primitive/primordial version, so there is the added disadvantage of having to judge which isoform to probe with, and whether this will actually hybridise successfully with the *Xenopus* homologue.

The second method involves using antibodies to screen an expression library and given that in this particular case we had purified antibodies that we knew crossreacted well with *Xenopus*, this was an option. An advantage of this method is that, whereas the DNA sequences may be quite different across species, the amino acid sequences will be less divergent, with epitopes conserved sufficiently for the antibodies to recognise them i.e. less identity is required between homologous/orthologous molecules for antibodies to be effective at identifying putative clones than when using DNA. However, the disadvantages include the fact that the reading frame is important.
for this method. In the making of any cDNA expression library, sequences are ligated into the vector with a 1 in 3 chance of being in the correct reading frame, and a 1 in 2 chance of being in the correct orientation, resulting in only a 1 in 6 chance of a sequence yielding the molecule coded for correctly when expression is induced, effectively reducing the library's complexity.

The libraries that were available for screening could be probed using either method. Given the advantages and disadvantages of both methods of screening over such a large evolutionary distance, it was decided to use both methods in parallel to try to identify any putative *Xenopus* desmoglein clones (sections 3.4.2 and 3.4.4). The more recent approach of "touchdown PCR" using degenerate primers was also attempted (section 3.4.5).

### 3.4.1 Southern blot analysis of *Xenopus* genomic DNA.
To determine which of the three human desmoglein isoforms to use for DNA screening of the library, *Xenopus* genomic DNA (a kind gift from Dr M. Sargent) was digested with either *BamHI* or *HindIII* and Southern blotted as set out in section 2.4.3. cDNA probes generated using the three different human isoforms of desmoglein as templates were made (section 2.3.1) and used to probe the blot under low stringency conditions (section 2.4.4). As can be seen from Figure 15 a faint signal was seen with the DSG3 probe, so this isoform was chosen for screening the library.

### 3.4.2 Screening of a λZap2 *Xenopus* liver library using a cDNA probe.
#### 3.4.2.1 Initial screening process
An adult male *Xenopus* liver λZap2 library was available (a kind gift from Dr J.R. Tata.) and, as liver is an organ one would expect desmosomes (and so desmoglein) to be present in, it was thought to be an ideal candidate library for screening. A Western blot of *Xenopus* liver samples using the pan-desmoglein antiserum, was performed before screening began to confirm the presence of a desmoglein. The library had been constructed by Dr A. Kawahara (Hiroshima University, Japan) using *EcoRI* oligodT primed sequences, and the insert size was 1-2kb with multiple inserts in each phage. It
FIGURE 15: Southern blot of Xenopus genomic DNA using the three human desmoglein isoforms as probes.

Xenopus genomic DNA obtained from blood, kindly supplied by Dr M. Sargent, was digested with either Bam HI or HindIII for 1.5 hours at 37°C as set out in section 2.3.5 and anti-desmoglein probes prepared as set out in section 2.3.1 from clones present in the laboratory. To make the probes the following templates were used:

- Dsg1: Full length human Dsg1 (EMBL accession number X56654)
- Dsg2: bp numbers 2663-3455 (EMBL accession number Z26317; Full sequence not known at the time).
- Dsg3: bp numbers 447-1670 (EMBL accession number M76482)

The blots were probed under low stringency conditions (hybridisation at 60°C without formamide followed by the first wash at 50°C in 0.1% SDS, 1x SSC, and the second in 1xSSC). The faint signals that were seen when the and blot was probed for Dsg3 are indicated by the arrows on the right hand side. The large amount of signal seen on the left of each blot corresponds to the lambda markers of 1.0kb, 0.517bp and 0.396 bp indicated on the left of the diagram. EtBr is a picture of the ethidium bromide stained gel before blotting.

I lambda markers
II BamHI digested Xenopus genomic DNA
III HindIII digested Xenopus genomic DNA

plasmid and how much?Bluescript; Dsg2 in XXXsame again; and Dsg3 as a full length clone?3' bit p56 in the pGEM®-11Zf(+/−) vector.

DSG3 is bp 447-1670 EMBL accession number M76482
Dsg1 whole length EMBL accession number X56654
Dsg2 2663(Ja156)-3455(Ja157) EMBL accession number Z26317 - whole clone available at the time.
had been used previously by Dr Q. Xu, during her studies with Dr Tata at the Institute, to obtain two clones (the *Xenopus* C/EBP gene [Xu et al., 1992], and *Xenopus* liver-type arginase [Xu et al., 1993]) and so was thought to be a good library to screen. It was titred at 4.2x10^10 pfu/ml, and a primary round plated out as described in section 2.3.2 with a total of 2x10^6 pfu screened.

A primary round of screening yielded approximately 30 potential clones which were all replated and screened again (examples of autoradiographs from successive rounds of screening are shown in Figures 16, 17, and 18). From this secondary screen, a number of putative positives were then plated out for a third round at which time they seemed to have been isolated to homogeneity. However, when the *in vivo* excision protocol was followed to subclone the putative clones (section 2.3.4) a number of problems prevented successful subcloning; these included severe bacterial contamination problems, and even when some uncontaminated colonies were obtained following the excision process, DNA was difficult to obtain. The protocol was attempted a number of times, over a period of 4 months, with different batches of host bacteria (different stocks of XL1-Blue and also TG1) and different helper phage stocks (both R408 and ExAssist from different laboratories) without success, although when a positive control was used (a known clone from a different library, kindly supplied by Dr A. Chambers) ampicillin resistant colonies were produced.

3.4.2.2 Attempts to isolate the insert DNA by alternative methods.

In an attempt to isolate putative clones by conventional methods, the plug stocks were also used for λ DNA preparations (section 2.3.5). When samples of undigested and digested (EcoRI) phage DNA were separated by agarose gel electrophoresis and stained, an unusual set of results were observed (Figure 19A). In both the undigested and digested DNA lanes, bands were seen at approximately 3kb as well as larger bands, presumably representing the λ arms. It was thought that the band at 3kb might be the single stranded version of the Bluescript plasmid with insert (corresponding to the 3kb Bluescript plasmid with 3kb of insert when double stranded), or the double stranded Bluescript plasmid with a small insert or without any insert (so representing false
FIGURE 16: Examples autoradiographs from primary round of screening using radiolabelled DNA probes.

A and B are replica lifts from one of four plates used for the primary round of library screening. The plaques picked are identified by circles shown on A. The circles without a question mark beside them appeared the most promising and were amongst the first to be replated for the secondary round of screening. A total of thirty plaques were picked from the primary round of screening.
FIGURE 17: Examples of autoradiographs from the secondary round of screening.

Examples from three plates from the secondary round of screening that yielded positive plaques. A and B are relipca lifts. Circled "dots" were picked and a selection of these replated for the tertiary round of screening.
FIGURE 18: Examples of autoradiographs from the tertiary round of screening.

These are replica lifts that were generated when certain of the plaques from plates 3 or 16 (circled in Figure 18), were plated out. The vast majority of plaques correlate between the two autoradiographs correlate, and represent the majority of the plaques on the plate, indicating that the putative clone was isolated to homogeneity. Some of these plaques were picked and used for the in vivo excision process as described in the text.
positives). Both possibilities would imply that the phagemid had somehow self-excised during the λ phage prep. To test whether these bands of approximately 3kb did contain Bluescript sequence, a Southern blot of the phage prep was probed with radiolabelled probes generated against Bluescript. These bands reacted strongly with the probes as shown in Figure 19B. When these same blots were probed for DSG3 all bands were positive, including a negative control band (Figure 19C) probably indicating that the stringency used was sufficiently low that all bands would hybridise making interpretation of the results difficult.

Samples of the putative Bluescript-containing bands were separated by agarose electrophoresis, GENECLEANed and used to transform competent cells. Ampicillin-resistant colonies grew, tending to support the hypothesis that the bands identified were in fact Bluescript-derived, carrying ampicillin resistance, somehow excised from the λ arms. When DNA mini-preps were performed using these colonies, and both undigested and digested (EcoRI) DNA separated by agarose gel electrophoresis, it seemed that there were small inserts of approximately 0.75kb for a number of the mini-preps (Figure 20A) supporting the hypothesis that the approximately 3kb band identified from the λ prep was probably the double stranded Bluescript with a small insert. When the gel was Southern blotted and probed for Dsg3 the vector appeared to hybridise, probably non-specific as the bands that react were quite strong, but the inserts did not react (Figure 20B).

Samples of the DNA isolated by mini-prep were then used for PCR using the T3 and T7 primer sequences. These sequences are located either side of the polylinker region of Bluescript, and the intention was to PCR the insert (annealing conditions 51°C) and perform Southern blot analysis on any products. The PCR gave a smear and a number of faint bands as a result, and when the Southern blot was probed for DSG3, no hybridisation was evident. Aliquots of phage from the secondary screen plugs were also PCRed using both M13 forward and reverse primers, and T3 and T7 primers. The reactions were separated by agarose gel electrophoresis, but no bands could be identified by ethidium bromide staining, and when the gel was blotted and probed with DSG3 radiolabelled probes, no bands hybridised except the positive control, suggesting
FIGURE 19: Lambda DNA preparations from putative clones/Odd results from lambda prep of λzap2 library.

A. Ethidium bromide stained agarose gel showing bands at approximately 3kb in both the undigested and digested DNA.

B. Southern blot of A, probed for bluescript sequence, showing a reaction with all the bands of approximately 3kb.

C. Southern blot of A probed for Dsg3 sequence, showing a reaction with all the bands of approximately 3kb.

i or other roman numerals are putative DP containing clone and so are negative controls.
FIGURE 20: Results of mini-preps of putative clones.

A. Ethidium bromide stained gel showing presence of inserts of approximately 0.75kb.

B. Southern blot of A probed with Dsg3. Note that the vector at 3kb lights up but the inserts of 0.75kb do not
that the isolated plaques were probably false positives. At this point the library was abandoned as it seemed that all possible courses of action to isolate the clones had been pursued without success.

3.4.3 Attempted isolation of DSG clone from other libraries using cDNA.
A number of different libraries were then obtained (Figure 21), λ preparations performed, the DNA digested with the enzymes used to make the libraries, samples separated by agarose gel electrophoresis and Southern blotted with probes directed against Dsg3. As can be seen from Figure 22, only the λ arms lit up for all the libraries except the stage 24 λgt10 library prepared by Kintner & Melton (1987). Although this was probably a non-specific reaction because the ethidium bromide stained band was relatively intense, there was the possibility that this could be a real reaction with the gene being represented well (especially as other strong bands did not hybridise - e.g. lanes in H2 and H4, Figure 22B). Therefore, samples of the λ DNA were digested as before and the band GENECLEANed, the DNA ligated into Bluescript and transformed into competent XL1 blue cells. Colonies were picked, mini-preps performed, the gels blotted and probed with DSG3, but all bands hybridised suggesting they were non-specific bands and making interpretation of the results impossible (Figure 23A and B). To double check that the libraries did not contain an isolatable insert, PCR was also performed using the original λ prep DNA (Figure 23C) and a Southern blot probed with DSG3 without success. At this point cloning using cDNA technology was abandoned.

3.4.4 Screening of a λZap2 expression library (stage 52 tail) using antibodies.
A number of the libraries available were constructed in λgt11 or λZap2, and so could be screened using antibodies. Western blots were performed on adult heart extracts and on stage 52 tail extracts using the purified pan-desmoglein antibody to determine which of the libraries to screen. Both were equally good by Western blot, and it was decided to probe the λZap2 tail library as this included skin in its preparation and so would be more likely to include the skin-specific isoform (the laboratory has a specific interest in
FIGURE 21: Table of libraries tested for screening.

<table>
<thead>
<tr>
<th>organ</th>
<th>stage</th>
<th>vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>head</td>
<td>tailbud</td>
<td>λzap2</td>
</tr>
<tr>
<td>heart</td>
<td>adult</td>
<td>λzap2</td>
</tr>
<tr>
<td>whole</td>
<td>st 24</td>
<td>λgt10</td>
</tr>
<tr>
<td>whole</td>
<td>oocyte</td>
<td>λgt11</td>
</tr>
<tr>
<td>whole</td>
<td>oocyte</td>
<td>λgt11</td>
</tr>
<tr>
<td>whole</td>
<td>st17</td>
<td>λgt11</td>
</tr>
<tr>
<td>tail</td>
<td>st 52</td>
<td>λzap2</td>
</tr>
<tr>
<td>liver</td>
<td>adult</td>
<td>λzap2</td>
</tr>
<tr>
<td>whole</td>
<td>10-11</td>
<td>λzap2</td>
</tr>
</tbody>
</table>
FIGURE 22: Southern blots of λ preparations from different libraries.

See text for details.

A. Ethidium bromide stained gel of digested λ DNA prepared from different libraries.

B. Southern blot of A probed for DSG3.

Key:

λH  λ DNA digested with HindIII as markers
λHE λ DNA digested with HindIII and EcoRI as markers
1  λZap2 library from tailbud head (Figure 21 library 1)
2  λZap2 library from adult heart (Figure 21 library 2)
3  λgt10 library from whole stage 24 embryo (Figure 21 library 3)
4  λgt11 library from whole oocyte (Figure 21 library 4)
5  λgt11 library from whole oocyte (Figure 21 library 5)
6  λgt11 library from whole stage 17 embryo (Figure 21 library 6)
7  λZap2 library from stage 52 tails (Figure 21 library 7)
8  λZap2 library from adult liver (Figure 21 library 8)

C negative control of no virus added to bacteria for overnight culture

H2 and control DNA of upstream sequence of Dsc2 in λfix2 vector (from M. Marsden)

Marker values given on the right.
FIGURE 23: Results using alternative methods in an attempt to isolate a Dsg clone.
The gels and blots presented here are raw data and unfortunately have writing on them (as it was not thought at the time that this data would not be presented). Please ignore all writing on the figures other than the labels.

A. Ethidium bromide stained gel of DNA obtained following mini-preps of bacteria transformed with Bluescript containing the insert band seen in lane 3 on figure 22.

B. Southern blot of A probed for DSG3.

Key:
λH λ DNA digested with HindIII as markers
λHE λ DNA digested with HindIII and EcoRI as markers
1, 4, 7, 9 DNA from mini-preparations of different individual colonies.
cut DNA digested with EcoRI (enzyme used to make the library)
uncut DNA not digested

C. Ethidium bromide stained gel of DNA obtained following PCR using λ DNA prepared as for Figure 22 using T3 and T7 sequences as primers for the λZap2 and λgt10 libraries and specific λgt11 sequences as primers for libraries made using those vectors. Southern blot not shown.

Key:
λH λ DNA digested with HindIII as markers
λHE λ DNA digested with HindIII and EcoRI as markers
+ PCR positive control for T3 and T7 primers
1 λZap2 library from tailbud head (Figure 21 library 1)
2 λZap2 library from adult heart (Figure 21 library 2)
3 λgt10 library from whole stage 24 embryo (Figure 21 library 3)
4 λgt11 library from whole oocyte (Figure 21 library 4)
5 λgt11 library from whole oocyte (Figure 21 library 5)
6 λgt11 library from whole stage 17 embryo (Figure 21 library 6)
7 λZap2 library from stage 52 tails (Figure 21 library 7)
8 λZap2 library from adult liver (Figure 21 library 8)
skin) as opposed to the heart-specific isoform if *Xenopus* did indeed have multiple isoforms of desmogleins. The library had a complexity of $1.6 \times 10^6$ (with an average insert size of 1.5kb) and was plated out on 150mm dishes at a density of $5 \times 10^4$ pfu/plate, with a total of 30 plates. 21 putative clones were picked from the primary round of screening, a number of these were plated out for a second round of screening at very low density and individual plaques could be picked. (These data cannot be presented as the filters used for screening were developed using BCIP/NBT colour development and have faded since the time the experiments were done; work predates the routine use of ECL as a detection method). A λ preparation was performed on 5 putative clones, and when digested all gave inserts of approximately 1-1.5kb. The *in vivo* excision protocol was performed on some of the clones, and three colonies picked at random to go on and sequence using universal forward and reverse primers after mini- and maxi-preps. None of the three putative clones sequenced had significant homology to a desmoglein. One clone appeared to be Troponin T (90% homology using the BLAST program), a second had no significant homology to anything in the database, and the third appeared to be 60s ribosomal protein L7A.

3.4.5 Touchdown PCR.

In a final attempt to isolate a *Xenopus* desmoglein clone, touchdown PCR was performed on genomic DNA using degenerate primers present in the laboratory (designed using the human and bovine sequences by Dr G. Wheeler; Figure 24). One very small (approximately 0.28kb) faint band appeared following the PCR reaction, which was thought not to represent a real product of interest. Indeed, when the gel was Southern blotted and probed for Dsg3, the band did not react. Apart from the fact that the experiment was attempting to cross a large species gap, one of the reasons that this process did not work was probably because of the different codon usage between mammals and *Xenopus* which was not taken into account when the primers were made. At this stage (August 1993) all screening was abandoned.
FIGURE 24: Degenerate primer sequences.

Primers used for degenerate PCR:

GW6

\[\text{5'}\]
ACT GGA ATT CIA CIC GCT CIG TIA CAA TIA CAT T
TT G G
\[\text{3'}\]

GW3

\[\text{5'}\]
ACT GGA ATT CGT IGC IGG lA C lA T IGG ITG CTG CC AA C TG G
\[\text{3'}\]

GW3 is the sense primer corresponding to the conserved amino acid sequence V/P/L, A/N, G/A, S, V/I, G, C, C near the end of the homology to the classical cadherins, plus a 5' EcoRI site.

GW6 is the antisense primer corresponding to the fifth repeated domain (NVIVTERV) in the cytoplasmic region unique to the desmogleins, plus a 5' EcoRI site.
3.5 Summary and discussion.

3.5.1 Antibody studies.

A number of different antisera in the laboratory, generated against mammalian desmosomal components, were screened for crossreactivity with *Xenopus* lysates by Western blot analysis, and four found to crossreact well. Two of the antisera were then tested for their efficacy in immunofluorescence studies using both SVK14 cells and sections of tailbud-tadpole stage embryos, and fixation and embedding conditions were optimised. However when early stages of *Xenopus* development were examined, the antisera were found to be unusable as the conditions required for epitope recognition were incompatible with those needed to obtain good sections. This is due to the fact that it is technically difficult to maintain tissue integrity of *Xenopus* at early stages of development when cutting sections not using wax or historesin embedding techniques. *Xenopus* early stage embryos are predominantly composed of yolk platelets which, if not supported sufficiently by the embedding medium, are difficult to cut through cleanly, and which tend not to adhere well to the slide once sections are cut, removing areas of the embryo with them. For normal staining of tissues (haematoxylin, eosin etc.) both the wax and historesin embedding techniques are satisfactory as the stains work well on sections and tissue integrity is maintained, but for immunohistology the epitopes recognised by antibodies can be more delicate and destroyed using these methods, probably due to exposure to high temperatures. For this study, of the tissue preparations tested, the antisera required embedding directly in a medium which does not support cellular infrastructure well, resulting in very poor quality sections at the stages of interest (very early development), and the eventual abandonment of the project. However, there is now available a low melting point wax which could be tested if the project were to be attempted again, which might overcome the problems encountered here. Morphology would be maintained, and there is a possibility that, without exposure to high temperatures, the epitopes recognised by the antisera would be maintained, although it is possible that the chemicals involved in the de-waxing procedure might still alter the epitopes sufficiently to prevent antibody recognition. It
should also be noted that when sections were prepared using acrylamide embedding (no exposure to high temperatures), the antisera in this study did not give a good response, so it may be more than simply exposure to heat preventing epitope recognition.

A compounding problem when using *Xenopus* embryos for fluorescence immunohistology studies is the fact that the yolk platelets autofluoresce. It is therefore difficult to obtain good immunofluorescence results from when using FITC as a fluorophore (green), as the yolk platelets autofluoresce a yellowy-green, making interpretation of results difficult, particularly if the signal is weak. There is a method to quench this autofluorescence (incubation with eriochrome black, making the platelets appear an orange/yellow colour instead; see materials and methods), but the efficacy of this method is variable, making interpretation of results difficult especially at early stages of development when the platelets make up the bulk of the cell. Using Rhodamine or Texas Red as fluorophores overcomes this problem, as the platelets do not interfere so much with the signal at this excitation wavelength, but both of these fluorophores are more difficult to obtain good pictures from for detailed analysis as they are less sensitive, and this makes results interpretation more difficult when the signal is weak. For this study fluorescence immunostaining, as opposed to insoluble colour immunostaining (e.g. DAB or NBT/BCIP), was chosen to examine protein localisation as confocal studies were anticipated to complement and enhance the conventional studies. In retrospect, it seems that insoluble colour staining might have been a better technique to use, at least for initial studies (which if successful could be complemented at a later stage with conventional fluorescence and confocal studies), given the large amount of autofluorescence at the early stages of development we were interested in.

As mentioned in section 3.3, time was limiting and so whole mount studies were not performed, but this obviously presents an alternative method of attempting to determine the proteins' expression patterns. By staining the embryo before sectioning, this method offers a way of overcoming the problems of having to maintain epitope integrity whilst obtaining good sections. However, this method has other caveats, such as the problem of antibody penetration; with sections, the antibodies do not have to
penetrate very far (sections are usually approximately 6μm thick), and so incubation times are relatively short (e.g. 1-2 hours). For the whole embryo, however, to ensure that the antibodies penetrate deep into the interior of the embryo, much longer incubation times are required (overnight), and the majority of protocols also use methanol to help permeabilise the embryo before the antibodies are used, which with the antisera used in this study would cause a problem. No doubt a protocol could be found that did not involve the use of methanol, but this would then require much longer incubation times, which would probably result in a higher background complicating the interpretation of results.

Another problem inherent with whole mount staining followed by sectioning is that any stain used for detecting the antibodies has to be resistant to the conditions used to obtain sections. This rules out fluorescence, as the fluorophores would degrade during the post-staining processing, but good results would probably be obtained using for example DAB. Fluorescence could be used if a different type of analysis was performed: confocal analysis could be used to examine the whole embryo, but the depths that could be examined would be very limited, and sections would have to be cut if any detailed analysis was desired. These sections, however, could be quite thick (50-100μm) if used for confocal analysis, reducing the problems of tissue integrity maintenance encountered for thin sectioning at early stages of development.

3.5.2 Attempted desmoglein cloning.

Of the three human desmoglein isoforms, Dsg3 gave a faint reaction when used to probe Xenopus genomic DNA digests, and so was used to screen an adult Xenopus λZap2 liver library. Putative clones were identified during the screening process, but a number of problems were encountered when attempting to excise the inserts from these clones. A variety of different techniques were then attempted to isolate the inserts from these clones, all of which proved unsuccessful. λ DNA was then prepared from a number of different Xenopus cDNA libraries and probed with human Dsg3, to determine if any of them could be used for screening. For all the libraries except one, the probe reacted with the λ vector without reacting with any of the inserts, and so it
was decided that these would not be screened using radiolabelled DNA probes. For the library that did give a reaction for the insert, the \( \lambda \) arms also reacted, so this was not screened by conventional methods either. The insert band from the \( \lambda \) preparation was, however, isolated and subcloned into Bluescript, but when the inserts were isolated from individual clones and blotted again, they seemed not to give a specific positive result, and so were not pursued.

It was possible to screen a number of the libraries using antibodies so, after testing samples of the tissue used to make the library by Western blot, one library was selected and probed this way. A number of putative clones were isolated, the inserts subcloned using the *in vivo* process (without problem this time) and sequenced. The clones, however, proved to be false positives. Finally, touchdown PCR was attempted using degenerate primers present in the laboratory, again without success.

**3.5.2.1 Southern blot.**
The result of the Southern blot to determine which isoform to use to screen the library, detecting DSG3 without detecting DSG2, in retrospect are somewhat surprising. This is because it has been shown, since these experiments were performed, that Dsg2 is the most widely expressed desmoglein isoform, and so one would expect it to be present, or more similar to an ancestral type *Xenopus* isoform, even if the other desmoglein isoforms were not (see section 1.1.4.3.2 and Legan *et al.*, 1994; Schäffer *et al.*, 1994; Nuber *et al.*, 1995). This lack of DSG2 detection, however, is probably due to the fact that at the time the project was undertaken, only partial sequence was available for both DSG2 and DSG3, and the portion of DSG2 used to generate probes is located in the intracellular region of least conservation (both between isoforms and across species), possibly resulting in a lack of hybridisation even if the orthologue was present. The DSG3 hybridisation is consistent with what is now known concerning this isoform and so likely to be a true result. As mentioned in section 1.1.4.3.2, Dsg3 is found in the basal layers of stratified epithelia, and so one might expect to find this isoform in frog epidermis. Also, the sequence used to generate the DSG3 probe is located in the extracellular region where there is most similarity (again both between isoforms and
across species) enhancing the probability of detecting an orthologue. The lack of
detection of DSG1 is also consistent with data produced since this study; it has been
shown to be present in only the uppermost layers of the human epidermis (the stratum
corneum and granular layers) and so is unlikely to be an isoform expressed in the less
stratified frog skin.

It would be interesting to repeat the Southern blot with the probes now available
to determine if the lack of hybridisation with DSG2 was simply due to lack of
hybridisation because of a less conserved region of the molecule being used as a probe,
or whether it is real, implying that Dsg2 may not be the ancestral/ubiquitous form of
the desmogleins as currently proposed (Schäfer et al., 1994), and that Dsg3 may be
more closely related to the ancestral type of desmoglein.

3.5.2.2 DNA screening.
The fact that problems were encountered when attempting to "in vivo excise" the insert
from putative clones obtained in section 3.4.2.1, but that a positive control from a
different library could be easily excised, implies that either there was something wrong
with the original aliquot of the library obtained for screening, or that somehow the
plugs picked from the screening process were affected during their storage such that the
excision process could no longer be performed easily. The latter possibility seems
more likely as Dr Q. Xu had used the library previously to obtain clones on two
occasions (Xu et al., 1992; Xu et al., 1993). A plausible explanation for this is that the
plug stocks may have become contaminated with helper phage. As the ratio of helper
phage to library-containing phage is important during the excision process, an excess of
the helper phage during this process might prevent the library phage excising the
phagemid. Alternatively, if the phagemid does get excised then it might well be
outcompeted at the rescue stage by helper phage, and so not generate ampicillin-
resistant colonies. This explanation would also explain the unexpected band observed
at approximately 3kb following the λ preparation described in section 3.4.2.2, as M13
helper phage would be present in the overnight growth period of the λ preparation,
resulting in a certain amount of "in vivo excision" occurring.
A possible explanation for the compounding difficulties experienced in isolating DNA from the few colonies obtained following the *in vivo* excision process, is *λ* phage infection. Although only bacteria infected with the phagemid grow into colonies at the rescue stage of the excision process (due to the presence of the ampicillin resistance sequence in the Bluescript phagemid) a number of the cells will still have *λ* phage attached to the outside of them. This means that when colonies are picked and mini-preps prepared, the phage can re-infect the cells during the overnight growth process and cause the cells to lyse, thus making isolation of plasmid DNA more difficult as it will be present in much lower amounts than one would expect from a normal mini-prep. To combat this possibility, Stratagene have since developed a slightly different method of *in vivo* excision process; they now suggest that the single stranded phagemid is rescued using a *λ*-resistant bacterial strain (lacking the maltose permease *λ* receptor, e.g. SOLR cells) thus preventing the *λ* phage co-infection problem. Stratagene now also recommend the use of the Ex-Assist helper phage to perform the excision process, rather than the R408 helper phage used in this study; Ex-Assist phage contains an amber mutation that prevents replication of the M13 phage genome in a non-suppressing *E.coli* strain (such as SOLR cells). Therefore, when the single stranded phagemid is rescued using these cells, the helper phage is unable to replicate, allowing the excised phagemid to replicate without competition, again promoting maximal clone DNA isolation.

In retrospect, it seems quite possible that one of the plugs isolated from the cDNA screening might well have contained at least a portion of the *Xenopus* homologue of a desmoglein, and that sequencing should have been attempted using some of the DNA prepared from the *λ* preparation, and some of the DNA obtained from the putative rescued phagemid prepared in section 3.4.2.2. If the project were to be attempted again, the λgt10 Kintner library would be a good starting point for screening given the results from section 3.3, and also as desmosomal staining was observed by immunofluorescence during the studies described in section 3.4.3. It may be that, if full length probes are generated as discussed in section 3.5.2.1, more
stringent conditions could be used, reducing background hybridisation with the \( \lambda \) arms, and possibly enabling the isolation of a *Xenopus* clone.

### 3.5.2.3 Antibody screening

The lack of success in obtaining a clone using this method is not entirely unexpected given the inherent problems when screening with antibodies outlined at the beginning of section 3.4, especially when the complexity of the library does not represent the entire genome (genome size is \( 3.07 \times 10^9 \)bp [Graf and Kobel, 1991]; library complexity is \( 1.6 \times 10^6 \) with an average insert size of 1.5kb giving \( 2.4 \times 10^9 \)bp). However, given that most libraries are of a similar complexity and insert size, it would probably be difficult to obtain a library that represents the entire genome to improve the likelihood of detecting a clone. In retrospect, it may have been better to screen the library more fully (i.e. more than one complexity of the library), for if the desmosomal cadherins are not well represented, the clone may have been present but missed. It might also have been worth sequencing more of the putative clones isolated, although it would be difficult to determine at which stage to stop given that the first clones sequenced were so discouraging, and time was limiting. If the project were to be attempted again, it is possible that, with the improved ECL techniques available now, fewer false positives would be isolated and that the method might work, but the cDNA approach of screening should probably be attempted first.

### 3.5.2.4 PCR

In this study, this approach was used at the end of a prolonged and abortive attempted cloning process, and so when a negative result was obtained, the experiment was not extensively repeated, and the project was abandoned. However, given the sequence data now available concerning different vertebrate desmoglein homologues, this would represent a good approach to pursue if the project were to be attempted again, designing specific primers to highly conserved regions of the desmoglein molecules (taking into account *Xenopus* codon preferences) and attempting touchdown PCR with
them; or using them in combination with primers to the library vector again for touchdown PCR.

3.5.3 Current state of knowledge about desmosomal cadherins during early vertebrate development.

Little is still known about the role of desmosomal proteins in vertebrate development, and to my knowledge nothing in Xenopus development, other than the original observation in the Franz et al. paper (1983) showing their presence at stage 7. The last few years have, however, revealed some information, mainly from mouse development, and this is discussed below.

Desmosomes first assemble at the 32-cell stage in mouse development, coincident with blastocyst cavitation, and appear to be regulated by the onset of glycoprotein synthesis (Fleming et al., 1991, 1993); the desmocollin present at this stage is Dsc2, transcription of which seems to require the presence of a contact-free cell surface (Collins et al., 1995). In the blastocyst the desmosomes are confined to the trophectoderm and are absent from the inner cell mass. Dscl and Dsc3 are absent in the early embryo, being first upregulated in the epidermis on embryonic days 15 and 12 respectively (Garrod et al., 1996).

King et al. (1996) examined the expression pattern of Dscl and Dsg1, the so called "skin-type" desmosomal cadherins, during mouse epidermis development, to determine if their expression is synchronised and whether their expression is associated with stratification or with keratinization. Using RNA in situ analysis, both Dsg1 and Dsc1 showed considerable up-regulation in general epidermis at 15.5 days which is after the onset of stratification (day 13.5), but before the start of keratinization (day 17.5). However, Dsg1 expression was observed in some epithelial tissues in which Dsc-1 was absent, and was consistently observed before Dsc1 both in development and in differentiation (epithelium morphogenesis), showing that expression of the two desmosomal cadherins is not tightly coupled. This was an unexpected finding, as previous reports (Schäfer et al., 1994; Nuber et al., 1995) had tended to indicate that particular Dsg and Dsc isoforms were expressed constitutively in pairs (i.e. Dsg3 with
Dsc3 etc.). It now seems, however, that at least in epithelial morphogenesis, desmosomes do not change their composition in discrete pairs but probably in a complex pattern of overlapping Dsc and Dsg isoforms, resulting in gradually changing adhesive properties.

Perhaps the most exciting work recently done is that described by Allen et al. (1996), in which a transgenic mouse was made expressing a dominant negative mutant form of Dsg3 (lacking most of the extracellular domain) under the control of the keratin 14 promoter and hence expressed in the more basal epidermal layers. The mice have interesting skin phenotypes even though expression of the transgene product is low (swollen paws and digits; flaky skin particularly on the dorsolateral surface of the back; the tip of each animal's tail blackens and progressively becomes amputated until the adult has only a stub for a tail). By light microscopy, the epidermis was markedly thickened and changes in morphology occurred extending into upper differentiating layers, due to an upregulation in keratinocyte proliferation. When sections were examined by electron microscopy, the desmosomes in areas where the transgene was expressed (i.e. the basal and lower spinous cell layers of neonatal mice) were markedly reduced and found to have a perturbed morphology. There were also large intercellular gaps, however, neither the intercellular adhesions nor the integrin mediated cell-substratum adhesions were completely disrupted. There appeared to be reduced numbers of adherens type junctions too, probably because in areas of desmosomal disruption the adherens type junctions are not sufficiently stable to withstand the stresses of the environment. Both males and females were smaller than litter mates, and the adult mice exhibited a bizarre behaviour and appearance, constantly grooming themselves and drooling, resulting in a wet matted coat. It is thought that this may be due to the K14 promoter driving the transgene remaining active in the adult salivary gland, whereas it gets down regulated in the skin 4-7 days after birth. The fact that disruption of the epidermal desmosomes resulted in an increase in proliferation and hyperplasia within the epidermis lends support to the hypothesis that observed reductions in desmosomes associated with squamous cell carcinomas may actively contribute to the enhanced proliferation of these tumours.
It would be interesting to see if Dsg2 and/or Dsc2 knockout or dominant negative experiments produce viable offspring, as these are the most widely expressed isoforms and so could well cause irreparable damage during development if misexpressed, similar to that produced by with E-cadherin misexpression (Larue et al., 1994; Riethmacher et al., 1995).
Chapter 4: Production of GST-fusion proteins XPGN, XPGC, HPGN and generation of antibodies.

4.1 Introduction.

Following the negative results obtained described in Chapter 3, it was decided to pursue a new project with more focused aims. At the time this decision was being made, McCrea et al. (1993) published the first work showing the importance of β-catenin in early *Xenopus* pattern formation. As β-catenin is related to the desmosomal plaque protein PG, it was decided to investigate this latter molecule further, to determine if it too had a role to play in early pattern formation, by adopting an approach similar to that used by McCrea et al. This involved generating specific antibodies to the N-terminal region of PG, purifying and making F\textsubscript{ab} fragments from them, microinjecting these into blastomeres at the 4 cell stage and determining if they disrupted pattern formation.

To make the antibodies for microinjection, a fusion protein of the unique N-terminal domain of *Xenopus* PG had to be constructed and purified. It was decided that the opportunity should be taken to also make antibodies to the unique C-terminal domain of *Xenopus* PG and to a larger N-terminal region of human PG to generate a useful set of tools for the laboratory, which might also be used for microinjection studies. The construction of the fusion proteins, and the production and characterisation of the antibodies generated therefrom are described in this chapter.

4.2 Production of fusion proteins.

Unique N- and C-terminal domains of PG were isolated by PCR from a *Xenopus* cDNA library (stage 17 λgt10 library; Kintner and Melton, 1987), and an N-terminal domain of human PG amplified from a clone (HPG Ca 2.1) kindly supplied by Dr. W.W. Franke using the primers described in Figure 25. These PG domains named XPGN, XPGC, and HPGN correspond to amino acids 1-106, 666-738, and 5-304 respectively. The PCR products were purified using the GENECLEAN® kit, cut with appropriate enzymes and ligated into pGEX fusion protein vectors. The *Xenopus* PG products were cut with *EcoR*I and *BclI* then ligated into pGEX 2T previously cut with *BamHI* and
EcoRI, and the human PG fragment was cut with BclI and ligated into pGEX 3X previously cut with BamHI; the human PG PCR product includes an internal BclI site, at amino acid 304, that makes this ligation possible. The plasmids were then transformed into competent DH5α cells, clones picked, and the orientation of the human PG determined by digestion with EcoRI and SphI; in the correct orientation a 200bp fragment would be produced, in the incorrect orientation an 800bp fragment would be produced. Putative positive clones were sequenced along their entire length (using the Sequenase® kit) to ensure that the PCR products were inserted correctly, and that there were no mistakes introduced from the PCR reaction.

Clones that contained the correct sequence were cultured and fusion protein expression induced by the addition of IPTG as set out in materials and methods. The bacteria were lysed and the fusion proteins purified from the endogenous bacterial proteins using glutathione agarose beads to which the GST-portion of the fusion protein bound (see Figure 26). Once purified the fusion proteins were concentrated to 200μg/ml, dialysed into MTPBS and each used to immunise two rabbits in order to generate polyclonal antibodies according to the protocol set out in materials and methods.

The antibodies generated against the fusion proteins were designated as follows : XPGN, 2006 and 2008; XPGC, 1 and 2; HPGN, 7 and 8.

4.3 Specificity of antibodies by Western blot.

To ensure that the antibodies generated against the fusion proteins would detect PG specifically, an attribute necessary if the antibodies were to be useful in microinjection experiments later, samples of adult Xenopus heart and human A431 cell extracts were run out by SDS-PAGE and electro-blotted onto nitrocellulose. These blots were then probed using dilutions of the sera obtained from the rabbits immunised with the fusion proteins, or with purified antibodies against β-catenin (directed against the C-terminal most 31 amino acids of mouse β-catenin, a kind donation from Dr K. Herrenknecht, Eisai Research, London). As can be seen from Figure 27, all the anti-XPGN and -XPGC antisera do not to cross react with β-catenin even at high concentrations, the
FIGURE 25: Schematic outline of cloning strategy for production of plakoglobin fusion proteins.

N- and C-terminal domains of *Xenopus* or human plakoglobin (solid green bars) were isolated by PCR and cloned into pGEX-3X or pGEX-2T respectively using the PCR primers and restriction enzymes described in this diagram:

Primers HC1 and HC2 for the unique N-terminal domain of *Xenopus* plakoglobin
Primers HC3 and HC4 for the unique C-terminal domain of *Xenopus* plakoglobin
Primers JA100 and JA 102 for the N-terminal domain of human plakoglobin
See text for details.

Purple boxes represent armadillo repeats. Amino acids residue numbers are shown above the diagrams, and below the green bars. Hashed green bar indicates where the polypeptide would extend to if the whole PCR product were ligated into the vector without first digesting with Bcl 1.
Human Plakoglobin

**PCR product has internal Bcl1 site**

Xenopus Plakoglobin

**Stop codone**
FIGURE 26: Purification of fusion proteins.

A: Schematic of fusion protein isolation and purification protocol.

Key:
green line: plakoglobin fusion protein
pink dot: glutathione-Sepharose beads
black dot: glutathione
purple and black crosses: miscellaneous endogenous proteins from bacteria

B: Coomassie stained PAGE of products obtained during protein purification of XPGN fusion protein.

Key:
supernatant after beads: sample of protein that did not bind to glutathione-Sepharose beads
wash: sample of protein from wash of glutathione-Sepharose beads
glutathione elution: sample of protein that is specifically bound to glutathione-Sepharose beads
empty pGEX expression: sample of whole cell lysate obtained when the empty vector is transformed into bacteria and protein expression induced
F XPGN fusion protein
G GST

C: Coomassie stained PAGE showing purity of fusion proteins used for generating antibodies and for in vitro assays. Position of fusion proteins indicated on right of diagram, molecular weight marker values indicated on left.
O vernight culture 37° C

Dilute 1/10 grow 37°C

Induce 0.1 mM IPTG grow 5 1/2 hours 37° C

Spin down bacteria resuspend pellet + protease inhibitors 4° C

Sonicate on ice

Add more protease inhibitor

Spin down cell debris

Supernatant containing fusion protein

Supernatant containing fusion protein + Glutathione-sepharose beads

Mix RT 30 min → 1 hr for binding then spin down

Wash then elute with 10 mM reduced Glutathione (in Tris pH 8.0)

Spin

Supernatant

Pellet

Glutathione sepharose beads

Purified fusion protein

Supernatant

Glutathione sepharose beads

B

C

Supernatant after beads

Glutathione Elution

Empty pGEx expression

F

G

120

76

66

58

43

33

HPGN

XPGN

XPGC

GST
anti-HPGN serum 8 recognises PG but also detects a higher molecular weight protein that could well be another armadillo repeat containing protein (such as one of the p120 isoforms), and the anti-HPGN serum 7 cross reacts with β-catenin in both Xenopus and A431 cell extracts. The lack of specificity of the latter two sera is probably due to the fact that the HPGN fusion protein extends into the armadillo repeat region, an area with much higher homology to β-catenin and other armadillo family members than the unique terminal domains.

4.4 Zooblot of antibodies.
Samples of cultured cells or tissue lysates from different species were run out on SDS-PAGE, electro-blotted and probed with dilutions of the antisera generated against the fusion proteins to determine their ability to recognise PG across species. As can be seen from Figure 28, the antisera have good cross-species reactivity with antibodies against each of the fusion proteins recognising PG from human, cow, dog, rat, mouse and Xenopus, and with antiserum 7 possibly cross-reacting with Drosophila and Sf9 cells too.

4.5 Conclusion and discussion.
GST fusion proteins of the unique N- and C-terminal domains of Xenopus PG have been made, as has a fusion protein of the N-terminal 2/5ths of human PG. These have been used to generate antibodies; those raised against the Xenopus PG domains have good specificity and cross-species reactivity, those raised against the human PG domain have better cross-species reactivity but less specificity. This means that a useful set of tools have been generated which can be used for future experimentation: for example the anti-XPGN and -XPGC antibodies could be used to isolate PG clones from different species and should be useful for Western blot and possibly immunofluorescence, whereas the anti-HPGN antibodies could be used for identifying armadillo repeat-containing proteins from a wider variety of species such as Drosophila.
FIGURE 27: Specificity of anti-fusion protein sera.

Western blots of A431 cell NP40 extracts, and Xenopus heart extracts probed with anti plakoglobin-fusion protein sera and anti β-catenin antibodies to determine antisera cross-reactivity with β-catenin:

- **anti XPGN 2006:** specifically recognises plakoglobin in *Xenopus* extracts but does not cross react with A431 cell extracts.
- **anti XPGN 2008:** specifically recognises plakoglobin in *Xenopus* extracts, and recognises plakoglobin, but also cross-reacts with a 70kD protein, in A431 extracts.
- **anti XPGC 1:** specifically recognises plakoglobin in both A431 and *Xenopus* extracts.
- **anti XPGC 2:** specifically recognises plakoglobin in both A431 and *Xenopus* extracts.
- **anti HPGN 7:** recognises plakoglobin and β-catenin in both A431 and *Xenopus* extracts.
- **anti HPGN 8:** recognises plakoglobin in both A431 and *Xenopus* extracts as well as higher molecular weight proteins that may be other armadillo repeat containing proteins.
A431 Extract

XPGN XPGC HPGN

108 - 206 2008 γ 2 8 8

β-catenin

plakoglobin

Coomassie

Xenopus Heart Extract

XPGN XPGC HPGN

108 - 206 2008 γ 2 8 8

myosin

β-catenin

plakoglobin

Coomassie
FIGURE 28: Ability of antisera to recognise plakoglobin across species: zooblot results.

Western blots using extracts from a variety of species to determine the ability of the antisera to recognise plakoglobin across species:

anti XPGN 2008: cross reacts with human, cow, dog, rat, mouse and *Xenopus* (the band seen in the fission yeast lane is non-specific).

anti XPGC 1: cross reacts with human, cow, dog, rat, mouse, *Xenopus* (the bands seen in the *Drosophila* and fission yeast lanes are non-specific).

anti HPGN 7: cross reacts with human, cow, dog, rat, mouse, *Xenopus*, and possibly with *Drosophila* and Sf9 cells, although these last two could be cross reactions to other armadillo repeat containing proteins especially as the antiserum detects β-catenin in *Xenopus* extracts.

Key:

B: Budding Yeast (*S. cerevisiae*)
F: Fission Yeast (*S. pombe*)
S: Sf9 insect cell line
D: *Drosophila* adult
X: *Xenopus* heart
M: Mouse tongue
R: Rat tongue
MD: MDCK dog cell line
D: Dog skin
C: Cow muzzle (desmosome preparation)
H: Human heart
and other insect species, and are useful to show the positions of both β-catenin and PG by Western blot analysis.
5.1 Introduction.

This chapter describes the experiments (briefly outlined at the beginning of Chapter 4) performed to determine if PG, like β-catenin, has a role in early *Xenopus* pattern formation. Firstly, however, section 5.2 presents and compares the expression patterns of PG and β-catenin during early development as determined by Western blot analysis. Section 5.3 describes the purification and F\textsubscript{ab} fragment generation from the anti-PG antibodies, and section 5.4 describes the results observed when these F\textsubscript{ab} fragments were microinjected into ventral *Xenopus* blastomeres at the four cell stage, to determine if pattern formation could be disrupted.

5.2 PG expression in early *Xenopus* development.

To examine the expression pattern of PG and confirm that it was present at the stage of interest for planned microinjection experiments (i.e. at the 4 cell stage), Western blots of *Xenopus* NP40 lysates from different developmental stages (from fertilised egg to swimming tadpole) were prepared. Duplicate blots were probed in parallel with anti-β-catenin antibody (from Dr K. Herrenknecht) to allow a comparison of the two protein expression patterns during early development. Initial blots (with samples from stages 1 [1 cell], 2 [2 cell], 3 [4 cell], 4 [8 cell], 5 [16 cell], 9 [late blastula], 11 [mid-gastrula], 19 [neurula], 25 [early tailbud], and 35 [early tadpole]) showed that both proteins were present throughout these stages of development, and indicated an interesting dip in levels of both proteins for the stage 9 time point (results not shown). The results were repeated twice, then the phenomenon investigated more thoroughly (repeated four times) with more time points taken to map this expression pattern more fully. Two examples of these blots can be seen in Figures 29 and 30. PG is present in an NP40-soluble form throughout early development with levels increasing until the 16 cell stage, dipping at stage 6.5, pulsing at stage 8.5, rising by stage 10 and staying relatively constant through gastrulation, then rising again at the early neurula stage 15 and tailbud stage 24, before dipping again at tadpole stage 35. A similar expression pattern can be
seen for β-catenin from stages 1-11, but then the levels of protein increase again at stage 12 (mid-late gastrula) and remain relatively constant through the neurula, tailbud, and tadpole stages.
FIGURE 29: Expression of PG and β-catenin in early *Xenopus* development (Example 1).

One example of Western blots of NP40 extracts from different developmental stages (from 1 cell to swimming tadpole) probed for plakoglobin (antiserum 2006) and β-catenin showing that the levels of expression for both proteins dip from stages 6 to 9 then increase again at the beginning of gastrulation.

Molecular weight markers given on the left.
FIGURE 30: Expression of PG and β-catenin in early *Xenopus* development (Example 2).

Second example of Western blots of NP40 extracts from different developmental stages (from 1 cell to swimming tadpole) probed for plakoglobin (antiserum 2006) and β-catenin showing that the levels of expression for both proteins dip from stages 6 to 9 then increase again at the beginning of gastrulation.

Molecular weight markers given on the left.
5.3 Generation and purification of F_{ab} fragments.

The anti-fusion protein sera were depleted of GST-reactive antibodies by mixing with GST-Affi10 beads overnight. The remaining antibodies were then purified by running the supernatant over a Protein A column and collecting the eluate, which was dialysed and concentrated to 5mg/ml in 100mM sodium acetate. F_{ab} fragments were then generated from the "purified" antibodies by digestion with papain, and purified by running the digest over a Protein A column and taking the supernatant (the eluate contained F_{c} fragments and undigested antibodies; see Figure 31). The pre-immune sera from the appropriate rabbits were also treated in the same way to be used as negative controls in the microinjection experiments. The F_{ab} fragments, once produced, were dialysed and concentrated to approximately 5mg/ml in XIB Buffer (as determined by SDS-PAGE analysis with control concentrations of BSA as standards, and Bio-Rad Bradford assay), aliquoted out and stored at -70°C until ready to use.

An alternative method of complete purification of the antibodies was also tried (i.e. the sera were mixed with GST-Affi10 beads, the supernatant mixed with fusion protein-Affi10 beads and the eluate taken which would contain only antibodies reactive to the PG portion of the fusion proteins) but the final quantities of antibodies obtained by this method were so low that it was abandoned and the one described above used. This may have been due to the fact that the antibodies, once bound to the fusion protein on the column, would not actually elute off again. This is supported by the fact that the fusion protein columns seemed to "go off" very quickly, i.e. would not bind significant amount of antibody after one or two uses.
FIGURE 31: Example antibody purification and F<sub>ab</sub> production.

Schematic of F<sub>ab</sub> production protocol and PAGE of F<sub>ab</sub> generation and purification. Gels show purification and F<sub>ab</sub> generation of anti-XPGN 2008 antiserum; similar results were obtained for both the other antisera used (anti-XPGN 2006 and anti-XPGC 1).

Top gel: Papain digest of purified antibodies separated by SDS-PAGE in the presence or absence of DTT:
- M: marker lane
- no Ab: all digest buffers and enzyme without antibody present
- Ab: sample of antibody alone
- T<sub>0</sub>: sample of digest at time 0 (antibody reduced without DTT due to presence of cysteine in reaction buffer)
- T<sub>2</sub>: sample of digest after 2 hours incubation

Middle gel: Fractions obtained after running the papain digest over protein A beads and separating samples by SDS-PAGE under non-reducing conditions showing that the majority of F<sub>ab</sub> fragments were collected in the supernatant and first two washes:
- M: marker lane
- S/N: supernatant
- W1-2: washes with 100mM Tris pH 8.0
- W3-4: washes with 10mM Tris pH 8.0
- E1&2: elutions with 100mM glycine pH 3.0
- W1e: wash with 100mM Tris pH 8.0

Bottom gel: Samples of purified F<sub>ab</sub> fragments separated by SDS-PAGE under non-reducing conditions before and after volume reduction by lyophilisation to show an approximately five-fold increase in protein concentration.
Serum

GST-Affi10 column

 Supernatant

 eluate

 Protein A column

 Dialyse vs 1/5 NaAc

 Lyophilise to 1/5 volume

 Digest with papain RT 2hrs

 Protein A beads

 Supernatant +1st 2 washes

 Dialyse vs 1/5 XIB

 Lyophilise to 1/5 volume so Fabs ~ 5mg/ml in 1x XIB

+DTT

-DTT

unreduced Ig

H chain unreduced Fab

red Ferduced Fab

unreduced Fab

Fab

Fc

Fab before after

M cone. conc.
5.4 Microinjections of anti-XPGN, anti-XPGC, pre-immune and anti-β–catenin Fab fragments into Xenopus embryos.

Xenopus embryos were obtained and allowed to develop to the 4 cell stage in 1/10 NAM buffer, then transferred into 3/4 NAM with 4% Ficoll and microinjected into one of the two ventral blastomeres.

Batches of embryos (30-45 per concentration point) were injected with a dilution series of Fab fragments generated from the GST-depleted pre-immune and terminal bleed antibodies prepared as described above, and scored at stage 34 (for an experiment to be scorable the uninjected embryos had to survive to the tadpole stage). The experiments were repeated a number of times with Fab fragments generated from rabbits immunised with the XPGN fusion protein (rabbits 2008 and 2006, repeated approximately 10 and 17 times with 3 and 10 scorable results respectively), or XPGC fusion protein (rabbit 1, repeated approximately 15 times with 8 scorable results), or with purified Fab fragments against the N-terminal portion of β-catenin (a kind gift from Dr P. McCrea, used for five experiments due to lack of reagent) which were used as a positive control (McCrea et al., 1993).

Embryos that were injected with anti-β-catenin Fab fragments generated secondary axis phenotypes as expected (Figure 32) although the amount needed to produce the phenotype was much lower than that published (equivalent to 2nl as opposed to the 25nl published), and the percentage of secondary axis phenotype embryos produced was lower than that published (approximately 62% as opposed to 95%). If the published volume was injected into embryos, the dose was lethal. This is probably due to different sensitivities of different stocks of frogs.

Embryos that were injected with anti-fusion protein Fab fragments gave a variety of phenotypes that appeared to be non-specific as the non-immune negative control injected embryos also gave similar phenotypes (Figure 33). These effects became more severe as the amount of protein injected increased until the majority of embryos injected died (Figures 34, 35, and 36). These data indicate that the anti-PG Fab fragments generated here have no specific effect when injected into Xenopus
FIGURE 32: Secondary axis embryos produced by microinjection of anti-β-catenin F\textsubscript{ab} fragments.

Examples of double axis embryos produced when diluted and reduced volumes of anti β-catenin F\textsubscript{ab} fragments were microinjected. Bar represents 1mm; arrows indicate secondary axis; normal tadpole of same stage shown in (i).

(a): dorsal view of tadpole with partial secondary axis stained with 12/101 antibody showing muscle blocks.
(b): lateral view of same tadpole as shown in (a) before staining.
(c): dorsal view of tadpole with partial secondary axis stained with 12/101 antibody showing muscle blocks.
(d): lateral view of same tadpole as shown in (c) before staining. Arrow indicates position of secondary cement gland.
(e): dorsal view of tadpole with more complete secondary axis (three eyes and two cement glands) stained with 12/101 antibody showing muscle blocks.
(f): dorsal view of same tadpole as shown in (e) before staining.
(g): lateral view of tadpole with pronounced secondary axis (two cement glands and 4 eyes).
(h): dorsal view of same tadpole as shown in (g).
(i): lateral view of one tadpole with two secondary axes (upper tadpole) compared with one normal tadpole of the same stage (lower tadpole).
(j): lateral view of tadpole with pronounced secondary axis.
FIGURE 33: Examples of phenotypes produced after microinjection of anti-fusion protein F\textsubscript{ab} fragments.

Figure showing the variety of phenotypes, both mild and severe, produced when embryos were injected with anti-fusion protein F\textsubscript{ab} fragments. Bar represents 1mm; normal tadpole of same stage shown in (a).

(a): the upper tadpole is normal, the lower tadpole has mild "smaller" phenotype.
(b): both tadpoles have "bubble belly" phenotype (mild defect).
(c): both tadpoles have "spinabifida" phenotype (i.e. incomplete closure of the neural tube), the one on the left also having a "bubble belly".
(d): tadpole with slightly reduced head and tail, also shorter than normal.
(e): very anteriorised tadpole with multiple cement glands and essentially no anterior-posterior axis.
(f): tadpole with "spinabifida" phenotype combined with reduced anterior features, only the cement gland remains (the tadpole is curled back on itself so the cement gland is towards the top of the picture).
(g): tadpole with slight "bubble belly" and much reduced head (left of picture) and tail (right of picture).
(h): anteriorised tadpoles with reduced body axis, the one on the left also having a "bubble belly" phenotype.
(i): tadpole with "spinabifida" phenotype and much reduced body axis.
(j): tadpole with much reduced body axis and "bubble belly" phenotype (anterior of embryo to left).
(k): tadpole with reduced anterior structures and much shortened axis (anterior to left).
(l): left tadpole as (j), right tadpole with reduced body axis and slight "spinabifida" phenotype.
FIGURE 34: Bar charts illustrating results of microinjection experiments using Fab fragments derived from antiserum 2006

Compilation of 8 scorable experiments. Different ranges of concentrations were used in different experiments due to logistical difficulties (it was rare that a good set of embryos was obtained in which a large range of concentration points could be used; also there was the problem of embryos developing past the point at which injections could be performed), however, as 30-45 embryos were used per concentration point in an experiment, even when n=1 there is still a reasonable size group for data.

Although there is variability in specific points, the trend for both the PI and the TB injections is that there is an increase in the percentage of embryos that die as the amount of protein injected increases. There is not a usable window of difference between the TB and PI effects to be able to determine a biological difference between the two.

Within the defective embryos are any secondary axis embryos generated. However, these were negligible (only 1 or 2 in occasional sets of injections with similar quantities being obtained for both the control and terminal bleed Fab fragments) and were never as numerous as those generated when use β-catenin was used.

Key:

- normal
- defective: embryos alive when scored at stage 34 but not normal; see Figure 33 for examples of phenotypes. This category includes any secondary axis embryos produced.
- dead

X axis: ng of Fab fragments microinjected
P: Fab fragments generated from pre-immune serum
T: Fab fragments generated from terminal bleed serum
uninj: uninjected
XIB: Xenopus injection buffer (10nl)
β-cat: 10ng purified anti-β-catenin antibody
FIGURE 35: Bar charts illustrating results of microinjection experiments using $F_a b$ fragments derived from antiserum 1.

Compilation of 10 scorable experiments. Different ranges of concentrations were used in different experiments due to logistical difficulties (it was rare that a good set of embryos was obtained in which a large range of concentration points could be used; also there was the problem of embryos developing past the point at which injections could be performed), however, as 30-45 embryos were used per concentration point in an experiment, even when n=1 there is still a reasonable size group for data.

Although there is variability in specific points, the trend for both the PI and the TB injections is that there is an increase in the percentage of embryos that die as the amount of protein injected increases. There is not a usable window of difference between the TB and PI effects to be able to determine a biological difference between the two.

Within the defective embryos are any secondary axis embryos generated. However, these were negligible (only 1 or 2 in occasional sets of injections with similar quantities being obtained for both the control and terminal bleed $F_a b$ fragments) and were never as numerous as those generated when use $\beta$-catenin was used.

Key:

- normal
- defective: embryos alive when scored at stage 34 but not normal; see Figure 33 for examples of phenotypes. This category includes any secondary axis embryos produced.
- dead

X axis: ng of Fab fragments microinjected

P: Fab fragments generated from pre-immune serum
T: Fab fragments generated from terminal bleed serum
uninj: uninjected
XIB: Xenopus injection buffer (10nl)
$\beta$-cat: 10ng purified anti-$\beta$-catenin antibody
FIGURE 36: Table showing example of data obtained from one microinjection experiment (anti-XPGN 2006 Fab fragments).

Similar results were obtained when either of the two other Fab fragments (anti-XPGN 2008 or anti-XPGC 1) were used for microinjection. Sample sizes of 30-45 embryos were used per set of injections, and embryos were scored at approximately stage 34. The "defective" classification encompasses any embryos still alive at that stage that were not completely normal (examples of the most common non-specific phenotypes are shown in Figure 33) and includes any secondary axis embryos. The secondary axis embryos were also scored separately in the right hand columns.

10nl of solution microinjected for each set of injections; approximately 10ng anti-β-catenin Fab fragments microinjected per embryo. PI: Fab fragments generated from pre-immune anti-sera; TB: Fab fragments generated from terminal bleed anti-sera.
<table>
<thead>
<tr>
<th></th>
<th>NORMAL</th>
<th>DEFECTIVE</th>
<th>DEAD</th>
<th>secondary axis</th>
<th>sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>un.injected</td>
<td>77.8</td>
<td>22.2</td>
<td>0.0</td>
<td>0.0</td>
<td>45</td>
</tr>
<tr>
<td>β-catenin</td>
<td>2.2</td>
<td>86.7</td>
<td>11.1</td>
<td>62.2</td>
<td>45</td>
</tr>
<tr>
<td>0.1 ng PI</td>
<td>42.2</td>
<td>55.6</td>
<td>2.2</td>
<td>0.0</td>
<td>45</td>
</tr>
<tr>
<td>0.5 ng PI</td>
<td>40.0</td>
<td>55.6</td>
<td>4.4</td>
<td>2.2</td>
<td>45</td>
</tr>
<tr>
<td>1.0 ng PI</td>
<td>35.6</td>
<td>60.0</td>
<td>4.4</td>
<td>6.7</td>
<td>45</td>
</tr>
<tr>
<td>2.5 ng PI</td>
<td>2.2</td>
<td>77.8</td>
<td>20.0</td>
<td>17.8</td>
<td>45</td>
</tr>
<tr>
<td>5.0 ng PI</td>
<td>2.2</td>
<td>66.7</td>
<td>31.1</td>
<td>6.7</td>
<td>45</td>
</tr>
<tr>
<td>0.1 ng TB</td>
<td>46.7</td>
<td>46.7</td>
<td>6.7</td>
<td>0.0</td>
<td>45</td>
</tr>
<tr>
<td>0.5 ng TB</td>
<td>20.0</td>
<td>73.3</td>
<td>6.7</td>
<td>2.2</td>
<td>45</td>
</tr>
<tr>
<td>1.0 ng TB</td>
<td>8.8</td>
<td>82.2</td>
<td>8.8</td>
<td>11.1</td>
<td>45</td>
</tr>
<tr>
<td>2.5 ng TB</td>
<td>4.4</td>
<td>73.3</td>
<td>22.2</td>
<td>13.3</td>
<td>45</td>
</tr>
<tr>
<td>5.0 ng TB</td>
<td>0.0</td>
<td>77.8</td>
<td>22.2</td>
<td>4.4</td>
<td>45</td>
</tr>
</tbody>
</table>
embryos, suggesting that they do not affect signal transduction in early *Xenopus* development. Whole mount experiments using HRP-conjugated anti-rabbit antibodies to follow the path of the injected F\textsubscript{ab} fragments, showed that the injections had targeted the areas that gave rise to the ventral and posterior portion of the embryo, confirming the site of microinjection was correct (results not shown), and also confirming that the F\textsubscript{ab} fragments had not been degraded (Cho *et al.*, 1988, showed that antibodies last at least two days after microinjection into *Xenopus* embryos).

### 5.5 Conclusion and discussion.

#### 5.5.1 PG expression pattern.

Anti XPGN 2006 has been used to show that PG is present at the 4 cell stage of *Xenopus* development, and the expression patterns of PG and β-catenin appear similar through the early stages of development until the beginning of gastrulation. Both proteins have an intriguing "blip" of expression at stage 8.5 and, given that β-catenin has been shown to play a role in pattern formation in the Nieuwkoop centre (Guger and Gumbiner, 1995), it is interesting to speculate that this pulse of both proteins may be involved in determining the developing embryo's body plan at this stage too. Unfortunately, these data were generated at the end of the project, and so with time limiting, this phenomenon was not investigated further.

#### 5.5.1.1 Comparison with other PG and β-catenin expression data.

Two groups have published work examining the expression pattern of PG and/or β-catenin (DeMarias and Moon, 1992; Schneider *et al.*, 1993); this work is described below and compared with the results obtained in this thesis. However, it should be noted that in neither study were as many time points examined as the work described here, allowing for the possibility that the groups may well have missed the phenomenon observed in this study, and that also the protein extraction techniques used vary between studies.

DeMarias and Moon (1992) examined PG and β-catenin expression at six stages of early *Xenopus* development both at the RNA and protein level; however, the precise
stages (or time elapsed post-fertilisation) for the samples are not given, only that the samples are from the fertilised egg, blastula, gastrula, neurula, tailbud or tadpole stages. They found RNA present for both molecules from fertilised egg through to the tadpole stage of development with β-catenin levels increasing three-fold at the gastrula stage onwards and those of PG doubling at the blastula stage then again at the neurula stage. These data correlate reasonably well with the observations seen in Figures 29 and 30 (depending on which particular time point on the blot correlates with the one they used), particularly the later stages where PG protein levels appear to increase at the neurula stage whilst those of β-catenin increase earlier at the gastrula stage.

When DeMarias and Moon (1992) examined protein expression, they concluded that PG was present in the fertilised egg, gradually increased in abundance to the neurula stage but declined at the tailbud and tadpole stages, whereas β-catenin was present in the fertilised egg through to tadpole stages (although the antibody they used seems to have had much poorer reaction that the one used in this study, with the result that the expression levels are not very clear). These results presented the authors with a conundrum for PG expression; reconciling the high levels of RNA transcripts throughout development with the apparent decline in protein levels. One explanation they suggested was that PG might become modified at the tailbud stage such that the monoclonal antibody they used no longer detected it. If this is the case, then the data presented here indicate that the antibodies generated in the course of this research can recognise modified forms of PG that their monoclonal antibody could not. If this is true, and if there are modified forms of PG at early stages of development too, this may provide one explanation for the apparent discrepancies observed between their data and that given here for the earlier stages. Another explanation for the differences between their protein expression results and those presented here may be due to the method of isolation; they used total protein extract after Freon extraction (to remove yolk platelets) whereas the samples used in this study are NP40-soluble extract only (after yolk platelet removal by centrifugation). It may be that there are real differences in the expression pattern of PG depending on the extraction technique; to test this hypothesis,
extracts using both techniques from multiple time points similar to those used in this study would have to be run in parallel.

Schneider et al. (1993) studied the expression pattern of β- and α-catenin in *Xenopus* embryos (from the egg to stage 23), and concluded that both proteins are present throughout early development. Their data for expression of β-catenin in the early stages (stages 2, 7, 9, and 11) directly contrast with the data presented above; they show a gradual increase of protein levels throughout these stages, without the "blip" of expression found in this thesis. However, they may simply have missed the phenomenon described above as, again, fewer time points were used for their analysis than were used in this study. Another possible explanation for the differences between their data and those produced here, is a slight difference in assigning stages; each batch of embryos varies in the precise timing of its development, and staging of embryos is somewhat subjective, so it is possible that the samples they obtained and labelled 7 and 9 might actually correspond to the ones labelled 6.5 and 8.5 respectively in this thesis. These latter samples still show a high level of protein expression, as opposed to the lower levels of expression obtained for the stages labelled 7 and 9 here, so this could well account for the apparent differences in observed protein levels. Perhaps given this possibility for discrepancy in staging, if the experiments were to be repeated or the phenomenon investigated further, samples should be taken at specific times post fertilisation (with incubation temperature specified), and the stages merely noted for confirmation.

**5.5.1.2 Possible interpretations of data.**

There are a number of ways of interpreting the observed expression pattern. For example it could be that a certain amount of translation from maternal RNA occurs early in development, peaking at the 8 cell stage, and that after this (about 2h30-2h45 hours after fertilisation, stage 16 onwards) the pool depletes simply due to the half-life of the protein and normal turnover. There then appears to be a pulse of expression above this depleting background level at stage 8.5 which could be due to an increase of translation from maternal RNA or, more likely at this stage, an increase of expression
due to the beginning of zygotic gene transcription (one of the changes that occurs at the mid-blastula transition that takes place around stage 8-9). If either of these explanations is true, then the dip of expression at stage 9 is most intriguing. Alternatively, the increase of protein at stage 8.5 could be due to the release of protein from an NP40-insoluble junction-bound complex at the cell membrane into the cytoplasmic pool, with zygotic transcription of these particular proteins delaying until stage 10 onwards.

A different explanation could be that translation from maternal RNA remains fairly constant throughout early development, and that what is being observed is a dip in the levels of cytoplasmic protein at stages 6.5-7, and again at 9 due either to a transitory increase in protein turnover, or an increase in the amounts of these proteins being sequestered to the NP40-insoluble pool at these stages, with zygotic gene transcription beginning from stage 10 onwards so bringing levels up again for later developmental stages.

To determine how the modulation of protein levels arises a number of experiments suggest themselves. To investigate if the increase of protein level at stage 8.5 or 10 is due to the start of zygotic transcription, Northern blots of the developing embryos could be performed and the relative levels of RNA and protein compared to see if there is a correlation; also new RNA synthesis could be inhibited by incubating embryos with actinomycin D, and the expression of the proteins examined, but there is the likelihood that the embryos will fail to develop as they cannot make other RNA required for normal development. Alternatively a variation of RNAse protection could be performed and compared to the protein profiles: after initially incubating the embryos with tritiated uridine, RNA could be isolated and hybridised with non-radiolabelled anti-sense PG, exposed to RNAse, a gel run and fluorographed to show when transcription from the zygotic version of the gene begins (although whether there would be enough signal to register on the film and generate useful data in practice is debatable).

To test if the proteins are being newly synthesised the embryos could be incubated with radioactively-labelled methionine and an immunoprecipitation of the
relevant proteins performed to determine if the increase of expression is due to new synthesis or simply a re-distribution of older protein. One could determine if the proteins are being selectively stabilised in the cytoplasm at particular times (due to post-translational modifications or selective complexing with other proteins in an NP40-unstable complex) by performing pulse-chase experiments at different stages of development and seeing whether the proteins half-life alters significantly. Cellular fractionation experiments at stages 6.5 - 10 would help determine if the proteins are being released from, or sequestered into, complexes at the cell surface so causing the changes in NP40-soluble expression. Preliminary experiments that were performed to address this for PG, in which the membranes from 2-cell or stage 12 embryos were isolated, as well as NP40-soluble proteins, suggested that at these stages all the protein was localised to the NP40-soluble fraction only (results not shown, and not repeated and extended to other time points due to experimental difficulties and lack of time). This compares with cell fractionation data from Fagotto and Gumbiner (1994) in which β-catenin at the 32-cell stage is predominantly membrane-bound, and sucrose density gradient data by Schneider et al. (1993) which suggests that most β-catenin at stage 10 is present in a fraction consistent with it being complexed to cadherin (possibly EP-cadherin) although these were NP40 extracts implying the complex was not linked to the cytoskeleton network.

5.5.1.3 Possible functional significance of data.
A number of changes occur around stages 8.5-10 which both proteins might well be involved in. The mid-blastula transition not only involves the beginning of zygotic transcription, but also increased cellular adhesion has been reported (Slack, 1991) which would correlate with the proteins being sequestered away from the NP40-soluble pool to form new or stronger cell-cell junctions. Cells in the animal hemisphere begin a process of epiboly/expansion, during which they thin and spread laterally starting to move down the sides of the embryo beginning the cellular movements required for gastrulation, and within the embryo pre-gastrula movements begin (Gerhart, 1988). Both of these processes require a plasticity of adhesion, with cells binding tightly to

203
some neighbours so that they move in a co-ordinated sheet together, and adhering more weakly to others as they move over them. The fluctuations in expression of both proteins may be involved in somehow modulating these adhesions. Immunohistology studies by Fagotto and Gumbiner (1994) found that β-catenin was present at high levels in regions of the *Xenopus* embryos undergoing active morphogenetic movements, which would support the idea that both PG and β-catenin might be involved in the dynamic adhesion events occurring at this stage of development.

Although the extracts prepared for the experiment reported here are whole embryo extracts, it is possible that the increase in protein levels is restricted to particular areas of the embryo. In the pre-gastrula embryo there is an interesting set of expression patterns of certain genes in the presumptive mesoderm; for example in the area that will become the future blastopore lip (the organiser area), there is a crescent of expression of goosecoid (Cho et al., 1991), whilst all around the rest of the meridian at this level XWnt-8 is expressed (Christian et al., 1991). It is thought that XWnt-8 might be involved in signalling a ventral fate to the mesodermal cells expressing and surrounding it, probably by attenuating the dorsalising signal emanating from the organiser (Christian and Moon, 1993) and it is possible that the increase of cytoplasmic protein expression seen in the Westerns shown could be involved in this signalling pathway. This hypothesis could be tested by dissecting embryos at appropriate stages and performing Western blots on the different portions to determine if the protein signal observed is localised to a particular area of the embryo, or by immunohistology of embryo sections. Whole mount staining experiments that were performed for PG did not help clarify whether the increase of cytoplasmic protein is localised as the protein is present in all cells of the embryo, and it was difficult to determine whether a specific area had higher levels of signal than another (results not shown).

5.5.2 Microinjection results.

Antibodies to the unique terminal domains of *Xenopus* PG were purified and Fab fragments generated from them. When these were microinjected into *Xenopus* blastomeres at the 4-cell stage and the embryos allowed to develop they gave no
specific phenotype although secondary axis embryos were produced when \( F_{ab} \) fragments against the N-terminal region of \( \beta \)-catenin were microinjected.

The results obtained indicate that either the anti-PG \( F_{ab} \) fragments generated here do not interfere with normal PG function, or that PG does not have a signalling role in early *Xenopus* development. The latter explanation seems unlikely as experiments by Karnovsky and Klymkowsky (1995) that were published during the course of this investigation show that over-expression of PG by microinjection of RNA into *Xenopus* embryos can induce secondary axis embryos. This indicates that PG can have a role in signalling during early *Xenopus* development, although whether this is direct or indirect, or indeed normally occurs *in vivo* remains to be proven. For example it may be the case that, when in excess, PG signals indirectly by displacing \( \beta \)-catenin from one of its complexes within the cell (for example the cadherin-catenin complex or its complex with APC), so freeing \( \beta \)-catenin to fulfil its signalling role. It seems probable however that PG has a direct signalling role to play as the Karnovsky and Klymkowsky paper shows that PG itself localises to the nucleus, a phenomenon already described for \( \beta \)-catenin when it causes axis duplication (Funayama *et al.*, 1995), tending to indicate that it is producing the effect itself. The Funayama *et al.* paper showed that the \( \beta \)-catenin armadillo repeat region alone targets to the nucleus and is sufficient for axis duplication effects, so it is interesting to speculate whether all armadillo repeat-containing proteins, if sufficiently over-expressed, might overcome the normal constraints upon them, which tether them to a particular cellular location, and target to the nucleus. If they do this might result in abnormal signalling and secondary axis phenotypes in this assay, although the results might get quite confusing as at least some of these proteins have proven signalling function in other pathways. One could also test if there is a lower limit to the number of armadillo repeats required to cause a secondary axis, as so far both the molecules that do induce a secondary axis have 13 repeats, whilst proteins such as plakophilin, p120 and smgGDS have 10 or 11 (depending on particular sequence alignments), and APC has 7. It would also be interesting to see if particular armadillo repeats are required for secondary axis induction as the conservation between repeats on the same molecule is quite low (about
although the conservation of the same repeat across species can be quite high (for example repeat 1 of armadillo is 90% identical to repeat 1 of β-catenin; Peifer et al., 1994a) suggesting that different repeats might have different functions.

From the data obtained here it would appear that the Fab fragments produced and injected into Xenopus do not adversely affect normal PG function. There could be a number of reasons why these Fab fragments apparently have no effect whilst those against essentially the same region of β-catenin do. A trivial explanation could be that the anti-PG Fab fragments do potentially have the same effect as those against β-catenin, but the phenotype cannot be generated because the non-specific Fab fragments that are co-injected, due to the method of purification, have toxic effects and ultimately kill the embryos before the specific Fab fragments can reach a high enough concentration to produce a secondary axis; it may be that if one could isolate the anti-PG Fab fragments and concentrate them specifically that the secondary axis phenotype would be observable above background non-specific toxic effects. As mentioned above, a complete purification of anti-PG antibodies was attempted but was unsuccessful.

If this trivial explanation is not the case then there are other theories needed to account for the observed data. As can be seen from Figures 37 and 38, the anti-β-catenin Fab fragments used by McCrea et al. (1993) extend into the region of α-catenin binding, but those generated here against PG do not (supported by data obtained in section 6.3). This means that the phenotype observed by McCrea et al. (1993) could be due to the inhibition or disruption of the β-catenin/α-catenin complex, probably by steric hindrance effects, somehow allowing β-catenin to signal. It has been shown that α-catenin links to the actin cytoskeleton (Rimm et al., 1995), so the disruption of the β-catenin/α-catenin complex could liberate β-catenin and enable it to diffuse in the cytoplasm and function differently. This would also explain how two apparently opposite experiments, the over-expression of β-catenin (Funayama et al., 1995) and the presumably negative regulation of β-catenin by Fab fragment injection (McCrea et al., 1993), could give rise to the same phenotype. It may be that PG if released from
FIGURE 37: Amino acid sequence comparison of plakoglobin (human and *Xenopus*) and β-catenin (*Xenopus*).

Amino acid residues indicated on right side of diagram (data from Fouquet *et al.*, 1992).

Hpg: human plakoglobin  
Xpg: *Xenopus* plakoglobin  
Xbc: *Xenopus* β-catenin  
* identical amino acid residues  
• conservative amino acid exchange  
- gap introduced to optimise alignment

- extent of HPGN  
- extent of XPGN  
- extent of McCrea anti-β-catenin fusion protein  
- extent of XPGC  
- armadillo repeats  
- α-catenin binding site of plakoglobin (Aberle *et al.*, 1996)  
- α-catenin binding site of β-catenin (Aberle *et al.*, 1994)
FIGURE 38: Schematic indicating reported protein binding domains of plakoglobin.

Armadillo repeat. Amino acid residue numbers are given.

Key:

§ Aberle et al. 1996.
Various domains of plakoglobin were expressed as GST- or MBP-fusion proteins and affinity precipitation assays performed using Triton X-100 extracts of SW480 cells or histidine-tagged recombinant α-catenin.

• Sacco et al. 1995.
Deletion mutants of plakoglobin were transfected into HT-1080 cells, NP40 extracts prepared and immunoprecipitations performed using anti-N-cadherin antibodies.

† Wahl et al. 1996.
Deletion mutants of plakoglobin were transfected into A431 cells, NP40 extracts prepared and immunoprecipitations performed using anti-P-cadherin, anti-desmoglein or anti-desmocollin antibodies.

¶ Witcher et al. 1996.
Yeast two hybrid analysis was used to test the interaction of plakoglobin deletion mutants with Dsg-1 cytoplasmic domain. The deletion constructs were also co-transfected into 293T cells (along with full length Dsg-1 or Dsc-1a) and co-precipitation assays performed. The third repeat of plakoglobin is essential for binding Dsg-1 with repeats 1 and 2 enhancing this binding (represented by lighter hashed region), both ends of the repeat region appear necessary for binding Dsc-1a.

• Chitaev et al. 1996.
Histidine-tagged fragments of plakoglobin were immobilised on a 96 well dish and incubated with recombinant cadherin fragments differing only in the C domain (desmoglein, desmocollin or E-cadherin) and binding assessed. Overlay assays were also performed with the plakoglobin fragments bound to nitrocellulose and incubated with the cadherin fusion proteins. The central and more C-terminal regions of the repeat region bind equally well the three C-domains tested, with the more N-terminal region of the repeat domain also binding the desmoglein C domain. (The binding of all regions to desmoglein repeat region could be equally competed using the C-terminal most region tending to indicate that each plakoglobin fragment recognises the same, or overlapping, sequences in the C domain of desmoglein.)

209
various complexes with α-catenin could also signal. Unfortunately this hypothesis
cannot be tested with the antibodies generated here as the anti-HPGN antibodies cross
react with β-catenin, so any data generated if they were injected would be unspecific
and difficult to interpret (the anti-β-catenin antibodies react specifically with β-catenin;
McCrea et al., 1993). In the future it would be possible to address whether the
disruption of the β-catenin/α-catenin complex is the cause of phenotype by generating
antibodies specific to the α-catenin binding site and using these for microinjection
experiments, although there would always be the question of whether this was
interacting with PG or β-catenin. From the literature it seems that more consistent
results are obtained with RNA or plasmid injections as opposed to protein injections, so
perhaps a better way to test this hypothesis would be to co-inject α-catenin with β-
catenin or PG RNA constructs and see if the secondary axis phenotype is rescued.

Recent work by Rubinfeld et al. (1996) has shown that APC and GSK-3β have
a role in modulating β-catenin function in the Wnt signalling pathway, and perhaps the
alteration of the relative levels of β-catenin complexed with these molecules or simply
free in the cytoplasm affects signalling. It could be that the anti-β-catenin Fab
fragments prevent binding of GSK-3β to the APC-β-catenin complex and so regulation
is derailed. If this is the case and PG acts in a similar fashion, which seems likely as it
has been shown to bind APC and be similarly phosphorylated (Rubinfeld et al., 1995;
Shibata et al., 1994), it would appear that the anti-PG Fab fragments produced here do
not affect this complex formation, and allow signalling to continue as usual.

If these secondary axis phenotypes are produced simply by steric hindrance
effects disrupting the stability of certain complexes, then it is worth noting that the anti-
XPGC Fab fragments that overlap one of the proposed sites of PG-desmosomal
cadherin interaction (Wahl et al., 1996) do not affect PGs normal function, in that no
secondary axis embryos are produced. It should however be noted that unlike the α-
catenin binding site which has been quite precisely localised (Aberle et al., 1996; Sacco
et al., 1995) a number of papers recently published seem to identify a variety of
different domains on PG that appear to be involved in interacting with the desmosomal
cadherins (Chitaev et al., 1996; Wahl et al., 1996; Witcher et al., 1996), so disruption of one site could possibly be insufficient to cause displacement effects (Figure 38).
Chapter 6: *In vitro* assays.

6.1 Introduction.

6.1.1 PG tyrosine phosphorylation.

As outlined in section 1.3.1, it is clear that both serine/threonine and tyrosine phosphorylation events play important roles in regulating the function of the armadillo family proteins armadillo, β-catenin and PG, yet to date the sites of phosphorylation have not been mapped. The majority of tyrosine phosphorylation data available for the vertebrate molecules concern β-catenin alone (for example Matsuyoshi *et al.*, 1992; Behrens *et al.*, 1993; Hamaguchi *et al.*, 1993; and Kinch *et al.*, 1995) and indeed it is for β-catenin that most is known about the location of the tyrosine phosphorylation site(s). Hoschuetzky *et al.* (1994) performed *in vitro* kinase assays using recombinant EGF-receptor and GST-fusion proteins of either the central armadillo repeat region of β-catenin, or the whole β-catenin molecule, and showed that the site(s) of phosphorylation lie(s) in one or both of the two unique end regions of the molecule, as the whole molecule was phosphorylated, but the core region was not. Moreover, this paper (as well as work performed by Shibamoto *et al.*, 1994) goes on to show that PG also becomes tyrosine phosphorylated when human carcinoma cells are stimulated with EGF. As the two molecules are so similar, it seems likely that the EGF-inducible tyrosine phosphorylation sites will be similarly located.

Given the data described above, and the availability of the terminal fusion proteins created during the generation of the anti-PG antiserum (XPGN, XPGC and HPGN described in chapter 4), it was decided to use the fusions to perform *in vitro* kinase assays in an attempt to map the EGF-inducible tyrosine phosphorylation site of PG to one end of the molecule or the other. This work was carried out using both EGF receptors isolated from A431 cells and recombinant EGF receptor, and is described in section 6.2.
6.1.2 Binding studies.

At the time these experiments were performed (early 1995) little was known about which proteins bound directly with PG, nor, for the proteins that were known to bind directly, where they had their binding sites on the molecule. Another logical use of the fusion proteins available was, therefore, to perform in vitro binding studies (co-precipitation assays using the fusion proteins as "bait", and also blot overlay assays) to determine which (if any) proteins co-precipitated with either of the unique terminal domains. This work could then be extended and the site(s) more fully mapped using deletion constructs of the fusions in future binding assays.

Also present in the laboratory at the time, generated in the course of a different project (by Dr H. Thirlwell), was a fusion protein encoding part of the cytoplasmic domain of human Dsg1. The particular domain encoded was of the desmoglein-specific repeat region (amino acids 751-911; Figure 39), a domain of intense interest as it is one of the few areas of the desmogleins without similarity to the classical cadherins, and whose function remains to be elucidated. It was not known if any proteins bind to this region, possibly affecting desmosomal stability or function, so it was decided to use this fusion, in parallel with the PG fusion proteins, in the in vitro binding assays. Unlike the PG fusion proteins, the Dsg fusion was generated using the pET 21a expression vector, resulting in an N-terminal 6xHis-tagged protein which was specifically isolated using nickel-agarose beads (protocol set out in Materials and Methods).

The binding assay experiments are described in section 6.3.

6.2 Examination of tyrosine phosphorylation of fusions.

Membranes of A431 cells were obtained and an in vitro kinase assay carried out, as described in Materials and Methods, to determine whether or not the PG fusion proteins XPGN, XPGC, and HPGN were phosphorylated in an EGF-dependent manner. A431 cells were chosen because they have a large number of EGF receptors present on their cell surface (Waterfield et al., 1982) which can be relatively easily isolated with their activity remaining intact. The isolated membranes were incubated with the different fusion proteins with or without the presence of EGF, the samples run out on SDS-
FIGURE 39: Human desmoglein 1 cytoplasmic domain amino acid sequence.

Amino acids numbers indicated on either side of diagram.

- classical cadherin-like region (C domain)
- plakoglobin binding domain
- proline rich domain
- Dsg-1 fusion protein
- repeat domain (black bars indicate extent of individual repeats)
PAGE, electro-blotted and probed with anti-phosphotyrosine antibody (monoclonal antibody 4G10 of the IgG2b class a kind gift from Dr S. Ley, NIMR, London/from TCS Biologicals Ltd, Buckingham; Druker et al., 1989) to detect phosphorylated proteins. As can be seen from Figure 40A, the fusion proteins were not tyrosine phosphorylated under these conditions, although the receptor is intact and can be activated in the assay as evidenced by the anti-phosphotyrosine antibody recognising the autophosphorylated EGF receptor at 175kD only in the presence of EGF.

In an attempt to confirm this result, EGF receptor was expressed in vitro using the baculovirus system and the kinase assay repeated (virus encoding EGF receptor, and R1 antibody were kind gifts from Dr G. Panayotou, Ludwig Institute, London). The receptor was isolated by immunoprecipitation with specific monoclonal antibody (R1 antibody of the IgG2b class; Waterfield et al., 1982), bound to Protein A-beads, and incubated with fusion protein with or without EGF. Unfortunately the receptor appears to have been isolated in either a constitutively active or inactive state, probably the latter, as evidenced by the fact that the presence of EGF had no effect on it's tyrosine phosphorylation state (Figure 40B) indicating that for this experiment and for future use, the receptor cannot be obtained in this manner for EGF-dependent kinase assays. Hoschuetzky et al. (1994) used the same baculovirus and obtained the receptor in an active form for β-catenin kinase assays, although their isolation procedure was somewhat different to that used here. If the receptor has been obtained in its active state, then the data obtained support the A431 membrane results, namely that none of the fusion proteins become tyrosine phosphorylated by the active EGF receptor. These Western blots were also probed with anti-mouse antibodies which only lit up the bands at 50kD and 25kD showing that the band observed at ~180kD was not unreduced antibody precipitated with the Protein A-beads.

6.3 Fusion protein binding studies.
6.3.1 Precipitations.
A431 cells were incubated overnight with $^{35}$S-TRAN label, and NP40 lysates prepared in the presence of protease inhibitors and phosphatase inhibitors to keep all proteins intact and phosphorylated as in vivo. After pre-clearing the lysates, fusion proteins
FIGURE 40: *In vitro* kinase assays.

A. *In vitro* kinases assay using A431 membranes.

Western blot of *in vitro* kinase assay probed with anti-phosphotyrosine antibody (black bands) overlying amido-black stained blot to indicate position of fusion proteins (blue bands and marked on right of gel). The figure shows that none of the fusion proteins are tyrosine phosphorylated using this assay, but that the EGF receptor autophosphorylates in the presence of EGF.

B. *In vitro* kinase assay using recombinant EGF receptor bound to Protein A beads.

Western blot of *in vitro* kinase assay probed with anti-phosphotyrosine antibody showing that neither the fusion proteins or EGF receptor were phosphorylated in an EGF-dependent manner in this assay.

no protein: kinase assays without fusion protein added
GST: kinases assays with GST protein added
HPGN: kinase assays with HPGN fusion protein added
XPGN: kinase assays with XPGN fusion protein added
XPGC: kinase assays with XPGC fusion protein added
 -: kinase assay performed in the absence if EGF
 +: kinase assay performed in the presence of EGF
A431: sample of A431 SDS lysate
EGFR: epidermal growth factor receptor
H chain: heavy chain of antibody molecule
L chain: light chain of antibody molecule
were added and any newly formed complexes precipitated by the addition of glutathione-agarose or, in the case of the Dsg1 fusion protein, nickel-agarose beads. These complexes were run out on SDS-PAGE and a fluorograph obtained. The results of this are shown in Figure 41, and indicate that a protein of approximately 102kDa binds to HPGN, one of approximately 75kDa binds to the Dsg1 cytoplasmic domain, and that the other fusion proteins seem only to bind proteins that also bind the pre-clear beads.

To confirm this result, and identify the proteins that bind HPGN and the Dsg1 fusion protein, NP40 extracts were made from A431 cells that were pre-treated either with or without EGF. Fusion protein precipitations were carried out as above, run out on SDS-PAGE, electro-blotted and the blots probed with antibodies to various proteins to which it was thought the fusion proteins might bind. As can be seen in Figure 42 the only fusion protein that binds any of the molecules represented in this panel of antibodies is HPGN which binds α-catenin in an EGF-independent manner, tallying well with the result obtained by radiolabelling above.

6.3.2 Overlay assays.
As an alternative and supplementary method of determining which proteins the fusion proteins might bind, overlay assays were attempted. NP40 lysates of A431 cells were run out on SDS-PAGE and Western blotted as usual. After blocking, instead of a primary antibody being incubated with the blot, fusion protein (or GST protein alone as a negative control) at 10μg/ml was laid on top of the blot. The blot was then washed and probed sequentially with a primary antibody against the GST portion of the fusion protein (or in the case of the Dsg1 cytoplasmic domain fusion an antibody generated against it), and a secondary antibody against the first (goat-anti-rabbit alkaline phosphatase-conjugated antibody). Upon development the aim was to identify specific bands to which the fusion proteins bound, i.e. that were not seen in the GST overlay, or when the Western blots were probed with primary and secondary antibodies only. Unfortunately, results were uninterpretable due to the large number of bands that developed even in the negative control when blots were overlaid with GST protein.
FIGURE 41: Fusion protein precipitations of $^{35}$S-labelled A431 cells.

A: Schematic of GST-fusion protein precipitation protocol. For the Dsg-1 fusion protein precipitation, the protocol was essentially the same substituting Ni$^{2+}$-agarose beads for glutathione agarose beads.

B: Fluorographs of fusion protein precipitations:

- **Left panel:** a protein of approximately 102kD binds specifically with HPGN, position indicated with arrow.

- **Right panel:** a protein of approximately 70-75kD binds specifically with Dsg-1 repeat region fusion protein, position indicated with arrow.

- **GST:** GST used for precipitation.
- **XPGC:** XPGC fusion protein used for precipitation.
- **XPGN:** XPGN fusion protein used for precipitation.
- **HPGN:** HPGN fusion protein used for precipitation.
- **Pre-clear:** sample of pre-clear beads Western blotted.
A431 cells +/- EGF
NP40 lysis

Glutathione-agarose beads
preclear overnight

beads
supernatant

add fusion protein beads supernatant
mix 1 hr

wash NP40 buffer x4
Tris pH 6.8 x1
run on SDS-PAGE
Western blot
and probe

B

GST
XPGC
XPGN
HPGN
Pre-clear

217-
110-
71-
44-

217-
110-
71-
44-

Pre-clear Dsg-1
FIGURE 42: Western blot analysis of fusion protein precipitations.

A: Western blot of fusion protein precipitation probed with anti α-catenin antibody giving a positive result for HPGN with or without the pretreatment of the cells with EGF. Position of α-catenin marked on right hand side.

- HPGN: HPGN fusion protein used for precipitation
- GST: GST used for precipitation
- XPGN: XPGN fusion protein used for precipitation
- XPGC: XPGC fusion protein used for precipitation
- +: cells pre-treated with EGF before lysis
- -: cells nor pre-treated with EGF before lysis
- A431: sample of A431 SDS lysate

B: Table of results showing that the only protein giving a positive result for binding a fusion protein from this panel of antibodies was α-catenin which binds HPGN.
**A**

**Fusion protein used for precipitation**

<table>
<thead>
<tr>
<th>Protein probed for</th>
<th>HPGN</th>
<th>XPGN</th>
<th>XPGC</th>
<th>GST</th>
<th>DSG-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plakoglobin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-catenin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>p120</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α-catenin</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phospho-tyrosine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>APC</td>
<td>-</td>
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<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Desmoplakin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Western blots - no difference if cells treated with EGF before lysis
- no EGF induced tyrosine phosphorylation dependent interactions
alone, and reducing the concentration of the overlay solution did not reduce this background significantly (results not shown).

6.4 Conclusion and discussion.
6.4.1 Kinase assays.
These results could indicate a number of possibilities: that the fusion proteins used do not contain the sites for tyrosine phosphorylation of PG, that the fusion proteins are not complexed tightly enough with the EGF receptor to become phosphorylated, or that other cytoplasmic proteins, not isolated with the EGF receptor in the membrane pellet, are required in conjunction with the EGF receptor for phosphorylation of these polypeptides to occur. By analogy with β-catenin, the first suggestion seems unlikely; Hoschuetzky et al. (1994) obtained tyrosine phosphorylation of β-catenin, but not of a fusion of the core region alone, with the recombinant EGF receptor indicating that the phosphorylation sites are in the terminal domains of the protein. Also the only consensus tyrosine phosphorylation sites appear to be in these domains, not in the armadillo repeat region (see Figure 43).

Again by analogy with β-catenin the third possibility seems unlikely; as mentioned above Hoschuetzky et al. (1994) isolated active recombinant EGF receptor from the same source as the one used in this thesis, and observed tyrosine phosphorylation of β-catenin (without the presence of EGF), and although their isolation method was quite different (not specifically precipitating the EGF receptor) it is unlikely that even if the receptor could sequester certain cytoplasmic molecules from the Sf9 cells, that these would remain complexed after their isolation protocol. They lysed the cells at a density of 10^7 cells/ml in a Triton buffer [10mM HEPES pH 7.4, 200mM KCl, 1% Triton X-100, 10μg/ml leupeptin, 10μg/ml PMSF, 10μg/ml soybean trypsin inhibitor, 0.1U/ml α2-macroglobulin], centrifuged the lysate at 14,000g for 10 min, and removed low molecular weight components by gel filtration on a PD10 column (elution buffer 10mM HEPES pH 7.4, 200mM KCl, 0.1% TritonX-100) before using the remaining lysate for the in vitro kinase assay. It is possible, however, that interactions with the EGF receptor could be one of the areas where β-catenin and PG
FIGURE 43: Amino acid sequences of plakoglobin and β-catenin with potential tyrosine phosphorylation sites marked.

Tyrosine residues of human plakoglobin (Hpg), *Xenopus* plakoglobin (Xpg), or *Xenopus* β-catenin (Xbc) highlighted according to the likelihood of them being phosphorylation targets:

- **Pink**: unlikely phosphorylation site
- **Green**: possible phosphorylation site
- **Blue**: probable phosphorylation site

Predictions made according to the following criteria:

1. presence of acidic residues upstream (D or E)
2. conservation of residue across species or across proteins.

See Kemp and Pearson (1990) and references therein for further information on protein kinase phosphorylation site motifs.
differ, with the former binding the EGF receptor directly, and the latter possibly not. It could also be the case that the EGF-dependent tyrosine phosphorylation seen on PG is caused by downstream events from the EGF receptor itself, requiring other cytoplasmic components not obtained in either of the receptor isolation procedures here, whereas that of β-catenin is not.

It would appear, therefore, that the second explanation is the most likely. Again this is supported by the Hoschuetzky et al. (1994) paper which shows that the armadillo repeat region of β-catenin binds to the EGF receptor. Both the XPGN and XPGC fusion proteins do not extend into this region and so could simply not be brought into close enough proximity during the course of the kinase assay for phosphorylation to occur. The HPGN fusion protein, however, does cover the N-terminal most 4.6 armadillo repeats, suggesting that these alone are insufficient for binding the protein to the EGF receptor and obtaining phosphorylation if the phosphorylation site is in the N-terminal domain. To determine if direct binding to the EGF receptor is what is lacking, fusion proteins of the whole armadillo region with either the N- or C-terminal domains could be used for kinase assays, and if it were found to be necessary then the number of repeats needed for this interaction could be determined by sequential deletion analysis.

6.4.2 Binding assays.

In conclusion for this section, XPGN, XPGC and the Dsg1 fusion protein do not bind to PG, β-catenin, α-catenin, APC, desmoplakin, p120 or E-cadherin, and HPGN does not bind to all of the above barring α-catenin as detected on Western blot with the antibodies used. This maps the α-catenin binding site to the armadillo repeat region present in HPGN but not XPGN. This result is in agreement with recent results (Aberle et al., 1994 and 1996; Sacco et al., 1995).

A possible candidate for the protein that binds Dsg1 could be plakophilin 1 as it has the correct molecular weight, has been shown to bind desmogleins directly in overlay assays (Mathur et al., 1994), and is present in A431 cells as well as a larger variety of cell types than previously thought (W.W. Franke, personal communication UCL seminar 1995; Heid et al., 1994). One might suppose plakophilin 1 would bind to
the C-domain of desmoglein, as PG binds there via its armadillo repeat region. However plakophilin 1 has relatively low homology with PG, has most homology with p120, and has incompletely conserved armadillo repeats (Hatzfeld et al., 1994) perhaps allowing it to interact with the protein in a different manner at a different location on the molecule. We intend to test this hypothesis by fusion protein precipitations and Western blot analysis as soon as we have access to anti-plakophilin 1 antibodies.
Chapter 7: Summary.

Both projects described in this thesis are concerned with the role of desmosomal components in early *Xenopus* development.

The initial project was concerned with examining the expression and function of desmosomal cadherins in early *Xenopus* development. To do this I first determined which of a panel of antisera present in the laboratory cross-reacted with *Xenopus* desmosomal proteins by Western blot analysis. Attempts were made to perform immunohistology on sections prepared from *Xenopus* embryos at various stages of development. However, due to technical difficulties, this expression study was not feasible with the antisera available. Attempts were also made to isolate a *Xenopus* desmoglein for use in *in situ* studies and for possible intervention experiments. Both cDNA- and antibody-based techniques were used to screen libraries, however, again due to a variety of problems encountered, no clone was obtained. This project was then abandoned.

The second project was concerned with the potential signalling function of plakoglobin in early *Xenopus* development. GST-fusion proteins including the unique N- and C-terminal domains of *Xenopus* PG (XPGN and XPGC), and of the N-terminal two fifths of human PG (HPGN) were made, purified and used to generate rabbit polyclonal antisera. These antisera were shown to react well with PG in a variety of species with varying specificity.

One of these antisera (2006) was used to demonstrate the expression pattern of PG throughout early *Xenopus* development by Western blot analysis. When compared with the expression pattern of β-catenin, the initial pattern appeared similar, but varied at later stages of development. There was an intriguing dip, followed by a pulse of expression of both proteins around stage 7-10.

The antisera were then used to test for a possible involvement of PG in positional signalling. Three of the antisera (1, 2006 and 2008) were depleted for anti-GST antibodies, the remaining antibodies isolated then used to make F\text{ab} fragments and microinjected into *Xenopus* ventral blastomeres at the 4-cell stage. The resultant
embryos did not exhibit specific phenotypes above background (i.e. secondary axes were not routinely obtained), although these were produced by a positive control injection of anti-β-catenin Fαβ fragments.

An attempt was made to use these fusion proteins to map the reported EGF-dependent tyrosine phosphorylation sites. When the purified fusion proteins were used for in vitro kinase assays, using both A431 membranes and recombinant EGF receptor produced using the baculovirus system, none of the fusions became phosphorylated, although the assay itself was seen to work as demonstrated by the EGF receptor becoming auto-phosphorylated in the presence of EGF. This may have been due to the fact that the fusions had none (in the case of XPGN and XPGC), or only about one third (in the case of HPGN), of the armadillo repeats present, and so may not have been able to couple closely enough with the receptor for phosphorylation to occur.

The fusion proteins were also used for affinity precipitation assays to determine which proteins bound with the different domains of PG. α-catenin was shown to bind HPGN but none of the other fusion proteins, which confirms published data localising the α-catenin binding site to between amino-acids 109 and 137 on human PG (Aberle et al., 1996). Neither XPGN nor XPGC bound any of the panel of proteins assayed for, nor did they appear to bind any unidentified specific proteins. The histidine-tagged Dsg1 cytoplasmic repeat fusion protein bound none of the proteins assayed for, but appeared specifically to bind a protein of approximately 75kD, which might be plakophilin 1, another known desmosomal component and armadillo family member.
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