Specificity of Gap Junction Formation.

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ABSTRACT.

This thesis describes an investigation into the control of specificity of gap junctional intercellular communication, the mechanism which underlies the formation of communication compartments. Ductin, connexins and cadherins are all thought to contribute to gap junction formation, and members of the connexin and cadherin families because of their tissue specific expression may play a role in the control of specificity.

Two cell culture systems were used as models for the molecular analysis of junction formation and specificity. The *mec*$^-$ (defective in junction formation and *me*tabolic *c*ooperation) cell lines (L, MCF-7 and S180) provide a system for expression studies aimed at identifying proteins required for gap junction formation. The epithelial *mec*$^+$ cell line BRL and either of the fibroblast *mec*$^+$ cell lines BHK or BICR sort out when grown in mixed culture and show a specificity of gap junctional communication, providing a system for the identification of proteins which can break specificity.

L and S180 cells express ductin and connexin 43 but do not express the cell adhesion molecules E- ,N- or P-cadherin or N-CAM. Stable S180 transfectants expressing E- or P-cadherin protein, have a flatter morphology at confluency than untransfected cells and show increased levels of gap junctional communication, as demonstrated by microinjection of the dye Lucifer Yellow CH. S180 cells therefore appear to have a defect which reduces gap junction formation and this can be restored by expression of two different members of the cadherin family. The transfection of L cells with either E- or P- cadherin causes cells to grow in tight colonies and to show increased calcium dependent cell adhesion, however the transfected cells do not form gap junctions. Western analysis of connexin 43 expression is consistent with transfected S180 cells expressing phosphorylated connexin 43 and transfected L cells non-phosphorylated connexin 43. This suggests

that the phosphorylation of connexin 43, which is reported to occur in *mec*$^{+}$ cells is important for gap junction formation.

The causes of specificity are unknown. The expression and phosphorylation of connexin 43 may be essential for gap junctional communication but it does not account for the specificity of gap junctional communication seen between BRL and BHK or BICR cells, as western analysis of connexin 43 expression is consistent with these cell types expressing phosphorylated connexin 43. The analysis of cell adhesion molecule expression shows that the differential expression of cell adhesion molecules may contribute to specificity. The transfection of BICR but not BHK cells with E- or P-cadherin expression constructs causes marked morphological change and accumulation of exogenous cadherin at points of cell contact. When BICR cells transfected with E-cadherin and BRL cells also transfected with E-cadherin are grown in mixed cultures specificity is broken and dye transfer occurs between the cell types in 50% of injections, (5% in mixed cultures of parental cells). The mechanism by which E-cadherin breaks specificity is unknown. The cell types which show specificity form functional homologous gap junctions and it may be that E-cadherin induced cell-cell adhesion per se increases membrane apposition between different cell types which would not normally occur and this increases the frequency of close membrane apposition required for junction formation. Alternatively E-cadherin induced cell-cell interaction could cause signal transduction which affects proteins involved in gap junction formation.

To my family and Meggie

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Declaration.

The work described in this thesis was performed personally unless otherwise acknowledged.

Abbreviations.

A_x	$Absorbance_{wavelength}$
amp	ampicillin resistance marker
ATP	adenosine 5'triphosphate
bFGF	basic fibroblast growth factor
bp	base pairs
C	centigrade
cAMP	3',5'cyclic adenosine monophosphate
CIP	calf intestinal alkaline phosphatase
cm	centimetre
cpm	counts per minute
CTP	cytidine 5'triphosphate
Da	Dalton
DMEM	Dulbecco modified Eagles medium
DNA	deoyribonucleic acid
ECL	enhanced chemiluminescent
ECM	extracellular matrix adhesion molecule
EDTA	ethylenediaminetetra-acetic acid, disodium salt
EGF	epidermal growth factor
EM	electron microscopy
FAK	focal adhesion kinase
FTIR	Fourrier transformed infra-red
G418	geneticin
g	gram
GAPDH	glceraldehyde-3-phosphate dehydrogenase
GJIC	gap junctional intercellular communication
GMEM	Glasgow modified Eagles medium
HBS	HEPES buffered saline
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HMCF	HEPES buffered magnesium and calcium free saline
HPRT	hypoxanthine phosphoribosyltransferase
hr	hour
HSV	Herpes simplex virus
IMPs	intramembranous particles
IPTG	isopropylthiogalactoside
k	kilo
l	litre
LTR	long terminal repeat
µ	micro
m	milli
M	molar
mA	milliamps
mec$^-$	defective in gap junction formation and metabolic cooperation
mec$^+$	effective in gap junction formation and metabolic cooperation
min	minute
mol	mole
M_r	molecular weight
mRNA	messenger RNA
n	nano
N-CAM	neural cell adhesion molecule
neo	neomycin resistance marker
nm	nanometre

oligo	oligonucleotide
o/n	overnight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonylfluoride
pS	picosiemens
rpm	revolutions per minute
R	resistance
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate
sec	second
SLM	special liquid media
SSCHC	squamous cell carcinomas of the head and neck
TEMED	teramethylenediamine
tk	thymidine kinase
TPA	12-O-tetradecanoylphorbol-13-acetate
tris	2-amino-2-(hydroxymethyl) propane-1,3-diol
UV	ultraviolet
V	Volts
W	Watts
v/v	volume for volume
w/w	weight for volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Amino acid one and three letter codes

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

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Chapter 1.

Introduction.

1.1 General Introduction.

Gap junctions are apparently universal features of metazoan animals. With the exception of some cell types which become uncoupled during terminal differentiation (for example lymphocytes and skeletal muscle) nearly all cells *in vivo* can form gap junctions. Gap junctions are transmembrane channels which occur at regions of cell-cell contact. They enable cells to communicate directly with one another by the exchange of small cytoplasmic molecules. Gap junctional intercellular communication (GJIC) plays an important role in electrical tissues allowing synchronization and propagation of electrical activity. In non-excitable tissues gap junctional communication coordinates cellular activities and has the potential to regulate tissue growth within coupled cell populations.

1.2 Structure of Gap Junctions.

The structural unit of the gap junction is the connexon (Goodenough 1975), a cylinder of protein approximately 7nm in diameter with a central aqueous pore that spans the plasma membrane and protrudes (approximately 1nm) into the extracellular space. Connexons in apposed plasma membranes align end to end to form a continuous aqueous channel allowing the intercellular movement of ions and small cytoplasmic molecules between coupled cells.

1.2.1 A brief history of gap junction structure.

The early observations of electrical coupling (Furshpan and Potter 1959), dye coupling (Loewenstein and Kano 1964) and metabolite exchange (Subak-Sharpe et al 1966 and 1969) between neighbouring cells gave support to the idea that a specialised membrane structure was involved in direct cell-cell communication

(reviewed Azarnia and Larsen 1977). Hexagonally packed connexons on the extracellular face of the plasma membrane were first observed by electron microscopy (EM) in Mauthner cell synapse's after permanganate fixation (Robertson et al 1963). Subsequently using lanthanum as a tracer of extracellular space Revel and Karanovsky in 1967 described a separation or "gap" of 2nm between closely apposed plasma membranes in mouse liver or heart, which was bridged by connexons. The description of the gap gave rise to the name gap junction and distinguishes it from the tight junction which is impermeable to lanthanum. However, it is the connexon which spans the plasma membrane and protrudes into the gap that enables direct cell-cell communication. The term gap junction to describe this structure is therefore slightly misleading. Other names have been proposed, nexus (Dewey and Barr 1962) and maculae or communicating junction (Simionescu et al 1975) but gap junction has become universally used. Revel and Karanovsky also observed that the hexagonally packed connexons have a lanthanum permeable central pore. The permeability of intact gap junctions to extracellular lanthanum is unexpected since lanthanum fills the extracellular space between the membranes and would not be expected to enter a channel only open to the cytoplasm, if as predicted the wall of the channel is impermeable to aqueous solutes. The leakiness to lanthanum may be caused by the preparation steps necessary for electron microscopy.

The presence of gap junctions as determined by electron microscopy correlates well with functional measurements of intercellular communication (Gilula et al 1972, Caveney 1985). The gap junction has been found in a wide variety of mammalian tissues (Goodenough and Revel 1970, McNutt and Weinstein 1970, Peracchia 1978, Zervos et al 1985) as well as in invertebrates (Perrachia 1973, Lane and Swales 1980, Pitts and Finbow 1986). GJIC has been observed between cells in

intact tissue (Kam et al 1986), embryonic cells (Griepp and Revel 1977, Lo and Gilula 1979) and in primitive metazoan organisms (Fraser and Bode 1981).

Gap junction morphology has been determined for different tissues and species by analysis of gap junctions in intact tissue (by electron microscopy of thin section and freeze fracture preparations) and further structural details have been obtained by analysis of isolated gap junctions (by electron microscopy, electron diffraction, X-ray diffraction and atomic force microscopy).

1.2.2 Gap junctions in intact tissues.

The characteristic gap of approximately 2nm between closely apposed plasma membranes can be visualised by thin section electron microscopy. It appears conserved between different tissues and species, (Goodenough and Revel 1970, McNutt and Weinstein 1970, Perrachia 1973). The junctional thickness, (the overall width of both apposed membranes and the gap) is typically 14nm to 16nm in rodent liver (Goodenough and Stoeckenius 1972, Kensler and Goodenough 1980), rodent heart (Dupont et al 1988) and lobster hepatopancreas (Pitts and Finbow 1986).

Freeze fracture replicas of vertebrate gap junctions reveal intramembranous particles (IMPs) in a roughly hexagonal pattern on the cytoplasmic face (P-face) with corresponding pits on the extracellular face (E-face; Chalcroft and Bullivant 1970). In contrast, in arthropods the connexon particles mostly partition to the E-face leaving corresponding P-face pits (Peracchia 1973, Lane and Swales 1980, Pitts and Finbow 1986). The reason for this difference is not clear. A central depression in the IMPs and an occasional central "bump" in the pit can be observed in crayfish gap junctions (Peracchia 1973). A recent freeze fracture analysis of rat liver gap junctions using high angled shadowing reveals dimples in the P-face IMPs (interpreted as representing the channel pore) and a 2-3nm "peg" in the corresponding E-face pit

(Rash and Yasumura 1992). The peg may represent the frozen aqueous channel left after extraction of the connexon to the P-face.

Deep etch freeze-fracture preparations of fractured tissue reveal not only the cleaved faces (like the fracture replicas previously discussed) but also the natural cytoplasmic face of the junction. However, in regions where the cytoplasmic face is exposed there are no connexons visible (Hirokawa and Heuser 1982). The channel may extend only a short distance into the cytoplasm, be obscured by other proteins or its detection may be prevented by technical limitations.

The size of the junctional plaque observed by freeze fracture can vary from a few connexon pairs to several thousand. The reason for this clustering is unknown. The plaque may represent a focal point for apposition of connexons in adjacent plasma membranes, it may reduce the area of unstably close membrane apposition which occurs at these junctions (Braun et al 1984) or alternatively the plaque may be important for gap junction internalisation during turnover (Larsen and Tung 1978).

1.2.3 The structure of isolated gap junctions.

The morphology of the gap junction plaque is relatively resistant to detergent or alkali treatment. Gap junctions have been separated from other membrane components by a variety of methods which rely on this resistance. The characteristic gap of approximately 2nm between closely apposed plasma membranes can be visualised by taking thin sections through pellets of isolated gap junctions. It is similar in size to that observed *in situ*. The thickness of isolated gap junctions can vary from 14nm to 20nm (Goodenough and Stoeckenius 1972, Kensler and Goodenough 1980, Berdan and Gilula 1988, Leitch and Finbow 1990). Thin junctions (average width 14.1nm) or thick junctions (average width 18.4nm) have been found in gap junction preparations from mouse liver plasma membranes prepared by either triton X-100 extraction or by alkali extraction respectively. The use of detergents and urea during

extraction may cause the loss of lipid and/or associated protein from the gap junction, giving rise to a thinner appearance (Finbow and Meagher 1992).

The individual connexons of isolated rat liver gap junctions imaged by electron diffraction of unstained preparations (Unwin and Zampighi 1980), by atomic force microscopy (Hoh et al 1991a) or by X-ray diffraction (Makowski et al 1977, Caspar et al 1977) have similar dimensions, approximately 7nm in diameter and 7nm in length. The negative stained images of connexons often have a central dot (1-2nm in diameter) which is thought to represent the stain penetratable channel (Unwin and Zampighi 1980). The superimposition of these negative stained images suggests that individual connexons have a hexagonal morphology (Baker et al 1985). The X-ray diffraction data is consistent with this and shows each connexon is composed of six subunits (Makowski et al 1977, Caspar et al 1977, Makowski et al 1982) that have a protein component which is 60% α-helical (Tippetts et al 1990). A very similar α-helical content is suggested by FTIR (fourrier transformed infra-red) spectroscopy of isolated *Nephrops* gap junctions (Holzenburg et al 1993). The α-helical content best fits a model of each connexon being a hexamer of six similar protein subunits, with each subunit having four transmembrane domains arranged as an α-helical bundle (Tippetts et al 1990, Holzenburg et al 1993). The predicted structure of the gap junction channel protein should be consistent with this model (section 1.5).

Connexon structure appears highly conserved between rodent liver (Makowski et al 1977, Unwin and Zampighi 1980), rodent heart (Yeager and Gilula 1992) and lobster hepatopancreas (Sikerwar et al 1991). Although a description of thin (14nm) gap junctions, isolated using triton X-100 from the hepatopancreas of crayfish which were poorly penetrated by anionic stain led these structures to be called pseudo-gap junctions suggesting that they are different cellular structures (Berdan and Gilula 1988). However, the degree of penetration and hence staining of the channel can vary with the type of negative stain used, uranyl acetate a cationic

stain is reported to be better at penetrating the channel than phosphotungstate or ammonium molybate which are anionic stains (Baker et al 1985). The structures isolated by Berdan and Gilula are similar in appearance to gap junctions isolated from lobster hepatopancreas which have a central pore penetrated by cationic but not anionic negative stain (Leitch and Finbow 1990).

1.3 Permeability of gap junctional channels.

Gap junctional channels are freely permeable to ions and small cytoplasmic molecules, including metabolites and second messengers but are impermeable to large molecules such as proteins and nucleic acids (Pitts and Simms 1977, Lawrence et al 1978, Finbow and Pitts 1981, Saez et al 1989a).

GJIC between coupled cells can be demonstrated by microinjection of a fluorescent dye, a technique originally used by Loewenstein and Kano (1964). A fluorescent dye molecule widely used is Lucifer Yellow, which has a molecular radius of 0.7nm (M_r 457). It readily diffuses through gap junctions, demonstrating "dye coupling" between communicating cells.

The exclusion limit of the channel has been defined by dye injection studies using fluorescent probes of different molecular size and charge. The channels exhibit little charge selectivity and the permeability can be defined in terms of molecular weight. The exclusion limit is about M_r 900 for mammalian cells (Flagg-Newton et al 1979) and M_r 1500 for arthropod cells, although the exclusion limit has been determined for only a few arthropod cell types, *Chironomas* salivary gland and an insect cell line (Simpson et al 1977).

The measurement of gap junctional conductance, the flow of ions between coupled cells, is the electrical equivalent to dye transfer. The average single channel conductance for gap junctions in rat lacrimal gland is 120pS (Neyton and Trautmann 1985). However, the interpretation of single channel conductance is complicated by

the observation of multiple conductances in the same cell type. For example, in ventricle cell pairs isolated from seven day chick embryo heart six reproducible conductance levels of approximately 40, 80, 120, 160, 200 and 240ps have been reported which are not thought to result from random simultaneous opening of several 40ps channels (Chen and DeHaan 1992). These conductances could result from cooperative opening of several 40ps channels, several different channels each with its own conductance or a single population of large channels with a maximal conductance near 240ps and five smaller substates. This poses the question of whether individual gap junctional channels open in an "all or nothing" manner or in steps (permeable to progressively larger molecules and having larger conductances). The experimental results have been conflicting, either consistent with an all or nothing opening (Zimmerman and Rose 1983, Verselis et al 1986) or a stepped opening (Loewenstein and Rose 1978). The structural analysis of isolated gap junctions can resolve two different states open and closed (Unwin and Zamphigi 1980, Sikerwar and Unwin 1988) but no channel substates, although they may occur *in situ*. However, differences in the morphology of native gap junctions have not been observed (Hanna et al 1985).

1.4 Functions of gap junctional intercellular communication.

Gap junctional intercellular communication allows the equilibration of ions and small cytoplasmic molecules between cells in a coupled cell population. In excitable tissues gap junctional channels form electrical synapses which allow the direct propagation of action potentials (Furshpan and Potter 1959). In heart this electrical transmission synchronises myocyte contraction (Fawcett and McNutt 1969, Rooks et al 1990). In non-excitable tissues GJIC can coordinate metabolic activity and has the potential to regulate cell proliferation and differentiation during tissue growth, embryogenesis and tumorigenesis.

1.4.1 Metabolic cooperation.

Within a coupled cell population small cytoplasmic molecules will tend to equilibrate between cells and can support cell types that lack a full range of metabolic activities (Pitts 1980). For example, when mixed cultures of $HPRT^-$ cells (deficient in the enzyme hypoxanthine phosphoribosyl transferase and therefore unable to make purine nucleotides from hypoxathine) and TK^- cells (lacking thymidine kinase and therefore unable to make thymidine nucleotides from thymidine) are cultured in HAT medium survival and growth are dependant on mutual nucleotide exchange and only occur if the two cell types form gap junctions (Pitts 1971). Enzymatic activities that respond to changes in metabolite concentration can also be coordinated by cells within a coupled population (Sheridan et al 1979). Cross-feeding may be important *in vivo* for the maintenance of the normal phenotype of Lesch-Nyhan hemizygotes who have a proportion of $HPRT^-$ cells due to random X-inactivation (Migeon et al 1968). Metabolic cooperation has also been observed to occur between cumulus cells and oocytes (Moor et al 1980). It is likely that most coupled cell populations are functional syncitica with respect to their metabolites.

1.4.2 Gap junctional intercellular communication and the regulation of cell proliferation and differentiation.

The intercellular signalling mechanisms which regulate cell proliferation and differentiation are largely unknown. There are several reasons for considering a role for GJIC in the regulatory process.

The gap junctional pathway can coordinate the response of coupled cells to stimuli via the exchange of second messengers. For example, mouse myocardial cells normally respond to noradrenaline and rat ovarian granulosa cells to follicle stimulating hormone but in mixed cultures both cell types respond to either hormone (Lawrence et al 1978). This demonstrates that the common second messenger

(cAMP) triggered in either cell type can be transferred via gap junctions and induce a response. Gap junctions are permeable to other second messengers, inositol 1,4,5-triphosphate and Ca^{++} (Saez et al 1989a, Charles et al 1992), even though elevated intracellular calcium levels are known to block gap junctional communication (Rose and Loewenstein 1975). This apparent discrepancy may be because the concentration of calcium required to block communication (>1μM) is much higher than the normal resting level (Spray et al 1985). The identity of other gap junction permeable signalling molecules has remained elusive. This may be because they are intrinsically difficult to identify, due to their low concentration in small cell populations.

A number of proliferation control models have been proposed to show how GJIC might regulate cell growth in a density dependant manner (Sheridan 1976, Loewenstein 1979). The models are intrinsically the same and involve the rare asynchronous production of a small control molecule by individual cells that promotes cell division and diffuses throughout a coupled cell population via gap junctions. In order to trigger division the concentration of the control molecule must exceed a threshold value within an individual cell. At sparse cell densities, where gap junction formation is rare, individual cells will be asynchronously triggered to divide by the concentration of this cell cycle dependant control molecule. However, at higher cell densities, where the asynchronously growing cells form gap junctions with numerous other cells, the rarely produced control molecule will tend to dissipate throughout the coupled cell population, (which will act as a "sink"). If there are sufficient cells at different cell cycle stages coupled to the control molecule producing cell, then the control molecule will be diluted, such that its concentration falls below the threshold value required to promote cell division. Thus the growth of cells within a coupled cell culture or tissue may be affected by GJIC. In order to express new growth rates the model predicts that it is necessary for individual cells to become isolated in terms of GJIC from the cell population, or to produce sufficient control

molecules to prevent suppression by transfer to neighbours. Aberrant GJIC may therefore be important during tumorigenesis (see section 1.6).

The level of GJIC depends on the permeability of each individual channel and the number of channels formed. For most cell types these values are unknown but a number of observations show that GJIC enables a rapid, functionally significant exchange of small molecules (Safranyos and Caveney 1985, Kam et al 1986). This exchange could provide a mechanism to establish gradients of diffusable morphogens, such as those proposed to occur during pattern formation (Crick 1970, Wolpert 1978).

Support for the idea that GJIC contributes to the control of cell proliferation and differentiation comes from observations correlating the patterns of communication with cell morphology and developmental fate in mammalian skin (Kam et al 1986, Salomon et al 1988) and hair follicles (Kam and Hodgins 1992), insect epidermis (Warner and Lawrence 1982), and during the embryonic development of molluscs (Serras and van den Biggelaar 1987, Serras et al 1989), teleost fish (Gevers and Timmermans 1991) and mice (Lo and Gilula 1979, Kalimi and Lo 1988). For example, in mouse skin the pathways of GJIC have been mapped by microinjecting the dye Lucifer yellow and observing the patterns of dye spread (Kam et al 1986). This reveals a large dermal communication compartment of 500^{+} dermal fibroblasts and smaller epidermal compartments of 5-12 keratinocytes. Within each communication compartment cells are well coupled homologously via gap junctions as demonstrated by the spread of the dye Lucifer yellow but between communication compartments (for example, epidermal and dermal compartments) cells are poorly coupled heterologously and dye only rarely spreads. Similar results have been reported for human skin (Salomon et al 1988). A specificity of GJIC is occurring which restricts the diffusion via gap junctions of ions and small cytoplasmic molecules between communication compartments. The epidermal compartments

closely resemble the proposed epidermal proliferative units (Mackenzie 1975) in both size and organization, suggesting that communication compartmentalisation may contribute to epidermal proliferative control by regulating second messenger concentration within each epidermal proliferative unit (Kam and Pitts 1988, Pitts et al 1988).

The formation of communication compartments, which limit the spread of control molecules, may also be a requirement for the expression of different phenotypes. In the mammalian hair follicle (Kam and Hodgins 1992), insect epidermis (Warner and Lawrence 1982) and during embryonic development (Lo and Gilula 1979, Serras et al 1989, Kalimi and Lo 1988, Gevers and Timmermans 1991) the formation of communication compartments as demonstrated by dye transfer correlates temporally and spatially with the formation of subpopulations of cells having different developmental fates. Although, in the insect epidermis (Warner and Lawrence 1982) and the mouse embryo (Kalimi and Lo 1988) electrical coupling was also measured and observed to occur between cells that show a restriction of dye spread. This inconsistency has not been observed between communication compartments *in vitro* (Pitts and Kam 1986) but it may reflect a low level of gap junctional communication, the functional significance of which is uncertain.

Communication compartments are characterised by observations of restricted dye transfer, often demarking boundaries between cells that have different developmental fates. This restriction is unlikely to be due to a physical boundary such as a basement membrane because communication can occur between cumulus cells and oocytes through the zona pellucida (Gilula et al 1978) and communication compartments can form in places where there is no basement membrane, for example in the epidermis. In the fifth instar larvae of *Oncopeltus fasciatus*, cells within segments are well coupled but dye spread is restricted across segmental borders (Warner and Lawrence 1982) due to a discrete population of

communication incompetent cells that do not form gap junctions (Blennerhassett and Caveney 1984). Communication compartments in other systems (Fentiman et al 1976, Pitts and Burk 1976, Serras and van den Biggelaar 1987, Kalimi and Lo 1988, Kam and Hodgins 1992) may be due to the inability of communication competent cells to form heterologous gap junctions, a specificity of gap junction formation that could be caused by the differential expression of gap junctional channel proteins (section 1.5) or cell adhesion molecules (section 1.6.1). Alternatively, communication compartments could be a consequence not of gap junction formation but of gating, the regulated opening/closing of existing gap junctional channels (section 1.6.3).

1.5 Identity of the gap junctional channel protein.

It is widely held that connexins are the sole components of connexons, the structural unit of the gap junction (Dermietzel et al 1990, Bennett et al 1991, Haefliger et al 1992). This belief is challenged by the observation that a 16kD protein, recently named ductin is also present in gap junction preparations from a wide variety of animal tissues (Finbow et al 1983 and 1984, Buultjens et al 1988, Holzenburg et al 1993). The unrelated MIP 26 protein has also been proposed to have a role in GJIC in vertebrate lens (Bok et al 1983, Sas et al 1985), although this too is controversial (Zamphigi et al 1989, Paul et al 1991). The evidence concerning the identity of the gap junction channel protein is discussed below.

1.5.1 Isolation of gap junction proteins.

The gap junction was one of the first membrane structures to be isolated (Benedetti and Emmelot 1967). The early analysis of preparations rich in gap junctions by sodium doecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) generated a large number of candidate proteins of different molecular weight (reviewed by Robertson 1981). This variation in protein size can be attributed to the

use of different isolation techniques and the difficulty of assessing the purity of gap junction preparations by EM, which gives an unquantifiable impression of contamination. For example, in two separate studies of rat liver gap junction protein, (Hertzberg and Gilula 1979, Fallon and Goodenough 1981) similar isolation procedures were employed and gap junctions purified to homogeneity, as determined by EM but the SDS-PAGE gel profiles revealed no major proteins in common. However, a consensus did emerge at this time. A number of laboratories independently reported that the molecular weight of a major gap junction protein, present in the SDS-PAGE profiles of gap junction preparations made from rodent liver without enzyme treatment or solubilization by boiling in SDS, is 27kD (Hertzberg and Gilula 1979, Henderson et al 1979, Finbow et al 1980). The trypsinization of rat liver gap junction preparations isolated without enzyme treatment causes the disappearance of the 27kD band and the appearance of a 10kD band (Finbow et al 1980), previously reported as a component isolatable from trypsinized gap junction preparations and named connexin (Goodenough 1974). Two other proteins can also be observed in rodent liver gap junction preparations, a 21kD protein (Henderson et al 1979) and a 16kD protein (Finbow et al 1983). The 27kD, 21kD and 16kD proteins can occur in the same liver gap junction preparation prepared by different procedures (Willecke et al 1988, Hertzberg et al 1988), although the proportion of the 16kD protein, in mouse liver gap junction preparations can show considerable variation (5-60%; Willecke et al 1988, Finbow et al 1992). The 16kD protein (Buultjens et al 1988) and a higher molecular weight protein of 44-47kD (Manjunath et al 1987) are also observed in SDS-PAGE profiles of rat heart gap junction preparations. A number of different molecular weight proteins are therefore present in gap junction preparations. This raises the controversial question of which protein(s) forms the gap junctional channel.

1.5.2 The connexin family.

The 27kD protein from rodent liver and the 44-47kD protein from rodent heart were first shown to be related by protein sequencing (Nicholson et al 1981, 1985). The cDNA for the 27kD protein was subsequently cloned (Paul 1986, Kumar and Gilula 1986, Heynkes et al 1986) and the complete amino acid sequence determined, giving a predicted molecular weight of 32kD. Consequently this protein is called connexin 32 (Beyer et al 1987). Using the rat connexin 32 cDNA Beyer et al (1987) were able to clone the cDNA for the 44-47kD protein from rat heart and Zhang and Nikolson (1989) the cDNA for the 21kD protein from rat liver. The predicted molecular weight of the 44-47kD protein is 43kD and of the 21kD protein is 26kD, consequently these proteins are called connexins 43 and 26 respectively. The discrepancy between the observed molecular weight in SDS-PAGE profiles and the predicted molecular weight of connexins 26 and 32 is most likely to be due to anomalous migration during SDS-PAGE, a phenomenon also reported for other proteins, particularly those with hydrophobic domains (Weber and Osborn 1975, Isaac et al 1985), although other explanations were initially proposed, including proteolysis (Paul 1986, Kumar and Gilula 1986). The higher than predicted molecular weight of connexin 43 in SDS-PAGE profiles is due to post-translational phosphorylation (Musil et al 1990a and b), a modification also reported to affect the electrophoretic mobility of other proteins (Ralston and Bishop 1985, Moria et al 1989, Buchkovich et al 1989). The role of connexin phosphorylation in gap junction formation and regulation is discussed further in section 1.6.

Connexins 26, 32 and 43 are part of a family of proteins with molecular weights from 26 to 54kD. Currently nine rat connexin cDNAs have been cloned, (table 1). They have sequence homology which is particularly high (50-80%) in four regions, predicted from hydropathy plots (Kyte and Dolittle 1982) to be membrane spanning, and contain conserved motifs, including two cysteine rich regions

Table 1. The rat connexin family.

Table 1.

	Connexin.	Reference.
Group I or β	26	Zhang & Nicholson 1981
	31	Hoh et al 1991
	31.1	Haefliger et al 1992
	32	Paul 1986
Group II or α	33	Haefliger et al 1992
	37	Haefliger et al 1992
	40	Beyer et al 1992
	43	Beyer et al 1987
	46	Paul et al 1991

predicted to be extracellular domains (Beyer et al 1987, Haefliger et al 1992). On the basis of amino acid sequence similarities the connexins have been divided into classes, designated Group I or β (connexins 26, 31, 31.1 and 32) and Group II or α (connexins 33, 37, 40, 43 and 46; Risek et al 1990, Bennett et al 1991, Haefliger et al 1992). Between Group I and II the homology is approximately 40% but within each Group it is higher (42-65%). Highly conserved homologs of the rat connexins have been found in other species, including chicken (Beyer 1990, Musil et al 1990a, Rup et al 1993), human (Kumar and Gilula 1986, Fishmann et al 1990), mouse (Willecke et al 1991, Hennemann et al 1992, White et al 1992) and *Xenopus* (Gimilich et al 1990). A comparison of connexin 43 cloned from chicken and rat shows that 92% of the predicted amino acids are identical, with the majority of changes being conservative with respect to charge (Musil et al 1990a). There are also connexins cloned from chicken (Beyer et al 1990) and *Xenopus* (Ebihara et al 1989) that do not have rat homologs, suggesting that the total number of connexins in rat may be higher.

Connexins are expressed in wide variety of tissues. The pattern of expression of individual connexins is often overlapping with particular connexins exhibiting relatively broad distributions, for example connexins 32 and 43 (Dermietzel et al 1990), or highly restricted, for example connexins 31 and 46 (Hoh et al 1991b, Paul et al 1991). A punctate pattern of immunofluorescence is seen at points of cell contact between vertebrate cells expressing connexins (Paul 1986, Beyer et al 1989, Traub et al 1989, Paul et al 1991, Risek et al 1992, Bruzzone et al 1993) and anti-connexin antibodies also bind to a component of plant plasmodesmata (Yahalom et al 1991). The punctate pattern is not specific to connexins as antibodies directed against the amino-terminal domain of basic fibroblast growth factor (bFGF) label intercalated discs in heart sections, in an pattern indistinguishable from that obtained with antibodies against connexin 43 (Kardami et al 1991). Antibodies to connexin 26

(Nicholson et al 1987, Zhang and Nicholson 1989, Traub et al 1989), connexin 32 ((Paul 1986, Nicholson et al 1987, Traub et al 1989), connexin 40 (Kanter et al 1992), connexin 43 (Beyer et al 1989, Yancey et al 1989, Kardami et al 1991, Kanter et al 1992) and connexin 45 (Kanter et al 1992) have been shown to localise to *in situ* or isolated gap junction regions by immunoelectron microscopy. The anti-bFGF antibodies also heavily label gap junction regions (Kardami et al 1991), showing that these regions can contain more than one type of protein. The functional significance of this association of bFGF with gap junction regions is however unknown. The immunogold labelling of mouse liver ultrathin sections shows that connexins 26 and 32 can also occur in the same junctional plaque (Nicholson et al 1987, Traub et al 1989). However, the degree of interaction and the contribution made to gap junction channel formation of connexins co-expressed in the same gap junctional plaque is uncertain.

The structure of the isolated gap junction is highly resistant to proteases, such as trypsin, presumably due to the inaccessibility of the component protein (Goodenough 1976, Zimmer et al 1987). Therefore, only those proteins that survive protease treatment of isolated gap junctions, intact or in sufficient sized fragments to maintain the integrity of gap junction structure, are potentially gap junctional in origin. Connexins 32 and 43 have been shown to meet this requirement (Zimmer et al 1987, Hertzberg et al 1988, Yancey et al 1989). Protease treatment of isolated liver gap junctions (containing predominantly a 27kD protein, connexin 32, a 21kD protein, connexin 26 and a 16kD protein, ductin) generates peptides of 10kD and 17kD (Hertzberg et al 1988). The 17kD peptide band effectively masks the 16kD protein, which is protease resistant in intact gap junctions (Finbow et al 1983). Microsequencing of the 10kD and the 17kD peptides shows that they are fragments of connexin 32. The 21kD protein (connexin 26) is also sensitive to protease treatment but the size of the peptide fragments generated have not been reported,

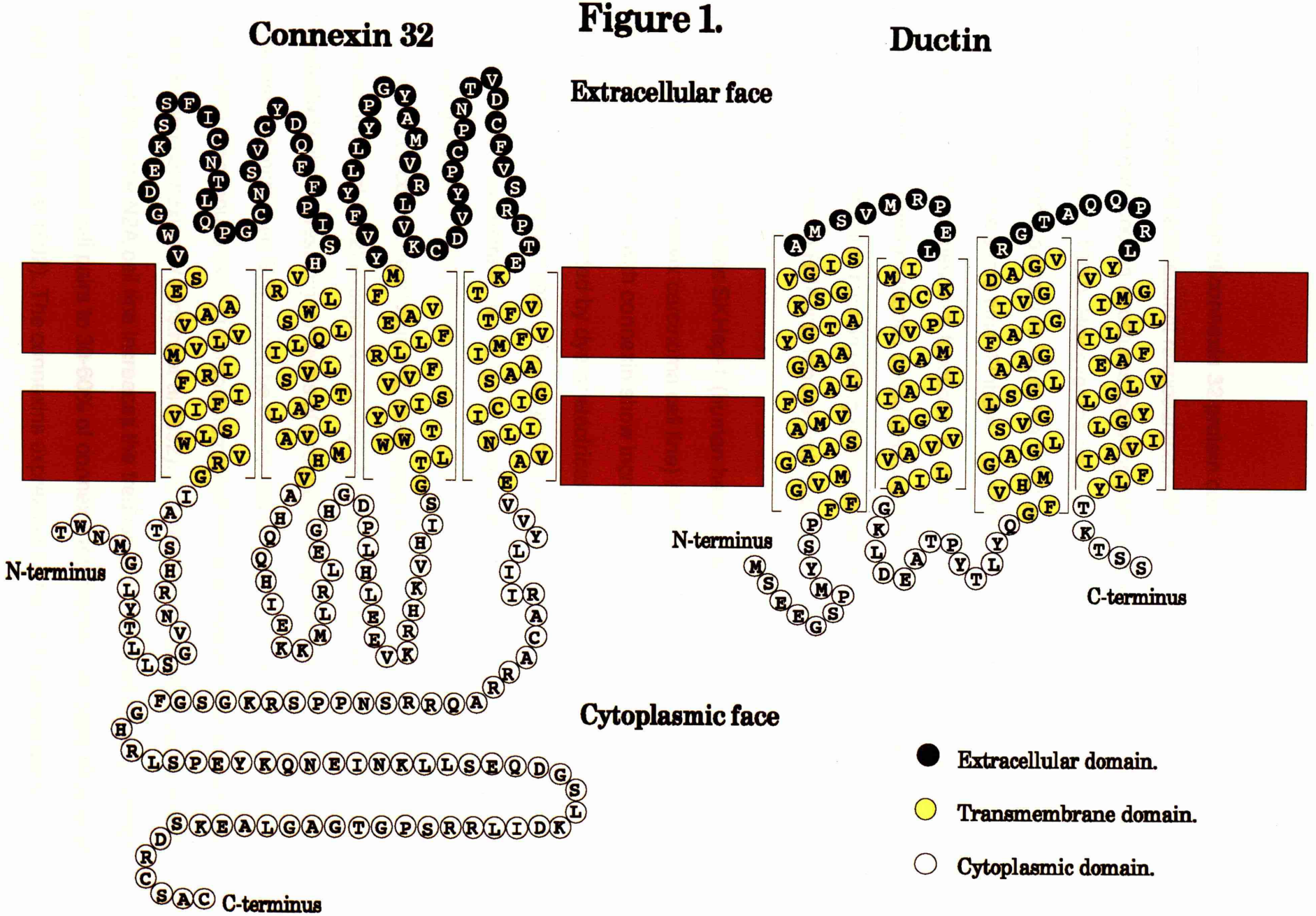
(presumably they are 10kD or smaller). The microsequencing suggests that proteolysis of connexin 32 removes the N-terminal and C-terminal domains and cleaves at a site between transmembrane domains II and III (Hertzberg et al 1988). This predicts that if connexin 32 is the major rat liver gap junctional channel protein, the integrity of the gap junction is maintained following protease treatment by interaction between the transmembrane regions of the 10kD and the 17kD peptides. Whether such an interaction occurs has not been investigated. The accessibility to protease of the N-terminus, the C-terminus and the loop connecting transmembrane domains II and III of connexin 32 indicates that these sites are likely to be cytoplasmic. This proposed topology (figure 1) is supported by immunological studies using site direct antibodies (Milks et al 1988, Goodenough et al 1988, Rahman and Evans 1991). A similar topological model for connexin 43 has been proposed (Beyer et al 1987), which is also supported by immunological studies (Yancey et al 1989, Laird and Revel 1990, Yeager and Gilula 1992).

The intact gap junction is not recognised by antibodies raised against peptides corresponding to putative extracellular loop regions of connexins 32 (Goodenough et al 1988) or 43 (Laird and Revel 1990). Procedures which split gap junctions enable immunofluorescent labelling of cells in a punctate pattern at the light microscope level (Goodenough et al 1988, Laird and Revel 1990) but not immunogold labelling of all isolated gap junctions at the electron microscopy level (Goodenough et al 1988). This inconsistency may be due to non-uniform splitting or degradation, which is only apparent at the electron microscopy level. Post-embedding immunogold labelling of thin sections, with an antibody directed against the second extracellular domain of connexin 43 has also been reported (Meyer et al 1992). Presumably thin sectioning prior to labelling exposes the extracellular domains. However, the immunogold labelling is of regions of close cell-cell membrane apposition and not necessarily specific to gap junctions.

Figure 1. Disposition of connexin 32 and ductin in the membrane.

Each molecule consists of four predominantly hydrophobic regions separated by hydrophyllic regions of various lengths. The N- and C-termini of each polypeptide are predicted to be cytoplasmic. The hydrophobic regions are of sufficient length to pass through the membrane and are predicted to be α-helical. Connexin 43 is homologous to connexin 32 but the C-terminus extends for an additional 80 residues on the cytoplasmic face. Based on a figure of Finbow and Pitts 1993.

Figure 1.


Connexin 32
Ductin
Extracellular face
N-terminus
C-terminus
N-terminus
C-terminus
Cytoplasmic face
Extracellular domain.
Transmembrane domain.
Cytoplasmic domain.

The expression of connexin 32 protein correlates with the loss (24-28 hours postoperative) and subsequent reappearance (36-44 hours postoperative) of recognisable gap junction structures following partial hepatectomy in rats (Yancey et al 1979, Finbow et al 1980). However the process of liver regeneration is complex and any correlation may be fortuitous, the 16kD protein for example also disappears and reappears at the same time (Finbow et al 1983). Direct evidence for the role of connexins in GJIC comes from functional studies: transfecting *mec*$^-$ (defective in junction formation and *me*tabolic *c*ooperation) cell lines with constructs for the expression of connexins; microinjecting sense connexin RNA into *Xenopus* oocytes or anti-sense connexin RNA into mouse embryos; and using anti-connexin antibodies to block GJIC.

The *mec*$^-$ cell lines SKHep-1 (human hepatoma cell line), C6 (rat glioma cell line), HeLa (human cervix carcinoma cell line) and N2A (mouse neuroblastoma cell line) when transfected with connexin show increased levels of gap junctional communication as detected by dye or electrical coupling (Eghbali et al 1990, Fishman et al 1990b and 1991, Zhu et al 1991 and 1992, Hennemann et al 1992, Rup et al 1993, Veenstra et al 1992). The connexin expression studies using SKHep-1 cells (Eghbali et al 1990, Fishman et al 1990, Hennemann et al 1992) are complicated by conflicting reports that these cells do (Beyer et al 1992) or do not (Hennemann et al 1992) express low levels of endogenous connexin 40. If the transfected cells express higher levels of endogenous connexin than the parental cells then the increased GJIC could be due to this and not exogenous connexin expression. The mouse (N2A) neuroblastoma cell line is reported not to express nine of the known connexins (Veenstra et al 1992). The expression of chick connexins 42, 43, 45 or 56 in the N2A cell line increases the frequency of electrical communication from 0% of parental cell pairs to 30-60% of connexin transfected cell pairs (Rup et al 1993, Veenstra et al 1992). The connexins expressed in the N2A cell line cause

different average channel conductances. Interestingly, chick connexins 42, 43 and 45 are all found in embryonic chick heart (Beyer 1990), which may account for the multiple channel conductances recorded in cells isolated from this tissue (Chen and DeHaan 1992). The dye coupling ability of N2A cells transfected with connexin has only been determined for cells transfected with connexin 56. Of 51 microinjections 25% were positive for dye transfer, typically to only one other cell (Rup et al 1993). The results while significant are not dramatic, as in a well coupled cell line such as BRL (Buffalo Rat Liver) dye spreads to approximately 20-40 other cells in 94% of microinjections (Pitts and Kam 1985).

An increased level of connexin 43 expression in C6 glioma cells is reported to affect not only GJIC but also the growth rate of transfected cells and co-cultured parental cells (Zhu et al 1991 and 1992). However, direct cell-cell contact is unnecessary for this growth inhibition as conditioned media from connexin transfectants also reduces proliferation. The growth inhibition is therefore due to soluble growth-regulatory factors and not GJIC. It was observed that for three connexin 43 transfected C6 clones, the clone with the lowest connexin 43 RNA levels produced conditioned media that has the least pronounced affect on cell proliferation. However, whether the conditioned media from a number of clones expressing low levels of connexin inhibits growth or not needs to be established before any correlation can be made in this system between connexin 43 expression and the production of soluble growth-regulatory factors. As the increased production of these factors may be a fortuitous consequence of cloning.

Connexins have also been expressed in the *Xenopus* oocyte system (Werner 1985, Swenson et al 1989). When oocytes, denuded of their covering layers are manually paired, a low endogenous gap junctional conductance is observed (Werner 1985). Two connexins are known to be expressed in the *Xenopus* oocyte, *Xenopus* connexin 38 which is a group II connexin unique to *Xenopus* and *Xenopus* connexin

43 which has high homology to rodent connexin 43 (Ebihara et al 1989, Gimilch et al 1990). The injection of antisense *Xenopus* connexin 38 oligonucleotides (but not antisense *Xenopus* connexin 43 oligonucleotides or random oligonucleotides) into *Xenopus* oocytes prior to pairing eliminates the background low endogenous conductance (Willecke et al 1991), presumably due to the breakdown of connexin 38 mRNA causing lower levels of endogenous *Xenopus* connexin 38 protein. Interestingly, when an oocyte microinjected with connexin 43 or connexin 37 mRNA is manually paired with a water injected oocyte (connexin 43/water pairs or connexin 37/water pairs), the electrical coupling is 100 fold higher than that observed between water/water pairs (Swenson et al 1989, Bruzzone et al 1993). This electrical coupling between connexin 43/water and connexin 37/water pairs can be greatly reduced if antisense connexin 38 oligonucleotides are co-injected with the connexin mRNA into the oocytes (Bruzzone et al 1993). Electrical coupling is not observed between connexin 32/water or connexin 40/water pairs (Swenson et al 1989, Bruzzone et al 1993). Suggesting that connexins 43 and 37 but not connexins 32 or 40 are able to interact with endogenous *Xenopus* connexin 38.

The ability to suppress background endogenous conductance by co-injection of antisense connexin 38 oligonucleotides has enabled a number groups to investigate whether rodent connexins can induce conductance in the *Xenopus* expression system when co-injected oocytes are paired either homologously (eg. connexin 43/43 pairs) or heterologously (eg. connexin 43/37 pairs). The homologous pairing of oocytes injected with rodent connexin 26, 32, 37, 40 or 43 mRNA induces conductances which are 100-500 times greater than the suppressed endogenous conductance (Barrio et al 1991, Willecke et al 1991, Hennemann et al 1992, Bruzzone et al 1993). The exception to this is connexin 46 which causes individual oocytes to swell and eventually burst, as if open water permeable channels are formed in the oocyte plasma membrane (Paul et al 1991).

The heterologous pairing of oocytes injected with different rat connexin mRNAs reveals a specificity of gap junction formation. Connexin 37/40 and connexin 37/43 pairs show increased electrical coupling whereas connexin 40/43 pairs do not (Bruzzone et al 1993). Connexin 37 is therefore more promiscuous than connexins 40 and 43 with respect to the ability to interact with other group II connexins and cause gap junction formation. The expression by adjacent cells of incompatible connexins may be a mechanism by which communication compartments are established in other systems and *in vivo*. However, the process by which connexins recognise and discriminate in their interactions with other connexins in adjacent cells is uncertain. The putative extracellular domains of connexins 37, 40 and 43 are likely to be the regions that physically interact and they are highly conserved, which could account for the promiscuity of connexin 37 (with respect to its ability to interact with both connexins 40 and 43) but not the incompatibility of connexins 40 and 43. The interaction of connexins could also be regulated by post-translational modifications, such as phosphorylation. The ability of *Xenopus* oocytes to carry out these modifications may therefore be limiting and this raises the possibility that connexin interactions in other systems are different.

The sensitivity of electrical coupling to applied transjunctional voltage differs for each connexin. When oocytes are paired that express different connexins the voltage dependence can be asymmetrical (Swenson et al 1989, Werner et al 1989, Barrio et al 1991, Bruzzone et al 1993). Each connexin therefore confers a different voltage sensitivity on electrical coupling.

Evidence for the role of connexins in GJIC also comes from studies showing that the microinjection of antisense rat connexin 32 RNA or anticonnexin 32 antibodies into early mouse embryos interferes with gap junctional communication and embryo compaction (Lee et al 1987, Bevilacqua et al 1989). GJIC can also be inhibited by anticonnexin antibodies either microinjected into cells (Warner et al

1984, Hertzberg et al 1985, Lee et al 1987, Traub et al 1989, Yancey et al 1989, Allen et al 1990), loaded into permeabilized cells (Fraser et al 1987) or incubated with cells dissociated with EDTA (Meyer et al 1992). The microinjection of anti-connexin antiserum into a cell of a *Xenopus* embryo at the 8-cell stage disrupts GJIC and often gives rise to tadpoles that lack eye structures normally derived from the microinjected cell (Warner et al 1984), which has led to the suggestion that GJIC may play a role in neural induction (Warner 1985). However, the disruption of GJIC is unlikely to be because of anti-connexin 32 activity because *Xenopus* connexin 32 RNA is not expressed at this embryonic stage (Gimlich et al 1990). The antiserum used by Warner et al 1984 and also by Fraser et al 1987 was raised against purified connexin 32 protein and it detects both connexin 32 and a 54kD protein (the β chain of the extracellular matrix protein fibronectin). The disruption of GJIC may therefore be due to activities other than that against connexin 32. In addition, the anti-connexin antiserum used by Hertzberg et al 1985, although affinity purified contains activity to ductin.

Well characterised, affinity purified polyclonal antibodies raised against either mouse connexin 26 or 32 protein block GJIC in almost all microinjected primary hepatocytes. The controls, microinjected rabbit preimmune IgG and monoclonal antidesmoplakin I and II antibodies have no effect on communication (Traub et al 1989), suggesting that the inhibition of dye coupling is not due to a nonspecific event of antibody binding to membrane proteins. Interestingly, the negative absorption of affinity purified polyclonal anticonnexin 26 antibodies with connexin 32 protein (to remove antibodies that crossreact) reduces the effectiveness of the polyclonal anticonnexin 26 antibodies to completely block GJIC, weak dye transfer is now seen in 60% of injections (Traub et al 1989). This partial blockage of communication suggests that both connexins contribute to gap junction function and that connexin 32 can independently maintain some GJIC in the presence of specific anti-connexin

26 antibodies. Alternatively the negative absorption of anti-connexin 26 antibodies may have decreased the activity of the antiserum to connexin 26 and thus directly reduced its ability to block dye coupling.

Microinjection of *mec*$^{+}$ neonatal cardiac myocytes with an affinity purified anti-connexin 43 amino terminus peptide antiserum or an affinity purified anti-connexin 43 protein antiserum blocks dye transfer whereas preimmune serum or antibodies to other membrane proteins do not, suggesting that connexin 43 plays an important role in dye coupling (Yancey et al 1989). The results are also consistent with the topological model of connexin 43, where the N-terminus is cytoplasmic and therefore accessible to the microinjected site specific antibody. The affinity purified anti-connexin 43 protein antiserum is reported to recognise epitopes in the cytoplasmic loop and the C-terminus but not the N-terminus, which suggests that either several domains of the protein regulate gap junction channel permeability or that binding of an antibody to a cytoplasmic domain is sufficient to induce confirmational change that will block dye transfer.

Affinity purified rabbit polyclonal antibodies raised against peptides specific to the extracellular domains of connexins 32 or 43 can prevent gap junction formation and adherens junction formation when added to the media of reaggregating Novikoff hepatoma cells which have been dissociated in EDTA (Meyer et al 1992). The functional interactions of the extracellular domains of connexin are therefore necessary for gap junction formation. The ability of antibodies raised against the extracellular domains of connexin 32 to inhibit gap junction formation presumably reflects the high conservation of this region as Novikoff hepatoma cells express connexin 43 not connexin 32. Interestingly antibodies to N-cadherin but not the mouse major histocompatibility complex also disrupted gap junction formation, suggesting that cell-cell adhesion is important in the gap junction formation process (section 1.6.1).

There is therefore a wealth of data showing that connexins play an important role in gap junction function: connexins are found in gap junction preparations (but see below); antibodies to connexins localise to isolated gap junction plaques and gap junction regions in tissue sections, they can also rapidly inhibit GJIC when microinjected into cells or prevent gap junction formation when added to the media of reaggregating cells; the transfection of mammalian *mec*$^-$ cell lines with constructs for the expression of connexins can increase GJIC, as does the microinjection of *Xenopus* oocytes with connexin RNA. These observations are widely interpreted as showing that connexins are the sole components of the connexon and therefore the gap junctional channel protein (for reviews see Dermietzel et al 1990, Bennett et al 1991). However, this belief can be criticised on a number of grounds:

a) Connexins or related proteins have not been found in arthropods, although gap junction structures can readily be isolated (Finbow et al 1984, Berdan and Gilula 1988, Ryerse 1989, Sikerwar et al 1991, Holzenburg et al 1993).

b) Connexon dimensions and structure are highly conserved between rodent liver (Makowski et al 1977, Unwin and Zampighi 1980) and rodent heart (Yeager and Gilula 1992) despite the large differences in molecular weight of the putative gap junction proteins, connexins 26 and 32 from liver and connexin 43 from heart. This discrepancy has been explained by assuming that the cytoplasmic C-terminal domain, which accounts for much of the variation in molecular weight is disordered and therefore undetectable by the imaging techniques used (Sosinsky et al 1990), although the cytoplasmic domain of the acetylcholine receptor can readily be visualised (Unwin 1993).

c) The gross appearance of negative stained heart gap junctions is unaltered by protease treatment, even though this removes 78kD of protein from each connexon (Yeager and Gilula 1992).

d) Gap junctions which lack connexins can be isolated from mouse liver by triton extraction and this has led to the controversial proposal that there are two gap junction-like structures, with similar morphologies formed from different proteins (Finbow and Meagher 1992).

These observations introduce some uncertainties into the accepted view that the gap junctional channel protein is made of connexin. The evidence that ductin and MIP26 are gap junctional channel proteins is considered in sections 1.5.3 and 1.5.4 respectively.

1.5.3 Ductin.

The 16kD protein observed in the SDS-PAGE profiles of gap junction preparations from rodent liver and heart is also found in preparations from a variety of other mammalian tissues and cell lines (Finbow et al 1983, Buultjens 1988), chicken and *Xenopus* liver (Buultjens 1988) and crustacean hepatopancreas (Finbow 1984, Holzenburg 1993). Gap junction preparations from the hepatopancreas of the crustacean *Nephrops* yield predominantly 16kD protein for which the amino acid sequence has been determined and the cDNA cloned (Finbow et al 1992). It is not related to connexins 32 or 43, representatives of the two major connexin classes, but instead has very high homology to the ubiquitous 16kD proteolipid subunit of the vacuolar H^+-ATPase (V-ATPase; Mandel et al 1988, Dermietzel et al 1989, Nelson 1992), to a 16kD component of the "mediatophore" complex present in the *Torpedo* electric organ (Birman et al 1990) which transports acetyl choline in response to an influx of calcium (Israel et al 1986) and to two other proteins identified in yeast (Sigh et al 1988, Apperson et al 1990).

The V-ATPases are a class of endomembrane proton pumps, found in all eukaryotic cells which acidify various endomembrane compartments including vacuoles (Nelson and Taiz 1989, Nelson 1992). The 16kD proteolipid is the principle

subunit of the V-ATPase proton channel and has sequence homology to the 8kD subunit c protein of the F_0 complex of the eubacterial F_1F_0ATP synthase (Mandel et al 1988, Nelson and Nelson 1990). In the yeast *S. cerevisiae*, (which does not form gap junctions) the inactivation of the endogenous VAM3 gene encoding the yeast 16kD proteolipid abolishes V-ATPase activity and prevents growth at pH 7.5 (Nelson and Nelson 1990). Growth at pH 7.5 can be restored by expression of the yeast VAM3 gene (Umemoto et al 1991, Noumi et al 1991) or the *Nephrops* gap junctional 16kD protein cDNA (Finbow et al 1992). The ability of the *Nephrops* 16kD protein to substitute for the yeast V-ATPase 16kD proteolipid, the presence of a 16kD protein in the "mediatophore" of *Torpedo* and the existence of other homologous proteins in yeast has led Holenzburg et al (1993) to propose that the 16kD proteins are a family of multifunctional channel proteins, which they have named "ductins", to signify a role in membrane transport.

The evidence that ductin is a gap junctional channel protein is similar but less extensive than that presented for connexins, it includes antibody localisation and blocking, structural and expression studies.

The primary sequence of ductin is highly conserved and hydropathicity plots suggest that there are four membrane spanning hydrophobic regions, each of 25-30 residues in length which are probably α-helical in structure (figure 1; Finbow et al 1992). The predicted α-helical content of ductin is approximately 60% and this is similar to the value obtained from the FTIR spectroscopy of isolated Nephrops gap junctions, which contain predominantly ductin (Holenzburg et al 1993). The predicted structure of ductin agrees well with the model of the connexon described in section 1.2.3, and the protease resistance of ductin is consistent with it being a hydrophobic transmembrane protein (Finbow et al 1983, Finbow et al 1992).

Ductin is found in gap junction preparations from a wide variety of tissues and species (Finbow et al 1983, Buultjens et al 1988). In preparations from the

hepatopancreas of *Nephrops norvegicus* or the closely related *Homarus americanus* hepatopancreas ductin predominates (Holzenburg et al 1993). The connexins or related proteins have not been identified in arthropods, although antisera raised against mammalian gap junctions recognise proteins in a variety of vertebrates, molluscs and coelenterates (Hertzberg and Skibbens 1984, Berdan 1987). Gap junction structures isolated from rodent liver by triton X-100 extraction are also found to contain predominantly ductin. (Finbow and Meagher 1992). However, ductin is present in vacuolar membranes as the channel component of the V-ATPase and it is therefore possible that the isolated gap junction structures, which contain predominantly ductin are derived from vacuolar membranes and not "true" gap junctions in the plasma membrane (Dermietzel et al 1989). This seems improbable as the isolated gap junction structure, plaques of hexagonally packed particles in paired membranes is distinctive and unlikely to arise spontaneously during isolation from the membrane unit of the V-ATPase. The gap junction isolation procedure would have to disrupt the detergent stable complex between the membrane and catalytic units of the V-ATPase (Moriyama and Nelson 1989, Parry et al 1989) and cause the individual membrane units to aggregate into plaques of hexagonally packed particles, that subsequently pair with other single membrane plaques of the same size and shape to give the characteristic gap junction structure. Consistent with this is the observation that gap junction structures cannot be isolated from yeast which have functional V-ATPases but do not form gap junctions (Dr P.Jones and Prof J.B.C.Findlay personal communication).

Antisera have been raised against chicken and *Nephrops* gap junctions that recognise a 16kD-18kD protein and an unrelated 54kD glycoprotein protein on immunoblots of gap junction preparations from a variety of mammalian tissues, chicken and *Xenopus* liver and crustacean hepatopancreas (Buultjens et al 1988, Finbow et al 1988, Leitch and Finbow 1990, Holenzburg et al 1993). The complete

antisera against *Nephrops* gap junctions (AN1 and AN2) and an antiserum affinity purified against *Nephrops* ductin (a.p.AN2, which only has activity to ductin) also specifically recognise a 29kD protein from *Drosophila* ovaries and bind to *Drosophila* follicle cell membranes in a punctate pattern (Bohrmann 1993). The 29kD protein of *Drosophila* and the 18kD *Nephrops* ductin therefore share epitopes. The *Drosophila* ductin cDNA has also been cloned, it is homologous to the *Nephrops* ductin cDNA, and the predicted molecular weight of the *Drosophila* ductin protein is 16kD. Interestingly, the native *Nephrops* ductin is a 18kD protein and its dimer a 29kD, suggesting that the 29kD protein of *Drosophila* could be a more stable ductin dimer. The complete antisera and a.p.AN2 bind to isolated *Nephrops* gap junctions and gap junction regions in plasma membrane preparations. However, only the complete antisera localize to regions of close plasma membrane apposition between epithelial cells of the *Nephrops* hepatopancreas. The complete antisera presumably contain antibodies to epitopes that recognise ductin in tissue sections but are lost during affinity purification with an SDS denatured protein (Leitch and Finbow 1990).

The complete antisera raised against either chicken or *Nephrops* gap junctions but not preimmune serum when microinjected into mammalian cells in tissue culture inhibits GJIC (Finbow et al 1988, Finbow et al 1993). The complete antisera against *Nephrops* gap junctions (AN1 or AN2) can also inhibit dye coupling in *Patella vulgata* (mollusc) embryos and *Drosophila* ovarian follicles (Serras et al 1988, Bohrmann 1993). However, the inhibition of dye coupling by these antisera is not necessarily due to activity against ductin as they also recognise a 54kD glycoprotein in gap junction preparations and other proteins in total preparations of *Drosophila* ovaries (Buultjens et al 1988, Bohrmann 1993). The affinity purified antiserum (a.p.AN2) appears to specifically recognise a 29kD protein in *Drosophila* ovaries and when microinjected into oocytes it also inhibits dye transfer to the neighbouring follicle cells (Bohrmann 1993). This is consistent with the inhibitory

action of the complete antisera being due to activity against ductin or the immunologically related 29kD protein. The microinjected antisera could affect GJIC directly by binding to gap junctions, or indirectly by interfering with V-ATPase function and thereby causing cytoplasmic acidification, which can block gap junctional communication in other cell types (Turin and Warner 1978, Spray et al 1981). However, bafilomycin which inhibits all detectable V-ATPase function does not affect dye coupling in tissue cultured mammalian cells (Finbow et al 1993). The inhibition of GJIC by these antisera is therefore consistent with anti-ductin antibodies directly blocking or closing gap junctional channels.

The ductin protein has also been reconstituted into lipid bilayers where it forms a channel permeable to the small molecule acetyl choline (Israel et al 1986, Birman et al 1990). Reconstitution experiments with connexins have shown that single channels are permeable to ions (Young et al 1987). However unlike the connexins, a role for ductin in gap junction function has not been demonstrated by expression in cell lines or *Xenopus* oocytes.

On the basis of its structure, co-isolation with gap junctions and the antibody localisation and blocking experiments ductin should be considered as a potential gap junctional channel protein.

1.5.4 Other putative gap junctional channel proteins.

A protein has been identified in vertebrate lens, MIP26 ("major intrinsic protein" of lens, M_r=26,000; Benedetti et al 1976, Gorin et al 1984) that is unrelated to the connexins or ductin but which has homology to other proteins with known channel activities (Baker and Saier 1990, Van Aelst et al 1991). The lens is composed of fibre cells, (which have lost their cellular organelles and contain high concentrations of lens specific proteins, the crystallins) and an anterior layer of epithelial cells at the lens surface (Goodenough 1992). The lens fibre cells are

coupled via gap junctions to themselves and the epithelial cells, which provide metabolites and ions (Scheutze and Goodenough 1982, Mathias and Rae 1985). The lens junctions are similar in morphology to gap junctions from other tissues but the lens fibre junctions lack the characteristic gap and particles imaged in freeze-fracture preparations are more loosely packed (Hertzberg et al 1982). The gel profiles of gap junction preparations from lens show a preponderance of MIP26 and this led to the hypothesis that MIP26 is the principal component of lens fibre gap junctions (Alcala et al 1975, Goodenough 1979). The immunolocalisation of MIP26 to lens fibre gap junctions has been reported by some groups (Bok et al 1982, Sas et al 1985) but not others (Paul and Goodenough 1983, Zamphigi et al 1989). The reconstitution of HPLC-purified MIP26 into planar lipid bilayers is reported to induce a channel conductance (Ehring et al 1990) and this has led to the proposal that MIP26 forms open channels in the interior of the lens, which permit intercellular communication via a small intercellular space, and also prevent water accumulation in this space through the establishment of an osmotic gradient. The lens fibre intercellular spaces are by necessity small in order to reduce light scattering and so this second function of MIP26 may be essential. Members of the connexin family and the unrelated MP20 have also been shown to localise to lens fibre junctions (Kistler et al 1985, Louis et al 1989, Paul et al 1991). The molecular nature of lens gap junctions is therefore not understood but proteins unrelated to connexins or ductin are potentially important in their formation and function.

1.5.5 Summary- the identity of the gap junctional channel protein.

The structural studies of isolated gap junctions show that the connexon is formed from six similar protein subunits. The identity of this protein is controversial, as members of the connexin family and ductin are both present in gap junction preparations from a variety of vertebrate tissues. The unrelated MIP26, which is only

expressed in vertebrate lens may also contribute to GJIC between lens fibre cells. Antibodies against either the connexins or ductin bind isolated gap junction plaques and block all detectable dye coupling when injected into cells, suggesting that both are necessary for gap junction function. The structural studies and modelling of ductin suggest that it is a transmembrane channel protein with dimensions very similar to those reported for the connexon. The connexins are also widely thought to be gap junctional channel proteins but they are much larger than ductin and harder to fit into the accepted model of the connexon (Finbow and Pitts 1993). On the basis of stoichiometric analysis it is not thought likely that connexins and ductin are subunits of the same gap junctional channel (Finbow and Meagher 1992). There may therefore be two different types of gap junctional channel, those composed of connexins and those composed of ductins, although the data are consistent with the gap junctional channels being composed solely of ductin, with the connexins having an essential role in their formation, maintenance and regulation.

1.6 Control of gap junctional intercellular communication.

GJIC can be regulated by a range of apparently unrelated agents which include phorbol esters, oncogenes, cyclic AMP (cAMP) and retinoids (see section 1.6.4). The regulation of GJIC can be rapid, electrophysiological recordings show that a reduction in channel conductance can occur within approximately 10-20 seconds of treating horizontal cells with dopamine (McMahon et al 1989) and this could involve gating, (a reversible change in the channel permeability; section 1.6.3). Slower changes in GJIC, such as the increased dye coupling seen in cultures of C3H10T1/2 cells 12-18hrs after treatment with a stable synthetic retinoid (TTNPB at 10^{-8}M; Rogers et al 1990), or the transient downregulation of GJIC between rat liver epithelial cells which occurs 2-3hrs following TPA treatment (Asamoto et al 1991),

may involve changes in gap junction formation (section 1.6.1) or turnover (section 1.6.2).

1.6.1 Gap junction formation.

Coupling via gap junctions can occur within minutes of cells being pushed together and in the presence of protein synthesis inhibitors, suggesting that pre-existing structures in the plasma membrane interact to cause junction formation (Loewenstein 1981, Epstein et al 1977, Chow and Young 1987, Rooks et al 1992). However, whether the connexon subunits assemble prior to reaching the plasma membrane is unknown. The association of subunits of other multimeric proteins can occur in the endoplasmic reticulum (Singh et al 1990). The subunits of the connexon may therefore occur in monomers, small oligomers or hexamers in the plasma membrane prior to gap junction formation. Open unpaired connexons which cause cell lysis (due to an osmotically driven influx of water) appear to form in *Xenopus* oocytes following the expression of connexin 46 (Paul et al 1991), suggesting that the connexon subunits can assemble prior to gap junction formation. If the connexon subunits occur as hexamers in the plasma membrane prior to gap junction formation they must normally be closed, in order to prevent this influx of water and the leakage of small cytoplasmic molecules. The open probability of connexons may increase following the association of the extracellular domains of connexons in apposed plasma membranes. The formation of closed hexamers followed by opening upon association would also overcome the problem of removing a central annulus of phospholipid from the assembled hexamer.

Gap junction formation requires adjacent cell plasma membranes to be in close proximity as the connexon subunits only protrude 1-2nm into the extracellular space. Therefore cell-cell adhesion, increasing the frequency of close membrane apposition is likely to proceed connexon alignment and the formation of functional

gap junctions (figure 2). The interactions of cell adhesion molecules have been proposed to be early recognition events between cells that are required for the formation of a number of specialised intercellular junctions (Edelman 1988), including the adherans junction (Volk and Geiger 1986), the tight junction (Gumbiner 1987, Fleming et al 1989) and the gap junction (Meyer et al 1992) as well as for the fusion of myoblasts during myogenesis (Knudson et al 1990, Mege et al 1992). Cell adhesion molecules have been classified into several major groups, the selectins (Bevilacqua et al 1991, Cummings and Smith 1992), the integrins (Buck and Horwitz 1987, Hynes 1992a), the immunoglobulin (Ig) superfamily (Cunningham et al 1987, Williams and Berkley 1988) and the cadherin superfamily (Geiger and Ayalon 1992, Hynes 1992b), and their role in gap junction formation is considered below.

1.6.1.1 The selectins.

The selectins (also called LECAMs) bind to specific carbohydrate groups on adjacent cells via their lectin domain in a Ca^{++} dependant manner. They also have an epidermal growth factor-like domain and consensus repeats for complement binding proteins, which have unknown functions (Bevilacqua et al 1991, Cummings and Smith 1992). The three selectins so far identified are expressed by haematopoetic cells and/or endothelial cells and are believed to mediate heterotypic interactions during lymphocyte homing and leukocyte adhesion (Watson et al 1991, von Andrian et al 1991). Their roles in other cell types, if any, have not been determined.

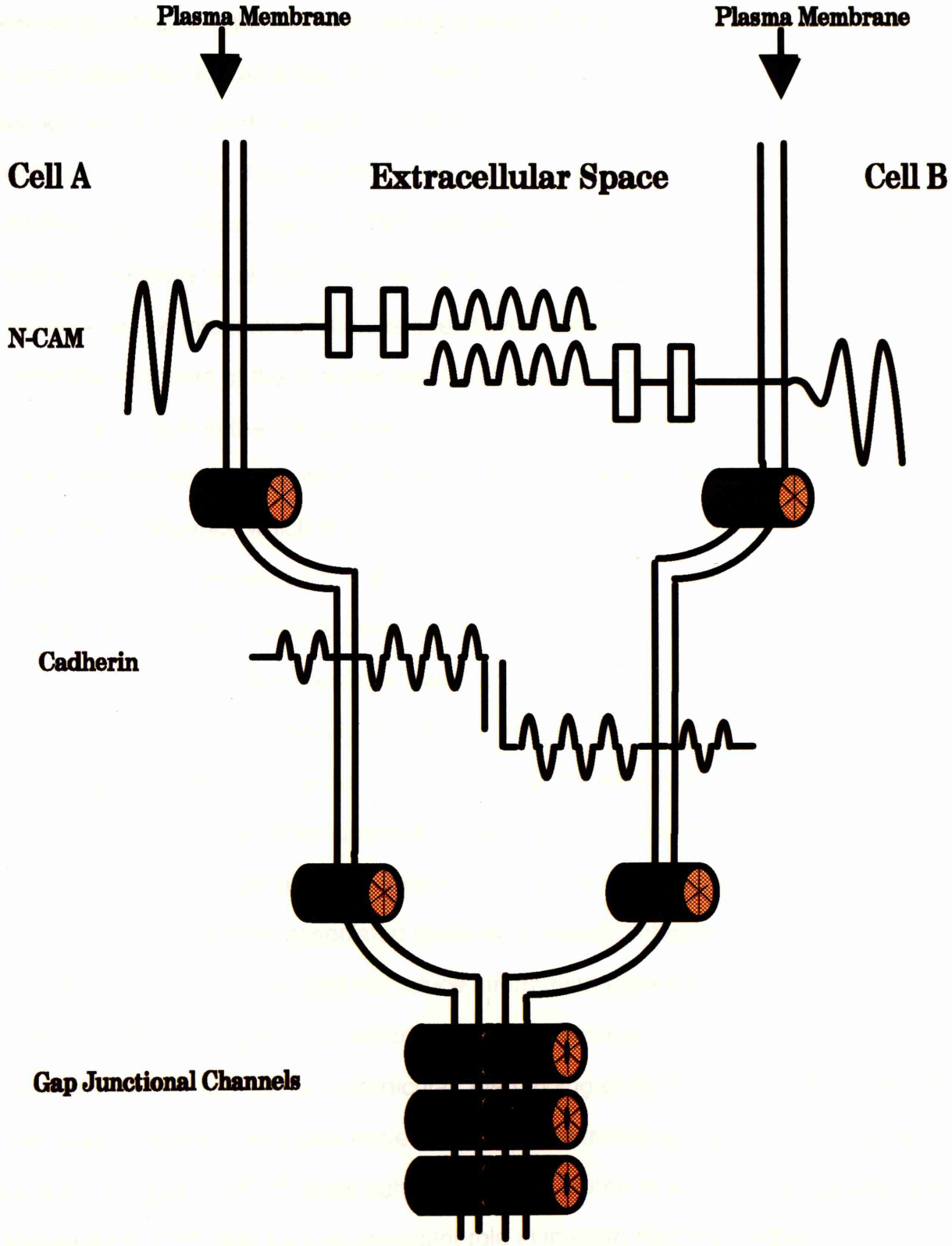
1.6.1.2 The integrins.

The integrins are found in a wide variety of cell types and are heterodimers consisting of noncovalently linked α and β subunits. They were originally divided into three sub-families, each with a common β subunit capable of associating with a

Figure 2. A schematic model of gap junction formation.

The membranes (double lines) are drawn closer together by the interactions of cell adhesion molecules. This increased frequency of close membrane apposition is likely to proceed connexon alignment and the formation of gap junctional channels joining the cytoplasms of cell A and B.

Figure 2.


Plasma Membrane
Plasma Membrane
Cell A
Extracellular Space
Cell B
N-CAM
Cadherin
Gap Junctional Channels

specific subset of α subunits (Hynes 1987). However, certain α subunits are now known to combine with more than one β subunit (Sonnenberg et al 1990) and this complicates the original groupings. Currently, about 20 known integrin heterodimers are known, made up of various pairings of 8 β and 13 α subunits (Albelda and Buck 1990, Hynes 1992a). The majority of integrins function as cell-substratum receptors, binding to extracellular matrix (ECM) molecules including fibronectin, laminin and tenascin (Argaves et al 1987, Ghelsen et al 1988, Bourdon and Ruoslahti 1989). However, a few integrins function as specific cell-cell adhesion receptors, binding to particular members of the Ig super family expressed on the surface of certain cell types, particularly in the immune system (Springer 1990). The binding of several but not all integrin receptors is mediated in part by the tripeptide Arg-Gly-Asp (RGD) recognition sequence which is found in a number of ECM and Ig family molecules (Ruoslahti and Pierbacher 1987, Anderson 1990, Kishimoto et al 1989). Additional binding sites, such as those located on integrin $\alpha_{11b}B_3$ (also termed glycoprotein 11b-111a complex; which mediates platlet aggregation) probably regulate which RGD-containing proteins bind to a particular integrin (Phillips et al 1991).

Several integrins localise to focal contacts, areas where the cell membrane is closely apposed to the ECM substrate and where actin bundles terminate in close association with vinculin and talin. Integrin interaction with the cytoskeleton appears to be through these actin associated proteins. However, integrins do not merely have an adhesive role, they can also transduce information from the outside to the inside of the cell, the consequence of which is often the tyrosine phosphorylation of cellular components (Hynes 1992a). In particular, the binding of ligand to integrin receptor in both platlets and NIH 3T3 cells correlates with an increase in the intrinsic tyrosine kinase activity of $p125^{FAK}$ (focal adhesion kinase; Lipfert et al 1992), suggesting that activated $p125^{FAK}$ may play an important role in integrin mediated signal transduction (Zachary and Rozengurt 1992).

The growth and differentiation of connective tissue and nervous system cells can be affected by their substrates, through integrins (Ruoslahti and Pierbacher 1987) and neural specific cell-cell adhesion molecules (discussed below; Doherty and Walsh 1989). For example, the outgrowth of retinal ganglion cell axons over glial cells or laminin is inhibited by antibodies against integrins (Cohen et al 1987, Neugebauer et al 1988). However, neither integrin mediated adhesion or signal transduction have been reported to directly affect GJIC, nor have antibodies against integrins been shown to block GJIC. In contrast, antibodies against the neural cell adhesion molecule (N-CAM; Keane et al 1988) or the cadherins (Mege et al 1988, Meyer et al 1992) can inhibit GJIC.

1.6.1.3 The immunoglobulin (Ig) super family.

The proteins belonging to Ig superfamily have Ig domains, a sequence of approximately 100 amino acids that contains a conserved disulphide bond which stabilises the interactions between other conserved amino acids within the sequence to form two β sheets folded together around a hydrophobic interior. These domains, first identified in immunoglobulin molecules are found in a variety of proteins which have adhesive or binding functions (Cunningham et al 1987, Williams and Barkley 1988). Typically the members of this family also have fibronectin type III repeats, which where first detected in the adhesive ECM protein fibronectin and also occur in cytokine receptors (Bazan 1990). The binding and adhesion functions of the Ig superfamily are Ca^{++} independent.

The neural cell adhesion molecule (N-CAM) is one of the best characterised members of the Ig superfamily. It is a polymorphic glycoprotein which has both Ig domains and fibronectin repeats. The most distal Ig domains are thought to determine binding specificity (Rutishauser 1986, Cunningham et al 1987), while the functions of the additional Ig domains and fibronectin type III repeats are unknown.

N-CAM is generally considered to be a homophyllic cell adhesion molecule (Cunningham et al 1987), although there is also evidence that it binds to heparin sulphate proteoglycans (Reyes et al 1990), which are widely distributed in the ECM and on cell surfaces. The alternative splicing of N-CAM RNA generates multiple isoforms (Santoni et al 1989), those isoforms with cytoplasmic domains may anchor to the cytoskeleton, although this has not been convincingly demonstrated. Others lacking cytoplasmic domains are attached to the membrane via glycosylphophatidyl tails or secreted and deposited into the ECM (Cole and Glaser 1986). Anchorage to the cytoskeleton is presumably necessary for N-CAM mediated cell-cell adhesion, while unanchored isoforms could function as cell-cell recognition receptors.

N-CAM is expressed by a variety of cells during embryonic development but in the adult it is largely restricted to neural cells (Edelman 1985, Rutishauser 1986). N-CAM contributes to the adhesion of neurons to a variety of cell types and can promote or support the outgrowth *in vitro* of neurites over monolayers of muscle cells, astrocytes or 3T3 cells transfected with N-CAM cDNA (Bixby et al 1987, Neubebauer et al 1988, Doherty et al 1989). This neurite outgrowth could be directly due to increased cell adhesion, although polysialic acid, a negative regulator of N-CAM mediated cell adhesion also promotes outgrowth (Rutishauser et al 1988, Doherty et al 1990). In addition, the N-CAM (and also N-cadherin) induced neurite outgrowth depends on the activity of a pertussis-toxin-sensitive G protein and the consequent opening of both L- and N-type neuronal calcium channels (Doherty et al 1991, Saffell et al 1992). This suggests that the function of these cell adhesion molecules in promoting outgrowth is not simply to induce cell-cell adhesion *per se* but also to activate a transmembrane signalling pathway.

Antibodies against N-CAM block neurite regeneration (Doherty and Walsh 1989) and decrease the fusion rate of myoblasts in culture (Knudsen et al 1990). In addition, chronic treatment of chick embryo neuroectoderm *in vitro* with Fab

fragments against N-CAM can interfere with the development of GJIC, as measured by dye transfer to second-order neighbours (Keane et al 1988), suggesting that N-CAM mediated cell adhesion or signal transduction contributes to gap junction formation.

1.6.1.4 The cadherin superfamily.

The cadherin superfamily are Ca^{++} dependant cell adhesion molecules which promote adhesion between cells, typically through homotypic binding interactions. They are thought to play a role in embryonic morphogenesis, in the maintenance of tissue morphology and in normal growth behaviour (Takeichi 1991, Magee and Buxton 1991, Geiger and Ayalon 1992). The original cadherin family of epithelial (E), neuronal (N) and placental (P) cadherins (Takeichi 1988 and 1990) has recently been extended to include brain (B) cadherin (Napolitano et al 1991), muscle (M) cadherin (Donalies et al 1991), retinal (R) cadherin (Inuzuka et al 1991), truncated (T) cadherin (Ranscht and Bonner 1991), EP cadherin (Ginsberg et al 1991) and the desmosomal glycoproteins, desmoglein and desmocollins (Buxton and Magee 1992, Garrod 1993). The cDNAs of eight, as yet uncharacterized cadherins have also been isolated from nervous tissue (Suzuki et al 1991). The cadherins are highly homologous and have similar sub-domains which include, a presequence that is cleaved upon processing, a large extracellular domain that has five homologous repeats, a single hydrophobic transmembrane region and a cytoplasmic domain (Geiger and Ayalon 1992). The desmoglein and desmocollin proteins have a similar overall structure which includes conserved extracellular sequences related to the cadherins, but their cytoplasmic domain has no homology to the highly conserved cytoplasmic domain of the cadherins. This may be because the desmosomal proteins associate via cytoplasmic plaques with intermediate filaments whereas the cadherins are anchored to actin filaments (Magee and Buxton 1991).

Cadherin mediated adhesion appears to be homophilic where each cadherin binds to the same cadherin on an adjacent cell, as L-cells expressing E-, N- or P-cadherins when mixed *in vitro* sort out (Nose et al 1988, Miyantani et al 1989), although segregation can also occur between cells expressing different amounts of the same type of cadherin (Friedlander et al 1989). In addition, the binding properties of chimeric E- and P-cadherin molecules suggest that the extracellular NH_2-terminal 113 amino-acid region confers binding specificity (Nose et al 1990, Takeichi 1990). Within this region there is a highly conserved amino acid sequence His-Ala-Val (HAV; Nose et al 1990, Geiger and Ayalon 1992) which may be involved in cadherin interaction as synthetic peptides that contain this sequence can block cadherin mediated adhesion (Blashuk et al 1990, Mege et al 1992) and the HAV flanking sequences are thought to confer binding specificity (Takeichi 1990). Interestingly chicken R- and N-cadherin have similar flanking sequences and may interact heterologously.

Localisation studies show that cadherins accumulate at points of cell-cell contact and that they are integral components of the adherans junction (Volk and Geiger 1986, Hirano et al 1987). When cadherins accumulate at cell boundaries they become resistant to extraction with non-ionic detergents, presumably due to interaction with the cytoskeleton. This association appears to be a prerequisite for cadherin mediated cell adhesion as L-cells expressing mutant E-cadherin, lacking the cytoplasmic domain fail to aggregate (Nagafuchi and Takeichi 1988, Ozawa et al 1990). Surprisingly T-cadherin which lacks a cytoplasmic domain is reported to induce Ca^{++}-dependant homophyllic cell adhesion when expressed in CHO cells (Vestal and Ranscht 1992), although the molecular mechanism of this adhesion is not understood. The normal association of cadherins with the actin filaments of the cytoskeleton is thought to occur via the cytoplasmic cadherin binding proteins,

catenins or a 220kD (ankryin or β-spectrin like) protein (Tsukita et al 1992). These cytoplasmic proteins may therefore regulate cadherin function.

The catenins are cytoplasmic proteins that co-precipitate with cadherin (Ozawa et al 1989). α-catenin has homology to vinculin, to which it may bind and vinculin in turn binds α-actinin which associates with actin filaments (Tsukita et al 1992). When neural α-catenin is transfected into cells of the poorly aggregating lung carcinoma cell line PC9, (which expresses E-cadherin and β-catenin but lacks α-catenin) this causes an increase in cell-cell adhesion (Hirano et al 1992), which is consistent with the hypothesis that α-catenin is necessary for cadherin function. β-catenin, has also recently been cloned and is homologous to plakoglobin, the major constituent of the desmosomal plaque (Mcrea and Gumbiner 1991), but its role in adherens junctions is uncertain. γ-catenin has not been cloned, it is similar in size to zyxin which also associates with adherens junctions (Crawford and Berkerle 1991), although a recent study suggests that it also has homology to plakoglobin, or an 83kD plakoglobin like protein (Knudson and Wheelock 1992).

Cadherins have also been proposed to interact with actin filaments independently of α-catenin and vinculin via a 220kD protein that may associate with α and β spectrin. These interactions have been less well characterised but may be especially important in fibroblasts, where the association between cadherins and vinculin is less pronounced (Itoh et al 1991).

Cadherin mediated interactions appear to not only affect adherens junction formation, (of which they are a major component) but also the distribution of Na^+,K^+-ATPase (McNeill et al 1990), the integrity of tight junctions (Gumbiner 1987, Gumbiner et al 1988) and the formation of gap junctions (Mege et al 1988, Matsuzaki et al 1990, Jongen et al 1991, Meyer et al 1992).

Meyer et al (1992) have reported that antibody Fab fragments against either N-cadherin or the extracellular domains of the connexins 32 and 43 when added to

the media of reaggregating Novicoff hepatoma cells inhibit adherens and gap junction formation (as determined by EM and dye transfer observations). This inhibition appears specific, as Fab fragments against the major histocompatibility complex, which also bind to the Novicoff cell surface fail to inhibit junction formation. The anti-cadherin and anti-connexin Fab fragments may prevent cell-cell recognition/adhesion and therefore the close membrane apposition associated with these junctions. Evidence for the role of cadherins in gap junction formation has also come from expression studies.

The cell lines S180 (mouse tumorigenic cell line of undefined tissue origin) and P3/22 (mouse epidermal cell line) are poorly coupled via gap junctions. The expression of E-cadherin in these cell types can increase the number of recognisable gap junctions (as determined by EM) and the level of dye coupling (Mege et al 1988, Jongen et al 1991). N-cadherin has also been expressed in S180 cells with similar effects (Matsuzaki et al 1990). The frequency of dye coupling in these cadherin transfected cells was also found to be Ca^{++} dependant and this is consistent with cadherin mediated cell-cell interaction playing an important role in gap junction formation.

Musil et al (1990b) have reported that the poorly coupled, untransfected S180 cells express two connexin 43 protein species, predominantly a non-phosphorylated 42kD species (cx43-NP) and also a slower migrating serine phosphorylated (~44kD) species (cx43-P_1). Whereas the better coupled, E-cadherin expressing S180 cells express more of the cx43-P_1 form and an additional heavily serine phosphorylated species cx43-P_2 (~46kD). This post-translational phosphorylation of connexin 43 appears to be a general phenomenon of communication competent cell types (Musil et al 1990b, Crow et al 1990, Kadle et al 1991, Lau et al 1991, Meyer et al 1992) and this suggests that phosphorylation is not simply fortuitous but that it plays a role in gap junction formation or regulation. Phosphorylation can affect a protein's metabolic

stability, intracellular transport or activity (Yarden and Ullrich 1988, Sibley et al 1987, Cooper and King 1986). However, the metabolic stability of connexin 43 appears unaffected by increased serine phosphorylation (Musil et al 1990b), the cx43-NP form is transported to the plasma membrane and when communication competent NRK cells are uncoupled by heptanol or cytoplasmic acidification there is no associated loss of the cx43-P_2 form, suggesting that phosphorylation does not directly affect channel opening or closing (Musil and Goodenough 1991). The phosphorylation of connexin 43 to the P_2 form may be important for gap junction formation or maintenance as cx43-P_2 is triton insoluble and accumulates in gap junctional plaques (Musil and Goodenough 1991). The molecular mechanism by which E-cadherin affects connexin 43 phosphorylation is however unknown.

1.6.1.5 Summary- gap junction formation.

Gap junction formation is not only dependant on the expression of ductin and/or the connexins, it is also affected by cell-cell interactions mediated by N-CAM, E-cadherin and N-cadherin. These molecules may influence gap junction formation by increasing the frequency of close membrane apposition and the interaction of connexons in apposing membranes, as results quoted by Mege et al (1988), using wheat germ agglutinin to link S180 cells suggest that cell adhesion *per se* is sufficient to increase the frequency of gap junction formation. However, Doherty et al 1991 have reported that cell-cell interaction between PC12 cells and 3T3 cells transfected with either N-CAM or N-cadherin (but not control untransfected 3T3 cells), activates a pertussis toxin sensitive neuronal Ca^{++} channel and thereby causes signal transduction. Therefore cell-cell interactions in other cell types may cause signal transduction, which could influence gap junction formation.

The observation that GJIC can be increased by the expression of E- or N-cadherin shows that cells may be poor communicators not because of aberrant

expression of the gap junctional channel protein but because they lack cell adhesion molecules. The aberrant expression of cell adhesion molecules, which is proposed to occur during tumorigenesis and metastisis (Fearon and Vogelstein 1990, Vleminckx et al 1991) may therefore influence not only cell-cell adhesion but also GJIC (Mesnil and Yamasaki 1993).

The differential expression of cell adhesion molecules by communication competent cells, may also cause a specificity of gap junction formation, which leads to the establishment of communication compartments (section 1.7).

1.6.2 Turnover.

In cases where uncoupling and recoupling are slow the turnover of gap junctions may also regulate GJIC. The apparent half life of connexins 26 and 32 in primary hepatocytes (Traub et al 1989) and of connexin 43 in various cell cultures (Musil et al 1990b, Crow et al 1990, Laird et al 1991) is only several hours, which is consistent with turnover playing an important role in the regulation of GJIC. However, the direct evidence that turnover plays an influential role in GJIC is limited. The mechanism of gap junction disappearance has been reported to occur via a process of internalization, whereby the junctions are taken in by endocytosis, and are recognised because they appear in annular form (Larsen 1985). However, it has also been observed that during metamorphosis in the insect *Manduca sexta* gap junction particles are not internalized but are dispersed over the membrane during the *in vivo* disappearance of gap junctions (Lane and Swales 1980).

1.6.3 Gating of the gap junctional channel.

Gating refers to the regulation of the permeability of gap junctional channels, it involves the switching of structurally intact channels between the open and closed conformational state. The fraction of open gap junctional channels can be estimated

from measurements of single channel conductance, the number of gap junctional particles and the measured junctional conductance. The results are variable and range from the normal state of native gap junctions is an open one allowing cell-cell communication in the crayfish septate axon (Zamphigi et al 1988), to only 1% of gap junctional channels are open at any one time in the club endings of Mauthner cell synapses (Lin and Faber 1988). In addition, the single channel electrophysiological data suggests that individual channels can flicker between open and closed states, with sub-millisecond transition times (DeHaan 1988). Gating may influence both the conductance level and open probability of individual channels.

Changes in gap junctional conductance that occur in seconds and are reversible are thought to be due to gating. A rapid inhibition of GJIC can occur in response to a number of treatments which include increasing either cytoplasmic H^+ or Ca^{++} ion concentrations, application of large transjunctional voltages, treatment with lipophylic agents such as heptanol, octanol and halothane and the microinjection of cAMP into cells (DeMello 1984, Kolb and Somogyi 1991, Bennett and Verselis 1992). However, whether these agents cause gating, or influence permeability in a less direct way is uncertain. In addition, the inhibition of GJIC shown to occur upon cytoplasmic acidification (Turin and Warner 1978, Spray et al 1981) or increasing cytoplasmic Ca^{++} ion concentrations (Rose and Loewenstein 1975) may not occur under non-pathological conditions, given the large increase in either H^+ or Ca^{++} ion concentration that is required to inhibit GJIC (Neyton and Trautmann 1986). Gap junctional channels may close in response to high intracellular calcium ion levels because such a change would only normally occur when cells are damaged. Closure of junctions would isolate dead or dying cells thus preventing the leakage of ions and small cytoplasmic molecules from surrounding normal cells through the damaged one.

1.6.4 Regulators of gap junctional intercellular communication.

The incidence and extent of GJIC can be influenced by unrelated agents which include cAMP, phorbol esters, retinoids and oncogenes. The mechanisms of actions of each of these agents is considered below.

1.6.4.1 cAMP.

The elevation of intracellular cAMP levels can up or downregulate GJIC depending on the cell type (Hax et al 1974, Radu et al 1982, Teranishi et al 1983, Piccolino et al 1984, Cole and Garfield 1986). The stimulation of embryonic mouse hepatocyte cultures with cAMP upregulates GJIC over the course of several hours, a process which is sensitive to a blocker of protein synthesis (Traub et al 1987) and similar findings have been reported with other cell types (Azarnia et al 1981). Traub et al 1987 demonstrated that embryonic hepatocytes showed increased levels of GJIC within hours of being stimulated with cAMP, and this correlated with increased expression (within three hours) and also increased phosphorylation (within 15 minutes) of connexin 32 protein. The elevation of intracellular cAMP levels has also been shown to cause increased transcription of connexin 43 mRNA in a number of cultured cell lines, which correlates with increased GJIC (Mehta et al 1992). Interestingly, in teleost horizontal cells dopamine treatment, which increases intracellular cAMP levels causes a reduction in conductance (Teranishi et al 1983, Piccolino et al 1984, McMahon et al 1989). There is no evidence of direct phosphorylation but the rapid uncoupling reported by McMahon et al 1989 would be consistent with this mechanism. The downregulation of GJIC following cAMP stimulation can also occur between cells in the uterine muscle, which express connexin 43 (Cole and Garfield 1986).

Elevated intracellular cAMP levels are known to stimulate the activity of cAMP dependant protein kinase A (PKA; Taylor et al 1990), which in turn activates by

phosphorylation the transcription factor cAMP-response element-binding factor CREB, causing increased transcription of cAMP responsive genes (Brinble and Montiminy 1992). Sequences corresponding to the cAMP response element (CRE) are located near the transcription site of the connexin 32 gene (Miller et al 1988), which could account for the increased connexin 32 transcription. However, whether CREs are also located near the transcription site of connexin 43 or the transcription site of other genes that can influence GJIC has not yet been reported. The activation of cAMP dependant PKA may also directly affect GJIC as:

a) cAMP stimulation induces phosphorylation of connexin 32 at Ser-233, which is in the sequence Lys-Arg-Gly-Ser that conforms to the A (and G) kinase consensus sequences. However the physiological relevance of this Ser-233 phosphorylation has not been established and connexin 43 lacks similar consensus sequences and may not be phosphorylated by cAMP dependant kinases (Saez et al 1990). cAMP dependant PKC may also phosphorylate other connexins or proteins that facilitate gap junction formation or function.

b) The addition of a PKA catalytic subunit to a communication deficient cell culture increases GJIC after several hours (Wiener and Loewenstein 1983).

1.6.4.2 Retinoids.

Retinoic acid and other derivatives of retinol (vitamin A) occur naturally, and are collectively called retinoids. They are capable of exerting profound effects on cell proliferation and differentiation (Maden 1982, Tickle et al 1982). Retinoids can also influence GJIC, although like their effects on growth this influence varies and depends on cell type, retinoid type and concentration (Pitts et al 1986, Yamasaki and Katoh 1988, Mehta et al 1989, Rogers et al 1990, Mehta and Loewenstein 1991). For example, retinoic acid blocks homologous communication in a variety of cell types at high concentrations (10^{-4}M; Pitts et al 1986) and in cultures of C3H10T1/2 cells at

low concentrations (10^{-10}-10^{-9}M; Mehta et al 1989), whereas at physiological concentrations (10^{-8}M; Thaller and Eichele 1987) homologous communication in C3H10T1/2 or chemically transformed C3H10T1/2 cell cultures is enhanced (Mehta et al 1989, Mehta and Loewenstein 1991) but surprisingly, heterologous communication between chemically transformed C3H10T1/2 cells and their normal counterparts is reduced (Mehta and Loewenstein 1991). The molecular mechanisms underlying this complex regulation of GJIC are not understood. Models have been proposed to explain the opposite responses of homologous and heterologous communication to retinoic acid, which involve the differential expression and/or regulation of cell adhesion molecules (Mehta and Loewenstein 1991) or gap junctional channel proteins (Loewenstein and Rose 1992). Retinoic acid is thought to exert its effects by binding to members of a family of nuclear receptors (Giguere et al 1987, Brand et al 1988, Zelent et al 1989), which are homologous to the steroid and thyroid hormone receptors (Evans 1988), suggesting that retinoids function by regulating gene transcription. Consistent with this is the observation that in cultures of C3H10T1/2, treated with the stable benzoic acid derivative of retinoic acid (TTNPB at 10^{-8}M) homologous gap junctional communication increases as does transcription of connexin 43 (Rogers et al 1990). Whether retinoids regulate the transcription of other genes important for gap junction formation and function is unknown.

1.6.4.3 Phorbol esters.

The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is a potent mouse skin tumour promoting agent (Slaga 1983), which activates the protein kinase C (PKC) family of serine- and threonine-specific protein kinases (Clemens et al 1992). This activation of PKC can cause a cascade of changes in cellular signalling, including the stimulation of mitogen activated protein (MAP) kinase activity (Nori et al 1992) and can also elevate directly the activity of the transcription factor NF-*kB*

(which is a member of the c-rel family; Ghosh and Baltimore 1990, Gosh et al 1990) and indirectly the activity of the nuclear proto-oncogene c-jun (Boyle et al 1991). The consequences of PKC activation are therefore complex, (and include the post-translational modification of proteins by phosphorylation and changes in gene expression), and they also vary between different cell types (Clemens et al 1992).

The inhibition of gap junctional communication by TPA treatment has been correlated, in separate studies using different cultured cell lines with changes in the levels of ductin (Finbow et al 1983) and connexin (Brissette et al 1991), as well as with changes in connexin 43 localisation and phosphorylation (Asamoto et al 1991, Reynhout et al 1992). The treatment of primary keratinocytes with TPA or their transformation with an activated *ras* oncogene inhibits dye coupling (Dotto et al 1989) , and this correlates with increased serine phosphorylation of connexin 43 and decreased connexin 26 and 43 expression (Brissette et al 1991). Interestingly, epidermal growth factor (EGF) stimulation also transiently inhibits GJIC in primary keratinocytes (Madhukar et al 1989) and liver epithelial cells (Lau et al 1992). In the liver cells this is also associated with an increase in serine phosphorylation of connexin 43 (Lau et al 1992). The correlation of serine phosphorylation of connexin 43 with inhibition of GJIC is in contrast to the observations of Musil et al (1990b) who reported that increased serine phosphorylation of connexin 43 correlates with increased GJIC. A possible explanation for this discrepancy is that the sites of serine phosphorylation are different, (as there are 23 serine residues in connexin 43 and the positions of the phosphorylated residues have not been determined), and specific phosphorylation events are known to differentially affect the function of other proteins. For example, the activity of $p34^{cdc2}$ (the catalytic subunit of a protein complex that regulates the progression of eukaryotic cells through the cell cycle) is regulated in part by both phosphorylation and dephosphorylation of certain tyrosine and threonine residues (Draetta 1990, Lorca et al 1992).

1.6.4.4 Oncogenes.

The first suggestion that oncogenes might influence GJIC came from observations showing that the transformation of mammalian cells with the *v-src* oncogene inhibits homologous GJIC (Atkinson et al 1981, Chang et al 1985), although contradictory results have subsequently been reported showing that transformation of rodent fibroblast cell lines affects heterologous but not homologous GJIC (section 1.7; Bignami et al 1988, Katoh et al 1993).

The inhibition of homologous communication following transformation with the *v-src* oncogene depends on the tyrosine kinase activity and membrane localisation of the protein product of the viral *src* gene, $pp60^{v\text{-}src}$ (Azarnia et al 1988, Crow et al 1992) and also correlates with the tyrosine phosphorylation of connexin 43 (Crow et al 1990, Filson et al 1990, Swenson et al 1990). However, the increased tyrosine phosphorylation of connexin 43 may be fortuitous, as $pp60^{v\text{-}src}$ also indirectly induces increased serine phosphorylation of connexin 43 (Filson et al 1990) and TMB-8 treatment prevents $pp60^{v\text{-}src}$ induced inhibition of communication (Rose et al 1986) but does not affect the level of tyrosine phosphorylation of connexin 43 (Rose et al 1986, Hyrc and Rose 1990). The mechanism(s) by which $pp60^{v\text{-}src}$ inhibits GJIC may also involve the disruption of cell-cell adhesion, as the transformation of different cell types with $pp60^{v\text{-}src}$ can alter cell morphology, reduce cell aggregation, disrupt adherans junction formation and induce the tyrosine phosphorylation of cadherins, catenins and vinculin (Sefton et al 1981, Warren and Nelson 1987, Volberg et al 1991, Matsuyoshi et al 1992, Behrens et al 1993). The mechanism(s) by which $pp60^{v\text{-}src}$ inhibits GJIC is therefore unresolved, partly because of its diverse biological activities. This is also true of the other oncogenes that are reported to inhibit GJIC, which include SV40, PyMT and *ras* (Dotto et al 1989, Yamasaki 1990, Katoh et al 1993).

There is only one example of an oncoprotein directly associating with a putative gap junction channel protein. The E5 oncoprotein of bovine papillomavirus type 1 (BPV-1) is a hydrophobic 44 amino acid protein, which induces the transformation of immortalised fibroblasts, it binds to ductin (Goldstein et al 1991). However, there is only preliminary evidence to suggest that expression of E5 homologs can reduce GJIC (Dr W.D.Pennie and Dr J.D.Pitts personal communication). The transforming activity of E5 involves the activation of growth factor receptors, such as EGF probably by inhibition of down modulation (Martin et al 1989). A potential mechanism of E5 action is the inactivation of V-ATPase through binding of E5 to the V-ATPase 16kD proteolipid, thereby preventing the acidification of endocytotic vesicles, which is thought to be critical for dissociating the ligand-receptor complex. The consequence of which could be prolonged growth factor/receptor interactions (Goldstein et al 1991). In addition, if expression of E5 or its homologs is confirmed to inhibit GJIC, this inhibition may not be due to the direct binding of E5 to gap junctions but could instead be an indirect consequence of growth factor receptor activation (Madhukar et al 1989, Lau et al 1992).

The growth and phenotype of certain transformed rodent fibroblasts can be suppressed when they are grown in contact with an excess of untransformed cells. This contact inhibition was first reported by Stoker et al (1966), who showed that the growth of polyoma virus-transformed hamster fibroblast cells could be inhibited by an excess of surrounding normal cells. Subsequent studies have suggested that heterologous GJIC between virally or chemically transformed cells and their normal counter parts plays an important role in regulating this suppression and preventing focus formation and this is discussed in section 1.7.

1.6.5 Summary- Control of gap junctional intercellular communication.

The incidence and extent of GJIC may be regulated by formation, turnover or gating of gap junctions. The mechanisms which control each of these processes are however poorly characterised. Rapid changes in GJIC, which are measured electrophysiologically may involve gating, a change in the permeability of the channel. This probably occurs through a conformational change in the channel protein and may be induced by phosphorylation, although there is no evidence to suggest that ductin is phosphorylated. Slower changes in GJIC, occurring in minutes or beyond could be a consequence of changes in the formation or turnover of gap junctions, which could be affected by the expression, localisation or degradation of ductin, connexins and cell adhesion molecules.

The downregulation of GJIC, isolating potentially oncogenic cells from intracellular growth control is also thought to play a role in tumorigenesis, as many types of tumour cells are aberrantly coupled via gap junctions (Loewenstein and Kano 1966, Loewenstein 1979, Yamasaki 1990), transformation of cells with viral oncogenes (*src*, SV40, PyMT and *ras*) can inhibit GJIC (Atkinson et al 1981, Azarnia and Loewenstein 1987, Dotto et al 1989, Yamasaki 1990, Katoh et al 1993) and treatment with the potent mouse skin tumour promoting agent TPA can reduce communication *in vitro* (Murray and Fitzgerald 1979, Yotti et al 1979, Dotto et al 1989, Madhukar et al 1989, Asamoto et al 1991), although contradictory results have been reported *in vivo* and the role that TPA induced inhibition of communication plays in tumour promotion questioned (Kam and Pitts 1988, Pitts et al 1988).

1.7 Specificity of gap junctional intercellular communication.

A specificity of GJIC has been observed *in vivo*, in the mammalian skin and hair follicle and during embryonic development, it may contribute to the control of cell proliferation and differentiation (section 1.4.2). The observations of restricted dye

spread, which characterise specificity, are often associated with groups of cells following different developmental pathways (Warner and Lawrence 1982, Kalimi and Lo 1988, Serras et al 1989) or, in the adult skin with subpopulations of differentiated cells (Kam et al 1986, Salomon et al 1988, Kam and Hodgins 1992). The developmental boundaries of the *Drosophila* wing imaginal disc have also been reported to coincide with communication compartments (Weir and Lo 1982), but conflicting results have been reported by Fraser and Bryant (1985), who observed dye spread across the developmental boundaries and suggested that the restricted dye spread observed by Weir and Lo (1982) occurred because of folds in the imaginal disc epithelium.

Specificity has also been observed *in vitro*, in mixed cultures of epithelial and fibroblast cells (Pitts and Burk 1976, Taylor-Papadimitriou et al 1976). These cell types tend to sort out, forming separate domains which have been termed communication compartments (Pitts and Kam 1985). Within each compartment cells are well coupled homologously (95-100% of contiguous cell pairs), but the frequency of heterologous gap junction formation between cells in different compartments is low (about 5%). The compartment boundaries are also exaggerated, because infrequent heterologous coupling is followed by more rapid dissipation into the well coupled population in an adjacent domain.

The transformation of rodent fibroblasts (NIH3T3 or BALB/c3T3 cells) with the oncogenes *src*, *ras* or PyMT or by chemical transformation is reported not to affect the homologous communication ability of the transformed cells, but to inhibit their ability to communicate heterologously with untransformed BALB/c3T3 cells (Bignami et al 1988, Katoh et al 1993). The ability of these transformed cells to communicate homologously is in contrast to previous reports of reduced dye transfer in NIH3T3 cells transformed by v-src (Chang et al 1985, Azarnia et al 1988) or rat fibroblasts transformed by PyMT (Azarnia and Loewenstein 1987). The reason for this

difference has not been determined, it could be due to subtle differences in the level of oncogene expression or differences in the methods used to assess communication.

A consequence of this altered specificity of GJIC is that these oncogene or chemically transformed cells escape contact inhibition by an excess of untransformed cells (Mehta et al 1986, Bignami et al 1988, Yamasaki and Katoh 1988, Katoh et al 1993). Contact inhibition was first reported by Stoker et al (1966) and subsequent analysis has suggested that heterologous GJIC between virally or chemically transformed cells and their normal counter parts is important in preventing focus formation. In co-culture experiments, involving BALB/c3T3 or NIH3T3 cells transformed with myc, fos or PyLT and untransformed BALB/c3T3, there is a high frequency of heterologous communication between the transformed and normal cells, the proliferation of the transformed cells is suppressed and few foci form. Treatment with phorbol ester (section 1.6.4.3) rescues these transformed cells and also inhibits the heterologous communication (Bignami et al 1988, Katoh et al 1993). While in co-cultures of BALB/c3T3 or NIH3T3 cells transformed with *ras* or *src* and untransformed BALB/c3T3 the frequency of heterologous communication is low, the proliferation of the transformed cells is not suppressed and numerous foci form (Bignami et al 1988, Katoh et al 1993). Rare heterologous coupling and the formation of numerous foci has also been observed in co-cultures of chemically transformed C3H10T1/2 cells and their normal counterparts. The treatment of these co-cultures with forskolin (which stimulates adenylate cyclase and raises intracellular cAMP levels; section 1.6.4.1) is reported to increase heterologous communication and this correlates with a decrease in the number of foci formed (Mehta et al 1986), which is consistent with GJIC contributing to the control of proliferation. However, recent experiments reported by Katoh et al (1993) show that focus formation is also inhibited when *myc*, *fos*, PyLT transformed BALB/c3T3 are co-culture with a non-transformed BALB/c3T3 line that loses its ability to dye couple at confluency, and

they have suggested that factors other than GJIC are involved in the suppression of the oncogene transformed cells by the surrounding normal cells, although a low level of undetected coupling may occur.

The molecular basis of specificity of GJIC is not understood. Ductin, which is present in gap junction preparations made from both invertebrate and vertebrate tissues (Buultjens et al 1988) appears to play a role in GJIC (section 1.5.3), but it is unlikely to contribute to the determination of specificity. The expression of connexin mRNAs in *Xenopus* oocytes shows that coupling depends on the expression of particular connexin combinations (section 1.5.2), which suggests that connexin expression may play a role in specificity. However, Risek et al 1992 have recently shown that connexin 43 is expressed by different cell lineages within the hair follicle, even through each lineage forms a separate communication compartment (Kam and Hodgins 1992). Cell adhesion molecules which affect gap junction formation may contribute to specificity, as mixed cultures of epithelial and fibroblast cells which show specificity sort out, suggesting that these cell types differentially express cell adhesion molecules.

The molecular mechanisms by which the oncogenes *src*, *ras* or PyMT cause a specificity of GJIC between transformed NIH3T3 or BALB/c3T3 cells and the surrounding normal BALB/c3T3 cells, (which may enable these transformed cells to escape from normal growth control) are unknown. Transformation may influence the expression, localisation, degradation or function of ductin, connexins or cell adhesion molecules and thereby affect gap junction formation (section 1.6.4.4), although Katoh et al 1993 have recently reported that the oncogene transformed cells express a level of connexin 43 mRNA which is comparable to the parental cells.

1.8. Summary and aims.

Gap junctions are transmembrane channels that occur at regions of cell-cell contact. They enable cells to communicate directly with one another via the exchange of small cytoplasmic molecules. In electrical tissues GJIC allows the synchronization and propagation of electrical activity, while in non-excitable tissues it coordinates metabolic activity and enables the exchange of ions and second messengers, which may contribute to the control of cell proliferation and differentiation.

A specificity of GJIC results in the formation of communication compartments, groups of cells which communicate homologously with each other via gap junctional channels but do not communicate heterologously with cells in adjacent compartments. Within a communication compartment the levels of small cytoplasmic molecules will tend to equilibrate and this may suppress the expression of new phenotypes by individual cells. The expression of different phenotypes during embryogenesis and tissue differentiation is often associated with the sub-compartmentalisation of GJIC. *In vitro* the growth and phenotype of transformed cells can be suppressed by contact with an excess of normal cells (Stoker 1967). This contact dependant suppression of the transformed phenotype is thought to mediated by heterologous GJIC (Mehta et al 1986), and changes in specificity, isolating potentially tumorigenic cells from normal intercellular growth control may contribute to tumorigenesis.

The aim of this thesis is to investigate the control of specificity of GJIC, the mechanism that underlies the formation of communication compartments, which in turn may influence embryogenesis, tissue differentiation and tumorigenesis. Ductin, connexins and cell adhesion molecules are all thought to play a role in gap junction formation. The connexins and the cadherins because of their tissue specific expression are most likely to control the formation of communication compartments.

I have used two cell culture systems as models for the molecular analysis of gap junction formation and specificity. The *mec*$^-$ (defective in junction formation and *m*etabolic *co*operation) cell lines (L, MCF-7 and S180) provide a system for expression studies aimed at identifying proteins required for gap junction formation. Mixed cultures of the epithelial *mec*$^+$ cell line BRL and either of the fibroblast *mec*$^+$ cell lines BHK or BICR show a specificity of GJIC and I have attempted to identify proteins which will break this specificity.

Chapter 2.

Materials and Methods.

2.1 Materials.

2.1.1 Chemicals.

All chemicals were of "AnalaR" grade and obtained from BDH Chemicals Ltd., Poole, Dorset, England or Sigma Chemical Co. Ltd., Poole, Dorset, England, except those obtained from the suppliers listed below.

Supplier- Amersham International PLC, Amersham, Bucks, England.

$\alpha[^{32}P]$ dCTP [3000Ci/mmol] Hybond-ECL

$[^{35}S]$ dATP αS [600Ci/mmol] Hybond-N+

Rainbow protein markers (30-200kD)

Supplier- B.R.L.(UK), Gibco Ltd, Paisley, Scotland.

All restriction enzymes, T4 DNA ligase and buffer concentrates were obtained from BRL except were otherwise indicated.

RNA markers

Supplier- Biogenesis Ltd., Bournemouth, England.

RNAzol B

Supplier- Biological Industries, Haemek, Israel.

10x RPMI 1640 (pH3.0-3.5)

Supplier- Boehringer Mannheim UK Ltd., Lewes, East Sussex, England.

Caesium Chloride Proteinase K

Calf intestinal alkaline phosphatase

DNA molecular weight markers (1-6)

Supplier:- J.Burrough (FAD) Ltd., Witham Essex, England.

Ethanol

Supplier- Central Services, Beatson Institute.

CT buffer (NaCl 6g, trisodium citrate 2.96g, tricine 1.76g, phenol red 5mg, water 700ml, pH7.8.

L-broth

Sterile glycerol

Sterile distiled water

Sterile PBS

Supplier- Chance Propper Ltd., Warley, England.

16mm diameter microscope glass coverslips

Supplier- Clarke Electromedical Instruments, Reading, England.

"Kwik fill" thin will glass capillaries

Supplier- Difco Labs., Detroit, Michigan, USA.

Bacto-tryptone

Bacto-yeast extract

Supplier- Eastman Kodak Co., Rochester, New York, USA.

Duplicating film

X-ray film (XAR-5)

Supplier- Fuji Photo Co. Ltd., Japan.

X-ray film (RX)

Supplier- Gateway PLC, Glasgow, Scotland.

Marvel, dried non-fat milk powder.

Supplier- Gibco Europe, Life Technologies Ltd, Paisley, Scotland.

10x DMEM

penicillin

Foetal calf serum

1x SLM

G418, Geneticin

7.5% sodium bicarbonate

200mM Glutamine

100mM sodium pyruvate

10x GMEM

streptomycin

hydrocortisone

2.5% Trypsin

5x non-essential amino acids

Tryptose phosphate broth

Supplier- Northumberland Biochemicals Ltd., Cramlington, England.

Restriction enzymes *Sma*I and *Bam*HI and appropriate buffer concentrates.

Supplier- Oxoid Ltd., Basingstoke, England.

PBS tablets

Supplier- Pharmacia Ltd., Milton Keynes, England.

Restriction enzymes *Sal*I, *Pst*I and *Bgl*II and appropriate buffer concentrates.

Nick columns.

Supplier- Rathburn Chemicals Ltd., Walkerburn, Scotland

Phenol (water saturated)

Supplier- Vector Laboratories Inc, Burlington, USA.

Vector shield mounting media for fluorescence

Supplier- Whatman International Ltd., Maidstone, England.

3MM chromatography paper

2.1.2 Kits.

Supplier- Amersham International PLC, Amersham, Bucks, England.

ECL Western Blotting analysis system

Supplier- Bio 101 Inc., Stratech Scientific, Luton, England.

Geneclean kit

Supplier- Pharmacia Ltd., Milton Keynes, Bucks., England.

Oligo-labelling kit

Supplier- Sigma Chemical Co. Ltd., Poole, England.

Bicinchroninic acid kit for protein concentration determination

Supplier- United States Biochemical, Cleveland, Ohio, USA.

Sequenase version 2.0

2.1.3 Equipment and Plasticware.

Main pieces of equipment are referred to in the appropriate sections. Plasticware was obtained from the Beatson Institute stores, and the following companies are the suppliers of the most commonly used items:

Supplier- Becton Dickinson Labware, Plymouth, Devon, England.

Tissue culture dishes (35, 60, 90mm)

Supplier- Bibby-Sterilin Ltd, Stoney, Staffs., England.

Bacteriological dishes (90mm)

Supplier- Gibco Europe, Life Technologies Ltd, Paisley, Scotland.

Nunc 1ml cryotubes

Supplier- Griener Labortechnik Ltd., Dursley, England.

Eppendorf tubes

Supplier- Labsystems, Basingstoke, England.

Pipette tips (200 and 500µl).

2.1.4 Plasmids.

pβact-Pcad	3.2kb P-cadherin cDNA fragment cloned into the *Eco*RI site of the vector pβact-Pcad (Nose et al 1988). A gift from Prof.M.Takeichi, Kyoto Uni., Japan.
pBabehygro	A gift from Dr.S.Jamieson, Beatson Institute, (Morgenstern and Land 1990).
pBATEM2	3.1kb E-cadherin cDNA fragment cloned into the *Eco*RI site of the vector pBATEM2 (Nose et al 1988). A gift from Prof.M.Takeichi, Kyoto Uni., Japan.
pBluescript SK	Stratagene, San Diego, USA.

pBluescript-Pcad	P-cadherin cDNA fragment cloned into the *Eco*RI site of pBluescript SK. A gift from Prof.M.Takeichi, Kyoto Uni., Japan (Nose et al 1987).
pGEM-cx32	1.5kb connexin 32 cDNA fragment cloned into the *Eco*RI site of Promega's pGEM.3 vector (Paul 1986). A gift from Dr.D.Paul, Boston Uni., USA.
pBluescript-cx43	1.4kb connexin 43 cDNA fragment (G2) cloned into the *Eco*RI site of pBluescript SK (Beyer et al 1987). A gift from Dr.D.Paul, Boston Uni., USA.
pGEM-NCAM	0.6kb N-CAM cDNA fragment (pM1.3) cloned into the *Eco*RI site of Promega's pGEM-1 (Goridis et al 1985). A gift from Dr.C.Goridis, Centre d'Immonologie, de Marseille-Luminy, France.
pHSG274	A gift from Dr.K.Hawker, Beatson Institute, (Brady et al 1984).
pIC20H	A gift from P.McLean, Beatson Institute, (Marsh et al 1984).
pIC20H-ductin	0.8kb ductin cDNA fragment cloned into the *Eco*RI site of pIC20H. A gift from P.McLean, Beatson Institute.
pUC-Ncadherin	4.3kb N-cadherin cDNA fragment cloned into the *Eco*RI site of pUC (Miyatani et al 1989). A gift from Prof.M.Takeichi, Kyoto Uni., Japan.
pZipneo.SVX	A gift from Dr.S.Jamieson, Beatson Institute, (Cepko et al 1984).

2.1.5 DNA probes.

The following DNA probes for E-, N- and P-cadherin, connexins 32 and 43, ductin, N-CAM and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used in this study. The origin of each probe is shown schematically in figure 3.

E-cadherin	1.1kb *Acc*I fragment of mouse E-cadherin cDNA.
N-cadherin	1.1kb *Pst*I fragment of mouse N-cadherin cDNA.
P-cadherin	0.65kb *Hinc*II fragment of mouse P-cadherin cDNA.
connexin 32	1.5kb *Eco*RI fragment of rat connexin 32 cDNA.
connexin 43	1.4kb *Eco*RI fragment of rat connexin 43 cDNA.
ductin	0.8kb *Eco*RI fragment of bovine ductin cDNA.
GAPDH	0.7kb PCR fragment of rat GAPDH a gift from B.Hennigan, Beatson Institute.
N-CAM	0.6kb fragment of mouse N-CAM cDNA.

2.1.6 Antiserum.

Mouse monoclonal anti-N-cadherin (GC4)

Sigma Chemical Co. Ltd., Poole, Dorset, England.

Mouse monoclonal anti-NCAM

Sigma Chemical Co. Ltd., Poole, Dorset, England.

Rat monoclonal anti-E-cadherin (ECCD-2)

A gift from Prof.M.Takeichi, Kyoto Uni, Japan (Shirayoshi et al 1986).

Rat monoclonal anti-P-cadherin (PCD-1)

A gift from Prof.M.Takeichi, Kyoto Uni, Japan (Nose and Takeichi 1986).

Rabbit anti-N-cadherin polyclonal

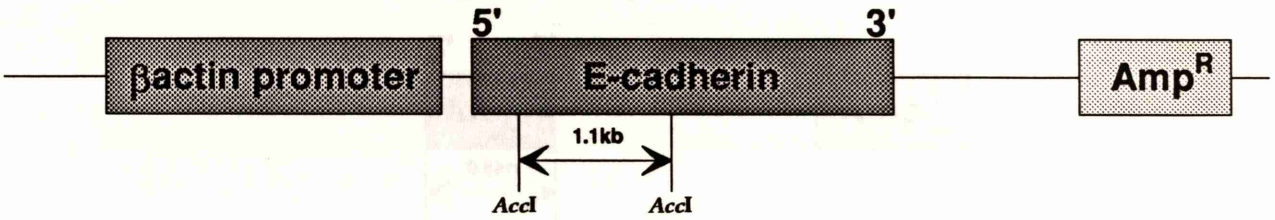
A gift from Dr.P.Doherty, UMDS, Guys Hospital, London (Doherty et al 1991).

Figure 3. Origin of DNA probes.

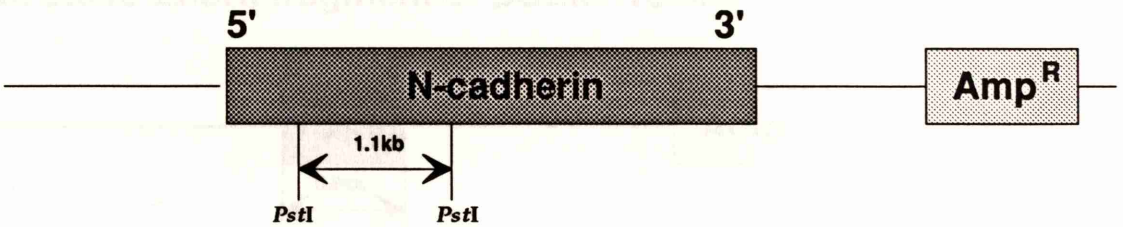
Specific DNA probes for E-, N- and P-cadherin, connexins 32 and 43, ductin and N-CAM were prepared by digesting plasmids containing cDNA fragments with the appropriate restriction enzymes, as described in materials and methods.

Figure 3.

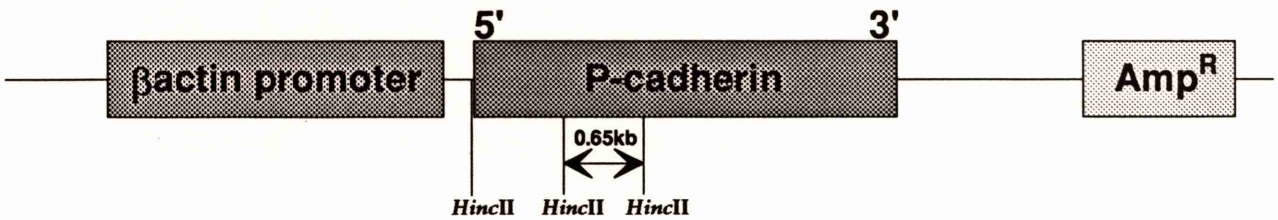
A. E-cadherin: 1.1kb *Acc*I fragment of pBATEM2.



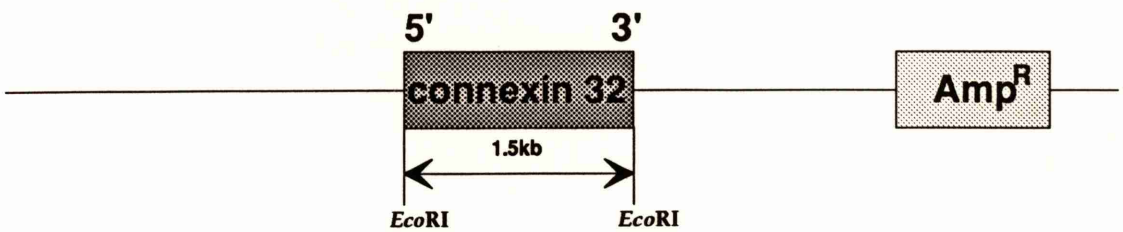
B. N-cadherin: 1.1kb *Pst*I fragment of pUC-Ncadherin.



C. P-cadherin: 0.65kb *Hinc*II fragment of pβact-Pcad.



D. Connexin 32: 1.5kb *Eco*RI fragment of pGEM-cx32.



E. Connexin 43: 1.4kb *Eco*RI fragment of pBluescript-cx43.

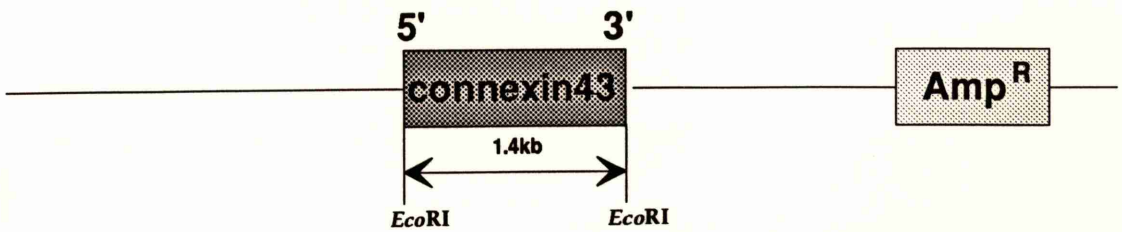
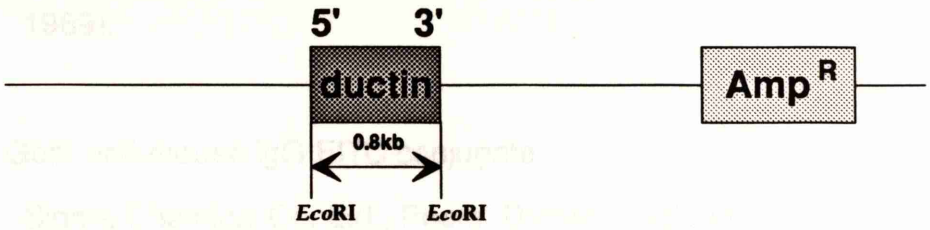
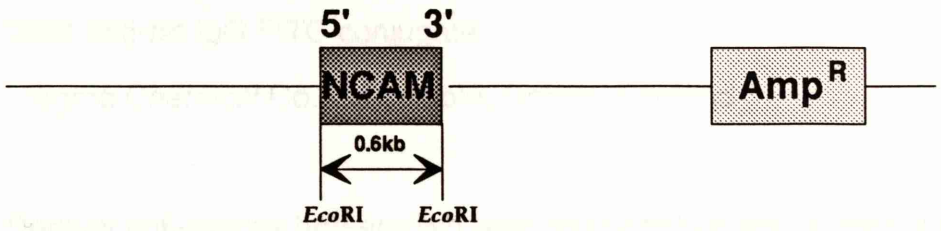


Figure 3.

F. Ductin: 0.8kb *Eco*RI fragment of pIC20H-ductin.



G. N-CAM: 0.6kb *Eco*RI fragment of pGEM-NCAM.



Rabbit anti-connexin 43 polyclonal ("Petunia")

A gift from Prof.D.Goodenough, Harvard Uni, Boston, USA (Beyer et al 1989).

Goat anti-mouse IgG FITC conjugate

Sigma Chemical Co. Ltd., Poole, Dorset, England.

Goat anti-rabbit IgG FITC conjugate

Sigma Chemical Co. Ltd., Poole, Dorset, England.

Goat anti-rat IgG FITC conjugate

Sigma Chemical Co. Ltd., Poole, Dorset, England

Donkey anti-mouse horseradish peroxidase-linked whole antibody

Amersham International PLC, Amersham, Bucks, England.

Sheep anti-rabbit horseradish peroxidase-linked whole antibody

Amersham International PLC, Amersham, Bucks, England.

Sheep anti-rat horseradish peroxidase-linked whole antibody

Amersham International PLC, Amersham, Bucks, England.

2.1.7 Bacterial Host.

Competant *E.coli* DH5α were obtained from B.R.L., Gibco Ltd.

2.1.8 Cell lines.

BHK 21/C13 Syrian Baby Hamster Kidney fibroblast line (Macpherson and Stoker 1962). Maintained in GMEM.

BICR	Mammary carcinoma fibroblast line established at the Biophysics Department, Institute for Cancer Research, Chester Beatty (Rajewsky and Gruneisen 1972). Maintained in SLM.
BRL	Buffalo Rat Liver epithelial line (Coon 1968). Maintained in GMEM.
L	Mouse fibroblast line (Sanford et al 1948). Maintained in GMEM.
MCF-7	Human mammary epithelial tumour cell line (Rose and McGrath 1975). Maintained in RPMI (Gibco).
S180	Mouse fibroblast sarcoma line (Foley et al 1960). Maintained in SLM.
SSCHN BICR 3-22	Human Squamous Cell Carcinomas of the Head and Neck established at the Beatson Institute for Cancer Research, a gift from K.Edington, Beatson Institute. Maintained in DMEM.
$3T3_I$	Irradiated mouse fibroblasts used as a feeder layer for growth of SSCHN BICR tumour cell lines, a gift from M.Nikolic, Beatson Institute.

2.1.9 Tissue culture media and buffers.

DMEM	Dulbecco's modified Eagles media supplemented with 2% or 10%FCS, 0.4mg/l hydrocortisone.
GMEM	Glasgow's modified Eagles media supplemented with 10%FCS, 5mM glutamine, 1mM sodium pyruvate, 0.375% sodium bicarbonate and 1x non-essential amino acids.

RPMI RPMI media supplemented with 10% FCS, 1mM glutamine and 0.3% sodium bicarbonate.

SLM Special liquid media supplemented with 10% FCS and 5mM glutamine.

2.1.10 Water

Distiled water for solutions was obtained from a Millipore MilliRO 15 system, and for nucleic acid, tissue culture and protein procedures it was further purified on a Millipore MilliQ system.

2.2 Methods.

2.2.1 Eukaryotic cell culture, cell adhesion assay and transfection.

2.2.1.1 Culture of cell lines.

The BHK, BRL, BICR, L, MCF-7 and S180 cell lines were maintained on plastic petri dishes in the appropriate media at 37^{o}C in an atmosphere of 5% CO_2. The SSCHN BICR tumour cell lines were cultured under the same conditions except that the cells were grown on irradiated 3T3 feeder layers in order to minimise the selection of fitter variants (Rheinwald & Beckett 1981). Stock cultures of each cell type were maintained by passaging sub-confluent 90mm plates at a ratio of 1:5-20 every 3-5 days (depending on cell type). Cells were passaged by aspirating off the growth media from the cell monolayer, adding 4mls of 0.25% trypsin in CT buffer (see section 2.1.1), immediately removing the majority of the trypsin solution and incubating the cells for 10-15min at room temperature or 37^{o}C until the cells detached. The detached cells were then resuspended in growth medium and replated at the appropriate dilution. Cell lines were typically maintained for 24-36 passages and then replaced from frozen stocks. The cell lines were also routinely found to be negative for mycoplasma infection in the laboratory mycoplasma test programme (B.McGuire and M.Freshney, Beatson Institute).

For dye coupling analysis a sub-confluent plate of cells was harvested, as described above and resuspended in appropriate growth medium at a density of approximately 2.5x10^{4} cells/ml, (cell numbers were determined using a Coulter Counter). 4ml aliquots of this cell suspension were then dispensed into 60mm tissue culture dishes and incubated for 48-72hr at 37^{o}C in an atmosphere of 5% CO_2. Mixed cultures for dye transfer analysis were established by harvesting a subconfluent plate of each cell type (BRL and BHK or BICR cells) and mixing the cells in a 1:1 ratio at a density of approximately 2.5x10^{4} cells/ml. Mixed cultures

of BHK and BRL cells were suspended and cultured in GMEM, whereas mixed cultures of BICR and BRL cells were suspended and grown in SLM. 4ml aliquots of each mixed cell suspension were dispersed into 60mm tissue culture dishes and incubated for 48-72hrs at 37^{o}C in an atmosphere of 5% CO_2. Sub-confluent or occasionally confluent cultures grown on these 60mm plates were subsequently used for dye coupling analysis as described in section 2.2.2. The communication ability of the BRL cells in mixed culture was unaffected by the choice of medium.

For immunofluorescence analysis cells were grown on sterile glass 16mm diameter coverslips. Sub-confluent plates of cells were harvested, (as described above) plated out in a 90mm plastic petri dish containing 4-6 sterile coverslips at a dilution of 1:5-20 depending on the cell type, in the appropriate media and incubated at 37^{o}C in an atmosphere of 5% CO_2 for 48-72 hr. The coverslip cell cultures were subsequently processed for immunofluorescence as described in section 2.2.4.1.

2.2.1.2 Frozen cell stocks.

Frozen cell stocks of each cell type were maintained in liquid nitrogen. The cell lines were trypsinized and resuspended in the appropriate growth media supplemented with 10% DMSO at approximately 1x10^{6} cells per ml. This cell suspension was immediately aliquoted into 1ml Nunc cryotubes and frozen at -70^{o}C in an insulated container. One to twos days later the cryotubes containing the frozen cells were transferred to liquid nitrogen for long term storage.

2.2.1.3 Calcium dependant cell adhesion assay.

Calcium dependant cell adhesion was assayed using a protocol based on a method reported by Takeichi 1977. Cells were cultured at approximately 0.5-

$1x10^5$ cells/90mm tissue culture dish, (5 dishes per cell type) and cultured for 48hrs at 37^oC in an atmosphere of 5% CO_2. The remaining subconfluent cell monolayers were divided into two groups, and single cell suspensions were prepared either in the presence of Ca^{++} (TC treatment) or the absence of Ca^{++} (TE treatment). The cell monolayers were washed 3x with Puck's saline (NaCl 7.40g, KCl 0.29g, $Na_2HPO_4.7H_2O$ 0.29g, KH_2PO_4 0.08g, $NaHCO_3$ 1.20g, glucose 1.10g and phenol red 0.001g in 1000mls H_2O) plus 1mM $CaCl_2$ (TC treatment) or without Ca^{++} (TE treatment), and incubated with 5mls of 0.01% trypsin in HCMF (8.0g NaCl, 0.4g KCl, 0.09g $NaH_2PO_4.7H_2O$ 0.09g, glucose 1g, HEPES 2.38g and 1N NaOH in 1000ml H_2O, pH 7.4) plus 1mM $CaCl_2$ (TC treatment) or 1mM EDTA (TE treatment) for 15 min at 37^oC. The trypsin was subsequently taken off, taking care not to remove cells (which if detached were spun down for 5 min at 1000rpm), and the cells resuspended in ice cold Pucks saline with 0.01% soybean trypsin inhibitor plus 1mM $CaCl_2$ (TC treatment) or 1mM EDTA (TE treatment). The TC and TE resuspended cells were pooled separately and then pelleted by centrifugation for 5 min at 1000rpm. The 0.01% soybean trypsin inhibitor in Pucks saline was then removed by aspiration and the cells resuspended in ice cold Pucks saline plus 1mM $CaCl_2$ (TC treatment) or 1mM EDTA (TE treatment). This washing procedure was repeated twice more and the cells finally resuspended as a single cell suspension in the experimental medium, HCMF plus 1mM $CaCl_2$ (TC treatment) or 1mM EDTA (TE treatment) at 4^oC. The resuspended TC treated and TE treated cells were then counted using a Coulter cell counter, diluted in cold HCMF plus 1mM $CaCl_2$ or 1mM EDTA to $2x10^5$ cells per ml, and 0.5mls of cells treated either way aliquoted into wells of a 24 well dish. The dishes were then shaken at 37^oC and the number of cells counted at different time points (0, 20, 30, 40, 60 and 90min) using a Coulter cell counter. If cells in either treatment group aggregated during the time

course of the experiment the total cell count decreased and this was represented graphically by plotting the number of counted cells at time t (N_t) divided by the initial number of counted cells (N_0) against time.

2.2.1.4 Transfection, selection and ring cloning of cell lines.

The cell lines BHK, BICR, BRL, L and S180 were co-transfected with a cadherin expression vector and a neomycin resistance conferring vector and clones selected in media containing G418. The L cell line was also transfected with connexin 43 in an expression vector which confers resistance to hygromycin B and clones selected in media containing hygromycin B. These transfections were carried out using a standard calcium phosphate precipitation protocol as described in Maniatis et al 1989. On day 1 exponentially growing cells were plated at $^1/_2$-2x10^6 cells/90mm petri dish, in 10mls of appropriate medium and cultured for approximately 24hrs at 37^{0}C in an atmosphere of 5% CO_2. On day 2 the precipitate was formed by slowly adding 0.5ml of 250mM $CaCl_2$, containing 18µg of cadherin expression vector and 2µg of neomycin conferring resistance vector (for co-transfections) or 20µg of connexin 43 expression vector to a sterile plastic bijou containing 500µl of 2xHBS solution (50mM HEPES, 280mM NaCl, 1.5mM $Na_2HPO_4.2H_2O$, pH7.1), over a period of 30sec while gently blowing air through the mixture to prevent a course precipitate forming. The final concentrations of the various components being 125mM $CaCl_2$, 20µg/ml DNA and 1xHBS. This solution was left to stand for 30min, during which time a fine precipitate formed. Mock transfections, with no DNA were also set up in parallel, to serve as controls. The old media was then removed from the exponentially growing cells to be transfected and 1ml of the gently mixed precipitate slowly added to the dish, and incubated for 15 min at room temperature. 9mls of fresh media were then added to each dish. Three to five hours later the cell

monolayers were washed once with 10mls of PBS, incubated with 1.5mls of 15% glycerol in 1xHBS at 37^{o}C for 30sec-3min and subsequently washed with 10ml PBS. Fresh growth media was then added and the cells incubated for 24-48hr at 37^{o}C in an atmosphere of 5% CO_2.

The transfected cells were then passaged at a 1:10 split into growth media containing G418 or hygromycin B at concentrations empirically determined for each cell line (table 2). This selection was applied for 2-3 weeks, with media containing G418 or hygromycin B being changed every 3-4 days. After approximately 10 days resistant colonies were observed in the DNA transfected dishes (but not in the mock transfected dishes), which were ring cloned as follows:

a) The position of isolated resistant colonies on each dish were marked.

b) The growth media removed by aspiration.

c) Sterile rings (prepared by cutting the wide end of yellow pipette tips with a scalpel, autoclaving and greasing with a small amount of sterile Vaseline prior to use) were carefully applied to the marked colonies.

d) 50µl of trypsin was added to each ring and left for 30secs at room temperature, then removed and the dish incubated for 2-5 min at 37^{o}C.

e) 50µl of growth media was then added to each ring, the trypsinized cells carefully resuspended and transferred to 4mls of media in 6 well plates.

Successfully ring cloned colonies were maintained in media containing G418 or hygromycin, and expanded by subcloning in 90mm dishes for further analysis and subsequent liquid nitrogen storage.

Table 2. Concentrations of G418 and hygromycin B used for selection of transfected cells.

Table 2.

Cell line.	Concentration of G418. (μg/ml)
L	400
S180	400
BHK	400
BICR	250
BRL	200

Cell line.	Concentration of hygromycin B. (μg/ml)
L	500

2.2.2 Dye transfer analysis.

2.2.2.1 Microinjection of Lucifer Yellow CH.

Sub-confluent or occasionally confluent cultures grown on 60mm dishes, (as described in section 2.2.1.1) were used for dye coupling analysis. The growth medium was removed from each culture dish and replaced with medium buffered with 25mM HEPES, and the dish transferred to a 37^{o}C stage in preparation for microinjection. Individual cells cultured on these 60mm dishes were iontophoretically injected with the dye, using microelectrodes made from "Kwik-fill" thin-wall glass capillaries, back filled with 4% Lucifer Yellow CH (as described by Pitts and Kam 1985). The process of microinjection was monitored on a Leitz Diavert inverted microscope with UV (epi-illumination) or visible (phase-contrast) light sources. The cells were injected with dye for 2 min with a current of 10nA in 0.5sec pulses at 1Hz. The extent of dye spread was recorded and a proportion of spreads immediately photographed, (the recorded result and the corresponding photographic record were in close agreement).

2.2.2.2 Statistical analysis.

The mean and standard deviation of the number of dye coupled cells in each injection were calculated for each cell type, and Students *t*-test used to show whether significant differences in the levels of dye coupling occurred between different cell types. *t*-values were calculated using the mean and standard deviation of the number of dye coupled cells, and the *P* value was determined from statistical tables. Values of $P<0.005$ were considered to be highly significant, $0.05>P>0.005$ significant and $P>0.05$ not-significant.

2.2.3 Nucleic Acid Procedures.

2.2.3.1 Growth, transformation and storage of competant cells.

The competant *E.coli* strain DH5α, (which is suitable for transformation with plasmid DNA) was purchased form B.R.L. and stored at -70°C.

Competant DH5α cells for transformation were thawed on ice, gently mixed and aliquoted 20ul/Eppendorf tube. Unused competant cells were refrozen on dry ice before returning to the -70°C freezer. To each 20ul of competant cells 1ul of diluted ligation mix (see section 2.2.3.3.C) or plasmid of interest were added, and incubated for 30 min on ice. The DH5α cells were then heat shocked at 42°C for 45sec and then incubated for 2mins on ice, after which 80ul of SOC medium (2% bacto-tryptone, 0.5% bacto-yeast extract, 9mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20mM glucose) was added and the cells shaken at 225rpm for 1hr in a 37°C incubator, to allow the expression of the antibiotic resistance marker (which for most plasmids was neomycin, conferring resistance to ampicillin). The DH5α cells were then spread onto agar plates (1.5% agar in L broth) containing 100ug/ml of ampicillin, and the plates incubated upside down at 37°C overnight to allow the formation of colonies. Colonies were picked using sterile toothpicks, transferred to a universal container containing 10 ml L-broth supplemented with 100ug/ml of ampicillin and incubated overnight at 37°C in a shaking incubator to enable subsequent plasmid preparations (see section 2.2.3.2) or storage (see below).

In order to distinguish between colonies of transformed cells containing the religated parental plasmid or recombinant plasmids containing exogenous cDNA ligated within the IPTG inducible β-galactosidase marker, transformed DH5α cells were plated on X-gal plates, (1.5% agar plates, prewarmed and on which 50µl of 100mM IPTG solution and 20ul of 2% X-gal had been spread prior to plating out). As the IPTG inducible β-galactosidase marker normally gives rise

to a blue colour when cells containing the religated parental plasmid are grown in the presence of the chromogenic substance X-gal, the cells containing recombinant plasmids having an insert within the β-galactosidase gene give rise to colourless plaques, enabling them to be distinguished and picked (as described above) for further analysis.

Glycerol stocks for the storage of DH5α cells found to contain the required plasmid, were prepared by mixing 1ml of an overnight DH5α culture with 1ml of glycerol and freezing in a sterile screw cap Eppendorf tube at -70^{o}C. Cells were recovered from these frozen stocks using sterile pipette tips.

2.2.3.2 Preparation of nucleic acids.

2.2.3.2.A Plasmid minipreparations.

Plasmid minipreparations were performed according to the boiling method of Holmes and Quigley (1981). Single bacterial colonies were picked from agar plates, used to inoculate 5ml L-broth supplemented with ampicillin at 50μg/ml and incubated overnight at 37^{o}C in a shaking incubator. 1.5ml of the overnight culture was transferred to an Eppendorf tube, pelleted at low speed for 5 min and the supernatant discarded. The pellet was then resuspended in 350μl of STET (5% triton X-100, 50mM EDTA, 50mM Tris, 8% sucrose, pH 8.0), vortexed for 3sec and 25μl of lysosyme solution added (10mg lysosyme, 10μl Tris pH8.0, 990μl dH_2O). The tube was then placed in boiling water for 40sec, and the lysate cleared by immediately microcentrifuging the sample for 10min at room temperature and removing the pellet with a sterile toothpick or yellow pipette tip. The DNA remaining in solution was then precipitated by adding 40μl of 3M NaAc (pH 7.0) and 420μl of isopropanol and incubating for 15min on dry ice. The precipitate was pelleted by centrifuging in a bench top microfuge for 15min. The pellet was briefly dried, resuspended in 50μl of TE buffer (1mM EDTA, 10mM

Tris, pH 8.0) containing RNase at 50µg/ml and incubated at 37°C for 10min, to allow the RNase to digest contaminating RNA that might otherwise mask small DNA fragments on the gel. Typically 10µl of this minipreparation DNA was used for restriction enzyme digestion and analysis. The remainder was stored at -20°C.

2.2.3.2.B Large scale plasmid preparations.

Large scale plasmid preparations were performed according to the alkaline lysis procedure described by Birnboim and Doly (1979). Overnight L broth cultures (500-1000ml) were pelleted in balanced 500ml polypropylene bottles by centrifuging at 8000 rpm for 5min at 4°C. The supernatant was decanted, the pellet resuspended vigorously in 20ml of solution I (10mM EDTA, 50mM glucose, 25mM Tris pH 8.0) containing lysosyme at 5mg/ml and incubated at room temperature for 10min. 40ml of solution II (0.2N NaOH, 1% SDS) was added, the solutions mixed by swirling and incubated for 5min on ice. The addition of 20ml of 5M acetate (pH4.8) and incubation for a further 15min on ice causes the formation of a heavy white precipitate, consisting of bacterial DNA and debris. This precipitate was collected by centrifugation at 8000rpm for 5min at 4°C and the supernatant filtered through a double layer of gauze into a measuring cylinder. An equal volume or at least 0.6 volume of isopropanol was added to the supernatant and the precipitated DNA pelleted by centrifugation in a Sorval GSA rotor at 8000rpm for 5min at 4°C in a 250ml polypropylene bottle. The pellets were then drained thoroughly, the sides of the centrifuge bottles wiped carefully to remove the remaining isopropanol and resuspended in 13.4mls of TE buffer (pH 8.0). To this 1.4ml of ethidium bromide (3mg/ml) and 14.4g of $CsCl_2$ was added. The refractive index was adjusted to 1.39 with CsCl or TE buffer and the solution transferred to the appropriate centrifuge tubes,

balanced to within 0.01g and centrifuged at either 40,000rpm for 40hrs at 20°C in a Sorval ultracentrifuge (T-1270 rotor) or at 80,000rpm overnight at 20°C in a Bechman benchtop ultracentrifuge (TLA 100.3 rotor). Plasmid DNA was carefully removed from the $CsCl_2$ gradients using a long needle and transferred to a plastic universal. 4mls of butan-1-ol was then added to this plasmid DNA, the universal gently shaken and the phases allowed to settle out. The upper pink phase, containing ethidium bromide was discarded and the extraction repeated 3-4x until the upper phase was clear. The lower phase was then transferred to a 50ml Corex tube, and 8ml of TE buffer and 16ml of ethanol added. The plasmid DNA was precipitated by incubating at -20°C for 30min and the precipitate collected by centrifuging in a SS-34 rotor at 10,000rpm for 20min at 4°C. The supernatant was decanted, the pelleted resuspended in 2ml of TE buffer (pH 8.0) and precipitated with 200µl of 3M NaAc (pH5.4) and 4.4ml ethanol for 15min at -70°C or overnight at -20°C. The plasmid DNA was then collected by centrifugation, washed with 70% ethanol, dried and resuspended in TE buffer (pH7.5 or 8.0). The concentration of the plasmid DNA was determined by measuring the OD (see section 2.2.3.5.D) and adjusted to 1mg/ml. This purified plasmid DNA was stored at 4°C or -20°C.

2.2.3.2.C Preparation of genomic DNA.

The following procedure was used to prepare DNA from cultured cells. It produces DNA that digests with most restriction enzymes and is of sufficiently high molecular weight for Southern blotting, the purpose for which it was prepared.

Confluent 90mm plates of 10^6-10^7 cells were harvested into 10ml of PBS plus 10% FCS, (to inactivate the trypsin). The cells were then pelleted by centrifuging at 1000rpm for 5min and washed twice with PBS, resuspending the

pellet the second time in 1ml of PBS and transferring to an Eppendorf tube. The cell suspension was then centrifuged for 5min in a microfuge, the pellet resuspended in 700µl of digestion buffer (100mM EDTA, 100mM NaCl, 1% SDS, 50mM Tris pH8.0) and 35µl of 10mg/ml proteinase K added. After incubating at 55^{o}C overnight the sample was gently mixed to disperse the DNA and extracted 3x with 700µl of a 1:1 phenol-chloroform solution (see section 2.2.3.5.B). The aqueous phase of the final extraction was separated by centrifuging for 10min in a bench top microfuge, transferred to a fresh Eppendorf tube, 0.6 vol of isopropanol was then added, mixed and the high molecular weight DNA which precipitated out collected by centrifuging for 30secs in a microfuge. The supernatant was discarded and the pelleted DNA washed with 70% ethanol and then 95% ethanol. This pellet was air dried and resuspended in 100-200µl of TE. Approximately 80µg of genomic DNA was obtained from each confluent plate of cultured cells, sufficient for 4-5 Southerns (see section 2.2.3.7). The genomic DNA was stored at 4^{o}C.

2.2.3.2.D Preparation of RNA.

Total cellular RNA was prepared from confluent 90mm plates of 10^{6}-10^{7} cells using a guandinium isothiocynate-phenol chloroform extraction method (Chomczynski and Sacchi 1987), which is marketed as RNasol B by Biogenesis Ltd. The manufacturers protocol was followed. Confluent plates were washed twice with ice cold PBS and the remaining PBS removed by aspiration. 0.8ml of RNazol/plate was added, the cells homogenised using a cell scraper and the resulting suspension transferred to an Eppendorf tube containing 80µl of chloroform. The samples were stored on ice for 5min and then the phases separated by centrifugation in a microfuge for 15min at 4^{o}C. The upper aqueous phases were collected and the RNA precipitated by adding 0.4ml of isopropanol

and incubating for 30min to overnight at 4°C. The RNA precipitates were collected by centrifugation in a microfuge at 4°C for 15min, the pellets washed once with 75% ethanol, briefly dried and resuspended in sterile double distiled water. Approximately 50µg of total RNA was obtained from each confluent plate of cultured cells, sufficient for 4-5 northerns (see section 2.2.3.8) and stored at -20°C.

The procedures used to prepare and analyse RNA were initially performed with diethyl pyrocarbonate treated plasticware and solutions. However, it was found that this treatment was unnecessary and that sterilized plasticware and solutions made with MilliQ water were sufficient, although care was taken to have separate solutions and equipment for RNA work where possible.

2.2.3.2.E Preparation of oligonucleotides.

Oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer. The 5'methyl-trityl groups were removed on the machine before the oligonucleotides were eluted from the synthesis column by "washing" 5-6x with approximately 2ml of concentrated ammonia solution (29%) over a 2-3hr period. The concentrated ammonia solutions containing the oligonucleotide were then transferred to glass vials, sealed with parafilm (to prevent evaporation of the samples) and incubated overnight at 55°C. The deprotected oligonucleotides were then ethanol precipitated by adding $^1/_{10}$vol NaAc (3M) and 2vol ethanol, incubating for 15min at -70°C and collecting the precipitate by centrifugation. The resulting pellets were briefly dried and resuspended in 200µl of dH_2O. 10µl of this solution was used for O.D. analysis (see section 2.2.3.5.D), the oligonucleotide concentrations adjusted to 1µg/ml and the oligonucleotide solutions stored at -20°C prior to their use for sequencing (see section 2.2.3.10).

2.2.3.3 Enzymatic manipulation of DNA.

2.2.3.3.A Restriction digests.

Small quantities of plasmid DNA, typically 1µg, were digested using 1-10 units of enzyme, (depending on the enzyme used and the number of restriction sites within the plasmid) in a total volume of 20µl which was buffered with buffer concentrates supplied by the manufacturers of the enzymes, (the volume of restriction enzyme solution never exceeded $^1/_{10}$ of the total volume). Digests were carried out at 37°C for 1-2hrs and were terminated by the addition of $^1/_6$vol of DNA gel loading buffer (0.25% bromophenol blue, 30% glycerol in water). Double digests were also performed using a suitable buffer concentrate, chosen on the basis of the manufacturers product information, and for larger scale preparative digests the volumes used were increased in proportion. Genomic DNA digests were carried essentially as described above, except 20µg of genomic DNA was digested overnight at 37°C.

2.2.3.3.B Dephosphorylation of 5' phosphate groups from plasmid DNA.

The dephosphorylation of 5' phosphate groups from 10ng linearised plasmid DNA with protruding ends which had been extracted with phenol/chloroform (see section 2.2.3.5.B) and resuspended in a minimum volume of 10mM Tris (pH 8.0) was achieved by incubating the linearised plasmid DNA with 0.1unit of calf intestinal alkaline phosphatase (CIP) in 1x CIP buffer (10x CIP buffer is 10mM $MgCl_2$, 1mM $ZnCl_2$, 10mM spermidine, 0.5M Tris, pH 9.0) for 30 minutes at 37°C. The CIP was then inactivated by heating the solution at 75°C for 10min. The dephosphorylated linear plasmid DNA was phenol/chloroform extracted and ethanol precipitated to recover the DNA. Diluted dephosphorylated linear plasmid DNA was then used for ligations.

2.2.3.3.C Ligation of DNA fragments.

Ligations of dephosphorylated vector (10-20ng in TE) and purified insert DNA (2-10 fold molar excess, in TE) were carried out using T4 DNA ligase (1µl) and 5x ligation buffer (4µl; supplied by the manufacturer of the enzyme) in a total volume of 20µl. Reactions were set up on ice, incubated overnight at 12-14^{o}C and diluted ligation mix used immediately to transform bacteria (see section 2.2.3.1). The unused ligation mix was stored at -20^{o}C.

2.2.3.4 Agarose gel electrophoresis.

Non-denaturing agarose gels of 0.8-1.2% were run to separate DNA fragments of >0.5kb. Gels were prepared by dissolving the agarose in either 50ml (mini) or 200ml (maxi) of 1x TAE (50x TAE buffer is 2M Tris base, 50mM EDTA, 57.1g/l glacial acetic acid, pH 8.15; for resolving DNA fragments for genecleaning) or 1x TBE (5x TBE is 0.45M Tris, 0.45M boric acid, 12.5mM EDTA, pH 8.3; for rapid resolution of DNA fragments) by heating until the solution boiled and the agarose dissolved. The gel mix was then allowed to cool to approximately 50^{o}C and ethidium bromide (10mg/ml) added to 1µg/ml. The gel was cast and allowed to set at room temperature before the comb was removed and the gel placed in the gel tank. Electrophoresis was carried out in the same buffer used to form the gel, either 1x TAE or TBE buffer at 1-5V/cm. The DNA samples (typically 1µg/10µl) and DNA standards (Boehringer Mannheim) for electrophoresis were mixed with $^{1}/_{6}$vol of DNA gel loading buffer, loaded and resolved by electrophoresis. On completion of electrophoresis the size of the DNA fragments was assessed by illuminating the gel on a 312nm transilluminator and comparing to the positions of the DNA standards. A photographic record of the UV illuminated gel was taken using a Polaroid camera on Polaroid Type 57 high speed film.

2.2.3.5 Purification and quantitation of nucleic acids.

2.2.3.5.A Recovery of DNA fragments from agarose gels.

DNA fragments of greater than 200bp were purified from TAE gels by "Genecleaning" (Bio 101 Inc., Stratech) according to the manufacturers instructions. Briefly, the desired band was excised from the gel under UV illumination and excess agarose removed. This gel piece was weighed, transferred to an Eppendorf tube and $2^1/_2$vol of NaI added. The tube was repeatedly heated to 55^{o}C for 2min until the agarose melted. 5µl of the manufacturers GLASSMILK solution was then added and the solution incubated at 4^{o}C for 5min with frequent mixing. The GLASSMILK was subsequently pelleted by centrifugation in a microfuge and the supernatant decanted. 450µl of manufacturers NEW wash buffer was added and the pellet resuspended by vortexing. The GLASSMILK was then pelleted again by centrifugation and fresh NEW wash buffer added. This washing procedure was repeated twice more and care taken to remove all NEW wash buffer after the final spin. The GLASSMILK pellet was then resuspended in 10µl of water, heated to 55^{o}C for 5min and pelleted by centrifugation. The supernatant containing the desired DNA fragment was transferred to a fresh Eppendorf tube and the pellet resuspended in water again. The previous step was then repeated and the supernatants pooled. 1µl of this solution ($^1/_{20}{}^{th}$ of the total) was then run on a mini gel to confirm the successful purification of the desired fragment.

2.2.3.5.B Purification of nucleic acids by phenol chloroform extraction.

Occasionally it was necessary to purify nucleic acids by extraction with phenol/chloroform in order to remove contaminants, such as enzymes, which might otherwise interfere with subsequent cloning steps. The method used was essentially that described by Maniatis et al (1989) and is as follows:

1) The DNA sample was mixed with an equal volume of phenol/chloroform, (freshly prepared from an equal volume of TE pH8.0 saturated phenol and chloroform/isoamyl alcohol, 24:1) until an emulsion formed.

2) The phases were then separated by centrifugation for 3min in a microfuge.

3) The aqueous phase was removed and the extraction repeated if necessary. The aqueous phase was then extracted with an equal volume of chloroform/isoamyl alcohol (24:1). The aqueous phase was collected again and transferred to a fresh Eppendorf tube. The DNA was then recovered by ethanol precipitation as described in section 2.2.3.5.C.

2.2.3.5.C Concentration of nucleic acids.

Ethanol precipitation was used to concentrate both DNA and RNA. The method used was essentially that described by Maniatis et al (1989) and is as follows:

1) To the aqueous nucleic acid solution $^{1}/_{10}$vol of 3M NaAc (pH5.2) and 2-2.5vol of ethanol were added.

2) The sample was then mixed well and the nucleic acid precipitated by chilling at -20^{o}C for 30min.

3) The precipitated nucleic acid was collected by centrifugation in a microfuge for 15min at 4^{o}C.

4) The supernatant was discarded, the pellet washed with 70% alcohol and briefly dried before resuspension in dH_2O or TE buffer at an appropriate concentration.

2.2.3.5.D Quantitation of nucleic acids.

The concentration of nucleic acid in a solution was determined spectrophotometrically by first calibrating the spectrophotometer using a dH_2O blank and then diluting the samples in dH_2O and reading the absorbency at wavelengths 260 and 280nm in a quartz cuvette with a pathway of 1cm. For plasmid and genomic DNA samples an A_{260} of 1 was taken to correspond to 50µg/ml, for oligonucleotides an A_{260} of 1 was taken to correspond to 20µg/ml, and for RNA an A_{260} of 1 was taken to correspond to 40µg/ml. The ratio of A_{260}/A_{280} ratio was also used as an measure of the contamination of the sample with protein and/or phenol. A_{260}/A_{280} values of 1.8-2.0 were taken to indicate that the preparation was sufficiently pure, lower values indicated the presence of contamination and the sample, if important was further purified by extraction/precipitation as described in sections 2.2.3.5.B/C.

2.2.3.6 Radiolabelling and purification of DNA probes.

DNA probes were prepared by random priming of appropriate DNA restriction fragments using a commercially available kit from Pharmacia and following the manufacturers instructions. The method is based on the hybridization of all possible hexanucleotides to the denatured DNA to be labelled. The complementary strand is synthesized from the free hydroxyl of the random hexanucleotide primer using the Klenow enzyme and the radioisotope (typically α[32P] dCTP) is incorporated into the newly synthesized cDNA strand during the course of the 1hr incubation. Unincorporated nucleotides are removed from the probe by purification on a column of Sephradex-G50 beads purchased from Pharmacia (Nick columns), and probes thus prepared were used to detect specific membrane bound nucleic acids as described in section 2.2.3.9.

2.2.3.7 Southern blotting.

Typically 20µg of restriction enzyme digested genomic DNA (see section 2.2.3.3.A) was run overnight on a 0.8% agarose TBE gel at a constant 25V (as described in section 2.2.3.4). This DNA was then denatured by soaking the suitably trimmed gel in several volumes of 1.5M NaCl and 0.5M NaOH for 1hr at room temperature. The denaturing solution was subsequently neutralized by soaking the gel for 1hr in several volumes of 1M Tris (pH 8.0) and 1.5M NaCl. After neutralization the DNA was transferred to Hybond N+ membrane by overnight capillary transfer with 20x SSC blotting buffer (3M NaCl, 0.3M Na citrate, pH 7.0) according to the membrane manufacturers instructions. After blotting the membrane was briefly washed in 2x SSC buffer to remove adhering agarose, dried at 80^{o}C for 10min and the DNA UV-crosslinked to the nylon membrane using a UV Stratalinker 1800 (Stratagene). This membrane was then probed as described in section 2.2.3.9.

2.2.3.8 Northern blotting.

10µg of total RNA (see section 2.2.3.2.A) and 6µl of RNA markers (B.R.L.) were ethanol precipitated, washed with 70% ethanol and briefly dried. The RNA was then resuspended in 25µl of MIX (6.7% formaldehyde, 50% formamide, 1x MOPS; 20x MOPS is 0.2M MOPS, 50mM NaAc, 10mM EDTA, pH7.0) and incubated for 15min at 65^{o}C. After this incubation the samples were cooled at 4^{o}C and 5µl of gel loading buffer (50% glycerol, 50mM EDTA, 0.45% bromophenol blue) added. To each sample 1µl of ethidium bromide (0.5 mg/ml) was also added. The RNA samples were then loaded onto a 1.2% agarose gel containing 2.2M formaldehyde and 1x MOPS buffer, and run overnight (at 20mA constant current) in 1x MOPS buffer which was continuously recirculated. On completion of electrophoresis the dye front had typically migrated 8-10cm, the

distance that the RNA markers and the ribosomal RNA in the sample lanes had migrated was determined by illuminating the gel on a 312nm transilluminator. A photographic record of the UV illuminated gel was also taken with a Polaroid camera on Polaroid Type 57 high speed film. The gel was then trimmed to remove the marker lane and transferred to Hybond N+ membrane by overnight capillary transfer with 20x SSC blotting buffer (3M NaCl, 0.3M Na citrate, pH 7.0) according to the membrane manufacturers instructions. After blotting the membrane was briefly washed in 2x SSC buffer to remove adhering agarose, dried at 80^{o}C for 10min and the RNA UV-crosslinked to the nylon membrane using a UV Stratalinker 1800 (Stratagene). The membrane was then probed as described in section 2.2.3.9.

2.2.3.9 Hybridization of DNA probes to membrane bound nucleic acids.

Radiolabelled DNA probes (see section 2.2.3.6) were used to detect specific DNA or RNA species which had been immobilised onto Hybond N+ membranes. The membranes were prehybridized at 42^{o}C for at least 30min in a sealed polythene bags containing 15-25ml of SSPE hybridization buffer (50% formamide, 0.5% SDS, 5x SSPE [20x SSPE is 3M NaCl, 0.2M $NaH_2PO_4.H_2O$, 0.8M EDTA pH7.4], 5x Denhardt's [100x Denhardt's is 1% bovine serum albumin, 1% Ficoll, 1% polyniyl pyrolidone, 0.5% SDS in dH_2O]) containing 30-50µl of boiled salmon sperm DNA (10mg/ml). This prehybridization solution was then replaced with an equal volume of fresh SSPE hybridization buffer containing 30-50µl of boiled salmon sperm DNA (10mg/ml) and a freshly boiled radiolabelled DNA probe, and hybridization were carried out overnight at 42^{o}C in a shaking water bath. The hybridization solution was then carefully disposed of by pouring into a designated sink and the membranes were washed at high stringency as described below, although the final wash (c) was often omitted if

monitoring of the membrane (with a Geiger counter) after wash (b) revealed a low number of background counts.

a) 2x 15min at 65^{o}C in 2x SSPE, 0.1% SDS.

b) 1x 30min at 65^{o}C in 0.5x SSPE, 0.1% SDS.

c) 1x 30min at 65^{o}C in 0.1x SSPE, 0.1% SDS.

The membranes were then drained to remove excess fluid, wrapped in Sarron wrap, placed in a suitable cassette with X-ray film and subjected to autoradiography at -70^{o}C.

2.2.3.10 Sequencing of double stranded DNA.

4µg of plasmid DNA in 18µl of dH_2O was first denatured by the addition of 2µl of 2M NaOH/20mM EDTA and incubating for 30min at 37^{o}C. This denatured DNA was then precipitated by adding 2µl of 3M NaAc and 60µl of ethanol, and incubating for 15min at -70^{o}C. The precipitated DNA was pelleted by centrifugation in a microfuge at 4^{o}C for 15min, and then washed with 70% ethanol and dried briefly. The pellet was then resuspended in 7µl of dH_2O. 1µl of oligonucleotide sequencing primers (50ng/µl; described in table 3) and 2µl of "Sequenase" reaction buffer (U.S.B. "Sequenase" kit) added to the denatured DNA solution. Annealing was achieved by incubating this mixture at 37^{o}C for 15-30min. The sequencing reactions were then performed using the reagents provided in the "Sequenase" version 2.0 kit (U.S.B.) according to the manufacturers instructions. The terminated, labelled fragments were resolved on 6% polyacrylamide denaturing gels (containing 42% urea) at 50W for 2-4hrs. The gels were fixed in a solution of 12% methanol and 10% acetic acid for 1hr at room temperature and then dried for approximately 2hr at 80^{o}C under vacuum, before autoradiography overnight at -70^{o}C.

Table 3. Oligonucleotide sequencing primers.

Table 3.

Oligo M13-20		5′ GTAAAACGACGGCCAGT 3′		
Oligo B (E-cad)	+2899	5′ TTGAGCTATGATCTGCC 3′	+3005
Oligo C (Cx43)	+23	5′ AGTGCTGATACCTCGTA 3′	+7
Oligo D (P-cad)	+3006	5′ CTGACATTTCTTGAGGC 3′	+3022
Oligo E (E-cad)	+184	5′ ATCTGGAGCCTTGACTT 3′	+168
Oligo J (Cx43)	+1355	5′ GCTTGAGCATCGAGCTG 3′	+1377
Oligo L (P-cad)	+265	5′ CGTATTACTACAGAGGT 3′	+249
Oligo P (P-cad)	+415	5′ ACTCGACTAAGTCGGAG 3′	+399

2.2.4 Protein procedures.

2.2.4.1 Immunofluorescence.

Subconfluent or occasionally confluent cultures of cells grown on sterile glass 16mm coverslips, (as described in section 2.2.1.1) were washed carefully with sterile PBS twice and either fixed by immersing in ethanol at -20^{o}C for 20min (cadherin and N-CAM antibody immunofluorescence analysis) or by immersing in 1% formaldehyde (freshly prepared from paraformaldehyde) in PBS (pH 7.4) for 1hr at room temperature (connexin 43 antibody immunofluorescence analysis). The coverslips were then air dried and processed as described below (all steps being performed at room temperature):

a) Block.	Wash 30 min with PBS containing 5% Marvel. For connexin 43 immunofluorescence analysis 0.2% Triton was also added to the milk solution.
b) 1^{o} antibody.	Incubate for 1hr with primary antibody (50-100µl per coverslip) diluted as appropriate in PBS containing 5% Marvel.
c) Wash.	Wash 4x 5min with PBS containing 5% Marvel
d) 2^{o} antibody	Incubate for 30min with appropriate FITC conjugated secondary antibody diluted 1:20 in PBS containing 5% Marvel.
e) Wash.	Wash 4x 5min with PBS containing 5% Marvel
f) Mount.	Drain coverslips, air dry at room temperature, and then mount inverted on a drop of Vectastain's anti-fade mountant and seal with clear nail varnish.
g) View.	The slides were examined using a Leitz Orthoplan microscope with UV epifluorescence or visible light sources, and photographed.

2.2.4.2 Protein preparation.

Protein samples were prepared from confluent 90mm plates of approximately 10^6-10^7 cells either by procedure (a) for cadherin and N-CAM western analysis, or procedure (b) for connexin 43 western analysis.

a) Detergent lysis.

Cell cultures were washed 3x with ice cold PBS and incubated on ice with 1ml of lysis buffer (100mM NaCl, 50mM Tris pH7.4, 1% Triton, 0.1% SDS, 0.5% Na deoxycholate, 2mM PMSF, 1% aprotinin). The plates were then scraped, the lysis buffer collected and transferred to an Eppendorf tube and centrifuged in a microfuge for 5min at 4^{o}C. The supernatant was then aliquoted into fresh tubes and stored at -20^{o}C.

b) Alkali lysis.

Cell cultures were washed 3x with ice cold PBS and 1ml of lysis buffer (1mM $HaHCO_3$, 1% aprotinin, 1mM PMSF, 1mM NaF, 1mM Na_3VO_4) added. The plates were then scraped, the lysis buffer collected, transferred to an Eppendorf tube and centrifuged in a microfuge for 10min at 4^{o}C. The supernatant was then discarded and the pellet stored at -20^{o}C.

2.2.4.3 Western analysis

Protein samples were prepared as described above and sodium doecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) utilised to resolve the proteins according to their molecular weight. 8cm and 15cm gels were employed to resolve cell adhesion molecule and connexin samples. Resolving gels (8% acrylamide/*bis*-acrylamide mix) were prepared with the following solutions, (values given are for two 15cm gel, for 8cm gels the proportion of the ingredients used was the same but the amount prepared was less):

30% acrylamide/*bis*-acrylamide mix	26.6ml
1.5 M Tris pH8.8	25.0ml
10% SDS	1.0ml
dH_2O	26.4ml
10% ammonium persulphate	1.0ml
TEMED	0.2ml

This resolving gel mix was poured between two glass plates, overlaid with isopropanol and allowed to polymerise for 1hr. The isopropanol was then removed using Whatman 3MM paper, the stacking gel mix added and the comb inserted. The stacking gels (4% acrylamide/*bis*-acrylamide mix) were prepared with the following solutions, (values given are for two 15cm gel, for 8cm gels the proportion of the ingredients used was the same but the amount prepared was less):

30% acrylamide/*bis*-acrylamide mix	2.6ml
1.0 M Tris pH 6.8	2.0ml
10% SDS	0.2ml
dH_2O	14.4ml
10% ammonium persulphate	0.2ml
TEMED	20.0µl

The stacking gel was allowed to polymerise for 1hr, before transfer to an electrophoresis tank and careful removal of the comb. 1x Tris-glycine electrophoresis buffer (5mM Tris, 50mM glycine pH8.3, 0.02% SDS in dH_2O) was then added, sufficient to fill both reservoirs and to cover the top of the gel plates. The wells were then flushed with 1x tank buffer by repeated pipetting and any bubbles which formed at the bottom of the gel plates were removed.

Approximately 20µg of cell adhesion protein samples, (as determined by protein concentration estimations using Sigma's Bicinchroninic acid kit) and 10µg

of prestained protein markers (30kD-200kD; Amersham) were aliquoted into Eppendorf tubes and sufficient dH_2O added to give a total volume of 30µl. An equal volume of 2x SDS gel loading buffer (4% SDS, 0.2% bromophenol blue, 20% glycerol, 1% β-mercaptoethanol, 100mM Tris pH 6.8) was added and the tubes placed into a boiling water bath for two minutes prior to loading onto the gel. The connexin samples (2.2.4.2.b) were prepared by resuspending each pellet in 250µl of 2x SDS gel loading buffer at 100^{o}C and immediately loaded onto the gel, (the amounts of protein were estimated from Comassie blue stained gels after electrophoresis).

The loaded proteins were then resolved by electrophoresis at a constant current of 30mA/gel. After the dye front had migrated to the bottom of the gel one gel was then stained for 2-3hr with Comassie blue stain (2.5g Comassie brilliant blue, 100ml glacial acetic acid, 450ml methanol, 450ml dH_2O) and then incubated with destain (100ml glacial acetic acid, 450ml methanol, 450ml dH_2O) until the protein bands were visible, in order to confirm equal loading. The other gel was wet blotted overnight in Towbin buffer (25mM Tris, 200mM glycine, 20% methanol, pH 8.3) using a Biorad Trans-Blot Cell according to the manufacturers instructions, in order to transfer the proteins onto a nitrocellulose membrane. The membrane was then used for western analysis using primary antibodies (section 2.1.6) and reagents provided in the Amersham ECL kit, as described below:

a) Block. Incubate membrane for 30 min at room temperature with PBS containing 5% Marvel and 0.05% Tween.

b) 1^{o} antibody. Incubate membrane for 4hr at 4^{o}C with primary antibody diluted 1:30-250 as appropriate, in PBS containing 5% Marvel and 0.05% Tween.

c) Wash. Briefly rinse membrane and then incubate 1x 15min, 2x 5min at room temperature with PBS containing 5% Marvel and 0.05% Tween.

d) 2^{o} antibody. Incubate membrane for 30min at room temperature with appropriate horseradish peroxidase labelled secondary antibody diluted 1:2000-5000 in PBS containing 5% Marvel and 0.05% Tween.

e) Wash. Briefly rinse membrane and then incubate 1x 15min, 4x 5min at room temperature with PBS containing 0.05% Tween.

f) Detection. Incubate membrane with an equal volume of ECL detection reagents 1 and 2 for 1min at room temperature. Drain membrane, wrap in Saran wrap and place in a cassette with X-ray film. Expose for 1sec-10min.

Chapter 3.

Characterization of *in vitro* model systems.

3.1 Introduction.

The review of the literature presented in sections 1.5 and 1.6 suggests that ductin, connexins and cell adhesion molecules contribute to gap junction formation and/or function. This raises the question as to what role these proteins play, if any in determining the specificity of GJIC which has been observed between epithelial and fibroblast cells *in vitro* (Pitts and Burk 1976, Taylor-Papadimitriou et al 1976) and *in vivo* (Kam et al 1986).

Two cell culture systems have been used as models in order to investigate the molecular basis of gap junction formation and specificity, firstly *mec*$^-$ cell lines which have only a low frequency of gap junction formation and secondly *mec*$^+$ cell lines which show specificity of gap junctional communication when grown in mixed culture. The initial aim of the work was to characterize the cell lines used in this study, with respect to their GJIC ability and their expression of ductin, connexin, cadherin and N-CAM.

3.2 GJIC in the cell lines.

The microinjection of the fluorescent dye Lucifer Yellow CH (as described in section 2.2.2) into MCF-7 cells (human mammary tumour cell line), L cells (mouse fibroblast cell line) or S180 cells (mouse sarcoma cell line) in confluent cultures reveals that these cell types are poorly coupled via gap junctions (figure 4). The incidence and extent of dye coupling is very low for MCF-7 and L cells but slightly higher for S180 cells, (MCF-7 $0^+/-0$, L $0.04^+/-0.2$, S180 $2^+/-2.4$, mean number of dye coupled cells per injection $^+/-$S.D.; table 4) and this is consistent with previous reports (Pitts 1971, Fentiman and Taylor-Papadimitriou 1977, Mege et el 1988). It is possible that these *mec*$^-$ cell lines share a single

defect that prevents gap junction formation, although the evidence from other expression and cell fusion studies suggests that communication incompetence can be caused by the aberrant expression of different genes (MacDonald 1982, Jongen et al 1991, Veenstra et al 1992). The *mec*$^-$ cell lines therefore provide an *in vitro* model for identifying proteins required for gap junction formation.

Mixed cultures (section 2.2.1.1) of epithelial BRL cells (rat liver cell line) and either fibroblast BHK cells (hamster kidney cell line) or BICR cells (rat mammary carcinoma cell line) tend to sort out, forming separate domains in which one or other cell type predominates. This leads to the establishment of cultures that consist of islands of epithelial cells surrounded by channels of fibroblast cells (figure 5 and 6). The characteristic morphologies of the different cell types enables the boundaries between domains to be located and studied. The fluorescent dye Lucifer Yellow CH microinjected into either cell type close to the boundary usually spreads homologously to cells of the same type but detectable fluorescence in the other cell type, indicating that heterologous GJIC is occuring is a rare event, even when homologous dye spread near the border between the two cell types is extensive (figure 5 and 6). These domains of coupled cells have been termed communication compartments (Pitts and Kam 1985).

The extensive dye spreads within the BICR or BRL compartments are rapid, (dye can spread to >30 cells during a 2 minute injection) and quantitatively similar (BICR 32$^+$/-18.8, BRL 30$^+$/-19.7, mean $^+$/- S.D.; table 4), while dye spreads within the BHK compartments are more restricted (BHK 10$^+$/-7.6, mean $^+$/- S.D.; table 4). The dye spreads within the BICR and BRL domains in mixed culture are smaller than the spreads seen when Lucifer Yellow CH is microinjected into cells into separate confluent cultures of either cell type (BICR

51+/-20.7, BRL 59+/-14.6, mean +/- S.D.) and this reflects the restrictions imposed on dye spread in mixed cultures by the compartmental boundaries.

The frequency of heterologous dye transfer in mixed cultures of epithelial BRL cells and fibroblast BHK or BICR cells is very low, (detectable trans-boundary spread in 6% of injections in BRL/BHK mixed cultures, 3% of injections in BRL/BICR mixed cultures), which is in contrast to the frequency of homologous communication in either culture (100%; table 5). In mixed cultures of BRL and BHK cells, only 6% of injections into BRL cells and 5% of injections into BHK cells resulted in heterologous dye transfer. While in mixed cultures of BRL and BICR cells, 0% of injections into BRL cells and 7% of injections into BICR cells were positive for heterologous dye transfer. The lack of heterologous dye spread from BRL to BICR cells, (when BRL cells are the primary receiver) may be a consequence of the low frequency of heterologous communication combined with the number of experimental observations. There are no theoretical grounds for expecting that heterologous dye spread can only occur in a single direction, (i.e. from BICR to BRL cells but not from BRL to BICR cells) and only the combined values are described in subsequent analyses.

The dye coupling results for the BRL/BHK mixed cultures are similar to those reported previously by Pitts and Kam (1985). The specificity of gap junctional communication observed in BRL/BICR mixed cultures has not been reported before and provides a second *in vitro* model system for the analysis of the molecular basis of specificity. Interestingly mixed cultures of BHK and BICR cells do not sort out or show a specificity of gap junctional communication (figure 7). Lucifer Yellow CH microinjected into either cell type spreads homologously in 95% of injections and heterologously in 91% of injections (table 5). However, because it is harder to distinguish between the fibroblast cell types and as fewer microinjections were made into BHK cells this result should be treated with

Figure 4. Homologous dye transfer in cultures of L, MCF-7 or S180 cells.

(A), (C) and (E) are phase contrast images of the fluorescence micrographs shown in (B), (D) and (F). Lucifer Yellow CH microinjected into an L (B) or MCF-7 (D) cell does not spread to adjacent cells, whereas Lucifer Yellow CH microinjected into an S180 cell (F) spreads to an adjacent cell. The injected cells are marked with a black dot. Magnification x1000.

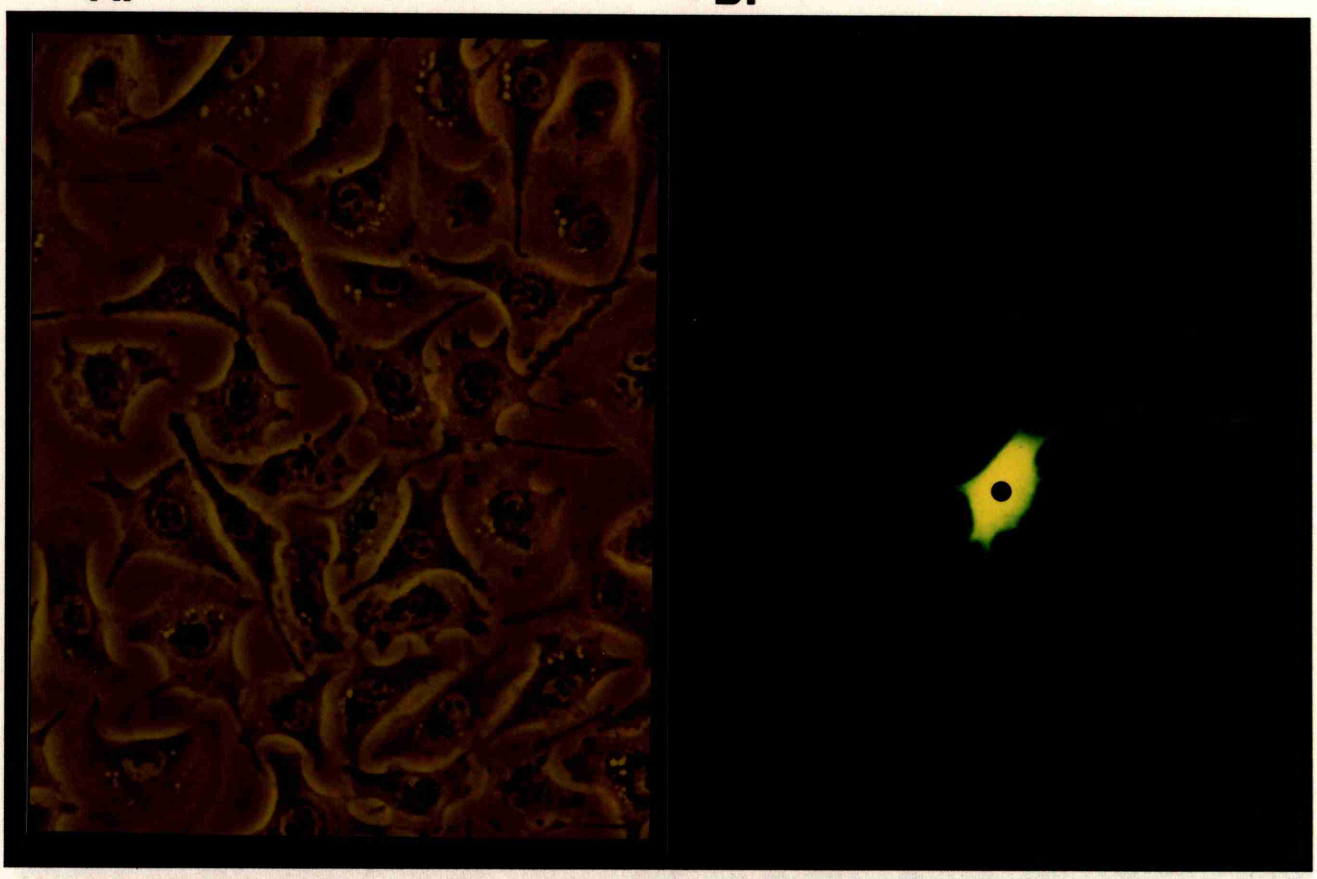
Figure 4.

Phase contrast.

Fluorescence.

A.

B.



C.

D.

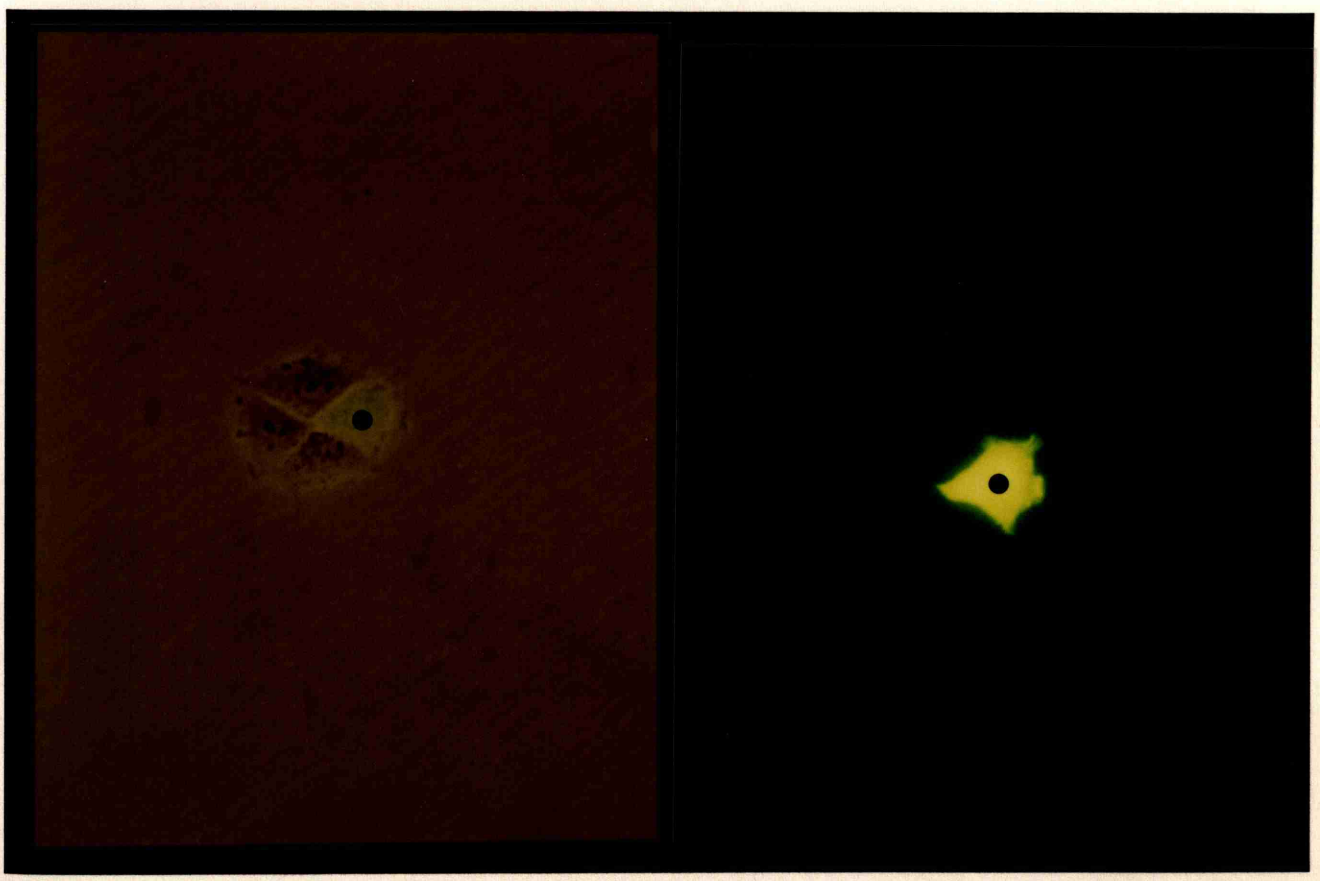


Figure 4.

Phase contrast. **Fluorescence.**

E. **F.**

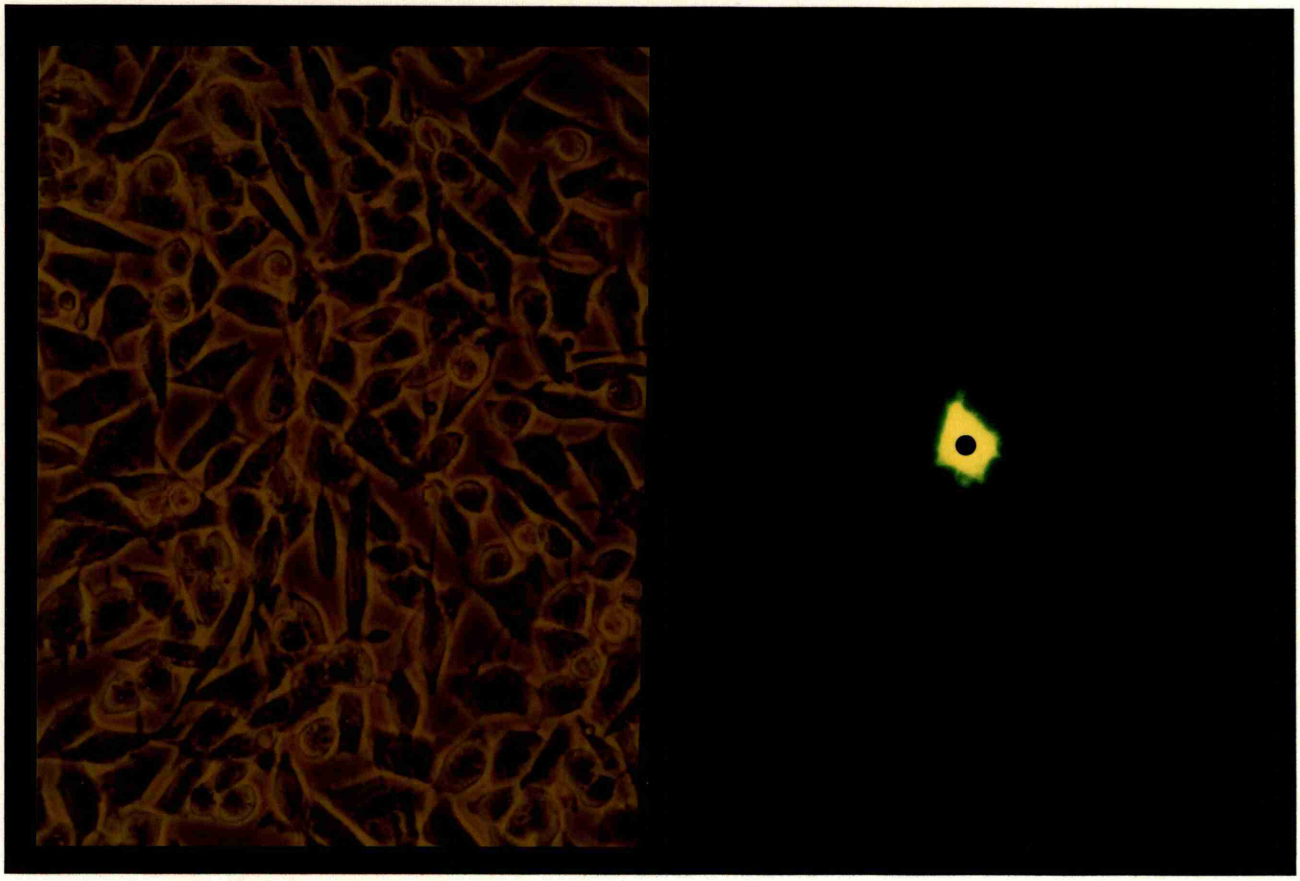


Table 4. Frequency of homologous dye transfer in cultures of MCF-7, L, S180, BHK, BICR and BRL cells.

Table 4.

Cell line	Number of injections.	Percentage positive.	Average number of coupled cells per injection, (S.D).
[1] MCF-7	26	0%	0 (0)
[1] L	55	4%	0.04 (0.19)
[1] S180	39	61%	2 (2.43)
[2] BHK	24	96%	10 (7.59)
[2] BICR	43	100%	32 (18.78)
[2] BRL	52	100%	30 (19.70)

[1] Homologous coupling in cultures of a single cell type.

[2] Homologous coupling in mixed culture.

Figure 5. GJIC in mixed cultures of BRL and BHK cells.

(A) and (C) are phase contrast images of the fluorescence micrographs shown in (B) and (D). Mixed cultures of BRL and BHK cells tend to sort out forming islands of epithelial BRL cells surrounded by channels of fibroblast BHK cells (A) and (C). Lucifer Yellow CH microinjected into a BRL cell spreads to other BRL cells within the island but not to the adjacent BHK cells (D), similarly Lucifer Yellow CH microinjected into a BHK cell spreads to other BHK cells but not to adjacent BRL cells (B). A specificity of GJIC occurs within these cultures. The injected cells are marked with a black dot. Magnification x1000.

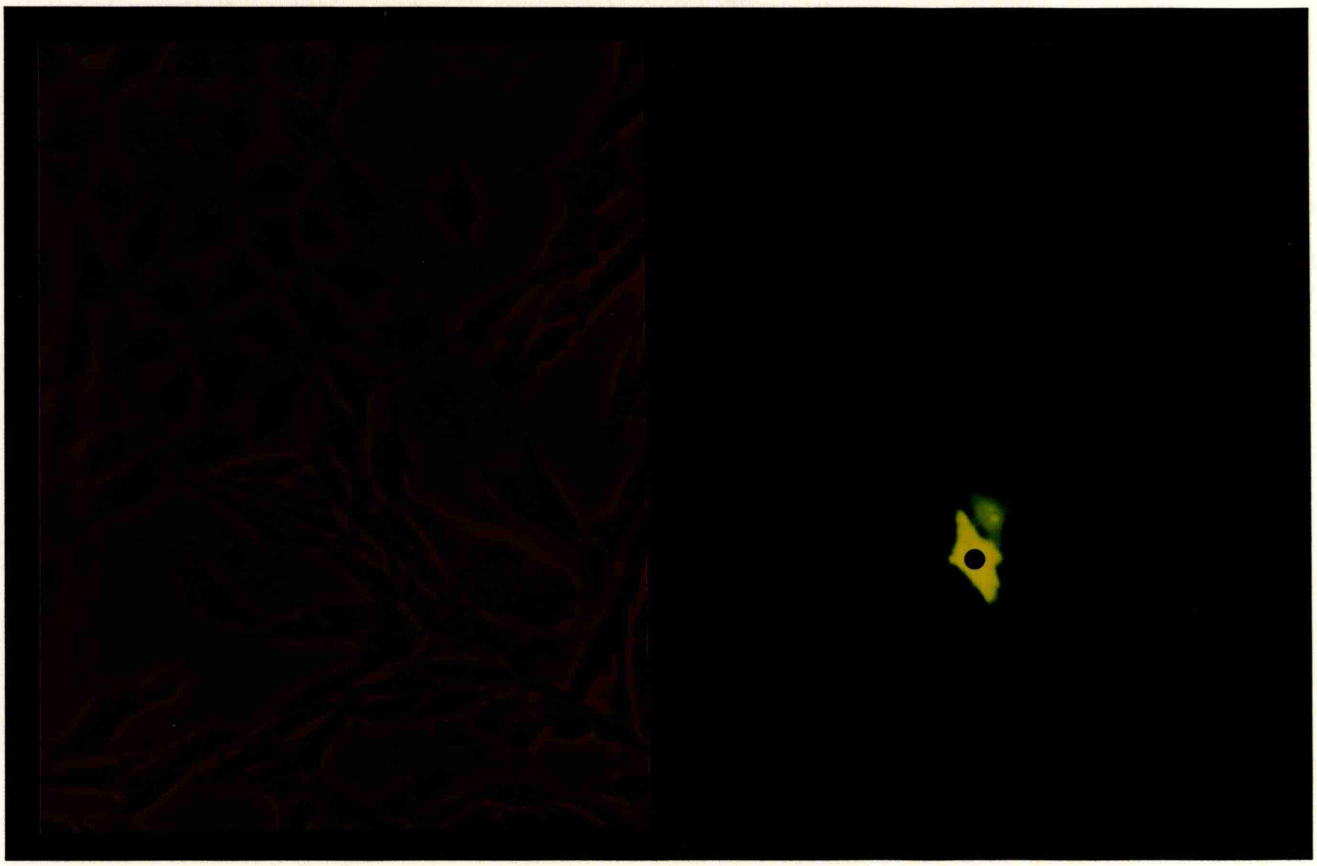
Figure 5.

Phase contrast.

Fluorescence.

A.

B.



C.

D.

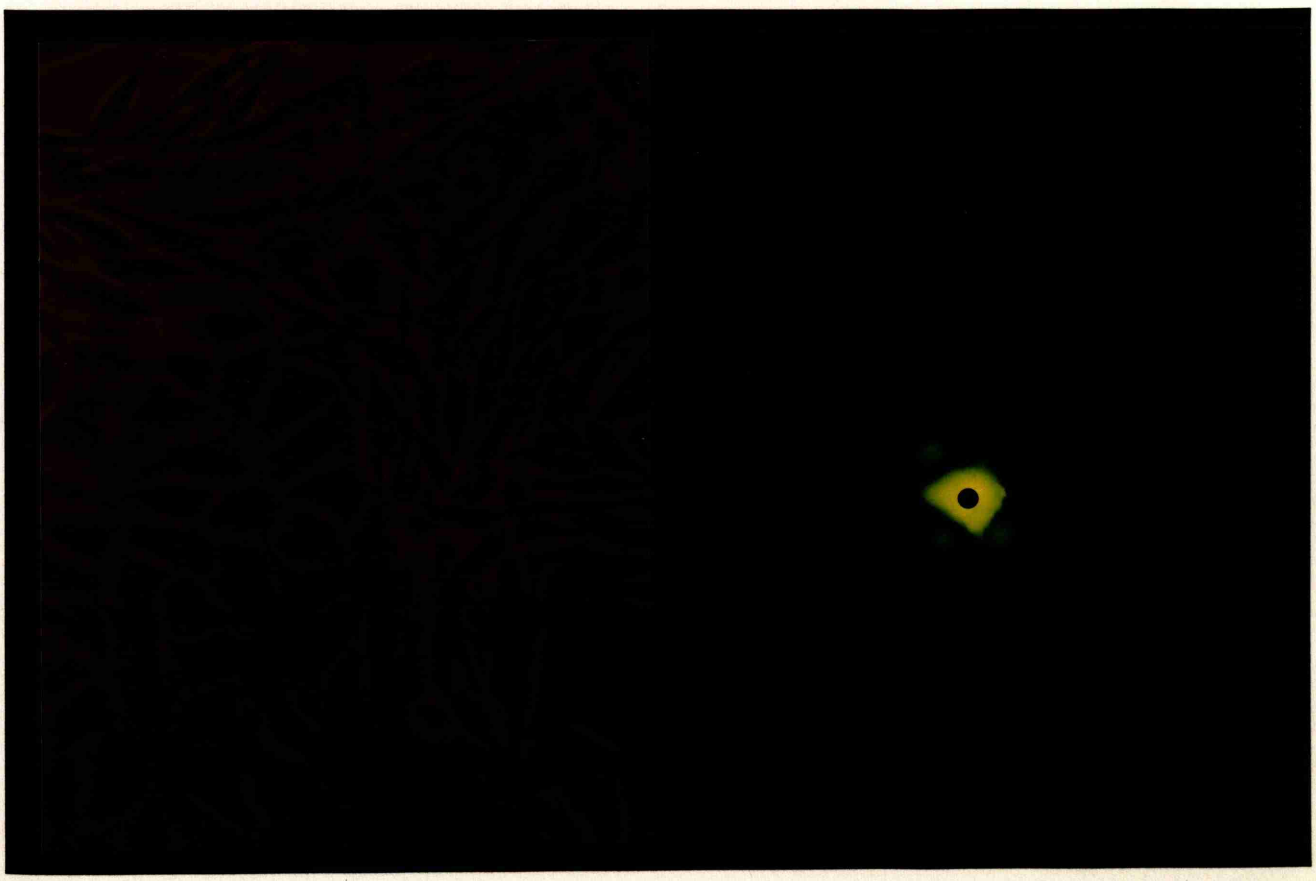


Figure 6. GJIC in mixed cultures of BRL and BICR cells.

(A) and (C) are phase contrast images of the fluorescence micrographs shown in (B) and (D). Mixed cultures of BRL and BICR cells were established. Lucifer Yellow CH microinjected into a BICR cell spreads to other BICR cells but not to the adjacent BRL cells (B), similarly Lucifer Yellow CH microinjected into a BRL cell spreads to other BRL cells but not to adjacent BICR cells, even when dye spread near the border between the two cell types is extensive (B,D). A specificity of GJIC occurs which causes the formation of communication compartments. The injected cells are marked with a black dot. Magnification x1000.

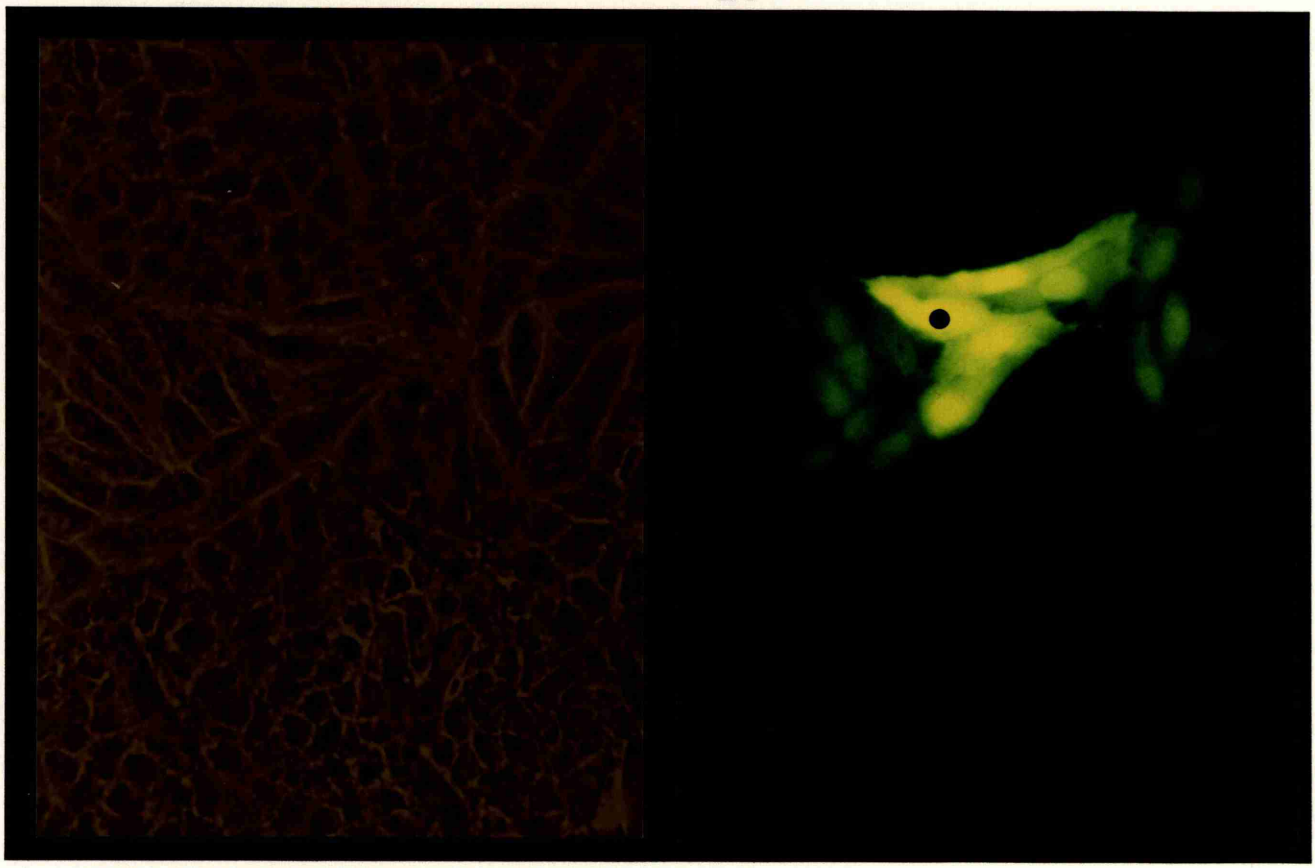
Figure 6.

Phase contrast.

Fluorescence.

A.

B.



C.

D.

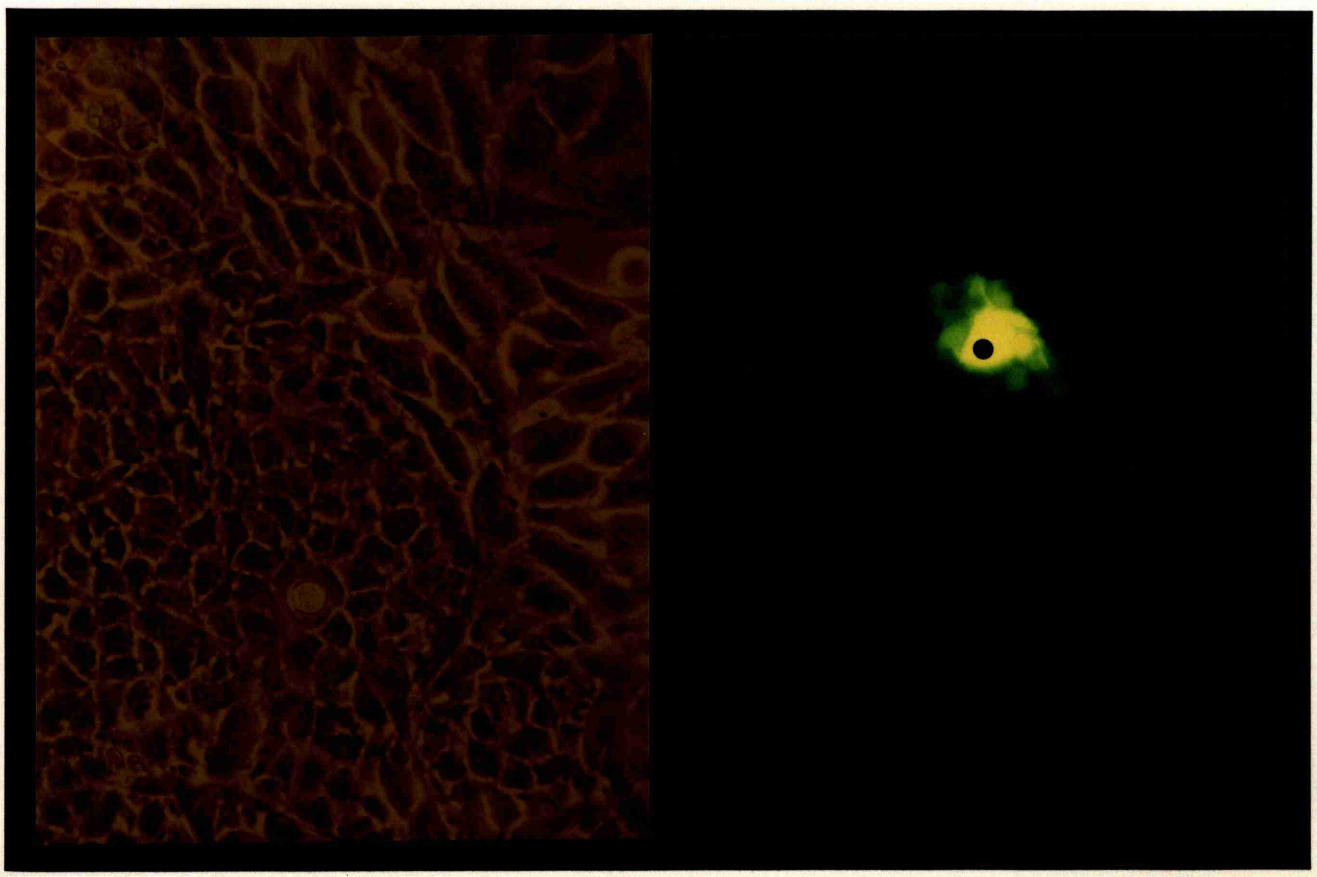


Figure 7. GJIC in mixed cultures of BHK and BICR cells.

(A) and (C) are phase contrast images of the fluorescence micrographs shown in (B) and (D). Mixed cultures of BHK and BICR cells were established. Lucifer Yellow CH microinjected into a BICR cell spreads to other BICR cells as well as to smaller BHK cells which are less well coupled (D), similarly Lucifer Yellow CH microinjected into a BHK cell spreads to other BHK cells as well as to adjacent BICR cells (B). The injected cells are marked with a black dot. Magnification x1000.

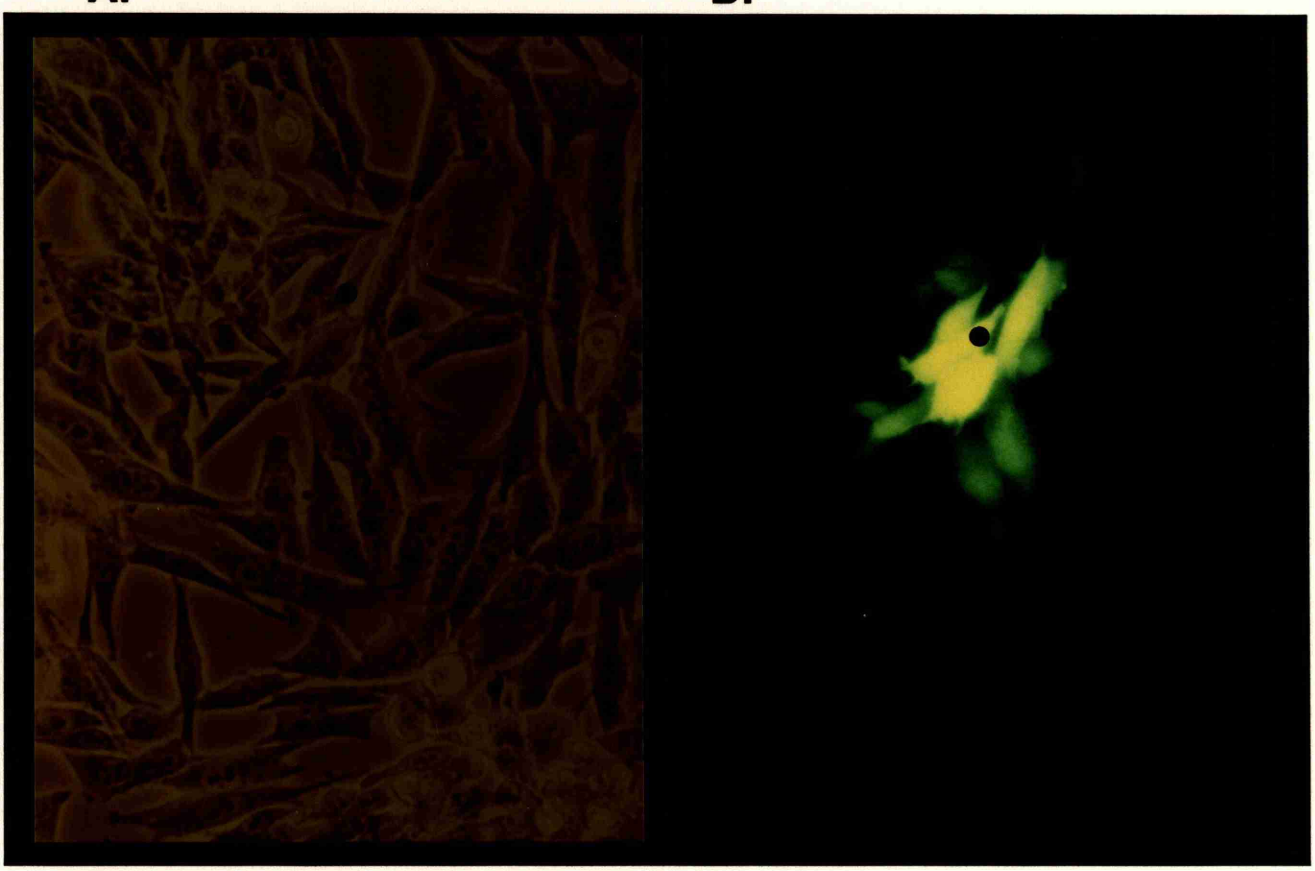
Figure 7.

Phase contrast.

Fluorescence.

A.

B.



C.

D.

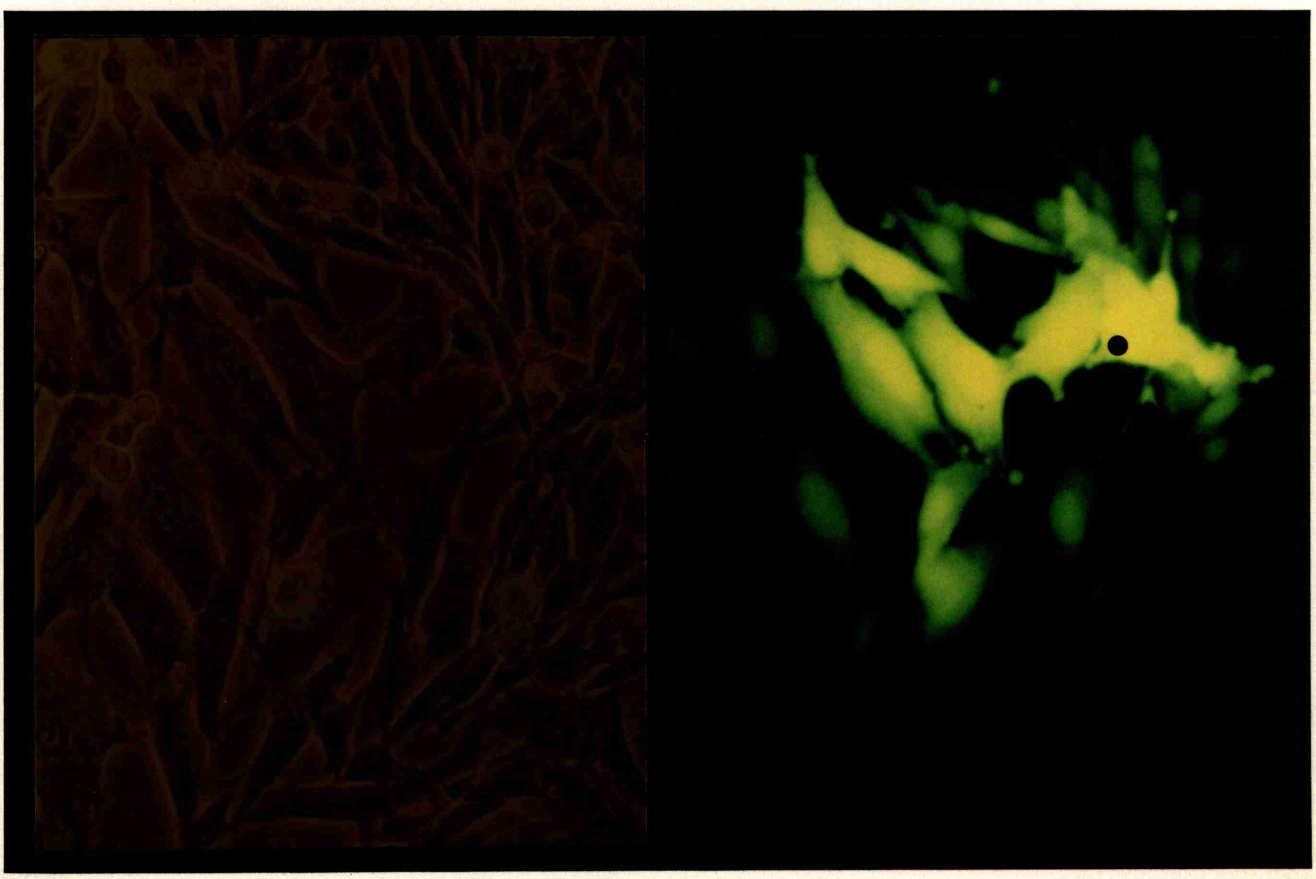


Table 5. Frequency of heterologous dye transfer in mixed cultures of BHK, BICR and BRL cells.

Table 5.

Cell types in mixed culture.	Injected cell.	Homologous dye transfer.	Heterologous dye transfer.
BRL/BHK.	BRL	17/17 (100%)	1/17 (6%)
	BHK	19/19 (100%)	1/19 (5%)
	BRL or BHK	36/36 (100%)	2/36 (6%)
BRL/BICR.	BRL	35/35 (100%)	0/35 (0%)
	BICR	27/27 (100%)	2/27 (7%)
	BRL or BICR	62/62 (100%)	2/62 (3%)
BHK/BICR.	BHK	4/5 (80%)	4/5 (80%)
	BICR	16/16 (100%)	15/16 (94%)
	BHK or BICR	20/21 (95%)	19/21 (91%)

caution, although it is consistent with the lack of specificity of GJIC previously reported in mixed cultures of different fibroblast cell types (Epstein and Gilula 1977).

3.3 Endogenous expression of ductin, connexin and cadherin in the cell lines.

a) Ductin.

The results obtained by northern analysis (as described in section 2.2.3.8) using a 0.8kb fragment of bovine ductin cDNA as a probe, show that all six of the cell lines endogenously express a 1.4kb ductin transcript (figure 8). A similar ductin transcript size is also reported to occur in *Nephrops* hepatopancreas and various rat tissues (Finbow et al 1992, Nezu et al 1992). It has previously been found that gap junction preparations, isolated by their resistance to triton X-100 from cultured BRL and BICR cells contain predominantly ductin (Finbow et al 1983, P.McLean personal communication) and this is consistent with the RNA expressed by the cell lines being translated. However, due to a lack of antibodies that recognise the mammalian form of ductin it is not possible to demonstrate that ductin protein localises to the plasma membrane at regions of close cell to cell contact between these cultured cells.

b) Connexin.

The levels of endogenous expression of connexin 32 and 43, representatives of the two major connexin sub-groups have been determined. The northern analysis of connexin 32 RNA expression, using a 1.5kb fragment of rat connexin 32 cDNA as a probe shows that none of the cell lines express detectable connexin 32 transcripts (figure 9). This result is surprising, given that the characteristic 1.7kb connexin 32 transcript is detected in mouse liver and the GAPDH control probe (a 0.7kb fragment of rat GAPDH cDNA) confirms that the

lanes are loaded evenly (figure 9), as it shows that the liver derived BRL cells do not express connexin 32 RNA. This result is consistent with western analysis which shows that BRL cells do not express detectable levels of connexin 32 protein (Dr S.Jamieson personal communication). The northern analysis of connexin 43 expression using a 1.4kb fragment of rat connexin 43 cDNA as the probe, shows that five (out of six) of the cell lines express a 3.0kb connexin 43 transcript, which is also detectable in mouse heart and kidney (figure 10). The autoradiograph of connexin 43 RNA expression shown in figure 10.C is over-exposed in order to show the lower level of connexin 43 expression in S180 cells, on shorter exposures a single 3.0kb band is also seen in the BHK, BICR and BRL lanes (figure 10.B). Western analysis (as described in section 2.2.4.3) using an antipeptide antiserum against amino acids 251 to 272 of connexin 43 (251-272) confirms that the connexin 43 mRNA expressed by the cell lines (L, S180, BHK, BICR and BRL) is translated (figure 11). A longer exposure is required to detect connexin 43 protein in the L cell lane and this also brings up non-specific binding to two larger proteins heavily over-expressed by MCF-7 cells (figure 11.B). The failure to detect connexin 43 protein in the MCF-7 lane is in agreement with the northern results. The level of connexin 43 protein expression by the cell lines correlates with their GJIC ability, poorly dye coupled cell lines (MCF-7 and L) express the least connexin 43 protein and well coupled lines (BICR and BRL) the most. In addition, the detection of two bands (of approximate molecular weight 43 and 45kD) in the BICR and BRL lanes (figure 11.A) is consistent with the presence of non-phosphorylated and phosphorylated forms of connexin 43 in these well coupled cells (Musil et al 1990a & b; Lau et al 1991). Immunofluorescence analysis (as described in section 2.2.4.1) confirms that the antipeptide antiserum (251-272) recognises connexin 43 in mouse heart sections (figure 12; Beyer et al 1989).

Figure 8. Northern analysis of ductin expression.

10μg of total cellular RNA prepared from the cell lines MCF-7, L, S180, BHK, BICR or BRL was separated on 1.2% agarose gels containing formaldehyde, blotted and probed with a 0.8kb fragment of bovine ductin cDNA, (as described in material and methods). The position of the 1.4kb ductin transcript and the ribosomal RNA are shown. The blot was subsequently reprobed with GAPDH (1.8kb transcript) as a loading control.

Figure 8.

Northern: Ductin.

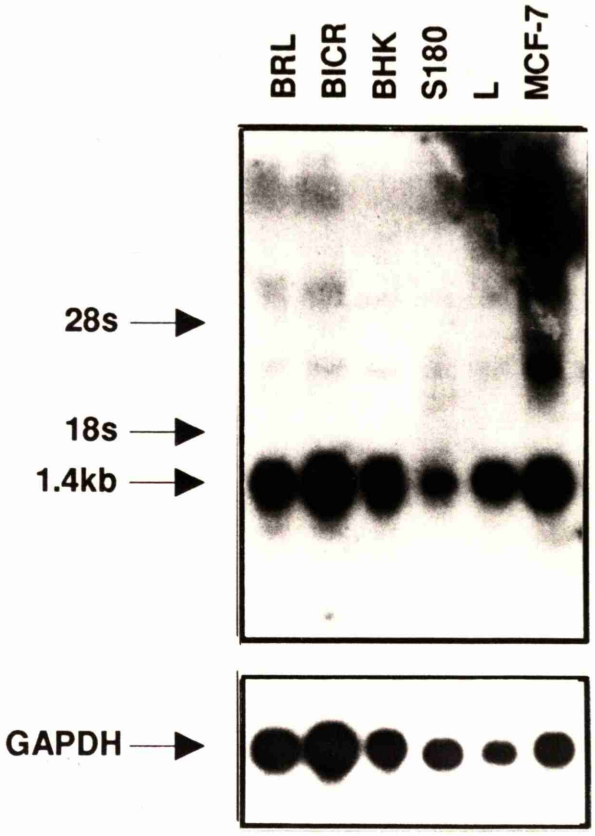


Figure 9. Northern analysis of connexin 32 expression.

10μg of total cellular RNA prepared from mouse liver and the cell lines MCF-7, L, S180, BHK, BICR or BRL was separated on 1.2% agarose gels containing formaldehyde, blotted and probed with a 1.5kb fragment of rat connexin 32 cDNA, (as described in material and methods). The position of the 1.7kb connexin 32 transcript and the ribosomal RNA are shown. The blot was subsequently reprobed with GAPDH (1.8kb transcript) as a loading control.

Figure 9.

Northern: Connexin 32.

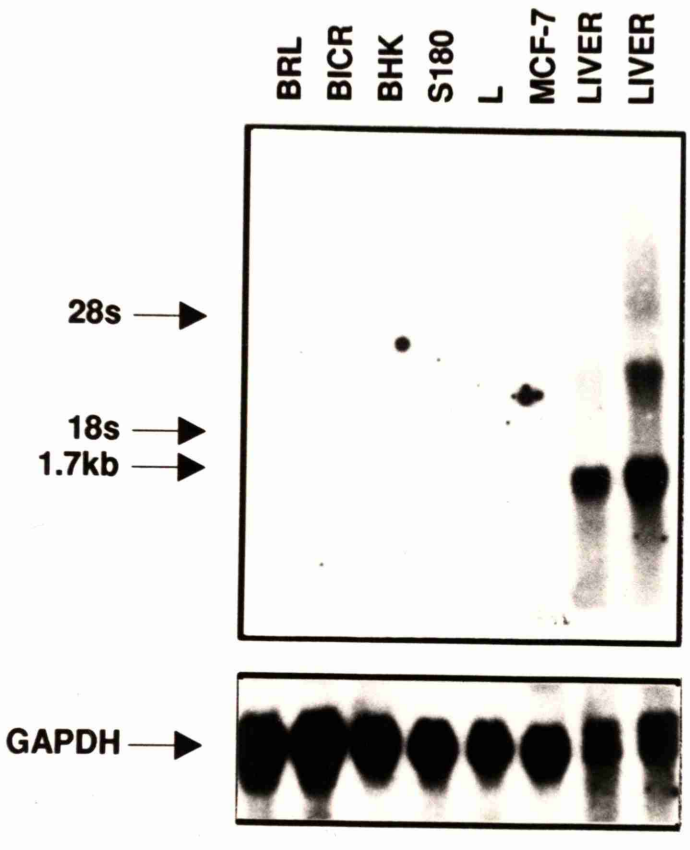


Figure 10. Northern analysis of connexin 43 expression.

10μg of total cellular RNA prepared from mouse kidney or heart, and the cell lines MCF-7, L, S180, BHK, BICR or BRL was separated on 1.2% agarose gels containing formaldehyde, blotted and probed with a 1.4kb fragment of mouse connexin 43 cDNA, (as described in material and methods). The positions of the 3.0kb connexin 43 transcript and the ribosomal RNA are shown. The expression of connexin 43 in kidney and heart is shown in (A), while the autoradiograph of connexin 43 expression shown in (C) is over-exposed in order to reveal low levels of connexin 43 expression. On shorter exposures a single 3.0kb band is also seen in the BHK, BICR and BRL lanes (C). The blots (B) and (C) were subsequently reprobed with GAPDH (1.8kb transcript) as a loading control.

Figure 10.

Northern: Connexin 43.

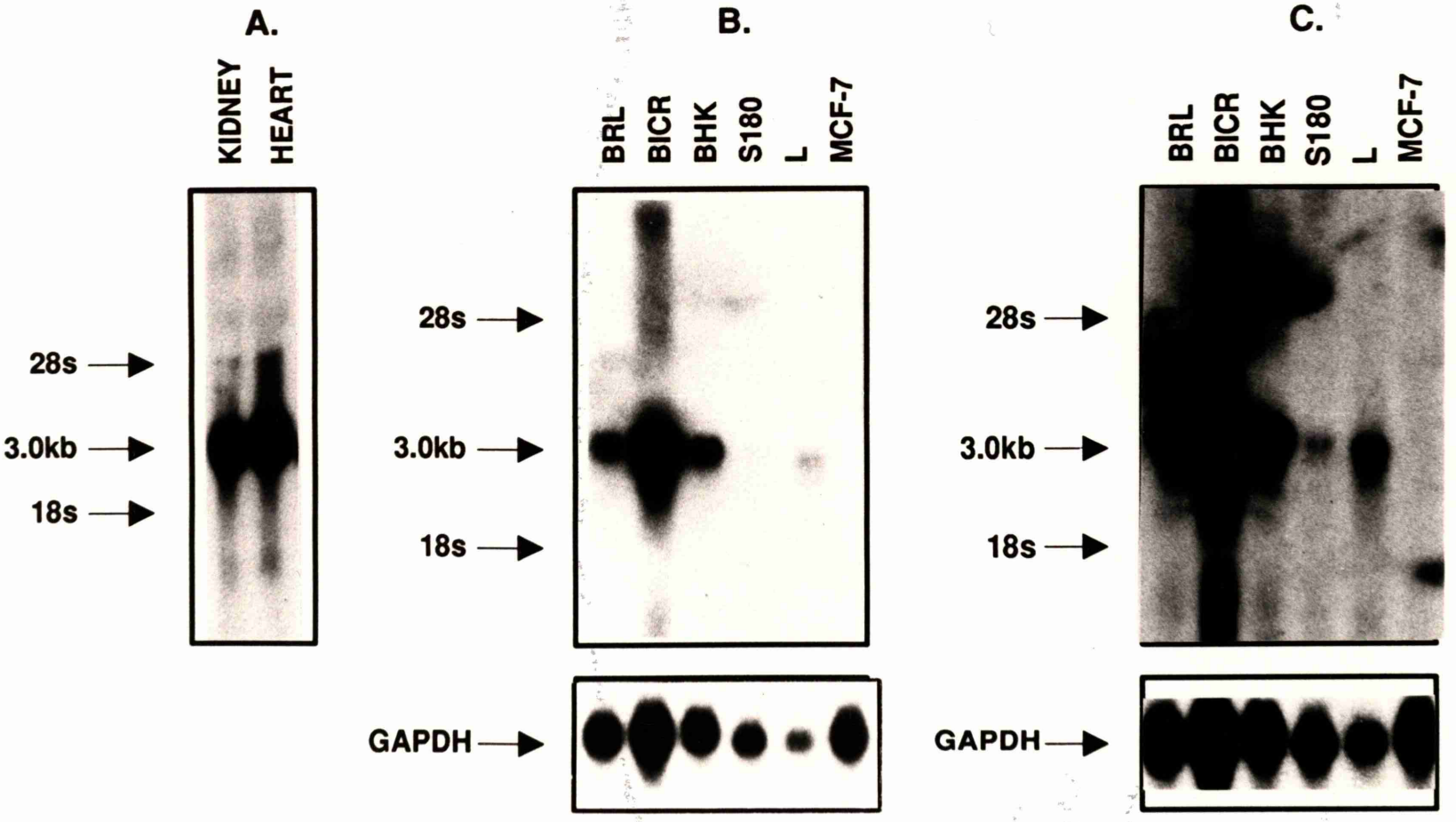
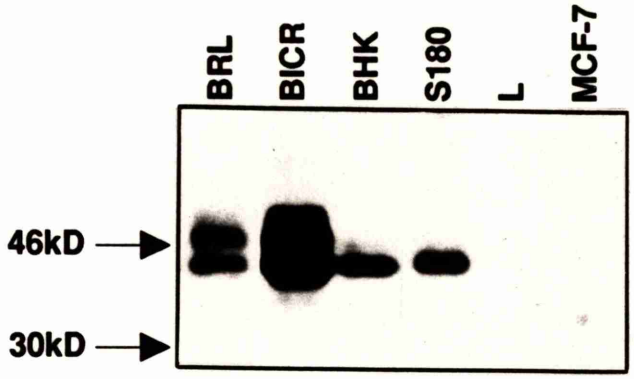


Figure 11. Western analysis of connexin 43 expression.

Protein samples were prepared from the cell lines and equivalent amounts of protein (approximately 20µg estimated from Coomassie blue stained gels) used for western analysis, as described in materials and methods. Primary antibody was rabbit anti-connexin 43 polyclonal "Petunia" at 1:500. The autoradiograph of connexin 43 expression shown in (B) is over-exposed in order to reveal low levels of connexin 43 expression. On shorter exposures a doublet is seen in BICR and BRL lanes (A).

Figure 11.

A. Western: Connexin 43.



B. Western: Connexin 43.

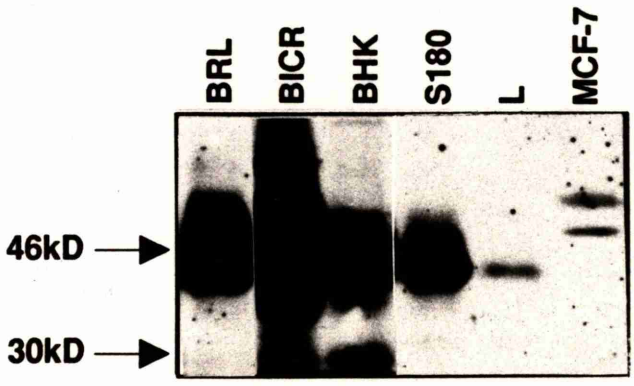


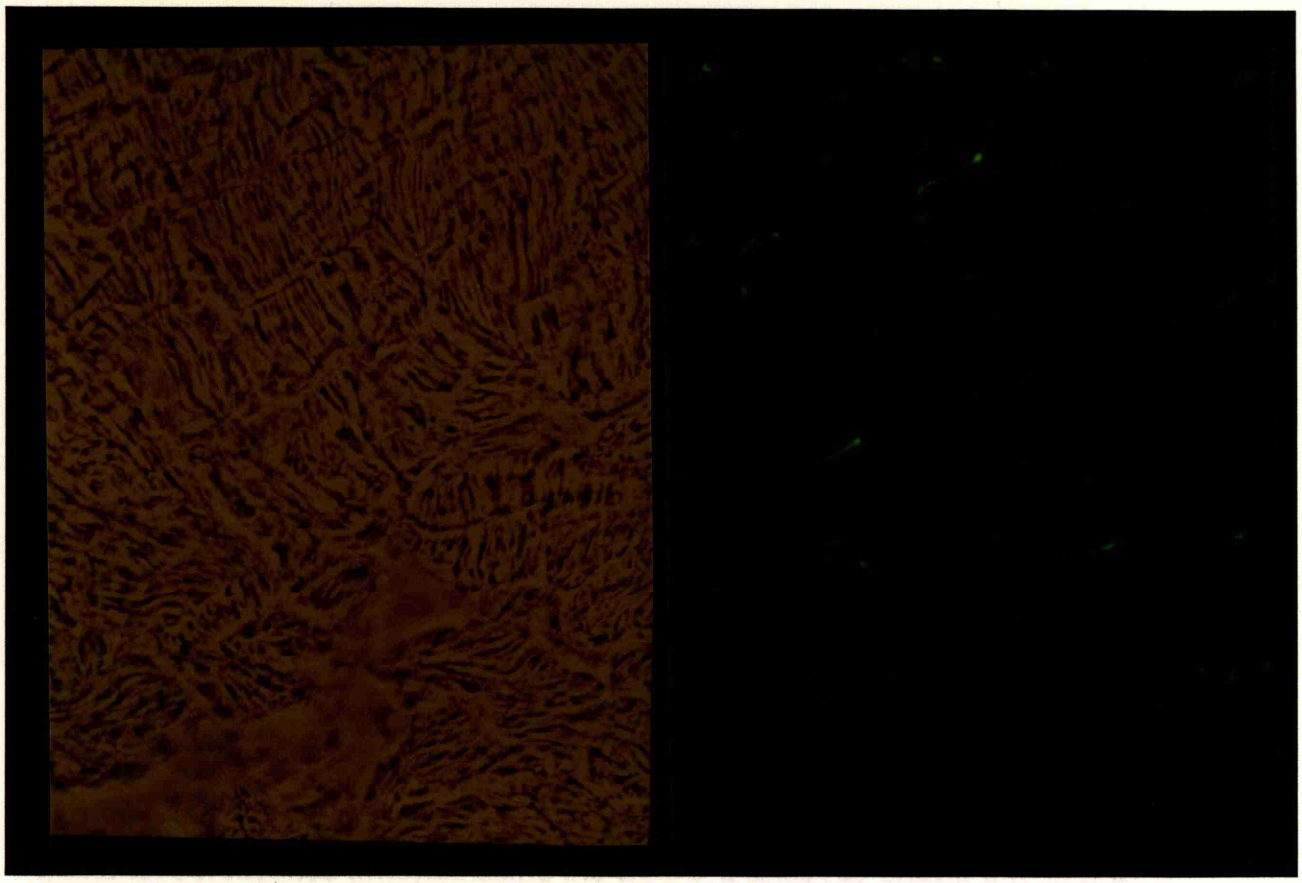
Figure 12. Immunofluorescence analysis of connexin 43 expression in mouse heart.

(A) is a phase contrast image of the fluorescence micrographs shown in (B). Mouse heart sections, (a gift from B. McGuire, Beatson Institute) were used for immunofluorescence analysis as described in materials and methods. Primary antibody was rabbit anti-connexin 43 polyclonal "Petunia" at 1:500. The antiserum stains the intercalated discs containing the cardiac gap junctions (Beyer et al 1989). Magnification x1000.

Figure 12.

Phase contrast. **Fluorescence.**

A. **B.**



c) Cadherins and N-CAM.

The endogenous expression of the cell adhesion molecules E-, N- and P-cadherin as well as N-CAM has also been determined by northern (figure 13 and 14), western (figure 15) and immunofluorescence analysis (figures 16-18). Northern analysis of E-cadherin RNA expression using a 1.1kb *Acc*I fragment of mouse E-cadherin cDNA as the probe, reveals that only MCF-7 cells express an E-cadherin transcript (figure 13.A). The 4.5kb transcript size is similar to that observed for E-cadherin in mouse and human keratinocytes (Jongen et al 1991, Nicholson et al 1991), rat kidney (Cyr et al 1992) and in mouse F9 cells (Nagafuchi et al 1987). Western analysis using the rat monoclonal antibody ECCD-2 to E-cadherin (Shirayoshi et al 1986) shows that the 4.5kb E-cadherin transcript expressed by MCF-7 cells is translated to give a 130kD protein (figure 15.A) and this is consistent with a previous report of E-cadherin protein expression in MCF-7 cells (Shinoyama et al 1989). In addition, immunofluorescence analysis using the ECCD-2 antibody shows that in MCF-7 cells the E-cadherin protein is expressed in the plasma membrane at points of cell contact between adjacent cells (figure 16).

The analysis of N-cadherin RNA expression using a 1.1kb *Pst*I fragment of mouse N-cadherin cDNA as the probe, reveals that both BRL and BICR cells express N-cadherin RNA transcripts of 4.0kb and 4.4kb (figure 13.B). An N-cadherin RNA doublet has also been reported in rat brain and testis (Cyr et al 1992), although only a single N-cadherin RNA transcript of approximately 4.3kb has been observed in chicken brain and heart (Hatta et al 1988). Western analysis using a polyclonal antibody raised amino acids 314-423 of human N-cadherin extracellular domain sequence fused to glutathione S-transferase (Doherty et al 1991) reveals that the predominant N-cadherin protein expressed by each of these cell lines is of a different molecular size (140kD in BICR cells,

125kD in BRL cells), although both species correspond to one of two proteins expressed in mouse heart (figure 15.B). However, the observation of two forms of N-cadherin protein is in contrast to previous studies, which have reported expression of a single N-cadherin protein of approximately 125kd in chicken heart (Hatta and Takeichi 1986) and PC12 cells (Doherty et al 1991). The interpretations of this result are considered in section 7.2. The polyclonal antibody against the N-cadherin fusion protein also recognises a number of uncharacterized proteins with molecular weights of 100kD or less in all the cell lines, including those which do not express N-cadherin RNA. Unfortunately, immunofluorescence analysis to investigate protein localisation with either the polyclonal antibody against the N-cadherin fusion protein (used for westerns), or the commercially available anti-N-cadherin antibody from Sigma has not been possible due to high non-specific activities of both these sera.

The northern analysis of P-cadherin expression using a 0.65kb *Hinc*II fragment of mouse P-cadherin cDNA as a probe, shows that only BRL cells express a P-cadherin transcript (figure 13.C). The 3.6kb P-cadherin transcript size is similar to that reported for P-cadherin in mouse and rat placenta, the mouse endoderm cell line PSA5-E and rat testis (Nose et al 1987, Cyr et al 1992). Western analysis using the rat monoclonal antibody PCD-1 to P-cadherin (Nose and Takeichi 1986) shows that the 3.6kb P-cadherin transcript expressed by BRL cells is translated to give a 120kD protein (figure 15.C) and this is consistent with the previously reported size for P-cadherin protein in mouse placenta, skin and lung (Nose and Takeichi 1986). In addition, immunofluorescence analysis using the PCD-1 antibody shows that BRL cells express the P-cadherin protein in the plasma membrane at points of cell contact between adjacent cells (figure 17).

Northern analysis of N-CAM RNA expression using a 0.6kb fragment of mouse N-CAM cDNA (clone pM1.3) as the probe, reveals that the hamster kidney BHK cells, (which does not endogenously express detectable levels of E-, N- or P-cadherin) express N-CAM transcripts of 6.7kb and 3.5kb and that BRL cells also express an N-CAM RNA transcript of 4.0kb (figure 14). Western analysis using a mouse monoclonal anti-N-CAM antibody (purchased from Sigma), which is reported to detect the 140kD and 180kD N-CAM isoforms shows that BHK cells express the 140kD N-CAM protein (figure 15.D) and this is consistent with a previous report suggesting that the 140kD mouse brain N-CAM isoform is the protein product of a 6.7kb mRNA (Santoni et al 1987). The larger band of >200kD in the BHK lane corresponds to N-CAM protein at the interface between the stacking and running gels. Smaller N-CAM isoforms arising from translation of alternatively spliced N-CAM mRNA are not detected in either of the BHK or BRL cell lanes (figure 15.D) but may be expressed given the reported inability of the mouse monoclonal anti-N-CAM antibody to detect smaller isoforms of N-CAM. Immunofluorescence analysis also reveals that the N-CAM protein expressed by BHK cells has a more diffuse pattern of immunofluorescence (figure 18) than that previously described for E- and P-cadherin expressed by MCF-7 or BRL cells respectively (figure 16 and 17).

A summary of the endogenous expression results is given in table 6 and their significance discussed below.

3.4 Discussion.

The ubiquitous expression of ductin transcripts by all six cell lines, regardless of their ability to form functional gap junctions is consistent with ductin forming the V-ATPase proton channel, (which is widely distributed in the endomembranes of eukaryotic cells and appears to be essential; section 1.5.3

Figure 13. Northern analysis of cadherin expression.

10μg of total cellular RNA prepared from the cell lines MCF-7, L, S180, BHK, BICR or BRL was separated on 1.2% agarose gels containing formaldehyde, blotted and probed with either a 1.1kb fragment of mouse E-cadherin cDNA, a 1.1kb fragment of mouse N-cadherin cDNA or a 0.65kb fragment of mouse P-cadherin cDNA,(as described in material and methods). The expression of the 4.5kb E-cadherin transcript is shown in (A), the 4.4kb and 4.0kb N-cadherin transcripts in (B), and the 3.6kb P-cadherin transcript in (C). The position of the ribosomal RNA is also given. The blots were subsequently reprobed with GAPDH (1.8kb transcript) as a loading control.

Figure 13.

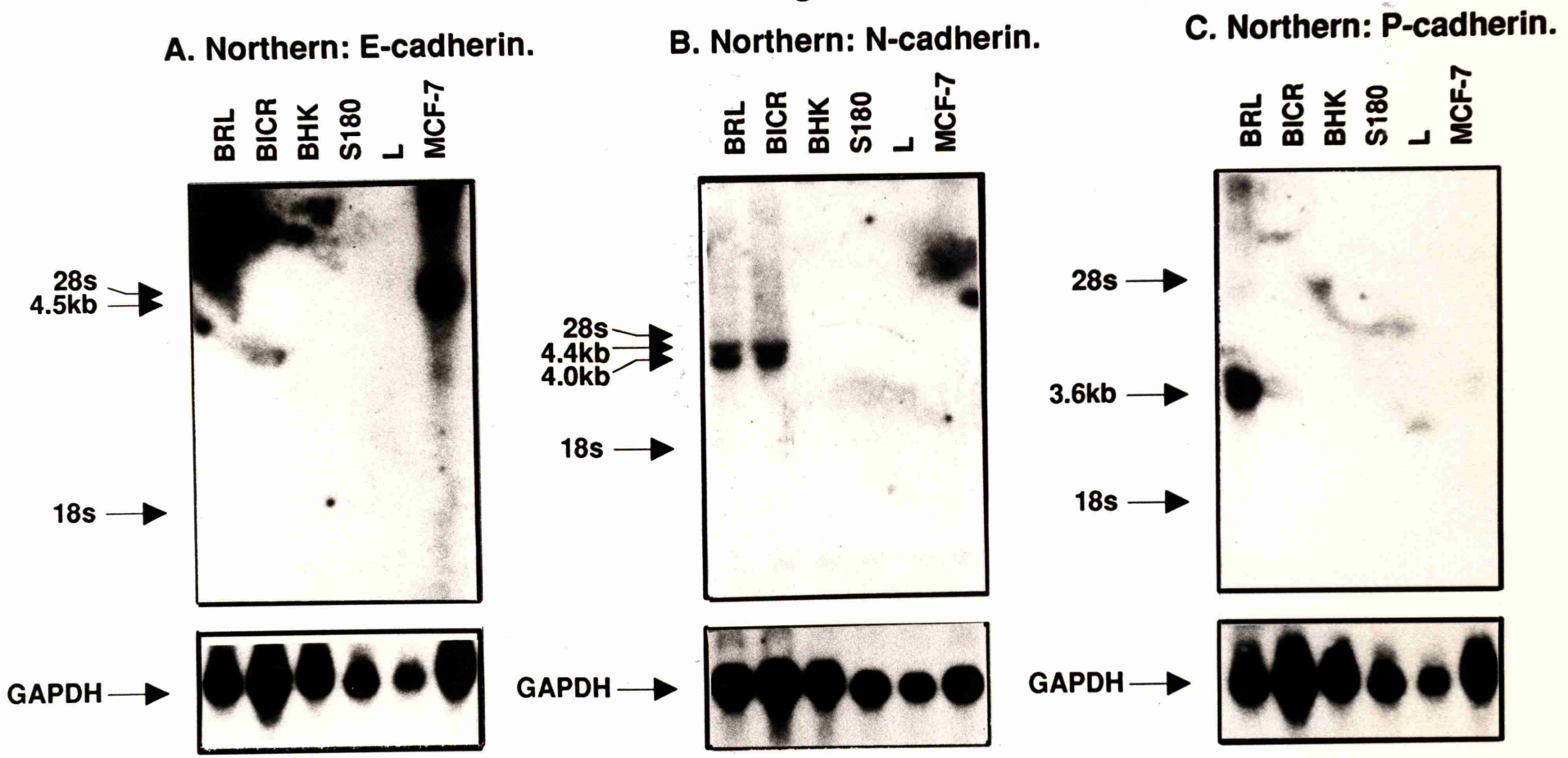

A. Northern: E-cadherin.
BRL
BICR
BHK
S180
L
MCF-7
28s
4.5kb
18s
GAPDH
B. Northern: N-cadherin.
BRL
BICR
BHK
S180
L
MCF-7
28s
4.4kb
4.0kb
18s
GAPDH
C. Northern: P-cadherin.
BRL
BICR
BHK
S180
L
MCF-7
28s
3.6kb
18s
GAPDH

Figure 14. Northern analysis of N-CAM expression.

10µg of total cellular RNA prepared from the cell lines MCF-7, L, S180, BHK, BICR or BRL was separated on 1.2% agarose gels containing formaldehyde, blotted and probed with a 0.6kb fragment of mouse N-CAM cDNA,(as described in material and methods). The position of the 6.7kb, 4.0kb and 3.5kb N-CAM transcripts and the ribosomal RNA are shown. The blots were subsequently reprobed with GAPDH (1.8kb transcript) as a loading control.

Figure 14.

Northern: N-CAM.

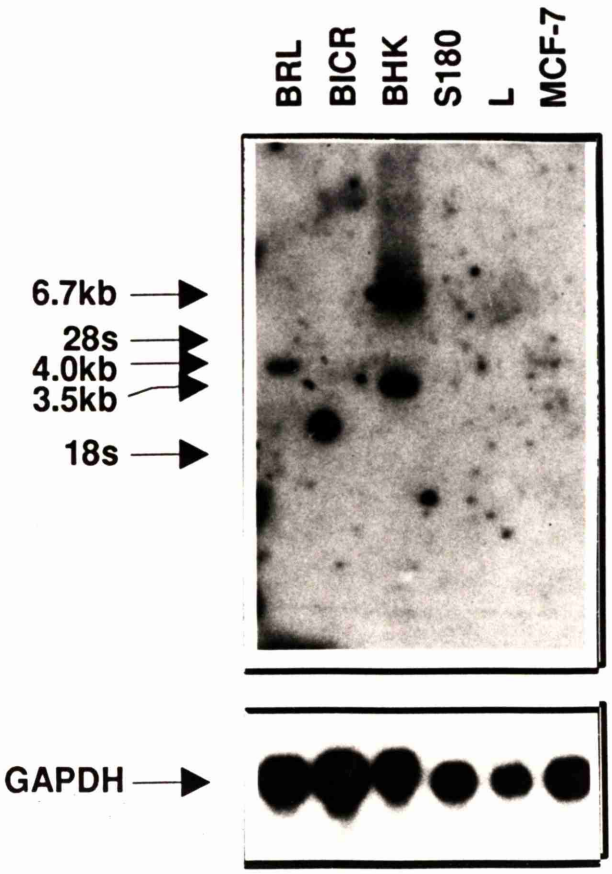
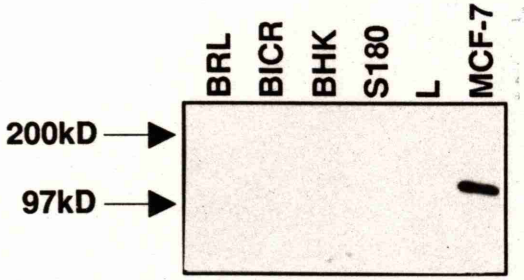


Figure 15. Western analysis of cadherin and N-CAM expression.

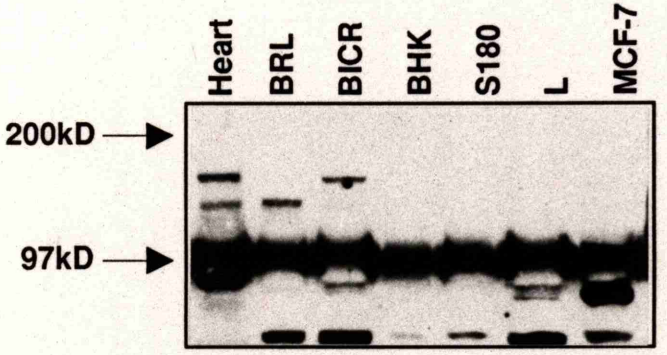
Protein samples were prepared from the cell lines and equivalent amounts of protein (approximately 20μg, as estimated from Coomassie blue stained gels) used for western analysis as described in materials and methods. Primary antibody was either ECCD-2 (rat monoclonal anti-E-cadherin) at 1:250 (A), rabbit anti-N-cadherin polyclonal at 1:500 (B), mouse monoclonal anti-NCAM at 1:250 (C) or PCD-1 (rat monoclonal anti-P-cadherin) at 1:30 (D). The position of the molecular weight markers are shown.

Figure 15.

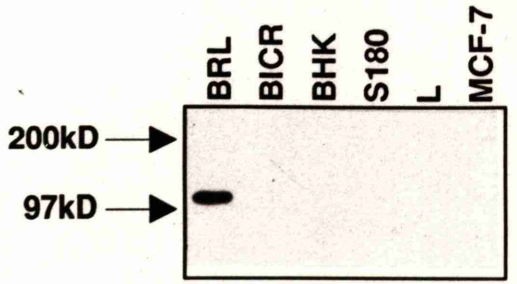
A. Western: E-cadherin.



B. Western: N-cadherin.



C. Western: P-cadherin.



D. Western: N-CAM.

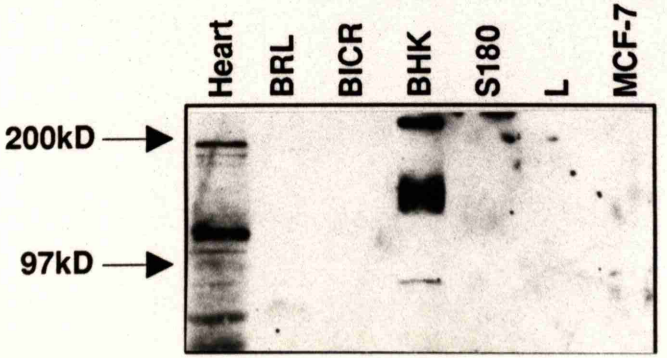


Figure 16. Immunofluorescence analysis of E-cadherin expression by MCF-7 cells.

(A) and (B) are phase contrast images of the fluorescence micrographs shown in (B) and (D). MCF-7 cells cultured on coverslips and fixed in ethanol were used for immunofluorescence analysis as described in materials and methods. Primary antibody was either ECCD-2 (rat monoclonal anti-E-cadherin) at 1:500 (B) or PCD-1 (rat monoclonal anti-P-cadherin) at 1:100 (D). E-cadherin protein is expressed at points of cell contact between MCF-7 cells (B). In contrast, no fluorescence was seen when the primary antibody was PCD-1 (rat monoclonal anti-P-cadherin; D). Magnification x1000.

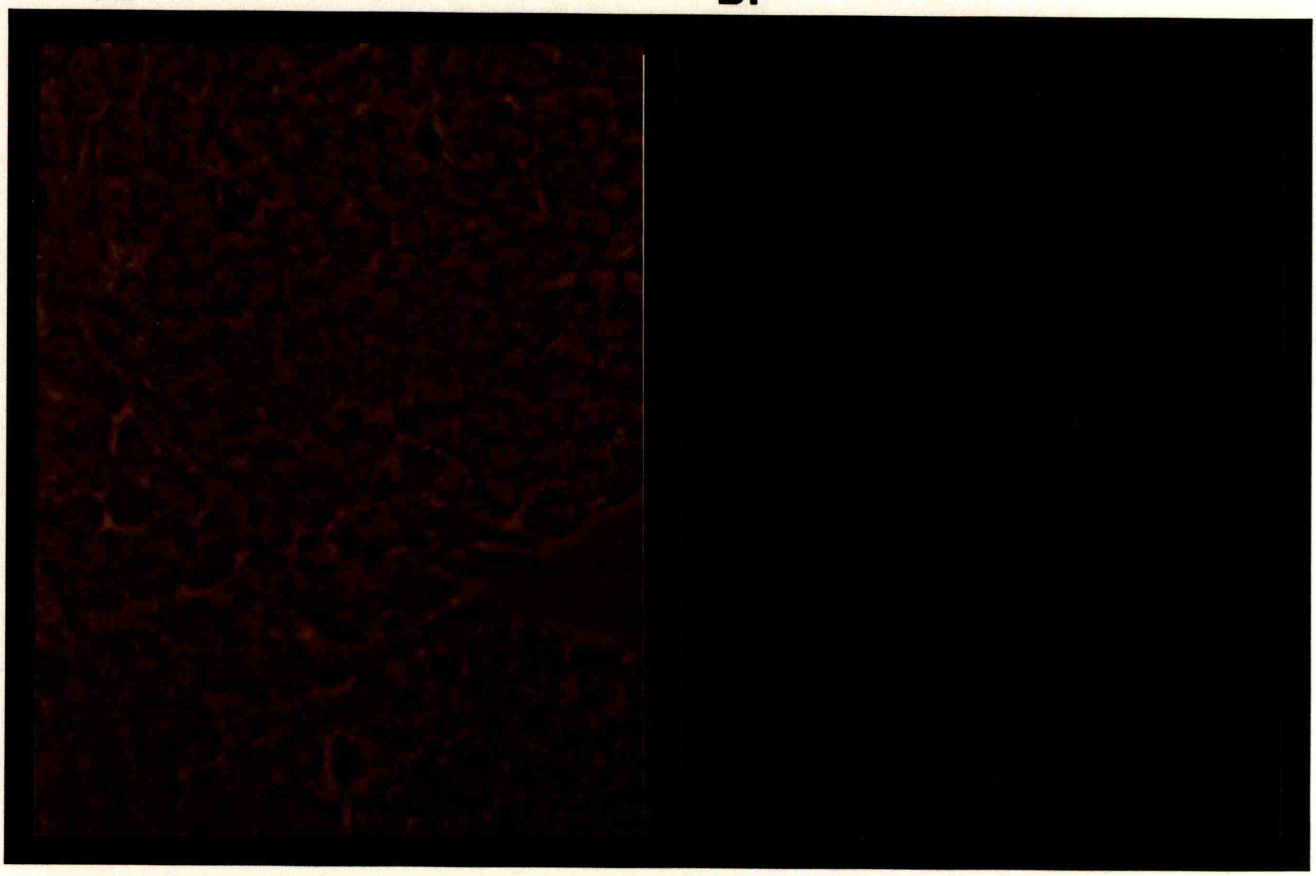
Figure 16.

Phase contrast.

Fluorescence.

A.

B.



C.

D.

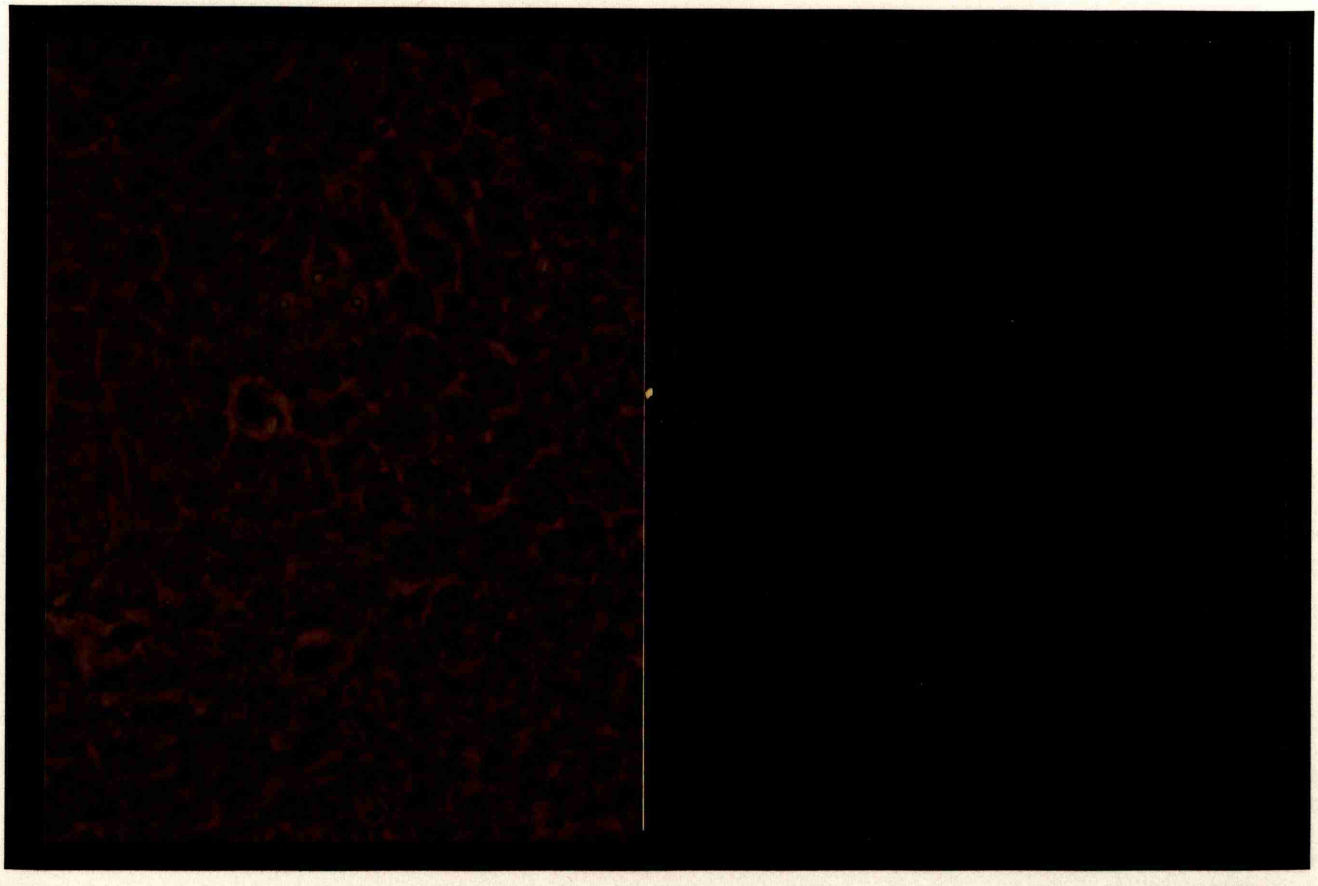


Figure 17. Immunofluorescence analysis of P-cadherin expression by BRL cells.

(A) and (B) are phase contrast images of the fluorescence micrographs shown in (B) and (D). BRL cells cultured on coverslips and fixed in ethanol were used for immunofluorescence analysis as described in materials and methods. Primary antibody was either ECCD-2 (rat monoclonal anti-E-cadherin) at 1:500 (B) or PCD-1 (rat monoclonal anti-P-cadherin) at 1:100 (D). P-cadherin protein is expressed at points of cell contact between BRL cells (D). In contrast, no fluorescence was seen when the primary antibody was ECCD-2 (rat monoclonal anti-E-cadherin; B). Magnification x1000.

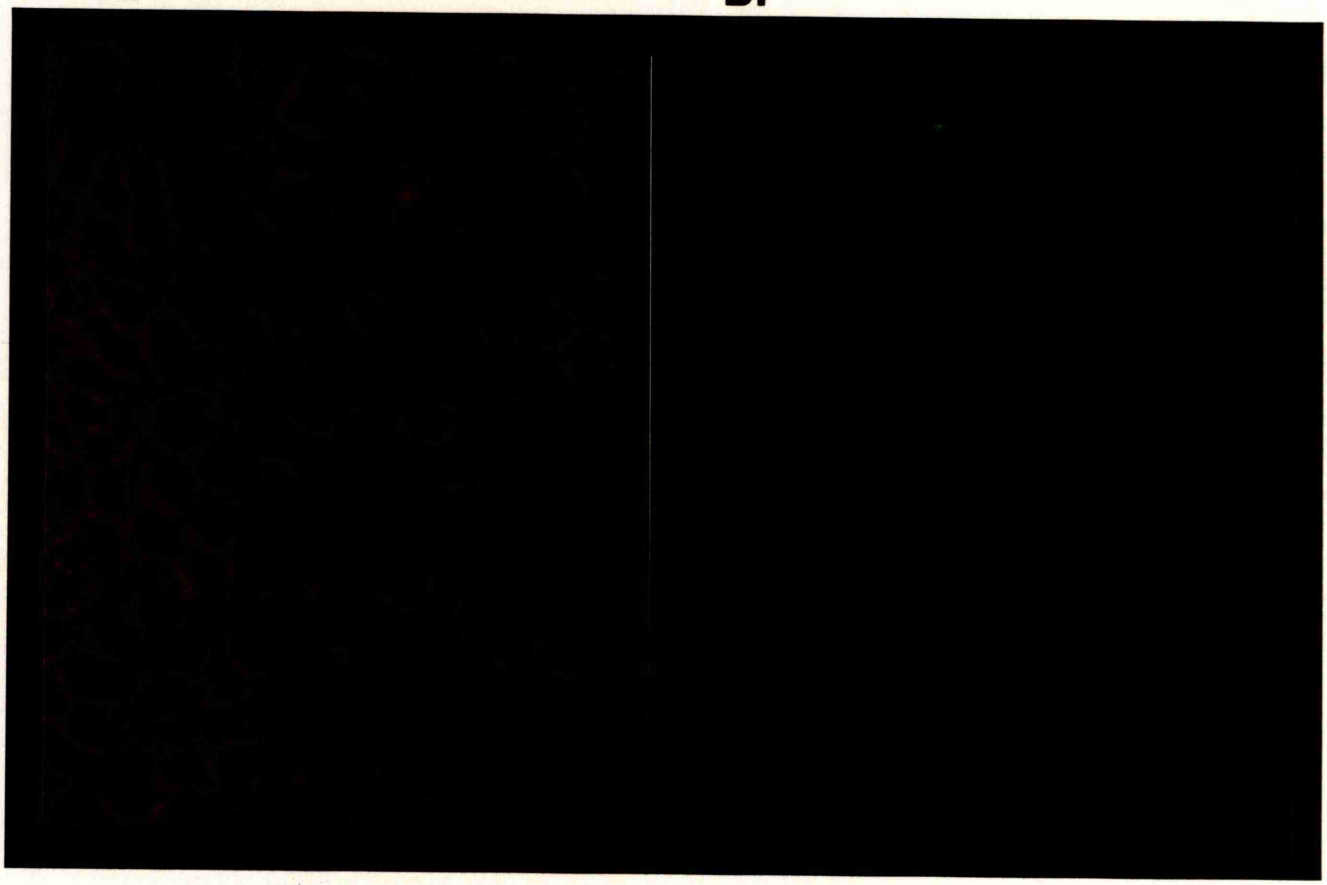
Figure 17.

Phase contrast.

Fluorescence.

A.

B.



C.

D.

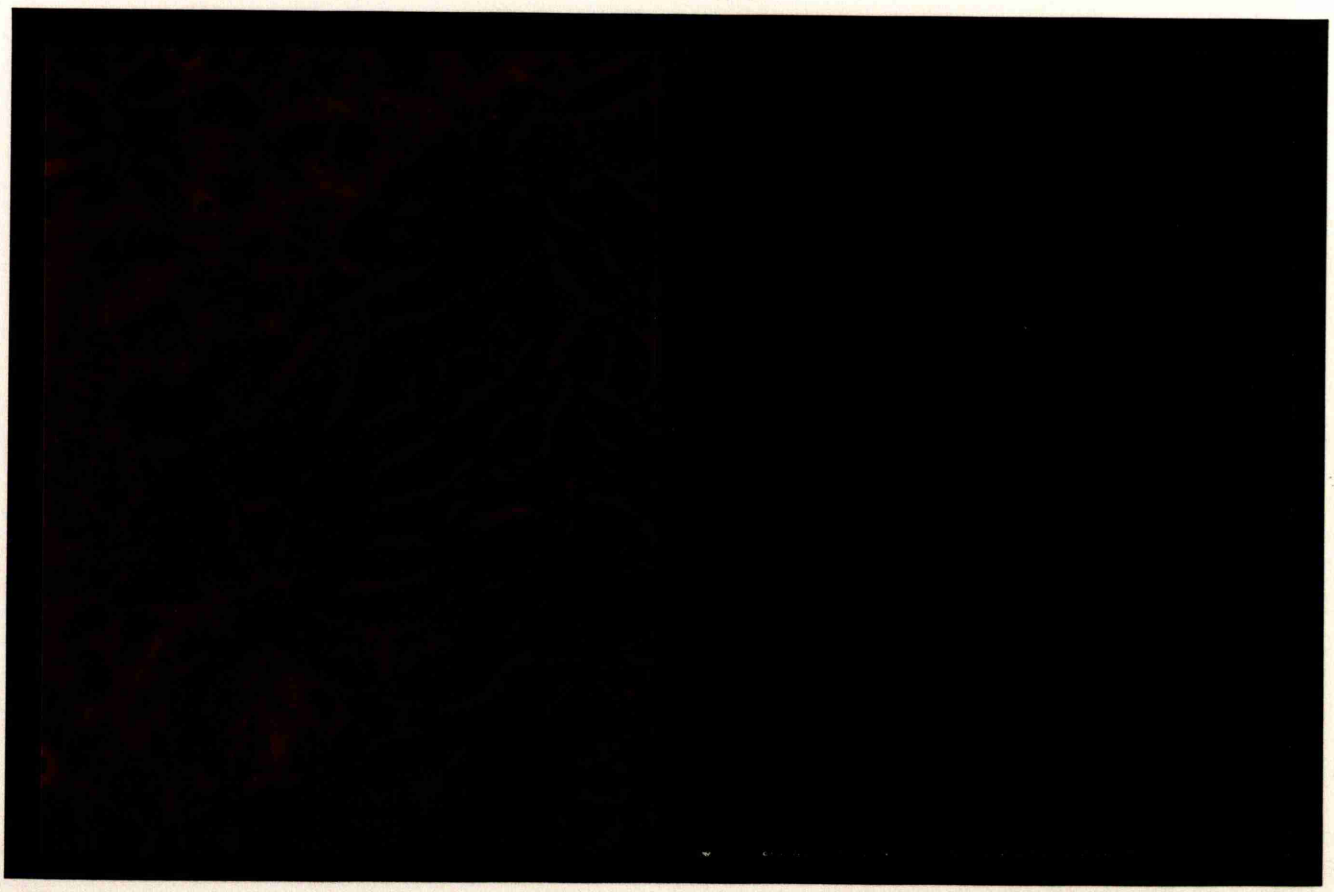


Figure 18. Immunofluorescence analysis of N-CAM expression by BHK cells.

(A) and (B) are phase contrast images of the fluorescence micrographs shown in (B) and (D). BHK cells cultured on coverslips and fixed in ethanol were used for immunofluorescence analysis as described in materials and methods. Primary antibody was mouse monoclonal anti-NCAM at 1:500 (B). No fluorescence was observed when the primary antibody was omitted (D). Magnification x1000.

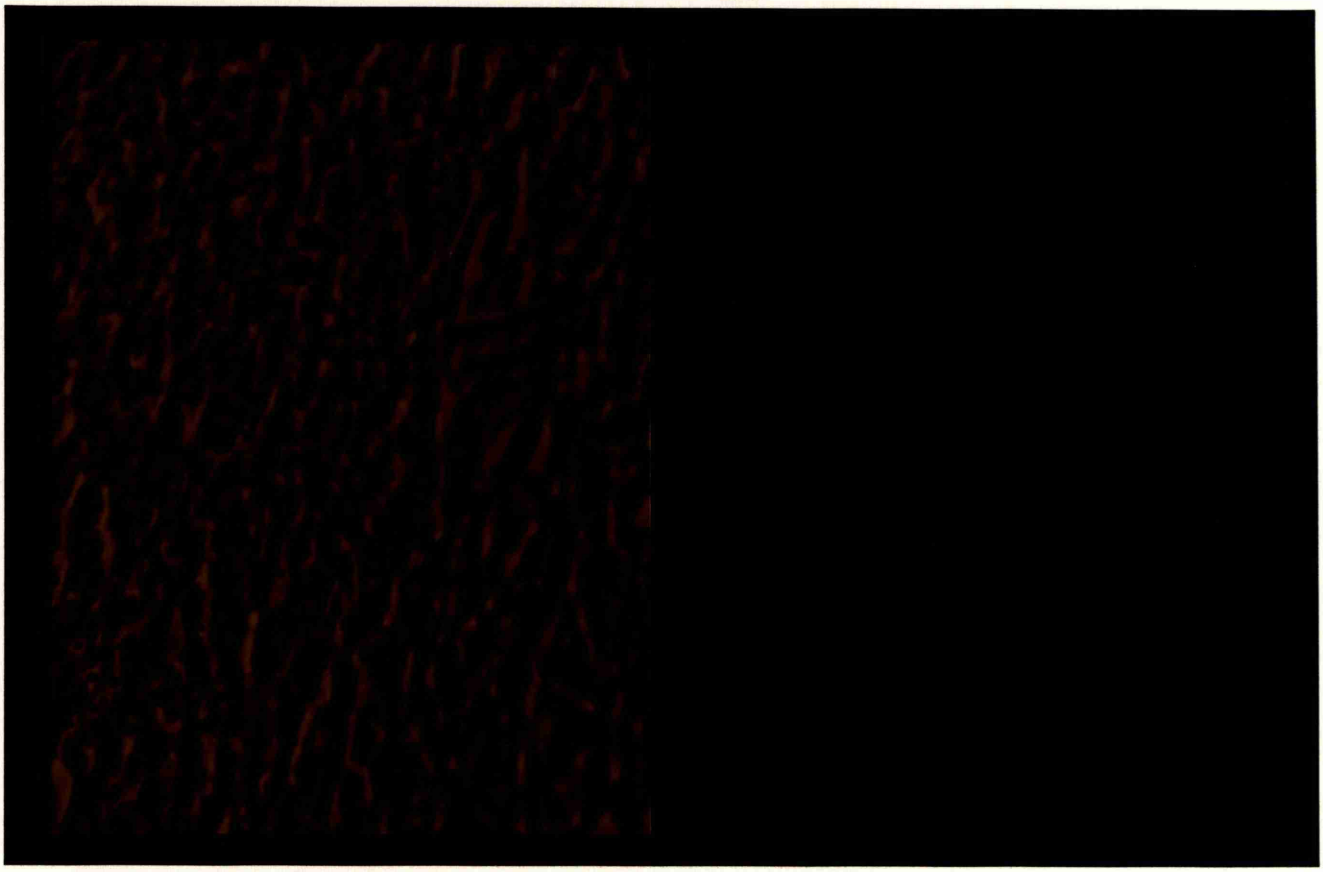
Figure 18.

Phase contrast.

Fluorescence.

A.

B.



C.

D.

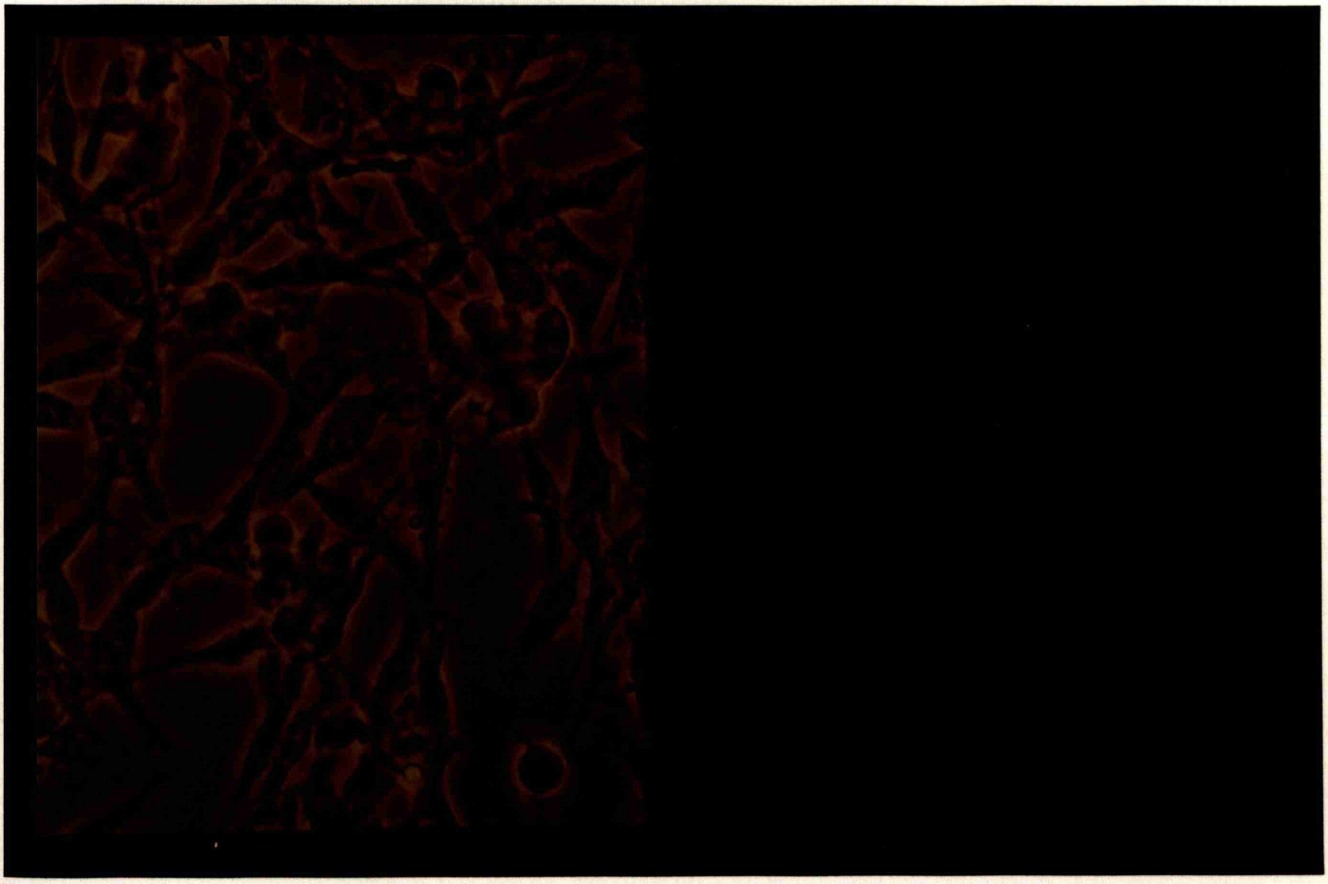


Table 6. Summary of endogenous expression of ductin, connexin 32 and 43, E-, N- and P-cadherin, and N-CAM by MCF-7, L, S180, BHK, BICR and BRL cells.

Table 6.

Cell Line.	Endogenous Expression.														
	Ductin	Cx 32	Cx 43		E-cad			N-cad		P-cad			N-CAM		
	N	N	N	W	N	W	I	N	W	N	W	I	N	W	I
MCF-7	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-
L	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-
S180	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-
BHK	+	-	+	+	-	-	-	-	-	-	-	-	+	+	+
BICR	+	-	+	+	-	-	-	+	+	-	-	-	-	-	-
BRL	+	-	+	+	-	-	-	+	+	+	+	+	+	-	-

N= Northern analysis
W= Western analysis
I= Immunofluorescence analysis

and Nelson 1992) and shows that ductin expression alone is not sufficient for gap junction formation or function. With the exception of the MCF-7 cell line, (which does not express either connexin 32 or 43), all the cell lines tested express connexin 43 but not connexin 32. This is particularly surprising for BRL cells which are derived from rat liver, where the major endogenous connexins expressed are connexins 26 and 32 (Traub et al 1989). The expression of connexin 43 by cell lines derived from diverse tissues has also been reported by others (Musil et al 1990b, Meyer et al 1992, Stutenkemper et al 1992, Katoh et al 1993). Stutenkemper et al (1992) have suggested that this altered connexin expression is a consequence of culturing cells in serum containing medium, as they found that immortalised hepatocytes in defined media express transcripts for connexin 32, connexin 26 and the liver specific proteins α-fetoprotein, tyrosine aminotransferase and albumin but that upon culturing the same cells in serum containing medium the expression of these transcripts decrease and the expression of connexin 43 transcripts dramatically increase.

The expression of cadherins and N-CAM by the cell lines is also unpredictable on the basis of their tissue of origin, for example rat liver derived BRL cells express P- and N-cadherin while hepatocytes express E-cadherin, which was originally identified as the chicken liver cell adhesion molecule (L-CAM; Gallin et al 1987). In addition, it is surprising that N-cadherin is expressed by BRL and BICR cells and N-CAM by BHK cells given that these cell adhesion molecules have previously been identified predominantly in nervous tissues (Edelman 1985, Rutihauser 1986, Takeichi 1988).

The *mec*$^{+}$ cell lines BHK, BICR and BRL either express a cadherin or N-CAM and given that the *mec*$^{-}$ MCF-7 cells also express E-cadherin, this is consistent with these cell adhesion molecules being necessary but not sufficient for gap junction formation. MCF-7 cells may be poor communicators because

they do not express connexins (figure 9 and 10; Lee et al 1992), although it is possible that these cells express other uncharacterized connexins and that other proteins are important. L and S180 cells which express ductin and connexin 43 may be poorly coupled via gap junctions because they do not express cell adhesion molecules and the effects of expressing exogenous cadherin in L and S180 cells are described in chapters 4 and 5.

The molecular basis of GJIC specificity is not understood but it may involve the differential expression of proteins that have been demonstrated to play a role in gap junction formation and function. As ductin is expressed in a wide variety of different tissues (Buultjens et al 1988) as well by each of the *mec*$^+$ lines BHK, BICR and BRL it is unlikely to have an important role in determining specificity. The connexins because of their cell specific expression and selective ability to cause gap junction formation when expressed heterologously in *Xenopus* oocytes pairs (section 1.5.2) are good candidates for a role in determining the specificity of GJIC seen in mixed cell cultures *in vitro*. However, as all three *mec*$^+$ lines express connexin 43 protein (figure 11), this shows that the expression of the same connexin is not sufficient for gap junction formation between the epithelial and fibroblast cell types, other proteins are necessary. Previous studies have suggested that the specificity of GJIC seen in mixed cultures of BRL/BHK cells is linked to the process of cell sorting (Pitts and Kam 1985). The results presented in section 3.2 are consistent with this as mixed cultures of BRL/BICR cells also sort out to form communication compartments whereas mixed cultures of BHK/BICR cells which do not sort out are heterologously coupled.

The process of sorting out can be mediated by the cadherins and N-CAM (Mege et al 1988, Nose et al 1988, Matsuzaki et al 1990) and as these molecules can also influence GJIC (Keane et al 1988, Mege et al 1988, Jongen

et al 1991, Meyer et al 1992) they are also good candidates for a role in determining specificity. Consistent with this is the observation that BHK and BRL cells express different cell adhesion molecules (table 6) and BICR and BRL cells express two transcripts of N-cadherin RNA and predominantly different forms of N-cadherin protein (figure 15.B; section 7.2). It is therefore possible that the differential expression of cell adhesion molecules influences the formation of communication compartments and this has been investigated by expressing exogenous E-cadherin or P-cadherin in these cells (chapters 4 and 6). Interestingly, BHK and BICR cells which communicate heterologously in mixed cultures do not share expression of cadherin or N-CAM. This suggests that either other uncharacterized cell adhesion molecules are present or the formation of gap junctions between fibroblast cells only depends on the type of connexin expressed, (and this is discussed in section 7.2).

Chapter 4.

Characteristics of cadherin transfectants.

4.1 Introduction.

The results presented in chapter 3 are consistent with published data showing that cadherins play a role in gap junction formation and suggest that their differential expression may also contribute to the specificity of GJIC seen in mixed cultures of BRL and BHK or BICR cells.

The *mec*$^-$ cell lines L and S180 were transfected with either a mouse E- or P-cadherin expression construct, in order to confirm that these vectors cause the expression of functional cadherin which affects the frequency of gap junction formation. The *mec*$^-$ cell lines L and S180 were chosen because previous studies have shown that the expression of exogenous cadherin in either cell type causes increased cell-cell aggregation (Hatta et al 1988, Mege et al 1988, Nose et al 1988, Matsuzaki et al 1990), which in the S180 E- or N-cadherin transfectants is also associated with increased GJIC (Mege et al 1988, Matsuzaki et al 1990). To investigate the role of cadherins in specificity the *mec*$^+$ cell lines BICR and BHK were transfected with mouse E- or P-cadherin expression constructs and BRL cells also transfected with the E-cadherin expression construct, (as this cell type endogenously expresses P-cadherin). The characteristics of the cadherin transfectants are described in this chapter.

4.2 Expression of exogenous cadherin in cell lines.

The cell lines L, S180, BHK, BICR and BRL were cotransfected (as described in section 2.2.1.4) with the E-cadherin expression construct pBATEM2, in which the expression of E-cadherin is under the control of the chicken β-actin promoter (figure 19; Nose et al 1988), and either the neomycin expression vector pZIPneoSVX, (which can also be used for the expression of

exogenous cDNAs inserted into the *Bam*HI site; Cepko et al 1984) or pHSG274 (Brady et al 1984) as dominant selection markers. A number of geneticin sulphate resistant ($G418^R$) colonies were obtained for each pBATEM2 cotransfected cell type after selecting in media containing G418. Cell lines were established by ring cloning representative colonies, (table 7A-C) and cell stocks stored in liquid nitrogen. Those pBATEM2 cotransfected cell lines which were distinguishable from the parental cell type because they grew in tight cell groups and/or were positive on an initial screen for E-cadherin expression by immunofluorescence analysis, have been used in further studies.

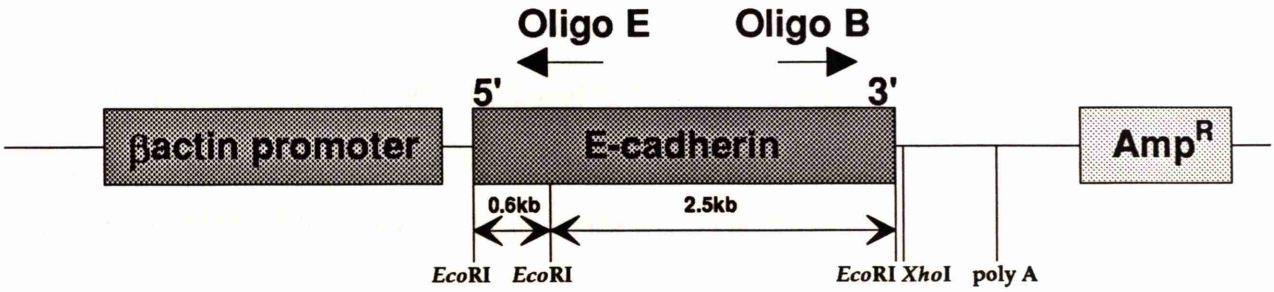
Genomic DNA was prepared from the parental cells and the pBATEM2 cotransfected cell lines (as described in section 2.2.3.2.C). The DNA was digested with *Eco*RI, which cuts the pBATEM2 construct as shown in figure 19, generating E-cadherin fragments of 0.6kb and 2.5kb. Southern blotting and subsequent hybridization using a 1.1kb *Acc*I fragment of mouse E-cadherin as the probe, reveals the presence of the 2.5kb and the 0.6kb fragments in the transfected lines but not the parental cells (figure 20.A). The intensity of the 0.6kb band is weaker than that of the 2.5kb band, and the 0.6kb band is only detectable in all the transfected cell lanes on longer exposures (data not shown). This is presumably because the 1.1 kb probe hybridizes less efficiently to a smaller region of the 0.6kb fragment. Those clones which contain copies of the pBATEM2 plasmid also expressed E-cadherin transcripts (figure 21.A) and the appropriate 130kD E-cadherin protein (figure 22.A). In the lanes where E-cadherin protein is most heavily expressed (BHK EB3, L E5.5 and L E10.10; figure 22.A) there is a less intense band that migrates slightly faster than the 130kD E-cadherin protein. A similar band has also been observed by Nagafuchi et al 1987 and it is thought to be due to degradation of the 130kD E-cadherin protein. Immunofluorescence staining with the monoclonal antibody ECCD-2 to

Figure 19. A schematic diagram of linearised E-cadherin expression construct pBATEM2.

Sequence analysis was performed with oligo E or B to confirm the identity of the construct and the integrity of part of the E-cadherin cDNA fragment.

Figure 19.

pBATEM2.



Sequence obtained with oligo E:

5'[62]**GTCCGCC ATG GGA GCC CGG TGC CGC AGC TTT TCC GCG CTC CTG CTC CTG CTG**

CAG GTC TCC TCA TGG CTT TGC CAG GAG CTG GAG CCT GAG TCC TGC AGT CCC

GGC TTC AGT TCC GAG GTC TAC ACC TTC CCG GTG CCG GAG AGG CAC[209] **3'**

Nucleotide sequence corresponds to mouse E-cadherin,

positions 62 to 209 (Nagafuchi et al 1987).

Sequence obtained with oligo B:

5'[3026]**CCCCTCACACCTCACTAATTTTTTACATTGTGTACTTGCCCTCAATTACCATGTTTGCTGTATTCTAAT**

AGTCACTCATCTTCCTGAATTC[3116]GAGCTCGGTACCCGGGGATCCTCTAG 3'

GAATTC *Eco*RI

GAGCTC *Xho*I

Nucleotide sequence in bold corresponds to mouse E-cadherin,

position 3026 to 3116 (Nagafuchi et al 1987).

Table 7. Summary of cadherin co-transfections.

The cell lines L, S180, BHK, BICR and BRL were co-transfected with the cadherin expression constructs, as described in materials and methods. A number of $G418^R$ lines were obtained for each cell type.

Table 7A.

Calcium Phosphate Transfection.

Cell Line.	Plasmid(s).	Approximate number of cells transfected ($\times 10^6$).	Number of $G418^R$ colonies.	Number of $G418^R$ cell lines obtained.	Cadherin expressing/ $G418^R$ clonal lines.
L	control (no DNA)	4	0		
	pZipneoSVX (2μg)	4	>100		
	pZipneoSVX (2μg) +pBATEM2 (20μg)	2	>100	28	L E5.5 L E10.10
	pZipneoSVX (2μg) +pβact-Pcad (20μg)	2	>100	20	L P1A L 15P
S180	control (no DNA)	1	0		
	pHSG274 (2μg)	1	>100		
	pHSG274 (2μg) +pBATEM2 (20μg)	1	>100	11	S180 E5&E5.5 S180 E1
	pHSG274 (2μg) +pβact-Pcad (20μg)	1	>100	14	S180 P11 S180 P17

Table 7B.

Calcium Phosphate Transfection.

Cell Line.	Plasmid(s).	Approximate number of cells transfected ($\times 10^6$).	Number of $G418^R$ colonies.	Number of $G418^R$ cell lines obtained.	Cadherin expressing/ $G418^R$ clonal lines.
BHK	control (no DNA)	3	0		
	pZipneoSVX (2μg)	1	>100	12	
	pZipneoSVX (2μg) +pBATEM2 (20μg)	1	>100	24	BHK EA4 BHK EB3
	pZipneoSVX (2μg) +pβact-Pcad (20μg)	2	>100	21	BHK P3B3 BHK P3B4 BHK PC3
BICR	control (no DNA)	1	0		
	pZipneoSVX (20μg)	1	>100		
	pZipneoSVX (1μg)	1	>100		
	pZipneoSVX (2μg) +pBATEM2 (20μg)	1	>100	25	BICR EA1.1 BICR EA1.5
	pZipneoSVX (2μg) +pβact-Pcad (20μg)	1	>100	21	BICR PI9

Table 7C.

Calcium Phosphate Transfection.

Cell line.	Plasmid(s).	Approximate number of cells transfected ($\times 10^6$).	Number of $G418^R$ colonies.	Number of $G418^R$ cell lines obtained.	Cadherin expressing/ $G418^R$ clonal lines.
BRL	control (no DNA)	5	0		
	pZipneoSVX (2μg)	4	1		
	pZipneoSVX (4μg)	1	12		
	pZipneoSVX (2μg) +pBATEM2 (20μg)	4	1	1	
	pZipneoSVX (4μg) +pBATEM2 (16μg)	1	11	9	BRL E5E

Figure 20. Southern analysis of cadherin transfectants.

20μg of genomic DNA prepared from the cell lines MCF-7, L, S180, BHK, BICR, BRL or the cadherin transfectants was digested with *Eco*RI and separated on 0.8% TBE gels. The DNA was subsequently transferred to Hybond-N+ membranes by Southern blotting and hybridized with either a labelled 1.1kb fragment of mouse E-cadherin cDNA (A) or a 0.65kb fragment of mouse P-cadherin cDNA (B; as described in material and methods). The position of the DNA molecular weight markers, the 0.6kb and 2.5kb pBATEM2 fragments (A), and the 3.2kb pβact-Pcad fragment (B) are shown.

Figure 20.

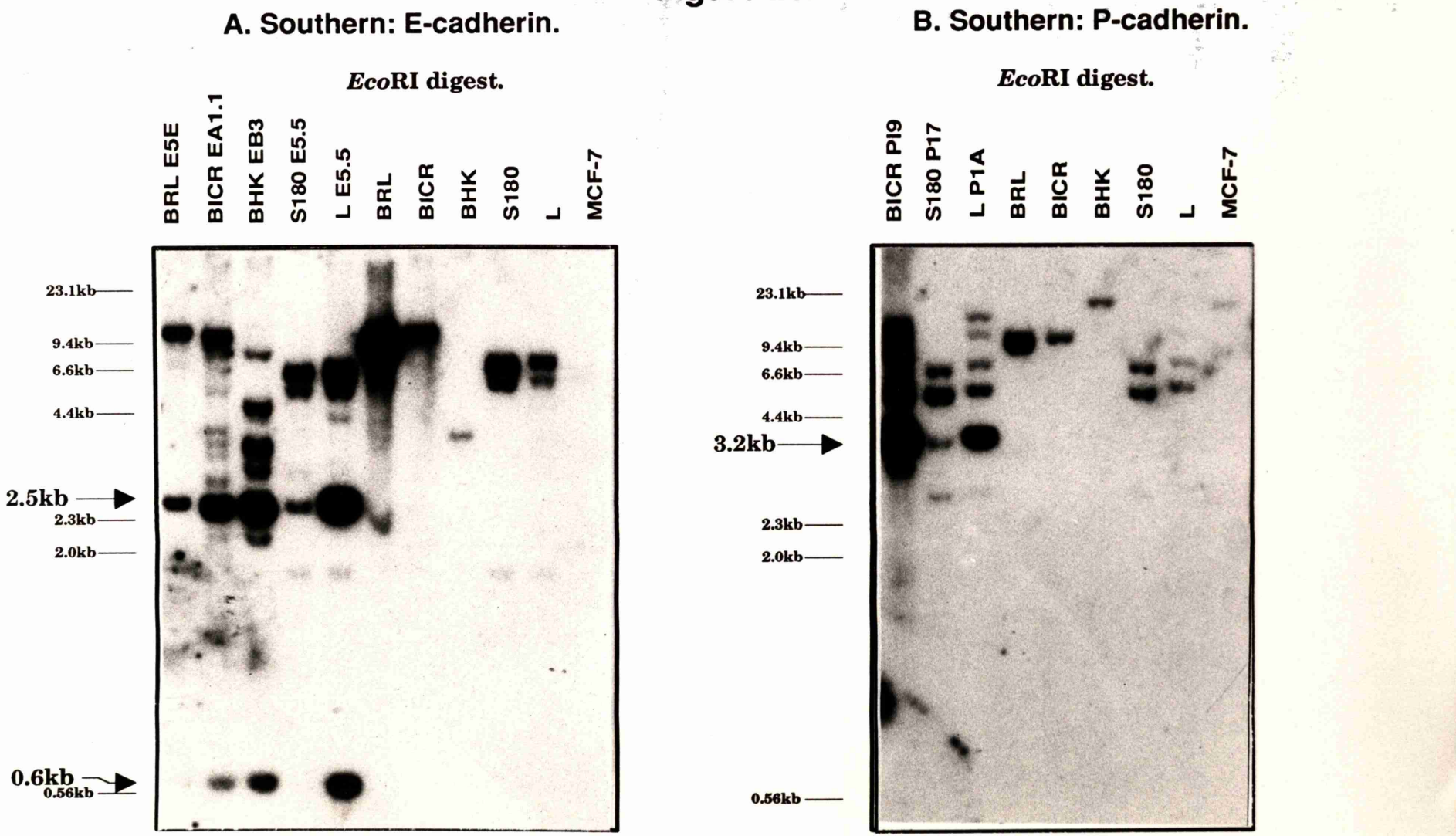

A. Southern: E-cadherin.
EcoRI digest.
BRL E5E
BICR EA1.1
BHK EB3
S180 E5.5
L E5.5
BRL
BICR
BHK
S180
L
MCF-7
23.1kb
9.4kb
6.6kb
4.4kb
2.5kb
2.3kb
2.0kb
0.6kb
0.56kb
B. Southern: P-cadherin.
EcoRI digest.
BICR PI9
S180 P17
L P1A
BRL
BICR
BHK
S180
L
MCF-7
23.1kb
9.4kb
6.6kb
4.4kb
3.2kb
2.3kb
2.0kb
0.56kb

Figure 21. Northern analysis of cadherin expression by the transfected cell lines.

10μg of total cellular RNA prepared from the cell lines MCF-7, L, S180, BHK, BICR, BRL or the cadherin transfectants was separated on 1.2% agarose gels containing formaldehyde, blotted and probed with either a labelled 1.1kb fragment of mouse E-cadherin cDNA or a 0.65kb fragment of mouse P-cadherin cDNA,(as described in material and methods). The expression of E-cadherin transcripts is shown in (A) and P-cadherin transcripts in (B). The position of the ribosomal RNA is also given. The blots were subsequently reprobed with GAPDH (1.8kb transcript) as a loading control.

Figure 21.

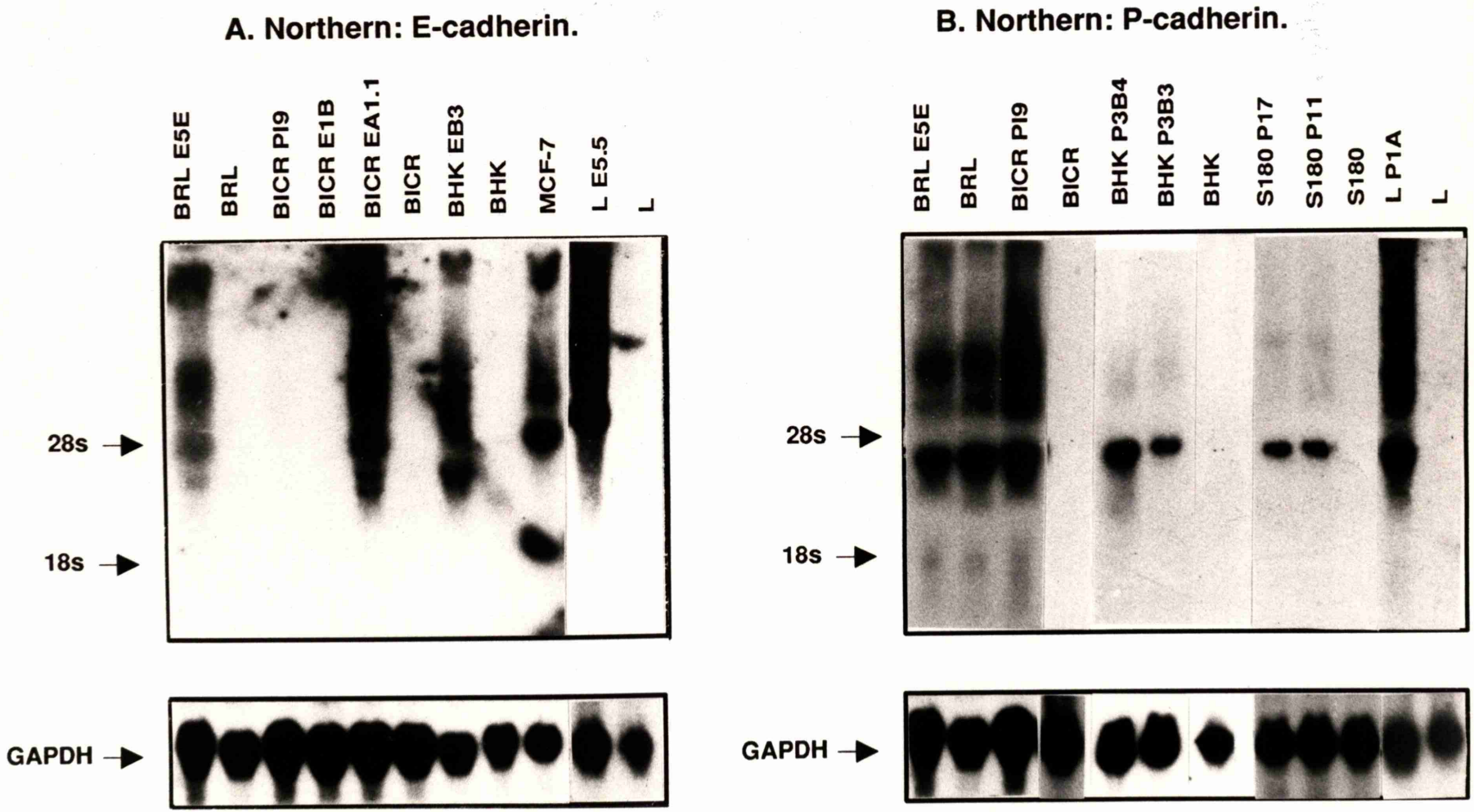

A. Northern: E-cadherin.
BRL E5E
BRL
BICR PI9
BICR E1B
BICR EA1.1
BICR
BHK EB3
BHK
MCF-7
L E5.5
L
28s
18s
GAPDH
B. Northern: P-cadherin.
BRL E5E
BRL
BICR PI9
BICR
BHK P3B4
BHK P3B3
BHK
S180 P17
S180 P11
S180
L P1A
L
28s
18s
GAPDH

Figure 22. Western analysis of cadherin expression by the transfected cell lines.

Protein samples were prepared from the cell lines and equivalent amounts of protein (approximately 20μg, as estimated from Coomassie blue stained gels) used for western analysis as described in materials and methods. Primary antibody was either ECCD-2 (rat monoclonal anti-E-cadherin) at 1:250 (A) or PCD-1 (rat monoclonal anti-P-cadherin) at 1:30 (B). The position of the molecular weight markers are shown.

Figure 22.

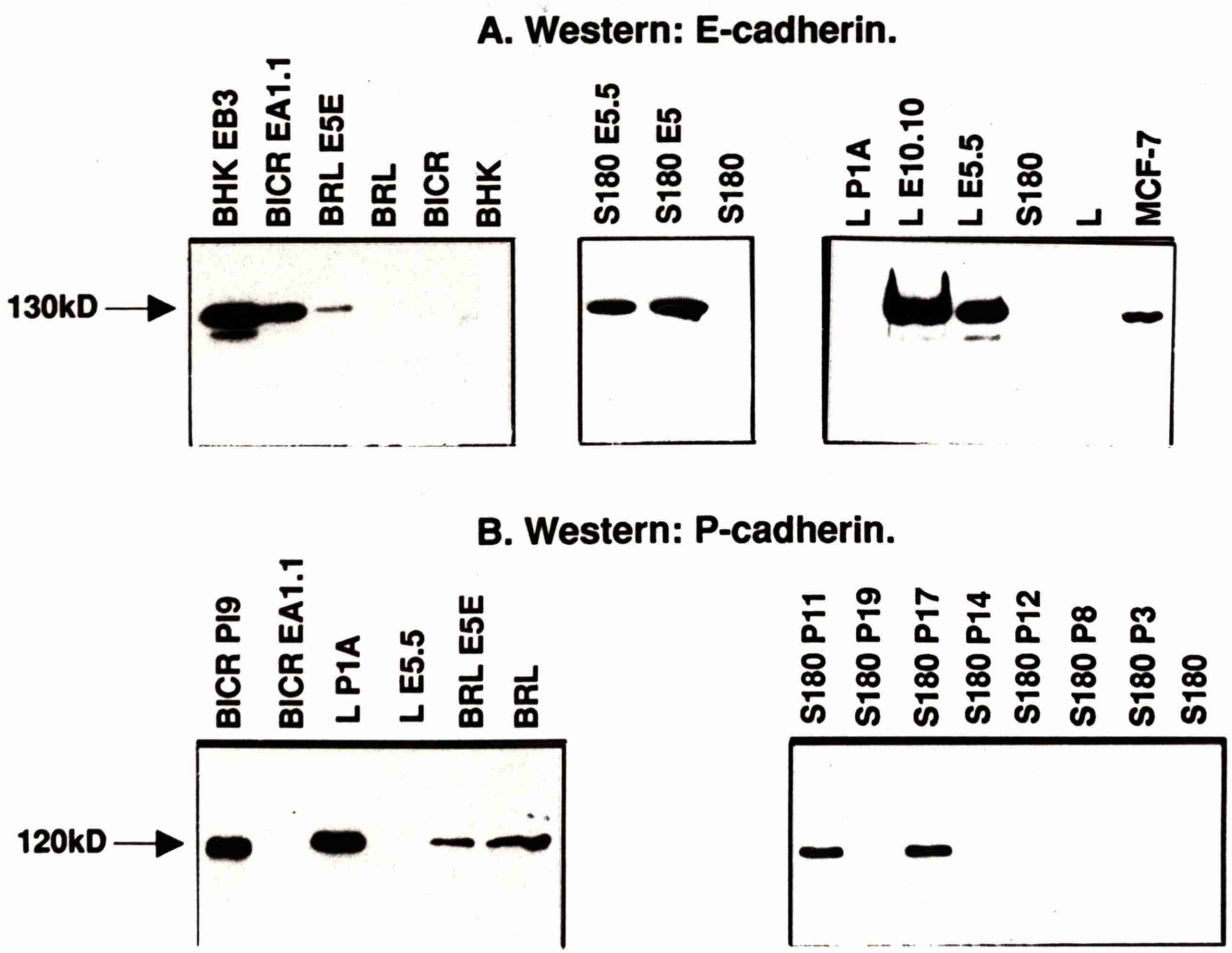

A. Western: E-cadherin.
BHK EB3
BICR EA1.1
BRL E5E
BRL
BICR
BHK
S180 E5.5
S180 E5
S180
L P1A
L E10.10
L E5.5
S180
L
MCF-7
130kD
B. Western: P-cadherin.
BICR PI9
BICR EA1.1
L P1A
L E5.5
BRL E5E
BRL
S180 P11
S180 P19
S180 P17
S180 P14
S180 P12
S180 P8
S180 P3
S180
120kD

Figure 23. Immunofluorescence analysis of E-cadherin expression by L E5.5 cells.

(A) is a phase contrast image of the fluorescence micrograph shown in (B). L E5.5 cells expressing exogenous E-cadherin, were cultured on coverslips, fixed in ethanol and used for immunofluorescence analysis as described in materials and methods. Primary antibody was ECCD-2 (rat monoclonal anti-E-cadherin) at 1:500 (B). E-cadherin protein is expressed at points of cell contact between the transfected cells (B). In contrast, no fluorescence was seen when the primary antibody was PCD-1 (rat monoclonal anti-P-cadherin; data not shown). Magnification x1000.

Figure 23.

Phase contrast.

Fluorescence.

A.

B.

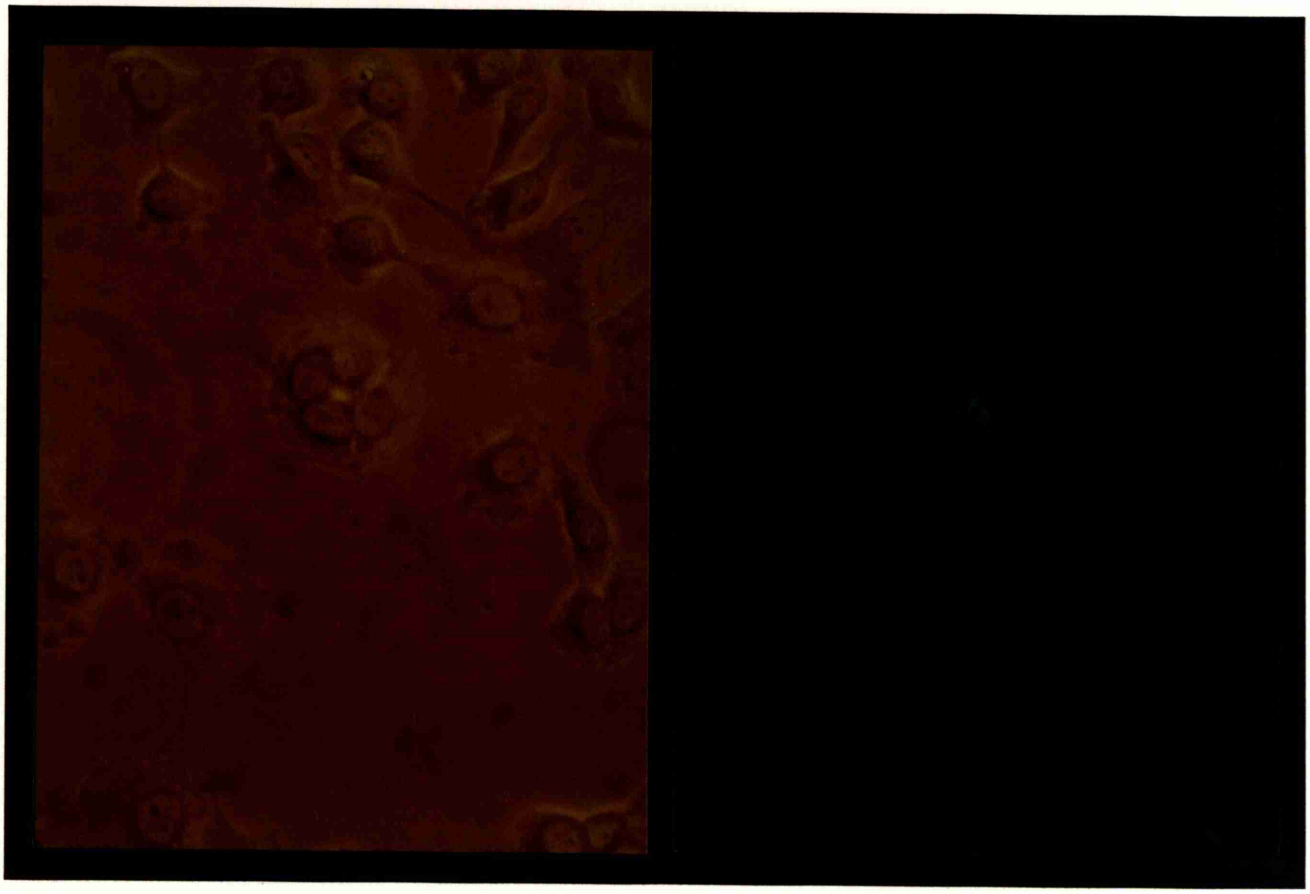


Figure 24. Immunofluorescence analysis of E-cadherin expression by S180 E5.5 cells.

(A) is a phase contrast image of the fluorescence micrograph shown in (B). S180 E5.5 cells expressing exogenous E-cadherin, were cultured on coverslips, fixed in ethanol and used for immunofluorescence analysis as described in materials and methods. Primary antibody was ECCD-2 (rat monoclonal anti-E-cadherin) at 1:500 (B). E-cadherin protein expression was detectable (B). In contrast, no fluorescence was seen when the primary antibody was PCD-1 (rat monoclonal anti-P-cadherin; data not shown). Magnification x1000.

Figure 24.

Phase contrast.

Fluorescence.

A.

B.

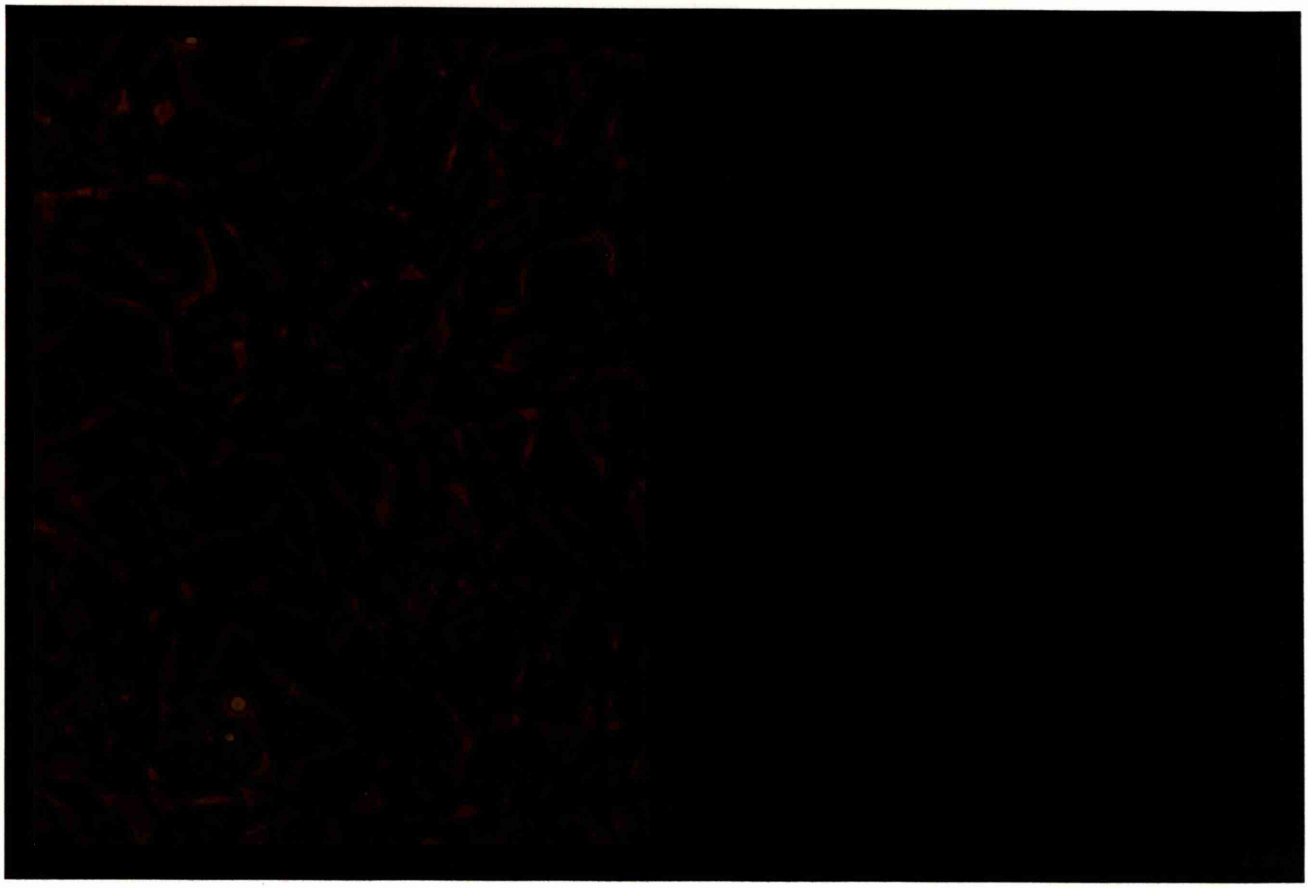


Figure 25. Immunofluorescence analysis of E-cadherin expression by BHK EB3 cells.

(A) is a phase contrast image of the fluorescence micrograph shown in (B). BHK EB3 cells expressing exogenous E-cadherin, were cultured on coverslips, fixed in ethanol and used for immunofluorescence analysis as described in materials and methods. Primary antibody was ECCD-2 (rat monoclonal anti-E-cadherin) at 1:500 (B). E-cadherin protein expression was detectable (B). In contrast, no fluorescence was seen when the primary antibody was PCD-1 (rat monoclonal anti-P-cadherin; data not shown). Magnification x1000.

Figure 25.

Phase contrast. **Fluorescence.**

A. **B.**

Figure 26. Immunofluorescence analysis of E-cadherin expression by BICR A1.1E cells.

(A) is a phase contrast image of the fluorescence micrograph shown in (B). BICR A1.1E cells expressing exogenous E-cadherin, were cultured on coverslips, fixed in ethanol and used for immunofluorescence analysis as described in materials and methods. Primary antibody was ECCD-2 (rat monoclonal anti-E-cadherin) at 1:500 (B). E-cadherin protein is expressed at points of cell contact between the transfected cells (B). In contrast, no fluorescence was seen when the primary antibody was PCD-1 (rat monoclonal anti-P-cadherin; data not shown). Magnification x1000.

Figure 26.

Phase contrast. **Fluorescence.**

A. **B.**

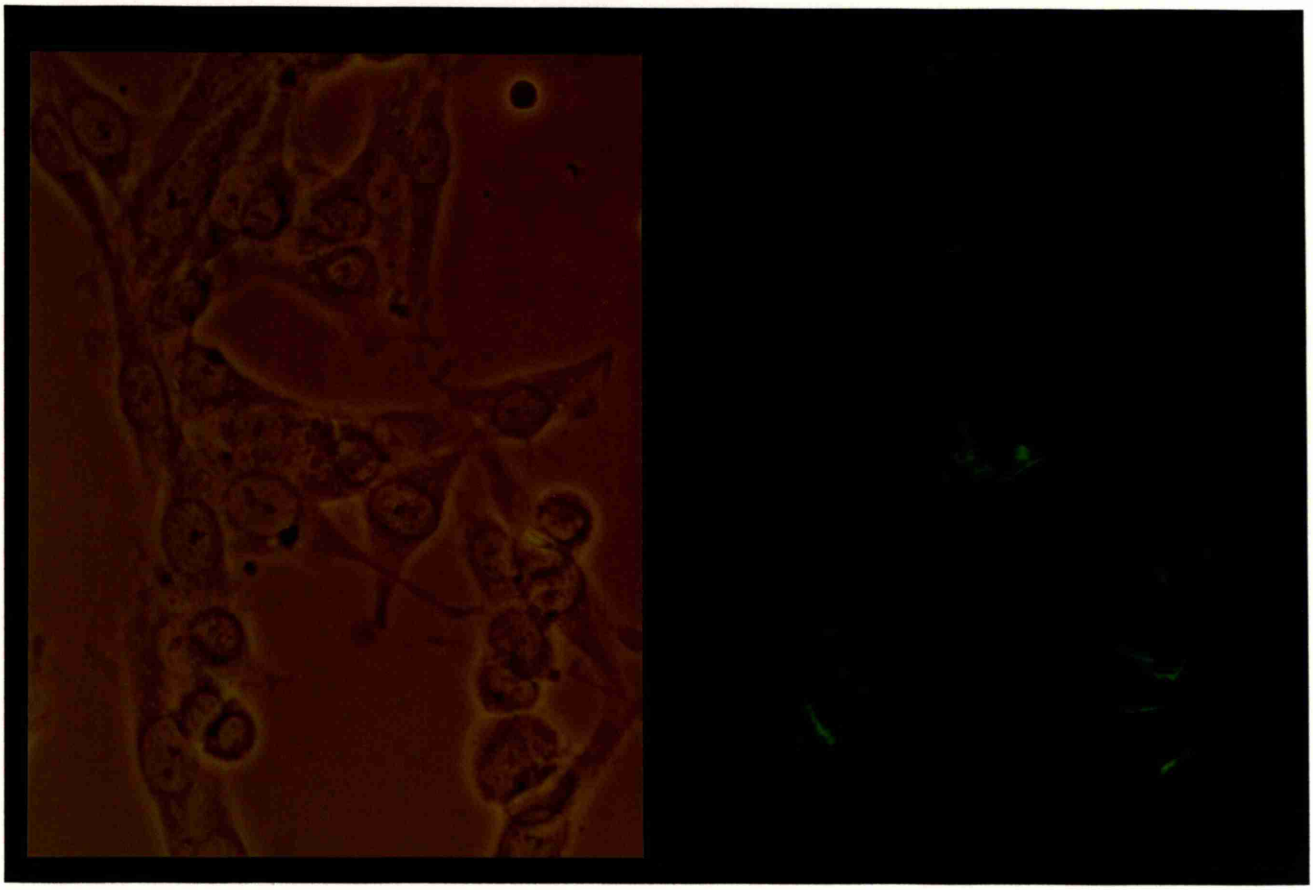


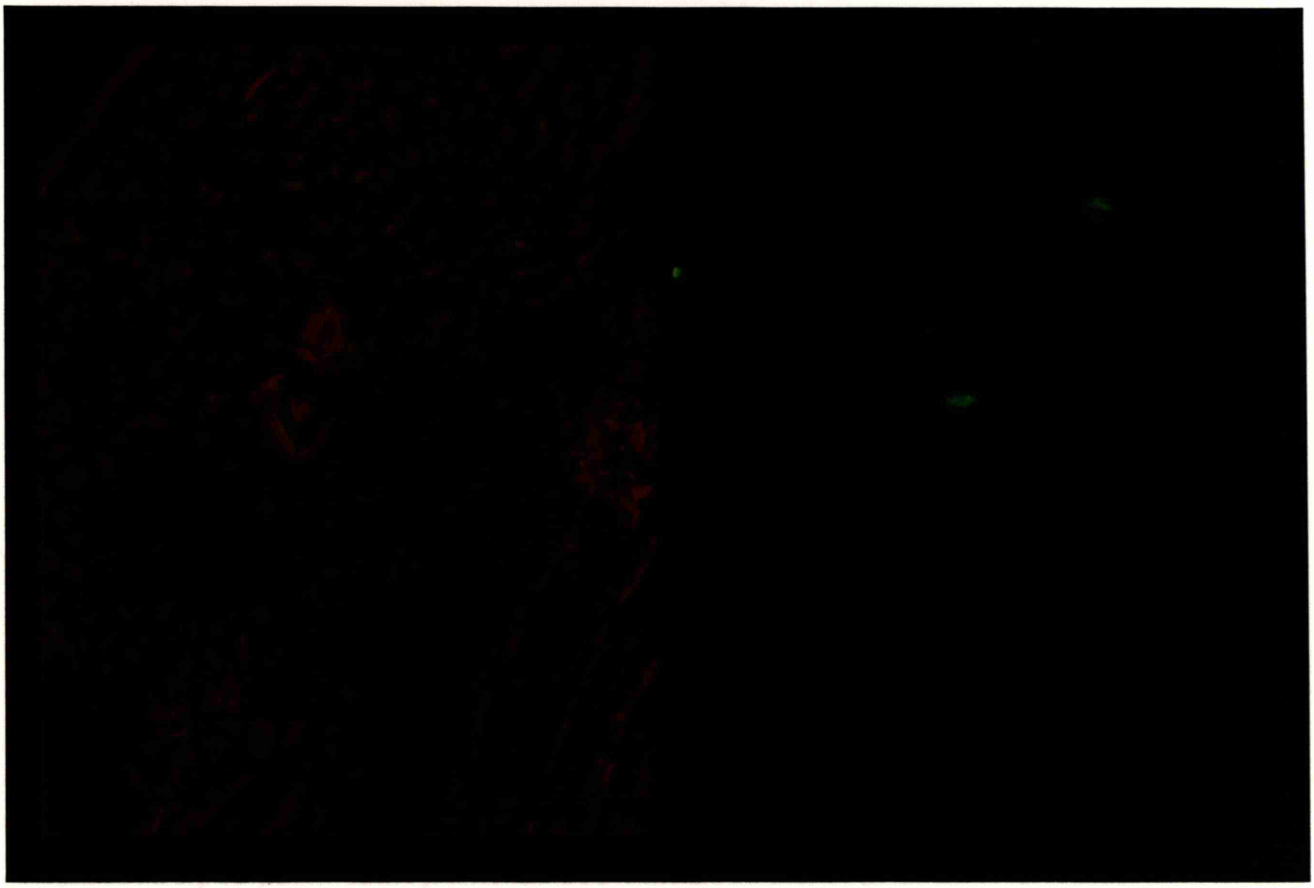
Figure 27. Immunofluorescence analysis of E-cadherin expression by BRL E5E cells.

(A) is a phase contrast image of the fluorescence micrograph shown in (B). BRL E5E cells expressing exogenous E-cadherin, were cultured on coverslips, fixed in ethanol and used for immunofluorescence analysis as described in materials and methods. Primary antibody was ECCD-2 (rat monoclonal anti-E-cadherin) at 1:500 (B). E-cadherin protein expression is detectable (B). In contrast, no fluorescence was seen when the primary antibody was PCD-1 (rat monoclonal anti-P-cadherin; data not shown). Magnification x1000.

Figure 27.

Phase contrast. **Fluorescence.**

A. **B.**



E-cadherin confirms that exogenous E-cadherin protein is expressed by L, S180, BHK, BICR and BRL transfected clones (figure 23, 24, 25, 26 and 27). The transfected clones expressing exogenous E-cadherin which have been used for further studies include L E5.5, S180 E5.5, BHK EB3, BICR A1.1E and BRL 5E5.

In order to express P-cadherin in the cell lines a mouse P-cadherin cDNA (of 3.2kb) cloned into the *Eco*RI site of pBluescript SK (obtained from Prof.Takeichi, Kyoto University, Japan; figure 28.A) was subcloned using standard DNA manipulations (as described in section 2..2.3.3) from pBluescript SK via pIC20H, (to enable the generation of 5'*Bgl*II and 3'*Bam*HI sticky ends) into the *Bam*HI site of the expression vector pZipneoSVX (figure 28.B). Sequence obtained using oligo L as a primer revealed that the subcloning was successful, (as shown by the presence of the correct pZipneo sequence 5' of the *Bam*HI/*Bgl*II hybrid site; figure 28.B). However, the sequencing also revealed that the P-cadherin cDNA contained a 47bp deletion in the 5' region, (from nucleotide position 1 to 47). The deletion included the A and T of the initiation codon, (at nucleotide positions 46 and 47 respectively) and this generated in its place the codon TCG which will not act as start site for translation. Sequence obtained with the primer M13-20 shows that the 47bp deletion is also present in the original P-cadherin cDNA cloned into the *Eco*RI site of pBluescript SK (figure 28.A), demonstrating that it is not a consequence of subcloning. Obtaining the P-cadherin expression construct pβact-Pcad (Nose et al 1988) from Prof.Takeichi proved to be the most efficient solution to this problem. Sequence data confirmed that the pβact-Pcad construct contains an intact ATG start site and that the P-cadherin cDNA is in the correct orientation for sense RNA expression (figure 28.C).

The pβact-Pcad construct, in which the expression of P-cadherin is under the control of the chicken β-actin promoter has been cotransfected with either the

neomycin expression vector pZIPneoSVX (lacking exogenous cDNA inserted into the *Bam*HI site) or pHSG274, into the cell lines L, S180, BHK and BICR. $G418^R$ cell lines were established by selecting in media containing G418 and ring cloning representative colonies (table 7A-C). Those pβact-Pcad cotransfected cell lines which grew in tight cell groups and/or were positive for P-cadherin expression by preliminary immunofluorescence analysis have been used for further studies. Genomic DNA was prepared from the parental and pβact-Pcad transfected cell lines and digested with *Eco*RI, which cuts pβact-Pcad to generate the 3.2kb P-cadherin cDNA insert (figure 28.C). Southern analysis using a 0.65kb HincII fragment of mouse P-cadherin cDNA as the probe, reveals the presence of the expected 3.2kb band in the transfected lines but not in the parental cells (figure 20.A). Those clones shown by Southern analysis to contain copies of the exogenous plasmid DNA also expressed P-cadherin transcripts (figure 21.B) and the appropriate 120kD P-cadherin protein (figure 22.B). Immunofluorescent staining with monoclonal antibody PCD-1 against P-cadherin confirmed that the exogenous P-cadherin protein was expressed by L, S180, BHK and BICR transfected clones (data not shown). The transfected clones expressing exogenous P-cadherin which have been used for further studies include L P1A, S180 P17, BICR PI9 and BHK P3B3.

4.3 Morphological changes induced by exogenous cadherin expression.

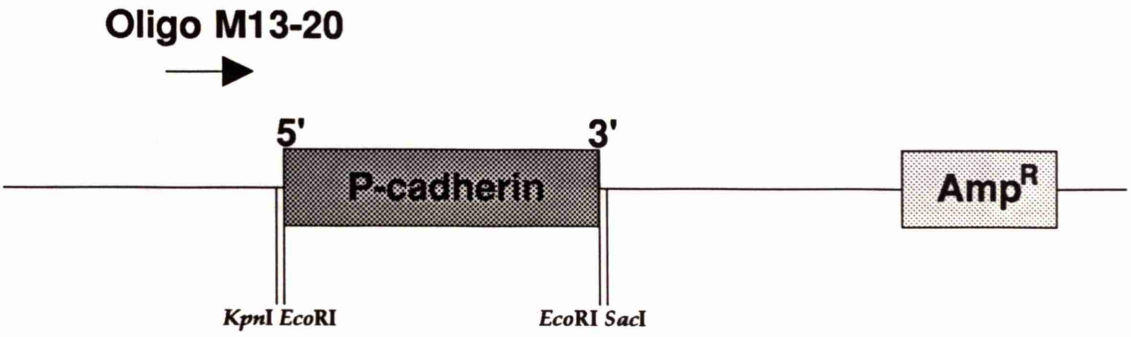
The morphological changes caused by the exogenous expression of either mouse E- or P-cadherin varies between the cell lines but not with the type of cadherin expressed. It is most dramatic for L and S180 cadherin transfectants, where the cadherin expressing clones grow in tight groups, in which the cells appear flatter and less fibroblastic than the parental cells (figure 29). The BICR

Figure 28. P-cadherin constructs.

Sequence analysis was performed to show the integrity of part of the P-cadherin cDNA fragment cloned into each construct.

Figure 28.A

pBluescript SK-Pcad.



Sequence obtained with oligo M13-20:

5'CCATGGCCCGGGGGGGAGCTCCAGCTGCCATAGCTATTCGAACTATAGCTTAAGGGG[48] **GAG CTT CTT**

AGT GGG CCT CAC GCC TTC CTG CTC CTC CTG CTC CAG GTT[102] **3'**

CCATGG *KpnI*

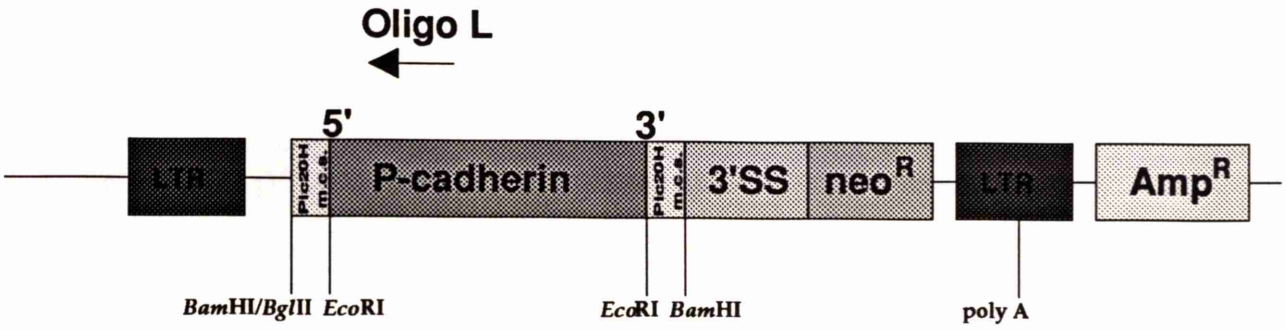
CTTAAG *EcoRI*

Nucleotide sequence in bold corresponds to mouse P-cadherin,

positions 48 to 102 (Nose et al 1987).

Figure 28.B

pZipneoSVX-Pcad.



Sequence obtained with oligo L:

5'TTTGCTTTCGGTTTGGGACCGAAGCCGCGCCGCGCGCGTCTTGGTCGCCAGATCCGGATCTAGATATCG

ATGAATTCG[48] **GAG CTT CTT AGT GGG CCT CAC GCC TTC CTG CTC CTC CTG CTC CAG**[96] 3'

GGATCT *BamHI/BglII*

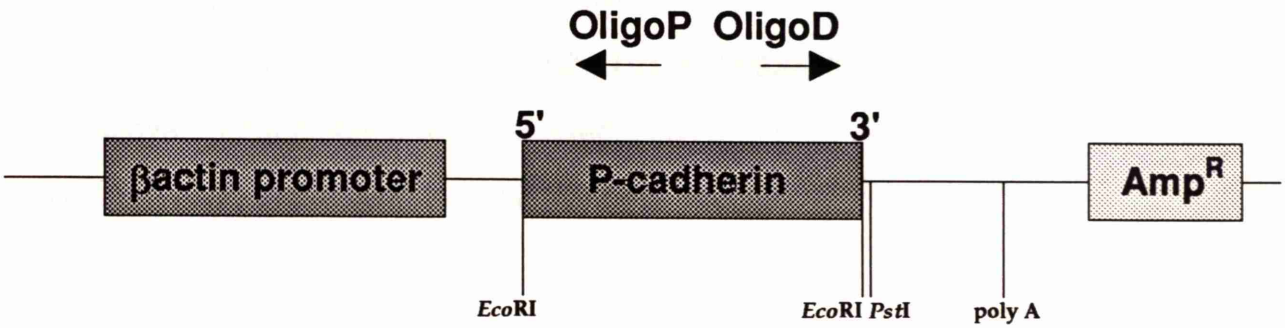
GAATTC *EcoRI*

Nucleotide sequence in bold corresponds to mouse P-cadherin,

positions 48 to 96 (Nose et al 1987).

Figure 28.C

pβact-Pcad.



Sequence obtained with oligo P:

5'[39]TTCAGTT ATG GAG CTT CTT AGT GGG CCT CAC GCC TTC CTG CTC CTC CTG CTC

CAG GTT TGC TGG CTA CGC AGC GTG GTC TCC GAG CCC TAC CGA GCG GGC TTC

ATC GGG GAG[150] 3'

Nucleotide sequence corresponds to P-cadherin,

position 39 to 150 (Nose et al 1987).

Sequence obtained with oligo D:

5'[3089]TATTTTTTTATTTTCCCTGTTATGTGCTGTAGATGGAGAGTGATGACAATCGTGTAAATGTACTAGAAT

TTTTTTATTAAAATGAACTTTTTTTTTTCC[3187]CCCCGGAATTCCTGCAGCCCACTGTA 3'

GAATTC *EcoRI*

CTGCAG *PstI*

Nucleotide sequence in bold corresponds to P-cadherin,

positions 3089 to 3187 (Nose et al 1987).

Figure 29. Morphological changes induced in L and S180 cells by exogenous cadherin expression.

Monolayer morphology of untransfected (A,D) and E-cadherin (B,E) or P-cadherin (C,F) expressing L and S180 cells. Magnification x500.

Figure 29.

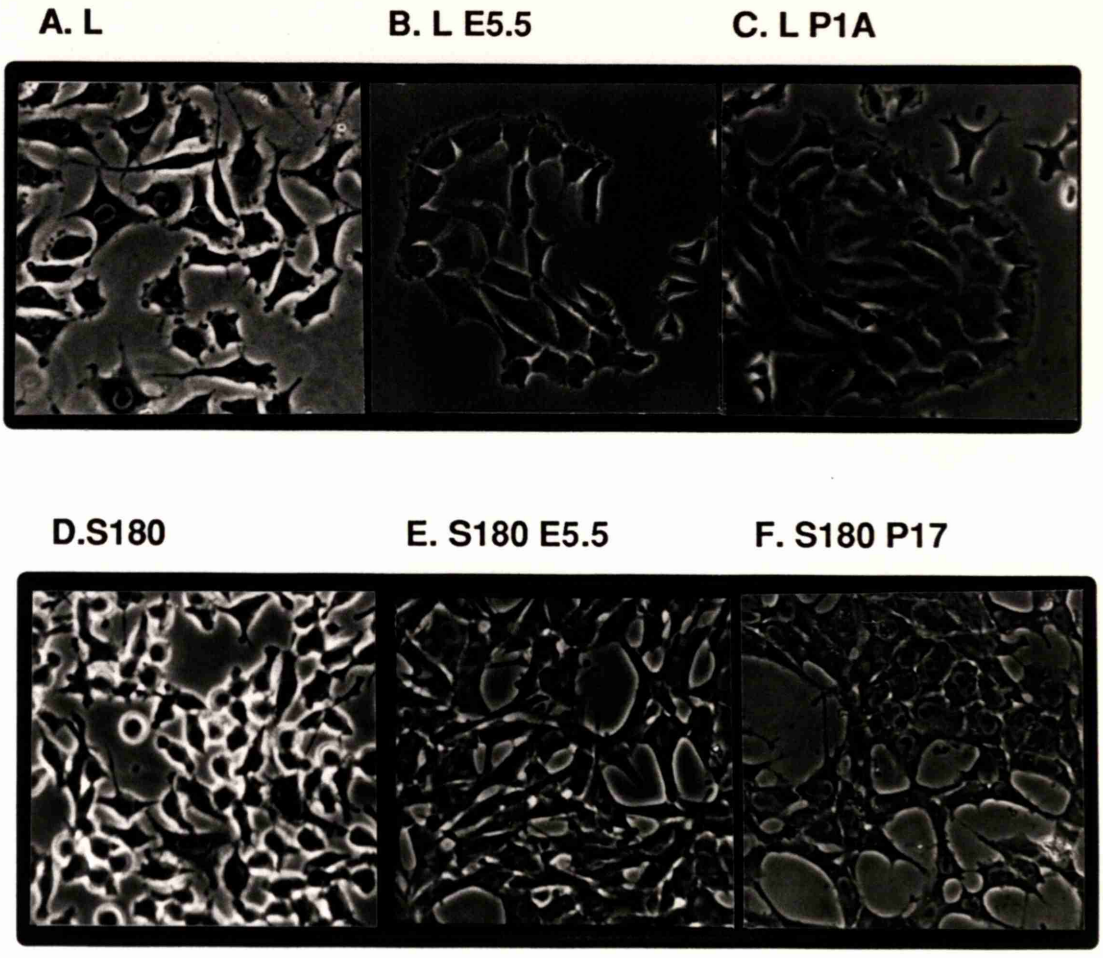


Figure 30. Morphological changes induced in BHK, BICR and BRL cells by exogenous cadherin expression.

Monolayer morphology of untransfected (A,D,H) and exogenous E-cadherin (B,E,H) or exogenous P-cadherin (C,F) expressing BHK, BICR cells and BRL cells. Magnification x500.

Figure 30.

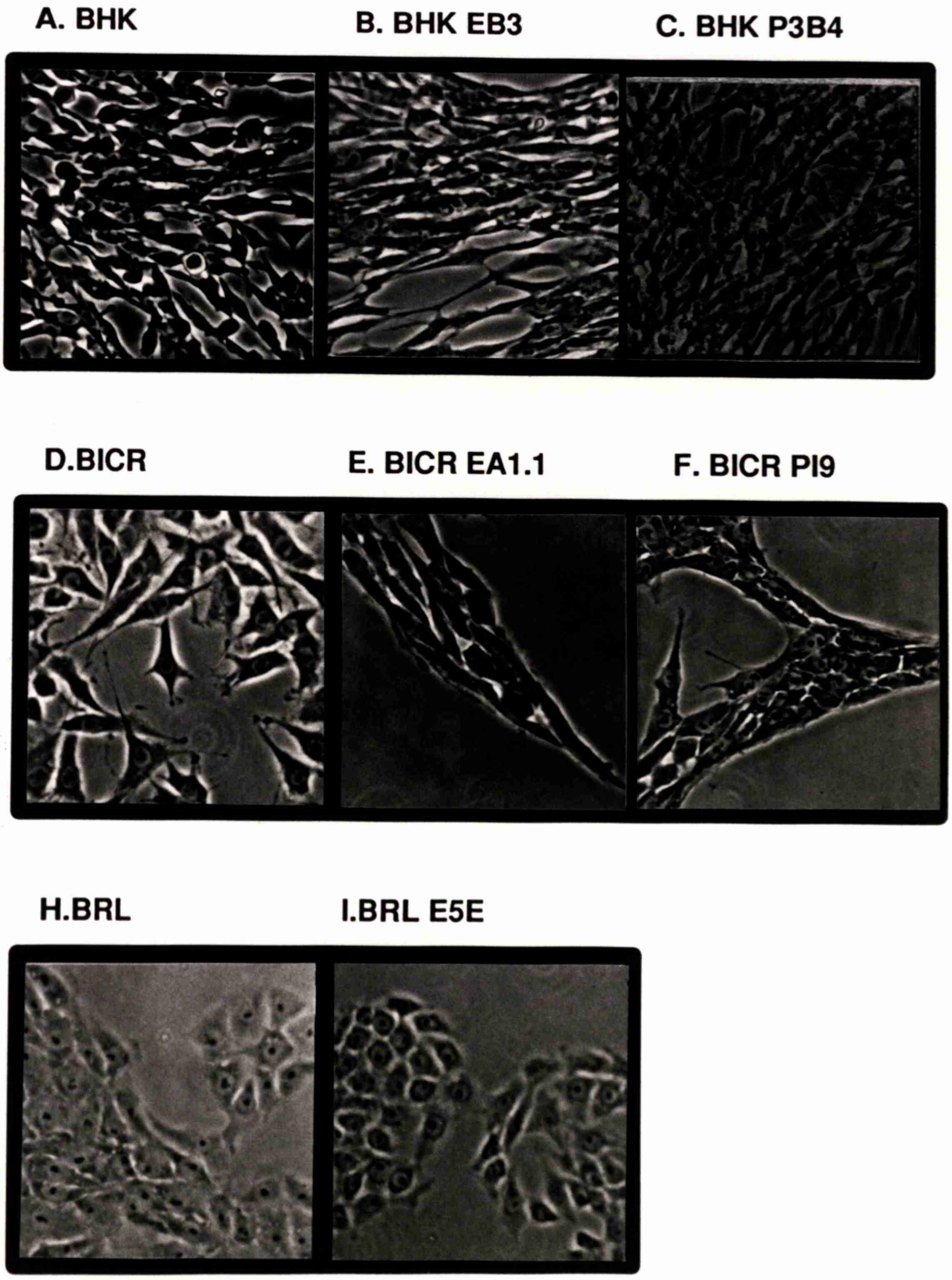
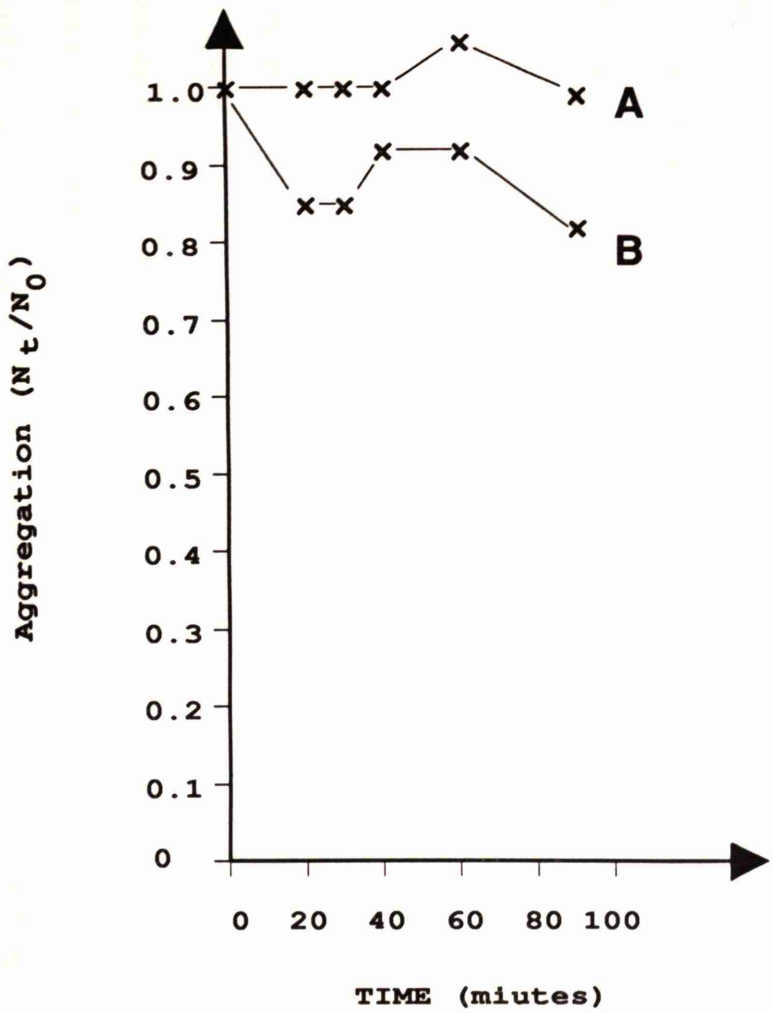
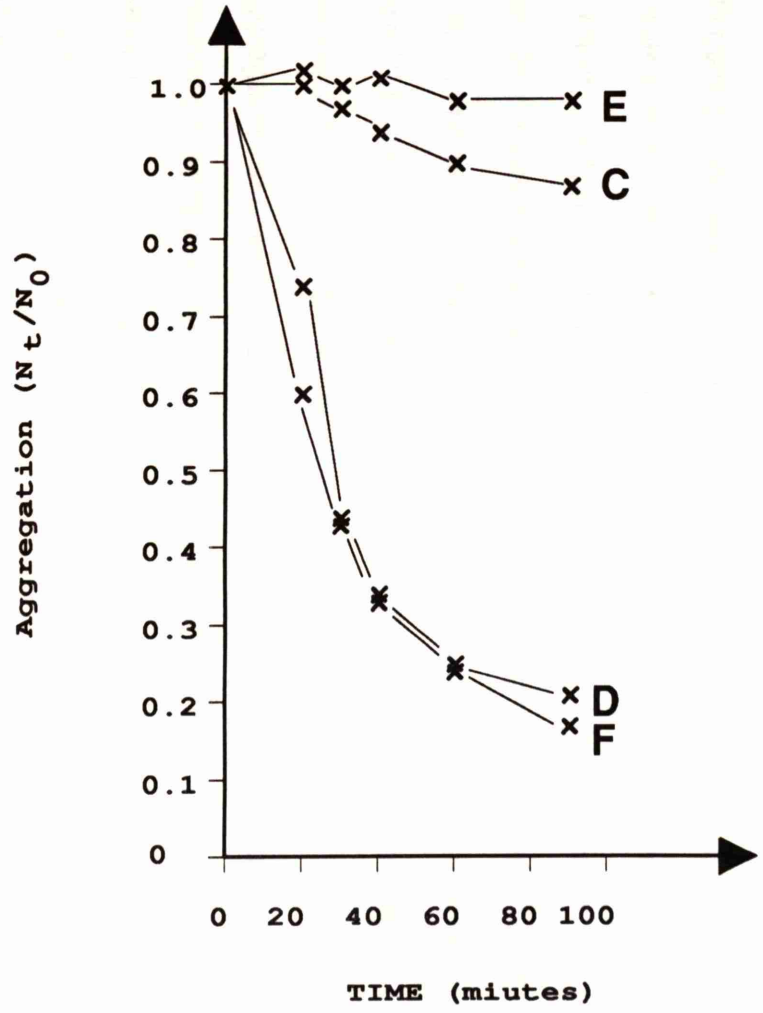

A. BHK
B. BHK EB3
C. BHK P3B4
D.BICR
E. BICR EA1.1
F. BICR PI9
H.BRL
I.BRL E5E

Figure 31. Calcium dependant cell adhesion.

Cell aggregation assays were performed (as described in materials and methods) to show the adhesive ability of untransfected and transfected L cells expressing exogenous cadherin. The number of cells counted at time t (N_t) divided by the initial number of cells (N_0) was plotted against time. (A) L +EDTA, (B) L +Ca^{++}, (C) L E5.5 +EDTA, (D) L E5.5 +Ca^{++}, (E) L P1A +EDTA, (F) L P1A +Ca^{++},

Figure 31.


Aggregation (N_t/N_0)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A
B
0 20 40 60 80 100
TIME (miutes)


Aggregation (N_t/N_0)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
E
C
D
F
0 20 40 60 80 100
TIME (miutes)

cadherin expressing transfectants retain the fibroblastic appearance of the parental BICR cells but adhere more strongly to each other, forming smooth edged, often star shaped colonies (figure 30.A). The BHK cadherin expressing transfectants retain the fibroblastic appearance of the parental BHK cells, at lower densities they appear better grouped together than the parental cells but at higher densities the features of the transfected and parental cells are similar as shown in figure 30.B. The epithelial morphology of the BRL cell line is unaltered by the exogenous expression of E-cadherin (figure 30.C).

Cell aggregation assays to show the adhesive ability of parental and cadherin transfected L cells were performed to confirm that cadherin expression influences cell-cell interaction. Monolayer cultures of either parental or cadherin transfected L cells were trypsinized in the presence of 1mM calcium, dissociated into single cell suspensions and allowed to associate over the course of a ninety minute incubation (as described in section 2.2.1.3). During this time course, as shown in figure 31 the cadherin expressing clones L E5.5 or L P1A but not the parental L cells aggregated into clumps. However, if the trypsinization and subsequent incubation were performed in the presence of 1mM EDTA the aggregation was greatly reduced. These results are similar to those previously reported by Nose et al (1988) and provide evidence for functionally active cadherin expression.

4.4 Discussion.

Cell lines were established that contain copies of the E- or P-cadherin expression constructs and express the appropriate cadherin transcripts and proteins, as shown by Southern, northern and western analysis. The immunofluorescent staining of the transfected cells generally reveals that the exogenous cadherin is expressed at points of cell-cell contact, consistent with a

role for cadherin in mediating cell adhesion. BHK transfectants appear to have a more diffuse pattern of exogenous cadherin expression, which may be due to the failure of the exogenous cadherin expressed in this cell line to associate with one or more of its normal cytoplasmic partners, the catenins. As a similar pattern of E-cadherin immunofluorescence is seen in L cells expressing mutant E-cadherins containing cytoplasmic deletions that prevent association with catenins and therefore anchorage to the cytoskeleton (Nagafuchi and Takeichi 1988, Takeichi 1991).

The L and S180 cadherin transfectants have a more epithelial morphology, which is consistent with the results of others (Mege et al 1988, Nose et al 1988, McNeill et al 1990), whereas BICR and BHK cadherin transfectants remain fibroblastic. The mechanisms underlying cadherin induced morphological changes and the establishment of ordered cell arrangements are not understood, cadherin-cadherin interaction and the transmission of a signal via the catenins to the cytoskeleton may be crucial in the reorganisation of the latter system. The effects of cadherin expression may vary between each cell type because of variation in catenin expression or cytoskeletal organisation. Interestingly, as E- and P-cadherin expression has similar effects on cell phenotype this suggests that these cell adhesion molecules interact with the same cytoplasmic proteins.

Cadherins can therefore affect cell morphology and cell-cell adhesion and the question of whether they also influence gap junction formation is addressed in chapters 5 and 6.

Chapter 5.

The control of gap junction formation in *mec*$^-$ cells.

5.1 Introduction.

The cell lines L and S180 are poorly coupled via gap junctions. These cell types express ductin and connexin 43 but unlike the *mec*$^+$ cell lines (BHK, BRL or BICR) they do not express either of the cell adhesion molecules E-, N- or P-cadherin or N-CAM. The expression of ductin and connexin 43 by L and S180 cells is therefore not sufficient for extensive GJIC and this suggests that cell adhesion molecules may also contribute to gap junction formation. The dye transfer results showing the frequency of homologous GJIC between L or S180 cells transfected with either E- or P-cadherin are described in this chapter.

5.2 The influence of cadherin and connexin expression on the frequency of homologous GJIC in L cells.

L cell transfectants expressing either exogenous E-cadherin (L E5.5 and L E10.10) or P-cadherin (L P1A and L P15) were microinjected with Lucifer Yellow CH for 2 minutes and the extent of dye coupling observed (table 9). Representative dye spreads are shown in figures 32 and 33. The cadherin expressing L cell clones, despite the obvious phenotypic changes are poorly coupled via gap junctions (L E5.5 0%, L E10.10 0%, L P1A 10%, L P15 10%; percentage of injections positive for dye transfer). This incidence of dye coupling is comparable to that of the parental L cells (4% of injections positive for dye transfer). The restoration of cell adhesion by the expression of E- or P-cadherin is therefore not sufficient by itself to correct the poor communication ability of L cells, similar unpublished observations have also been made by Rose, Takeichi and Loewenstein and are quoted by Loewenstein and Rose (1992). This

suggests that other proteins, required for gap junction formation and/or function are also aberrantly expressed by L cells.

An obvious candidate is connexin 43 which is only expressed at low levels and in the non-phosphorylated form by both parental and cadherin transfected L cells (figure 36). To investigate whether exogenous connexin 43 expression is required for communication the $G418^R$, cadherin expressing L cell lines L E5.5 and L P1A were transfected with the connexin 43 expression vector pBabehygro-cx43. This vector was constructed using standard DNA manipulations by subcloning a 1.4kb fragment of rat connexin 43 cDNA (clone G2; Paul 1986) containing the entire open reading frame, from the *Eco*RI site of pBluescript SK into the *Eco*RI site of the expression vector pBabehygro (Morgenstein and Land 1990). Sequence analysis revealed that the subcloning was successful, (as shown by the ability to the read the correct pBabehygro sequence 5' and 3' of the connexin 43 insert; figure 34). The expression vector pBabehygro-cx43 should express connexin 43 mRNA from a promoter within the Moloney murine leukaemia virus (MoMuLV) long terminal repeat (LTR) and also mRNA conferring resistance to hygromycin B from the internal SV40 early region promoter, thereby allowing the selection of hygromycin B resistant clones. Cell lines were obtained by selecting in media containing G418 and hygromycin B and ring cloning colonies with the characteristic morphology of cadherin expressors.

Genomic DNA was prepared from the parental and pBabehygro-cx43 cotransfected cell lines and digested with either *Eco*RI or *Xba*I, which cut the pBabehygro-cx43 construct as shown in figure 34 and generate fragments of 1.4kb and 5.3kb respectively. Southern blotting and subsequent hybridization using the 1.4kb fragment of connexin 43 cDNA as the probe, reveals the presence of the expected 1.4kb and 5.3kb fragments in the pBabehygro transfected cell lines L E43 2A2, L E43 2A4 and L E43 2A5 but not in the

Figure 32. Dye transfer analysis in cultures of L E5.5 cells expressing E-cadherin.

(A) is a phase contrast image of the fluorescence micrographs shown in (B). Lucifer Yellow CH microinjected into an L E5.5 cell transfected with the E-cadherin expression construct pBATEM2 does not spread to adjacent cells. The injected cells are marked with a black dot. Magnification x1000.

Figure 32.

Phase contrast.

Fluorescence.

A.

B.

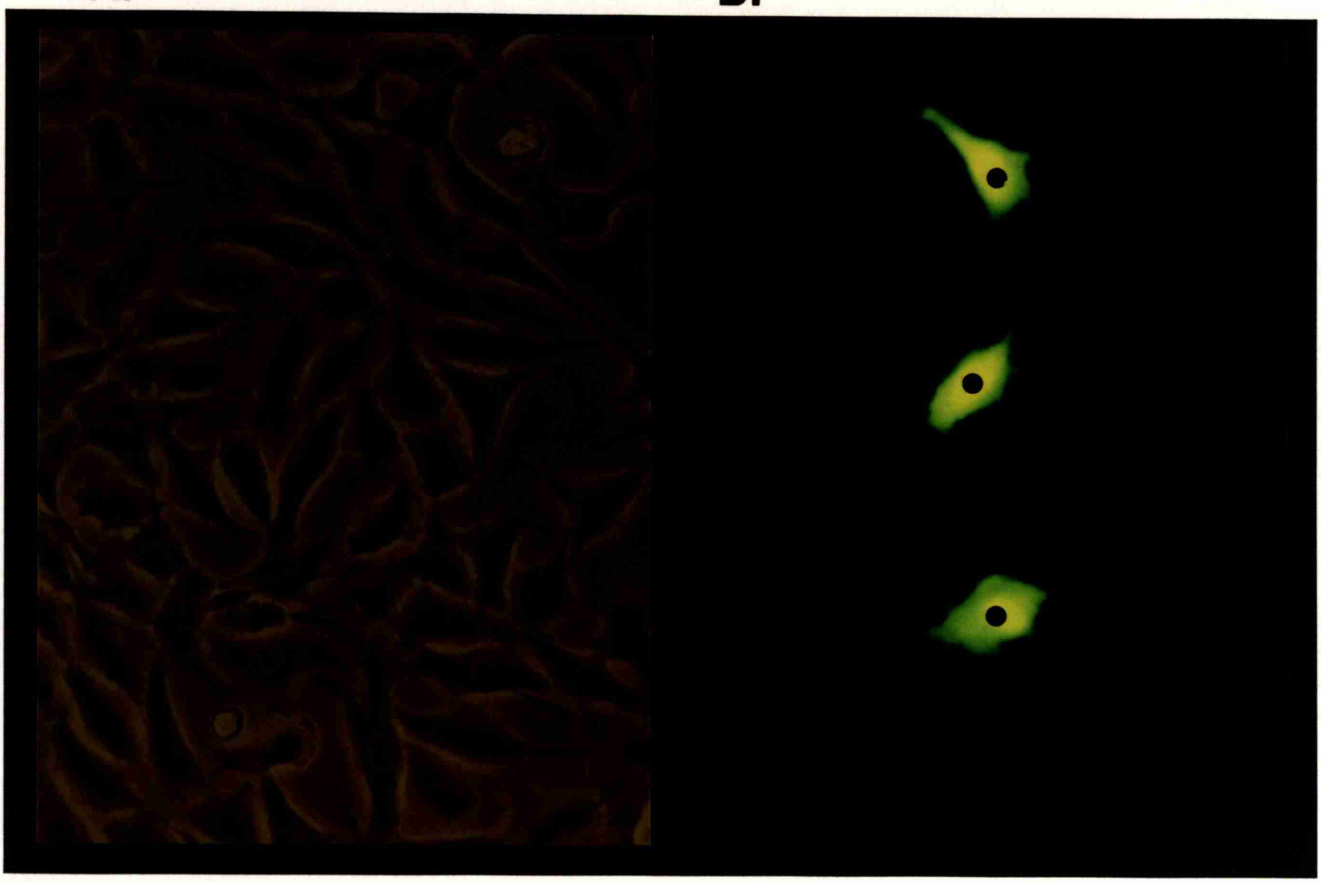


Figure 33. Dye transfer analysis in cultures of L P1A cells expressing P-cadherin.

(A) is a phase contrast image of the fluorescence micrographs shown in (B). Lucifer Yellow CH microinjected into an L P1A cell transfected with the P-cadherin expression construct pβact-Pcad does not spread to adjacent cells. The injected cells are marked with a black dot. Magnification x1000.

Figure 33.

Phase contrast.

Fluorescence.

A.

B.

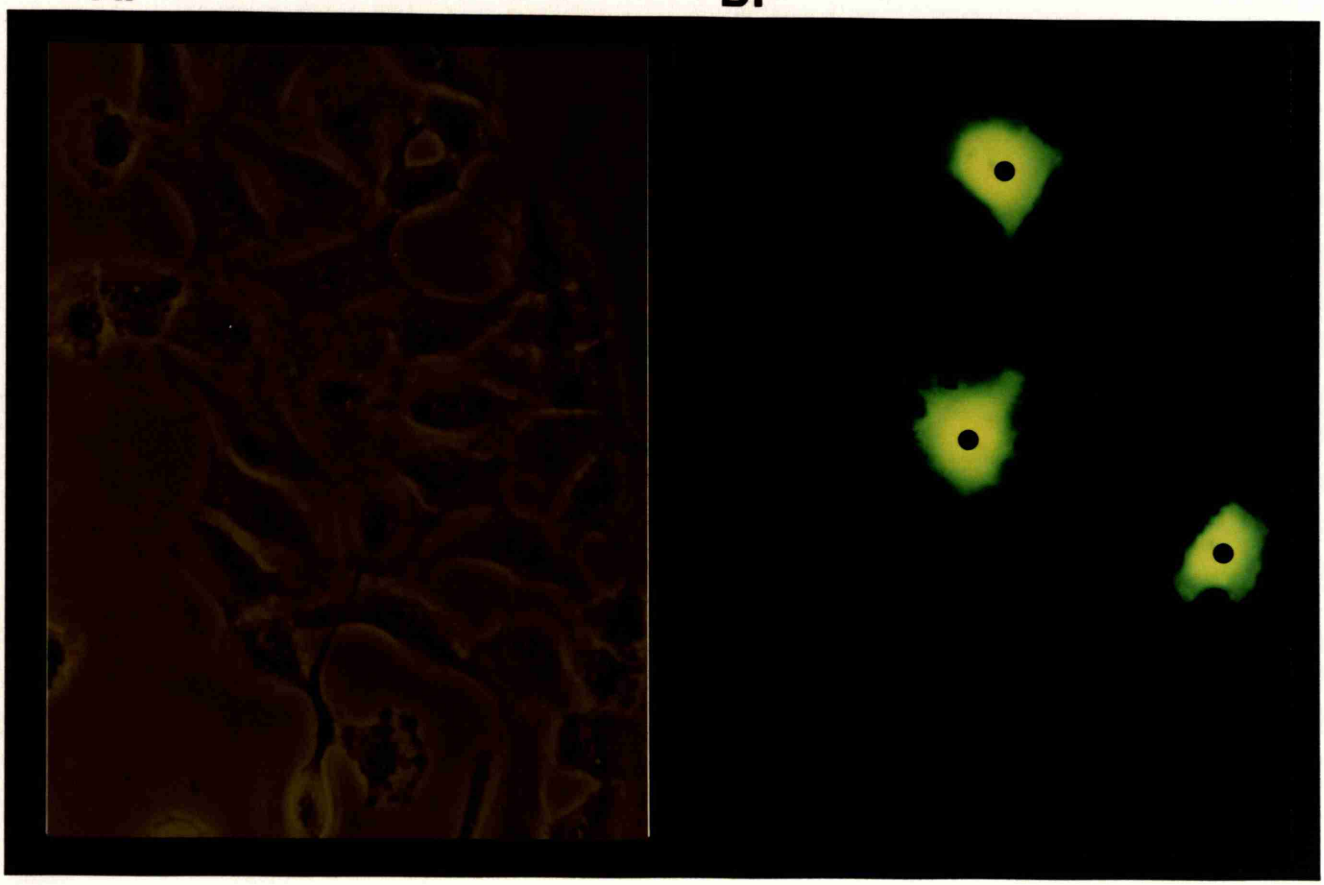


Table 8. Frequency of homologous dye transfer in cultures of L cells expressing exogenous cadherin.

L cell lines expressing exogenous E- or P-cadherin were examined for their dye coupling ability. The increased dye coupling ability of L P1A and L P15 cells was not significantly higher than that of the parental cells.

Table 8.

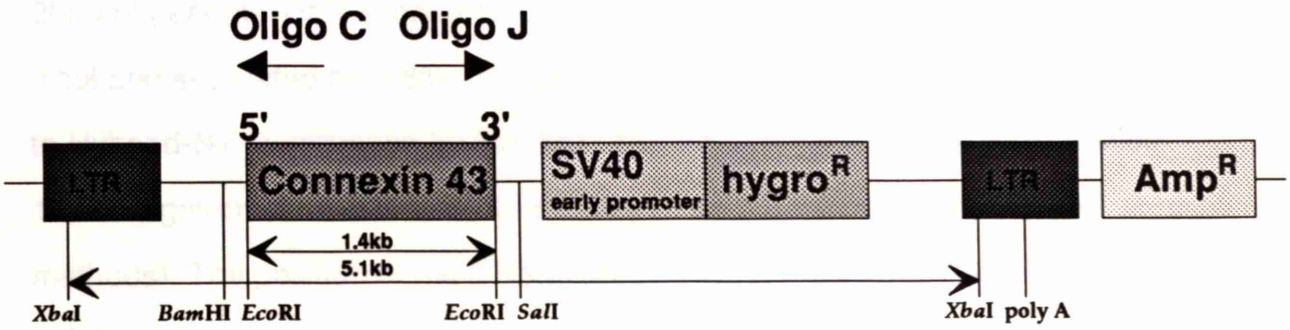
Cell line	Number of injections.	Percentage positive.	Average number of coupled cells per injection, (S.D.).	t.	P.
L	55	4%	0.04 (0.19)		
L E5.5	30	0%	0 (0)		
L E10.10	10	0%	0 (0)		
L P1A	29	10%	0.1 (0.40)	1.04	P>0.05
L P15	10	10%	0.1 (0.32)	0.61	P>0.05

Figure 34. A schematic diagram of linearised connexin 43 expression construct pBabehygro-cx43.

Sequence analysis was performed to confirm the integrity of the cloning site.

Figure 34.

pBabehygro-Cx43.



Sequence obtained with oligo C:

5'TTGAACCTCCTCGGTTCGACCCCGCCTCGATCCTCCCTTTATCCAGCCCTCACTCCTTCTCTAGGCGCC

GGCCGGATCCCAGTGTGGTGGTACGTAGGAATTCG 3'

GGATCC *BamHI*

GAATTC *EcoRI*

Nucleotide sequence corresponds to pBabehygro 5' of connexin 43 insert.

Sequence obtained with oligo J:

5'CCAGCACAGTGGTCGACCCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCA

GGCAGAAGTATGCAAAGCAT 3'

GTCGAC *SalI*

Nucleotide sequence corresponds to pBabehygro 3' of connexin 43 insert.

Figure 35. Southern analysis of pBabehygro-cx43 transfectants.

20µg of genomic DNA prepared from the cell lines was digested with *Eco*RI or *Xba*I and separated on 0.8% TBE gels. The DNA was subsequently transferred to Hybond-N+ membranes by Southern blotting and hybridized with a labelled 1.4kb fragment of rat connexin 43 cDNA (A), as described in material and methods). The position of the DNA molecular weight markers, the 0.6kb and 2.5kb pBabehygro-cx43 bands are shown. The L E43 2A5 lanes are overloaded and a shorter exposure is shown in (B).

Figure 35.

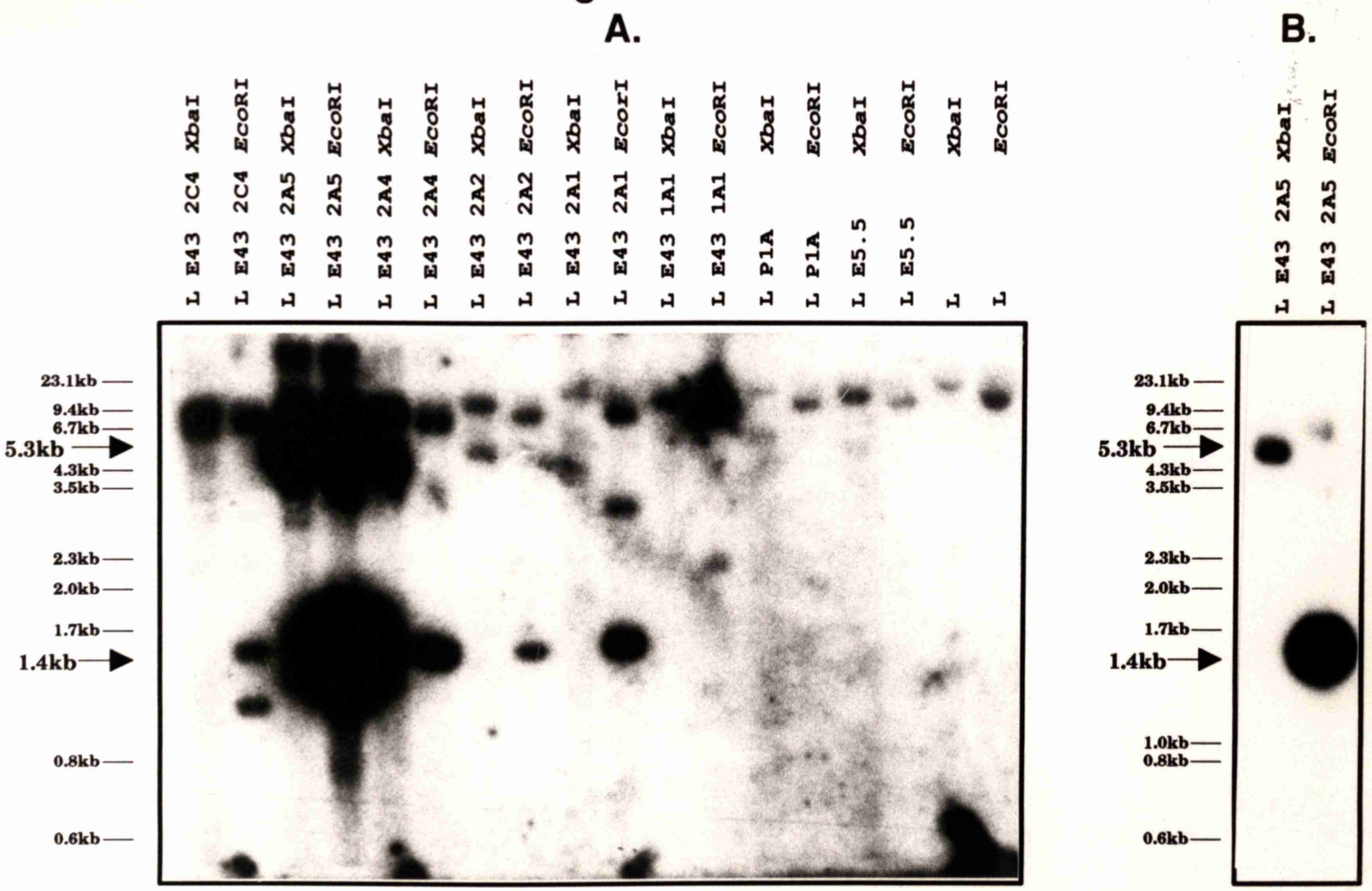

A.
B.
L E43 2C4 XbaI
L E43 2C4 EcoRI
L E43 2A5 XbaI
L E43 2A5 EcoRI
L E43 2A4 XbaI
L E43 2A4 EcoRI
L E43 2A2 XbaI
L E43 2A2 EcoRI
L E43 2A1 XbaI
L E43 2A1 EcorI
L E43 1A1 XbaI
L E43 1A1 EcoRI
L P1A XbaI
L P1A EcoRI
L E5.5 XbaI
L E5.5 EcoRI
L XbaI
L EcoRI
23.1kb
9.4kb
6.7kb
5.3kb
4.3kb
3.5kb
2.3kb
2.0kb
1.7kb
1.4kb
0.8kb
0.6kb
L E43 2A5 XbaI
L E43 2A5 EcoRI
23.1kb
9.4kb
6.7kb
5.3kb
4.3kb
3.5kb
2.3kb
2.0kb
1.7kb
1.4kb
1.0kb
0.8kb
0.6kb

Table 9. Frequency of homologous dye transfer in cultures of L cells transfected with cadherin and connexin 43 expression constructs.

The dye coupling ability of L P43 2C4 cells was significantly higher than that of the parental cells.

Table 9.

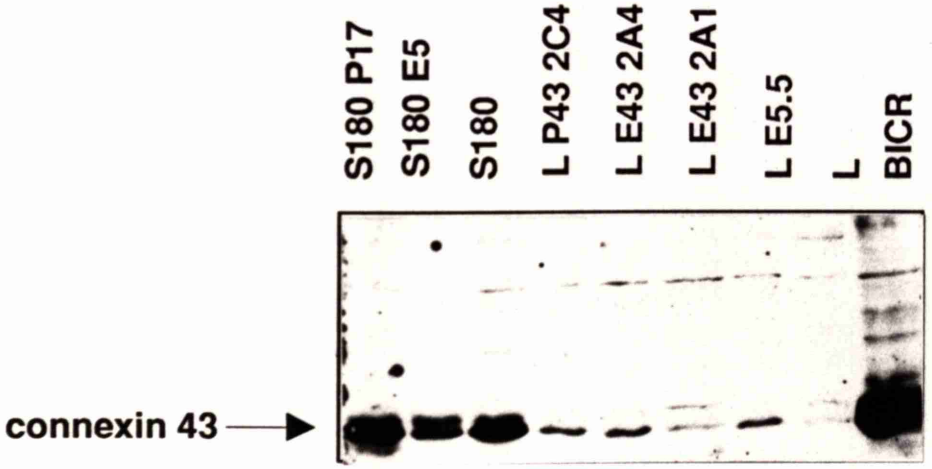
Cell line	Number of injections.	Percentage positive.	Average number of coupled cells per injection, (S.D.).	t.	P.
L	55	4%	0.04 (0.19)		
L E43 2A1	19	15%	0.3 (0.73)	1.34	P>0.05
L E43 2A2	20	25%	0.5 (0.89)	2.06	0.05>P>0.01
L E43 2A4	20	5%	0.2 (0.67)	0.75	P>0.05
L P43 2C4	37	70%	2 (2.18)	5.42	P<0.001

Figure 36. Western analysis of connexin 43 expression in L cell pBabehygro-cx43 transfectants.

Protein samples were prepared from the cell lines and equivalent amounts of protein (approximately 20μg estimated from Coomassie blue stained gels) used for western analysis, as described in materials and methods. Primary antibody was rabbit anti-connexin 43 polyclonal "Petunia" at 1:500. The position of connexin 43 is indicated.

Figure 36.

Connexin 43 western.



parental cell line L E5.5 (figure 35). The Southern analysis also shows that the 1.4kb *Eco*RI but not the *Xba*I 5.3kb fragment is present in the pBabehygro-cx43 transfected cell line L P43 2C4, suggesting that deletions or rearrangements have occurred affecting the *Xba*I site within the MoMuLV LTR which may also affect the expression of exogenous connexin 43 from this promoter.

The L cell pBabehygro-cx43 transfected lines show considerable variability in their ability to dye couple (table 9). The line L P43 2C4, a P-cadherin and connexin 43 double transfectant has the highest incidence of dye coupling; of 37 injections 26 (70%) resulted in dye transfer. However, western analysis shows that this increased frequency of dye coupling does not correlate with detectable changes in the expression of connexin 43 protein, as similar levels of connexin 43 protein are observed in all the double transfectants and in the parental E-cadherin expressing clone L E5.5 (figure 36). Nor can the increased GJIC ability of double transfectant L P43 2C4 be accounted for by detectable changes in connexin 43 phosphorylation, as the only connexin 43 band apparent in the L cell lanes in figure 36 corresponds to the non-phosphorylated form of connexin 43, although the failure to detect a small level of exogenous connexin 43 expression could account for the increased coupling between the transfected cells. The reason for the poor expression of exogenous connexin 43 in the hygromycin B resistant clones is considered in section 5.4.

5.3 The influence of cadherin expression on the frequency of homologous GJIC in S180 cells.

S180 transfectants expressing either exogenous E-cadherin (S180 E5 and its sub-clone S180 E5.5) or P-cadherin (S180 P17) were microinjected with Lucifer Yellow CH for 2 minutes, in order to determine their ability to communicate (table 11) and representative dye spreads are shown in figures 37

and 38. The parental S180 cells transfer dye in 60% of injections and on average the injected cell is coupled to 2^{+}/-2.4 ($^{+}$/-S.D.) neighbouring cells. The E-cadherin expressing clone S180 E5 shows only a slight increase in the incidence of dye coupling (to 77%) and no change in the extent of coupling compared to the parental cells. However this clone did not show as dramatic a morphological change as its subclone S180 E5.5, (obtained by single cell cloning S180 E5) which is also better coupled, transferring dye in 30 out of 30 injections (100%), to an average of 8^{+}/-5.5 ($^{+}$/-S.D.) neighbouring cells. Statistical analysis (using Students t-test) confirms that the extent of dye coupling between S180 E5.5 cells is significantly higher than the level observed between the untransfected S180 cells ($P<0.001$). A similar significant increase in GJIC is also seen in the P-cadherin transfected clone S180 P17, which is coupled in 69 out of 70 injections (98%) to an average of 10^{+}/-7.9 ($^{+}$/-S.D.) neighbouring cells. Consistent with a role for cadherin mediated cell adhesion in causing this increased frequency of gap junction formation is the observation that preincubating, (for 3-4 hours before microinjecting) the P-cadherin expressing transfectant S180 P17 with the monoclonal antibody PCD-1 to P-cadherin causes a reduction in both the extent and incidence of dye transfer (2^{+}/-2.4, mean number of coupled cells per injection $^{+}$/-S.D.). This reduced level of dye coupling is not significantly different from the level of coupling observed between the parental S180 cells ($P>0.05$; table 11). The western analysis of connexin 43 expression (figure 36) shows that the S180 cell lines express both non-phosphorylated and phosphorylated forms of connexin 43, although the resolution of the gel is not sufficient to reveal the presence of multiple phosphorylated forms of connexin 43 which have been reported by Musil et al 1990b.

Figure 37. Dye transfer in cultures of S180 E5.5 cells expressing E-cadherin.

(A) is a phase contrast image of the fluorescence micrographs shown in (B). Lucifer Yellow CH microinjected into a S180 E5.5 cell transfected with the E-cadherin expression construct pBATEM2 spreads to a number of adjacent cells. The injected cells are marked with a black dot. Magnification x1000.

Figure 37.

Phase contrast. Fluorescence.

A. B.

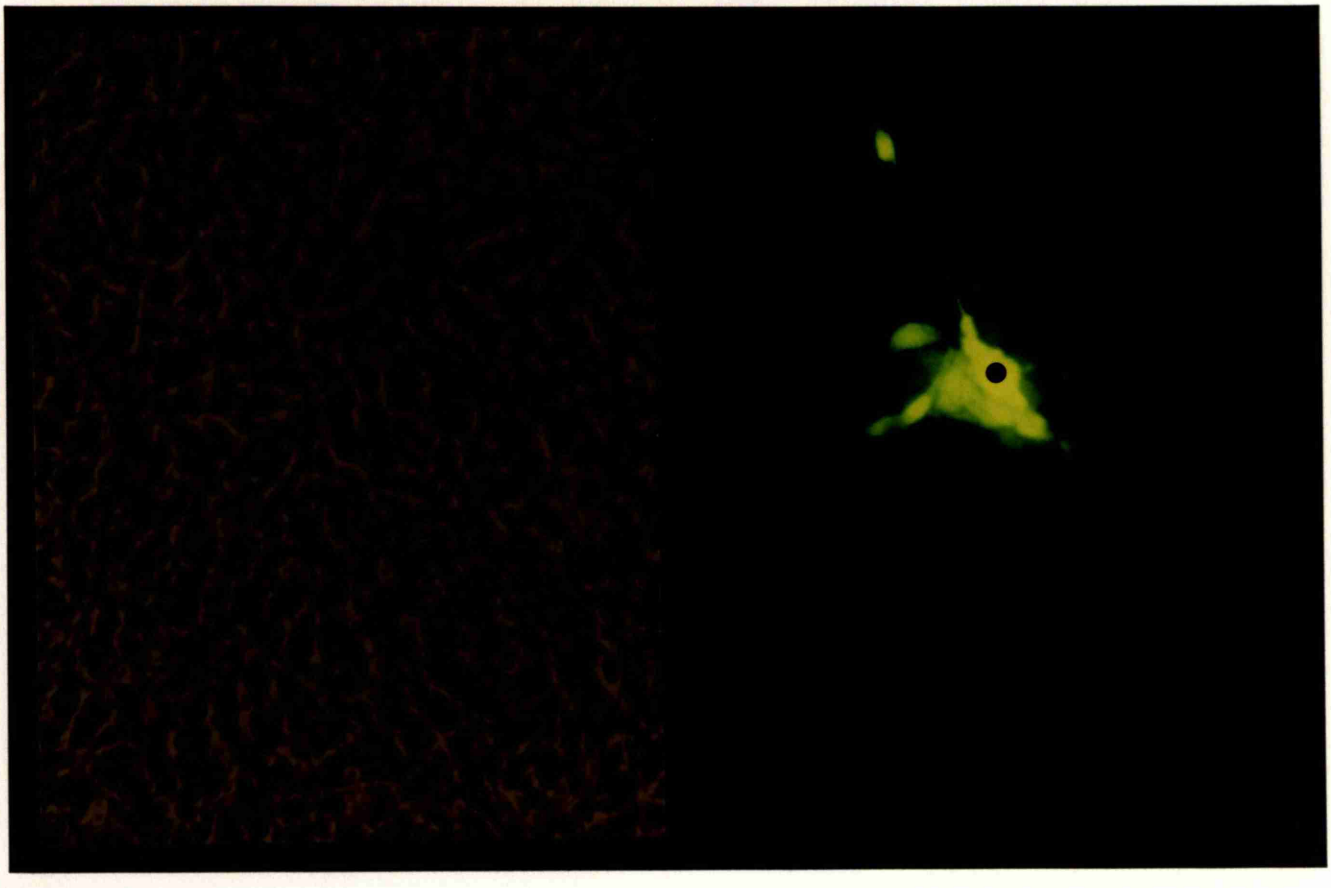


Figure 38. Dye transfer in cultures of S180 P17 cells expressing P-cadherin.

(A) is a phase contrast image of the fluorescence micrographs shown in (B). Lucifer Yellow CH microinjected into a S180 P17 cell transfected with the P-cadherin expression construct pβact-Pcad spreads to a number of adjacent cells. The injected cells are marked with a black dot. Magnification x1000.

Figure 38.

Phase contrast. Fluorescence.

A. B.

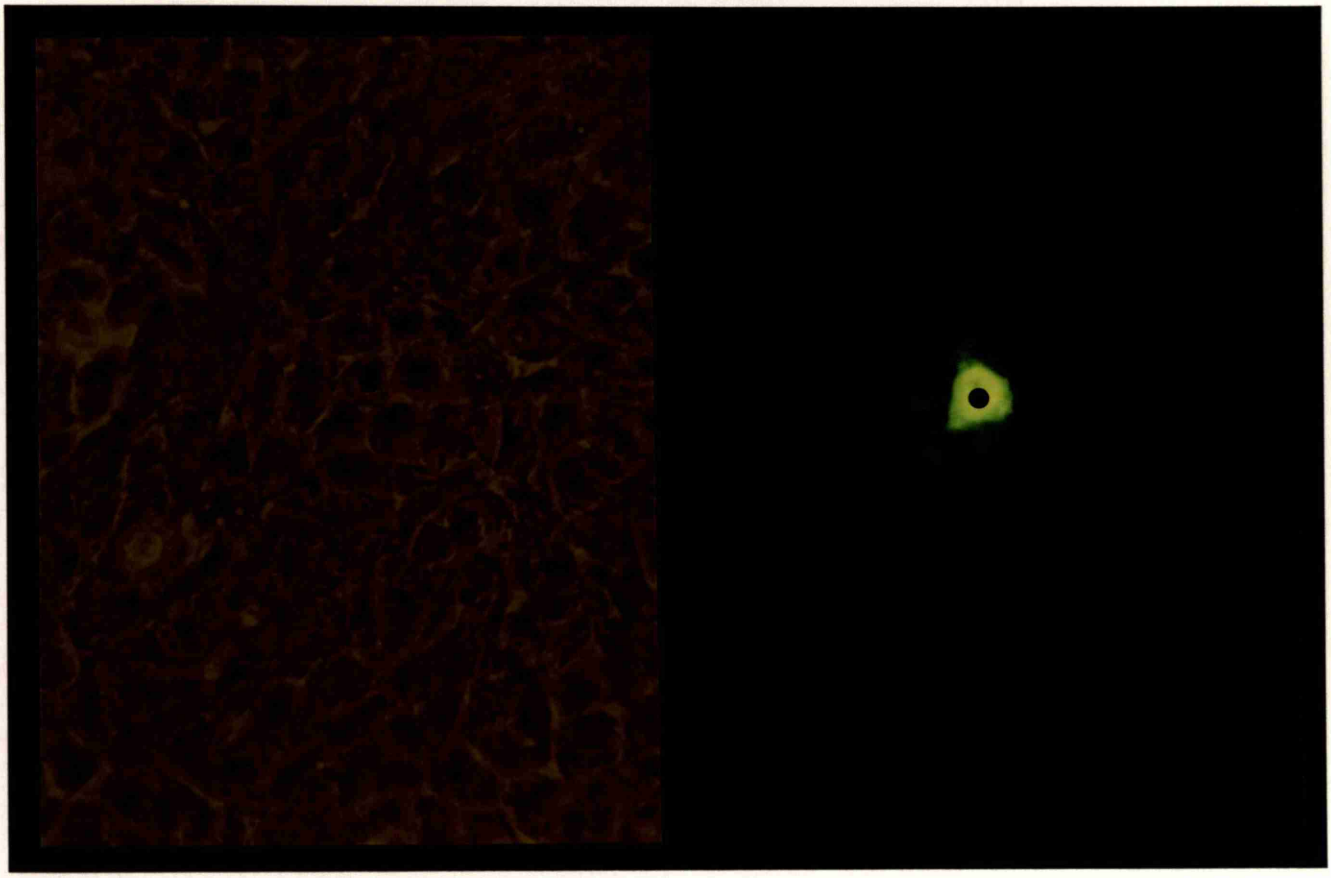


Table 10. Frequency of homologous dye transfer in cultures of S180 cells expressing exogenous cadherin.

S180 cell lines expressing exogenous E- or P-cadherin were examined for their dye coupling ability. The dye coupling ability of S180 E5.5 and S180 P17 cells was significantly higher than the parental cells. Pretreatment of cultures of S180 P17 cells with anti-P-cadherin antibody PCD-1 (but not anti-E-cadherin antibody ECCD-2) reduced the level of coupling.

Table 10.

Cell line	Number of injections.	Percentage positive.	Average number of coupled cells per injection, (S.D.).	t.	P.
S180	39	61%	2 (2.40)		
S180 E5	30	77%	2 (1.83)	0	P>0.05
S180 E5.5	30	100%	8 (5.46)	5.62	P<0.001
S180 P17	70	98%	10 (7.93)	7.82	P<0.001
S180 P17 + P-cad antibody.	30	63%	2 (2.39)	0	P>0.05
S180 P17 + E-cad antibody.	22	95%	9 (4.84)	6.35	P<0.001

5.4 Discussion.

The *mec*$^-$ cell lines L and S180 appear to share a defect in cell-cell adhesion molecule expression that can be corrected in both cell types by the expression of either E- or P-cadherin (chapter 4). Interestingly, this expression of cadherin affects the ability of S180 transfectants but not L transfectants to communicate via gap junctions. The cadherin expressing S180 clones (S180 E5.5 and S180 P17) show an increased frequency of dye transfer compared to the S180 parental cells, whereas the dye transfer ability of the cadherin expressing L clones (L E5.5, L E10.10, L P1A and L P15) is unaltered compared to the parental L cells.

S180 cells therefore appear to have a single adhesion defect which also contributes to the low frequency of GJIC between this cell type. The exogenous expression of E- or P-cadherin corrects the adhesive deficiency, (giving rise to clones that grow in tight groups in which the cells appear less fibroblastic) and enables a higher incidence of GJIC. The preincubation of confluent monolayers of S180 P17 cells with PCD-1, a monoclonal antibody against P-cadherin which has previously been shown to disrupt P-cadherin mediated cell-cell adhesion between L cells (Nose et al 1988) causes the S180 P-cadherin transfectants to acquire a morphology similar to the parental cell type and this is associated with a reduction in the extent and frequency of dye coupling. The ability to disrupt GJIC with antibodies against P-cadherin is consistent with the results of Mege et al (1988) who found that antibodies against L-CAM the chicken homolog of E-cadherin also reduced dye coupling when added to the media of L-CAM transfected S180 cells. That the expression of exogenous P-cadherin influences GJIC has not previously been reported.

The increased frequency of GJIC between cadherin transfected cells may be a consequence of cadherin mediated adhesion *per se*, that is cadherin

mediated cell-cell interaction causing an increased frequency of close cell membrane apposition which increases the likelihood that connexons in apposing membranes interact to form gap junctional channels. However, Musil et al (1990b) have reported that by immunoprecipitating connexin 43 with an affinity purified connexin 43 antibody they find a correlation between increased GJIC in a L-CAM transfected S180 clone and the phosphorylation of connexin 43 to the P_2 form. They have suggested that L-CAM expression may contribute to increased GJIC by affecting the phosphorylation of connexin 43 and therefore its activity. Western analysis reveals that S180 parental cells and S180 cadherin transfectants express both non-phosphorylated and phosphorylated forms of connexin 43 and that levels of expression are unaltered by cadherin expression (figure 36). However, the western analysis does not resolve the level of expression of connexin 43 P_1 and P_2 phosphorylated forms, which presumably run together as the single higher molecular weight band (of approximately 46kD) and therefore the changes in phosphorylation of connexin 43 (involving additional serine phosphorylation and the conversion of connexin 43 P_1 to the higher phosphorylated form P_2; Musil et al 1990b) cannot be confirmed. Unfortunately, sufficient connexin 43 antibodies were not available to perform the immunoprecipitations necessary to demonstrate whether changes in phosphorylation occur. A role for connexin 43 phosphorylation in GJIC is suggested by the observation that transfection of L cells with E- or P-cadherin does not induce detectable phosphorylation of connexin 43, (as shown by the presence of only a single connexin 43 band in the L cell lanes in figure 36) or cause the cells to become coupled.

The expression of ductin, a low level of non-phosphorylated connexin 43 and cadherin by the L cell transfectants is not sufficient to increase the levels of GJIC, which is in contrast to S180 cells where the correction of an adhesive

deficiency by itself is sufficient to establish intermediate levels of GJIC, (8-10 cells coupled per injection compared with 2 cells/injection for the parental line). This suggests that L cells aberrantly express or regulate other proteins that are important for gap junction function. An obvious candidate is connexin 43, because the western analysis is consistent with both the parental and cadherin transfected L cells expressing a low level of non-phosphorylated connexin 43. However, the L cell expression studies, using connexin 43 cloned into the vector pBabehygro to test this hypothesis have proved to be inconclusive, as the significant increase in the levels of dye coupling in cultures of either L E43 2A2 or L P43 2C4 could be due to either an undetectable level of exogenous connexin 43 expression, (which would be in line with the low level of communication) or the fortuitous selection of better coupled clones. The reason for this surprisingly poor expression of exogenous connexin 43 protein is unclear. It could be because the MoMuLV LTR is a weak promoter in this cell type, although previous studies have successfully reported exogenous gene expression in a variety of cell types using this promoter (Wilson et al 1988, Morgenstern and Land 1990, Olsen et al 1993) or because of a decline in expression from the MoMuLV LTR promoter following continuous selection with hygromycin B. As using a similar expression construct conferring resistance to G418 Olsen et al (1993) have recently observed, following continuous selection with G418, the outgrowth of cells which express higher levels of G418 resistance mRNA from the internal SV40 promoter and decreased levels of the inserted cDNA mRNA, due to deletions or rearrangements that have affected transcription initiated from the MoMuLV LTR promoter.

Parallel studies attempting to express exogenous connexin 32 and connexin 43 in MCF-7, L, BHK and BRL cells using the expression vectors pZipneo.SVX and P289 have also yielded disappointing results (Dr S.Jamieson

personal communication) and the difficulties of expressing connexin and the role of connexin phosphorylation in GJIC are considered further in the discussion (section 7.1).

The results presented in this chapter are consistent with the model of gap junction formation presented in figure 2, where cell-cell interaction (mediated by the cadherins) precedes connexon alignment and the formation of gap junction channels between adjacent cells. In chapter 6 the question of whether cadherin expression will break the specificity of gap junctional communication seen between epithelial and fibroblast cell types that sort out is addressed.

Chapter 6.

The influence of cadherin expression on heterologous GJIC.

6.1 Introduction.

The results presented in section 3.2 show that mixed cultures of epithelial BRL and fibroblast BHK or BICR cells tend to sort out, forming separate domains consisting of islands of epithelial cells surrounded by channels of fibroblast cells. Within each domain cells are well coupled homologously via gap junctions but heterologous GJIC between cells in different domains, which have also been described as communication compartments is rare. The BHK, BICR and BRL cell lines endogenously express ductin and connexin 43, showing that the expression of these molecules is not sufficient to establish heterologous communication between the epithelial and fibroblast cell types in mixed cell cultures. Cell adhesion molecules can influence both cell sorting and gap junction formation (Mege et al 1988, Nose et al 1988, Meyer et al 1992) and their differential expression, (as discussed in section 3.4) may therefore contribute to the specificity of gap junction formation observed in these mixed cell cultures.

The transfection of *mec*$^-$ cell type S180 with the pBATEM2 or pβact-Pcad expression vectors confirms that these constructs can cause the expression of functional cadherin which affects the frequency of GJIC, as described in section 5.3. The *mec*$^+$ cell types BHK, BICR and BRL have also been transfected with these constructs (as described in section 4.2), in order to investigate whether the exogenous expression of E- or P-cadherin will break the specificity of GJIC which occurs in co-cultures of BRL and BHK or BICR cells. The dye transfer results, showing the frequency of heterologous communication between BRL and BHK or BICR cells transfected with cadherin are reported in this chapter.

6.2 The influence of cadherin and connexin expression on the frequency of heterologous communication in BHK/BRL and BRL/BICR mixed cultures.

Co-cultures of BRL and BHK or BICR cells expressing exogenous cadherins were established. Interestingly the cells retained the ability to sort out, forming domains consisting of islands of epithelial cells surrounded by channels of fibroblast cells. The affect of cadherin expression on specificity was examined by microinjecting cells adjacent to boundaries between the domains with Lucifer Yellow CH for two minutes. Dye spreads in mixed cultures of BICR and BRL cells expressing exogenous cadherin are shown in figure 39 and 40, and all the results are summarised in tables 11 and 12.

The frequency of heterologous communication in mixed cultures of BRL cells, which endogenously express P-cadherin and the P-cadherin transfected BHK lines BHK P3B3, BHK P3B4, and BHK PC3 was 10-11%, and comparable to the frequency of heterologous GJIC observed between BRL and untransfected BHK cells (6%; table 11). The expression of exogenous P-cadherin in BICR cells was also found not to break the specificity of GJIC observed between BRL and BICR cells, as in mixed cultures of BRL expressing endogenous P-cadherin and exogenous E-cadherin (cell line BRL E5E) and BICR cells expressing P-cadherin (cell line BICR PI9) the frequency of heterologous dye coupling was 3%, and comparable to the frequency of heterologous GJIC observed between untransfected BRL and BICR cells (4%; table 12). The expression of exogenous P-cadherin in these fibroblast cell lines is therefore not sufficient to break the specificity of GJIC communication. The microinjection of Lucifer Yellow CH into cells close to domain boundaries in mixed cultures of the E-cadherin expressing cell lines BRL E5E and BHK E3B3 also revealed no increase in the frequency of

heterologous communication (table 11), showing that the expression of E-cadherin by BHK and BRL cells is not sufficient to break specificity.

In contrast, the incidence of heterologous dye coupling increased from 4% (i.e. 2 out of 51 microinjections) in co-cultures of untransfected BRL and BICR cells to 52% (i.e. 33 out of 63 microinjections) in co-cultures of E-cadherin transfected cells, cell line BRL E5E and BICR A1.1E, an increase of approximately 10 fold (table 12). This heterologous GJIC between BICR A1.1E and BRL E5E cells was not restricted by the cell type injected, dye spread could occur form BICR to BRL or *vice versa*, as shown in figure 40.

A number of observations are consistent with E-cadherin playing a role in this increased incidence of heterologous GJIC. Firstly, in mixed cultures of BRL E5E and the independently established E-cadherin expressing line BICR EA1.5E the observed frequency of heterologous communication was also higher, but as fewer microinjections were performed and the incidence of heterologous dye coupling was 20% this result should be treated with caution. Secondly, no increase in heterologous communication was observed in mixed cultures of BRL E5E and BICR PI9 cells. Finally, no increase in the frequency of heterologous communication was observed between exogenous E-cadherin expressing BICR A1.1E cells and untransfected BRL cells, which is consistent with the expression of exogenous E-cadherin by both cell lines being required to increase the frequency of heterologous communication. However, specificity is only partially broken, as the greatest incidence of heterologous communication (52%; table 12) is still less than the frequency of homologous communication in either cell type (100%; table 4) or the frequency of heterologous communication between BHK and BICR cells (91%; table 5), suggesting that other, as yet unidentified factors are required for extensive GJIC between these cell types (see section 6.3).

Table 11. Frequency of heterologous dye transfer in mixed cultures of BHK and BRL cells expressing cadherins.

The frequency of heterologous communication between BHK and BRL cells was not affected by the expression of cadherin.

Table 11.

Cell lines	Number of injections.	Percentage positive for heterologous communication.
BRL /BHK	37	6%
BRL /BHKEA4	10	10%
BRLE5E/BHKEB3	10	10%
BRL /BHKP3B3	10	10%
BRL /BHKP3B4	9	11%
BRL /BHKPC3	28	11%

Figure 39. GJIC in mixed cultures of BRL E5E and BICR PI9 cells.

(A) and (C) are phase contrast images of the fluorescence micrographs shown in (B) and (D). Mixed cultures of BRL E5E cells, (which endogenously express P-cadherin) and BICR PI9 cells (which exogenously express P-cadherin) were established. Lucifer Yellow CH microinjected into a BICR P19 cell spreads to other BICR P19 cells but not to the adjacent BRL E5E cells (B), similarly Lucifer Yellow CH microinjected into a BRL E5E cell spreads to other BRL cells but not to adjacent BICR PI9 cells. The injected cells are marked with a black dot. Magnification x1000.

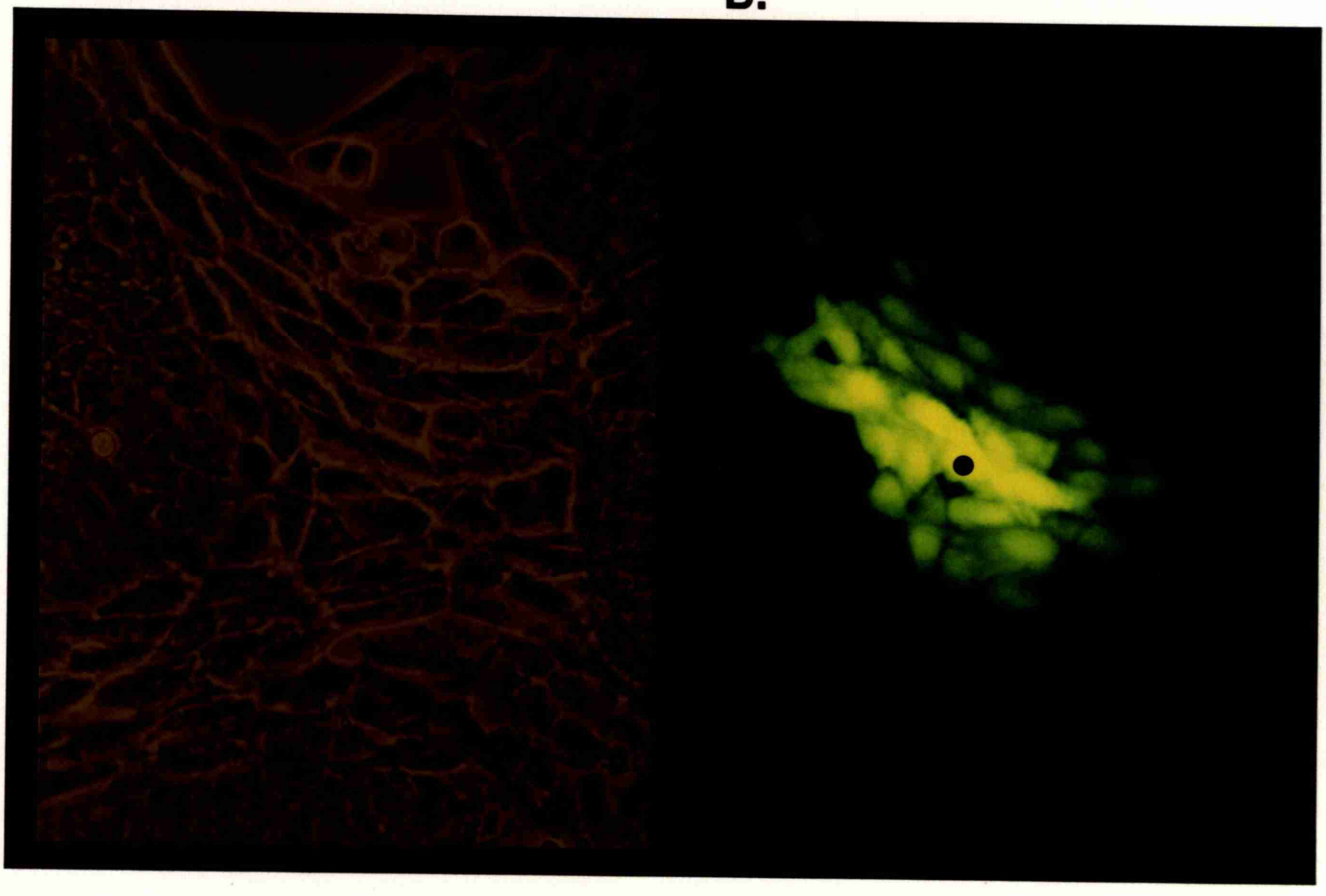
Figure 39.

Phase contrast.

Fluorescence.

A.

B.



C.

D.

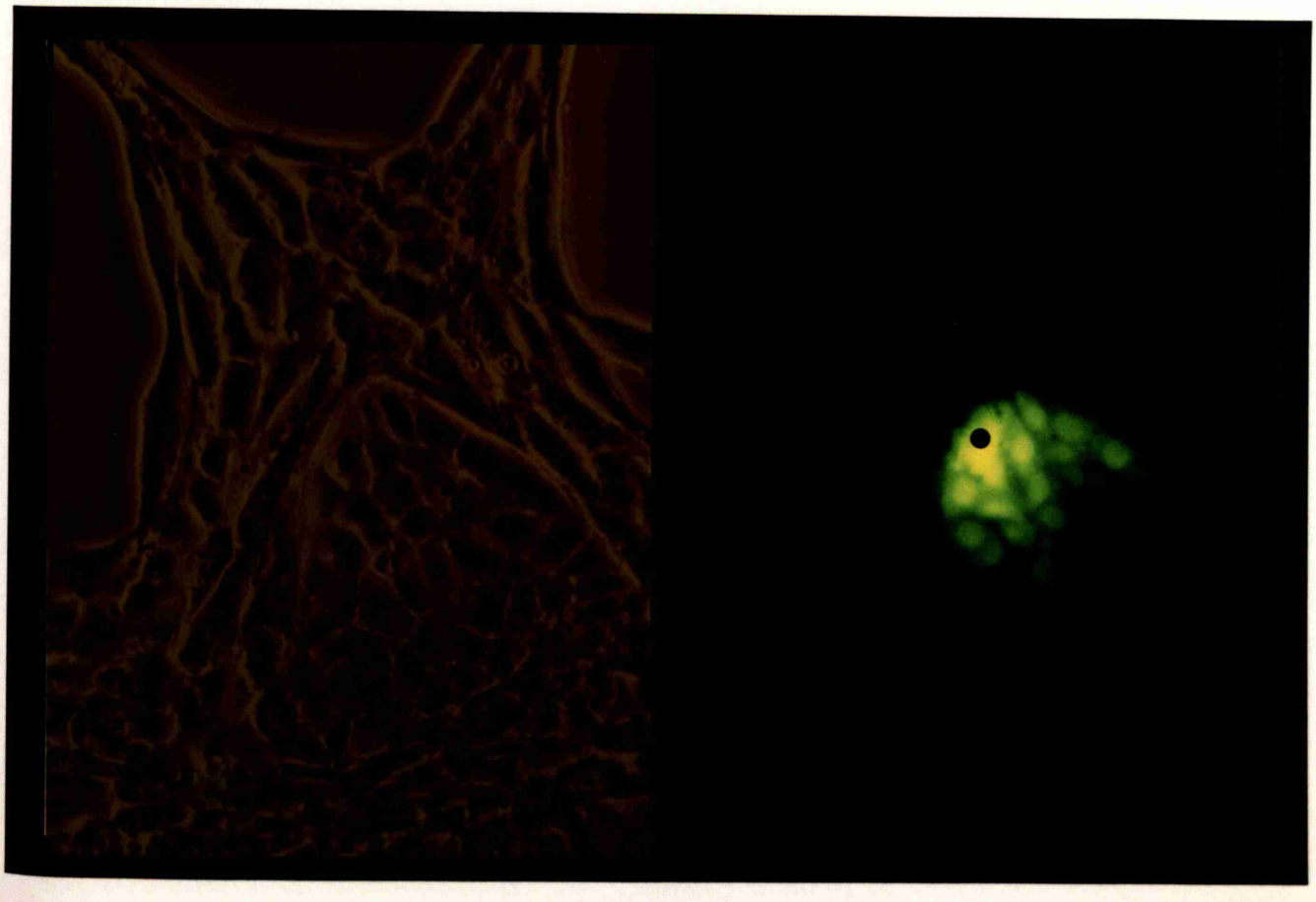


Figure 40. GJIC in mixed cultures of BRL E5E and BICR EA1.1 cells.

(A) and (C) are phase contrast images of the fluorescence micrographs shown in (B) and (D). Mixed cultures of BRL E5E and BICR EA1.1 cells, (which exogenously express E-cadherin) were established. Lucifer Yellow CH microinjected into a BICR A1.1E cell can spread heterologously to adjacent BRL E5E cells, as well homologously (B). Lucifer Yellow CH microinjected into a BRL E5E cell can also spread heterologously to adjacent BICR A1.1E cells, as well homologously (D). The injected cells are marked with a black dot. Magnification x1000.

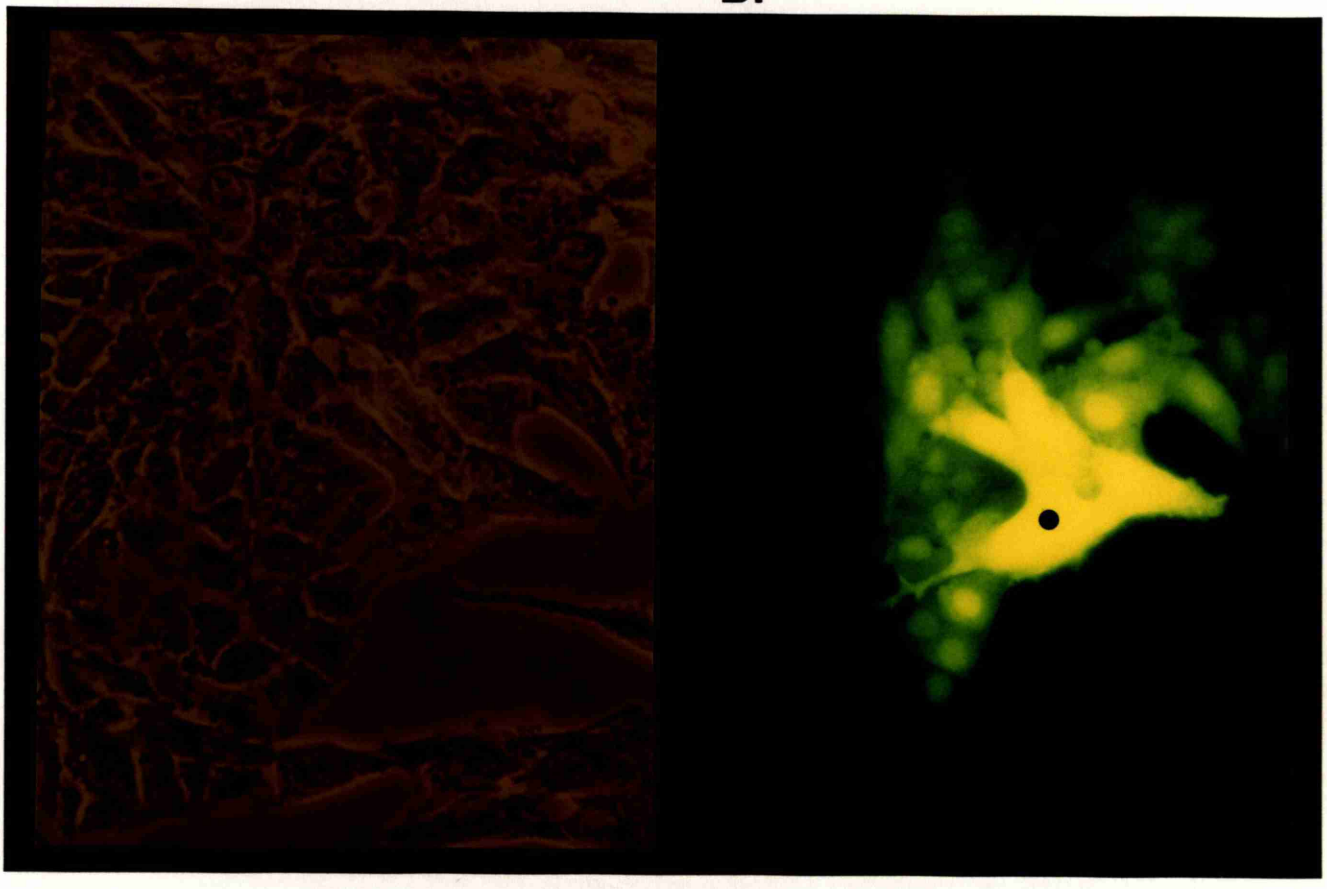
Figure 40.

Phase contrast.

Fluorescence.

A.

B.



C.

D.

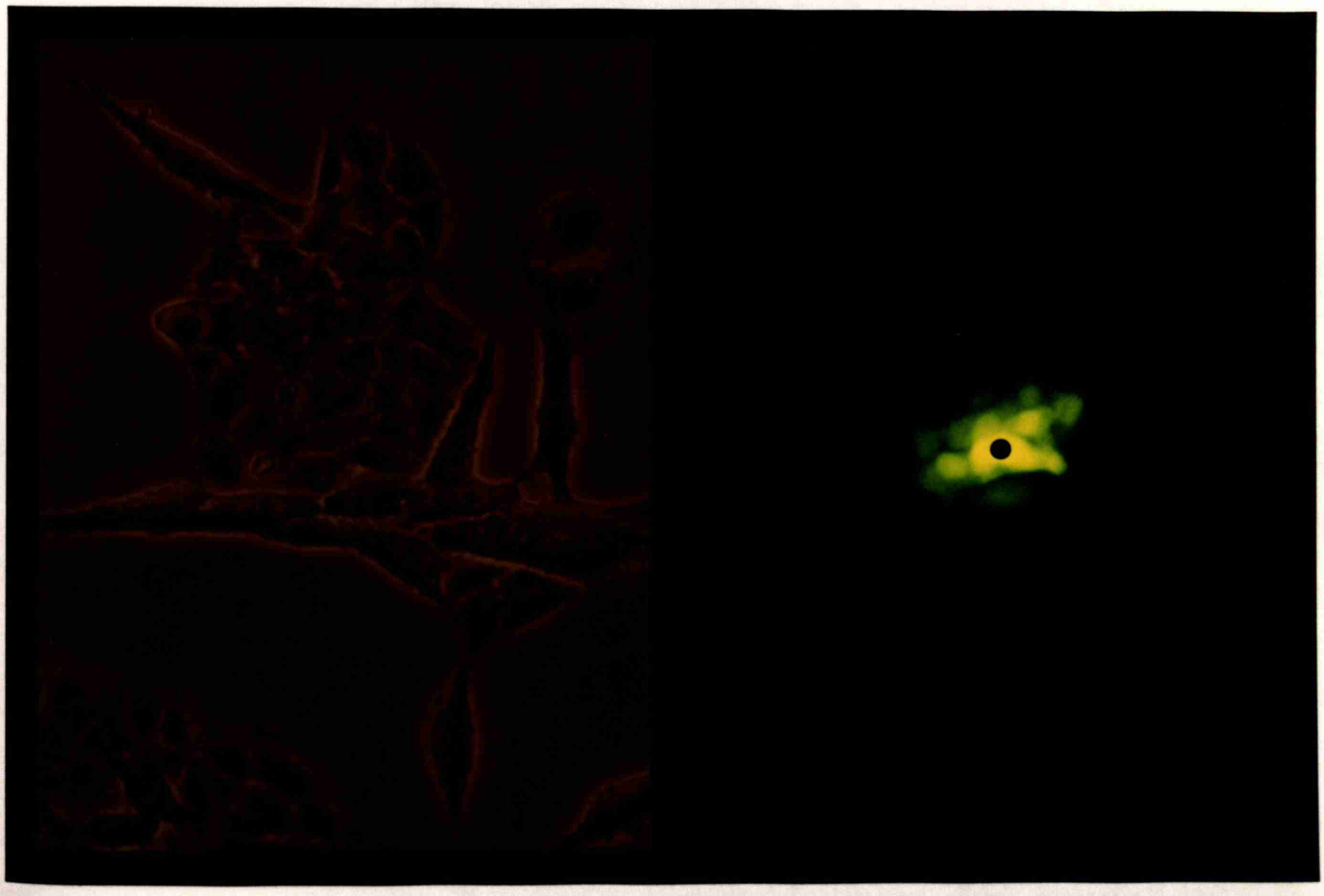


Table 12. Frequency of heterologous dye transfer in mixed cultures of BICR and BRL cells expressing cadherins.

The frequency of heterologous communication between BICR and BRL cells was influenced by the expression of E-cadherin but not P-cadherin.

Table 12.

Cell lines	Number of injections.	Percentage positive for heterologous communication.
BRL / BICR	51	4%
BRLE5E / BICRPI9	35	3%
BRL / BICREA1.1	20	5%
BRLE5E / BICREA1.1	63	52%
BRLE5E / BICREA1.5	10	20%

6.3 Discussion.

Cell adhesion molecules known to be involved in GJIC are differentially expressed by the *mec*$^+$ cell lines which show specificity. The transfection of these lines with vectors for the expression of E- or P-cadherin enables an investigation into the role of these molecules in specificity of GJIC.

BHK cells do not express detectable levels of E-, N- or P-cadherin express N-CAM (table 6), have an intermediate level of dye coupling (dye spreads to 12 cells on average during a two minute injection; table 4) and in co-culture with BRL cells show specificity of GJIC. The expression of exogenous E- or P-cadherin in these cells does not increase the level of homologous dye coupling (7^+/-5.7, mean number of cells coupled per injection$^+$/-S.D.) compared to the level of homologous coupling in the parental cells (10^+/-7.6, mean$^+$/-S.D.), nor does it affect the incidence of heterologous dye transfer in co-culture experiments with BRL cells (table 11). It is possible that the BHK cells are deficient in an additional factor required for increased GJIC or that the exogenous cadherins expressed by BHK cells are non-functional, which would be consistent with their diffuse pattern of expression (figure 25) and the lack of marked morphological change in the BHK cadherin transfectants (figure 30.A).

The expression of exogenous P-cadherin in BICR cells does not significantly influence the ability of these cells to heterologously communicate with BRL cells (table 12), which endogenously expresses P-cadherin (figure 13.C and 15.C). This result is surprising given that P-cadherin expression can influence gap junction formation, S180 cells transfected with the same expression vector show an increase in the level of homologous GJIC (section 5.3). It is possible that the P-cadherin expressed by either BICR or BRL cells is non-functional or that the level of P-cadherin expression is critical, as cells expressing different amounts of the same cell adhesion molecule retain the

ability to sort out (Friedlander et al 1989), which could affect the incidence of gap junction formation.

The expression of E-cadherin in both BRL and BICR cells partially breaks the specificity of GJIC between these two cell types, increasing (5-10 fold) the frequency of gap junction formation in this model system. Interestingly, the greatest incidence of heterologous communication (52%; table 12) is still less than the frequency of homologous communication in either cell type (100%; table 4) or the frequency of heterologous communication between BHK and BICR cells (91%; table 5). This suggests that either other unidentified factors are required for extensive GJIC between the cell types or that the level of cadherin expression also affects gap junction formation.

Chapter 7.

General Discussion.

7.1 Control of gap junction formation.

The published studies discussed in chapter 1 suggest that ductin, connexins and cell adhesion molecules all play a role in the establishment and maintenance of functional gap junctional channels. The initial aim of this project was to characterise the *mec*$^-$ and *mec*$^+$ cell lines used in this study with respect to their communication ability and their expression of these candidate genes.

Ductin transcripts are expressed by all six cell lines regardless of their GJIC capacity, showing that ductin expression alone is not sufficient for gap junction formation or function. However, the interpretation of the ductin expression data is complicated by the observation that ductin is a component of V-ATPase (section 1.5.3). The ubiquitous expression of ductin transcripts by the cultured cell lines used in this study and in various rat tissues (Nezu et al 1992), may therefore be a consequence of the essential role ductin plays as the proton channel component of V-ATPase. It is uncertain what proportion of ductin is normally present in each structure (gap junction or V-ATPase), and how ductin is processed to either the plasma membrane to form gap junctions, (where the N- and C-termini are cytoplasmic), or to the vacuolar membrane to form the proton channel of the V-ATPase, (where the N- and C-termini protrude into the lumen of the vacuole). Leitch and Finbow 1990 using polyclonal antibodies raised against *Nephrops* gap junctions, (which contain predominantly ductin) reported that ductin localises to the plasma membrane at points of cell-cell contact between epithelial cells of the *Nephrops* hepatopancreas but they did not observe appreciable immunolabelling of intracellular vacuoles, suggesting that antibodies in the serum may be ineffective at recognising the vacuolar ductin or that it is present in much lower amounts. The investigation into the localisation of ductin

in mammalian cells is hampered by the current lack of effective antibodies which recognise the mammalian form of ductin, (which is a poor immunogen due to its high degree of hydrophobicity), and the difficulty of expressing *Nephrops* ductin protein, (for which there are antibodies) in cultured mammalian cells (P.McLean personal communication). An alternative experimental approach would be to attach a short nucleotide sequence encoding for a normally nuclear immunogenic epitope to the N-terminus of a mammalian ductin cDNA, and then to express this novel cDNA in the mammalian cell lines and perform immunocytochemistry using antibodies against the immunogenic epitope, in order to localise the tagged ductin within the transfected cells. The technique of epitope tagging has already been successfully used by Goldstein et al 1992 to show that the E5 oncogene expressed in yeast co-precipitates with an epitope tagged yeast ductin, although it was not demonstrated whether the tagged ductin was also functional. The association of E5 with ductin in transformed mammalian cells may reduce V-ATPase activity and thereby inhibit receptor down modulation, providing an explaination for the increased receptor activity observed in E5 transformed cells (section1.6.4.4; Goldstein et al 1991). Preliminary results also suggest that the expression of E5 in mammalian cells decreases GJIC.

The expression of connexin 43 but not connexin 32 by five out of the six cell lines was surprising, given their diverse origins, but it may be a consequence of culturing the cell lines in serum containing medium (section 3.4; Stutenkemper et al 1992). The expression of connexin 43 is consistent with connexin being necessary but not sufficient for GJIC, because connexin 43 is expressed by the *mec*$^{+}$ cell lines BHK, BICR and BRL, as well as by the *mec*$^{-}$ cell lines L and S180. The MCF-7 cells which do not express connexin 43 may be poor communicators for this reason, as Lee et al 1992 have also recently reported

that MCF-7 cells do not express a variety of other connexin transcripts. However, expression studies to demonstrate a role for connexin in the establishment of GJIC between MCF-7 cells have not yielded convincing results, as only a low percentage of the transfected cells expressed detectable levels of exogenous connexin 32 or 43 protein (S.Jamieson personal communication). The reason for this low level of exogenous connexin protein expression is uncertain, but as the exogenous connexin RNA was expressed by the transfected MCF-7 cells this suggests that either translation of the exogenous RNA was inefficient or the exogenous protein was rapidly degraded.

It has previously been reported that the phosphorylated forms of connexin 43 migrate more slowly than the non-phosphorylated form of connexin 43 during SDS-PAGE and that there is a correlation between the expression and phosphorylation of connexin 43 and dye coupling ability (Musil et al 1990b). The western analysis shown in figure 11 is therefore consistent with well coupled BRL and BICR cells expressing both non-phosphorylated (lower band) and phosphorylated (upper band) connexin 43, and the poorly coupled L and MCF-7 cells expressing either low levels of non-phosphorylated or no connexin 43 protein respectively. However, direct evidence to demonstrate that differential phosphorylation gives rise to the 43 and 46kD bands in these cell lines requires further analysis.

Musil et al 1991 have reported that the connexin 43 expressed by *mec*$^+$ cultured cells is phosphorylated on serine residues, and that this phosphorylation of connexin 43 to the cx43-P_2 form does not influence the transport of connexin 43 to the plasma membrane, the stability of the protein or the gating of the gap junctional channel. However, they do associate the phosphorylation of connexin 43 to the cx43-P_2 form with increased resistance of connexin 43 to solubilization in 0.4% triton, which they suggest reflects changes in either the formation or

maintenance of junctional plaques. The increased phosphorylation of connexin 43 on serine residues has also been correlated with the downregulation of GJIC in epithelial cells (Brissette et al 1991, Lau et al 1992), suggesting that the influence of phosphorylation may vary with the cell type or that it may depend on which particular serine residues are phosphorylated.

The *mec*$^{+}$ cell lines used in this study express either cadherin or N-CAM and also connexin 43, which is in contrast to the *mec*$^{-}$ cell types L and S180 which express connexin 43 but do not express either E-, N- or P-cadherin or N-CAM, or MCF-7 cells which express E-cadherin but not connexin. This suggests that the expression of a cell adhesion molecule and a connexin is required for GJIC. The influence of cell adhesion molecule expression on GJIC is supported by the observation that the expression of either exogenous E- or P-cadherin in S180 cells significantly increases GJIC, although whether this increased dye coupling is associated with increased phosphorylation of connexin 43, (which has previously been reported to occur in an S180 clone transfected with L-CAM, the chicken homologue of E-cadherin; Musil et al 1990b) requires further analysis. However, a role for connexin 43 phosphorylation is suggested by the observation that L cell clones transfected with the same E- or P-cadherin expression constructs and expressing exogenous cadherin do not express increased levels of the 46kD connexin 43 band, or show a significant increase in the levels of dye coupling, even though cell morphology and cell-cell adhesion are altered. The reason for this lack of connexin 43 phosphorylation in L cells is uncertain, it is possible that L cells lack or do not activate the appropriate kinase or that the connexin 43 expressed is defective in targeting, assembly or phosphorylation sites. The role of connexin 43 in GJIC was investigated further by attempting to express exogenous rat connexin 43 in L cell cadherin transfectants using the vector pBabehygro-cx43. The results however were

inconclusive due to the poor expression of connexin 43, (the possible reasons for which have been discussed in section 5.4). An alternative approach, (aside from analysising more clones or repeating the transfections with a different expression vector) would be to clone and sequence the endogenous L cell connexin 43 mRNA. In order to compare it to the published rodent connexin 43 sequence and establish whether there are any differences caused by deletions or mutations which might affect connexin function.

The results do not directly address the question of whether either ductin and/or connexin forms gap junction channels. Kumar and Gilula 1992 have recently reported that the expression of connexin 32 in yeast, which does not form gap junctions or express members of the connexin family, leads to the formation of double membrane structures that resemble gap junctions isolated from rodent liver, (as determined by electron microscopy). This they have suggested shows that connexin 32 expression is sufficient for gap junction formation and that no other gap junction specific factors are required. However, it is not clear whether the structures isolated were present in plasma membranes or intracellular membranes, the latter being more likely given that yeast are surrounded by a cell wall that will prevent the association of adjacent cell plasma membranes. In addition, if gap junction like structures are formed in yeast following connexin 32 expression the gap junctional channel itself could be formed from the endogenous yeast ductin not connexin 32. An interesting experiment would therefore be to express connexin 32 in yeast where the endogenous VMA3 gene, which encodes yeast ductin has been knocked out, (the cells grow at pH5.5 not pH7.0; Nelson and Nelson 1990) and then to look for gap junction structures, although the interpretation of this experiment may be complicated because yeast cells also express ductin homologes (Sigh et al 1988, Apperson et al 1990, Umemoto et al 1991).

An approach which should provide further insights into the function of particular domains of ductin or connexin is to determine the consequences of expressing mutated or chimeric proteins in various cell types. For example, the expression of mutated or chimeric ductin proteins in the yeast VAM3 knockouts, may be informative about the molecular mechanism by which the V-ATPase channel forms and how the translocation of protons is achieved. Troyanovsky et al 1993 have recently reported that a chimeric connexin 32 protein, lacking the C-terminus and fused to the cytoplasmic domain of the long splice form of desmocollin but not its shorter splice form, or desmoglein, when expressed in cultured A-431 human carcinoma cells can cause the formation of gap junction like structures, which are associated with desmosome like plaques. The result suggests that the cytoplasmic domain of connexin 32 is not necessary for gap junction formation, (although the major differences between members of the connexin family are within this domain) but because changes in the communication ability of these cells expressing chimeric connexin 32 were not reported, it is not clear whether functional gap junctional channels were formed. Further analysis of these and other mutated or chimeric proteins will therefore be required in order to elucidate the function of connexins and ductin.

In summary, the results are consistent with the expression of ductin, phosphorylated connexin 43 and a cell adhesion molecule (cadherin or N-CAM) being necessary for homologous GJIC, whether heterologous coupling also requires the expression of the same cell adhesion molecule in each cell type is discussed in section 7.2. In other systems homologous GJIC has been associated with the expression of different members of the connexin family (Traub et al 1987, Bruzzone et al 1993, Veenstra et al 1992). The reason for this diversity of connexins, there are nine currently identified in rat (table 1) is unknown. The expression of alternate members of the connexin family may

enable the differential regulation of homologous GJIC or influence heterologous gap junction formation.

7.2 Specificity of gap junctional intercellular communication.

A major aim of the thesis was to address the question of the molecular basis of specificity of GJIC, a phenomena that may influence embryogenesis, tissue differentiation and tumorigenesis, and about which little is known. The co-culture of communication competent epithelial BRL cells and either fibroblast BHK or BICR cells leads to the formation of communication compartments, which consist of islands of epithelial cells surrounded by channels of fibroblast cells. Within these domains each cell type is well coupled homologously (100% of microinjections) but heterologous coupling between domains is rare (3-5% of microinjections). These cell types express both ductin and connexin 43, showing that the expression of ductin and the same member of the connexin family is not sufficient to establish a high frequency of heterologous communication, despite the relatively high levels of homologous coupling within BRL or BICR domains which give rise to well defined compartmental boundaries between these cell types (figure 5).

The cause of specificity is therefore likely to be the differential expression of other molecules involved in gap junction formation. The cell types which show specificity sort out, a process which can be influenced by the expression of cadherins or N-CAM, and as these cell adhesion molecules can affect gap junction formation (Keane et al 1988, Mege et al 1988 Meyer et al 1992) they are also candidates in the control of specificity of GJIC.

The analysis of cell adhesion molecule expression reveals that BHK cells express N-CAM, BICR cells express N-cadherin and BRL cells express N- and P-cadherin. It is interesting that BICR and BRL cells are both of rat origin and

express an N-cadherin RNA doublet but predominantly different sizes of N-cadherin protein, (140kD in BICR cells and 125kD in BRL cells). A previous study by Cyr et al 1992 has also shown that a N-cadherin RNA doublet is expressed by cells in the rat testis, and their western data also suggests that a N-cadherin protein doublet is expressed in this rat tissue, (which could be due to the expression of different forms of N-cadherin by a mixture of different cell types) although the authors only refer to a single 130kD N-cadherin protein. A 130kD N-cadherin protein is also reported to be expressed in chicken heart and brain (Hatta and Takeichi 1986).

The significance of the two N-cadherin RNA transcripts expressed by BICR and BRL cells is uncertain, they may arise from alternate N-cadherin genes, as the copy number of the N-cadherin gene in the genomes of these cell lines is unknown, or from the differential processing of transcripts from a single N-cadherin gene.

The significance of the two N-cadherin protein isoforms which are predominantly expressed by either BRL and BICR cells is also uncertain, they may arise from:

a) Differences in the extent of glycosylation of N-cadherin protein in each cell type, as glycosylation can account for large differences in a protein's apparent molecular mass on SDS-PAGE gels. Whether the differential glycosylation of N-cadherin is occuring could be investigated by treating cells with tunicamycin and looking for changes in the apparent molecular weight of the protein. Glycosylation may also affect the function of the protein in each cell line.

b) The differential phosphorylation of N-cadherin protein by each cell type. However, although members of the cadherin family are phosphorylated (Weider et al 1990), it might not be expected that phosphorylation could account for such a large difference (15kD) in the apparent molecular mass N-cadherin.

c) The differential regulation of either the N-cadherin transcripts, (if each transcript gives rise to a protein of different molecular size) or N-cadherin protein in each cell type could account for the predominance of an N-cadherin isoform. In order to determine whether there are differences in the size of the open reading frames between the N-cadherin transcripts expressed by these cell lines it would be necessary to clone and subsequently sequence each transcript.
d) The detection by the polyclonal antibody used in this study of both the precursor and mature N-cadherin protein. Weider et al 1990 have reported that using an affiniy purified polyclonal antiserum to E-cadherin they are able to detect both the precursor (135kD) and mature (125kD) forms of E-cadherin protein in MDCK epithelial cells. If the 140kD protein predominantly expressed by BICR cells is the precursor form of N-cadherin, this would suggest that these cells fail to cleave N-cadherin to the mature form, and this might affect N-cadherin function.
e) The degradation of cadherin can give rise to a band of less than 100kD (Matsuyoshi et al 1992), but as this is smaller than the 125-140kD N-cadherin protein observed it is unlikely to account for the N-cadherin expression pattern.
f) Finally, the result could be an antibody artefact, but the observation that only BICR and BRL cells express N-cadherin transcripts and proteins of the appropriate size, suggests that this N-cadherin antibody is detecting N-cadherin.

The expression of N-cadherin RNA by BICR and BRL cells is not sufficient to establish a high frequency of heterologous communication between these cell types in co-culture experiments. It is possible that one of the N-cadherin proteins expressed by either BRL or BICR cells is non-functional, which might be the case if BICR cells express uncleaved N-cadherin protein, or if the differential glycosylation of N-cadherin accounts for the different apparent molecular weights

of N-cadherin in each cell type and that glycosylation also affects N-cadherin function.

The differential expression of cell adhesion molecules by the cell lines may therefore influence the formation of communication compartments, which is consistent with the observations of Matsuzaki et al 1990, who found that in mixed cultures of S180 cells transfected with either chicken N- or E-cadherin (L-CAM), of eight microinjections of Lucifer yellow into mixed colonies of prelabelled cells, five had dye transfer exclusively and extensively to cells expressing the same cadherin as the injected cell. In the remaining three microinjections dye spread predominantly to cells expressing the same cadherin, although single cells expressing the other cadherin were labelled in each case. The authors concluded that this level of dye transfer between the different cadherin cell types was comparable to the level of background coupling and that the cadherin expressing cells were better coupled to cells expressing the same cadherins than to cells expressing different cadherins. However, the fibroblast cell types BHK and BICR which do not sort or show specificity, (although these results should be treated with caution due to the difficulty of identifying the different cell types) express different cell adhesion molecules (BICR cells N-cadherin, BHK cells N-CAM), suggesting that either the expression of connexin 43 by both cell types is sufficient to establish GJIC or that these fibroblast cell types express similiar levels of a common unidentified cell adhesion molecule.

To investigate whether the differential expression of cell adhesion molecules contributes to the specificity of GJIC in the epithelial/fibroblast co-culture experiments. The cell lines BHK, BICR and BRL were cotransfected with the cadherin expression constructs, (which had been previously used to successfully express exogenous cadherin in L and S180 cells) in order to try and break the observed specificity of GJIC. The results show that specificity between

BRL and BICR cells can be partially broken by the expression of E-cadherin but not P-cadherin, whereas specificity between BRL and BHK cells is unaffected by expression of these cadherins. The observation that specificity is unaffected by cadherin expression in BHK cells may be because the exogenous cadherin fails to accumulate at points of cell-cell contact, suggesting that it is non-functional or that BHK cells lack one or more of the catenins or other cytoplasmic modulators of cadherin mediated interaction. The partial break in specificity between BICR and BRL E-cadherin expressors and the failure of P-cadherin to affect heterologous gap junctional communication, suggests that either: exogenous P-cadherin expressed by the BICR transfectant or endogenous P-cadherin expressed by BRL cells is non-functional; that E- and P-cadherin affect specificity differently; or that the level of cadherin expression may be critical, as cells expressing different levels of the same cell adhesion molecules can still sort out (Friedlander 1989), which could influence the frequency of gap junction formation. The expression of E-cadherin only partially breaks specificity, (increasing the frequency of heterologous coupling between BRL and BICR cells 5-10 fold, to a maximum of 50% of microinjections) and this suggests that either other as yet unidentified factors are required to further increase the frequency of heterologous communication, or that the level of cadherin expression is again critical.

The specificity of GJIC that is observed in mixed cultures of epithelial and fibroblast cell types can therefore be influenced by cadherin expression. However, it is not clear whether specificity in other co-culture systems, (for example between transformed and non-transformed cells in culture; section 1.7) is also due to cadherin expression or regulation, or whether it depends on the expression or regulation of connexins, (which have been shown to cause a specificity of gap junction formation in the paired *Xenopus* oocyte expression

system; section 1.5.2) or other as yet unidentified factors. *In vivo* both connexins and cadherins may contribute to specificity, which is thought to influence embryogenesis and tissue differentiation (section 1.4.2). In addition, an altered specificity of GJIC may enable potentially tumorigenic cells to escape from normal GJIC mediated growth control, and this is discussed below.

7.3 Gap junctional intercellular communication and tumour suppression.

Tumour formation is believed to be a multistep process, requiring a number of sequential genetic changes, (which include point mutations, deletions, chromosomal translocations and gene amplifications) to convert a normal cell to a malignant cell (Hunter 1991). The statistical analysis of the age related incidence of cancer in humans is consistent with five or six independent genetic changes being required for the formation of a diagnosable tumour (Peto et al 1975), with each change potentially expanding the number of altered cells and creating a larger pool of cells for subsequent genetic changes. The types of genetic change which are thought to be important in the conversion of normal cells to cancer cells are of two sorts: dominantly acting mutations in normal cellular genes called proto-oncogenes, which can confer a transformed phenotype on certain immortalised cell types in culture (Bishop 1991); and recessive mutations in growth suppressor genes that result in loss of function (Knudson 1971, Marshall 1991).

The tumour suppressor genes infered from the analysis of cell hybrids, pRB (identified by analysis of the familial retinoblastoma) and p53 (identified through analysis of loss of heterozygosity in tumours) are thought to operate intracellularly (Friend et al 1986, Lee et al 1987, Harris 1988, Montenarh 1992). In contrast, the phenomemon of contact inhibition, observed in co-cultures of

normal and transformed rodent fibroblasts, operates intercellularly and has been correlated with GJIC (sections 1.6.4.4 and 1.7).

A role for GJIC in tumour suppression is also suggested by the observation that the expression of exogenous connexin 43 in chemically transformed mouse fibroblast 10T1/2 cells enhances GJIC, normalises transformed cell growth in culture (Mehta et al 1991) and reduces tumorigenicity in nude mice (Rose et al 1993). In addition many types of tumour cells are reported to have decreased levels of GJIC (Loewenstein 1979, Lee et al 1992), and abherrant connexin expression occurs in human tumours of the lung and myometrium, (which is associated with decreased levels of connexin 26, 32 and 43 protein expression; Wilgenbus et al 1992) and in a number of human mammary tumour cell lines, (which is associated with the transcriptional downregulation of connexins 26 and 43; Lee et al 1992). However, genetic abnormalities (which would be consistent with connexins playing a role in tumour suppression) have yet to be reported in tumour cell types at the recently described connexin loci on chromosomes 1, 6, 13 and X (Willecke et al 1990, Fishman et al 1991, Hsiech et al 1991), although the fine location of the connexin genes have yet to be reported.

The results presented in chapter 5 show that the introduction of exogenous E- or P-cadherin into S180 cells significantly increases GJIC, which is consistent with the published reports implicting cell adhesion molecules in gap junction formation (Mege et al 1988, Meyer et al 1992). Cell adhesion molecules, such as E-cadherin may therefore contribute to tumour suppression by affecting the establishment of GJIC, in addition to their cell-cell adhesion function, (which is considered important in the suppression of invasiveness; Frixen et al 1991, Mareel et al 1991, Vleminckx et al 1991). There are several lines of evidence which suggest that cell adhesion molecules contribute to tumour suppression *in*

vivo: the decreased expression of E-cadherin in poorly differentiated epithelial carcinomas (Schipper et al 1991, Umbas et al 1992, Mayer et al 1993, Oka et al 1993); the frequent LOH of chromosome 16q in human prostrate and hepatocellular carcinomas has been localised to 16q22.1 the site of the E-cadherin gene (Carter et al 1990, Tsuda et al 1990, Bergerheim et al 1991, Fugimori et al 1991), and in five out of six prostrate tumour samples the genetic changes at this locus have been correlated with abnormal E-cadherin expression (Giroldi and Schalken 1993); and the *DCC* gene which is deleted in a high proportion of colorectal carcinomas has homology to N-CAM (Fearon et al 1990). However, there has only been one report of reduced levels of a cell adhesion molecule expression in a tumour derived cell line correlating with decreased GJIC (Jongen et al 1991).

An investigation into the GJIC ability and E-cadherin expression levels of a panel of seven human squamous cell caricnoma derived cell lines of the head and neck (SSCHN) was therefore initiated. The SSCHN cell lines were recently derived at the Beatson Institue for Cancer Research and have been called BICR 3, 6, 10, 16, 18, 19 and 22 (K. Eddington pers. comm.), they are unrelated to the fibroblastic mammary cell line BICR described previously in the results section, which was established in the 1970's at the Biophysics Department of the Institute for Cancer Research (Rajewsky and Gruneisen 1972). To avoid confusion these squamous cell carcinoma lines are therefore refered to as SSCHN BICR 3-22. Prelimenary analysis reveals that the SSCHN BICR cells lines are dye coupled and express E-cadherin to various degrees (table 13). The GJIC and E-cadherin expression levels of the SSCHN BICR cell lines are consistent with reports that cell lines derived from mouse papillomas and carcinomas, (induced by a chemical initiation with DMBA and promotion with TPA) which have an epithelial morphology, are coupled via gap junctions and express cadherin (Jongen et al

Table 13. Preliminary analysis of the homologous dye transfer ability and the E-cadherin expression levels of a panel of human squamous cell carcinoma lines derived from tumours of the head and neck.

Table 13.

Cell line	Number of injections.	Percentage positive.	Average number of coupled cells per injection, (S.D.).	E-cadherin expression levels.
SSCHN BICR 3[NT]	15	87%	22 (18.37)	+++
SSCHN BICR 6	20	100%	10 (3.90)	+++++
SSCHN BICR 10[S/E]	20	45%	1 (1.49)	+
SSCHN BICR 16	11	100%	23 (17.76)	+++++
SSCHN BICR 18[M]	15	93%	9 (3.90)	+
SSCHN BICR 22[M]	22	91%	9 (6.98)	+++++
3T3I	N.D.			-

M Metastatic.

NT Non-tumorigenic.

N.D. Not Done.

S/E Spindle and epithelial morphology.

1991, Dr A.Stoller personal communication). Interestingly SCCHN BICR 10 cells have a mixed morphology consisting of both spindle and squamous cells, express a very low level of E-cadherin and are poorly coupled via gap junctions, as the conversion of a mouse squamous cell carcinoma to a spindle cell carcinoma, (which is thought to be a late event in skin tumorigenesis) is associated with a reduction in GJIC, and loss of E-cadherin and normal *ras* p21 protein (Dr A.Stoller and R.Crombie personal communication). However, changes in the GJIC ability of malignant cells may also occur *in vivo* which affect tumour promotion, progression and also metastasis.

Metastasis is a complex process involving a number of genetic changes which are poorly understood (Shekhar et al 1993). GJIC and altered cell-cell adhesion may contribute to each stage of metastasis: the release of malignant cells from the primary tumour into the blood stream; their attachment and infiltration at a secondary site; and proliferation at this site. The metastatic cell lines SCCHN BICR 18 and 22 are communication competant and express E-cadherin (to different degrees). The suppression of invasion *in vitro* and metastasis of Madin Darby canine kidney (MDCK) cells in nude mice has previously been linked to E-cadherin expression, as *in vitro* the invasive capacities of 25 carcinoma cell lines inversely correlated with E-cadherin expression (Frixen et al 1991), and *in vivo* primary tumours and large metastases arising from *ras* transformed MDCK cells (which in culture homogeneously express E-cadherin) are heterogeneous for E-cadherin expression but small metastases are negative for E-cadherin expression (Mareel et al 1991), suggesting that transient changes in E-cadherin may occur. El-Sabbin and Pauli 1991 have shown that GJIC can occur between metastatic rat cells and bovine aortic endothelial cells, and they suggest that GJIC may positively influence the infiltration of the metastatic cell, which is in contrast to the

potentially suppresive influence of GJIC on the subsequent proliferation of malignant cells at this secondary site. Further analysis of the SSCHN BICR cell lines is therefore required to determine the level of heterologous communication *in vitro* and *in vivo* and to correlate these results with a more extensive analysis of the expression of genes likely to influence communication.

An important mechanism by which potentially malignant cells may escape form GJIC mediated control of proliferation is by reduced communication with the surrounding normal cells. The tumor cells may achieve this reduction in communication by either becoming communication incompetent or showing an altered specifcity of GJIC, (where homologous communication between the tumour cells is unaffected but heterologous communication between the tumour cells and the normal cells is greatly reduced). Specifcicity may be regulated by the differential expression of members of the connexin (Bruzzone et al 1993) or the cadherin family (chapter 6), but (as discussed above for the SCCHN BICR cell lines) further investigation is required to demonstrate whether different tumour cell types show specificity and differentially express these genes. If normal GJIC acts a tumour suppressor *in vivo* and an altered specificity of GJIC contributes to escape from this growth control mechanism, then upregulating heterologous communication between tumour and normal cells is a potential mechanism by which the proliferation of the malignant cell can be controlled. Therefore certain tumour cell types, (those showing aberrant cadherin expression and reduced heterologous GJIC with normal cells), would be potentially treatable by the introduction and expression of exogenous cadherin. A gene therapy approach which is analogous to that recently proposed by Rose et al 1993 for certain human mammary, lung and myometrial tumours where a deficiency of connexin has been reported. An alternative approach to the treatment of tumours is to modify the malignant cells, (using a gene transfer

approach) such that they become sensitive to treatment with a non-toxic chemothrerapeutic agent. For example, cells modified to express the Herpes Simplex Virus thymidine kinase (HSV tk) gene become sensitive to treatment with the antiviral agent ganciclovir, a guanosine analog which is non-toxic to normal cells. The HSV tk expressing cells convert ganciclovir to an intermediate nucleotide that disrupts DNA synthesis and is lethal (Field et al 1983). A problem with this and the gene therapy approaches discussed previously is that the efficiency of *in vivo* gene transfer is currently less than 100% (Culver et al 1992), enabling unmodified malignant cells to escape. However, Culver et al 1992 have reported that there is a significant "bystander" effect which kills surrounding unmodified malignant cells but only minimally affects normal cells. The mechanism mediating this "bystander" effect is unknown but if the HSV tk expressing cells are communication competent it may be analogous to the "kiss of death", where the gap junction permeable hypoxanthine analogue thioguanine is converted by normal cells to its lethal nucleotide and spread via gap junctions to thymidine kinase lacking mutants (Fujimoto et al 1971). Further investigation is therefore required to establish whether GJIC plays a role in this "bystander" effect, as if the malignant cells show a specifcity of GJIC with the surrounding normal cells they would also be precisely targeted.

References

Albelda, S.M. & Buck, C.A. (1990) Integrins and other cell adhesion molecules. *Faseb Journal*, **4**, 2868-2880.

Alcala, J., Lieska, n. & Maisel, H. (1975) Protein component of bovine lens cortical fiber cell membranes. *Experimental Eye Research*, **21**, 581-595.

Allen, F., Tickle, C. & Warner, A. (1990) The role of gap junctions in patterning of the chick limb bud. *Development*, **108**, 623-634.

Anderson, H. (1990) Adhesion molecules and animal development. *Experientia*, **46**, 2-13.

Apperson, M., Jensen, R.E., Suda, K., Witte, C. and Yaffe, M.P. (1990) A yeast protein homologous to the proteolipid subunit of the chromaffin granule proton-ATPase is important for cell growth. *Biochemical & Biophysical Research Communications*, **168**, 574-579.

Argaves, W.S., Suzuki, S., Arai, H., Thompson, K., Piersbacher, M.D. and Ruoslahti, E. (1987) Amino acid sequence of the human fibronectin receptor. *Journal of Cell Biology*, **105**, 1183-1190.

Asamoto, M., Oyamada, M., el Aoumari, A., Gros, D. & Yamasaki, H. (1991) Molecular mechanisms of TPA-mediated inhibition of gap- junctional intercellular communication: evidence for action on the assembly or function but not the expression of connexin 43 in rat liver epithelial cells. *Molecular Carcinogenesis*, **4**, 322-327.

Atkinson, M.M., Menko, A.S., Johnson, G.G., Sheppard, J.R. & Sheridan, J.D. (1981) Rapid and reversible reduction of junctional permeability in cells infected with a temperature sensitive mutant of avian sarcoma virus. *Journal of Cell Biology*, **19**, 573-578.

Azarnia, R. & Larsen, W.J. (1977) Intercellular communication and cancer. In *Intercellular Communication*, ed. de Mello, W.C., pp 145-172. New York: Plenum Publishing Corporation.

Azarnia, R., Dahl, G. & Loewenstein, W.R. (1981) Cell junction and cyclic AMP: III. Promotion of junctional membrane permeability and junctional membrane particles in a junction-deficient cell type. *Journal of Membrane Biology*, **63**, 133-146.

Azarnia, R.& Loewenstein, W.R. (1987) Polyomavirus middle-T antigen downregulates junctional cell-cell communication. *Molecular & Cellular Biology*, **7**, 946-950.

Azarnia, R., Reddt, S., Kmiecik, T.E., Shalloway, D. & Loewenstein, W.R. (1988) The cellular src gene product regulates junctional cell-to-cell communication. *Science*, **239**, 398-401.

Baker, M.E. & Saier, M.H., Jr. (1990) A common ancestor for bovine lens fiber major intrinsic protein, soybean nodulin-26 protein, and E. coli glycerol facilitator. *Cell*, **60**, 185-186.

Baker, T.S., Sosinsky, G.E., Caspar, D.L.D., Gall, C. & Goodenough, D.A. (1985) Gap junction structures VII. Analysis of connexon images obtained with cationic and anionic negative stains. *Journal of Membrane Biology*, **184**, 81-98.

Barrio, L.C., Suchyna, T., Bargiello, T., Xu, L.X., Roginsky, R., Bennett, M.L.V. and Nicholson, B.J. (1991) Gap junctions formed by connexins 26 and 32 alone and in combination are differently affected by voltage. *Proceedings National Academy of Sciences (USA)*, **88**, 8410-8414.

Bazan, J.F. (1990) Structural design and molecular evolution of a cytokine receptor superfamily. *Proceedings National Academy of Sciences (USA)*, **87**, 6934-6938.

Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M.M. & Birchmeier, W. (1993) Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/beta- catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *Journal of Cell Biology*, **120**, 757-766.

Benedetti, E.L. & Emmelot, P. (1967) Hexagonal arrays of subunits in tight junctions separated from isolated plsma membranes. *Journal of Cell Biology*, **38**, 15-24.

Benedetti, E.L., Dunia, I., Bentzel, C.J., Vermorken, A.J.M., Kibbelaar, M. and Bloemendal, H. (1976) A portrait of plasma membrane specializations in eye lens epithelium and fibers. *Biochima et Biophysica Acta*, **457**, 353-384.

Bennett, M.V., Barrio, L.C., Bargiello, T.A., Spray, D.C., Hertzberg, E. & Saez, J.C. (1991) Gap junctions: new tools, new answers, new questions. *Neuron*, **6**, 305-320.

Bennett, M.V. & Verselis, V.K. (1992) Biophysics of gap junctions. *Seminars in Cell Biology*, **3**, 29-47.

Berdan, R.C. (1987) Intercellular communication in arthropods: biophysical, ultrastructural, and biochemical approaches. In *Cell-to-Cell Communication*, ed. de Mello, W.C. pp299-370. Plenum Publishing Corporation, New York.

Berdan, R.C. & Gilula, N.B. (1988) The arthropod gap junction and pseudo-gap junction: Isolation and preliminary biochemical analysis. *Cell and Tissue Research*, **251**, 257-274.

Bergerheim, U.S.R., Kunimi, K., Collins, V.P. & Ekman, P. (1991) Deletion mapping of chromosomes 8, 10, and 16 in human prostrate carcinoma. *Genes, Chromosomes and Cancer*, **3**, 215-220.

Bevilacqua, A., Loch-Caruso, R. and Erickson, R.P. (1989) Abnormal development and dye coupling produced by antisense RNA to gap junction protein in mouse preimplantation embryos. *Proceedings National Academy of Sciences (USA)*, **86**, 5444-5448.

Bevilacqua, M., Butcher, E., Furie, B., Gallatin, M., Gimbrone, M., Harlan, J., Kishimoto, K., Lasky, L., et al (1991) Selectins: a family of adhesion receptors. *Cell*, **67**, 233.

Beyer, E.C., Paul, D.L. & Goodenough,D.A. (1987) Connexin 43: A protein from rat heart homologous to a gap junction protein from liver. *Journal of Cell Biology*, **105**, 2621-2629.

Beyer, E.C., Kistler, J., Paul, D.L. & Goodenough, D.A. (1989) Antisera directed against connexin43 peptides react with a 43-kD protein localized to gap junctions in myocardium and other tissues. *Journal of Cell Biology*, **108**, 595-605.

Beyer, E.C. (1990) Molecular cloning and developmental expression of two chick embryo gap junction proteins. *Journal of Biological Chemistry*, **265**, 14439-14443.

Beyer, E.C., Reed, K.E., Westphale, E.M., Kanter, H.L. & Larson, D.M. (1992) Molecular cloning and expression of rat connexin40, a gap junction protein expressed in vascular smooth muscle. *Journal of Membrane Biology*, **127**, 69-76.

Bignami, M., Rosa, S., Falcone, G., Tato, F., Katoh, F. & Yamasaki, H. (1988) Specific viral oncogenes cause differential effects on cell-to-cell communication, relevant to the suppression of the transformed phenotype by normal cells. *Molecular Carcinogenesis*, **1**, 67-75.

Birman, S., Meunier, F.M., Lesbats, B., Le Caer, J.P., Rossier, J. & Israel, M. (1990) A 15 kDa proteolipid found in mediatophore preparations from Torpedo electric organ presents high sequence homology with the bovine chromaffin granule protonophore. *Febs Letters*, **261**, 303-306.

Birnboim, H.C. & Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research*, **7**, 1513.

Bishop, J.M. (1991) Molecular themes in oncogenesis. *Cell*. **64**, 235-248.

Bixby, J.L., Pratt, R.S., Lilien, J. and Reichardt, L.F. (1987) Neurite outgrowth on muscle cell surfaces involves extracellular matrix receptors as well as Ca^{++}-dependant and -independant cell adhesion molecules. *Proceedings National Academy of Sciences (USA)*, **84**, 2555-2559.

Blaschuk, O.W., Sullivan, R., David, S. and Pouliot, Y. (1990) Identification of a cadherin cell adhesion recognition sequence. *Developmental Biology*, **139**, 227-229.

Blennerhassett, M.G. and Caveney, S. (1984) Separation of developmental compartments by a cell type with reduced junctional permeability, *Nature*, **309**, 361-364.

Bok, D., Dockstader, J. and Horwitz, J. (1982) Immunocytochemical localization of the lens main intrinsic polypeptide (MIP) in communicating junctions. *Journal of Cell Biology*, **92**, 213-220.

Bohrmann, J. (1993) Antisera aggainst a channel-forming 16kDa-protein inhibit dye-coupling and bind to cell membranes in Drosophila ovarian follicles. *Journal of Cell Science*, **In press**.

Bourdon, M.A. & Ruoslahti, E. (1989) Tenascin mediates cell attachment through an RGD-dependent receptor. *Journal of Cell Biology*, **108**, 1149-1155.

Boyle, W.J., Smeal, T., Defize, L.H.K., Angel, P., Woodgett, J.R., Karin, M. & Hunter, T. (1991) Activation of protein kinase C decreases phosphorylation of c-*jun* at sites that negatively regulate its DNA-binding activity. *Cell*, **64**, 573-584.

Brady, G., Jantzen, H.M., Bernard, H.U., Brown, R., Schutz, G. & Hashimoto-Gotoh, T. (1984) New cosmid vectors developed for eukaryotic DNA cloning. *Gene*, **27**, 223-232.

Brand, N., Pertovich. M., Krust, A., Chambon, P., de The, H., Marchio, A., Tiollais, P. & Dejean, A. (1988) Identification of a second human retinoic acid receptor. *Nature*, **332**, 850-853.

Braun, J., Abney, J.R. & Owiki, J.C. (1984) How a gap junction maintains its structure. *Nature*, **310**, 316-318.

Brindle, P.K. & Montminy, M.R. (1992) The CREB family of transcription activators. *Current Opiniom in Genetics and Development*, **2**, 199-204.,

Brissette, J.L., Kumar, N.M., Gilula, N.B. & Dotto, G.P. (1991) The tumor promoter 12-O-tetradecanoylphorbol-13-acetate and the ras oncogene modulate expression and phosphorylation of gap junction proteins. *Molecular & Cellular Biology*, **11**, 5364-5371.

Bruzzone, R., Haefliger, J.A., Gimlich, R.L. & Paul, D.L. (1993) Connexin40, a component of gap junctions in vascular endothelium, is restricted in its ability to interact with other connexins. *Molecular Biology of the Cell*, **4**, 7-20.

Buchkovich, K., Duffy, L.A and Harlow, E. (1989) The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. *Cell*, **58**, 1097-1105.

Buck, C.A. & Horwitz, A.F. (1987) Cell surface receptors for extracellular matrix adhesion molecules. *Annual Review of Cell Biology*, **3**, 179-205.

Buultjens, T.E.J., Finbow, M.E., Lane, N.J. & Pitts, J.D. (1988) Tissue and species conservation of the vertebrate and arthropod forms of the low molecular weight (16-18000) proteins of gap junctions. *Cell & Tissue Research*, **251**, 571-580.

Buxton ,R.S. and Magee, A.I. (1992) Structure and interactions of desmosomal and other cadherins. *Seminars in Cell Biology*, **3**, 157-167.

Carter, B.S., Ewing, C.M., Ward, W.S., treiger, B.F., Aalders, T.W., Schalken, J.A., Epstein, J.I. & Isaacs, W.B. (1990) Allelic loss of chromosomes 16q and 10q in human prostrate cancer. *Proceedings National Academy of Sciences (USA)*, **87**, 8751-8755.

Caspar, D., Goodenough, D., Makowski, L. & Phillips, W. (1977) Gap junction structure I. Correlated electron microscopy and X-ray diffraction. *Journal of Cell Biology*, **74**, 605-628.

Caveny, S. (1985) The role of gap junctions in development. *Annual Review of Phsiology*, **47**, 319-355.

Cepko, C.L., Roberts, B.E. & Mulligan, R.C. (1984) Construction and Application of a highly Transmisable Murine Retrovirus Shuttle Vector. *Cell*, **37**, 1053-1062.

Chalcroft, P. & Buillivant, S. (1970) An interpretation of liver cell membrane and junction structure based on observation of freeze fracture replicas of both sides of the fracture. *Journal of Cell Biology*, **47**, 49-60.

Chang, C., Trosko, J.E., Kung, H., Bombick, D. & Matsummura, F. (1985) Potential role of the src gene product in inhibition of gap-junctional communication in NIH3T3 cells. *Proceedings National Academy of Sciences (USA)*, **82**, 5360-5364.

Charles, A.C., Naus, C.C.G., Kidder, G.M., Dirkensen, E.R. & Sanderson, M.J. (1992) Intercellular calcium signalling via gap junctions in glioma cells. *Journal of Cell Biology*, **118**, 195-201.

Chen, Y.H. & DeHaan, R.L. (1992) Multiple-channel conductance states and voltage regulation of embryonic chick cardiac gap junctions. *Journal of Membrane Biology*, **127**, 95-111.

Chomczynski, P. & Sacchi, N. (1987) Single-step method of RNA isolation by guanidinium thiocynate-phenol-chloroform extraction. *Annals of Biochemistry*, **162**, 156-159.

Chow, I. & Young, S.H. (1987) Opening of single gap junction channels during formation of electrical coupling between embryonic muscle cells. *Developmental Biology*, **122**, 322-337.

Clemens. M.J., Trayner, I. & Menaya, J. (1992) The role of protein kinase C isoenzymes in the regulaton of cell proliferation and differentiation. *Journal of Cell Science*, **103**, 881-887.

Cohen, J., Burne, J.F., McKinley, C. and Winter, J. (1987) The role of laminin and the laminin/fibronectin receptor complex in the out-growth of retinal ganglion cell axons. *Developmental Biology*, **122**, 407-418.

Cole, G.J. & Glaser, L. (1986) A heparin-binding domain from N-CAM is involved in neural cell-substratum adhesion. *Journal of Cell Biology*, **102**, 403-412.

Cole, W,C. & Garfield, R.E. (1986). Evidence for physiological regulation of myometrial gap junction permeability. *American Journal of Physiology*, **251**, C411-C420.

Coon, H.G. (1968) Clonal culture of differentiated rat liver cells. *Journal of Cell Biology*, **39**, 29a.

Cooper, J.A. & King, C.S. (1986) Dephosphorylation or antibody binding to the carboxyl terminus stimulates pp60c-src. *Molecular & Cellular Biology*, **6**, 446-447.

Crawford, A.W. & Beckerle, M.C. (1991) Purification and characterization of zyxin, an 82,000-dalton component of adherens junctions. *Journal of Biological Chemistry*, **266**, 5847-5853.

Crick., F. (1970) Diffusion in embryogenesis. *Nature*, **225**, 420-422.

Crow, D.S., Beyer, E.C., Paul, D.L., Kobe, S.S. & Lau, A.F. (1990) Phosphorylation of connexin43 gap junction protein in uninfected and Rous sarcoma virus-transformed mammalian fibroblasts. *Molecular & Cellular Biology*, **10**, 1754-1763.

Crow, D.S., Kurata, W.E. & Lau, A.F. (1992) Phosphorylation of connexin43 in cells containing mutant src oncogenes. *Oncogene*, **7**, 999-1003.

Culver, K.W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E.H. and Blaese, R.M. (1992) In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science*, **256**, 1550-1552.

Cummings, R.D. and Smith, D.F. (1992) The selectin family of carbohydrate-binding proteins: structure and importance of carbohydrate ligands for cell adhesion. *Bioessays*, **14**, 849-856.

Cunningham, B.A., Hemperly, J.J., Murray, B.A., Prediger, E.A., Brackenbury, R. and Edelman, G.M. (1987) Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science*, **236**, 799-806.

Cyr, D.G., Blaschuk, O.W. & Robaire, B. (1992) Identification and developmental regulation of cadherin messenger ribonucleic acids in the rat testis. *Endocrinology*, **131**, 139-145.

DeHaan, R.L. (1988) Dynamic behaviour of cardiac gap junctions. In *Gap Junctions*.ed. Hertzberg, E.L. and Johnson, R.G.pp 305-320. Alan Liss Inc., New York.

DeMello, W.C. (1984) Effect of intracellular injection of cAMP on the electrical coupling of mammalian cardiac cells. *Biochemical Biophysics Research Communications*, **119**, 1001-1007.

Dermietzel, R., Volker, M., Hwang, T.K., Berzborn, R.J. & Meyer, H.E. (1989) A 16 kDa protein co-isolating with gap junctions from brain tissue belonging to the class of proteolipids of the vacuolar H+-ATPases. *Febs Letters*, **253**, 1-5.

Dermietzel, R., Hwang, T.K. & Spray, D.S. (1990) The gap junction family: structure, function and chemistry. *Anatomy & Embryology*, **182**, 517-528.

Dewey, M.M. & Barr, L. (1962) Intercellular connections between smooth muscle cell: The nexus. *Science*, **137**, 670-672.

Doherty, P. & Walsh, F.S. (1989) Neurite guidance molecules. *Current Opinion in Cell Biology*, **1**, 1102-1106.

Doherty, P., Barton, C.H., Dickson, G., Seaton, P., Rowett, L.H., Moore, S.E., Gower, H.J. & Walsh, F.S. (1989) Neuronal process outgrowth of human sensory neurons on monolayers of cells transfected with cDNAs for five human N-CAM isoforms. *Journal of Cell Biology*, **109**, 789-798.

Doherty, P., Cohen, J. & Walsh, F.S. (1990) Neurite outgrowth in response to transfected N-CAM changes during development and is modulated by polysialic acid. *Neuron*, **5**, 209-219.

Doherty, P., Ashton, S.V., Moore, S.E. & Walsh, F.S. (1991) Morphoregulatory activities of NCAM and N-cadherin can be accounted for by G protein-dependent activation of L- and N-type neuronal Ca2+ channels. *Cell*, **67**, 21-33.

Donalies, M., Cramer, M., Ringwald, M. & Starzinski-Powitz, A. (1991) Expression of M-cadherin, a member of the cadherin multigene family, correlates with differentiation of skeletal muscle cells. *Proceedings National Academy of Sciences (USA)*, **88**, 8024-8028.

Dotto, G.P., el-Fouly, M.H., Nelson, C. & Trosko, J.E. (1989) Similar and synergistic inhibition of gap-junctional communication by ras transformation and tumor promoter treatment of mouse primary keratinocytes. *Oncogene*, **4**, 637-641.

Draetta, G. (1990) Cell cycle control in eukaryotes: molecular mechanisms of cdc2 activation. *Trends in Biochemical Sciences*, **15**, 378-383.

Dupont, E., El Aoumari, A., Roustiau-Severe, S., Briand, J.P. & Gros, D. (1988) Immunological characterization of mouse myocardial gap junctions: presence of common antigenic determinates in heart of other vertebrate species and in various organs. *Journal of Membrane Biology*, **104**, 229-240.

Ebihara, L., Beyer, E.C., Swenson, K.I., Paul, D.L. & Goodenough, D.A. (1989) Cloning and expression of a Xenopus embryonic gap junction protein. *Science*, **243**, 1194-1195.

Edelman, G.M. (1985) Cell adhesion and the molecular process of morphogenesis. *Annual Review of Biochemistry*, **54**, 135-169.

Edelman, G.M. (1988) Morphoregulatory molecules. *Biochemistry*, **27**, 3533-3543.

Eghbali, B., Kessler, J.A. & Spray, D.C. (1990) Expression of gap junction channels in communicatin incompetant cells after stable transfection with cDNA encoding connexin 32. *Proceedings of the National Academy of Sciences (USA)*, **87**, 1328-1331.

Ehring, G.R., Zampighi, G., Horwitz, J., Bok, D. & Hall, J.E. (1990) Properties of channels reconstituted from the major intrinsic protein of lens fiber membranes. *Journal of General Physiology*, **96**, 631-664.

el-Sabban, M.E. & Pauli, B.U. (1991) Cytoplasmic dye transfer between metastatic tumor cells and vascular endothelium. *Journal of Cell Biology*, **115**, 1375-1382.

Epstein, M.L. & Gilula, N.B. (1977) A study of communication specificity between cells in culture. *Journal of Cell Biology*, **75**, 769-787.

Epstein, M.L., Sheridan, J.D. & Johnson, R.G. (1977) Formation of low resistance junctions in vitro in the absence of protein synthesis and ATP production. *Experimental Cell Research*, **104**, 25-30.

Evans, R.M. (1988) The steroid and thyroid hormone receptor superfamily. *Science*, **240**, 889-895.

Fallon, R.F. and Goodenough, D.A. (1981) Five hour half-life of rat liver gap-junction protein. *Journal of Cell Biology*, **90**, 521-526.

Fawcett, D.W. & McNutt, N.S. (1969) The ultrastructure of the cat myocardium (I). *Journal of Cell Biology*, **42**, 1-45.

Fearon, E.R. & Vogelstein, B. (1990) A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759-767.

Fearon, E.R., Cho, K.R., Nigro, J.M., Kern, S.E., Simons, J.W., Ruppert, J.M., Hamilton, S.R., Preisinger, A.C., Thomas, G., Kinzler, K.W. & et, a.l. (1990) Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science*, **247**, 49-56.

Fentiman, I., Taylor-Papadimitriou, J. & Stoker, M.P.G. 1976) Selective contact dependant cell communication. *Nature*, **264**, 760-762.

Fentiman, I. & Taylor-Papadimitriou, J. (1977) Cultured human breast cancer lose selectivity in direct intercellular communication. *Nature*, **269**, 156-158.

Field, A.K., Davies, M.E., DeWitt, C. Perry, H.C., Germershausen, J., Karkas, J.D., Ashton, W.T. Johnson, D.B.R. & Tolman, R.L. (1983) {[2-hydroxy-1 (hydroxymethyl) ethoxy] methyl} guanine: a selective inhibitor of herpes virus replication. *Proceedings National Academy of Sciences (USA)*, **80**, 4139-4143.

Filson, A.J., Azarnia, R., Beyer, E.C., Loewenstein, W.R. & Brugge, J.S. (1990) Tyrosine phosphorylation of a gap junction protein correlates with inhibition of cell-to-cell communication. *Cell Growth & Differentiation*, **1**, 661-668.

Finbow, M.E., Yancey, S.B., Johnson, R. & Revel, J-P. (1980) Independant lines of evidence suggesting a major gap junctional protein with a molecular weight of 26000. *Proceedings of the National Academy of Sciences (USA)*, **77**, 970-974.

Finbow, M.E. & Pitts, J.D. (1981) Permeability of junctions between animal cells:transfer of vitamens and a vitamin derived cofactor. *Experimental Cell Research*, **131**, 1-13.

Finbow, M.E., Shuttleworth, J., Hamilton, A.E. & Pitts, J.D. (1983) Analysis of vertebrate gap junction proteins. *EMBO Journal*, **2**, 1479-1486.

Finbow, M.E., Eldrige, T., Buultjens, J., Lane, N,L., Shuttleworth, J. and Pitts, J.D (1984) Isolation and characterization of arthropod gap junctions. *EMBO Journal*, **3**, 2271-2278.

Finbow, M.E., Buultjens, J., Serras, F., Kam, E., John, S., and Meagher, L. (1988) Immunological and biochemical analysis of the low molecular weight gap junctional proteins. In *Gap Junctions*, Hertzberg, E.L. and Johnson, R.G.pp 53-68. Alan Liss Inc., New York.

Finbow, M.E. & Meagher, L. (1992) Connexins and the vacuolar proteolipid-like 16-kDa protein are not directly associated with each other but may be components of similar or the same gap junctional complexes. *Experimental Cell Research*, **203**, 280-284.

Finbow, M.E., Eliopoulos, E.E., Jackson, P.J., Keen, J.N., Meagher, L., Thompson, P., Jones, P. & Findlay, J.B. (1992) Structure of a 16 kDa integral membrane protein that has identity to the putative proton channel of the vacuolar H(+)-ATPase. *Protein Engineering*, **5**, 7-15.

Finbow, M.E. & Pitts, J.D. (1993) Is the gap junctional channel-the connexon-made of connexin or ductin? *Journal of Cell Science*, **in press**.

Finbow, M.E., John, J. Kam, E., Apps, D.K. & Pitts, J.D. Disposition and orientation of ductin (DCCD reactive vacuolar H^+-ATPase subunit) in mammalian membrane complexes. (1993) *Experimental Cell Research*, **in press**.

Fishman, G.I., Spray, D.C. & Leinwand, L.A. (1990) Molecular characterization and functional expression of the human cardiac gap junction channel. Journal of Cell Biology, 111, 589-598.

Fishman, G.I., Eddy, R.L., Shows, T.B., Rosenthal, L. & Leinwand, L.A. (1991a) The human connexin gene family of gap junction proteins: distinct chromosomal locations but similar structures. *Genomics*, **10**, 250-256.

Fishman, G.I., Moreno, A.P., Spray, D.C. & Leinwand, L.A. (1991b) Functional analysis of human cardiac gap junction channel mutants. *Proceedings National Academy of Sciences (USA)*, **88**, 3525-3529.

Flagg-Newton, J., Simpson, I. & Loewenstein, W.R. (1979). Permeability of cell-cell membrane channels in mammalian cell junctions. *Science*, **205**, 404-409.

Fleming, T.P., McConnell, J., Johnson, M.H. & Stevenson, B.R. (1989) Development of tight junctions de novo in the mouse early embryo: control of assembly of the tight junction-specific protein, ZO-1. *JournalofCell Biology*, **108**, 1407-1418.

Foley, G.E., Drolet, B.P., McCarthy, R.E., Goulet, K.A., Dokos, J.M. & Filler, D.A. (1959) Isolation and serial propagation of malignant and normal cells in semi-defined media. *Cancer Research*, **20**, 930-931.

Fraser, S.E. & Bode, H.R. (1981) Epithelial cells of hydra are dye coupled. *Nature*, **294**, 356.

Fraser, S.E. & Bryant, P.J. (1985) Patterns of dye coupling in the imaginal wing disk of *Drosophila melanogaster*. *Nature*, **317**, 533-536.

Fraser, S.E., Green, C.R., Bode, H.R. and Gilula, N.B. (1987) Selective disruption of gap junctional communication interferes with the patterning process in hydra. *Science*, **237**, 49-55.

Friedlander, D.R., Mege, R.M., Cunningham, B.A. & Edelman, G.M. (1989) Cell sorting-out is modulated by both the specificity and amount of different cell adhesion molecules (CAMs) expressed on cell surfaces. *Proceedings National Academy of Sciences (USA)*, **86**, 7043-7047.

Friend, S.H., Bernards, R., Rogelj, S., Weinberg, R.A., Rapaport, J.M., Albert, D.M. & Dryja, T.P. (1986) A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature*, **323**, 642-646.

Frixen, U.H., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., Lochner, D. & Birchmeier, W. (1991) E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *Journal of Cell Biology*, **113**, 173-185.

Fugimori, M., Tokino, T., Hino, O., Kitagawa, T., Imamura, T., Okamoto, E., Mitsunobu, M., Ishikawa, T., et al. (1991) Alleotype study of primary hepatocellular carcinoma. *Cancer Research*, **51**, 89-93.

Fujimoto, W.J., Subak-Sharpe, J.H. & Seegmiller, J.E. (1971) Hypoxanthine-guanine phosphoribosyltransferase deficiency: chemical agents selective for mutant or normal cultured fibroblasts in mixed and heterozygote cultures. *Proceedings National Academy of Sciences (USA)*, **68**, 1516-1519.

Furshpan, E.J & Potter, D.D. (1959) Transmission at the giant motor synapses of the crayfish. *Journal of Physiology*, **145**, 289-325.

Garrod, D.R. (1993) Desmosomes and hemidesmosomes. *Current Opinion in Cell Biology*, **5**, 30-40.

Geiger, B. & Ayalon, O. (1992) Cadherins. *Annual Review of Cell Biology*, **8**, 307-332.

Gevers, P. & Timmermans, L.P.M. (1991) Dye coupling and the formation and fate of the hypoblast in the teleost fish embryo. *Development*, **112**, 431-438.

Ghelson, K.R., Dillner, L., Engvall, E. & Ruoslahti, E. (1988) The human laminin receptor is a member of the integrin family of cell adhesion receptors. *Science*, **241**, 1228-1229.

Giguere, V., Ong, E., Segui, P. & Evans, R.M. (1987) Identification of a receptor for the morphogen retinoic acid. *Nature*, **330**, 624-629.

Gilula, N.B., Reeves, R.O. and Steinbach, A. (1972) Metabolic coupling, ionic coupling and cell contacts. *Nature*, **235**, 262-265.

Gilula, N.B., Epstein, M.L. & Beers, W.H. (1978) Cell-to-cell communication and ovulation. *Journal of Cell Biology*, **78**, 58-75.

Gimlich, R.L., Kumar, N.M. & Gilula, N.B. (1990) Differential regulation of the levels of three gap junction mRNAs in Xenopus embryos. *Journal of Cell Biology*, **110**, 597-605.

Giroldi, L.A. & Schalken, J.A. (1993) Decreased expression of the intercellular adhesion molecule E-cadherin in prostate cancer: biological significance and clinical implications. *Cancer & Metastasis Reviews*, **12**, 29-37.

Goldstein, D.J., Finbow, M.E., Andresson, T., McLean, P., Smith, K., Bubb, V. & Schlegel, R. (1991) Bovine papillomavirus E5 oncoprotein binds to the 16K component of vacuolar H(+)-ATPases. *Nature*, **352**, 347-349.

Goldstein, D.J., Tayama, R., Dhar, R. & Schlegal, R. (1992) The BPV-1 E5 oncoprotein expressed in *Schizosaccharomyces pombe* exhibits normal biochemical properties and binds to the endogenous 16-kDa component of the vacuolar proton-ATPase. *Virology*, **190**, 889-893.

Goodenough, D.A. (1974) Bulk isolation of mouse hepatocyte gap junctions. *Journal of Cell Biology*, **61**, 557-563.

Goodenough, D.A. (1975) Methods for the isolation and structural characterization of hepatocyte gap junctions. In *Methods in Membrane Biology*,ed. Vol.III. Korn E.D. Vol.III, pp 51-80. Plenum Publishing Corporation, New York.

Goodenough, D.A. (1976) In vitro formation of gap junction vesicles. *Journal of Cell Biology*, **68**, 220-231.

Goodenough, D.A. (1979) Lens gap junctions: a structural hypothesis for non-regulated low resistance intercellular pathways, *Investigative Opthalmology & Visual Science*, **18**, 1104-1122.

Goodenough, D.A. (1992) The crystalline lens. A system networked by gap junctional intercellular communication. *Seminars in Cell Biology*, **3**, 49-58.

Goodenough, D.A. & Revel. J-P. (1970) A fine structural analysis of intercellular gap junctions. *Journal of Cell Biology*, **45**, 272-290.

Goodenough, D.A. & Stoeckenius, W. (1972). The isolation of hepatocyte gap junctions. Prelimenary chemical characterization and X-ray diffraction. *Journal of Cell Biology*, **74**, 605-628.

Goodenough, D.A., Paul, D.L. and Jesaitis, L. (1988) Topological distribution of two connexin 32 antigenic sites in intact and split rodent hepatocyte gap junctions. *Journal of Cell Biology*, **107**, 1817-1824.

Goridis, C., Hirn, M., Santoni, M.J., Gennarini, G., Deagostini-Bazin, H., Jordan, B.R., Kiefer, M. & Steinmetz, M. (1985) Isolation of mouse N-CAM-related cDNA: detection and cloning using monoclonal antibodies. *EMBO Journal*, **4**, 631-635.

Gorin, M.B., Yancey, S.B., Cline, J., Revel, J-P. and Horwitz, J. (1984) The major intrinsic protein (MIP) of the bovine fiber membrane: characterzation and structure based on cDNA cloning. *Cell*, **39**, 49-59.

Gosh, S. & Baltimore, D. (1990) Activation *in vitro* of NF-κB by phosphorylation of its inhibitor IκB. *Nature*, **344**, 678-682.

Gosh, S. Gifford, A.M., Riviere, L.R., Tempest, P., Nolan, G.P. & Baltimore, D. (1990) Cloning of the p50 DNA binding subunit of NF-κB: homology to *rel* and *dorsal*. *Cell*, **62**, 1019-1029.

Griep, E.B & Revel, J-P. (1977) Gap junctions in development. In *Intercellular Communication*, ed. de Mello, W.C. pp 1-32. Plenum Publishing Corporation, New York.

Gumbiner, B. (1987) Structure, biochemistry, and assembly of epithelial tight junctions. *American Journal of Physiology*, **253**, C749-C758.

Gumbiner, B., Stevenson, B. and Grimaldi, A. (1988) The role of cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *Journal of Cell Biology*, **107**, 1575-1587.

Haefliger, J.A., Bruzzone, R., Jenkins, N.A., Gilbert, D.J., Copeland, N.G. & Paul, D.L. (1992) Four novel members of the connexin family of gap junction proteins Molecular cloning, expression, and chromosome mapping. *Journal of Biological Chemistry*, **267**, 2057-2064.

Hanna, R.B., Ornberg, R.L & Simon, T.S. (1985) Structural details of rapidly frozen gap junctions. In *Gap Junctions*, ed. Bennett, M.V.L. and Spray, D.C. pp23-32. Cold Spring Harbour Laboratory.

Harris, H. (1988) The analysis of malignancy in cell fusion: the position in 1988. *Cancer Research*, **48**, 3302-3306.

Hatta, K. & Takeichi, M. (1986) Expression of N-cadherin molecules associated with early morphogenic events in chick development. *Nature*, **320**, 447-449.

Hatta, K., Nose. A., Nagafuchi, A. & Takeichi, M. (1988) Cloning and expression of cDNA encoding a neural calcium-dependant cell adhesion molecule: Its identity in the cadherin gene family. *Journal of Cell Biology*, **106**, 873-881.

Hax, W.M.A., van Venrooji, G.E.P.M., & Vossenberg, J.B.J. (1974) Cell communication: a cyclic-AMP mediated phenomenon. *Journal of Membrane Biology*, **19**, 253-266.

Henderson, D., Eibl, H. and Weber, K. (1979) Structure and biochemistry of mouse hepatocyte gap junctions. *Journal of Molecular Biology*, **132**, 193-218.

Hennemann, H., Suchyna, T., Lichtenberg-Frate, H., Jungbluth, S., Dahl, E., Schwarz, J., Nicholson, B.J. & Willecke, K. (1992) Molecular cloning and functional expression of mouse connexin 40, a second gap junction gene preferentially expressed in lung. *Journal of Cell Biology*, **117**, 1299-1310.

Hertzberg, E.L. & Gilula, N.B. (1979) Isolation and charaterization of gap junctions from rat liver. *Journal of Biological Chemistry*, **254**, 2138-2147.

Hertzberg, E.L., Anderson, D.J., Friedlander, M. & Gilula, N.B. (1982) Comparitive analysis of the major polypeptides from liver gap junctions and lens fiber membranes. *Journal of Cell Biology*, **92**, 53-59.

Hertzberg, E.L. and Skibbens, R.V. (1984) A protein homologous to the 27000 dalton liver gap junction protein is present in a variety of species and tissues. *Cell* **39**, 61-69.

Hertzberg, E.L., Spray, D.C. & Bennet, M.V.L. (1985) Reductional of gap junctional conductance by microinjection of antibodies against the 27kD liver gap junction polypeptide. *Proceedings of the National Academy of Sciences (USA)*, **82**, 2412-2416.

Hertzberg, E.L., Spray, D.C. & Cook, R. (1988) Biochemical, immunological and topological studies of gap junctions. In *Gap Junctions*, ed. Hertzberg, E.L. and Johnson, R.G. pp 9-28. Alan Liss Inc., New York.

Heynkes, R., Kozjek, R., Traub, O. and Willecke ,K. (1986) Identification of a rat liver cDNA and mRNA encoding for the 28kD gap junction protein. *Febs Letters*, **202**, 56-60.

Hirano, S., Nose, A., Hatta, K., Kawakami. A. and Takeichi, M. (1987) Calcium-dependant cell-cell adhesion molecules (cadherins): subclass specificities and possible involvement of actin bundles. *Journal of Cell Biology*, **105**, 2501-2510.

Hirano, S., Kimoto, N., Shimoyama, Y., Hirohashi, S. & Takeichi, M. (1992) Identification of a neural alpha-catenin as a key regulator of cadherin function and multicellular organization. *Cell*, **70**, 293-301.

Hirokawa, N. & Heuser, J. (1982) The inside and outside of gap junction membranes visualized by deep etching. *Cell*, **30**, 395-406.

Hoh, J.H., Lal, R., John, S.A., Revel, J.P. & Arnsdorf, M.F. (1991a) Atomic force microscopy and dissection of gap junctions. *Science*, **253**, 1405-1408.

Hoh, J.H., John, S.A. & Revel, J.P. (1991b) Molecular cloning and characterization of a new member of the gap junction gene family, connexin-31. *Journal of Biological Chemistry*, **266**, 6524-6531.

Holzenburg, A., Jones, P.C., Franklin, T., Pali, T., Heimburg, T., Marsh, D., Findlay, J.B. & Finbow, M.E. (1993) Evidence for a common structure for a class of membrane channels. *European Journal of Biochemistry*, **213**, 21-30.

Holmes, D.S. & Quigley, M. (1981) A rapid boiling method for the preparation of bacterial plasmids. *Annals of Biochemistry*, **114**, 193.

Hsieh, C.L., Kumar, N.M., Gilula, N.B. & Francke, U. (1991) Distribution of genes for gap junction membrane channel proteins on human and mouse chromosomes. *Somatic Cell & Molecular Genetics*, **17**, 191-200.

Hunter, T. (1991) Cooperation beteen oncogenes. *Cell.* **64**, 249-270.

Hynes, R.O. (1987) Integrins: a family of cell surface receptors. *Cell*, **48**, 549-554.

Hynes, R.O. (1992a) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell*, **69**, 11-25.

Hynes, R.O. (1992b) Specificity of cell adhesion in development: the cadherin superfamily. *Current Opinion in Genetics & Development*, **2**, 621-624.

Hyrc, K. & Rose, B. (1990) The action of v-src on gap junctional permeability is modulated by pH. *Journal of Cell Biology*, **110**, 1217-1226.

Inuzuka, H., Miyatani, S. & Takeichi, M. (1991) R-cadherin: a novel Ca(2+)-dependent cell-cell adhesion molecule expressed in the retina.*Neuron*, **7**, 69-79.

Isaac, P.G., jones, V.P. and Leaver, C.P. (1985) The maise cytochrome c oxidase subunit I gene. *EMBO Journal*, **4**, 1617-1623.

Israel, M., Morel, N., Lesbats, B., Birman, S. and Manaranche, R. (1986) Purification of a presynaptic membrane protein that mediates a calcium-dependent translocation of acetylcholine. *Proceedings National Academy of Sciences (USA)*, **83**, 9226-9230.

Itoh, M., Yonemura, S., Nagafuchi, A., Tsukita, S. and Tsukita, S. (1991) A 220-kD undercoat-constituive protein: its specific localization at cadherin-based cell-cell adhesion sites. *Journal of Cell Biology*, **115**, 1449-1462.

Jongen, W.M., Fitzgerald, D.J., Asamoto, M., Piccoli, C., Slaga, T.J., Gros, D., Takeichi, M. & Yamasaki, H. (1991) Regulation of connexin 43-mediated gap junctional intercellular communication by Ca2+ in mouse epidermal cells is controlled by E-cadherin. *Journal of Cell Biology*, **114**, 545-555.

Kadle, R., Zhang, J.T. & Nicholson, B.J. (1991) Tissue-specific distribution of differentially phosphorylated forms of Cx43. *Molecular & Cellular Biology*, **11**, 363-369.

Kalimi, G.H. & Lo, C.W. (1988) Communicating commpartments in the gastrulating mouse embryo. *Journal of Cell Biology*, **107**, 241-255.

Kam, E., Melville, L. & Pitts, J.D. (1986) Patterns of junctional communication in skin. *Journal of Investigative Dermatology*, **87**, 748-753.

Kam, E. & Pitts, J.D. (1988) Effects of the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate on junctional communication in intact mouse skin: persistance of homologous communication and increase in epidermal-dermal coupling. *Carcinogenesis*, **9**, 1389-1394.

Kam, E. & Hodgins, M.B. (1992) Communication compartments in hair follicles and their implication in differentiative control. *Development*, **114**, 389-393.

Kanter, H.L., Saffitz, J.E. & Beyer, E.C. (1992) Cardiac myocytes express multiple gap junction proteins. *Circulation Research*, **70**, 438-444.

Kardami, E., Stoski, R.M., Doble, B.W., Yamamoto, T., Hertzberg, E.L. & Nagy, J.I. (1991) Biochemical and ultrastructural evidence for the association of basic fibroblast growth factor with cardiac gap junctions. Journal of *Biological Chemistry*, **266**, 19551-19557.

Katoh, F., Klein, J-L., Bignami, M. & Yamasaki, H. (1993) Association of viral oncogene-induced changes in gap junctional intercellular communication and morphological transformation in BALB/c3T3 cells. *Carcinogenesis*, **14**, 435-440.

Keane, R.W., Mehta, P.P., Rose, B., Honig, L.S., Loewenstein, W.R. & Rutishauser, U. (1988) Neural differentiation, N-CAM mediated adhesion and gap junctional communication in neuroectoderm. A study *in vitro*. *Journal of Cell Biology*, **106**, 1307-1319.

Kensler, R.W. & Goodenough, D.A. (1980) Isolation of mouse myocardial gap junctions. *Journal of Cell Biology*, **86**, 755-764.

Kishimoto, T.K., O'Conner, K. and Springer, T.A. (1988) Leukocyte adhesion deficiency. Aberrant splicing of a conserved integrin sequence causes a moderate deficiency phenotype. *Journal of Biological Chemistry*, **266**, 4471-4477.

Kistler, J., Kirland, B. & Bullivant, S. (1985) Identification of a 70,000Da protein in lens membrane junctional domains. *Journal of Cell Biology*, **101**, 28-35.

Knudsen, A.G. (1971) Mutation and cancer: statistical study of retinoblastoma. *Proceedings National Academy of Sciences (USA)*, **68**, 820-823.

Knudsen, K.A. & Wheelock, M.J. (1992) Plakoglobin, or an 83-kD homologue distinct from beta-catenin, interacts with E-cadherin and N-cadherin. *Journal of Cell Biology*, **118**, 671-679.

Knudsen, K.A., McElwee, S.A. & Myers, L. (1990) A role for the neural cell adhesion molecule, NCAM, in myoblast interaction during myogenesis. *Developmental Biology*, **138**, 159-168.

Kolb, H-A. & Somogyi, R. (1991) Biochemical and biophysical analysis of cell-to-cell channels and regulation of gap junctional permeability. *Reviews of Physiology Biochemistry & Pharmacology*, **118**, 1-47.

Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic charater of a protein. *Journal of Molecular Biology*, **157**, 105-132.

Kumar, N.M. & Gilula, N.B. (1986) Cloning and characterization of human and rat cDNAs coding for a gap junction protein. *Journal of Cell Biology*, **103**, 767-776.

Kumar, N.M. & Gilula, N.B. (1992) Molecular biology and genetics of gap junction channels. *Seminars in Cell Biology*, **3**, 3-16.

Laird, D.W. and Revel, J-P. (1990) Biochemical and immunochemical analysis of the arrangement of connexin 43 in rat heart gap junction membranes. *Journal of Cell Science*, **97**, 109-117.

Laird, D.W., Puranam, K.L. & Revel, J.P. (1991) Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. *Biochemical Journal*, **273**, 67-72.

Lane, N.J. & Swales, L.S. (1980) Dispersal of junctional particles, not internalization, during the in vivo disappearance of gap junctions. *Cell*, **19**, 579-586.

Larsen, W.J. & Tung, H.N. (1978) Origin and fate of cytoplasmic gap junctional vessicles in rabbit granulosa cells. *Tissue Cell*, **10**, 585-598.

Larsen, W.J. (1985) Relating the population dynamics of gap junctions to cellular function.In *Gap Junctions*, ed. Bennett, M.L.V. and Spray, D.C. pp 289-306. Cold Spring Harbour Laboratory.

Lau, A.F., Hatch-Pigott, V. & Crow, D.S. (1991) Evidence that heart connexin43 is a phosphoprotein. *Journal of Molecular & Cellular Cardiology*, **23**, 659-663.

Lau, A.F., Kanemitsu, M.Y., Kurata, W.E., Danesh, S. & Boynton, A.L. (1992) Epidermal growth factor disrupts gap-junctional communication and induces phosphorylation of connexin43 on serine. *Molecular Biology of the Cell*, **3**, 865-874.

Lawrence, T.S., Beers, W.H. & Gilula, N.B. (1978) Hormonal stimulation and cell communication in cocultures. *Nature*, **272**, 501-506.

Lee, S.W., Gilula, N.B. & Warner, A.E. (1987) Gap junctional communication and compaction during preimplantation stages of mouse development. *Cell*, **51**, 851-860.

Lee, S.W., Tomasetto, C., Paul, D., Keyomarsi, K. & Sager, R. (1992) Transcriptional downregulation of gap-junction proteins blocks junctional communication in human mammary tumor cell lines. *Journal of Cell Biology*, **118**, 1213-1221.

Lee, W-H., Bookstein, R., hong, F., Young, L-J., Shew, J-Y. and Lee, E.Y-H.P. (1987) Human retinoblastoma susceptibility gene: cloning, identification, and sequence. *Science*, **235**, 1394-1399.

Leitch, B. & Finbow, M.E. (1990) The gap junction-like form of a vacuolar proton channel component appears not to be an artifact of isolation: an immunocytochemical localization study. *Experimental Cell Research*, **190**, 218-226.

Lin, J-W. & Faber, D.S. (1988) Synaptic transmissions mediated by single club endings on the goldfish Mauthner cell. *Journal of Neuroscience*, **8**, 1302-1312.

Lipfert, L., Haimovich, B., Schaller, M.D., Cobb, B.S., Parsons, J.T. & Brugge, J.S. (1992) Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125^{FAK} in platelets. *Journal of Cell Biology*, **119**, 905-912.

Lo, C.W. & Gilula, N.B. (1979) Gap junctional communication in the post-implantation mouse embryo. *Cell*, **18**, 411-422.

Loewenstein, W.R. (1979) Junctional intercellular communication and the control of growth. *Biochimica Biophysica Acta*, **560**, 1-65.

Loewenstein, W.R. (1981) Junctional intercellular communication: the cell to cell membrane channel. *Physiology Reviews*, **61**, 829-913.

Loewenstein, W.R. & Kano, Y. (1964) Studies on the epithelial gland junction. *Journal of Cell Biology*, **22**, 565-585.

Loewenstein, W.R. & Rose, B. (1978) Calcium in (junctional) communication a thought on its behaviour in intercellular communication. *Annals of the New York Academy of Sciences*, **301**, 285-307.

Loewenstein, W.R. & Rose, B. (1992) The cell-cell channel in the control of growth. *Seminars in Cell Biology*, **3**, 59-79.

Lorca, T., Labbe, J-C., Devault, A., Fresquet, D., Capony, J-P., Le Bouffant, F. & Doree, M. (1992) Dephosphorylation of cdc2 on threonine 161 is required for cdc2 inactivation and normal anaphase. *EMBO Journal*, **11**, 2381-2390.

Louis, C.F., Hur, K.C., Galvan, A.C., TenBroek, E.M., Jarvis, L.J., Eccleston, E.D. and Howard, J.B. (1989) Identification of an 18000-Dalton protein in mammalian lens fibers. *Journal of Biological Chemistry*, **264**, 19967-19973.

Mackenzie, I.C. (1975) Ordered structure of the epidermis. *Journal of Investigative Dermatology*, **65**, 45-51.

MacDonald, C (1982) Genetic complementation in hybrid cells derived from two metabolic cooperation defective mammalian cell lines. *Experimental Cell Research*, **138**, 303-310.

Macpherson, I.A. & Stoker, M.G.P. (1962) Polyoma transformation of hamster cell clones- an investigation of genetic factors affecting cell competance. *Virology*, **16**, 147-151.

Maden, M. (1982) Vitamen A and pattern formation in the regenerating limb. *Nature*, **295**, 672-675.

Madhukar, B.V., Oh, S.Y., Chang, C.C., Wade, M. & Trosko, J.E. (1989) Altered regulation of intercellular communication by epidermal growth factor, transforming growth factor-beta and peptide hormones in normal human keratinocytes. *Carcinogenesis*, **10**, 13-20.

Magee, A. and Buxton, R.S. (1991) Transmembrane assemblies regulated by the greater cadherin family. *Current Opiniom in Cell Biology*, **3**, 854-861.

Makowski, L., Caspar, D.L.D., Phillips, W.C. & Goodenough, D.A. (1977) Gap junction structures II. Analysis of the X-ray diffraction data. *Journal of Cell Biology*, **74**, 629-624.

Maniatis, T., Fritsch, E.F. & Sambrook, J. (1989) *Molecular Cloning: A laboratory mannual*. 2nd Edition. Cold Spring Harbor Laboratory Press.

Mandel, M. Moriyama, Y., Humes, J.D., Pan Y-C, E., Nelson, H., Nelson, N. (1988) Cloning of cDNA sequence encoding the 16kDa proteolipid of chromaffin granules implies gene duplication in the evolution of H^+-ATPases. *Proceedings National Academy of Sciences (USA)*, **85**, 20390-20393.

Manjunath, C.K., Nicholson, B.J., Teplow, D., Hood, L, Page, E. and Revel, J-P. (1987) The cardiac gap junction protein (Mr=47000) has specific cytoplasmic domain of Mr 17000 at its carboxyl-terminus. *Biochemical Biophysics Research Communications*, **142**, 228-234.

Mareel, M.M., Behrens, J., Birchmeier, W., De Bruyne, G.K., Vleminckx, K., Hoogewijs, A., Fiers, W.C. & Van Roy, F.M. (1991) Down-regulation of E-cadherin expression in Madin Darby canine kidney (MDCK) cells inside tumors of nude mice. *International Journal of Cancer*, **47**, 922-928.

Marsh, J.L., Erfle, M. & Wykes, E.J. (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. *Gene*, **32**, 481-485.

Marshall, C.J. (1991) Tumor suppressor genes. *Cell.* **64**, 313-326.

Martin, P., Vass, W.C., Schiller, J.T., Lowry, D.T. & Velu, T.J. (1989) The bovine papillomavirus E5 transforming protein can stimulate the transforming activity of EGF and CSF-1 receptors. *Cell*, **59**, 21-32.

Mathias, R.T. and Rae, J.L. (1985) Transport properties of lens. *American Journal of Physiology*, **249**, C181-C190.

Matsuyoshi, N., Hamaguchi, M., Taniguchi, S., Nagafuchi, A., Tsukita, S. & Takeichi, M. (1992) Cadherin-mediated cell-cell adhesion is perturbed by v- src tyrosine phosphorylation in metastatic fibroblasts. *Journal of Cell Biology*, **118**, 703-714.

Matsuzaki, F., Mege, R.M., Jaffe, S.H., Friedlander, D.R., Gallin, W.J., Goldberg, J.I., Cunningham, B.A. & Edelman, G.M. (1990) cDNAs of cell adhesion molecules of different specificity induce changes in cell shape and border formation in cultured S180 cells. *Journal of Cell Biology*, **110**, 1239-1252.

Mayer, B., Johnson, J,P., Leitl, F., Jauch, K.W., Heiss, M.M., Schildberg, F.W., Birchmeier, W. & Funke, I. (1993) E-cadherin expression in primary and metastatic gastric cancer: down-regulation correlates with cellular dedifferentiation and glandular disintegration. *Cancer Research*, **53**, 1690-1695.

McMahon, D.G., Knapp, A.G. and Dowling, J.E. (1989) Horizontal cell gap junctions: Single-channel conductance and modulation by dopamine. *Proceedings National Academy of Sciences (USA)*, **86**, 7639-7643

McNeill, H., Ozawa, M., Kemler, R. & Nelson, W.J. (1990) Novel function of the cell adhesion molecule uvomorulin as an inducer of cell surface polarity. *Cell*, **62**, 309-316.

McNutt, N.S. & Weinstein, R.S. (1970) The ultrastructure of the nexus. *Journal of Cell Biology*, **55**, 511-515.

McCrea, P. & Gumbiner, B. (1991) Purification of a 92-kDa cytoplasmic protein tightly associated with the cell-cell adhesion molecule E-cadherin (uvomorulin). Characterization and extrability of the protein complex from the cell cytoskeleton. *Journal of Biological Chemistry*, **266**, 4515-4520.

Mege, R-M., Matusuzaki, F., Gallin, W.J., Goldberg, J.I., Cunnigham, B.A. and Edelman, G.M. (1988) Construction of epithelioid sheets by transfection of mouse sarcoma cells with cDNAs for chicken cell adhesion molecules. *Proceedings of the National Academy of Sciences (USA)*, **85**, 7274-7278.

Mege, R.M., Goudou, D., Diaz, C., Nicolet, M., Garcia, L., Geraud, G. & Rieger, F. (1992) N-cadherin and N-CAM in myoblast fusion: compared localisation and effect of blockade by peptides and antibodies. *Journal of Cell Science*, **103**, 897-906.

Mehta, P.P., Bertram, J.S. & Loewenstein, W.R. (1986) Growth inhibition of transformed cells correlates with their junctional communication eith normal cells. *Cell*, **44**, 187-196.

Mehta, P.P., Bertram, J.S. & Loewenstein, W.R. (1989) The actions of retinoids on cellular growth correlate with their actions on gap junctional communication. *Journal of Cell Biology*, **108**, 1053-1065.

Mehta, P.P. & Loewenstein, W.R. (1991) Differential regulation of communication by retinoic acid in homologous and heterologous junctions between normal and transformed cells. *Journal of Cell Biology*, **113**, 371-379.

Mehta, P.P., Hotz-Wagenblatt, A., Rose, B., Shalloway, D. & Loewenstein, W.R. (1991) Incorporation of the gene for a cell-cell channel protein into transformed cells leads to normalization of growth. *Journal of Membrane Biology*, **124**, 207-225.

Mehta, P.P., Yamamoto, M. & Rose, B. (1992) Transcription of the gene for the gap junctional protein connexin43 and expression of functional cell-to-cell channels are regulated by cAMP. *Molecular Biology of the Cell*, **3**, 839-850.

Mesnil, M. & Yamasaki, H. (1993) Cell-cell communication and growth control of normal and cancer cells: evidence and hypothesis. *Molecular Carcinogenesis*, **7**, 14-17.

Meyer, R.A., Laird, D.W., Revel, J.P. & Johnson, R.G. (1992) Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. *Journal of Cell Biology*, **119**, 179-189.

Migeon, B.R., Kaloustian, V.M., Nyhan, W.L., Young, W.J. & Childs, B. (1968) X-linked hypoxanthine-guanine phosphoribosyl transferase deficiency: heterozygote has two clonal populations. *Science*, **160**, 425-427.

Milks, L.C., Kumar, N.M., Houghton, R., Unwin, P.N.T., Gilula, N.B. (1988) Topology of the 32-kD liver gap junction protein determined by site directed antibody localisation. *EMBO Journal*, **7**, 2967-2975.

Miller, T., Dahl, G. & Werner, R. (1988) Structure of a gap junction gene: rat connexin 32. *Bioscience Reports*, **8**, 455-464.

Miyatani, S., Shimamura, K., Hatta, M., Nagafuchi, A., Nose, A., Matsunaga, M., Hatta, K. & Takeichi, M. (1989) Neural cadherin: role in selective cell-cell adhesion. *Science*, **245**, 631-635.

Montenarh, M. (1992) Biochemical properties of the growth suppressor/oncoprotein p53. *Oncogene*, **7**, 1673-1680.

Moor, R.M., Smith, M.W. & Dawson, R.M.C. (1980) Measurement of intercellular coupling between oocytes and cumulus cells using intracellular markers. *Experimental Cell Research*, **126**, 15-29.

Morgenstern, J.P. & Land, H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Research*, **18**, 3587-3596.

Moria, A.O., Draetta, G., Beach, D. and Wang, J.Y.J. (1989) Reversable tyrosine phosphorylation of cdc2: dephosphorylation activation during mitosis. *Cell*, **58**, 193-203.

Moriyama, Y. and Nelson, N. (1989) Cold inactivation of vacuolar H^+-ATPases. *Journal of Biological Chemistry*, **264**, 3577-3582.

Murray, A.W. & Fitzgerald, D.J. (1979) Tumor promoters inhibit metabolic cooperation in cocultures of epidermal and 3T3 cells. *Biochemical Biophysics Research Communications*, **91**, 395-401.

Musil, L.S., Beyer, E.C. & Goodenough, D.A. (1990a) Expression of the gap junction protein connexin43 in embryonic chick lens: molecular cloning, ultrastructural localization, and post-translational phosphorylation. *Journal of Membrane Biology*, **116**, 163-175.

Musil, L.S., Cunningham, B.A., Edelman, G.M. & Goodenough, D.A. (1990b) Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. *Journal of Cell Biology*, **111**, 2077-2088.

Musil, L.S. & Goodenough, D.A. (1991) Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. *Journal of Cell Biology*, **115**, 1357-1374.

Nagafuchi, A. Sirayoshi, Y., Okazaki, K. & Takeichi, M. (1987) Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature*, **329**, 341-343.

Nagafuchi, A. & Takeichi, M. (1988) Cell binding of E-cadherin is regulated by the cytoplasmic domain. *EMBO Journal*, **7**, 3679-3684.

Napolitano, E.W., Venstrom, K., Wheeler, E.F. and Reichardt, L.F. (1991) Molecular cloning and characterization of B-cadherin, a novel chick cadherin. *Journal of Cell Biology*, **113**, 893-905.

Nelson, N. (1992) Organellar proton-ATPases. *Current Opinion in Cell Biology*, **4**, 654-660.

Nelson, N. & Taiz, L. (1989) The evolution of H+-ATPases. *Trends in Biochemical Sciences*, **14**, 113-116.

Nelson, H. & Nelson, N. (1990) Disruption of genes encoding subunits of yeast vacuolar H^+-ATPase causes conditional lethality. *Proceedings National Academy of Sciences (USA)*, **87**, 3503-3507.

Neugebauer, K.M., Tomaselli, K.J., Lilien, J. and Reichardt, L.F. (1988) N-cadherin, NCAM, and integrins promote retinal neurite out-growth on astrocytes *in vitro*. *Journal of Cell Biology*, **107**, 1177-1187.

Neyton, J. & Trautmann, A. (1985) Single-channel currents of an intercellular junction. *Nature*, **317**, 331-335.

Neyton, J. & Trautmann, A. (1986) Physiological modulation of gap junction permeability. *Journal of Experimental Biology*, **124**, 93-144.

Nezu, J., Motojima, K., Tamura, H. & Ohkuma, S. (1992) Molecular cloning of a rat liver cDNA encoding the 16 kDa subunit of vacuolar H(+)-ATPases: organellar and tissue distribution of 16 kDa proteolipids. *Journal of Biochemistry*, **112**, 212-219.

Nicholson, B.J., Hunkapiller, M.W., Grim, L.B., Hood, L.E. & Revel J-P. (1981) Rat liver gap junction protein: properties and partial sequence. *Proceedings National Academy of Sciences (USA)*, **78**, 7594-7598.

Nicholson, B.J., Gros, D.B., Kent, S.B.H., Hood, L.E. & Revel J-P. (1985) The Mr 28000 gap junction protein from rat heart and liver are different but related. *Journal of Biological Chemistry*, **260**, 6514-6517.

Nicholson, B.J., Dermietzel, R., Teplow, D., Traub, O., Willecke, K. & Revel, J-P. (1987) Two homologous protein components of hepatic gap junctions. *Nature*, **329**, 732-734.

Nicholson, L.J., Pei, X.F. & Watt, F.M. (1991) Expression of E-cadherin, P-cadherin and involucrin by normal and neoplastic keratinocytes in culture. *Carcinogenesis*, **12**, 1345-1349.

Nori, M., L'Allemain, G. & Weber, M.J. (1992) Regulation of tetradecanoyl phorbol acetate-induced responses in NIH 3T3 cells by GAP, the GTPase-activating protein associated with $p21^{c\text{-}ras}$. *Molecular & Cellular Biology*, **12**, 936-945.

Nose, A. & Takeichi, M. (1986) A novel cadherin cell adhesion molecule: its expression patterns associated with inplantation and organogenesis of mouse embryos. *Journal of Cell Biology*, **103**, 2694-2658.

Nose, A., Nagafuchi, A. & Takeichi, M. (1987) Isolation of placental cadherin cDNA: identification of a novel gene family of cell-cell adhesion molecules. *EMBO Journal*, **6**, 3655-3651.

Nose, A., Nagafuchi, A. & Takeichi, M. (1988), Expressed recombinant cadherins mediate cell sorting in model systems. *Cell*, **54**, 993-1001.

Nose, A., Tsuji, K. & Takeichi, M. (1990) Localization of specificity determining sites in cadherin cell adhesion molecules. *Cell*, **61**, 147-155.

Noumi, T., Beltran, C., Nelson, H. & Nelson, N. (1991) Mutational analysis of yeast H^+-ATPase. *Proceedings National Academy of Sciences (USA)*, **88**,1938-1942.

Oka, H., Shiozaki, H., Kobayashi, K., Inoue, M., Tahara, H., Kobayashi, T., Takatsuka, Y., Matsuyoshi, N., Hirano, S., Takeichi, M. et al. (1993) Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. *Cancer Research*, **53**, 1696-1701.

Olsen, J.C., Johnson, L.G., Wong-Sun, M.L., Moore, K.L., Swanstrom, R. & Boucher, R.C. (1993) Retrovirus-mediated gene transfer to cystic fibrosis airway cells: effect of selectable marker sequences on long-term expression. *Nucleic Acid Research*, **21**, 663-669.

Ozawa, M., Baribault, H. & Kemler, R. (1989) The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independant proteins structurally related in different species. *EMBO Journal*, **8**, 1711-1717.

Ozawa, M., Ringwald, M. & Kemler, R. (1990) Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. *Proceedings National Academy of Sciences (USA)*, **87**, 4246-4250.

Parry, R.V., Turner, J.C. and Rea, P.A. (1989). High purity preparations of higher plant vacuolar H^+-ATPase reveal additional subunits. *Journal of Biological Chemistry*, **264**, 20025-20032.

Paul, D.L. (1986) Molecular cloning of cDNA for rat liver gap junction protein. *Journal of Cell Biology*, **103**, 123-134.

Paul, D.L. and Goodenough, D.A. (1983) Preparation, localization and characterization of antiserum against bovine MP26, an integral protein from lens fiber plasma membrane. *Journal of Cell Biology*, **96**, 625-632.

Paul, D.L., Ebihara, L., Takemoto, L.J., Swenson, K.I. & Goodenough, D.A. (1991) Connexin46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membrane of Xenopus oocytes. *Journal of Cell Biology*, **115**, 1077-1089.

Peracchia, C. (1973) Low resistance junctions in crayfish. *Journal of Cell Biology*, **57**, 66-76.

Peracchia, C. (1978) Calcium effects on gap junction structure and coupling. *Nature*, **271**, 669-671.

Peto, R., Roe, F.L.C., Lee, P.N., Levy, L. & Clack, J. (1975) Cancer and aging in mice and men. *British Journal of Cancer*, **32**, 411-426.

Phillips, D.R., Charo, I.F. and Scarborough, R.M. (1991) GPIIb-IIIa: the responsive integrin. *Cell*, **65**, 359-362.

Piccolino, M., Neyton, J. & Gershenfeld, H.M. (1984) Decrease in gap junction permeability induced by dopamine and cyclic adenosine 3':5'-monophosphate in horizontal cells of turtle retina. *Journal of Neuroscience*, **4**, 2477-2488.

Pitts, J.D. (1971) Molecular exchange and growth control in tissue culture. In *Growth Control in Cell Cultures*, pp 89-105. Ciba Foundation Symposium.

Pitts, J.D. (1980) The role of junctional communication in animal tissues. *In Vitro*, **16**, 1049-1056.

Pitts, J.D. & Burk, R.R. (1976) Specificity of junctional communication between animal cells. *Nature*, **264**, 760-762.

Pitts, J.D. & Simms, J.W. (1977) Permeability of junctions between animal cells. Intercellular transfer of nucleotides but not of macromolecules. *Experimental Cell Research*, **104**, 153-163.

Pitts, J.D. & Kam, E. (1985) Communication compartments in mixed cell cultures. *Experimental Cell Research*, **156**, 439-449.

Pitts, J.D. & Finbow, M.E. (1986) The Gap Junction. *Journal of Cell Science*, **Supplement 4**, 239-266.

Pitts, J.D., Hamilton, A.E., Kam, E., Burk, R.B. & Murphy, J.P. (1986) Retinoic acid inhibits junctional communication between animal cells. *Carcinogenesis*, **7**, 1003-1010.

Pitts, J.D., Kam, E. and Morgan, D. (1988) The role of gap junctional communication in growth control and tumorigenesis. In *Gap Junctions*.ed. Hertzberg, E.L. and Johnson, R.G.pp 397-409. Alan Liss Inc., New York.

Radu, A, Dahl, G. and Loewenstein, W.R. (1982) Hormonal regulation of cell junction permeability: upregulation by catecholamine and prostaglandin E1. *Journal of Membrane Biology*, **70**, 239-251.

Rahmon, S. & Evans, H. (1991) Topology of connexin 32 in rat liver gap junctions: Evidence for an intramolecular disulphide linkage connecting the two extracellular peptide loops. *Journal of Cell Science*, **100**, 567-578.

Rajewsky, M.F. & Gruneisen A. (1972) Cell proliferation in transplanted rat tumors: Influence of host immune system. *European Journal of Immunology*, **2**, 445-447.

Ralston, R. & Bishop, J.M. (1985) The product of the protooncogene *c-src* is modified during the cellular response to platelet derived growth factor. *Proceedings National Academy of Sciences (USA)*, **82**, 7845-7849.

Ranscht, B. & Bronner-Fraser, M. (1991) T-cadherin expression alternates with migrating neural crest cells in the trunk of the avian embryo. *Development*, **111**, 15-22.

Rash, J.E. & Yasumura, T. (1992) Improved structural detail in freeze fracture replicas: high-angle shaddowing of gap junctions cooled below -170^{O}C and protected by liquid nitrogen-cooled shrouds. *Microscopy Research and Technique*, **20**, 187-204.

Revel, J-P. & Karnovsky, M.J. (1967) Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *Journal of Cell Biology*, **33**, 87-102.

Reyes, A.A., Akeson, R., Brezina, L. and Cole, G.J. (1990) Structural requirements for neural cell adhesion molecule-heparin interaction. *Cell Regulation*, **1**, 567-576.

Rheinwald, J.D.& Beckett, M.A. (1981) Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinomas. *Cancer Research*, **41**, 1657-1663.

Risek, B., Guthrie, S., Kumar, N. & Gilula, N.B. (1990) Modulation of gap junction transcript and protein expression during pregnancy in the rat. *Journal of Cell Biology*, **110**, 269-282.

Risek, B., Klier, F.G. & Gilula, N.B. (1992) Multiple gap junction genes are utilized during rat skin and hair development. *Development*, **116**, 639-651.

Robertson, D. (1981) Membrane structure. *Journal of Cell Biology*, **91**, 189s-204s.

Robertson, J.D., Bodenheimer, P.S. & Stage, D.E. (1963) The ultrastructure of Mauthner cell synapses and nodes in goldfish brain. *Journal of Cell Biology*, **19**, 159-199.

Rogers, M., Berestecky, J.M., Hossain, M.Z., Guo, H.M., Kadle, R., Nicholson, B.J. & Bertram, J.S. (1990) Retinoid-enhanced gap junctional communication is achieved by increased levels of connexin 43 mRNA and protein. *Molecular Carcinogenesis*, **3**, 335-343.

Rook, M.B., de Jonge, B., Jongsma, H.J. & Masson-Pevet, M.A. (1990) Gap junction formation and functional interaction between rat cardiocytes in culture: A correlative physiological ultrastructure study. *Journal of Membrane Biology*, **118**, 179-192.

Rose,B. & Loewenstein, W.R. (1975) Permeability of cell junctions depends on local cytoplasmic calcium activity. *Nature*, **254**, 250-252.

Rose, B., Yada, T. & Loewenstein, W.R. (1986) Downregulation of cell-to-cell communication by the viral *src* gene is blocked by TMB-8 and recovery of communication is blocked by vanadate. *Journal of Membrane Biology*, **94**, 129-142.

Rose, B., Mehta, P.P. & Loewenstein, W.R. (1993) Gap-junction protein gene suppresses tumorigenicity. *Carcinogenesis*, **14**, 1073-1075.

Ruoslathi, E. and Piersbacher, M.D. (1987) New perspectives in cell adhesion: RGD and integrins. *Science*, **238**, 491-497.

Rup, D.M., Veenstra, R.D., Wang, H.Z., Brink, P.R. & Beyer, E.C. (1993) Chick connexin-56, a novel lens gap junction protein. Molecular cloning and functional expression. *Journal of Biological Chemistry*, **268**, 706-712.

Rutishauser, U. (1986) Differential cell adhesion through spacial and temporal variations of N-CAM. *Trends in Neuroscience*, **9**, 374-378.

Rutishauser, U., Acheson, A., Hall, A., Mann, D.M. and Sunshine, J. (1988) The neural cell adhesion molecule (NCAM) as a regulator of cell-cell interactions. *Science*, **240**, 53-57.

Ryerse, J.S. (1989) Isolation and characterization of gap junctions from *Drosophila melanogaster*. *Cell & Tissue Research*, **256**, 7-16.

Saez, J.C., Connor, J.A., Spray, D.C. & Bennett, M.V.L. (1989) Hepatocyte gap junctions are permeable to the second messenger, inositol 1,4,5-trisphosphate, and to calcium ions. *Proceedings National Academy of Sciences (USA)*, **86**, 2708-2712.

Saez, J.C., Nairn, A.C., Czernik, A.J., Spray, D.C., Hertzberg, E.L., Greengard, P. & Bennett, M.V. (1990) Phosphorylation of connexin 32, a hepatocyte gap-junction protein, by cAMP-dependent protein kinase, protein kinase C and Ca2+/calmodulin-dependent protein kinase II. *European Journal of Biochemistry*, **192**, 263-273.

Saffell, J.L., Walsh, F.S. & Doherty, P. (1992) Direct activation of second messenger pathways mimics cell adhesion molecule-dependent neurite outgrowth. *Journal of Cell Biology*, **118**, 663-670.

Safranyos, R.G.A. & Caveny, S. (1985) Rates of diffusion of fluorescent molecules via cell-to-cell membrane channels in developing tissue. *Journal of Cell Biology*, **100**, 736-747.

Salomon, D., Saurat, J-H. & Meda, P. (1988) Cell-to-cell communication within intact human skin. *Journal of Clinical Inestigation*, **82**, 248-254.

Sanford, K.K., Earle, W.R. & Likely, G.D. (1948) The growth *in vitro* of single isolated tissue cells. *Journal of the National Cancer Institute*, **9**, 229.

Santoni, M.J., Bartheis, D., Vopper, G., Boned, A., Goridis, C. & Willie, W. (1989). Differential exon usage involving an unusual splicing mechanism generates at least eight types of NCAM cDNA in mouse brain. *EMBO Journal*, **8**, 385-392.

Sas, D.F., Sas, M.J., Johnson, K.R., Menko, A.S. & Johnson, R.G. (1985) Junctions between lens fiber cells are labelled with a monoclonal antibody shown to be specific for MP26. *Journal of Cell Biology*, **100**, 216-225.

Schipper, J.H., Frixen, U.H., Behrens, J., Unger, A., Jahnke, K. & Birchmeier, W. (1991) E-cadherin expression in squamous cell carcinoma of head and neck: inverse correlation with tumor dedifferentiation and lymph node metastasis. *Cancer Research*, **51**, 6328-6337.

Schuetze, S.M. & Goodenough, D.A. (1982) Dye transfer betwen cells of the embryonic chick lens become sensitive to CO_2 treatment with development. *Journal of Cell Biology*, **92**, 694-705.

Sefton, B., Hunter, T., Ball, E.H. & Singer, S.J. (1981) Vinculin: a cytoskeletal target of the transforming protein of Rous sarcoma virus. *Cell*, **24**, 165-174.

Serras, F. & van den Biggelaar, J.A.M. (1987) Is a mosaic embryo also a mosaic of communication compartments? *Developmental Biology*, **120**, 132-138.

Serras, F., Buultjens, T.E. & Finbow, M.E. (1988) Inhibition of dye coupling in *Patella* (Mollusca) embryos by microinjection of antiserum against *Nephrops* (Arthropoda) gap junctions. *Experimental Cell Research*, **179**, 282-288.

Serras, F., Damen, P., Dictus, W.J.A.G., Notenboom, R.G.E. & van den Biggelaar, J.A.M. (1989) Communication compartments in the ectoderm of *Patella vulgata*. *Roux's Archive of Developmental Biology*, **198**, 191-200.

Shekhar, P.V.M., Aslakson, C.J. & Miller, F.R. (1993) Molecular events in metastatic progression. *Cancer Biology*, **4**, 193-204.

Sheridan, J.D. (1976) Cell coupling and communication during embryogenesis. In *The Cell Surface in Animal Embryogenesis*. ed. Poste, G. and Nicholson, G.L. pp 409-447. Elsevier, New York.

Sheridan, J.D., Finbow, M.E. & Pitts, J.D. (1979) Metabolic interactions between animal cells through permeable intercellular junctions. *Experimental Cell Research*, **123**, 111-117.

Shimoyama, Y., Hirohashi, S., Hirano, S., Noguchi, M., Shimosato, Y., Takeichi, M. & Abe, O. (1989) Cadherin cell-adhesion molecules in human epithelial tissues and carcinomas. *Cancer Research*, **49**, 2128-2133.

Shirayoshi, Y., Nose, A., Iwasaki, K. & Takeichi, M. (1986) N-linked oligosaccharides are not involved in the function of a cell-cell binding glycoprotein E-cadherin. *Cell Structure and Function*, **11**, 245-252.

Sigh, C-K., Wagner, R., Feinstein, S., Kanik-Ennulat, C and Neff, N. (1988) A dominant trifluoroperozine resistant gene from *Sacchromyces cerevisiae* has homology with F_0F_1 ATP synthase and confers calcium-sensitive growth. *Molecular & Cellular Biology*, **8**, 3094-3103.

Singh, I., Doms, R.W., Wagner, K.R., Helenius, A. (1990) Intracellular transport of soluble membrane-bound glycoproteins: folding, assembly and secretion of anchor free influenza hammagglutinin. *EMBO Journal*, **9**, 631-639.

Sikerwar, S.S., Downing, K.H. & Glaeser, R.M. (1991) Three-dimensional structure of an invertebrate intercellular communicating junction. *Journal of Structural Biology*, **106**, 255-263.

Sikerwar, S.S. & Unwin, P.N.T. (1988) Three dimensional structure of gap junctions in fragmented plasma membranes from rat liver. *Journal of Biophysics*, **54**, 113-119.

Sibley, D.R., Benovic, J.L., Caron, M.G. and Lefkowitz, R.J. 1987 Regulation of transmembrane signalling by receptor phosphorylation. *Cell*, **48**, 913-922.

Simionescu, M., Simionescu, N. & Palade, G.E. (1975) Segmental differentiation of cell junctions in the vascular endothelium. *Journal of Cell Biology*, **67**, 863-885.

Simpson, I., Rose, B. & Loewenstein, W.R. (1977) Size limit of molecules permeating the junctional membrane channel. *Science*, **195**, 294-296.

Slaga, T. (1983) Cellular and molecular mechanisms of tumour promotion. *Cancer Surveys*, **2**, 596-612.

Sonnenberg, A., Linders, C.J.T., Modderman, P.W., Damsky, C.H., Aumailley, M. and Timpi, R. (1990) Integrin recognition of different cell-binding fragments of laminin (P1, E3, E8) and evidence that $\alpha_3\beta_1$ but not $\alpha_6\beta_4$ functions as a major receptor for fragment E8. *Journal of Cell Biology*, **110**, 2145-2155.

Sosinsky, G.E., Baker, T.S., Caspar, D.L. & Goodenough, D.A. (1990) Correlation analysis of gap junction lattice images. *Biophysical Journal*, **58**, 1213-1226.

Spray, D.C., Harris, A.L. & Bennett M.V.L. (1981) Gap junctional conductance is a simple and sensitive function of intracellular pH. *Science*, **211**, 712-715.

Spray, D.C., White, R.L., Mazet, F. & Bennett M.L.V. (1985) Regulation of gap junctional conductance. *American Journal of Physiology*, **17**, H753-H764.

Springer, T.A. (1990) Adhesion receptors of the immune system. *Nature*, **346**, 425-434.

Stoker, M.P.G., Shearer, M. & O'Neill (1966) Growth inhibition of polyoma-transformed cells by contact with static normal fibroblasts. *Journal of Cell Science*, **1**, 297-310.

Stoker, M.P.G. (1967) Transfer of growth inhibition between normal and virus transformed cells: autoradiographic studies using marked cells. *Journal of Cell Science*, **2**, 293-304.

Stutenkemper, R., Geisse, S., Schwarz, H.J., Look, J., Traub, O., Nicholson, B.J. & Willecke, K. (1992) The hepatocyte-specific phenotype of murine liver cells correlates with high expression of connexin32 and connexin26 but very low expression of connexin43. *Experimental Cell Research*, **201**, 43-54.

Subak-Sharpe, H., Burk, R. & Pitts, J.D. (1966) Metabolic cooperation by cell-cell transfer between genetically different mammalian cells in tissue culture. *Heredity*, **21**, 342.

Subak-Sharpe, H., Burk, R. & Pitts, J.D. (1969) Metabolic cooperation between biochemically marked mammalian cells in tissue culture. *Journal of Cell Science*, **4**, 353-367.

Suzuki, S., Sano, K. and Tanihara, H. (1991) Diversity of the cadherin family: evidence for eight new cadherins in nervous tissue. *Cell Regulation*, **2**, 261-270.

Swenson, K.I., Jordan, J.R., Beyer, E.C. & Paul, D.L. (1989) Formation of gap junctions by expression of connexins in Xenopus oocyte pairs. *Cell*, **57**, 145-155.

Swenson, K.I., Piwnica-Worms, H., McNamee, H. & Paul, D.L. (1990) Tyrosine phosphorylation of the gap junction protein connexin43 is required for the pp60v-src-induced inhibition of communication. *Cell Regulation*, **1**, 989-1002.

Takeichi, M. (1977) Functional correlation between cell adhesive properties and some cell surface proteins. Journal of *Cell Biology*, **75**, 464-474.

Takeichi, M. (1988) The cadherins: cell-cell adhesion molecules controlling animal morphogenesi. *Development*, **102**, 639-655.

Takeichi, M. (1990) Cadherins: A molecular family important in selective cell-cell adhesion. *Annual Review of Biochemistry*, **59**, 237-252.

Takeichi, M. (1991) Cadherin cell adhesion receptors as a morphogenic regulator. *Science*, **251**, 1451-1455.

Taylor, S.S., Buechler, J.A. & Yonemoto, W. (1990) cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. *Annual Review of Biochemistry*, **59**, 971-1005.

Teranishi, T., Negishi, K. and Kato, S. (1983) Dopamine modulates S-potential amplitude and dye-coupling between external horizontal cells in carp retina. *Nature*, **301**, 243-246.

Thaller, C. & Eichele, G. (1987) Identification and spacial distribution of retinoids in the developing chick limb bud. *Nature*, **327**, 625-628.

Tibbitts, T.T., Caspar, D.L., Phillips, W.C. & Goodenough, D.A. (1990) Diffraction diagnosis of protein folding in gap junction connexons. *Biophysical Journal*, **57**, 1025-1036.

Tickle, C., Alberts, B., Wolpert, L. and Lee, J. (1982) Local application of retinoic acid to the limb bud mimics the action of the polarising region, *Nature*, **296**, 564-566.

Traub, O., Look, J., Paul, D. & Willecke, K. (1987) Cyclic adensonine monophosphate stimulates biosynthesis and phosphorylation of the 26kDa gap junction protein in cultured hepatocytes. *European Journal of Cell Biology*, **43**, 48-54.

Traub, O., Look, J., Dermietzel, R., Brummer, F., Hulser, D. & Willecke, K. (1989) Comparative characterization of the 21-kD and 26-kD gap junction proteins in murine liver and cultured hepatocytes. *Journal of Cell Biology*, **108**, 1039-1051.

Troyanovsky, S.M., Eshkind, L.G., Troyanovsky, R.B., Leube, R.E. & Franke, W.W. (1993) Contributions of cytoplasmic domains of desmosomal cadherins to desmosome assembly and intermediate filament anchorage. *Cell*, **72**, 561-574.

Tsuda, H., Zhang, W., Shimosato, Y., Yokota, J., Terada, M., Sugimura, T., Miyamura, T. & Hirohashi, S. (1990) *Proceedings National Academy of Sciences (USA)*, **87**, 6791-6794.

Tsukita, S., Nagafuchi, A. & Yonemura, S. (1992) Molecular linkage between cadherins and actin filaments in cell-cell adherens junctions. *Current Opinion in Cell Biology*, **4**, 834-839.

Turin, L. and Warner, A.E. (1978) Carbon dioxide reversibly abolishes ionic communication between cells of early amphibian embryos. *Nature*, **270**, 56-57.

Umbas, R., Schalken, J.A., Aalders, T.W., Carter, B.S., Karthaus, H.F., Schaafsma, H.E., Debruyne, F.M. & Isaacs, W.B. (1992) Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer. *Cancer Research*, **52**, 5104-5109.

Umemoto, N., Yoshihisa, T., Hirata, R. & Anraku, Y. (1990) Roles of the VAM3 gene product, subunit of the vacuolar membrane H^+-ATPase on vacuolar acidification and protein transport. *Journal of Biological Chemistry*, **265**, 18447-18453.

Unwin, N. (1993) Nicotinic acetylcholine receptor at 9 A resolution. *Journal of Molecular Biology*, **229**, 1101-1124.

Unwin, P.N.T. & Zampighi, G. (1980) Structure of the junction between communicating cells. *Nature*, **283**, 545-549.

Van Aelst, L., Hohmann, S., Zimmermann, F.K., Jans, A.W. & Thevelein, J.M. (1991) A yeast homologue of the bovine lens fibre MIP gene family complements the growth defect of a Saccharomyces cerevisiae mutant on fermentable sugars but not its defect in glucose- induced RAS-mediated cAMP signalling. *EMBO Journal*, **10**, 2095-2104.

Veenstra, R.D., Wang, H.Z., Westphale, E.M. & Beyer, E.C. (1992) Multiple connexins confer distinct regulatory and conductance properties of gap junctions in developing heart. *Circulation Research*, **71**, 1277-1283.

Verselis, V., White, R.L., Spray, D.C. & Bennett, M.V.L. (1986) Gap junction conductance and permeability are linearly related. *Science*, **234**, 461-464.

Vestal, D.J. & Ranscht, B. (1992) Glycosyl phosphatidylinositol-anchored T-cadherin mediates calcium-dependent, homophilic cell adhesion. *Journal of Cell Biology*, **119**, 451-461.

Vleminckx, K., Vakaet, L., Jr., Mareel, M., Fiers, W. & Van Roy, F. (1991) Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell*, **66**, 107-119.

Volberg, T., Geiger, B., Dror, R. & Zick, Y. (1991) Modulation of intercellular adherens-type junctions and tyrosine phosphorylation of their components in RSV-transformed cultured chick lens cells. *Cell Regulation*, **2**, 105-120.

Volk, T. & Geiger, B. (1986) A-CAM: a135-kD receptor of intercellular adherans junctions. II. Antibody-mediated modulation of junction formation. *Journal of Cell Biology*, **103**, 1451-1464.

von Andrian, U.H., Chambers, J.D., McEvoy, L.M., Bargatze, R.F., Arfors, K-E. and Butcher, E.C. (1991) Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and leukocyte β_2 integrins *in vivo*. *Proceedings National Academy of Sciences (USA)*, **88**, 7538-7542.

Warner, A.E. & Lawrence, P.A. (1982) Permeability of gap junctions at the segmental border in insect epidermis. *Cell*, **28**, 243-252.

Warner, A.E., Guthrie, S.C. & Gilula, N.B. (1984) Antibodies to gap junctional protein selectively disrupt junctional communication on the early amphibian embryo. *Nature*, **311**, 127-131.

Warner, A.E. (1985) Antibodies to gap junction protein: probes for studying cell interactions during development. In *Gap Junctions*, ed. Bennett, M.L.V. and Spray, D.C. pp 275-288. Cold Spring Harbour Laboratory.

Warren, S.L. & Nelson, W.J. (1987) Nonmitogenic morphoregulatory action of $pp60^{v\text{-}src}$ on multicellular epithelial structures. *Molecular & Cellular Biology*, **7**, 1326-1337.

Watson, S.R., Fennie, C. and Lasky, L. (1991) Neutrophil influx into an inflammatory site inhibited by a soluble homing receptor-IgG chimaera. *Nature*, **349**, 164-167.

Weber, K. & Osborn, M. (1975) Proteins and sodium dodecyl sulfate: molecular weight determination on polyacrylamide gels and related procedures. In *The Proteins*, pp 180-221. Volume I, 3rd Edition, Neurath and Hill (editors).

Weider, K.M., Behrens, J., Vandekerckhove, J. & Birchmeier, W. (1990) Scatter factor: Molecular characteristics and effect on the invasiveness of epithelial cells. *Journal of Cell Biology*, **111**, 2097-2108.

Weir, M.P. & Lo, C.W. (1982) Gap junctional communication compartments in the *Drosophila* wing disk. *Proceedings National Academy of Sciences (USA)*, **79**, 3232-3235.

Werner, R., Miller, T., Azarnia, R. & Dahl, G. (1985) Translation and functional expression of cell-cell channel mRNA in Xenopus oocytes. *Journal of Membrane Biology*, **87**,253-268.

Werner, R., Levine, E., Rabadan-Diehl, C. & Dahl, G. (1989) Formation of hybrid cell-cell channels. *Proceedings National Academy of Sciences (USA)*, **86**, 5380-5384.

White, T.W., Bruzzone, R., Goodenough, D.A. & Paul, D.L. (1992) Mouse Cx50, a functional member of the connexin family of gap junction proteins, is the lens fiber protein MP70. *Molecular Biology of the Cell*, **3**, 711-720.

Wiener, E.C &Loewnestein, W.R. (1983) Correction of cell-cell communication defect by introduction of a protein kinase into mutant cells. *Nature*, **305**, 433-435.

Wilgenbus, K.K., Kirkpatrick, C.J., Knuechel, R., Willecke, K. & Traub, O. (1992) Expression of Cx26, Cx32 and Cx43 gap junction proteins in normal and neoplastic human tissues. *International Journal of Cancer*, **51**, 522-529.

Williams, A.F. & Barclay, A.N. (1988) The immunoglobulin superfamily-domains for cell surface recognition. *Annual Review of Immunology*, **6**, 381-405.

Willecke, K., Traub, O., Look, J., Stutenkemper, R. & Dermietzel, R. (1988) Different protein components contribute to the structure and function of hepatic gap junctions. In *Gap Junctions*, ed. Hertzberg, E.L. and Johnson, R.G. pp 41-52. Alan Liss Inc., New York.

Willecke, K., Jungbluth, S., Dahl, E., Hennemann, H., Heynkes, R. & Grzeschik, K.H. (1990) Six genes of the human connexin gene family coding for gap junctional proteins are assigned to four different human chromosomes. *European Journal of Cell Biology*, **53**, 275-280.

Willecke, K., Heynkes, R., Dahl, E., Stutenkemper, R., Hennemann, H., Jungbluth, S., Suchyna, T. & Nicholson, B.J. (1991) Mouse connexin37: cloning and functional expression of a gap junction gene highly expressed in lung. *Journal of Cell Biology*, **114**, 1049-1057.

Wilson, J.M., Johnson, D.E., Jefferson, D.M. & Mulligan, R.C. (1988) Correction of the genetic defect in hepatocytes fron Watanabe heritable hyperlipidemic rabbit. *Proceedings National Academy of Sciences (USA)*, **85**, 4421-4425.

Wolpert, L. (1978) Gap junctions: channels of commmunication in development. In *Intercellular junctions and synapses*, ed. Feldman, J., Gilula, N.B. and Pitts, J.D. pp 81-96. Chapman and Hall, London.

Yahalom, A., Warmbrodt, R.D., Laird, D.W., Traub, O., Revel, J.P., Willecke, K. & Epel, B.L. (1991) Maize mesocotyl plasmodesmata proteins cross-react with connexin gap junction protein antibodies. *Plant Cell*, **3**, 407-417.

Yamasaki, H. (1990) Gap junctional intercellular communication and carcinogenesis. *Carcinogenesis*, **11**, 1051-1058.

Yamasaki, H. & Katoh, H. (1988) Further evidence for the involvement of gap-junctional intercellular communication in the induction and maintenance of transformed foci in BALB/c 3T3 cells. *Cancer Research*, **48**, 3490-3495.

Yancey, S.B., Easter, D. and Revel, J-P. (1979) Cytological changes in gap junctions during liver regeneration. Journal of *Ultrastructure Research*, **67**, 229-242.

Yancey, S.B., John, S.A., Lai, R., Austin, B.J. & Revel, J-P. (1989) The 43-kD polypeptide of heart gap junctions: Immunilocalization(I), topology(II), and functional domains (III). *Journal of Cell Biology*, **108**, 2241-2254.

Yarden, Y., & Ullrich, A. (1988) Growth factor receptor tyrosine kinases. *Annual Review of Biochemistry*, **75**, 443-478.

Yeager, M. & Gilula, N.B. (1992) Membrane topology and quaternary structure of cardiac gap junction ion channels. *Journal of Molecular Biology*, **223**, 929-948.

Yotti, L.P., Chang, C-C. & Trosko, J.E. (1979) Elimenation of metabolic cooperation in Chinese hamster cells by a tumor promoter. *Science*, **206**, 1089-1091.

Young, J.D-E., Cohn, Z.A & Gilula, N.B. (1987) Functional assembly of gap junctional conductance in lipid bilayers: Demonstration that the major 27kd protein forms the functional channel. *Cell*, **48**, 733-743.

Zachary, I. & Rozengurt, E. (1992) Focal adhesion kinase ($p125^{FAK}$): a point of convergence in the action of neuropeptides, integrins, and oncogenes. *Cell*, **71**, 891-894.

Zampighi, G.A., Bosch, E., & Ramon, F. (1988) Some observations about the cellular mechanisms involved in gap junction regulation. In *Gap Junctions*, ed. Hertzberg, E.L. and Johnson, R.G. pp 151-164. Alan Liss Inc., New York.

Zampighi, G.A., Hall, J.E., Ehring, G.R. & Simon, S.A. (1989) The structural organization and protein composition of lens fiber junctions. *Journal of Cell Biology*, **108**, 2255-2275.

Zelent, A., Krust, A., Petkovich, M. & Chambon, P. (1989) Cloning of murine α and β retinoic acid receptors and a novel gamma receptor predominantly expressed in skin. *Nature*, **339**, 714-721.

Zervos, A.S., Hope, J. & Evans, W.S. (1985) Preparation of a gap junction fraction from uteri of pregnant rats: the 28kD polypeptide of uterus, liver, and heart gap junctions are homologous. *Journal of Cell Biology*, **101**, 1363-1370.

Zhang, J.T. & Nicholson, B.J. (1989) Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. *Journal of Cell Biology*, **109**, 3391-3401.

Zhu, D., Caveny, S., Kidder, G.M. and Naus, C.C.G. (1991) Transfection of C6 glioma cells with connexin 43 cDNA: Analysis of expression, intercellular coupling, and cell proliferation. *Proceedings National Academy of Sciences (USA)*, **88**, 1883-1887.

Zhu, D., Caveny, S., Kidder, G.M. and Naus, C.C.G. (1992) Growth retardation in glioma cells cocultured with cells overexpressing a gap junction protein. *Proceedings National Academy of Sciences (USA)*, **89**, 10218-10221.

Zimmer, D.B., Green, C.R., Evans, W.H. & Giula, N.B. (1987) Topological analysis of the major protein in isolated intact rat liver gap junctions and gapjunction derived single membrane structures. *Journal of Biological Chemistry*, **262**, 7751.

Zimmermann, A.L. & Rose, B. (1985) Permeability properties of cell-to-cell channels: kinetics of fluorescent tracer diffusion through a cell junction. *Journal of Membrane Biology*, **84**, 269-283.