PROTEIN PHOSPHORYLATION AND CELL DIVERSIFICATION IN THE MOUSE EARLY EMBRYO

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CONTENTS

PREFACE

The research described in this dissertation was carried out in the Department of Anatomy, Cambridge University, under the supervision of Dr. M. H. Johnson and by permission of Prof. Kuypers. In the preparation of cell pairs, described in section 4.4, I was assisted by Ms. S. J. Pickering. The remainder of this dissertation describes my own original work and has not been submitted previously in any form to this or any other University.

SUMMARY

This dissertation reports the results of studies into the control of compaction of the mouse preimplantation embryo. Compaction is a post-translationally controlled re-arrangement of cell contacts and the cytoskeleton that occurs at the 8-cell stage of development. This re-arrangement seems to be necessary for the differentiation of the two cell types present in the blastocyst. Protein phosphorylation is a post-translational modification believed to be important in the modulation of cell shape and cytoskeletal assembly. It is therefore feasible to propose a role for protein phosphorylation in compaction.

Two types of approach have been used to investigate the possible role of protein phosphorylation in compaction. Firstly, embryos have been treated with two drugs, 6-dimethylaminopurine (DMAP) and a phorbol ester (phorbol myristate acetate, PMA), each of which seems to affect both protein phosphorylation and compaction. DMAP is an adenine analogue and putative inhibitor of protein phosphorylation that was found to perturb the cell cycle of mouse embryos. In addition, DMAP caused rapid cellular flattening of 4-cell and 8-cell embryos. However, this flattening was not accompanied by cell polarisation and did not seem to be mediated by the cell adhesion molecule uvomorulin. It is therefore unlikely to be related directly to the flattening that occurs at compaction. Phorbol esters, such as PMA, are potent stimulators of the membrane-associated, Ca^{2+}- and phospholipid-dependent protein kinase, protein kinase C (PKC). Incubation in medium containing PMA had some effects on the cytoskeleton of oocytes and early embryos but caused severe, widespread disassembly of the cytoskeleton and reversal of flattening in 8-cell embryos. These effects of PMA, seen specifically at the 8-cell stage, may be related to the spatially restricted disassembly of the cytoskeleton that occurs naturally during compaction at the 8-cell stage. This interpretation provides indirect evidence for a possible role for PKC activity, and hence protein phosphorylation, in the process of compaction.

The relationship between protein phosphorylation and the events occurring at the 8-cell stage has been examined more directly by labelling 4-cell and 8-cell

embryos with [^{32}P]orthophosphate and examining the phosphoproteins obtained by one- and two-dimensional gel electrophoresis. By synchronising groups of embryos precisely to successive cleavage divisions prior to labelling, changes in phosphoprotein profile associated with passage through the 4-cell and 8-cell stages have been described. While many of the ^{32}P-labelled phosphoproteins detectable after electrophoresis in one or two dimensions are similar at each stage examined, there are some changes associated specifically with passage through the 8-cell stage which may be related to the cell flattening and polarisation occurring at this time. In addition, the profile of 8-cell embryos differed according to the duration of pulse-labelling with [^{32}P]orthophosphate or the inclusion of "chase" periods.

Finally, several treatments that affect features of compaction, including exposure to DMAP and PMA, have been used to assess the link between the observed changes in phosphoprotein profile and the events of compaction. Embryos were also incubated in protein synthesis inhibitors, which cause premature cell flattening in 4-cell embryos and in Ca^{2+}-free medium, which prevents intercellular flattening and delays polarisation of 8-cell blastomeres. In each case, the relative labelling intensity of some of the phosphoproteins characteristic of untreated 8-cell embryos was altered. The behaviour of these phosphoproteins suggests that they may be important in the mechanism by which cells flatten and polarise or in the maintenance of flattened, polarised, cells; they now provide a focus for future study.

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Abbreviations used:

BSA	bovine serum albumin
CCD	cytochalsin D
Con A	concanavalin A
dansyl PMA	11-((-5-dimethylaminonapthalene-1-sulphonyl) amino)undecanoylphorbol acetate
DMAP	6-dimethylaminopurine
DMSO	dimethylsulphoxide
EDTA	ethylenediaminetetra-acetic acid
FITC	fluoresceine isothiocyanate
H-7	1-(5-isoquinolinylsulfonyl)-2-methylpiperazine
hCG	human chorionic gonadotrophin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
ICM	inner cell mass
IEF	isoelectric focussing
M2	medium 2
M16	medium 16
mPMA	phorbol 12-myristate 13-acetate 4-0-methyl ether
M_r	relative molecular mass
NP-40	Nonidet-P40
OAG	oleoyl acetyl glycerol
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
4αPDD	4α-phorbol didecanoate
4βPDD	4β-phorbol didecanoate
PHA	phytohaemagglutinin
pI'	apparent isoelectric point
PIP_2	phosphatidylinositol bisphosphate
PIPES	piperazine-N, N'-bis[2-ethane-sulphonic acid]
PKC	protein kinase C

PMA	phorbol 12-myristate 13-acetate
PMS	pregnant mare's serum gonadotrophin
PVP	polyvinylpyrrolidone
S. D.	standard deviation
SDS	sodium dodecyl sulphate
TCA	trichloracetic acid
TE	trophectoderm
Tris	tris-(hydroxymethyl)-methyl amine

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PUBLICATIONS

The publications arising from work described in this thesis are listed below:

Bloom, T. L. (1989)

The effects of phorbol esters on mouse blastomeres: a role for protein kinase C in compaction ? *Development* **106** 159-171.

Bloom, T. L. & J. McConnell (1989)

Changes in protein phosphorylation accompanying compaction of the mouse embryo. *Submitted to Development.*

Bloom, T. L. (1989)

Experimental manipulation of compaction of the mouse embryo alters patterns of protein phosphorylation. *Submitted to Developmental Biology.*

CHAPTER ONE

INTRODUCTION

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This thesis examines the process of compaction of the mouse embryo. Compaction is a post-translationally regulated rearrangement of cell-cell contacts and intracellular organisation that occurs during the fourth cell cycle after fertilisation. This rearrangement seems to be a prerequisite for the subsequent differentiation of cells in the early embryo. The spatial and temporal controls of compaction are largely unknown. In this thesis, evidence of a close link between protein phosphorylation and compaction will be presented. The process of compaction can be studied in the context of strategies used by other organisms to produce differences between the cells of an early embryo. This Introduction will, therefore, briefly consider the general mechanisms of pattern formation and temporal control of early development, before moving on to a more detailed examination of the process of compaction of the mouse embryo.

1.1 The establishment of cellular diversity in early development

The ancestry of the hundreds of differentiated cell types of an adult organism can be traced to a single parent cell, the fertilised egg. To achieve this enormous diversity among the progeny cells of the egg, spatial heterogeneity must be generated initially either within the egg itself, or within and among early embryonic blastomeres. The basic mechanisms involved in achieving cellular diversity in most multicellular organisms can be divided into three groups: the localisation of materials asymmetrically within a single cell, the division of that cell to partition the localised material between daughter cells and cell-cell interactions between the resulting different types of progeny cells.

In most invertebrate and lower vertebrate species, early development is a race against time. A viable, self-sufficient larva must be produced before the environment or predation depletes embryo numbers. In many of these species, such as the nematode *Caenorhabditis elegans* (Strome & Wood, 1983), molluscs *Dentalium* (Verdonk, 1968) and *Lymnaea* (Raven, 1967), insects *Smittia* (Rau & Kalthoff, 1980) and

Drosophila (Anderson & Nusslein-Volhard, 1984), ascidians (Whittaker, 1973; Jeffrey, 1985) and amphibian *Xenopus* (Nieuwkoop, 1977), the unfertilised egg itself has some asymmetry of organisation that is important for later development. The asymmetry of these eggs seems to arise *via* positional information and localised materials received from maternal tissues during oogenesis. With fertilisation triggering the re-distribution of egg cytoplasm in many species (for example, ascidians, Whittaker, 1973; Jeffrey, 1985; *Xenopus*, Elinson, 1983), the fertilised egg already contains spatially organised "determinants" that pre-dispose cells inheriting particular regions of the egg cytoplasm to express certain genes and achieve particular fates. The pattern of cleavage of the zygote distributes these determinants into the progenitors of different lineages of cells. Later in development, cell-cell interactions are of major importance in achieving the final distribution and variety of cell types of the adult organism (Davidson, 1986).

In mammals, the rate of early development is slower than in most other animals, which may reflect the relative safety of the embryo from predation or environmental change, once implanted in the uterus. In these species, the unfertilised egg does not seem to house asymmetries that predetermine later development. Rather, the cells of the early embryo must generate asymmetry *de novo,* using positional cues generated between cells *via* cell-cell interactions. The generation of asymmetry, which occurs at compaction in the rodent embryo, can be considered to be equivalent to the spatial organisation of determinants in the eggs of other species, but occurring after fertilisation rather than before it (Johnson *et al.,* 1986a).

The generation of spatial asymmetry in the fertilised egg or early embryo is not wholly sufficient for subsequent cell diversification. Spatial information can only be correctly interpreted by eggs or early embryos if it is available at the appropriate time, when the developmental history of a cell has rendered it competent to respond to such information (Gurdon, 1987). A consideration of the mechanisms by which early embryos measure and record the passage of time is therefore an essential adjunct to any study of spatial pattern formation.

1.2 Timing the events of early development

Each of the key events in the early development of an organism, from the initiation of transcription through to morphogenesis, occurs at a predictable time after fertilisation. The approximately fixed duration of early cleavage cycles in each species results in such events occurring at reproducible chronological times as well as during particular cell cycles in "developmental time". There is little evidence that any developmental event is triggered according to the passage of real time. Rather, fundamental changes in spatial organisation or gene expression seem to be ordered in time with reference either to cyclically repeated time-points, such as DNA replication or cell division, or with reference to the unique time-point of fertilisation (Satoh, 1982b). The triggering of cell division cycles by the arrival of the sperm at the egg plasma membrane at fertilisation ensures that these two timing mechanisms are closely linked in early embryos. However, an understanding of the precise nature of these early embryonic timing mechanisms remains elusive; some current hypotheses are reviewed below.

The timing of many significant events in the development of early embryos seems to be dependent on the completion of cycles of DNA replication or cell division. The attainment of a critical nuclear:cytoplasmic ratio may therefore be of importance. After fertilisation, this ratio is altered as the volume of cytoplasm from one egg is divided into successively smaller blastomeres, each containing one diploid nucleus. "Nuclear" timing mechanisms may trigger complex developmental events occurring immediately on attainment of the correct ratio, such as the mid-blastula transition in *Xenopus* embryos. The initiation of transcription and cell motility at this time is apparently triggered by the nuclear:cytoplasmic ratio achieved after 11 cell divisions (Newport & Kirschner, 1982a, b; but see below for further interpretation of these experiments).

Nuclear timing mechanisms also seem to underlie less complex differentiative events that do not occur immediately on attainment of the correct nuclear:cytoplasmic ratio but, rather, require passage through further cycles of DNA replication before manifestation. The expression of genes characteristic of particular differentiated cell types in ascidians (Whittaker, 1973; Satoh & Ikegami, 1981a, b; Satoh, 1982a) and

nematodes (Laufer *et al.*, 1980) seem to be timed by such mechanisms. There is also some evidence that timing in differentiated adult tissues may depend on nuclear replication cycles (for example, glial cells, Temple & Raff, 1986), although cell growth prior to division ensures that the nuclear:cytoplasmic ratio is not altered with each cell cycle in adult tissues.

However, cycles of DNA replication and cell division are not the only means used to time gene expression in early development. Genes are expressed on schedule in *Chaetopterus* embryos when DNA synthesis has been partially inhibited, slowing down cell cycles and delaying the attainment of a particular nuclear:cytoplasmic ratio (Brachet *et al.*, 1981). In addition, timing of the mid-blastula transition of *Xenopus* and of transcriptional activation in *Drosophila* does not seem to be dependent directly on the nuclear:cytoplasmic ratio. These developmental events can be correlated more closely with the slowing and lengthening of the early embryonic cell cycle that occurs when certain, unidentified, maternally-inherited materials are depleted, after a certain number of cell cycles (Edgar *et al.*, 1986; Kimelman *et al.*, 1987). Recently, Edgar and McGhee (1988) have shown that in the nematode gut lineage, a single early burst of DNA replication is required for later gene expression but subsequent cycles of DNA replication or cell division are not required.

Timing mechanisms that involve "nuclear" cycles of DNA replication and division seem to be used in the control of gene expression and cellular differentiation, as described above. Satoh (1982b) has proposed that, by contrast, "cytoplasmic" mechanisms may be responsible for the regulation of pattern formation and morphogenesis. "Cytoplasmic" timing mechanisms include those that are wholly independent of the nucleus, operating even in enucleated cells. One manifestation of a "cytoplasmic" timing mechanism is the cortical contraction wave of activated but enucleated eggs, that occurs in synchrony with the division cycles of fertilised, nucleated eggs in many species, (ascidians: Bell, 1962; sea urchins: Kojima, 1969; Yoneda *et al.*, 1978; newt: Sawai, 1979; *Xenopus*: Hara *et al.*, 1980; mouse, Waksmundska *et al.*, 1984).

This distinction between "nuclear" and "cytoplasmic" timing mechanisms may be useful for some investigations. However, a fundamental cytoplasmic oscillation is

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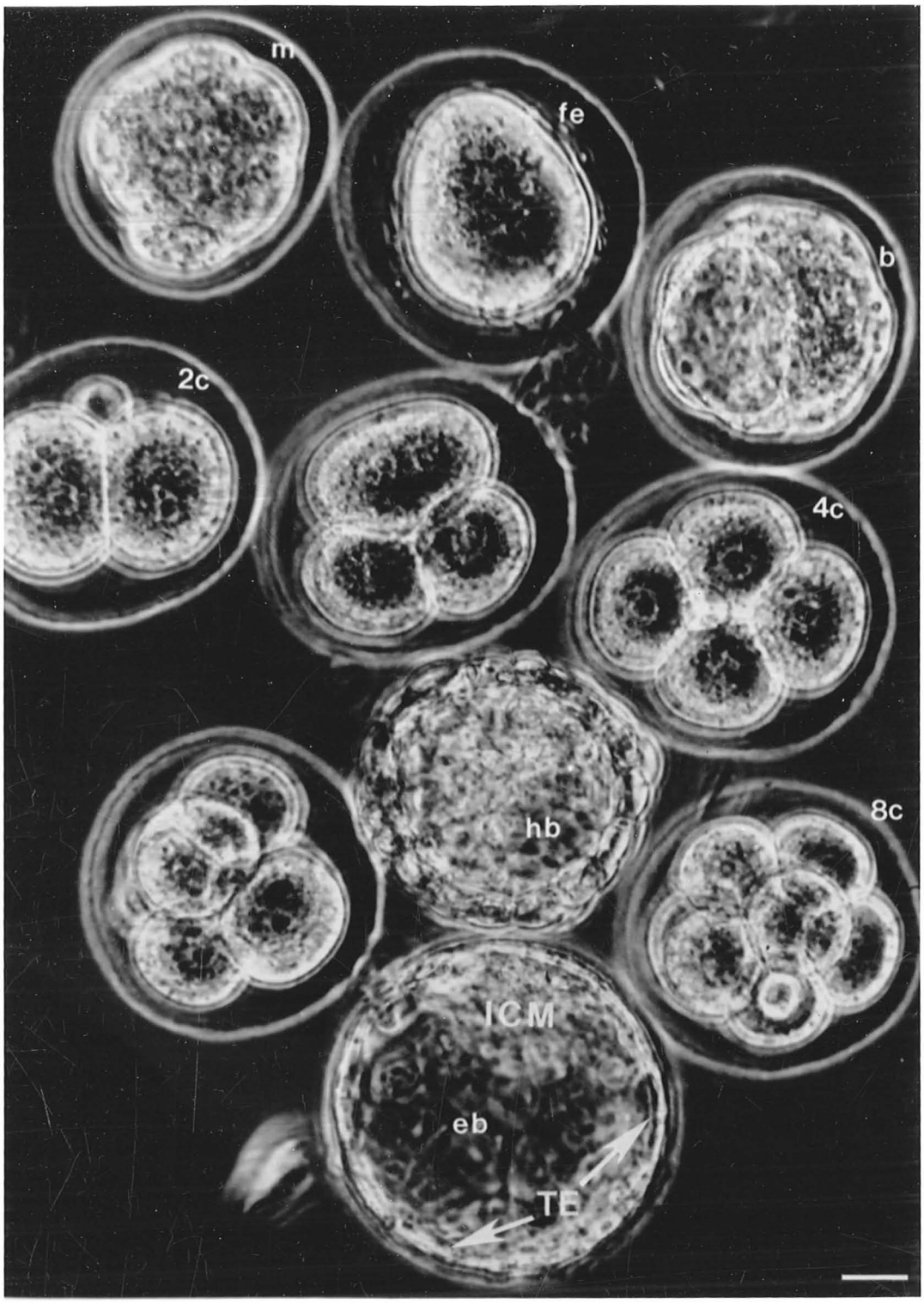

itself likely to control the initiation of DNA replication (Harland & Laskey, 1980) and mitosis (Gerhart *et al.*, 1984; Murray & Kirschner; 1989) in early embryonic cell cycles and so, indirectly, to time all those processes dependent on nuclear cycles. It seems most likely that a "master oscillator" provides the basis of timing for all these events although the nature of such an oscillator remains completely mysterious (Gerhart *et al.*, 1984). Although understanding of the timing mechanisms of early embryos remains poor, it is nevertheless important to consider the temporal background on which the spatial events of early development occur. Whatever the means of linking developmental time with real time, the availability of spatial information at the correct time during early development is essential to its successful utilisation.

1.3 The generation of two cell types in the mouse preimplantation embryo

The preimplantation development of the mouse occurs during the first (approximately) seven cell division cycles that follow fertilisation and results in the generation of a blastocyst, consisting of two clearly differentiated cell types (Fig. 1.1). Until the early 8-cell stage, blastomeres are apparently similar to each other in morphology (Ducibella & Anderson, 1975; Lehtonen, 1980) and developmental potential (Tarkowski & Wroblewska, 1967; Kelly, 1977). Early 8-cell blastomeres, like those of 2-cell and 4-cell embryos, are approximately radially symmetrical (Fig.

Figure 1.1 (facing page)

Phase contrast photomicrograph of preimplantation mouse embryos.

Shown are a newly fertilised egg (fe), 2-cell (2c), 4-cell (4c), early 8-cell (8c), compacted 8- to 16-cell morula (m), early blastocyst (b), expanded blastocyst (eb) and a blastocyst that has hatched from its zona pellucida (hb). The positions of the inner cell mass (ICM) and trophectoderm (TE) are indicated on the expanded blastocyst. This photograph was taken by Dr. P. R. Braude and is reproduced with his kind permission.

1.1). During the 8-cell stage, these rounded blastomeres increase the extent of their apposition and flatten onto each other while cytoskeletal elements and organelles become polarised along a radial axis, generating an asymmetry of organisation (Ducibella & Anderson, 1975; Lehtonen, 1980). This process of cell flattening and polarisation is called compaction.

Cells appear to differentiate on the basis of position after compaction (Mintz, 1965; Garner & McLaren, 1974; Graham & Deussen, 1978; Balakier & Pedersen, 1982). At the 32- to 64-cell stage, the fluid-filled blastocyst consists of an eccentrically located inner cluster of cells, the inner cell mass (ICM), surrounded by an outer, epithelial layer of trophectoderm (TE) cells (Fig. 1.1). ICM cells differ in morphology, gene expression and developmental fate from TE cells (Gardner & Papaioannou, 1975; Van Blerkom *et al.,* 1976; Dewey *et al.,* 1978; Howe & Solter, 1979; Brulet *et al.,* 1980; Handyside, 1981; Johnson & Ziomek, 1982; Randle, 1982; Fleming & Pickering, 1985). ICM cells are rounded and equally adhesive over their entire surface, which tends to keep them enclosed by contact with other cells (Nadjicka & Hillman, 1974; Fleming *et al.,* 1984). They have few microvilli (Ziomek & Johnson, 1981). The developmental fate of the ICM is the production of all tissues of the embryo itself in addition to some extra-embryonic tissues (Gardner, 1983). TE cells are characteristically epithelial, flattened cells with microvillous apical surfaces and a tight junctional seal between adjacent cells (Calarco & Brown, 1969; Calarco & Epstein, 1973; Ducibella *et al.,* 1975; Magnuson *et al.,* 1977; Wiley & Eglitis, 1981; Johnson & Ziomek, 1982; Fleming & Johnson, 1988). They are also more adhesive on their basolateral than their apical surfaces, which tends to maintain their outer position in the blastocyst (Kimber *et al.,* 1982; Surani & Handyside, 1983). The developmental fate of the trophectoderm is the production of extra-embryonic tissues that are responsible for the implantation, protection and nourishment of the developing embryo (Gardner, 1983).

The progenitors of these two populations of cells are already distinguishable in the 16-cell morula. The cells on the outside of the morula are polarised along a radial axis and are flattened (Ziomek & Johnson, 1982; Randle, 1982; Surani & Handyside, 1983). They tend to enclose the non-polarised, radially symmetrical inner cells of the

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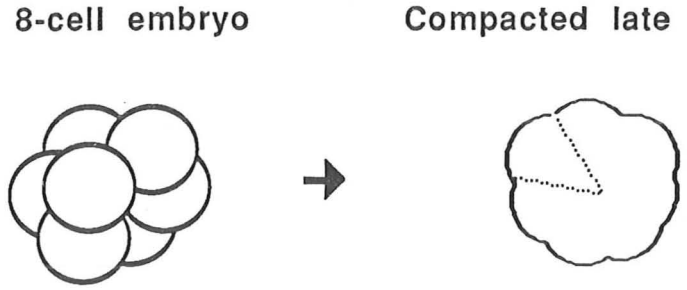

Division of an asymmetrical 8-cell blastomere:

(i) cleavage plane parallel to axis of polarity: equivalent daughter cells result

(ii) cleavage plane perpendicular to axis of polarity: non-equivalent daughter cells result

Figure 1.2
Cleavage of an asymmetrical blastomere can yield two different cell types.
Diagram to illustrate the cleavage of a flattened, polarised 8-cell blastomere (heavy line indicates material localised at apical pole); cleavage can give rise either to equivalent (i) or to non-equivalent (ii) daughter cells.

morula (Burgoyne & Ducibella, 1977; Ziomek & Johnson, 1981; Kimber *et al.,* 1982; Johnson & Ziomek, 1983). Experiments tracing cell lineage have shown that the inner cells of the morula tend to give rise to the ICM and outer cells to the TE (Mintz, 1965; Garner & McLaren, 1974; Graham & Deussen, 1978; Johnson & Ziomek, 1981b, 1982; Balakier & Pedersen, 1982; Fleming *et al.,* 1984; Pedersen *et al.,* 1986; Johnson *et al.,* 1986b; reviewed by Johnson & Maro, 1986), although this tendency can be perturbed (Ziomek & Johnson, 1981; Balakier & Pedersen, 1982; Ziomek *et al.,* 1982; Johnson & Ziomek, 1983).

Asymmetrical, flattened, polarised cells like those on the outside of the 16-cell morula first become apparent at compaction at the 8-cell stage. Some aspects of the intracellular asymmetry generated at compaction seem to be relatively stable during mitosis (Johnson & Ziomek, 1981a; Johnson *et al.,* 1988). Division of a polarised 8-cell blastomere can therefore generate either two equivalent daughter cells, by division parallel to the radial axis of symmetry, or two non-equivalent daughters by division perpendicular to this axis (Fig. 1.2). The process of compaction at which blastomeres first polarise and flatten onto each other and the preservation of cellular asymmetry through mitosis therefore seem central to the subsequent differentiation of two cell types in the blastocyst.

1.4 The process of compaction

At compaction, cells become polarised and flatten onto each other. Polarised distributions of membrane lipid (Pratt, 1985), microvilli (Ducibella & Anderson, 1975; Lehtonen & Badley, 1980; Handyside, 1980; Reeve & Ziomek, 1981), cytoplasmic actin (Johnson & Maro, 1984), myosin (Sobel, 1983, 1984), spectrin (Sobel & Alliegro, 1985), microtubules and their organising centres (Houliston *et al.,* 1987), clathrin vesicles (Maro *et al.,*1985b), endosomes (Reeve, 1981a; Fleming & Pickering, 1985) and specialised junctions (Ducibella & Anderson, 1975; Ducibella *et al.,* 1975; Magnuson *et al.,* 1977) have been described.

During the 2-cell, 4-cell and early 8-cell stages, blastomeres show only very localised flattening adjacent to points of contact between cells (Fig. 1.1). In addition,

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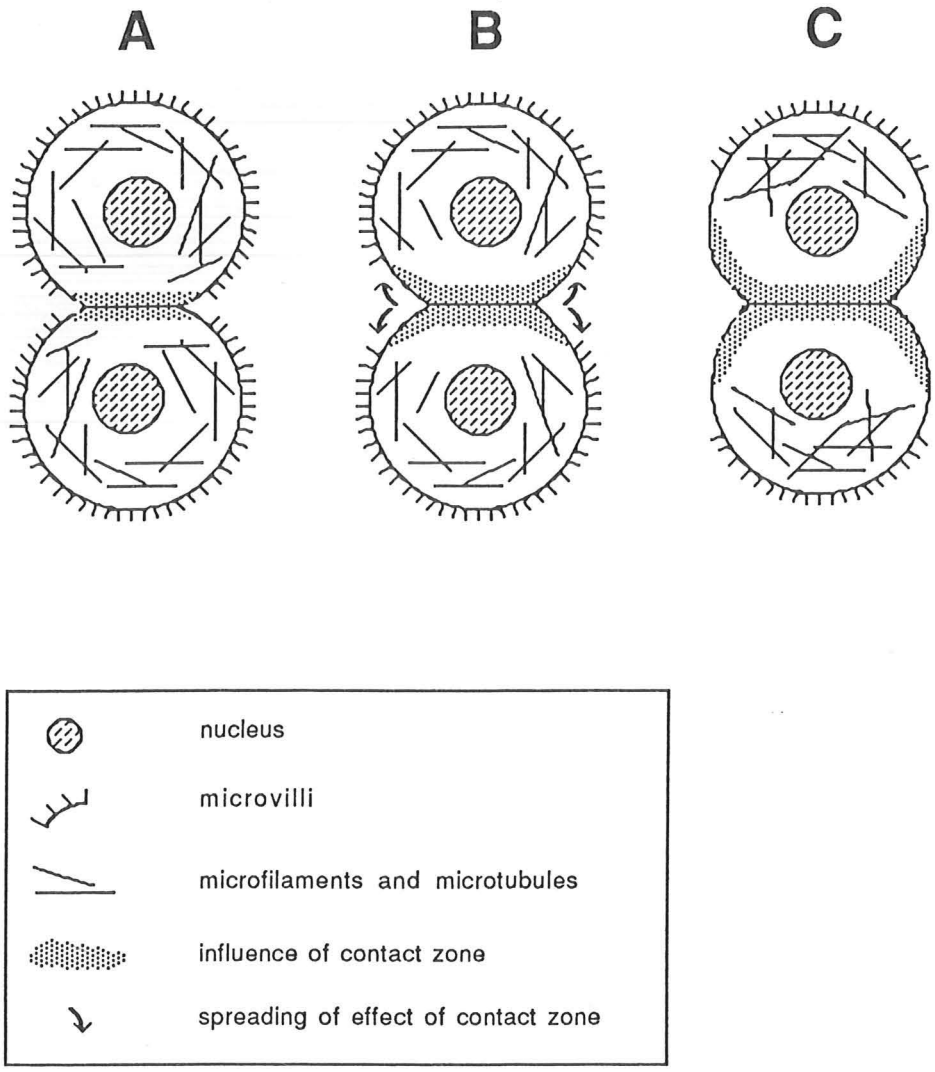


Figure 1.3
Schematic illustration of the progression of compaction.
A. Blastomeres of 2-cell, 4-cell and early 8-cell embryos show localised cell flattening and depletion of cytoskeletal elements and organelles in the region of cell-cell contact.
B. During the 8-cell stage, the influence of the contact zone, causing cell flattening and depletion, spreads beyond the immediate region of contact.
C. A flattened, polarised cell results.

cytoskeletal elements and organelles are depleted from the peripheral cytoplasm in this contact region (Sobel, 1983; Johnson & Maro, 1984; Maro & Pickering, 1984; Maro *et al.*, 1985b; Houliston *et al.*, 1987). This localised cytoplasmic depletion is dependent on continuing cell contact; it is lost if cells are isolated from each other (Johnson & Ziomek, 1981a). During the 8-cell stage, both cell flattening and cytoplasmic depletion spread from focal contact points to involve more of the cell circumference. This process results in flattened cells, each with an apical "pole" of assembled cytoskeletal elements and organelles (Fig. 1.3). Also, during the 8-cell stage, the positions of the cytoplasmic depletion and corresponding pole become independent of continuing cell contact (Johnson & Ziomek, 1981a; Ziomek & Johnson, 1981). The processes of cell flattening and polarisation can be therefore be considered as consisting of three phases which, although naturally continuous and interdependent, will be considered separately here, for clarity.

Firstly, at the appropriate time in development, cells must be triggered to "start" compaction. The timing of this, as of many developmental processes, remains largely mysterious. The second phase is the progression of cell flattening and cytoplasmic depletion, from the restriction to regions adjacent to cell-cell contacts in early embryos, to a more widespread phenomenon in late 8-cell embryos. This phase has proved most amenable to study. The third phase is the stabilisation of the asymmetrical organisation within each blastomere such that it is no longer dependent on cell-cell contact and is, in some way, resistant to re-organisation at mitosis.

(i) Initiation: In order to try and understand the timing of the initiation of compaction, many researchers have used drugs to perturb elements of the first four cell cycles of mouse development. These have shown that no known cell-cycle event is required from the late 2-cell stage onwards to allow intercellular flattening and surface polarisation to occur at the same time as in untreated embryos. Inhibition of cleavage with cytochalasin (Surani *et al.*, 1980; Pratt *et al.*, 1981) or of DNA replication with aphidicolin (Smith & Johnson, 1985) from as early as the late 2-cell stage fails to prevent compaction. Inhibition of transcription from the early 4-cell stage

also has no effect on the timing or incidence of compaction (Kidder & McLachlin, 1985). In fact, compaction appears to be triggered post-translationally and to use proteins that are present in the embryo from at least the 4-cell stage onwards. Inhibition of protein synthesis can even result in cell flattening and some cell surface polarisation occurring prematurely, during the 4-cell stage (Levy *et al.*, 1986).

While the control of the timing of the initiation of cell flattening and polarisation remains hard to understand, some of the spatial aspects are better understood. It seems likely that asymmetry of organisation develops first in a region that has been described as the cytocortex, comprising the cell membrane and associated proteins (Johnson *et al.*, 1986a). Thus, although a polarised organisation is first apparent in the cytoplasm, poles of microvilli can develop at the cell surface under conditions in which cytoplasmic polarisation is not evident and both microfilaments and microtubules are severely disrupted (Ducibella, 1982; Maro & Pickering, 1984; Fleming & Pickering, 1985; Johnson & Maro, 1985; Maro *et al.*, 1985b; Fleming *et al.*, 1986a, b; Houliston *et al.*, 1987, 1989b). The mechanisms that initiate blastomere polarisation must therefore be largely independent of cytoplasmic or cytoskeletal organisation.

The position of the cellular "pole" in an intact embryo or isolated groups of cells is determined by the pattern of cell-cell contacts (Ziomek & Johnson, 1980; Johnson & Ziomek, 1981a). However, although contact seems to be needed for the orientation of cellular asymmetry it is not necessary for the initiation of the process of cell polarisation. Isolated cells do polarise, although, as a population, they do so more slowly than cells in intact embryos (Ziomek & Johnson, 1980). Blastomeres from 8-cell embryos can also flatten and polarise onto 2-cell or 4-cell blastomeres (Johnson & Ziomek, 1981a), and will flatten onto lectin-coated beads (Kimber & Surani, 1982). Specific, co-operative cell-cell interactions are therefore unlikely to be involved in the initiation or progression of compaction. As initiation seems independent of cell cycle progression or cell-cell interactions, it seems likely to be controlled, either directly of indirectly, by a "cytoplasmic" mechanism that may start running at fertilisation (see 1.2).

(ii) Progression: Attempts to define the molecules involved in the progression of compaction have so far resulted in the characterisation of only one with a central role. This is the Ca^{2+}-dependent cell-cell adhesion molecule uvomorulin (Kemler *et al.*, 1977, 1988; Hyafil *et al.*, 1980, 1981; Peyrieras *et al.*, 1983; Vestweber & Kemler, 1984) that has also been identified as E-cadherin (Shirayoshi *et al.*, 1983; Yoshida-Noro *et al.*, 1984), L-CAM (Gallin *et al.*, 1983), gp120/80 (Damsky *et al.*, 1983) and arc-1 (Behrens *et al.*, 1985). Uvomorulin is one of a class of Ca^{2+}-dependent membrane glycoproteins called "cadherins" that are involved in cell-cell adhesion in various tissues (Takeichi, 1987). It seems likely that cadherin molecules in the plasma membrane of one cell interact with homologous molecules in the membrane of an adjacent cell to achieve adhesion. Transfection with E-cadherin (uvomorulin) cDNA is sufficient to confer Ca^{2+}-dependent adhesivity on fibroblasts (Nagafuchi *et al.*, 1987). Uvomorulin also seems to be wholly responsible for the increased apposition of basolateral surfaces of 8-cell blastomeres at compaction (Vestweber & Kemler, 1984 ; Johnson *et al.*, 1986b).

The progression of the zone of cytoplasmic depletion and manifestation of cell polarity also seems to involve uvomorulin to some extent. Thus, neutralisation of cell-cell contacts, either by physical isolation of cells (Ziomek & Johnson, 1980) or by incubation of cells in antibodies to uvomorulin (Shirayoshi *et al.*, 1983; Johnson *et al.*, 1986b) results in cells that polarise but more slowly than controls and with an axis of polarity no longer dependent on the positions of cell contacts. Uvomorulin-mediated cell contact is therefore essential for the orientation of poles opposite points of contact although it is not needed for the initiation phase (Shirayoshi *et al.*, 1983; Johnson *et al.*, 1986b). Similarly, microfilaments are required for cell flattening and seem to be involved in the orientation of cell polarity relative to points of contact (Johnson & Maro, 1984, 1985, Fleming *et al.*, 1985; Houliston *et al.*, 1989b), although they do not seem to be necessary for the initiation of polarisation (see (i), above). Cells do not flatten in drugs that disrupt microfilaments (Pratt *et al.*, 1981); the difference in rigidity between the microfilament-rich cell apex and the microfilament-depleted basolateral domain of polarised 8-cell blastomeres may be a prerequisite of cell flattening. Microtubules seem to be involved in determining the rate

of progression and final extent of flattening and cytoplasmic depletion, but also appear unnecessary for initiation (Ducibella, 1982; Maro & Pickering, 1984; Johnson & Maro, 1985; Houliston *et al.*, 1987, 1989a)

As neither an intact cytoplasmic cytoskeleton nor cellular flattening is required for some manifestation of radial asymmetry at the cell surface, it seems likely that the progression of the unidentified, fundamental feature of compaction involves signalling in the plane of the cell cortex itself. A propagated change in, for example, the phosphorylation of cytoskeletal organising proteins might be sufficient to produce the observed changes in cell organisation (Johnson & Maro, 1986; Fleming & Johnson, 1988). Intracellular second messengers are likely to be involved in the mediation of such a propagated change.

(iii) Stabilisation: Within the first half of the fourth cell cycle, the asymmetrical organisation of 8-cell blastomeres becomes stabilised, such that isolated cells retain their polar phenotype (Johnson & Ziomek, 1981a; Ziomek and Johnson, 1981; Nucitelli & Wiley, 1985). Many polarised features, such as cytoplasmic organelles (Reeve, 1981b; Fleming & Pickering, 1985, Maro *et al.*, 1985b) and cytoskeletal elements (Johnson & Maro, 1984; Houliston *et al.*, 1987) become redistributed at mitosis, presumably due to involvement in the process of mitosis and to ensure allocation to both daughter cells. Nevertheless, some fundamental element of cellular asymmetry seems to be resistant to re-organisation at mitosis. A "memory" of pole position can be revealed even after extreme artificial prolongation of mitosis, in the distribution of microvilli that are resistant to cytochalasin (Johnson *et al.*, 1988). The basis of this "memory" seems to reside in the cytocortex and may therefore be established as compaction progresses. This cytocortical memory of pole position seems to be the feature that determines a polar phenotype for daughter cells inheriting the apical domain of polarised 8-cell blastomeres (Johnson *et al.*, 1988).

The mechanism of stabilisation, like that of initiation, remains largely unknown. One postulated mechanism underlying the transition from apolar to polarised cell is the observed flow of positive ions through the cell from apex to base (Nuccitelli &

Wiley, 1985). However, it seems likely that this current flux reflects a polarised phenotype and is not causal (Wiley & Obasaju, 1988, 1989). It seems more likely that the pole "memory" resides in the asymmetrical organisation or modification of proteins either in the membrane itself or in the peripheral cytoplasm of the apical or basal region of polarised 8-cell blastomeres. The stable memory of pole position could therefore be established by similar mechanisms to those producing the progression phase. The achievement of a stable pole and its retention during mitosis seem to be essential to the determination of two cell types in later development (see 1.3).

In summary, compaction seems likely to be timed by a mechanism that is independent of the cell cycle, possibly initiated at fertilisation. It seems to be triggered by a change in competence in the cells that allows both cell flattening and cytoplasmic depletion to spread from localised contact points to involve more of the cell periphery. Both cell flattening and polarisation involve the cell adhesion molecule uvomorulin to some extent and may also involve signalling *via* second messengers and/or the propagation of a change such as post-translational modification in the cytocortex. The result of compaction is an embryo composed of flattened cells, each with a stably asymmetrical distribution of cytoskeletal elements and organelles (Fig. 1.3). Some elements of this cellular asymmetry are maintained through mitosis and can be inherited by daughter cells. The establishment of intracellular asymmetry at compaction seems to be necessary for the subsequent differentiation of two cell types in the blastocyst.

This thesis reports the results of two types of investigation into the possible role of intracellular second messengers and the post-translational modification of proteins in compaction. Initially, the effects on blastomere adhesion and organisation of two drugs, 6-dimethylaminopurine (DMAP) and a phorbol ester (phorbol myristate acetate, PMA), are described. Each drug affects both an intracellular second messenger signalling pathway and compaction. DMAP is an adenine analogue and putative inhibitor of protein phosphorylation that is found to perturb the cell cycle of mouse embryos. In addition, DMAP causes rapid cellular flattening in 4-cell and 8-cell embryos. However, this flattening is not accompanied by blastomere polarisation and does not seem to be

mediated by uvomorulin. It is therefore unlikely to be related to the flattening that usually occurs at compaction but may resemble the flattening characteristic of 16- to 32-cell morulae. Phorbol esters such as PMA are potent stimulators of the membrane-associated, Ca^{2+}- and phospholipid-dependent protein kinase, protein kinase C (PKC). The effects of PMA, occurring specifically at the 8-cell stage, can be interpreted as extreme but spatially disorganised versions of some of the features of compaction. This interpretation suggests a possible role for protein kinase C activity, and hence protein phosphorylation, in the normal progression of compaction.

In the second part of this thesis, the possible role of protein phosphorylation in compaction is examined more directly. The changes in phosphoprotein profile associated with passage through the 4-cell and 8-cell stages are described, both after brief pulse labelling of embryos with [^{32}P]orthophosphate and following longer incubations and "chase" periods. While many of the radiolabelled polypeptides detectable after electrophoresis in one or two dimensions are similar at each stage examined, there are some changes associated specifically with arrival at, and passage through, the 8-cell stage that may be related to the cell flattening and polarisation occurring at this time.

Finally, several treatments that affect features of compaction, including exposure to DMAP and PMA, have been used to assess the link between the observed changes in phosphoprotein profile and the events of compaction. Embryos have also been incubated in protein synthesis inhibitors, that cause premature intercellular flattening and surface polarisation in 4-cell embryos (Levy *et al.*, 1986) and in Ca^{2+}-free medium that prevents intercellular flattening and delays polarisation of 8-cell blastomeres (Ducibella & Anderson, 1975; Fleming *et al.*, 1989). In each case, the relative intensity of labelling of a group of the phosphoproteins found to be characteristic of untreated 8-cell embryos was altered. The behaviour of these phosphoproteins therefore seems closely linked to compaction. Particular patterns of protein phosphorylation within this group can be correlated with the competence of cells to polarise or flatten (initiation), or with cell flattening or polarisation (progression). The phosphoproteins concerned may therefore reflect, or be involved in, the initiation and progression phases of compaction. In addition, some proteins are phosphorylated at the same time as the stabilisation of the phenotype of flattened, polarised cells.

CHAPTER TWO

MATERIALS AND METHODS

CHAPTER TWO MATERIALS AND METHODS

2.1 Recovery and culture of oocytes and embryos

2.1.1 Superovulation

MF_1 female mice (3-4 weeks; Central Animal Services, Cambridge, U.K.) were superovulated by intraperitoneal injections of 5iu each of pregnant mares' serum gonadotrophin (PMS) and human chorionic gonadotrophin (hCG, Intervet) 48h apart. 50iu/ml stocks of PMS and hCG in 9% NaCl were stored at -20°C for up to 1 month prior to use. To obtain embryos, females were paired individually overnight with HC-CFLP males (Interfauna) and inspected for vaginal plugs the next day as an indication of successful mating.

2.1.2 Recovery of oocytes and embryos

Unfertilised oocytes were recovered from unmated females at $12^1/2$-$13^1/2$h post-hCG by release from dissected oviducts into warmed (37°C) Medium 2 (M2; Fulton & Whittingham, 1978) containing 4mg/ml BSA (M2+BSA). Oocytes were then exposed briefly to 0.1M hyaluronidase (Sigma) to remove cumulus cells before being washed and returned to M2+BSA for at least 1h before any further manipulations. One-cell, 2-cell and 4-cell embryos were flushed from oviducts into warmed M2+BSA and washed before culturing in drops of Medium 16 (M16; Whittingham & Wales, 1969) containing 4mg/ml BSA (M16+BSA) under paraffin oil (Martindale), in Falcon tissue culture dishes, in 5% CO_2 in air. One-cell embryos were recovered at 20h post-hCG, early 2-cell embryos at 40h post-hCG, late 2-cell and early 4-cell embryos at 46-50h post-hCG; 8-cell and 16-cell embryos were derived by overnight culture of 2-cell and 4-cell embryos. All microscopes had heated stages to maintain embryos near 37°C.

2.1.3 Depleted media

2.1.3.1 Ca^{2+}-free M16 or M2

$CaCl_2$ was was omitted and the osmolarity corrected with NaCl; 6mg/ml BSA was added.

2.1.3.2 Phosphate-free M16

KH_2PO_4 was omitted and the osmolarity corrected with NaCl.

2.1.3.3 Protein-free M2

6mg/ml polyvinylpyrollidone (PVP, M_r 44,000; Sigma) replaced BSA.

Aliquots of depleted media containing no BSA or PVP were stored frozen at -20°C for up to 3 months. On thawing, BSA or PVP was added, the pH adjusted and the medium Millipore filtered before use.

2.1.4 Synchronisation of embryos

2.1.4.1 4-cells

Populations of 2-cell embryos were inspected at hourly intervals and any embryos with 3 or 4 cells were selected and cultured for up to 2h. Those that had not completed division to 4-cells in this period were discarded. All the remaining 4-cell embryos were cultured as synchronised groups and the time at which the last blastomere was seen to have cleaved was designated the time of division; times are expressed as hours post-division to 4-cells.

2.1.4.2 8-cells

Populations of 4-cell embryos were inspected at hourly intervals and any embryos with 5-7 blastomeres were selected and cultured for up to 3 hours. Those that did not complete division to 8-cells during that time were discarded. All remaining 8-cell embryos were cultured together as synchronised groups with the time of last blastomere cleavage designating the time of division; times are expressed as hours post-division to 8-cells.

2.2 Manipulation of oocytes and embryos

2.2.1 Removal of the zona pellucida

After washing in M2+BSA, oocytes or embryos were exposed briefly to acid Tyrode's solution (Nicolson *et al.*, 1975) to remove the zona pellucida, then washed thoroughly in M2+BSA and returned to M16+BSA for 1-6h before exposure to drugs

(see 4.4).

2.2.2 Preparation of single blastomeres and 2/8 cell pairs

The zonae pellucidae were removed and embryos were rinsed and incubated in M2+BSA for 1h prior to disaggregation in Ca^{2+}-free M2 + 6mg/ml BSA, using flame-polished micropipettes, to yield single blastomeres. Isolated blastomeres were cultured in Sterilin tissue culture dishes in drops of M16+BSA under oil at 37°C in 5% CO_2 in air. To generate 2/8 cell pairs, cultures of isolated late 4-cell blastomeres were inspected each hour for evidence of division of blastomeres. All newly formed 2/8 cell pairs were removed and designated 0h post-division. Pairs were then cultured in Sterilin dishes in individual drops of M16+BSA under oil. All manipulations were carried out at 37°C on heated stages or in incubators.

2.3 Assessment of intercellular flattening

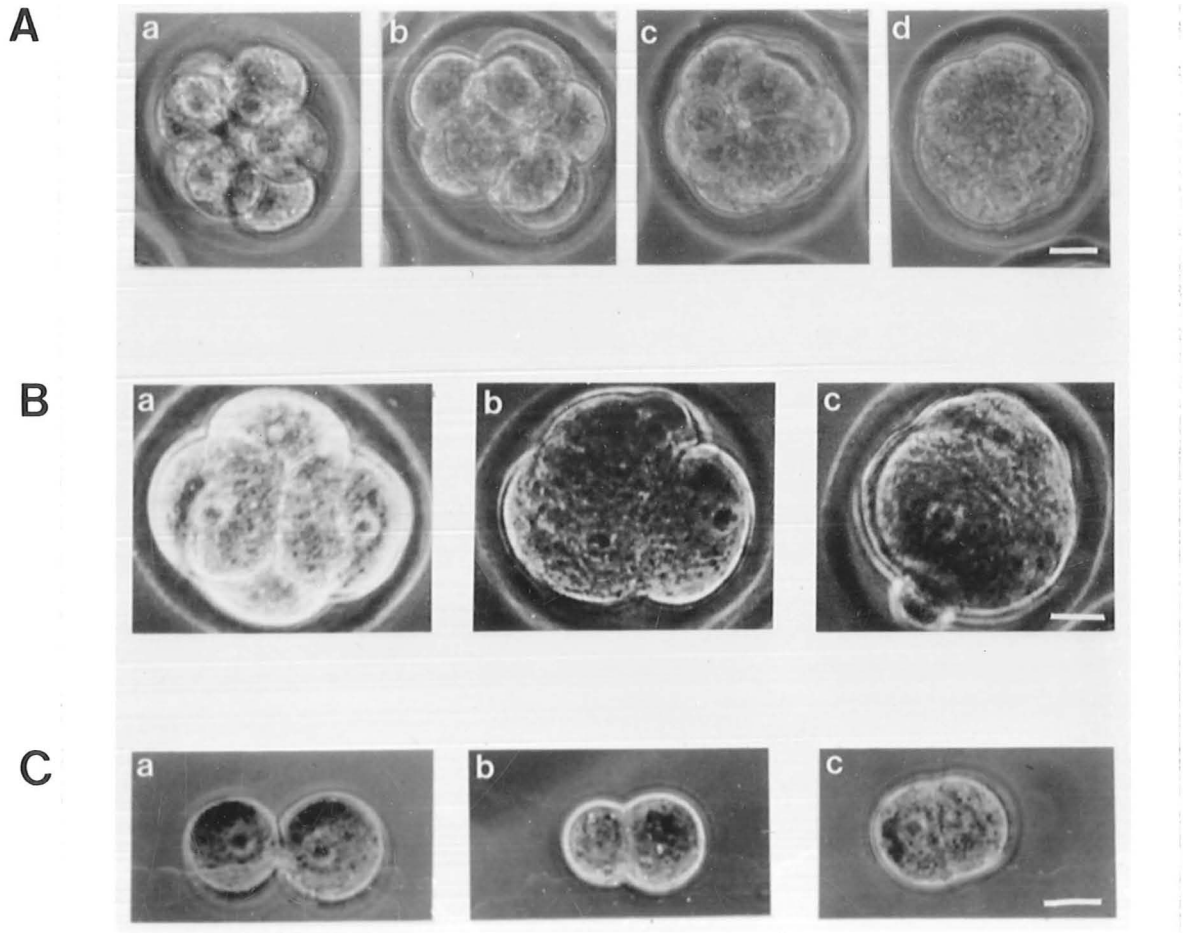
The degree of flattening of embryos was determined by examination using a Wild dissecting microscope. Eight-cell embryos were compared with standards illustrated in Figure 2.1A and assigned a score of 0 (a), 1 (b), 2 (c) or 3 (d) according to the degree of flattening. Four-cell embryos were compared with standards illustrated in Figure 2.1B and assigned a score of 0 (a), 1 (b) or 2 (c). The degree of flattening in 2/8 pairs of blastomeres was assessed similarly by comparison to standards (Fig. 2.1C a, b, c) and assigned a value of 0, 1 or 2 (maximum). For a population of embryos or cell pairs, the extent of flattening is expressed as a percentage of the maximum possible score, were all embryos or pairs to be fully flattened. Unless otherwise indicated in the text, a score such as 33% flattening indicates that most embryos were partially flattened, rather than that one third of embryos were fully flattened.

2.4 Additions to culture media

2.4.1 6-dimethylaminopurine (DMAP)

6-dimethylaminopurine (DMAP; Sigma) was added to M16+BSA to a final

2.1



concentration of 0.25-4.0mM, from a refrigerated (4°C) stock solution of 40mM in M16, and the medium pre-incubated for 3-4h before addition of embryos. In the experiments described in Chapter 6, medium containing DMAP was pre-incubated for 16-20h before addition of embryos. For discussion of the mode of action of DMAP see Chapter 3 and 6.5.

2.4.2 Antibodies to uvomorulin

Monoclonal antibody ECCD-1 (ascites fluid; Yoshido-Noro *et al.*, 1984) directed against E-type cadherin was diluted 1/50 in M16+BSA. An affinity purified polyclonal antiserum (anti-uvomorulin; Peyrieras, 1984; gift of N. Peyrieras and F. Jacob) directed against uvomorulin was diluted 1/50 in M16+BSA. Both ECCD-1 and anti-uvomorulin are active in preventing or reversing flattening of 8-cell blastomeres at dilutions in excess of 1/200 (Johnson *et al.*, 1986b). Both were centrifuged before use.

2.4.3 Trypsin and EGTA

Embryos were incubated for 15min in 0.1mg/ml trypsin (Gibco), with or without the addition of 0.1mM EGTA, in Ca^{2+}-free M2+PVP. They were then washed in M2+BSA before culturing further.

Figure 2.1 (facing page)

Photomicrographs of embryos and pairs of blastomeres, used as standards for flattening scores.

(A) Whole 8-cell embryos

a non-flattened, scored 0;
b. partially flattened, scored 1;
c extensively flattened, scored 2 ;
d. fully flattened, scored 3;

(B) 4-cell embryos and (C) 2/8 pairs of cells

a. non-flattened, scored 0;
b partially flattened, scored 1;
c. fully flattened, scored 2;
Bar=20μm in each

2.4.4 Phorbol esters and diacylglycerol

Stock solutions were made in dimethylsulphoxide (DMSO; BDH) of 25μg/ml phorbol 12-myristate 13-acetate (PMA), 100μg/ml phorbol 12-myristate 13-acetate 4-0-methyl ether (mPMA), 25μg/ml 4α phorbol didecanoate (4αPDD), 25μg/ml 4β phorbol didecanoate (4βPDD) and 200mM oleoyl acetyl glycerol (OAG) all from Sigma and all stored at -70°C. PMA, 4βPDD and OAG stimulate Ca^{2+}- and phospholipid-dependent protein kinase, protein kinase C (PKC); mPMA and 4αPDD are analogs of PMA and 4βPDD, respectively, that do not stimulate PKC (Castagna *et al.*, 1982; Niedel *et al.*, 1983). Phorbol esters were diluted in M2+BSA and embryos cultured in 5ml of medium in Sterilin tissue culture dishes at 37°C in humidified air. DMSO was added to all incubations, including controls, to a final concentration of 0.1%. An overlay of oil was not used, as many of the drugs would partition to the oil phase.

PMA has been reported to inhibit cytokinesis in fertilised oocytes and 2-cell embryos and may effectively reverse the process if applied before the cleavage furrow is completed. This generates triploid zygotes and binucleate blastomeres as karyokinesis is apparently unaffected (Niemerko & Komar, 1985; Sawicki & Mystkowska, 1981; Mystkowska & Sawicki, 1987). In the experiments described in Chapter 4, blastomeres of all stages showed a low incidence (7% or less) of binucleate blastomeres if close to mitosis when treated with PMA. Binucleate and fused blastomeres, where apparent, were not included in morphological scores.

2.4.5 Protein synthesis inhibitors

2.4.5.1 Cycloheximide

Cycloheximide (Sigma) from a stock of 40mM in water was used at 400μM in M16+BSA. Cycloheximide blocks the movement of ribosomes (Baliga & Munro, 1971, Schneiderman *et al.*, 1971).

2.4.5.2 Puromycin

Puromycin (Sigma) from a stock of 20mM in water was used at 20μM in M16+BSA. Puromycin acts as an amino acyl-tRNA analogue and causes premature release of nascent polypeptides (Yarmolinski & de la Haba, 1959; Hultin, 1966).

2.4.5.3 *Anisomycin*

Anisomycin (gift from Pfizer) from a stock of 10mM was used at 10μM in M16+BSA. Anisomycin inhibits peptide bond formation (Battaner & Vasquez, 1971).

All stocks were stored at -20°C.

2.4.6 *Cytochalasin D*

A stock solution of 1mg/ml cytochalasin D (Sigma) in dimethylsulphoxide (DMSO) was stored at –20°C. It was diluted in M16+BSA to a final concentration of 0.5μg/ml. Cytochalasin disrupts the organisation of polymerised actin (Brown & Spudich, 1979; Schliwa, 1982).

2.4.7 *Nocodazole*

A stock solution of 10mM nocodazole (Aldrich) in DMSO was stored at 4°C. It was diluted in M16+BSA to give a final concentration of 10μM. Nocodazole induces depolymerisation of microtubules (Hoeboke *et al.*, 1976). Cells can be arrested at metaphase by disruption of the mitotic spindle using nocodazole (Johnson *et al.*, 1988).

2.4.8 *Taxol*

A stock solution of 12mM taxol in DMSO (gift of N.I.H.; Lot T-4-112, N.I.H., Bethesda, USA) was stored at 4°C. For treatment of oocytes and embryos it was diluted in M16+BSA to a final concentration of 1.5μM. It was also included in PHEM buffer at a final concentration of 2μM (see 2.5.2.3, below). Taxol promotes microtubule assembly *in vitro* and stabilises microtubules *in vivo* (Schiff *et al.*, 1979).

2.4.9 *H-7*

The protein kinase inhibitor H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; Seikagaku America) was used at 18μg/ml or 36μg/ml (50μM or 100μM) in M16+BSA from a refrigerated (4°C) stock of 3.6mg/ml (10mM) in distilled water (pH≈4). H-7 is a kinase inhibitor (Hidaka *et al.*, 1984) that is reported to inhibit protein kinase C preferentially (Kawamoto & Hidaka, 1984).

2.5 Cytochemistry and light microscopy

2.5.1 Feulgen staining and nuclear DNA quantitation by microdensitometry

Glass microscope slides were scored with a grid approximately 10 x 0.1 x 0.1mm, washed in acetic acid and acetone and polished. The cut edge of a freshly dissected mouse liver was smeared on the bottom of the unscored side of each slide to provide diploid (2C) and tetraploid (4C) nuclei as standards. Timed embryos were pipetted onto the unscored side of the grid and air dried. Cells were then fixed in ethanol and acetic acid (3:1) for 5min followed by ethanol, acetic acid and formaldehyde (85:5:10) for 1h and stored at -20°C before staining.

Preparation of Schiff's basic stain: 2.0g basic fuchsin (Hopkin & Williams) was dissolved in 400ml boiling distilled water. The solution was left to cool for 30min before addition of 6g potassium metabisulphite (BDH) in 60ml 1N HCl. This mixture was allowed to stand overnight and then decolourised for 1h by stirring with activated charcoal, filtered and stored in a darkened bottle at 4°C.

For Feulgen staining, cells (on slides) were hydrolysed in 5N HCl for 55min at 26°C, washed in distilled water and then stained using Schiff's basic stain, for 2h in the dark. Slides were then washed three times in freshly prepared sulphorous acid (1:1 mixture of 1% (w/v) potassium metabisulphite and 0.1N HCl) followed by tap water. Slides were dehydrated through a graded alcohol series (5min each of 40%, 60%, 80%, 90%, 100% x 3) and xylene, mounted in Depex (Gurr) and stored in the dark at -20°C for up to 1 week before scoring.

The nuclear DNA content of individual cells was measured using a Vickers M86 scanning microdensitometer from absorption at 560nm (method adapted from Dietch *et al.*, 1967; Bolton *et al.*, 1984). Two scores for each nucleus were taken and the mean ± S. D. calculated for each sample. Photomicrographs were taken using bright field illumination using a Leitz Vario-Orthomat photographic system as described below (2.5.2.8).

2.5.2 Fluorescence microscopy

2.5.2.1 Use of chambers to support embryos or pairs of cells

Embryos or 2/8 pairs of cells were pipetted onto the coverslips of specially designed chambers as described by Maro *et al.* (1984). In most cases the chambers were coated first with a solution of 0.1mg/ml Con A in phosphate buffered saline (PBS) to increase adhesion of cells to chambers. When cells had been labelled with FITC-Con A, 1/10 phytohaemagglutinin (Gibco) in PBS was used to coat chambers. Chambers also contained appropriate drugs throughout processing until fixation. Chambers containing samples were centrifuged at 150 x g for 10min at 37°C (or 22°C for cells only labelled with FITC-Con A).

2.5.2.2 Labelling microvillous poles of unfixed cells with Con A

Cells were incubated for 1-2min in 700µg/ml FITC-labelled Concanavalin A (FITC-Con A; Polysciences) in M2+BSA at room temperature, followed by two washes in M2+BSA and then pipetted into chambers and fixed.

2.5.2.3 Extraction and fixation for tubulin staining

After a recovery period of 10min at 37°C, cells were washed quickly in PHEM buffer (10mM EGTA, 2mM MgCl2, 60mM PIPES, 25mM HEPES, pH6.9; described by Houliston *et al.*, 1987) containing 0.6mM taxol (PHEM-taxol), extracted for 5min in PHEM-taxol buffer containing 0.25% Triton X-100, washed in PHEM-taxol buffer and fixed for 45min with 2% formaldehyde (BDH) in PHEM-taxol buffer. All these steps were carried out in chambers, at 30°C on heated blocks. Tubulin was visualised with a monoclonal anti-αtubulin antibody (YL1/2; Kilmartin *et al.*, 1982) followed by FITC anti-rat IgG (Miles).

2.5.2.4 Routine fixation

Embryos or pairs of cells were fixed in chambers immediately after centrifugation, using 4% formaldehyde in PBS, for 45min. Chambers were then washed in 0.05M ammonium chloride in PBS for 10min, to neutralise the fixative, and washed in PBS.

2.5.2.5 Actin visualisation

Microfilamentous actin was stained with FITC-phalloidin (Sigma) after permeabilising cells with 0.25% Triton X-100 for 10min.

2.5.2.6 Visualisation of PMA-binding sites

Embryos were incubated in medium containing 25ng/ml 11-((-5-dimethylaminonapthalene-1-sulphonyl) amino) undecanoylphorbol acetate (dansyl PMA, Molecular Probes) for 1h prior to fixation (see 4.2).

2.5.2.7 Chromatin visualisation

Chromatin was visualised by incubating fixed cells in Hoechst dye 33258 (5µg/ml in PBS) for 45min.

PBS containing 0.1% Tween 20 (Sigma) was used routinely in washing steps.

2.5.2.8 Photomicroscopy

The coverslips were removed from chambers and samples were mounted in "Citifluor" (City University, London) and viewed on a Leitz Ortholux II microscope with filter sets L2 for FITC-labelled reagents and dansyl PMA, N2 for rhodamine-labelled reagents, and A for Hoechst dye. Photographs were taken on Kodak Tri-X film using a Leitz Vario-Orthomat photographic system. The three-dimensional structure of the cells is preserved in the whole mounts, but it is not possible to photograph the whole cell in the same focal plane; optical sections with only one plane through the cell in sharp focus are shown.

2.6 Electron microscopy

2.6.1 Transmission electron microscopy

The procedure used was that described by Fleming and Pickering (1985). Embryos were fixed in 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.3) for 30min at room temperature, post-fixed in 1% osmium tetroxide then washed in

distilled water, dehydrated though 40%, 60%, 80% and 100% ethanol and embedded in TAAB resin. Ultrathin sections were cut using an LKB Ultrotome III, stained with alcoholic uranyl acetate and lead citrate and viewed in a Phillips EM300 electron microscope at 80kV.

2.6.2 Scanning electron microscopy

The procedure used was modified from that used by Pickering *et al.* (1988). Alcohol-cleaned glass coverslips were coated with poly-L-lysine (Sigma, M_r >300,000; 1mg/ml in water) for at least 20min and washed three times in 0.1M cacodylate buffer pH 7.3 before being placed in wells of a Nunclon 24-well tissue culture dish containing cacodylate buffer. Cells were fixed in 3% glutaraldehyde in cacodylate buffer for 45min at room temperature in drops under oil and then washed in cacodylate buffer and transferred to the centre of freshly prepared coverslips. Samples were dehydrated through graded alcohols (30min each in 20%, 40%, 60%, overnight in 70%, 30min each in 80%, 90%, 95% and dry 100%) and then critical point dried from absolute alcohol via CO_2 in a Polaron E3000 critical point drying apparatus. Coverslips were mounted on stubs with Agar silver paint (Agar Aids), left to dry and coated with a 60nm layer of gold in a Polaron E5000 Diode sputtering system. Cells were examined in a JSM-35CF Jeol microscope under 5-20kV.

2.7 Radiolabelling of polypeptides

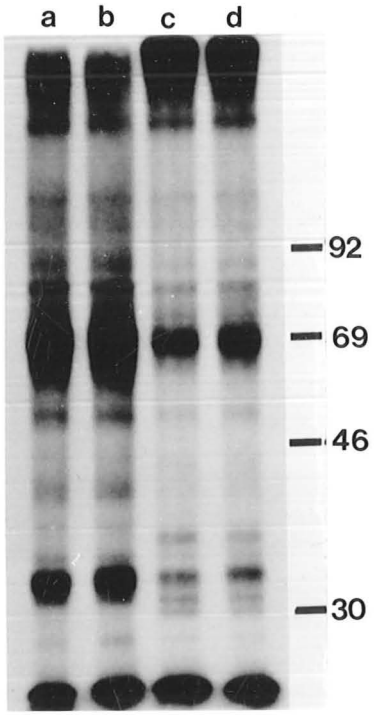
2.7.1 [^{35}S]methionine

Oocytes and embryos were incubated for 1h in 50µl M16+BSA containing 1.5mCi/ml of [^{35}S]methionine (specific activity: 1300-1400Ci/mmol; Amersham) and then washed in protein-free M2 containing 6mg/ml polyvinylpyrrolidone (M2+PVP) before harvesting.

2.7.2 [^{32}P]orthophosphate

Preliminary experiments were undertaken to assess the effect of pre-

2.2



incubation of embryos in medium depleted of phosphate for up to 14h prior to radiolabelling. These showed no effect on the pattern of ^{32}P-labelled phosphoprotein bands seen after one-dimensional electrophoresis of 4-cell or 8-cell embryos (Fig. 2.2). Incubating embryos in phosphate-free medium for up to 14h also did not affect the timing or proportion of embryos subsequently developing to blastocysts (data not shown).

To assess the toxicity of [^{32}P]orthophosphate on embryos in culture, groups of 50 embryos were cultured from the late 2-cell stage (50h post-hCG) for 0h, 1h, 3h, 6h, or 12h in phosphate-free M16+BSA supplemented with 1mCi/ml [^{32}P]orthophosphate ("^{32}P-medium"; [^{32}P]orthophosphate carrier-free; Amersham; used for up to 2 weeks after assay date) and then washed and transferred to complete, non-labelled medium. For incubations of 1h or 3h, there was no apparent effect on the survival rate or timing of development to blastocysts (controls, 1h and 3h incubations: 90-98% blastocysts 90h post-hCG). After 6h in ^{32}P-medium, only 46% of embryos developed to blastocysts, the remainder arresting as 2- and 4-cells. After 12h incubation, no embryos divided beyond 2-cells.

In the experiments described in Chapters 5 and 6, oocytes and embryos were

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Figure 2.2 (facing page)

Pre-incubation in phosphate-free medium does not appear to affect the pattern of ^{32}P-labelled phosphoprotein bands seen after one-dimensional electrophoresis.

One-dimensional SDS-PAGE separation of [^{32}P]orthophosphate-labelled polypeptides from late 4-cell embryos (a, b) and early 8-cell (c, d) embryos (62h post-hCG). Embryos were flushed from oviducts as 2-cells and 4-cells at 48h post-hCG and cultured in either M16+BSA (a, c) or phosphate-free M16+BSA (b, d) for 14h before addition of [^{32}P]orthophosphate for 6h. Different bands are detectable in 4-cells and 8-cells (compare lanes a & b with lanes c & d and see Chapter 5) but there are no detectable differences between embryos pre-incubated in the presence or absence of phosphate (compare a with b, c with d).

routinely incubated for 1h in 50µl phosphate-free M16+BSA containing 1mCi/ml of [^{32}P]orthophosphate (^{32}P-medium, as above). In cold-chase experiments, embryos were transferred from ^{32}P-medium to M16+BSA via 3 large wash drops (approximately 300µl each) of M16+BSA. Samples were washed through M2+PVP, as in 2.7.1, before harvesting. In experiments in which oocytes and embryos were labelled with ^{32}P in the presence of PMA (6.5), 50µl ^{32}P-medium containing 25ng/ml PMA was incubated in the well of a MicroTest II tissue culture plate (Falcon), in humidified air at 37°C, with no overlay of oil.

2.8 Quantitative analysis of [^{35}S]methionine uptake and incorporation into polypeptides

Groups of 10 embryos were labelled as described in 2.7.1 and washed through 6 x 100µl wash drops of M2+PVP. To assay uptake of [^{35}S]methionine, embryos were pipetted directly into 5ml scintillant (Ecoscint) in a scintillation vial. A 5µl aliquot of the final wash drop was sampled to ensure that no [^{35}S]methionine was carried over into samples. At the end of each experiment, 3 x 5µl aliquots of each labelling drop were each diluted in 5 x 500µl distilled water and 5 x 5µl aliquots counted.

Uptake was then calculated using the method of Holmberg & Johnson (1979):

$$\text{uptake per embryo (moles)} = \frac{\{(\text{cpm/embryo}) - (\text{cpm in wash})\}}{\text{cpm in labelling solution}} \times [\text{labelling drop molarity}] \times 5\mu\text{l}$$

To assay incorporation of [^{35}S]methionine into polypeptides, embryos were pipetted into 20µl distilled water, 20µl of 1mg/ml BSA was added as a carrier and 160µl ice-cold 10% trichloracetic acid (TCA) was added to precipitate polypeptides. This mixture was then allowed to stand overnight at 4°C, mixed and washed, with 10% TCA containing 10mM unlabelled methionine, through a pre-washed Whatman glassfibre filter. After a final rinse with alcohol, filters were dried under an infra-red lamp and then put into scintillation vials with 5ml scintillant. Samples were counted in a Packard CL/D liquid scintillation counter for 5min. At least 8 replicates of each sample were counted and the mean ± S. D. calculated.

2.9 Qualitative analysis of polypeptides

2.9.1 One-dimensional polyacrylamide gel electrophoresis

For 1-dimensional analysis, groups of 10 embryos were transferred to 10µl SDS sample buffer (Laemmli, 1970), boiled for 1min and stored at -70°C. Polypeptides were separated using uniform 10% SDS polyacrylamide slab gels containing 0.1% SDS and 0.5M Tris-HCl (pH 8.8), with a stacking gel of 4.5% acrylamide containing 0.1% SDS and 0.125M Tris-HCl (pH 6.8; method of Laemmli, 1970). Gels were run for 3.5-4h at a constant voltage of 175V. Gels containing ^{32}P-labelled samples were always run until the dye front had run completely off the bottom of the gel to ensure the elimination of low M_r, heavily-labelled non-protein material that can diffuse through the gel after fixation to produce a high background.

2.9.2 Two-dimensional polyacrylamide gel electrophoresis

For 2-dimensional analysis, groups of 50 embryos were transferred to 10µl sample lysis buffer (O'Farrell, 1975) and stored at -70°C. For separation by isoelectric focussing, samples were saturated with urea, frozen and thawed three times and applied to pre-equilibrated, cylindrical 4% acrylamide gels. These were run for a total of 6000Vh with the last hour at 800V and then equilibrated with SDS sample buffer (O'Farrell, 1975) before being placed onto 10% acrylamide slab gels, as described in 2.9.1, to separate proteins by relative molecular mass.

2.9.3 Gel fixation and autoradiography

Gels were fixed for 30-60min in 45% methanol, 10% acetic acid before drying down onto filter paper and exposure to Fuji RX X-ray film at –70°C for 3-10 days. Intensifying screens (Ilford) were used with ^{32}P-labelled gels.

Figures show representative gels, obtained on three or more occasions from samples collected during separate experiments.

CHAPTER THREE

6-DIMETHYLAMINOPURINE AFFECTS CELL CYCLE PROGRESSION AND COMPACTION

CHAPTER THREE 6-DIMETHYLAMINOPURINE (DMAP) AFFECTS CELL CYCLE PROGRESSION AND COMPACTION

3.1 Introduction

The events of compaction that precede cell diversification in the mouse embryo seem to be controlled entirely post-translationally (see 1.4; Kidder & McLachlin, 1985; Levy *et al.*, 1986). The inhibition of protein synthesis in 4-cell embryos can even lead to certain manifestations of compaction, cell flattening and surface polarisation, occurring prematurely (Levy *et al.*, 1986). This result led to the suggestion that cells are capable of compaction from at least the early 4-cell stage but are prevented from compacting by a rapidly-destroyed inhibitor. According to this hypothesis, preventing protein synthesis blocks the production of the inhibitor, allowing compaction to proceed (Levy *et al.*, 1986). More complex explanations of these experiments are clearly possible. It is noteworthy that the premature "compaction" induced in 4-cell embryos in the presence of protein synthesis inhibitors is only partial. A maximum of approximately 75% flattening and 40% cell surface polarisation is achieved in late 4-cell embryos following exposure to a protein synthesis inhibitor earlier in the third cell cycle (Levy *et al.*, 1986).

Two inhibitors of protein synthesis were used in the study by Levy *et al.* (1986): anisomycin, which is believed to lock nascent polypeptides onto ribosomes (Battaner & Vasquez, 1971), and puromycin, which acts as an amino acyl-tRNA analogue and causes premature release of nascent polypeptides (Yarmolinski & de la Haba, 1959; Hultin, 1966; Fig. 3.1). Both of these drugs are associated with effects on cells other than the arrest of protein synthesis (see below). It is therefore possible that the observed premature "compaction" of 4-cell embryos was due not to the inhibition of protein synthesis but to some other effect of these drugs. In previous studies, puromycin and related adenine analogues, that affect protein synthesis to varying degrees, have been documented to affect glycogenolysis in the liver with a reversed order of potency to their effects on protein synthesis (Hoffert & Boutwell, 1963). The effects on glycogenolysis are likely to be due, at least in part, to the inhibition of cAMP phosphodiesterase by

3.1

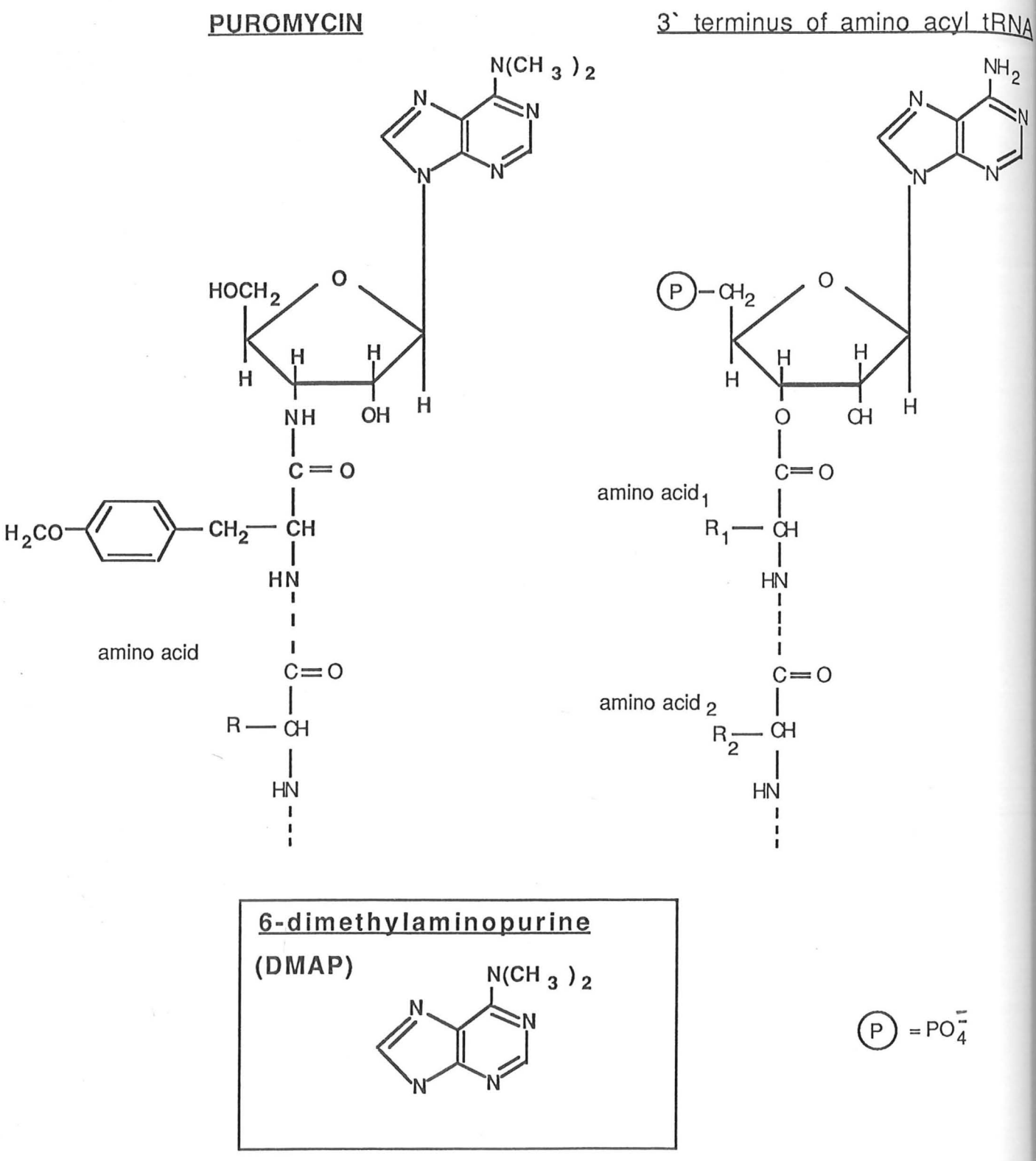


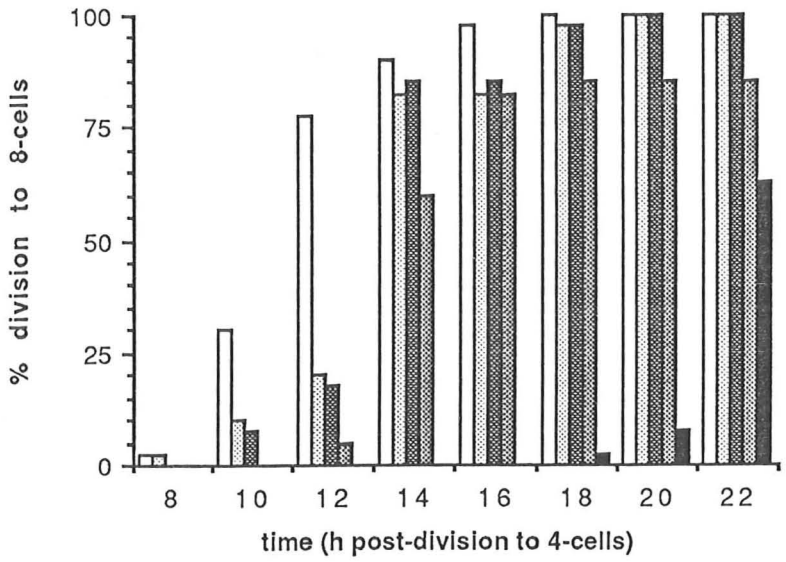
Figure 3.1
Diagram to illustrate the structure of puromycin (Yarmolinski & de la Haba, 1959; Hultin, 1966), the 3' terminus of an amino acyl -tRNA and 6-dimethylaminopurine (DMAP).

puromycin and related adenine analogues (Appleman & Kemp, 1966).

In order to distinguish the effects of puromycin on protein synthesis from any other effects it may have on cells, several workers have compared its effects with those of 6-dimethylaminopurine (DMAP). DMAP is analogous to a purine base, but, as DMAP has no other similarity to an amino acyl-tRNA, it is not expected to affect protein synthesis (Fig. 3.1). DMAP has been shown to arrest cleavage in marine embryos, probably in G_2 of the cell cycle. It has no detectable effect on either the incorporation of radio-labelled amino acids into proteins or on intracellular cAMP levels in these species (Rebhun *et al.*, 1973). DMAP has also been reported to decrease the recruitment of histone and other mRNAs onto polysomes in sea urchin eggs (Showman *et al.*, 1982).

Adenine-containing compounds have numerous roles in cells, both structurally, for example in nucleic acids, and dynamically, in the regulation of many proteins. Compounds such as DMAP could, therefore, disrupt cellular metabolism in many different ways. Of particular interest to this study are potential effects on regulatory enzymes which could be achieved by competition with cAMP or ATP. Among the classes of regulatory enzymes dependent on ATP and known to be affected by adenine analogues are protein kinases (Iwai *et al.*, 1972). Recently, DMAP has been reported to inhibit all detectable protein phosphorylation in starfish eggs (Neant & Guerrier, 1988). This effect does not seem to be due to any alteration in phosphoprotein phosphatase activity and is therefore presumed to be a result of inhibition of kinase activity (Neant & Guerrier, 1988). However, DMAP has a less severe effect on protein phosphorylation in maturing mouse oocytes in which the extent of inhibition of protein phosphorylation seems to vary with meiotic progression (Rime *et al.*, 1989). In this chapter, the effects of DMAP on cell cycle progression and on the events of compaction and the rate of protein synthesis in mouse embryos are described. The effects of DMAP on protein phosphorylation in compacting embryos will be considered further in Chapter 6.

3.2



control
n=142, 5 experiments

0.25mM DMAP
n=60, 2 experiments

0.5mM DMAP
n=39, 1experiment

1.0mM DMAP
n=33, 1experiment

2.0mM DMAP
n=29, 1 experiment

Figure 3.2
Dose-dependent inhibition of cell division by DMAP.
Groups of 4-cell embryos were incubated in medium containing DMAP from the time of division and scored for subsequent division to 8-cells.
Embryos were then fixed, stained with Hoechst dye and numbers of nuclei counted. Cytokinesis and karyokinesis were always prevented concomitantly. 4mM DMAP inhibited cleavage in 100% of 122 embryos in 5 experiments.

3.2 Effects of DMAP on the cell cycle

3.2.1 DMAP blocks cell division

4-cell and 8-cell embryos were incubated immediately post-division in M16+BSA containing DMAP in the concentration range 0.25mM - 4.0mM. Cytokinesis and karyokinesis were delayed and prevented together in a dose-dependent manner (Fig. 3.2). 4.0mM DMAP was sufficient to prevent all cytokinesis and karyokinesis. This concentration was therefore used in all the experiments described below.

3.2.2 The cell cycle is blocked very late in G_2

Timed groups of 4-cell and 8-cell embryos that had been incubated since division in untreated medium or medium containing 4.0mM DMAP were dried onto slides. The DNA content of their nuclei was assessed microdensitometrically and compared to the DNA content of diploid and tetraploid liver nuclei. The relative DNA content of samples taken at intervals through the third and fourth cell cycles is shown in Figure 3.3. As previously reported by Smith & Johnson (1986), both these cell cycles have a short G_1 phase (1-2h) followed by a prolonged S phase (approximately 7-8h). In both 4-cell and 8-cell embryos incubated in DMAP, the DNA content per nucleus increased with time similarly to controls, reaching the tetraploid value (4C) by 10-12h post-division. At this time blastomeres of control embryos began to cleave, each yielding two diploid (2C) nuclei; DMAP treated embryos failed to cleave and the DNA content per nucleus remained high (Fig. 3.3).

DMAP has previously been reported to block cell division in sea urchin embryos in G_2 (Rebhun *et al.*, 1973). The chromatin of DMAP-treated 4-cell and 8-cell embryos stained with Hoechst dye or Feulgen stain appeared like that of controls until 10-12h post-division, after which time nuclei showed a characteristic "semi-condensed" appearance within an intact nuclear envelope (Feulgen stained, Fig. 3.4). This appearance did not change with prolonged incubation (up to 40h post-division) and seems likely to correspond to a state transitional between interphase and mitosis, supporting the hypothesis that DMAP-treated cells are arrested very late in G_2 (Rebhun *et al.*, 1973).

3.3

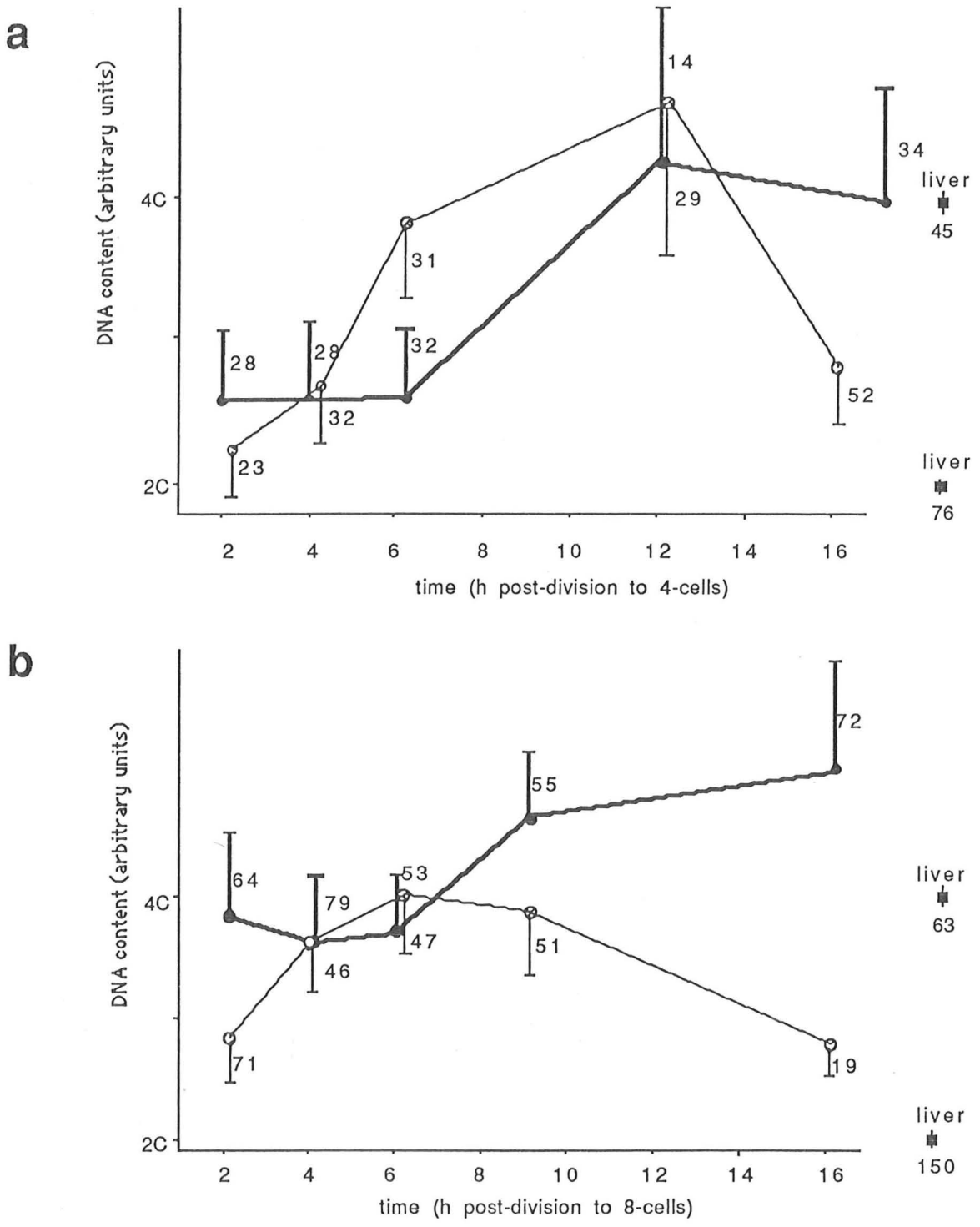


Figure 3.3
DMAP blocks the cell cycle in G_2
DNA content of nuclei measured by microdensitometric analysis at 560nm after Feulgen staining plotted against age of embryos post-division to (a) 4-cells and (b) 8-cells. Solid circles and heavy lines show mean scores for embryos incubated in 4mM DMAP from the time of division, hollow circles and fine lines show controls and squares show the mean scores for diploid (2C) or teraploid (4C) liver nuclei. The number of nuclei scored for each time point is indicated; bars indicate 1/2 S.D.

3.2.3 Cells can also be blocked in mitosis by DMAP

To establish whether the only possible effect of DMAP on cell cycle progression is at the G_2/M phase boundary, mitotic cells were incubated in medium containing DMAP. Mitotic blastomeres in 2-cell and 3-cell embryos were identified by hourly scoring for the absence of a nuclear envelope, using a dissecting microscope (Johnson *et al.*, 1988). Some embryos with one or more mitotic blastomeres were transferred directly to medium containing 4mM DMAP for 1h or 14h before harvesting. Other groups of similar embryos were transferred first to medium containing 10μM nocodazole for 3h, to accumulate cells at metaphase (Johnson *et al.*, 1988) and then either harvested immediately or transferred to DMAP for 1h or 14h before harvesting.

Nuclei were then Feulgen stained and examined. As shown in Table 3.1, approximately 20% of all cells scored as mitotic, however treated, were later shown to have only one interphase nucleus, and were therefore presumed to have been incorrectly scored as mitotic. A slightly higher proportion of cells placed in DMAP, approximately

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Table 3.1

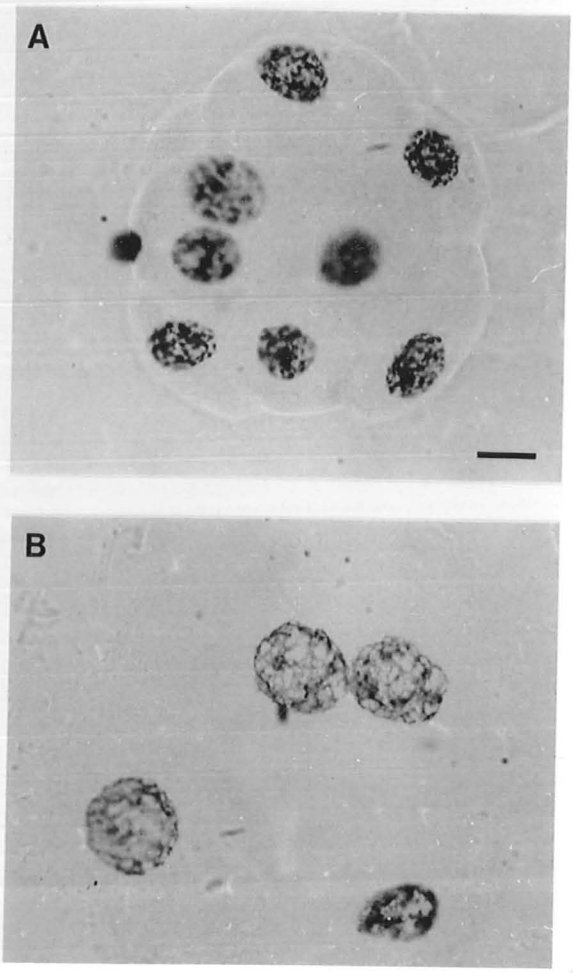
DMAP can block cells in either mitosis or interphase

	Scored after 1h treatment (%)				Scored after 14h treatment (%)					
	n (nuclei)	*interphase 1	*interphase 2	mitotic	n (nuclei)	*interphase 1	*interphase 2	mitotic	semi-condensed+ 1	semi-condensed+ 2
Treatment:										
none	35	20	69	11	30	23	77	0	0	0
nocodazole (10μM)	19	16	11	74	11	18	18	64	0	0
DMAP (4mM)	46	35	36	29	45	11	7	33	20	29
DMAP after 3h nocodazole	22	9	45	45	23	13	39	4	9	35

* blastomeres with 1 or 2 morphologically normal interphase nuclei

\+ blastomeres with 1 or 2 nuclei with semi-condensed chromatin (see Fig. 3.4)

3.4



1/3, showed a single interphase nucleus after 1h, with "semi-condensed" chromatin in most of these after 14h. This is consistent either with some mitotic nuclei having reverted to interphase in DMAP, and subsequently arrested at the G_2/M boundary, or with a higher level of incorrect scoring of mitotic cells in this group. In a further 1/3 of DMAP-treated blastomeres, the mitotic blastomere had undergone cytokinesis and karyokinesis and entered interphase of the next cell cycle. Nuclei of this type also had semi-condensed chromatin after 14h. In the final group of DMAP-treated embryos, one or more blastomeres contained mitotic chromosomes in prophase or prometaphase, suggesting arrest later than the G_2/M boundary but before the next interphase.

In nuclei accumulated in metaphase with nocodazole before treatment with DMAP, slightly more blastomeres had divided and entered the next interphase by 14h, and fewer had arrested in mitosis than in embryos treated with DMAP without prior metaphase arrest. Although the numbers of nuclei scored in each group are small, these data are consistent with DMAP causing arrest at two points in the cell cycle, one at the G_2/M boundary and the second very early in mitosis, before the metaphase block caused by nocodazole.

3.3 Effects of DMAP on compaction

3.3.1 DMAP can cause premature intercellular flattening

3.3.1.1 DMAP-induced flattening is very rapid and reversible

Timed groups of 4-cell or 8-cell embryos of different ages post-division were incubated in medium containing 4mM DMAP. In embryos of all ages post-division, intercellular flattening developed to close to the maximum score possible within 1-2h,

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Figure 3.4 (facing page)

Appearance of chromatin in 4-cell embryos incubated for 16h post-division in (a) control medium, divided to 8-cells, normal interphase morphology (b) 4mM DMAP, arrested as late 4-cells, chromatin appears "semi-condensed". Bar = 20μm

3.5

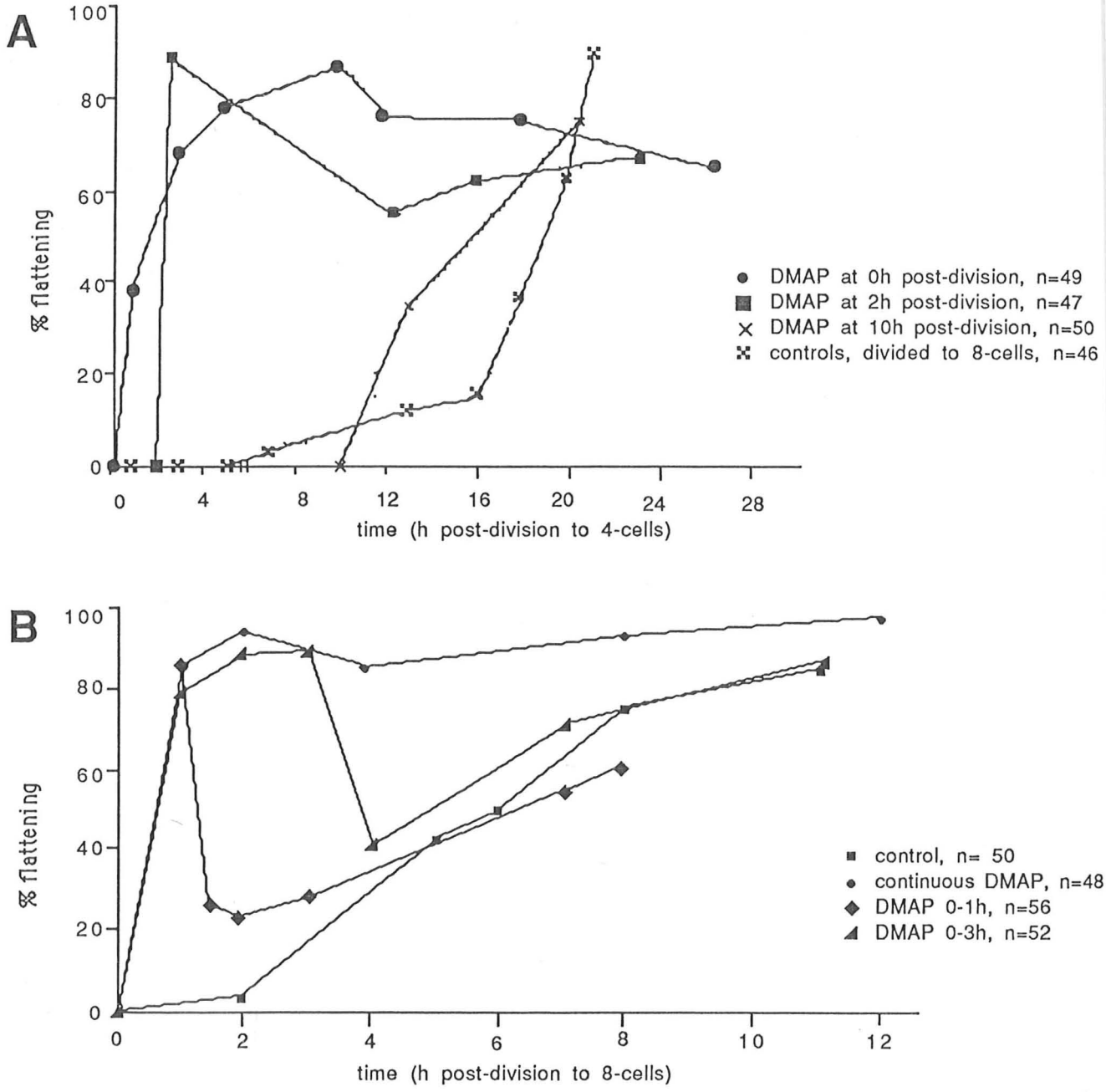


Figure 3.5
A. Premature flattening occurs rapidly in 4-cell embryos
4-cell embryos were placed in 4mM DMAP at the times indictated post-division and scored for premature intercellular flattening. The time course of flattening in untreated embryos (division to 8-cells at 10-16h post-division to 4-cells) is shown for comparison.
B. Premature flattening also occurs in 8-cell embryos and is rapidly reversible
8-cell embryos were placed in 4mM at 0h post-division, either continuously or for 1h or 3h before washing and returning to M16+BSA.

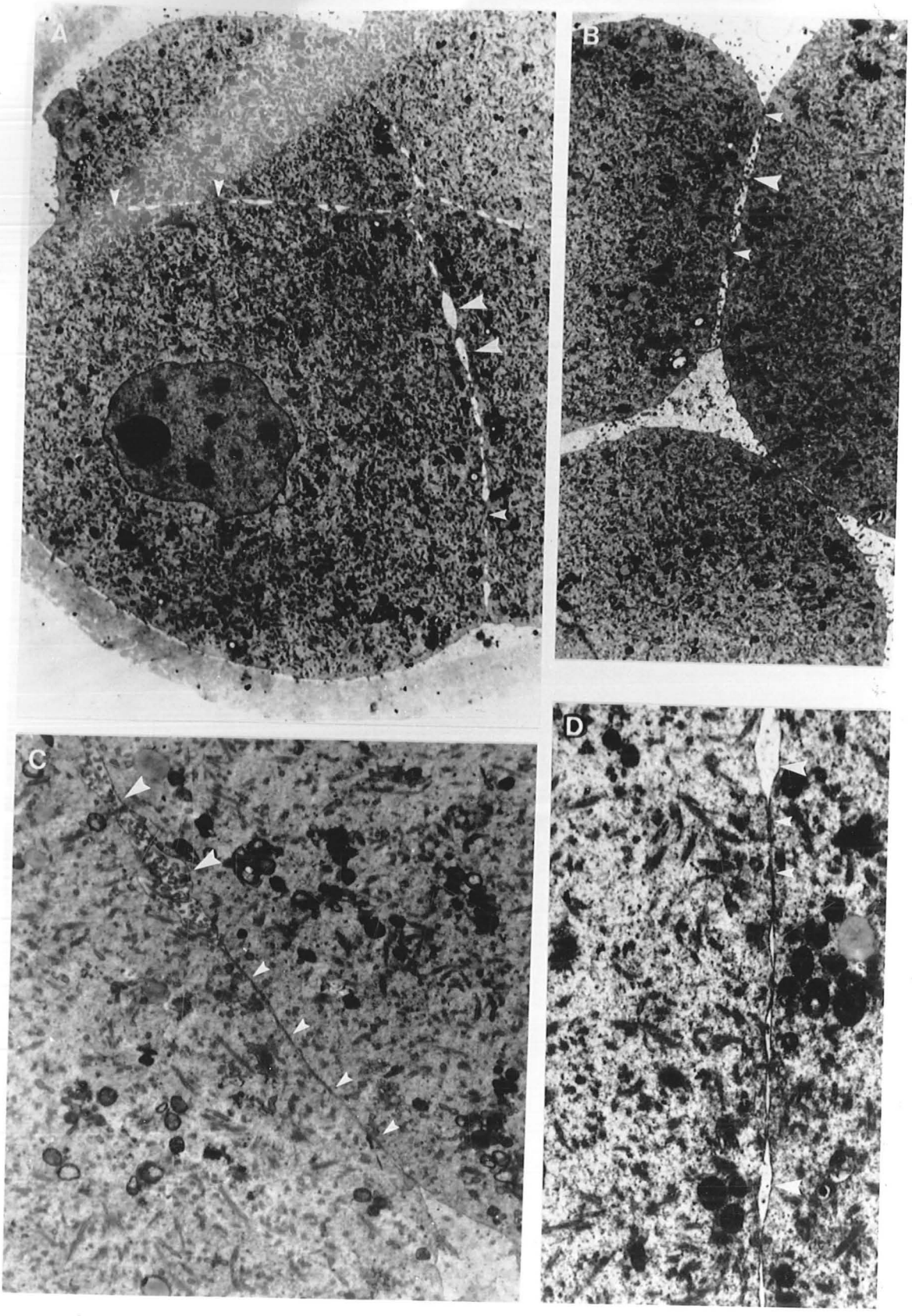
and was sustained as long as embryos were in DMAP (4-cell embryos, Fig. 3.5a, 8-cells, Fig. 3.5b). When embryos were exposed to DMAP for 3h or less and then washed out of DMAP, flattening was reversed rapidly to the level of contemporaneous controls (0% in 4-cells, partial flattening in early 8-cells, see Fig. 3.5b). Longer pulses of DMAP were associated with some residual flattening after washing embryos out of the drug (data not shown). In 8-cell embryos exposed to a 1-3h pulse of DMAP, development continued to the blastocyst stage at similar rates to untreated embryos (controls, 100% blastocysts, n=40; 4mM DMAP 0-1h post-division to 8-cells, 94% blastocysts, n=38; 4mM DMAP 0-2h post-division to 8-cells, 100% blastocysts, n=34; 4mM DMAP 0-3h post-division to 8-cells, 94% blastocysts, n=34; scored 46h post-division to 8-cells).

3.3.1.2 DMAP-induced flattening does not seem to be mediated by uvomorulin

Various agents can prevent and reverse the uvomorulin-mediated intercellular flattening of 8-cell embryos (see 1.4; Hyafil *et al,.* 1980, 1981; Ogou *et al.,* 1982; Pratt *et al.,* 1982, 1983). Some are specific to uvomorulin-mediated flattening while others are less specific. Incubation in medium containing polyclonal (Peyrieras, 1984) or monoclonal (ECCD-1; Yoshido-Noro *et al.,* 1984) antibodies to uvomorulin specifically prevents or reverses only uvomorulin-mediated flattening (Hyafil *et al,.* 1980, 1981; Ogou *et al.,* 1982; Johnson *et al.,* 1986b). Incubation in Ca^{2+}-free medium or in medium containing trypsin in the presence of EGTA are less specific, reversing and preventing any Ca^{2+}-dependent flattening or digesting the extracellular domains of integral membrane proteins that may be protected by the presence of Ca^{2+}, respectively (Hyafil *et al.,* 1980, 1981; Ogou *et al.,* 1982; Peyrieras *et al.,* 1983). Finally, incubation in cytochalasin D (CCD; Schliwa, 1982) also reverses the flattening of 8-cell embryos by causing cells to round up *via* disruption of the actin cytoskeleton (Ducibella *et al.,* 1977; Surani *et al.,* 1980; Pratt *et al.,* 1981, 1982; Johnson & Maro, 1984).

To assess whether DMAP-induced flattening could be reversed by any of these treatments, groups of 4-cell and 8-cell embryos were incubated in DMAP immediately

3.6



post-division and then transferred to Ca^{2+}-free medium or medium containing polyclonal anti-uvomorulin antibodies, ECCD-1, CCD or trypsin+EGTA, each in addition to DMAP. Similar groups of embryos were placed directly into these media with DMAP present but with no prior incubation in DMAP. Untreated, non-flattened and fully flattened 8-cell embryos (0h and 7-8h post-division, respectively) were incubated in the same range of media but containing no DMAP. Table 3.2 (overleaf) shows the results of scoring for flattening 1h after each treatment. Only those agents that are not specific to uvomorulin-mediated flattening could prevent or reverse DMAP-induced flattening. Unlike the natural intercellular flattening of 8-cell embryos, DMAP-induced flattening is therefore unlikely to be mediated by uvomorulin.

3.3.2 The morphology of intercellular contacts in embryos exposed to DMAP

4-cell and 8-cell embryos that had been incubated in DMAP for 1h immediately post-division and scored as fully flattened were examined by transmission electron microscopy and the ultrastructure of intercellular contacts compared with those of contemporaneous controls. Untreated 4-cell embryos consisted of rounded blastomeres with only small areas of apparently non-specialised intercellular contact (small arrowheads, Fig. 3.6B). Microvilli were present over the entire blastomere

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Figure 3.6 (facing page)

Ultrastructure of intercellular contacts after flattening in DMAP

Transmission electron micrographs of (A) 4-cell embryo, 1h post-division, after 1h incubation in 4mM DMAP, magnification x 1250; (B) untreated 4-cell embryo, 1h post-division, magnification x 1250; (C) DMAP-treated 4-cell, as (A), magnification x 4800; (D) untreated 8-cell embryo, 6h post-division, 1h after full flattening, magnification x 6100. In each frame, small arrowheads indicate regions of apparently non-specialised, close membrane apposition. Large arrowheads indicate intercellular spaces, which enclose microvilli in DMAP-treated (A, C) or untreated (B) 4-cells but do not enclose microvilli in untreated 8-cell embryos (D).

surface, including regions of contact (large arrowheads, Fig. 3.6B). Untreated 8-cell embryos (6h post-division), that had flattened within the last 1h, showed extensive areas of close membrane apposition between blastomeres, particularly basally (small arrowheads, Fig. 3.6D). Only small intercellular spaces were enclosed between cells and few microvilli were apparent in regions of contact (large arrowheads, Fig. 3.6D;

Table 3.2

Do agents that prevent or reverse flattening in 8-cell embryos affect DMAP-induced flattening?

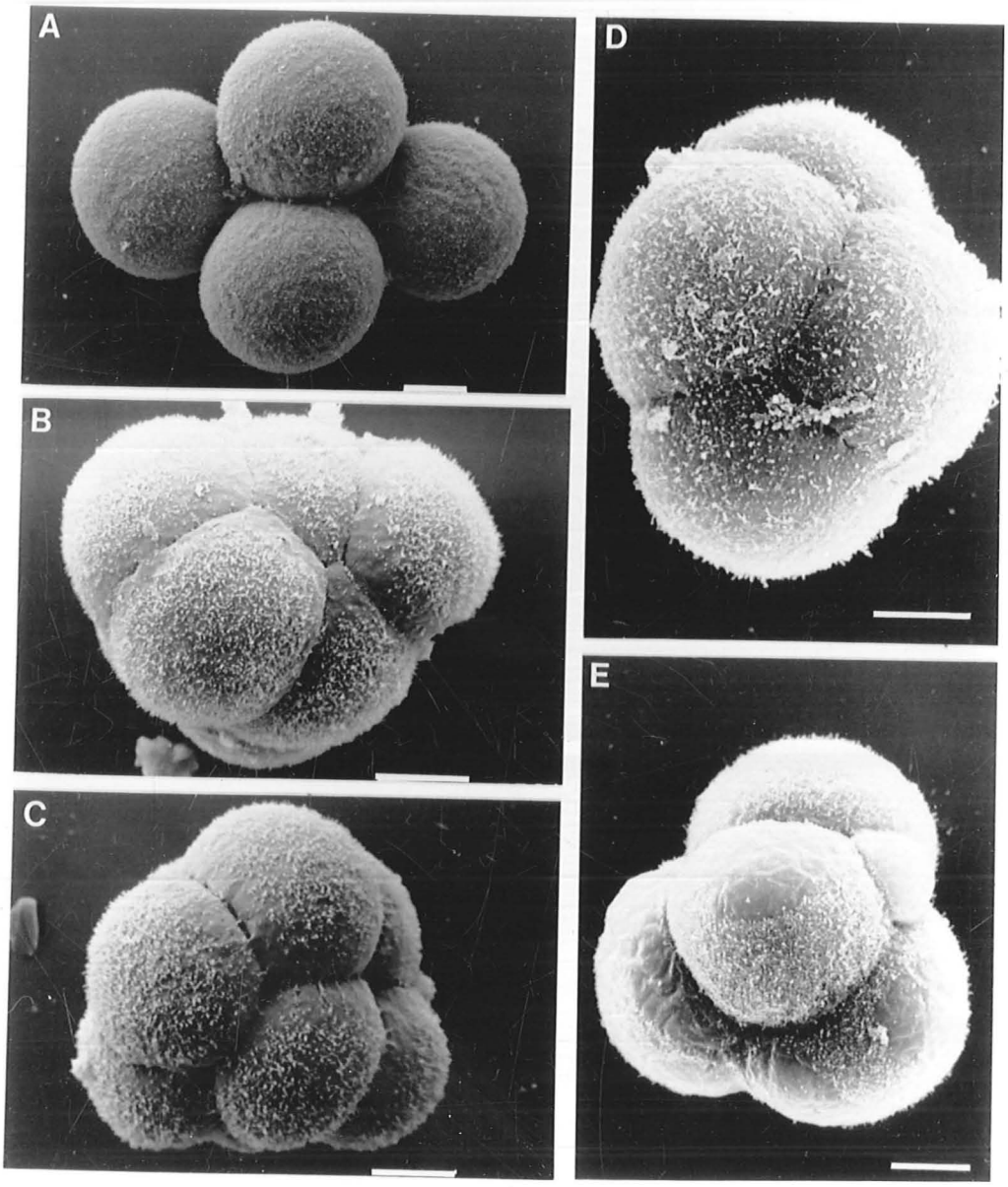
Treatment:	Ca^{2+}-free medium	polyclonal anti-uvomorulin (1/50)	ECCD-1 (1/50)	CCD (0.5µg /ml)	trypsin alone	trypsin +EGTA (0.1mM)
A. Reversal of pre-existing flattening						
Developmental stage						
control 8-cells (7-8h post-division)	x(0;22)	x(0;17)	x(0;23)	x(0; 26)	√(63;11)	x(0;26)
DMAP-treated 4-cells	√(63;19)	√(72;18)	√(68;19)	x(0;38)	√(67;15)	x(0;22)
DMAP-treated 8-cells	√(79;19)	√(63/19)	√(68;19)	x(0;19)	√(58;12)	x(0;15)

x....flattening reversed in presence of agent. √...flattening persisted in presence of agent. Flattening scores (%) and numbers of embryos are shown in brackets (% flattening;n) beside each point.

B. Prevention of the development of flattening						
Developmental stage						
control 8-cells (0h post-division)	x(0;25)	x(0;24)	x(0;30)	x(0;19)	√(33;24)	x(0;25)
DMAP-treated 4-cells	√(75;26)	√(63;15)	√(73;20)	x(0;20)	√(67;15)	x(0;15)
DMAP-treated 8-cells	√(63;19)	√(68;19)	√(63;19)	x(0;14)	√(60;10)	x(0;12)

x: flattening prevented in presence of agent. √: flattening developed in presence of agent. Flattening scores (%) and numbers of embryos are shown in brackets (% flattening;n) beside each point.

3.7



Ducibella & Anderson, 1975). DMAP-treated 4-cell and 8-cell embryos showed more extensive regions of contact that appeared punctate, enclosing intercellular spaces that contained microvilli at both stages (large arrowheads, Fig. 3.6A, C). The intercellular contacts of DMAP-treated embryos were therefore morphologically similar to those of newly-flattened untreated 8-cell embryos but with microvilli trapped within the regions of contact.

3.3.3 DMAP does not appear to affect cell polarisation

The observation of microvilli enclosed by punctate intercellular contacts in DMAP-treated 4-cell embryos suggests that the microvilli on blastomeres of these embryos were not in a polarised distribution, unlike those of untreated, flattened 8-cells (see 1.3, 1.4). To assess this more directly, 4-cell embryos were incubated immediately post-division in DMAP and compared to contemporaneous controls, using scanning electron microscopy. Untreated 4-cell embryos have microvilli evenly distributed over their surfaces (Fig. 3.7A). Microvilli are cleared basolaterally in untreated early 8-cell embryos (Fig. 3.7B) and only a tight pole of microvilli remains, at the apical cell surface, in late 8-cells (Fig. 3.7C).

After 6h or 18h incubation in DMAP and irrespective of the extent of intercellular flattening, the microvilli of DMAP-treated embryos appeared morphologically normal and usually were distributed evenly over the exposed surface of

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Figure 3.7 (facing page)

Do cells polarise after flattening in DMAP?

Scanning electron micrographs of untreated (A) 4-cell embryo, 6h post-division; (B) non-flattened 8-cell embryo, 6h post-division, note clearing of microvilli adjacent to regions of contact; (C) late 8-cell embryo, 9h post-division, note polar distribution of microvilli; (D) 4-cell embryo, 6h post-division, 0-6h DMAP, microvilli evenly distributed; (E) 4-cell embryo, 6h post-division, 0-6h DMAP (as D), patchy distribution of microvilli. Bars =10μm

each blastomere (Fig. 3.7D). A small proportion of blastomeres (0-18% in separate experiments) had zones cleared of microvilli (Fig 3.7E) but these did not appear in any reproducible shape or relationship to cell contacts. Embryos treated with CCD in addition to DMAP, to reverse flattening, still revealed no polarisation of microvilli (data not shown). Blastomeres of embryos treated in this way did show the characteristic patchy, clumped distribution typical of CCD-treated cells (data not shown; see Fleming & Pickering, 1985).

3.4 Effects of DMAP on protein synthesis

3.4.1 DMAP inhibits the incorporation of [^{35}S]methionine into protein

In order to establish that the effects of DMAP on cell flattening were not due simply to mimicking the effects of puromycin on protein synthesis, groups of 4-cell and 8-cell embryos were incubated for 2h immediately post-division in control medium or 4mM DMAP and then transferred to control medium or 4mM DMAP supplemented with 1.5mCi/ml [^{35}S]methionine for a further 1h. Embryos were then washed thoroughly through protein-free medium and treated in one of two ways. To assess uptake of [^{35}S]methionine, embryos were pipetted directly into scintillation vials for counting. To assess incorporation of [^{35}S]methionine into polypeptides, only TCA-precipitable material was counted (see 2.8). As shown in Table 3.3 (overleaf), DMAP has no significant effect on the uptake of [^{35}S]methionine but does significantly reduce its incorporation into protein ($p < 0.01$; Fisher-Behrens test). This effect does not appear specific to particular proteins. Samples containing the same number of untreated or DMAP-treated embryos were separated by SDS-PAGE (Fig. 3.8). A reduction in the intensity of lanes from DMAP-treated samples is apparent but the pattern of protein bands obtained is not detectably different from that of controls. (Fig. 3.8, lane a, control, lane d, 4mM DMAP).

Table 3.3

DMAP inhibits the incorporation of [^{35}S]methionine by 4-cell embryos (3h post-division)

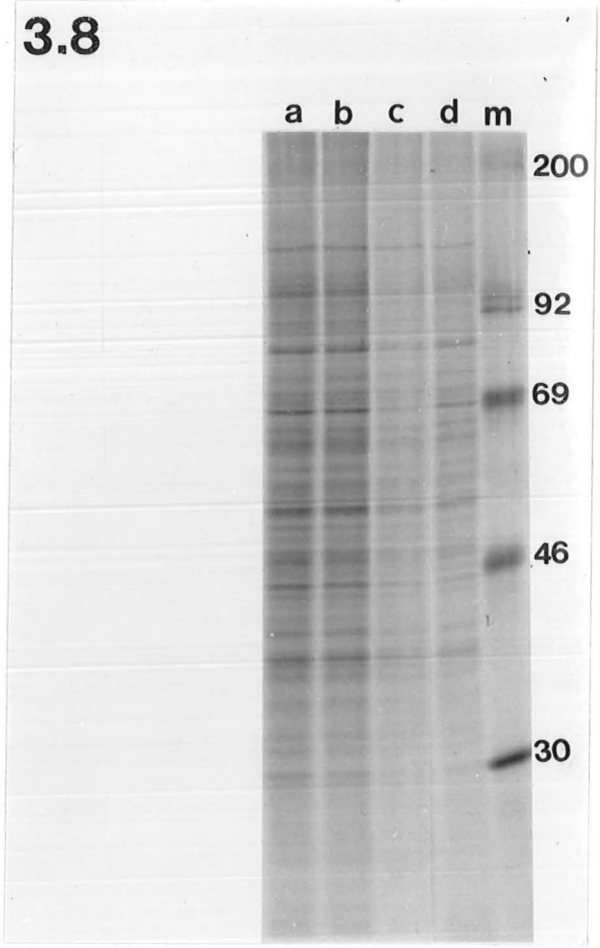
	Uptake (fmol methionine / embryo / h)	Incorporation
control	47.9 ± 10.8	3.2 ± 0.7
4mM DMAP	56.5 ± 20.1	1.8 ± 0.4

Each value represents the mean ± S. D. of 8 samples.

3.4.2 The inhibition of protein synthesis by DMAP is dependent on culture conditions

In all the experiments described so far, DMAP was added to cold culture medium from a refrigerated (4°C) stock solution of 40mM DMAP in M16. The medium was then equilibrated in 5% CO_2 at 37°C for 3-4h before culture of embryos. However, if 16h equilibration of medium containing DMAP had taken place before incubating embryos, no effect on the incorporation of [^{35}S]methionine into polypeptides was detectable (Fig. 3.8, compare lane a, control, and lane b, 16h pre-incubation). If DMAP was added directly to warmed culture medium and equilibrated for only 1h at 37°C before use, the inhibition of incorporation of [^{35}S]methionine into polypeptides was reduced to a level similar to that obtained following 4h pre-incubation (Fig. 3.8, compare lane c, 1h pre-incubation and lane d, 4h pre-incubation). Several different batches of DMAP were assessed in similar experiments. All had similar effects on the rate of protein synthesis, as assessed by SDS-PAGE, and similar temperature-sensitivity (data not shown). One of these batches of DMAP had been shown to have no effect on the rate of protein synthesis in starfish oocytes (gift of Dr. P. Guerrier).

3.8



Unlike the inhibition of incorporation of [^{35}S]methionine into protein, the effects of DMAP on intercellular flattening were apparently unaffected by prior incubation conditions. Groups of 4-cell and 8-cell embryos (50 each, 0h post-division) incubated in 4mM DMAP that had been equilibrated at 37°C for 24h, flattened to 42% and 38% within 30min of transfer to DMAP, achieved 92% and 87% flattening within 3h and remained flattened for at least 12h respectively (comparable to scores obtained in DMAP that had been pre-equilibrated for only 3-4h before addition of embryos, see 3.3.1).

Figure 3.8 (facing page)

The inhibition of [^{35}S]methionine incorporation into polypeptides does not alter the pattern of protein bands obtained by SDS/PAGE but is affected by prior incubation conditions.

One-dimensional SDS/PAGE of polypeptides synthesised by groups of 10 embryos (8-cell, 0-1h post-division) during 1h pulse-labelling with [^{35}S]methionine. The labelling medium used, containing 1.5mCi/ml [^{35}S]methionine, was (a) M16+BSA, (b) 4mM DMAP, pre-equilibrated at 37°C for 16h before adding [^{35}S]methionine and labelling embryos, (c) 4mM DMAP, pre-equilibrated at 37°C for 1h before adding [^{35}S]methionine and labelling embryos, (d) 4mM DMAP, pre-equilibrated at 37°C for 4h before adding [^{35}S]methionine and labelling embryos. All samples including (m), relative molecular mass markers: 200, 92 69, 46, 30 x 10^3, were run on a single gel and each lane was exposed for the same period.

3.5 Discussion

DMAP is an analogue of puromycin that is not predicted to affect the rate of protein synthesis (Fig. 3.1). Nevertheless, the results described here show that DMAP can affect the rate of incorporation of [^{35}S]methionine into TCA-precipitable material (Table 3.3), apparently without affecting either the rate of uptake of [^{35}S]methionine by embryos (Table 3.3) or the synthesis of particular protein bands (Fig. 3.8). This result presumably reflects a global reduction in the rate of protein synthesis in mouse embryos and is in direct contrast to the results obtained with sea urchin, clam and starfish embryos, in which the rate of protein synthesis is unaffected by DMAP. (Rebhun *et al.*, 1973; Neant & Guerrier, 1988).

Somewhat surprisingly, the effect of DMAP on the rate of protein synthesis in mouse embryos can be abolished by prior incubation of the drug at 37°C for several hours (Fig. 3.8). This temperature-sensitivity remains unexplained, but may represent the degradation of DMAP following incubation at 37°C. Alternatively, an unidentified impurity may be present in stocks of DMAP and this impurity may be either accumulated or degraded with time at 37°C. The complexity of culture medium renders difficult the identification of such a compound before and after incubation at 37°C. The temperature sensitivity of DMAP may explain the discrepancy between the results reported here and those of other workers. Although DMAP is unlikely to have been exposed to temperatures higher than room temperature for use with marine eggs and embryos, stock solutions may not have been refrigerated (Rebhun *et al.*, 1973; Neant & Guerrier, 1988). In the recent paper which reports that DMAP also has no effect on protein synthesis in maturing mouse oocytes (Rime *et al.*, 1989), the method of storage and pre-incubation of DMAP is not specified.

As DMAP does affect the rate of protein synthesis in the experiments described here, extreme caution is required in interpretation of the results obtained, as inhibition of protein synthesis itself has effects on progression through cleavage cell cycles (Hultin, 1961; Wilt *et al.*, 1967; Wagenaar, 1983; Harland & Laskey, 1980; Evans *et al.*, 1983; Miake-Lye *et al.*, 1983; Murray & Kirschner, 1989) and also on the events of compaction (Levy *et al.*, 1986). However, some of the effects of DMAP can be clearly distinguished from those of puromycin (see below). These may be due to DMAP

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competing with ATP for sites on protein kinases (see 3.1), or to other, unidentified actions. The inhibition of protein synthesis by DMAP may itself be due to an effect on a protein kinase(s) involved in the control of protein synthesis (Farrell *et al.*, 1977).

DMAP seems to have two distinguishable effects on the passage of blastomeres through the cell cycle. Cells seem to proceed through interphase in the presence of DMAP just as in controls until the point of entry into mitosis. At this point, DMAP-treated cells arrest with an intact nuclear envelope but with partially condensed chromatin (Figs. 3.3, 3.4). This condition is likely to correspond to the late G_2 arrest caused by DMAP in marine embryos (Rebhun *et al.*, 1973). If mitotic cells at metaphase (or later) are placed in DMAP, many proceed to the end of the next cell cycle before arrest late in G_2 (Table 3.3). The second effect of DMAP on passage through the cell cycle is seen in cells that have passed this point at which DMAP can cause arrest in G_2, but have not yet reached metaphase at the time of addition of DMAP. These cells can be arrested in late prophase or early metaphase by DMAP. Such arrest, early in mitosis, has not been described previously for this drug.

Cascades of protein kinase activity are now believed to be central to the control of the eukaryotic cell cycle (reviewed by Lee & Nurse, 1988). Genetic evidence, obtained mainly from studying the fission yeast *Schizosaccharomyces pombe,* has identified key points in the cell cycle at which the activity of particular protein kinases is required for progression, either through interphase (for example, *cdc2*, at "start" in G_1, Nurse & Bissett, 1981) or through mitosis (for example, *cdc2*, Nurse & Bissett, 1981; *wee1,* Russell & Nurse, 1987a; *nim1*, Russell & Nurse, 1987b; all required at the G_2/M transition). As homologs of many of these yeast gene products, with similar functions, have now been identified in higher eukaryotes (Draetta *et al.*, 1987; Lee & Nurse, 1987; Dunphy *et al.*, 1988; Gautier *et al.*, 1988; McConnell & Lee, 1989), protein kinase activity is likely to be important to progression through similar key points in higher eukaryotic cell cycles. DMAP may partially inhibit the activity of many protein kinases; alternatively, it may have different effects on different kinases. In either case, the major effects observed would be due to the inhibition of kinase-mediated, rate-limiting steps in cellular processes. It is possible that DMAP could

achieve the observed arrest points in the cell cycle of mouse blastomeres by affecting only a small number of kinase-mediated steps (such as those dependent on the activity of $p34^{cdc2}$; Simanis & Nurse, 1986).

The effects of DMAP on the processes of cell flattening and polarisation that occur at compaction can be separated from effects on the cell cycle by the use of carefully timed groups of embryos (see 2.1.4). Incubating embryos in medium containing DMAP causes rapid intercellular flattening of 4-cell and 8-cell embryos. However, this flattening is not like the flattening caused by inhibiting protein synthesis with puromycin in two respects. Firstly, DMAP-induced flattening is readily reversed if embryos are washed from DMAP (Fig. 3.5) whereas puromycin-induced flattening continues, and even increases, when embryos are washed from puromycin (Levy *et al.*, 1986). Secondly, and of more significance, DMAP-induced flattening does not seem to be mediated by Ca^{2+} and uvomorulin (Table 3.2). Thus, removal of Ca^{2+} or the addition of antibodies to uvomorulin to the medium, which prevents and reverses the intercellular flattening of compacted 8-cell embryos, does not prevent or reverse DMAP-induced flattening (Table 3.2). However, trypsin does reverse the flattening of DMAP-treated embryos in the presence of EGTA but not in the absence of EGTA (Table 3.2). DMAP is therefore likely to cause flattening *via* the action of a membrane protein with an extracellular Ca^{2+}-binding site that is protected from trypsin digestion by the binding of Ca^{2+} (Hyafil *et al.*, 1980, 1981; Ogou *et al.*, 1982; Peyrieras *et al.*, 1983).

It is possible that DMAP-induced flattening corresponds to the stabilised flattening of late 16-cell embryos which is also reversible using those agents that reverse DMAP-induced flattening but is not affected by antibodies to uvomorulin or by removal of Ca^{2+} from the medium (Kimber & Bird, 1985). This flattening may depend on quite a different system of cell-cell adhesion from that caused by uvomorulin at the 8-cell stage. Proposed mediators of this stabilised flattening include membrane bound galactosyl transferase (Shur, 1983; Bayna *et al.*, 1988), or lectins (Bird & Kimber, 1984; Kimber & Bird, 1985), each of which is likely to act in concert with the cytoskeleton to achieve changes in cell shape.

The establishment of cell polarity, like cell flattening, seems to be dependent on

uvomorulin to some extent (see 1.4). As DMAP-induced premature flattening of 4-cell embryos does not appear to be mediated by uvomorulin, it is perhaps not surprising that this flattening is not associated with polarisation of blastomeres (Fig. 3.7). It seems possible that DMAP has allowed the experimental dissociation of cell flattening from cell polarisation, bypassing the role of uvomorulin in the establishment of flattening and polarisation and achieving prematurely the stabilised cell apposition characteristic of 16-cell morulae. In Chapter 6, the possibility that the flattening caused by DMAP is mediated by altered protein phosphorylation will be considered.

CHAPTER FOUR

THE EFFECTS OF PHORBOL ESTER ON 8-CELL BLASTOMERES SUGGEST A ROLE FOR PROTEIN KINASE C IN COMPACTION

CHAPTER FOUR THE EFFECTS OF PHORBOL ESTER ON 8-CELL BLASTOMERES SUGGEST A ROLE FOR PROTEIN KINASE C IN COMPACTION

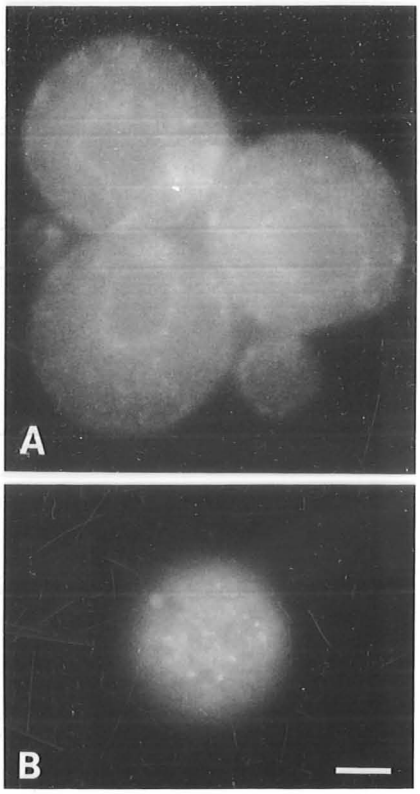
4.1 Introduction

The processes of cell flattening and polarisation can be considered to consist of initiation, progression and stabilisation phases. Both the initiation and progression of compaction appear to be controlled entirely post-translationally, using proteins that are present in the embryo from at least the early 4-cell stage (see 1.4). Once initiated, compaction seems to progress *via* spreading of a zone of cell flattening and of cytoplasm depleted of assembled cytoskeletal elements and cell organelles. Both cell flattening and cytoplasmic depletion are localised initially to the points of cell contact. The spreading of the zone of cytoplasmic depletion beyond the immediate region of contact involves an increasing proportion of the cell perimeter until the majority of cell organelles and assembled cytoskeletal elements are at the apices of late 8-cell "polarised" blastomeres (Fig. 1.3). In this chapter the effects on blastomeres of phorbol myristate acetate (PMA), a potent stimulator of the membrane-associated, Ca^{2+}- and phospholipid-dependent protein kinase, protein kinase C (PKC), are described.

Cell-cell contact can induce some aspects of cell polarity in late 8-cell blastomeres even in conditions in which the cytoskeleton is severely disrupted. It has therefore been suggested that the signal underlying the progression of compaction may reside in a propagated change in the plane of the membrane or cortical cytoskeleton. This change might affect either the membrane itself or membrane-associated proteins such as cytoskeletal-organising proteins (Johnson & Maro, 1986; Johnson *et al*, 1986a; Fleming & Johnson, 1988; see 1.4). Such a post-translational modification of proteins in the cytocortex might later become re-enforced and stabilised by additional processes such as the flow of positive ions through the cell from apex to base (Nucitelli & Wiley, 1985; Wiley & Obasaju, 1988, 1989; 1.4). Intracellular second messengers and associated post-translational modifications are obvious possible mediators of a propagated change in the region of the cell membrane.

PKC acts in the inositol phospholipid signalling pathway in a wide variety of cell types to achieve both immediate and long-term alterations to cell physiology (Nishizuka, 1984, 1988). The effects of PMA on cell flattening and the cytoskeleton in 8-cell blastomeres suggest that PKC activity may be involved in the mechanisms underlying the progression of cell flattening and polarisation.

4.1



4.2 PMA binds oocytes and embryos

In order to confirm binding of PMA to cells and to assess the localisation of any binding sites, oocytes and blastomeres were cultured for 1h in the presence of the biologically active, fluorescent derivative of PMA, dansyl PMA (Liskamp *et al.*, 1985), with or without competition by an excess of unlabelled PMA. Embryos were then fixed and observed by indirect immunofluorescence. Oocytes and blastomeres of all stages showed bright punctate staining around the cell perimeter after incubation with dansyl PMA, with patchy, diffuse staining throughout the cytoplasm but excluded from the nucleus (for example, 4-cell embryo, Fig. 4.1). Pre-incubation for 30min with 100- or 1000-fold excess of unlabelled PMA followed by the addition of dansyl PMA for a further 30min prevented any detectable fluorescent binding. If both dansyl and unlabelled PMA were present throughout incubation, 1000-fold excess of unlabelled PMA prevented any detectable binding of dansyl PMA.

4.3 PMA reverses and prevents intercellular flattening

4.3.1 Flattening of intact embryos

4.3.1.1 Prevention of the development of cell flattening

Completely non-flattened 8-cell embryos, 0h or 3h post-division, were incubated in control medium or medium containing 100ng/ml or 25ng/ml PMA and scored hourly for evidence of flattening. Some embryos were left in PMA-containing

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Figure 4.1 (facing page)

Fluorescent localisation of PMA-binding sites in 4-cell embryos assessed after 1h incubation in 25ng/ml dansyl PMA before fixation.

A. Optical section through blastomeres showing patchy, diffuse staining throughout the cytoplasm but excluded from nuclei.

B. Tangential view showing bright, punctate staining at the blastomere perimeter. Bar=10μm

4.2

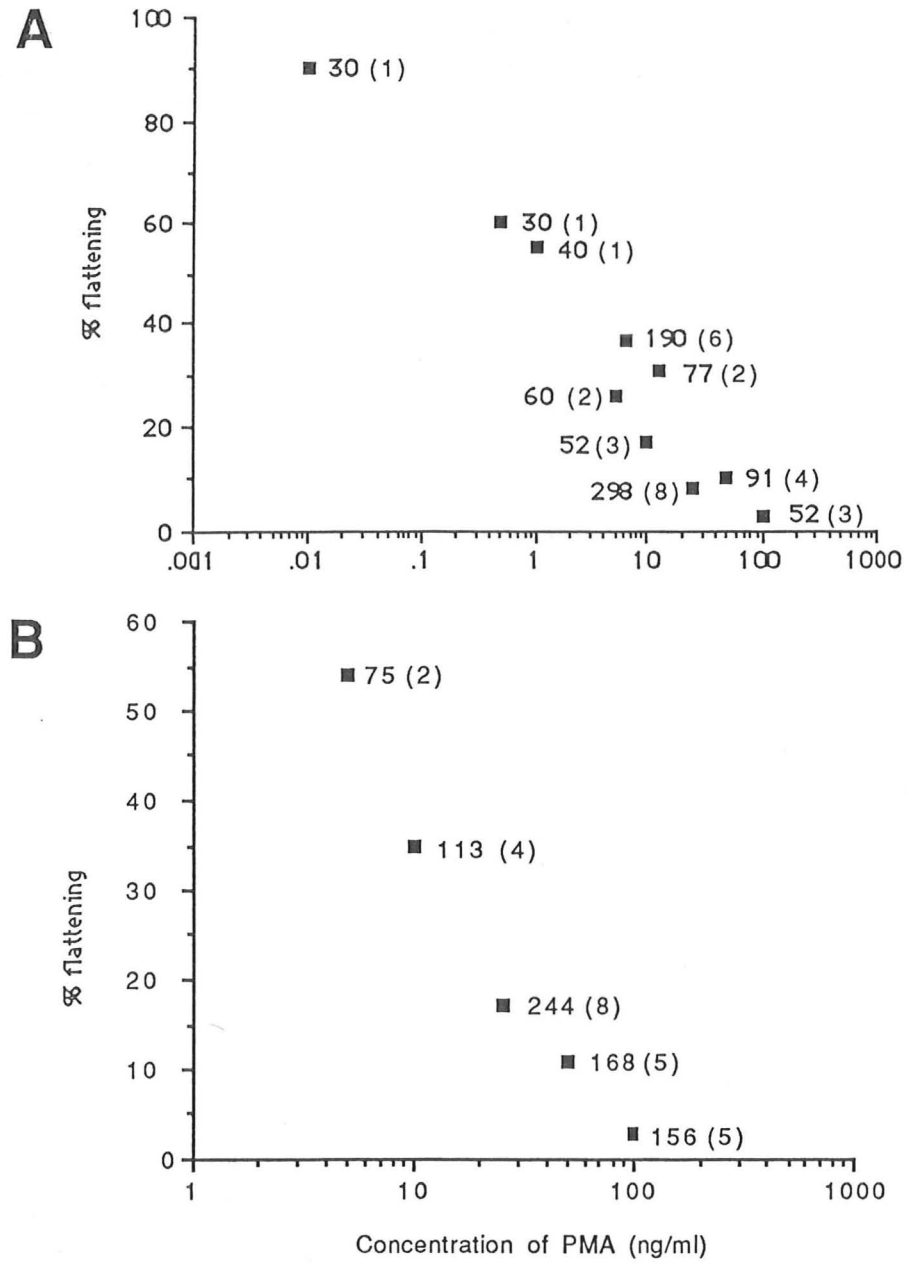


Figure 4.2
The effects of various concentrations of PMA on flattening in whole embryos and pairs of cells
A. Plot of the effects of PMA on flattening in fully flattened 8-cell embryos (7-8h post-division). The vertical axis expresses the degree of flattening as a percentage of the maximum possible were all embryos fully flattened. The figures beside each point indicate the number of embryos exposed to each condition and those in brackets show the number of experimental replicates. The scores from separate experiments were never more than ± 5% different from the averages shown. Embryos placed in medium containing no PMA scored 86% (between 80% and 100% in separate experiments; 218 embryos).
B. Plot of the effects of PMA on intercellular flattening in fully flattened 2/8 cell pairs (7-8h post-division). Pairs placed in medium containing no PMA scored 89% after 1h (n=182)

medium for up to 8h while others were washed and transferred into control medium after 1h. No embryo exposed to PMA, using any schedule, flattened within 8h of exposure to PMA (12 experimental groups, 386 embryos). Controls flattened to an average of 94% of the maximum possible (6 groups, 250 embryos).

4.3.1.2 Reversal of established cell flattening

Eight-cell embryos (7-8h post-division) that were scored as fully flattened were placed in control medium or medium containing PMA at various concentrations for 1h and scored again for flattening. The results are plotted in Figure 4.2A. In the concentration range over which PMA is known to activate PKC (Nishizuka, 1984), a dose-dependent reversal of intercellular flattening was seen. Only 10% of the maximum possible flattening remained after 1h exposure to 25ng/ml PMA.

4.3.2 Flattening of 2/8 pairs of cells

Fully flattened 2/8 pairs of blastomeres (8h post-division) were similarly exposed to various concentrations of PMA and the extent of flattening assessed. The results are plotted in Figure 4 .2B. The extent of intercellular flattening in 2/8 pairs is reduced by PMA similarly to intact embryos. The flattening score for each concentration of PMA is slightly higher in the case of 2/8 pairs. This is probably due to easier visualisation of contact zones and, hence, easier detection of any residual flattening in pairs of cells.

4.3.3 Protein synthesis is required to restore flattening after exposure to PMA

To determine whether the inhibition and reversal of flattening by activators of PKC was reversible, compacted 8-cell embryos (7h post-division) were exposed to PMA for 1h and then washed and cultured in control medium and scored for flattening over the ensuing 24h of culture. The results, plotted in Figure 4.3A, show that the reversal of flattening persisted for 6h. Flattening was then restored gradually over a period of 12h. This recovery of flattening was slower than that seen after exposure to medium depleted of Ca^{2+} (full flattening within 1h of restoring Ca^{2+}) and slower than

4.3

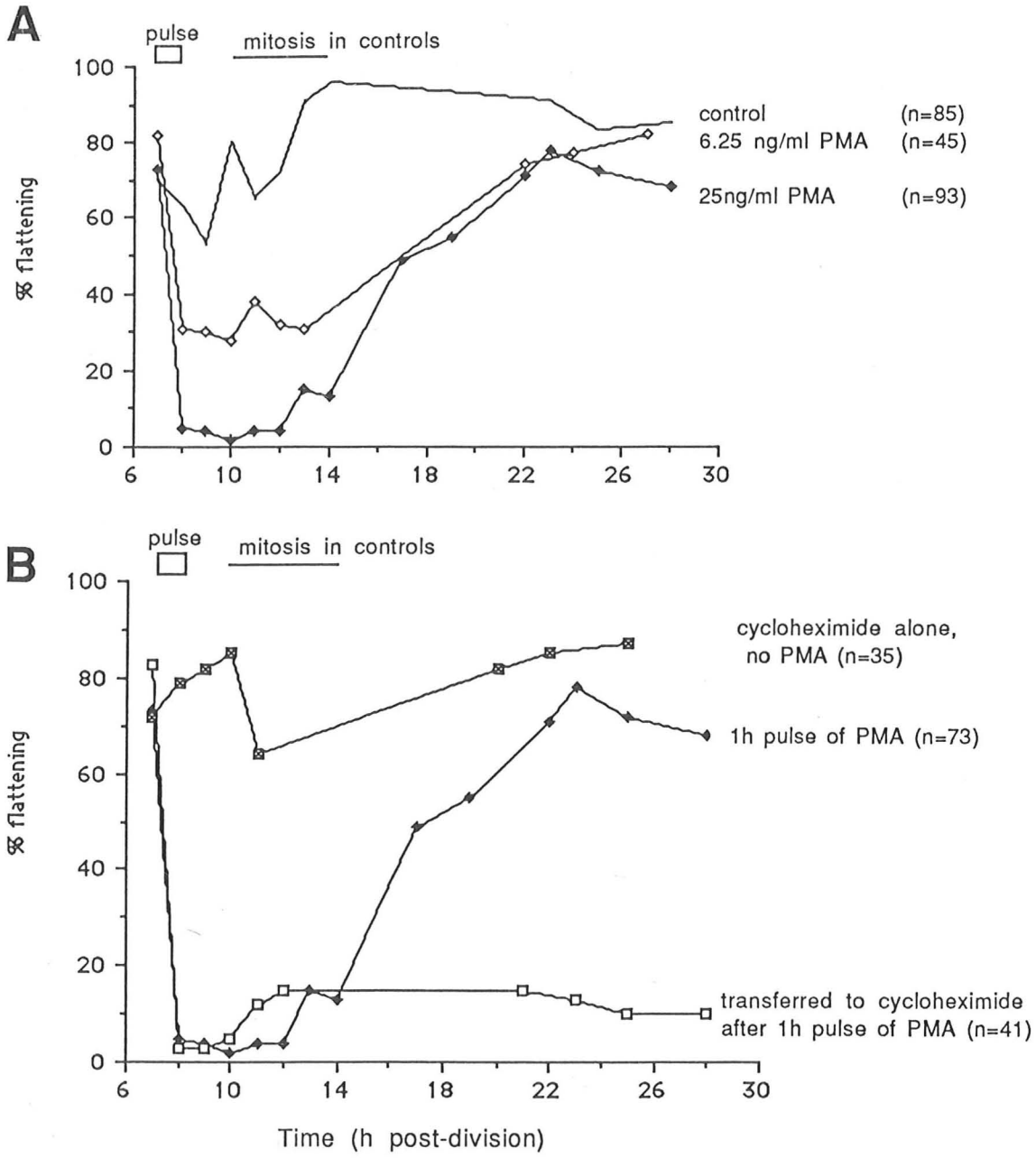


Figure 4.3
Time course of intercellular flattening in 8-cell embryos exposed to 25ng/ml PMA for a 1h period at 7h post-division, washed and incubated in (A) M16+BSA or (B) M16+BSA containing 400μM cycloheximide for 24h. The figures in brackets indicate the number of embryos examined under each condition. The period during which mitosis of 8- to 16-cell blastomeres occurred is indicated; this coincided with a transient reversal of flattening as shown (Lehtonen, 1980). Embryos incubated in PMA or cycloheximide did not cleave during observation.

the original process of flattening during the 8-cell stage (full flattening by 7-8h post-division). During the period of observation, blastomeres in control embryos continued to divide and cavitation began in these embryos.

Protein synthesis is required for the restoration of intercellular flattening after exposure to PMA. Embryos that were exposed to PMA for 1h and then transferred to medium containing 400μM cycloheximide failed to flatten within 24h (Fig. 4.3B). Cycloheximide alone had no detectable effect on intercellular flattening.

4.4 PMA causes specific disruption of the cytoskeleton of 8-cell embryos

4.4.1 PMA can cause oocyte activation and affect the organisation of the oocyte cytoskeleton under certain experimental conditions

PMA has been reported to cause activation of unfertilised oocytes at concentrations considerably higher than those used here (0.1-1μg/ml: Cuthbertson & Cobbold, 1985). However, at 25ng/ml, the extent of oocyte activation and the effects of PMA on the organisation of microfilaments and microtubules in both oocytes and embryos were found to depend on an interaction between the effects of PMA and the effects of other experimental manipulations.

Since immunocytochemical analysis requires removal of the zona pellucida, preliminary experiments were undertaken to determine the influence of this procedure on the outcome of experiments. Untreated oocytes are arrested in metaphase II of meiosis. Their chromatin is condensed into metaphase chromosomes on the equatorial plate of the meiotic spindle. Microtubules are found exclusively in the spindle (Fig. 4.4A, B; Wassarman & Fujiwara, 1978; Maro *et al.*, 1985a; Schatten *et al.*, 1985). Microfilaments are organised into two domains. In the region of the spindle, microfilaments form a dense cortical "cap" overlying the spindle. The region covered by this cap is depleted of microvilli. Microvilli, with microfilamentous cores, and a fine cortical mesh of microfilaments are evenly distributed over the remainder of the oocyte surface (Fig. 4.7A; Eager *et al.*, 1976; Maro *et al.*, 1984; Longo & Chen, 1985). In fertilised or parthenogenetically activated oocytes, the microtubules form a dense

cytoplasmic and cortical mesh after completion of meiosis (Fig. 4.4C, D; Maro *et al.*, 1985a; Schatten *et al.*, 1985). Microfilaments are distributed around the pronuclei, in a uniform cortical mesh and in the cores of microvilli (Fig. 4.7B; Maro *et al.*, 1984).

Groups of oocytes had their zonae removed with acid Tyrode's solution, were allowed a recovery period of 1h or 6h and were then exposed to PMA. Oocytes were then centrifuged onto microscope coverslips in chambers and treated in one of two ways. One group were fixed immediately after centrifugation and double-stained for the distribution of microfilamentous actin and chromatin. The second group were recovered for 10min at 37°C, extracted for 5min in 0.25% Triton X-100 and then fixed before double-staining for the distribution of microtubules and chromatin (see 2.5.2 for precise methods). If oocytes were allowed to recover for 6h after zona removal and then exposed to 25 ng/ml PMA, the distributions of microfilamentous actin, microvilli, microtubules and chromatin were all indistinguishable from similarly treated or untreated controls (as described above; see also below, Figs. 4.5-4.7). However if oocytes were exposed to PMA after only 1h recovery following zona removal, different results were seen in oocytes prepared for microfilament or microtubule visualisation.

In approximately 50% of oocytes that were exposed to PMA 1h after zona removal and immediately fixed for actin visualisation, some chromosomes were detached from the metaphase plate. The "cap" of microfilaments was enlarged or split to lie over the chromosomes in these oocytes (Table 4.1A, overleaf). The level of activation of oocytes, judged by the presence of non-metaphase chromatin, was similar to controls. Oocytes that had been exposed to PMA and were then extracted prior to fixation and stained for tubulin distribution, by contrast, showed a considerably higher level of activation than controls that had been treated similarly (Table 4.1B). Most of the non-metaphase oocytes in the PMA-treated group seemed to have been activated very recently prior to fixation as they were in anaphase or telophase (Table 4.1B). The microtubule organisation of these PMA-treated oocytes also suggested recent activation, as microtubules were present in both the cell cortex and the anaphase or telophase spindle (Table 4.1B; Fig 4.4G, H; Maro *et al.*, 1985a). In addition, in approximately 10% of PMA-treated oocytes, the chromatin seemed to remain in metaphase chromosomes that were arranged on the spindle but microtubules were present in both the cell cortex and

Table 4.1

Interaction of effects of PMA and experimental procedures on oocytes

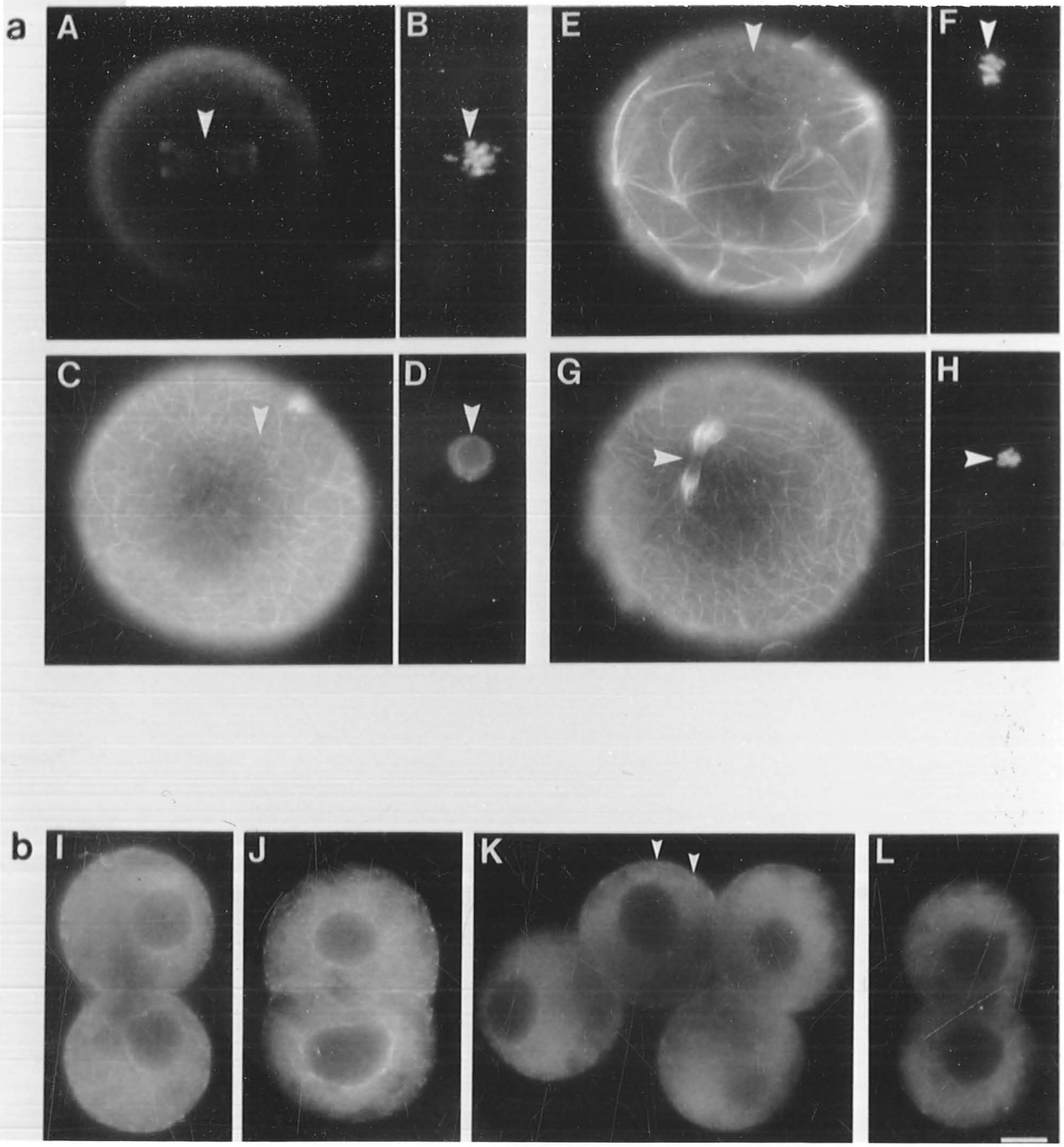
A. Oocytes prepared for microfilament visualisation.
Oocytes had their zonae removed, were allowed to recover for 1h, were incubated ± 25ng/ml PMA for 1h and were then fixed (see text and Fig. 4.7).

	n	Microfilaments (% oocytes)		
		cap	larger / split cap	none
Control:	38			
Chromatin				
Metaphase Plate		92	0	0
Pronucleus		5	0	3
25ng/ml PMA:	37			
Chromatin				
Metaphase Plate		43	0	0
Dispersed metaphase		0	51	0
Pronucleus		5	0	0

B. Oocytes prepared for tubulin visualisation
Oocytes had their zonae removed, were allowed to recover for 1h, were incubated ± 25ng/ml PMA for 1h and were then extracted before fixation (see text and Fig. 4.4).

	n	Microtubules (% oocytes)				
		spindle only	disrupted spindle	cortex only	spindle and cortex	none
Control:	55					
Chromatin						
Metaphase Plate		82	0	0	0	11
Pronucleus		0	0	7	0	0
25ng/ml PMA:	59					
Chromatin						
Metaphase Plate		5	7	0	10	7
Anaphase/telophase		0	13	17	22	0
Pronucleus		0	0	19	0	0

4.4



the meiotic spindle (Table 4.1B). The cortical microtubules in these oocytes tended to form large cortical asters comprising long, thick bundles (Fig. 4.4E, F). This phenomenon may represent an extremely early effect of activation of oocytes with PMA or may reflect effects of PMA on microtubule organisation separate from activation.

These results suggest that PMA can affect the meiotic arrest of oocytes and can affect their cytoskeletal organisation. However, PMA has no effect on oocytes unless it is either present at a very high concentration (Cuthbertson & Cobbold, 1985) or is combined with other experimental manipulations such as extraction or zona removal. It may be significant that removal of the zona with acid Tyrode's solution can itself cause activation of some oocytes (Johnson *et al.*, 1989). It seems likely that each of the routine procedures used in handling oocytes makes them prone to activation or cytoskeletal re-organisation; in combination, and after exposure to PMA, these procedures can have more severe effects.

Figure 4.4 (facing page)

Effects of PMA on microtubule organisation following 1h recovery from zona removal

a. Oocytes double-stained for microtubules (A, C, E, G) and chromatin (B, D, F, H). (A, B) Untreated metaphase oocyte with microtubules exclusively in the spindle and chromosomes arranged on the metaphase plate; (C, D) spontaneously activated oocyte with microtubules in a cortical mesh and chromatin in a single pronucleus; (E, F) PMA-treated "metaphase" oocyte with microtubules in cortical asters of long, thick bundles; (G, H) anaphase, PMA-activated oocyte with microtubules in both spindle and cortex. Arrowheads in each pair of frames indicate the position of the chromosomes or pronucleus

b. Blastomeres from untreated 2/8 pairs of cell, (I) 1h post-division, non-flattened; (J) 6h post-division, fully flattened; (K) PMA-treated 8-cell embryo, 6h post-division, with very few microtubules evident, arrowheads point to cortical microtubules. The staining in a 2/8 cell pair treated with 10μM nocodazole (L) is shown for comparison. Bar=10μm

4.4.2 The effects of PMA on microtubule organisation of blastomeres also reveal interaction with the effects of Acid Tyrode's solution

As was found for oocytes, the effects of PMA on microtubule organisation in blastomeres also depended on the sequence of treatments of the embryos. Some zona-intact embryos were exposed to PMA followed by removal of the zona using acid Tyrode's solution just before fixation and analysis. Other embryos had their zonae removed with acid Tyrode's solution followed by a recovery period of up to 6 hours before exposure to PMA. When exposure to PMA was followed by removal of the zona prior to fixation, almost all microtubules in fully flattened, late 8-cell embryos (6h post-division) were destroyed (Fig. 4.4K). By contrast, the cortical and cytoplasmic mesh of microtubules in 1-cell, 2-cell, 4-cell or early (1h post-division) 8-cell embryos were not obviously affected.

However, as was found for oocytes, when microtubules were examined in embryos that had been exposed to PMA after allowing a 6h recovery period after zona removal, the microtubule network was not detectably different from that of similar, untreated embryos at any stage examined. Moreover, the incidence of a polar distribution of microtubules in late 8-cell embryos was similar to that of controls (Table 4.2, overleaf). These results indicate that PMA may have some stage-dependent effect on microtubule organisation in compact 8-cell embryos, but that this effect is only revealed after exposure to acid conditions.

4.4.3 Effects of PMA on the organisation of microvilli and microfilaments

4.4.3.1 Microvilli of intact embryos examined by scanning electron microscopy

Zygotes and blastomeres of 2-cell, 4-cell and early 8-cell embryos have a dense, even covering of short microvilli over the entire blastomere surface, absent only from regions immediately adjacent to cell-cell contacts (Fig. 4.5B, C), and, in unfertilised oocytes, in the area overlying the meiotic chromosomes (Fig. 4.5A; Eager *et al.*, 1975). During the 8-cell stage, microvilli are lost progressively from regions further from cell contacts until the only microvilli remaining are in an apical pole (Fig. 4.5D).

Table 4.2

Effects of PMA and zona removal on the distribution of microtubules in late 8-cell embryos (7-8h post-division)

	Drug	No. of cells	Incidence of microtubules (% cells)				Polarity of microtubules (%)*		mitotic/ fused cells
			like controls	fewer	sparse	none	apolar	polar	
A	mPMA+	225	84	0	0	0	41	43	17
	Nocodazole	228	2	2	31	61	2	0	4
	PMA	266	1	34	52	8	1	0	6
B	mPMA+	121	94	0	0	0	74	20	6
	PMA	163	94	0	0	0	51	43	6

A Zona removal after exposure to PMA, immediately prior to fixation

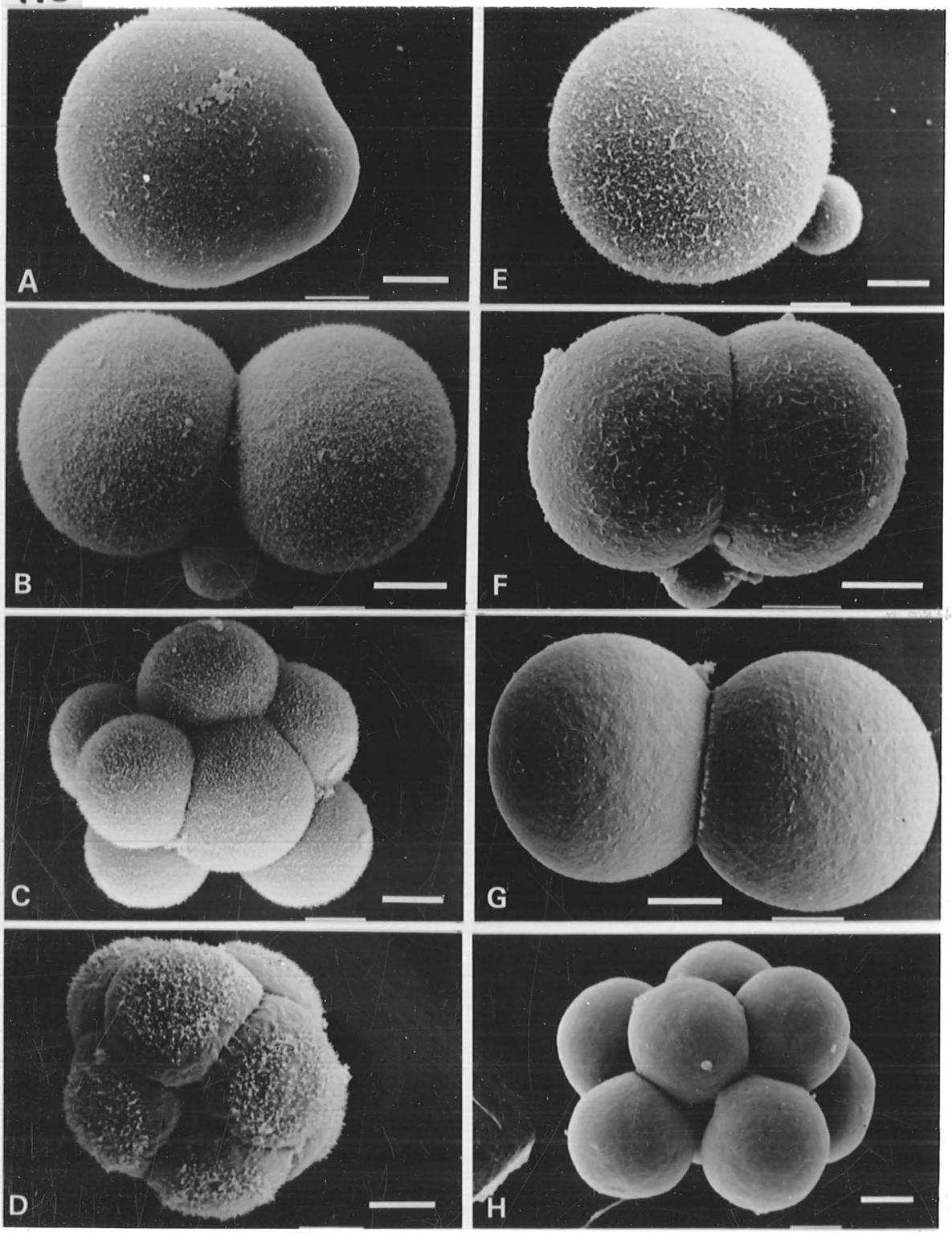
B Six hour recovery period allowed after zona removal before exposure to PMA and immediate fixation.

Drug concentrations: PMA and mPMA, 25ng/ml; nocodazole, 10µM

*Polarity is expressed only for those cells having similar numbers of microtubules to controls.

+mPMA is an inactive analogue of PMA (see p.58)

4.5



Oocytes and embryos at different developmental stages were exposed to PMA, and blastomere surfaces examined by scanning electron microscopy. As described above, PMA can affect microtubule and microfilament organisation in oocytes, but only if less than 6h recovery after zona removal has been allowed. Exposure to PMA following a 6h recovery period was also found to have little effect on the morphology or distribution of microvilli on oocytes (Fig. 4.5A, E). In embryos, observations of microvilli by scanning electron microscopy and of microfilamentous actin by immunofluorescence also revealed an interaction of the effects of PMA and zona removal with acid Tyrode's solution similar to that described for oocytes and for microtubules in embryos. If zona removal was followed by a recovery of less than 6h before exposure to PMA, a disruption of the actin cytoskeleton and of microvilli was detected in embryos of all stages (Tables 4.3 & 4.4, overleaf). Hence in all the experiments described below, a minimum of 6h recovery after zona removal was allowed.

Exposure of embryos to PMA following 6h recovery after zona removal caused a loss of microvilli that was more severe the later the developmental stage examined, with more blastomeres having fewer visible microvilli or none at all. From the 1-cell to early 8-cell stages, a small proportion of blastomeres had no microvilli and increasing numbers had fewer microvilli than controls (Fig. 4.5F, G). In late 8-cell and 16-cell embryos, most blastomeres had no microvilli at all (Fig. 4.5H). The morphology of microvilli also changed with the developmental stage when exposed to PMA. The

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Figure 4.5 (facing page)

Scanning electron micrographs of oocytes and embryos following 1h incubation in control medium (A-D) or 25 ng/ml PMA (E-H). The stages shown are oocyte (A, E), early 2-cell (B, F), late 2-cell (G), early 8-cell (C) and late 8-cell (D, H). The microvilli in (D) show a typically polar distribution, those in (F) are longer and thinner than those of controls and those in (G) are both shorter and more sparse than those of control embryos. A late 8-cell embryo exposed to PMA (H) has no detectable microvilli. Bar = 10μm in each frame

microvilli of oocytes exposed to PMA were indistinguishable from those of controls. One-cell embryos exposed to PMA were almost all covered in microvilli longer than those of control embryos and lying parallel to the cell surface rather than perpendicular as in controls (Fig. 4.5F). The incidence of this sort of microvilli was lower in later developmental stages which tended to have very short, sparse microvilli (Fig. 4.5G). The proportion of blastomeres with each type of microvilli at each stage is summarised in Figure 4.6. It is clear that major changes in the response to PMA occur after fertilisation and, in particular, at the 8-cell stage.

Table 4.3

Effect of recovery period after zona removal on microvilli of late 2-cell embryos

Recovery period (h)	Drug	n (cells)	Microvilli					
			Distribution (%)			Morphology (%)		
			normal	sparse	none	normal	long	short
0	mPMA*	26	50	0	50	50	0	0
0	PMA	28	0	50	50	0	50	0
3	mPMA*	28	75	25	0	75	25	0
3	PMA	22	0	59	41	0	0	59
6	mPMA*	12	83	17	0	83	17	0
6	PMA	33	6	94	0	6	94	0

Table 4.4

Effect of recovery period after zona removal on actin staining in late 2-cell embryos

Recovery period (h)	Drug	n (cells)	Staining (%)		Mitotic/fused cells
			fine+punctate	patchy	
0	mPMA*	15	60	20	20
0	PMA	31	61	29	10
3	mPMA*	20	50	5	45
3	PMA	36	67	6	28
6	mPMA*	40	95	0	5
6	PMA	45	93	0	7

*mPMA is an inactive analogue of PMA (see p.58)

4.6

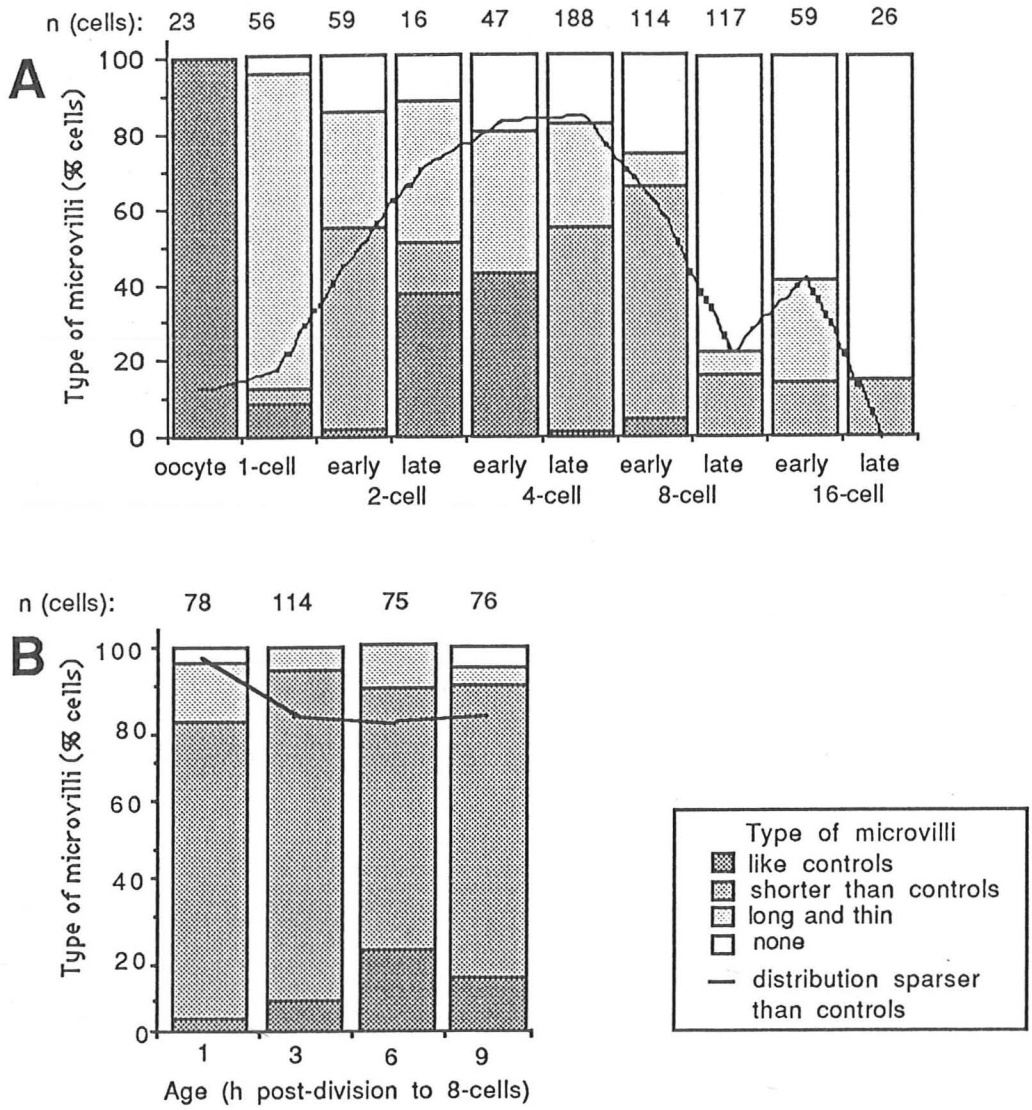


Figure 4.6
Summary of effects of incubation in 25ng/ml PMA on distribution and morphology of microvilli of embryos of different developmental stages and cell 2/8 pairs.
A. Whole embryos. The precise timings of the developmental stages are:
oocyte: 19-20h post-HCG; late 4-cell: 60h post-HCG;
1-cell: 25-28h post-HCG; early 8-cell: 3-4h post-division;
early 2-cell: 38h post-HCG; late 8-cell: 8-9h post-division;
late 2-cell: 54-58h post-HCG; early 16-cell: 6h post-compaction;
early 4-cell: 52-54h post-HCG; late 16-cell: 12h post-compaction.
B. 2/8 pairs of cells of different ages post-division.
The figures above each bar indicate the number of cells of each stage examined.

No PMA-treated blastomeres from 8-cell embryos were observed to have tightly polarised microvilli. Poles were observed in 87% (n=246) of blastomeres of control embryos and 86% (n=219) of blastomeres in embryos exposed to 25ng/ml mPMA at 8-9h post-division.

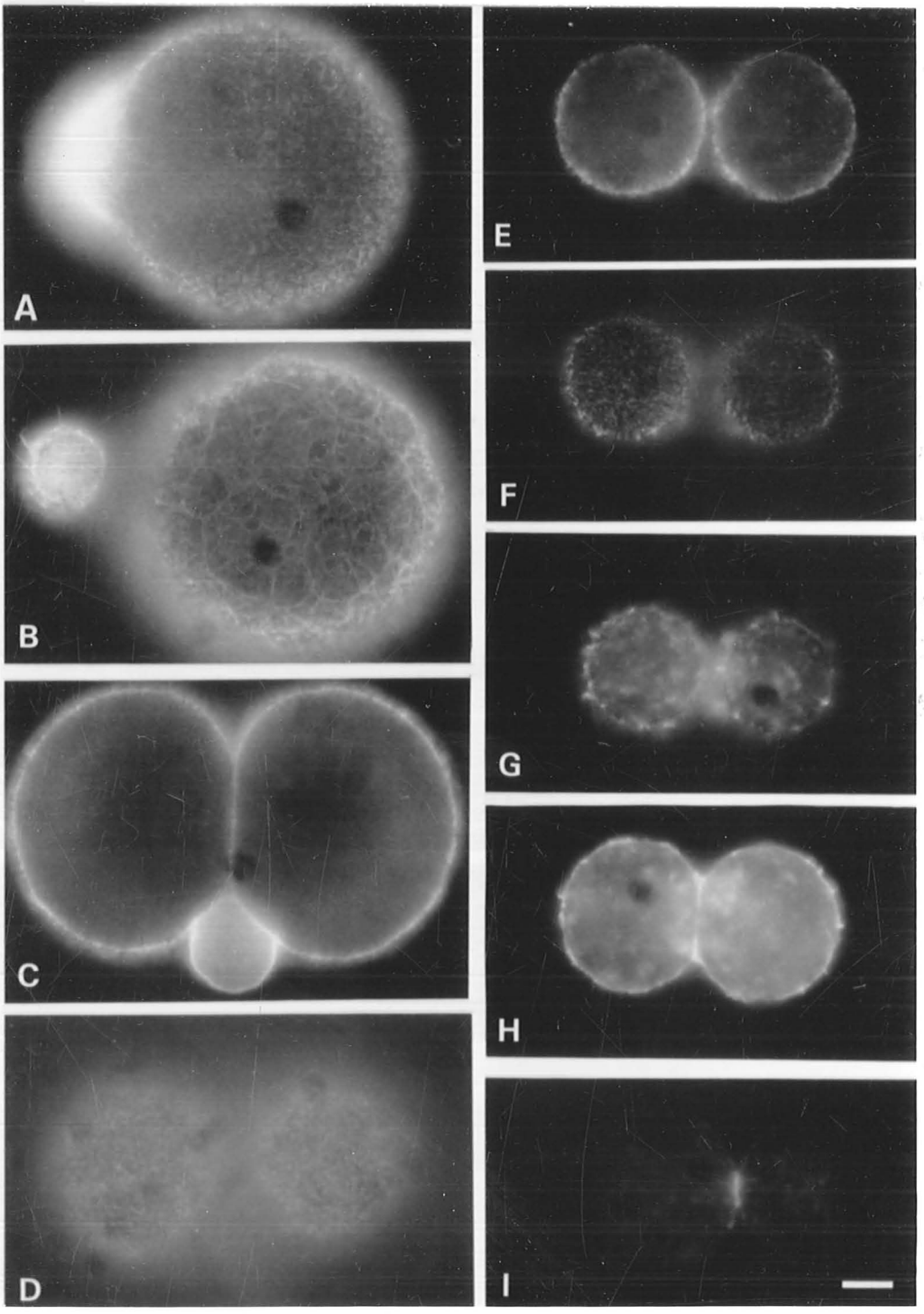
4.4.2.2 Distribution and morphology of microvilli on 2/8 cell pairs, assessed by scanning electron microscopy

In an attempt to identify more precisely the time during the 8-cell stage at which microvillous loss is caused by PMA, timed groups of 2/8 pairs of blastomeres were examined. These could be synchronised more precisely to the previous cleavage than blastomeres in intact embryos. The morphology and distribution of microvilli on 2/8 pairs exposed to PMA is summarised in Figure 4.6B. Most blastomeres of each age post-division had very short, sparsely distributed microvilli in a similar proportion of blastomeres to late 4-cell or early 8-cell embryos exposed to PMA (Fig. 4.6A). Few blastomeres had no microvilli, in contrast to cells of similar ages post-division that were exposed to PMA while part of an intact embryo. This result may be related to the different proportion of the blastomere surface that is covered with microvilli or that is involved in cell-cell contact in pairs of blastomeres compared to intact embryos (see 4.7 for discussion).

4.4.2.3 Fluorescent visualisation of microfilamentous actin with phalloidin

Microvilli can also be visualised by fluorescent labelling with FITC-phalloidin, which binds to polymerised actin, revealing the microfilamentous actin cores of microvilli. Oocytes, embryos and 2/8 pairs of blastomeres were exposed to PMA and then stained with phalloidin. At most stages, the pattern of staining was indistinguishable from that of controls. Oocytes stained intensely in a cortical cap overlying the meiotic spindle and also in microvilli over the remainder of the surface (Fig. 4.7A; contrast with data in Table 4.1 in which only 1h recovery following zona removal was allowed). In zygotes, microvilli and a cortical mesh of microfilaments were apparent (Fig. 4.7B). In later embryos, the staining appeared only as fine spots in the region of the cell cortex and membrane, presumably reflecting microvillous cores (Fig. 4.7C, D). After exposure

4.7



to PMA, a small proportion of blastomeres in late 4-cell embryos and early 8-cell blastomeres (1-6h post-division) had large patches of staining in the cell cortex and some had no staining at all, but most remained like controls (Fig. 4.7E, F). In later 8-cell blastomeres (8h post-division), 60% of cells did not stain at all with phalloidin and the remainder had large cortical patches of staining (Fig. 4.7G-I; summarised in Table 4.5). None of the PMA-treated blastomeres of any age were scored as having tight poles of phalloidin staining; 38% (n=86) of untreated blastomeres at 8h post-division were scored as polarised by this method.

Table 4.5

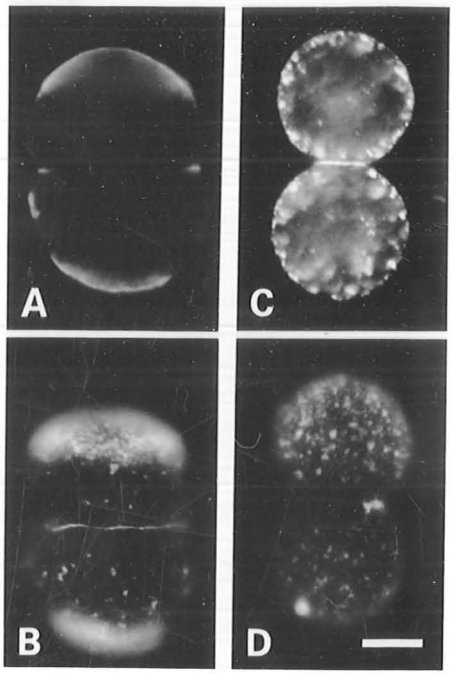
Effect of PMA on distribution of polymerised actin visualised with FITC-phalloidin

Developmental Stage (as Fig. 4.6)	No. of cells	Staining %			% Poles in controls of same age
		like controls	patchy	none	
oocyte	38	100			0
early 2-cell	31	100			0
late 2-cell	20	100			0
late 4-cell	57	86	4	10	0
2/8 pair:					
1h post-division	61	100			0
2h post-division	40	100			8
3h post-division	57	68	14	18	10
4h post-division	24	96	0	4	14
6h post-division	68	97	3	0	25
8h post-division	114	2	36	62	70

Figure 4.7 (facing page)

Distribution of polymerised actin in untreated (A) oocyte, (B) zygote and (C, D) late 2-cell embryo, that are indistinguishable from those in PMA-treated groups. (E-I) pairs of 8-cell blastomeres are aged (E, F) 1h post-division, appearing like controls, (G, H) 3h post-division, staining is patchy and (I) 8h post-division, very little staining evident. All are stained with FITC-phalloidin. Optical sections through cells (B, C, E, H, I) and tangential views (A, D, F, G) are shown. Bar=10µm

4.8



4.4.2.4 Fluorescent visualisation of Con A receptors

The final method used to visualise microvilli and the distribution of surface membrane was labelling with FITC-Con A. Con A binds to receptors in the plasma membrane and therefore highlights regions of high membrane density such as microvilli (Handyside, 1980). Late 2/8 pairs of cells (8h post-division) were exposed to various concentrations of PMA and mPMA before staining with Con A. Untreated and mPMA-treated cells all had fine, punctate staining over the surface of the cell that was absent basolaterally, consistent with the distribution observed for microvilli. All concentrations of PMA produced large patches of staining and reduced total staining (Fig. 4.8). Where any staining was evident, the incidence of polarity of Con A binding was also greatly reduced in cells exposed to PMA compared to controls (Table 4.6).

Table 4.6

Effect of PMA on distribution of Con A receptors on 2/8 pairs of cells, 8h post-division

Drug	Concentration (ng/ml)	No. of cells	Staining %			Poles %			
			like controls	*patchy	none	apolar	broad pole	tight pole	fused/ mitotic
none		125	97	2	0	19	12	67	1
mPMA	25	32	94	0	0	22	9	63	6
PMA	5	70	24	70	0	57	13	24	5
PMA	10	71	23	68	2	60	30	2	7
PMA	25	130	8	86	0	83	12	0	6
PMA	50	106	26	68	0	88	4	0	7
PMA	100	31	0	97	0	94	3	0	3

* See text and Fig. 4.8; poles were scored as "broad" if more than 1/3 cell was stained

Figure 4.8 (facing page)

Pairs of 8-cell blastomeres (8h post-division) examined for distribution of surface Con A receptors. Pairs of cells were incubated for 1h in control medium (A, B) or medium containing 25ng/ml PMA (C, D) then washed and exposed to FITC-Con A prior to fixation. Cells are shown in section (A, C) and in tangential view (B, D). Bar= 20μm

4.5 Effects of other activators of PKC are like those of PMA

4.5.1 Effects on intercellular flattening

To determine whether the effect of PMA on intercellular flattening correlates with the activation of PKC, embryos were exposed to other biologically active and inactive phorbol esters and a synthetic diacylglycerol (OAG) and the effects on flattening scored (Table 4.7). Only those drugs capable of activating PKC, namely PMA, 4β-phorbol-12,13-didecanoate (4βPDD) and OAG (40μg/ml but not 4μg/ml), reversed flattening of previously fully flattened embryos. 4βPDD showed very similar dose-response behaviour to that of PMA while 40μg/ml OAG partially reversed flattening. 4α-phorbol-12,13-didecanoate (4αPDD) up to 100ng/ml and phorbol 12-myristate 13-acetate 4-0-methyl ether (mPMA), that do not activate PKC (Niedel *et al.*, 1983), as well as the kinase inhibitor H-7, had no apparent effect on intercellular flattening.

Table 4.7

Effects on intercellular flattening of 1h incubation of compact 8-cell embryos in drugs that affect PKC

Drug	Concentration	No. of embryos	% flattening after 1h exposure
none		43	98
mPMA	25ng/ml	41	96
4αPDD	5ng/ml	30	91
4αPDD	25ng/ml	29	97
4αPDD	50ng/ml	20	80
4αPDD	100ng/ml	29	91
H-7	18μg/ml	47	85
OAG	4μg/ml	51	94
OAG	40μg/ml	46	57
4βPDD	5ng/ml	29	68
4βPDD	25ng/ml	30	25
4βPDD	50ng/ml	20	0
4βPDD	100ng/ml	29	11
PMA	25ng/ml	60	0

4.9

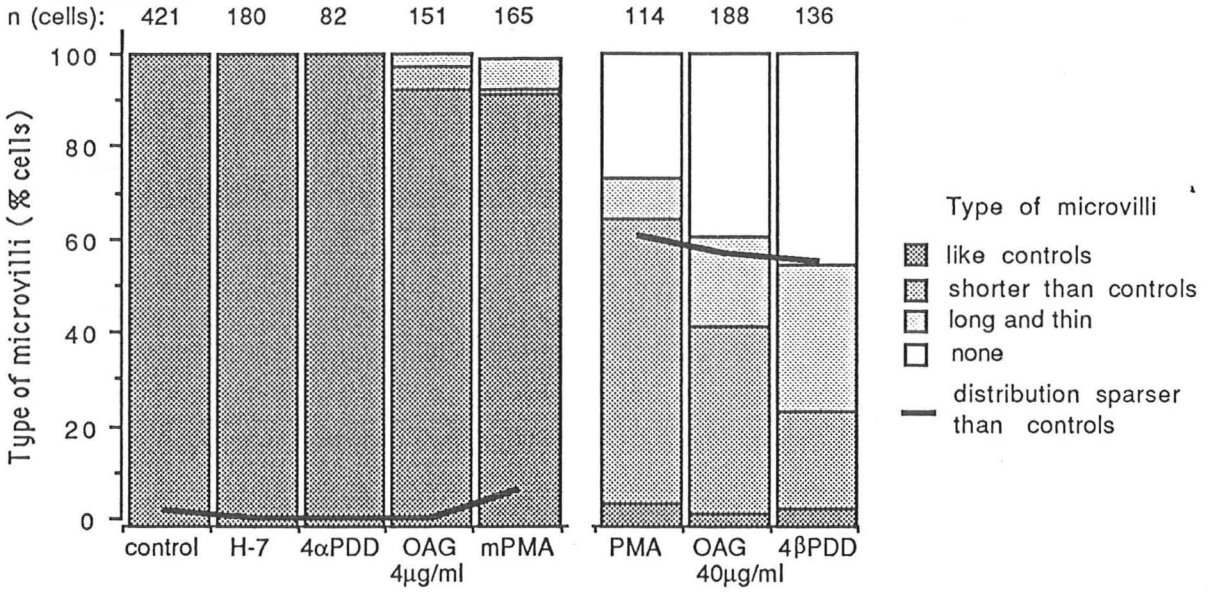


Figure 4.9
Summary of effects of 1h incubation in drugs that affect PKC on distribution and morphology of microvilli of early 8-cell embryos , 3-4h post-division. The concentrations of drug s used are: H-7, 18μg/ml; 4 αPDD, mPMA, PMA and 4βPDD, each 25ng/ml. See also Fig. 4.6.

4.5.2 Effects on microvilli

The effects of these biologically active and inactive phorbol esters and diacylglycerol on microvillous distribution and morphology were also assessed. Those drugs capable of activating PKC and reversing intercellular flattening, 4βPDD and 40μg/ml OAG, had similar effects to those shown for PMA on the distribution and morphology of microvilli of 8-cell embryos, while 4αPDD, mPMA, 4μg/ml OAG and H-7 had little obvious effect (Fig. 4.9).

4.6 Localised delivery of PMA to restricted regions of blastomere surfaces does not seem feasible

Activators of protein kinase C seem to prevent or disrupt the radial asymmetry of late 8-cell blastomeres (see below, 4.7). The distinction in cytoskeletal organisation and surface morphology and adhesivity that is normally apparent between apical and basolateral domains is no longer apparent after exposure to PMA. This could be due either to a global effect of PMA on cell physiology or to a localised effect on the cell surface. A cell surface-specific effect would be apparent over the entire cell surface after cells have been incubated in, and therefore surrounded by, medium containing PMA. To distinguish between these two possibilities, an attempt was made to apply PMA locally to a restricted region of the surface of a blastomere.

Isolated early 1/8 blastomeres were incubated in medium containing dansyl PMA for periods of 15min to 1h, washed through M2+BSA and then aggregated into pairs with untreated isolated 1/8 blastomeres. Such artificially generated 2/8 pairs of blastomeres were then either fixed immediately or incubated for up to 1h in M2+BSA before fixation and examination by fluorescence microscopy. The procedure of aggregation and transfer to fixative took less than 2min for a single pair of cells. However, even in samples in which the pairs of cells had been placed in fixative immediately after aggregation, fluorescence was detected all over the surfaces of both blastomeres. The staining appeared indistinguishable from that obtained by incubating intact 2/8 pairs of cells directly in dansyl PMA. This probably results from the solubility of PMA in lipid (Jacobson *et al.*, 1975; Deleers & Malaisse, 1982; Deleers *et al.*, 1982; Das & Rand, 1984) that presumably causes its rapid distribution

throughout the plasma membrane. It is, therefore, not possible to establish whether there is any effect of PMA that is restricted to a small region of the plasma membrane.

4.7 Discussion

At compaction, both blastomere polarisation and the accompanying cell flattening seem to be associated with a change in the capacity of blastomeres to respond to uvomorulin-mediated cell-cell contact. This change in capacity results in a propagated cytocortical reorganisation which, in turn, leads to cell surface and cytoskeletal changes (see 1.4). All the proteins necessary for this change in response to cell contact are present prior to the 8-cell stage and the trigger for their utilisation at compaction is also post-translational (Kidder & McLachlin, 1985; Levy *et al.*, 1986). It seems possible that maturation of some aspect of second messenger systems, such as messenger generation, stimulation or range of action, might be involved in the changing response to cell-cell contacts.

It has been reported that adenylate cyclase activity and responsiveness change during preimplantation development, although later than compaction (Manejwala *et al.*, 1986). In the experiments reported in this chapter, the possible involvement of the inositol phospholipid signalling pathway in compaction has been investigated indirectly. Stimulation of PKC by a variety of drugs at the time when polarisation and intercellular flattening normally occur leads to several changes in cell organisation that do not occur if blastomeres are exposed to such drugs at other times. This result suggests that such drugs reveal the otherwise hidden change in cell physiology at the 8-cell stage that causes the initiation of compaction (see 1.4). The effects of phorbol esters on cell surface and cytoskeletal organisation in 8-cell blastomeres may reflect a role for PKC in the normal progression of cell flattening and polarisation.

Phorbol esters bind specifically to mouse zygotes and blastomeres at sites close to the cell membrane (Fig. 4.1). Their application has profound effects on cytoskeletal organisation as assessed both indirectly and directly. Intercellular flattening was prevented and reversed in 8-cell embryos exposed to PMA and flattening was not restored in the absence of protein synthesis (Figs. 4.2, 4.3). PKC stimulation has therefore caused a change to the cells that is not simply reversed by removal of the stimulus, implying that required proteins have been destroyed or irreversibly modified in response to stimulation.

More direct evidence for effects of PMA on cytoskeletal organisation has come from examination of microtubule and microfilament organisation by immunofluorescence. The outcome of this analysis depended both on the developmental stage of cells examined and on the experimental protocol used. In oocytes, PMA could cause activation and cytoskeletal re-organisation but only if oocytes had recently been subjected to additional experimental procedures, namely zona removal with acid Tyrode's solution and detergent extraction (Table 4.1). Similarly, in embryos, if zonae pellucidae were removed with acid Tyrode's solution after exposure to PMA, destruction of microtubules was observed at the 8-cell stage but not at any other stage. By contrast, if embryos were allowed a 6h recovery period after removal of the zona before exposure to PMA, no effects were detectable on the number or distribution of microtubules (Table 4.2). Sobel (1983) found that the distribution of myosin in mouse blastomeres is disrupted by zona removal with acid Tyrode's solution and that normal organisation is only restored some six hours later. It therefore seems that zona removal has a profound effect on the cytoskeleton, presumably mediated *via* the associated pH change. PMA allows this change to be observed if exposure to PMA preceded zona removal or followed zona removal with an interval of less than six hours (Tables 4.1, 4.2, 4.3, 4.4).

In addition to these observations, in the absence of any effects of acid Tyrode's solution on the cytoskeleton, exposure to PMA had increasingly severe effects on the microfilament system with increasing developmental age. Thus, microvilli of zygotes and early 2-cell embryos seemed to collapse, becoming longer and thinner than those of controls (Figs. 4.5, 4.6). With further development to the 8-cell stage blastomeres tended to have fewer microvilli, a phenomenon probably due to their retraction since actin cores were still evident cytochemically although the microvilli appeared extremely short when examined by scanning electron microscopy (Figs. 4.5, 4.7). At the 8-cell stage, there were no microvilli on many cells after exposure to PMA and polymerised actin cores were no longer visible. These results suggest a change in the response of the cytoskeleton to PKC stimulation at the 8-cell stage, the time during development at which cell flattening and polarisation normally occur.

4.10

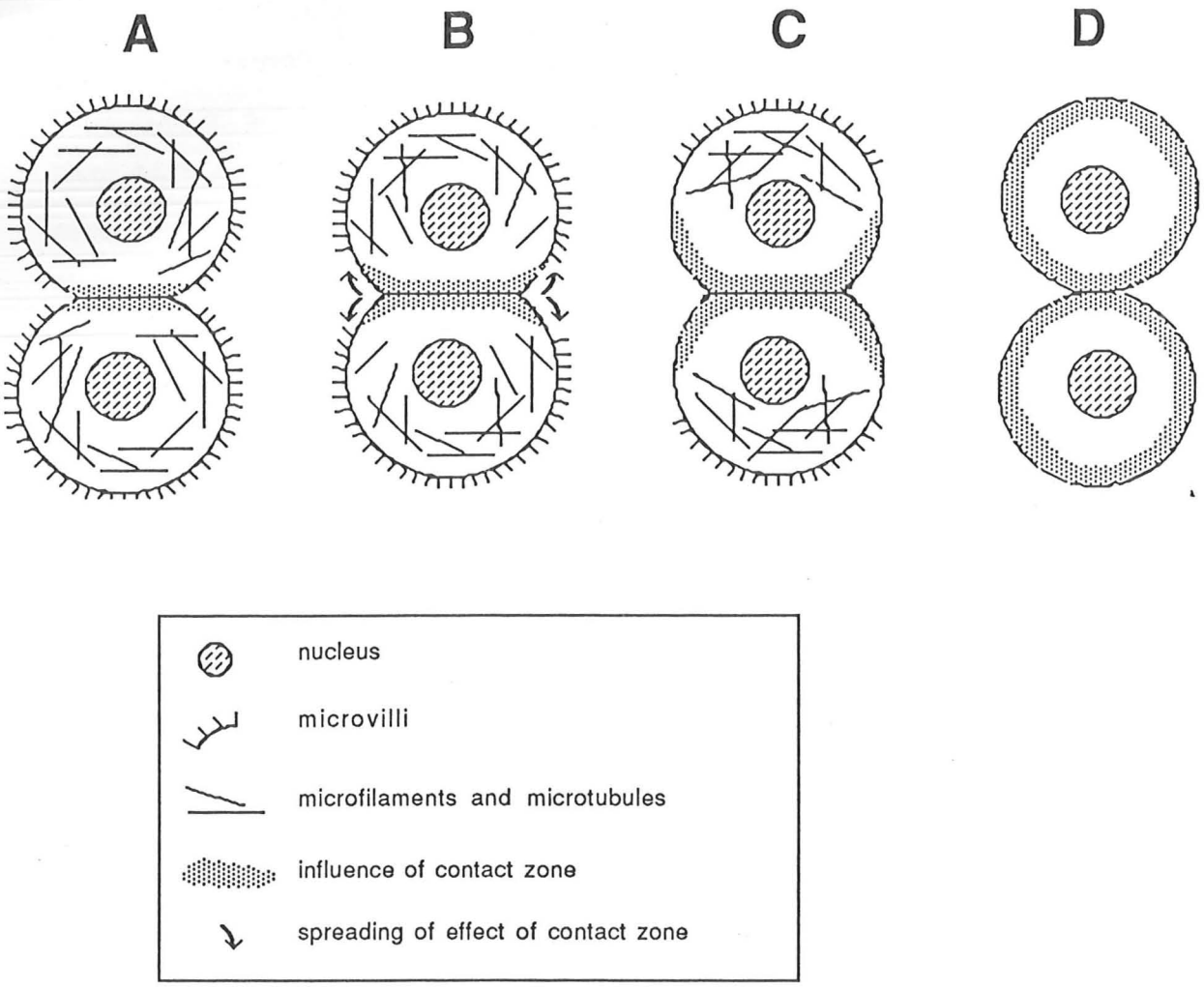


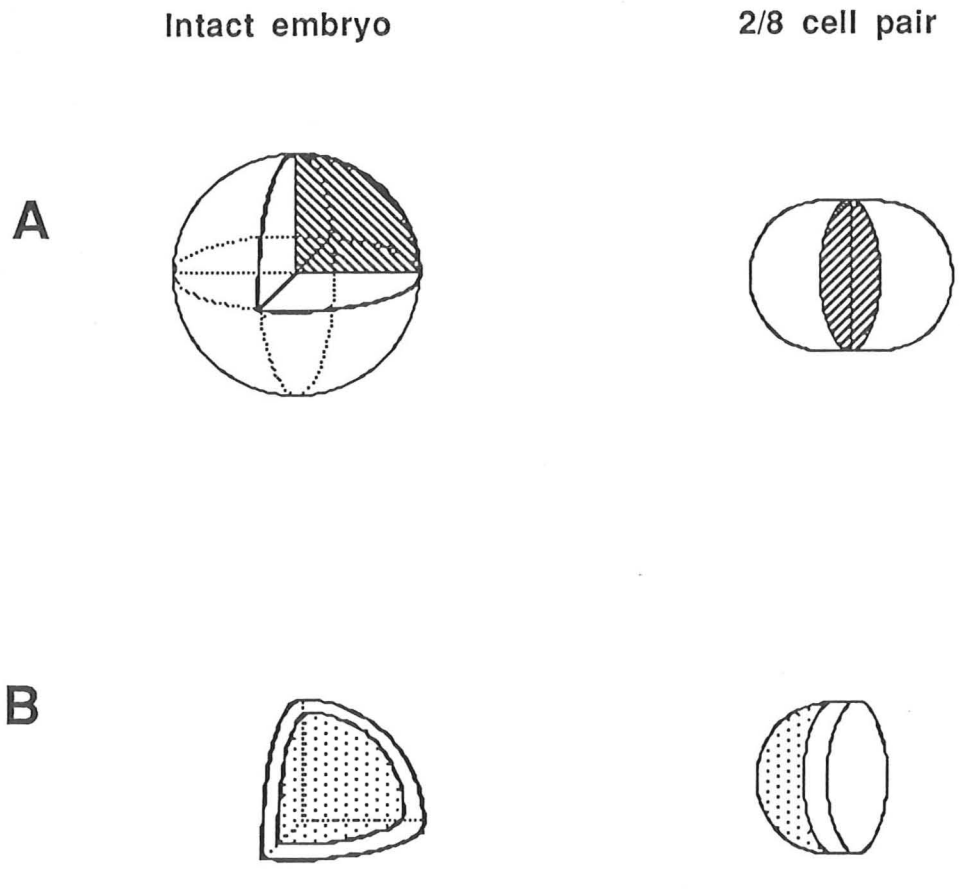
Figure 4.10
Schematic representation of a model for the effects of PMA on 8-cell blastomeres.
A. Two blastomeres from an early 8-cell embryo showing localised depletion of cytoskeletal elements from the cytoplasm adjacent to cell-cell contact. Flattening of the cells onto each other is also seen at the contact region.
B. During the 8-cell stage, both cell flattening and cytoskeletal depletion radiate from the region of cell apposition.
C. A polarised and fully flattened pair of cells results.
D. The cytocortex is competent to generate a widespread change in adhesive properties and cytoplasmic organisation at this stage of development. PMA acts over the entire plasma membrane to make the whole cell behave like the contact zone, with equal adhesivity and universal cytoskeletal depletion.

PMA has been reported to have a variety of effects on the actin cytoskeleton of various cultured cell lines (Rifkin *et al.*, 1979; Schliwa *et al.*, 1983; Meigs & Wang, 1986; Hedberg *et al.*, 1987; Burn *et al.*, 1988) and to cause changes in cell shape (Croop *et al.*, 1980; Phaire-Washington *et al.*, 1980; Robinson *et al.*, 1987). Increasing evidence is now available, particularly from studies of the red blood cell membrane and cytoskeleton, that the phosphorylation of constituent proteins can profoundly affect cell shape (reviewed by Backman, 1988). Just as the response to PMA in the early embryo shows stage-dependent changes, it seems likely that in other systems the particular effects of PMA are highly specific to cell type or differentiated state (Toutant & Sobel, 1987), reflecting the normal range of physiological response available to each cell type.

The change in response to PMA of mouse blastomeres during the 8-cell stage may reflect an altered physiological state that is relevant to the events of compaction. Both compaction and exposure to PMA are associated with the dismantling of microtubules, microfilaments and microvilli. At compaction these changes are spatially restricted in relation to the positions of intercellular contact. By contrast, following PKC stimulation with PMA, dismantling of the cytoskeleton occurs throughout the cell. Thus it is possible to hypothesize that the exposure of cells to PMA is equivalent to symmetrical and universal cell contact (see Figure 4.10). Indeed, if 8-cell blastomeres are surrounded by contacts on all sides, non-polar, non-microvillous cells that may be analogous to PMA-stimulated blastomeres result (Ziomek & Johnson, 1981). Attempts to mimic the effects of localised cell-cell contact by delivery of PMA to only a small portion of the cell surface have failed since PMA distributes extremely rapidly all around the cell surface from the initial point of application.

The results obtained after exposing 2/8 pairs of cells to PMA differ from those obtained using intact embryos. The difference between the two sets of results may be of significance to the hypothesis outlined above to explain the effects of PMA on 8-cell blastomeres. The microvilli on late 2/8 pairs of cells, assessed by scanning electron microscopy, appeared less severely affected by PMA than those on cells of the same developmental age that were exposed to PMA while in an intact embryo (Fig. 4.6). However, the microfilamentous actin that forms the core of each microvillus, stained

4.11



	Intact embryo	2/8 cell pair
% blastomere surface covered with microvilli:	24.1 ± 3.3	49.0 ± 3.2

Figure 4.11
Diagrams to illustrate the proportion of the surface of each blastomere involved in cell-cell contact (A) or covered with microvilli (B) in an intact embryo or 2/8 cell pair.
The shaded area in (A) indicates the region of contact with one other cell; the stippled area in (B) indicates the portion of the blastomere surface that is covered in microvilli. The figures for the proportion of the blastomere surface covered with microvilli in an intact embryo or 2/8 cell pair are shown as mean ± S. D. and are taken from the paper by Pickering et al. (1988).

with FITC-phalloidin, was not detectable in late 2/8 pairs after exposure to PMA (Fig. 4.7 & Table 4.5). The discrepancy between the results obtained with electron microscopy and those obtained by fluorescence microscopy for 2/8 pairs of cells might reflect the greater sensitivity of scanning electron microscopy for detecting residual microvilli, although this would be expected to apply to intact embryos as much as to 2/8 pairs of cells. Alternatively, the plasma membrane may retain some residual organisation into "short microvilli" in 2/8 cell pairs even though no actin cores remained. The observation that these "short microvilli" appear in PMA-treated late 2/8 cell pairs but not in intact embryos suggests a difference in the effect of PMA on blastomeres in intact embryos or 2/8 cell pairs.

There are two clear differences between blastomeres of similar age post-division that are in intact embryos or are part of 2/8 cell pairs that may be relevant to understanding their different responses to PMA. Firstly, the proportion of each blastomere engaged in cell-cell contact in a fully-flattened pair is much less than in a fully-flattened whole embryo (see Figs. 2.1, 4.11A) although the proportion involved in contact with one other blastomere may be similar in each case (Fig. 4.11A). Secondly, the proportion of the blastomere surface covered by the microvillous pole is significantly greater for blastomeres in 2/8 cell pairs than intact embryos (Pickering *et al.*, 1988; Fig. 4.11B). The progression of compaction occurs *via* the spreading of the influence of the contact zone and accompanying cell flattening and cytoplasmic depletion. Both of the observed differences between intact embryos and 2/8 pairs suggest that the progression of compaction has involved more of the surface of each blastomere in an intact embryo than in a 2/8 cell pair. The reduced severity of the effects of PMA on 2/8 cell pairs compared to intact embryos may therefore correspond to the reduced extent of cortical zone spreading achieved prior to exposure to PMA in 2/8 cell pairs, and suggests a limit to the proportion of the blastomere surface that can be made to resemble the contact zone using PMA alone.

A large number of studies of the physiological activation of PKC has not revealed any means of stimulation other than by diacylglycerol, which is most commonly produced by increased phosphoinositide turnover following occupation of ligand

receptors in the plasma membrane (Nishizuka, 1984; Kikkawa & Nishizuka, 1986; Bell, 1986). However, there are reports of hydrolysis stimulated by such diverse triggers as the action of light at photoreceptors (Fein, 1986) and the arrival of sperm at the egg plasma membrane (Ciapa & Whittaker, 1983; Bloom *et al.*, 1988). In translating patterns of cell contacts into an intracellular response at compaction, it is possible that material concentrated at cell contacts can activate PKC either directly or indirectly. Potential candidate molecules include uvomorulin (Kemler *et al.*, 1988) and components of the cytoskeleton (Sobel & Alliegro, 1985; Reima & Lehtonen, 1985; Lehtonen & Reima, 1985; Damjanov *et al.*, 1986; Sobel *et al.*, 1988) or plasma membrane (Pratt, 1985; Ziomek, 1987), which are known to be localised asymmetrically. However, such mechanisms of PKC activation have not been demonstrated previously.

The nature of the postulated permissive change in cell physiology that results in altered effects of PMA on blastomeres at the 8-cell stage remains unknown. The early embryonic cells described here only acquire the features of mature cells gradually, during successive cell division cycles (reviewed by Fleming & Johnson, 1988). Elements of the inositol phospholipid second messenger system may also be acquired progressively during this period. It is unlikely that availability of an activatable protein kinase C is the limiting factor, since phorbol esters both bind to and can have effects on oocytes and earlier embryos. It is possible that the pathways for diacylglycerol generation and response are only mature at the 8-cell stage; the response to phorbol esters at earlier stages does not yield any information about the availability of phosphatidylinositol bisphosphate (PIP_2), the substrate from which diacylglycerol is normally produced, at those earlier stages, or of the functioning of phospholipase C, the enzyme responsible for cleaving PIP_2. Alternatively a new PKC isotype with different stimulus and / or substrate specificity may be activated at the 8-cell stage (Carpenter *et al.*, 1987; Nishizuka, 1988). Perhaps the most likely explanation is that specific substrate(s) for phosphorylation by PKC become accessible at the 8-cell stage and not earlier. Experiments aimed at identifying the substrates for protein phosphorylation at the time of compaction are described in the next two chapters.

CHAPTER FIVE

CHANGES IN PHOSPHOPROTEIN PROFILE ASSOCIATED WITH COMPACTION

CHAPTER FIVE CHANGES IN PROTEIN PHOSPHORYLATION ASSOCIATED WITH COMPACTION

5.1 Introduction

The mechanisms responsible for the initiation and progression of both intercellular flattening and intracellular polarisation seem to be entirely post-translational (see 1.4). Possible post-translational mechanisms by which cell-cell contact could induce intracellular polarisation and variations in cell adhesivity include those dependent on the production of intracellular second messengers and subsequent post-tranlational modifications. In the previous chapter, embryos were exposed to phorbol esters, which mimic some actions of the second messenger diacylglycerol and are potent stimulators of the Ca^{2+}- and phospholipid-dependent protein kinase, protein kinase C. Phorbol esters cause various changes to the cytoskeleton and cell surface of 8-cell blastomeres that can be interpreted as an extreme but spatially disorganised version of compaction. This response presumably arises as a result of stimulation of protein kinase C activity. Phosphorylation of certain proteins by protein kinase C may therefore prove to have a role in the normal process of compaction.

In this chapter, the changes in phosphoprotein profile associated with passage through the 4-cell and 8-cell stages are described, both after brief pulse-labelling with [^{32}P]orthophosphate and following longer incubations and "chase" periods in non-labelled medium. Many of the radiolabelled polypeptides detectable after electrophoresis in one or two dimensions are similar using all protocols. However, several differences in phosphoprotein profile are apparent between pulse-labelled and pulse-chased 8-cell embryos. There are also several changes in profile associated specifically with passage through the 8-cell stage that may be related to the cell flattening and polarisation occurring at this time.

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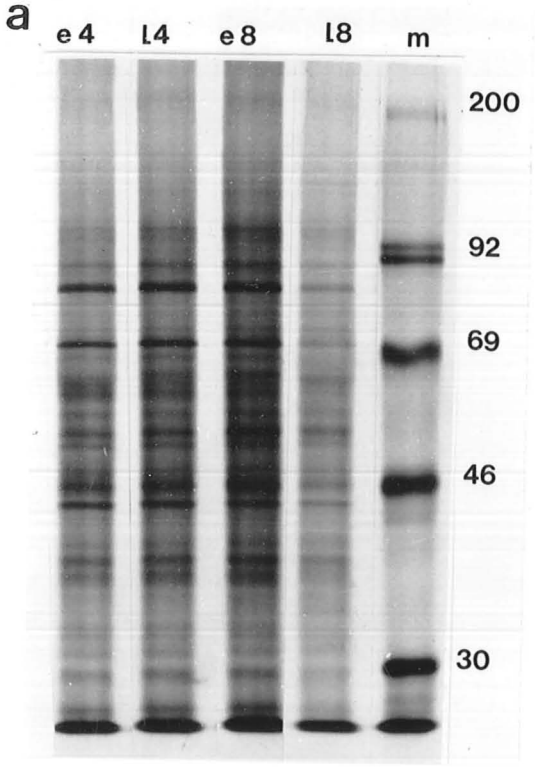
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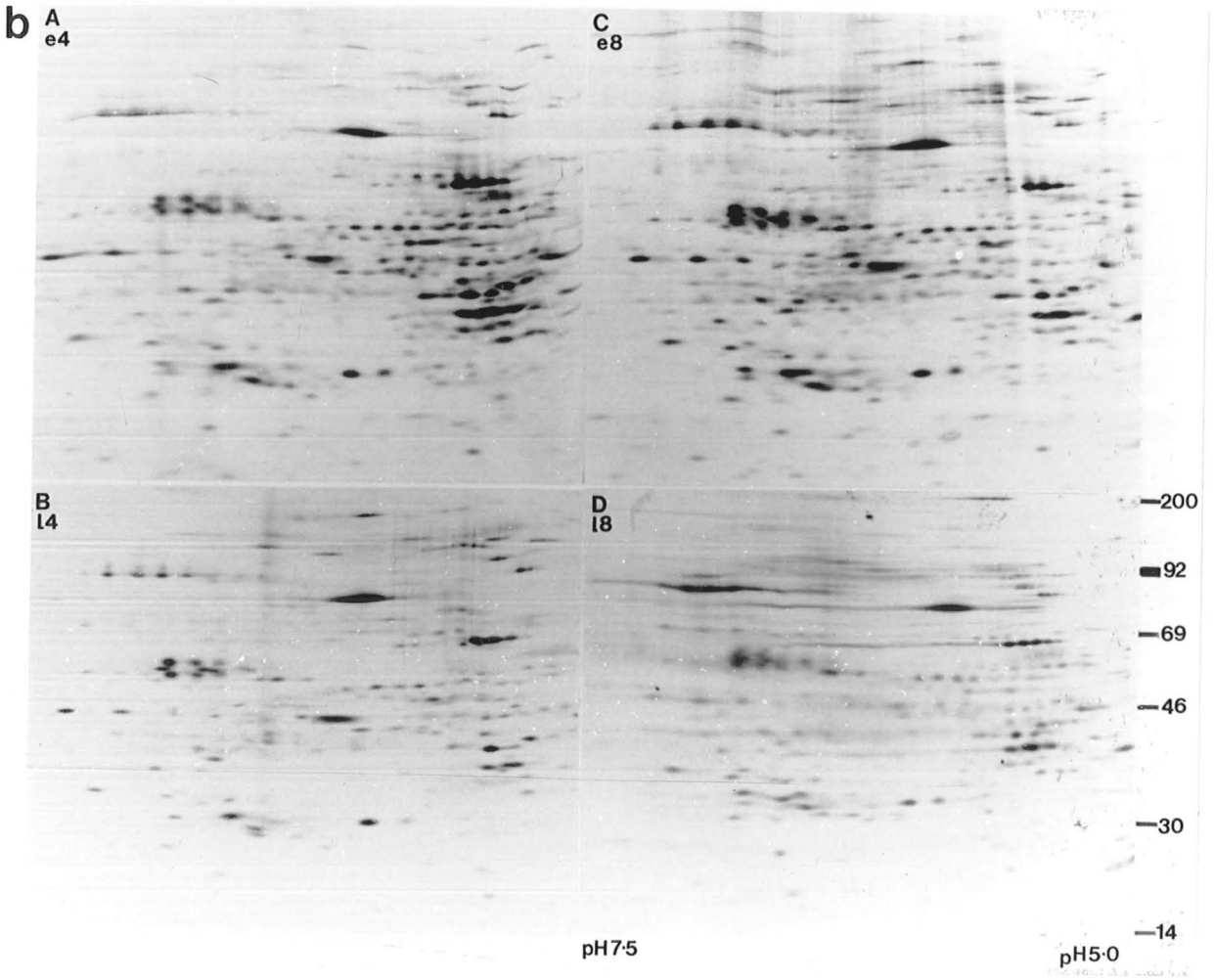
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5.2 The pattern of protein phosphorylation seems to change with compaction

5.2.1 The pattern of proteins synthesised does not appear to change

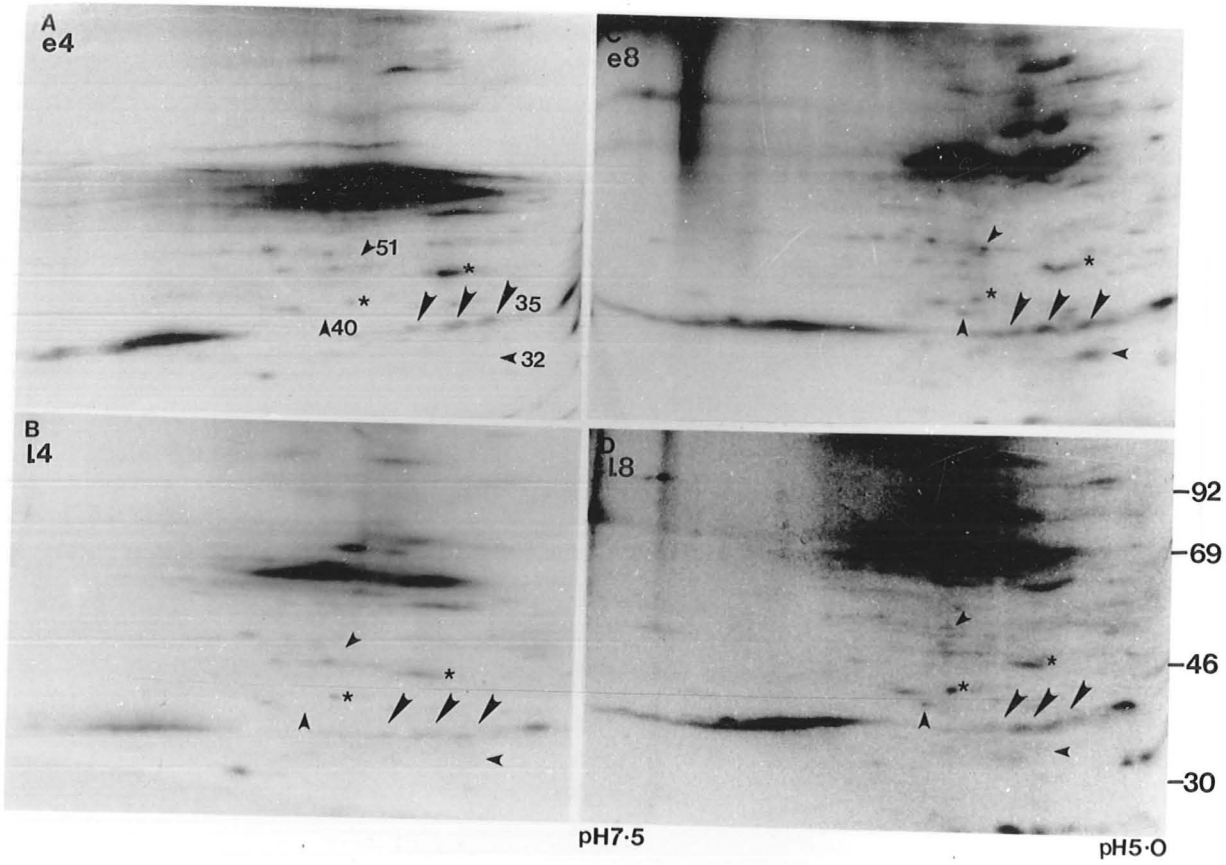
There are major changes apparent in the protein synthetic profile of mouse embryos during the first and second cell cycles of development (Van Blerkom, 1981; Flach *et al.*, 1982; Pratt *et al.*, 1983; Howlett & Bolton, 1985; Howlett, 1986; Endo *et al.*, 1986). To establish whether similar changes occur during subsequent cell cycles, newly synthesised proteins were labelled by incubating timed groups of 4-cell and 8-cell embryos in medium containing 1.5mCi/ml [^{35}S]methionine for 1h and were then separated either by one dimensional SDS-PAGE (Fig. 5.1A) or in two dimensions by IEF/SDS-PAGE (Fig. 5.1B). Examination of replicate gels revealed no reproducible differences, using either method, between embryos labelled during similar periods of the third and fourth cycles.

Figure 5.1

A. One-dimensional SDS-PAGE separation of [^{35}S]methionine-labelled polypeptides synthesised during the 4-cell and 8-cell stages. Embryos were cultured in M16+BSA containing 1.5mCi/ml [^{35}S]methionine for 1h at 0h (e4 and e8) and 6h (l4 and l8) post-division to 4-cells or 8-cells. Relative molecular mass markers (m) 200, 92, 69, 46, 30 x 10^3.

B. Two-dimensional IEF/SDS-PAGE separation of [^{35}S]methionine-labelled polypeptides synthesised by (A) early 4-cell embryos, 0h post-division, (B) late 4-cell embryos, 6h post-division, (C) early 8-cell embryos, 0h post-division, (D) late 8-cell embryos, 6h post-division. Isoelectric focussing is from the left (approx. pH 7.5) to right (approx. pH 5.0) as indicated. Bars indicate the migration of relative molecular mass markers 200, 92, 69, 46, 30 x 10^3 as in (A).

5.2



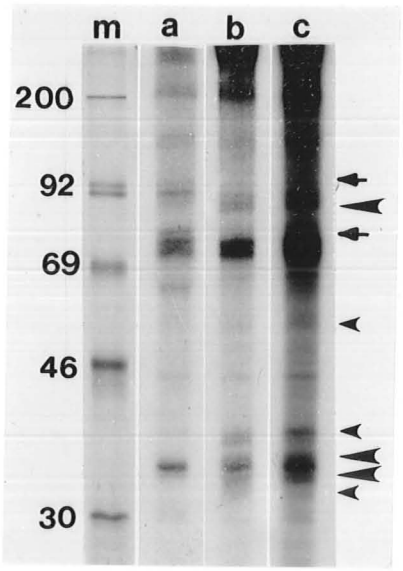
5.2.2 Pulse-labelling embryos with [^{32}P]orthophosphate reveals some differences between 4-cell and 8-cell embryos

Groups of 50 embryos of the same age post-division were incubated in medium containing 1mCi/ml [^{32}P]orthophosphate for 1h and then washed and harvested immediately. Groups of embryos timed at 0h, 3h, 6h, & 9h post-division to 4-cells (Fig. 5.5A, lanes a-d) or post-division to 8-cells (Fig. 5.5B, a-d) showed similar patterns of major phosphoprotein bands after electrophoresis in one dimension. After separation in two dimensions (Fig. 5.2), some phosphoprotein spots are detectable reproducibly at both the early and late 8-cell stages that are not present at the 4-cell stage (compare Fig. 5.2A & B with C & D). The positions of examples of such spots, M_r 32K, 40K and 51K are illustrated by small arrowheads in Figure 5.2. Additionally, a chain of phosphoprotein spots, M_r 35K, that are detectable at the early 4-cell stage show an increase in intensity relative to reference phosphoprotein spots by the early 8-cell stage (large arrowheads, Fig. 5.2; compare intensity with reference phosphoprotein spots marked with asterisks).

Figure 5.2

Two-dimensional IEF/SDS-PAGE separation of [^{32}P]orthophosphate-labelled polypeptides detected after 1h pulse-labelling of (A) early 4-cell embryos; (B) late 4-cell embryos; (C) early 8-cell embryos; (D) late 8-cell embryos. The positions of phosphoprotein spots that reproducibly alter with developmental age are indicated: small arrowheads indicate the position of phosphoprotein spots detectable in 8-cell (C, D) but not 4-cell (A, B) embryos, M_r 32K, 40K, 51K; large arrowheads indicate a chain of phosphoprotein spots, M_r 35K, detectable in 4-cell embryos (A, B) that increase in relative intensity in 8-cell embryos (C, D). Reference phosphoprotein spots that do not appear to alter in intensity are marked with asterisks. Timing of embryos, IEF and markers M_r 92, 69, 46, 30 x 10^3, as for Fig. 5.1.

5.3



5.3 Some phosphoproteins are detected only after prolonged incubation in [^{32}P]orthophosphate or pulse-chase

5.3.1 Longer pulses label more phosphoprotein bands

Incubation of cells in medium containing [^{32}P]orthophosphate does not cause all phosphoproteins to become labelled immediately (see 5.5 for discussion). Groups of 8-cell embryos (0h post-division) were incubated in ^{32}P-medium for 1h (Fig. 5.3, lane a) or 6h (Fig. 5.3, lane c), washed and harvested immediately. As expected, longer incubation in ^{32}P-medium is associated with a generally increased intensity of labelling. In addition, an increase in the relative intensity of several bands is detected. For example, phosphoprotein bands M_r 32K, 35K, and 84K (large arrowheads, Fig. 5.3) increased in relative intensity and novel bands M_r 31K, 40K, 54K appeared (small arrowheads, Fig. 5.3) after 6h pulse but were not detectable after to 1h pulse.

Figure 5.3

One-dimensional SDS-PAGE separation of [^{32}P]orthophosphate-labelled polypeptides detectable in 8-cell embryos after prolonged incubation or pulse-chase. Eight-cell embryos were incubated in ^{32}P-medium for (a) 1h, (b) 1h followed by 5h chase or (c) 6h. Large arrowheads indicate phosphoprotein bands M_r 32K, 35K and 84K that reproducibly increase in relative intensity after more than 1h incubation. Small arrowheads indicate the position of phosphoprotein bands M_r 31K, 40K and 54K that are reproducibly detected only after 6h incubation or 5h chase. Arrows indicate bands M_r 76K and 92K that reproducibly disappear (76K) or decrease in relative intensity (92K) after chase compared to pulse-labelling. All lanes are taken from a single gel. This was exposed to film for a shorter period for lane c than lanes a, b and m (relative molecular mass markers, as in Fig. 2), to allow comparison of embryos incubated for 6h in ^{32}P. See also Figs. 5.5 and 5.6

5.3.2 *"Pulse-chase" produces mostly similar phosphoprotein bands to a continuous pulse over the same time period*

As prolonged incubation in ^{32}P-medium decreases embryo viability (see 2.7.2), it is desirable to keep incubation periods to a minimum. Eight-cell embryos (0h post-division) were therefore labelled for 1h and "chased" in medium containing non-labelled phosphate for a further 5h before harvesting. Most of the pattern of phosphoprotein bands obtained on separation in one dimension after a 1h pulse and 5h chase was similar to that obtained after a 6h pulse (Fig. 5.3, compare lanes b and c). In particular, the phosphoprotein bands M_r 31K, 32K, 35K, 40K, 54K and 84K, referred to above (arrowheads, Fig. 5.3), all increased in relative intensity in the pulse-chased group as well as after a 6h pulse, compared to 1h pulse. However, a phosphoprotein band M_r 76K that is present in both 1h and 6h pulse-labelled samples was not detectable in the pulse-chased sample and a phosphoprotein band of 92K decreased in relative intensity in the pulse-chased sample (arrows, Fig. 5.3).

5.4 The effects on the phosphoprotein profile of the prolonged presence of ^{32}P can be distinguished from the effects of developmental age

5.4.1 *Experimental strategy*

When 8-cell embryos were labelled for prolonged periods or in pulse-chase regimes, many novel phosphoprotein bands were seen compared to 1h pulse-labelling (Fig. 5.3). However, labelling for 6h at the 8-cell stage occupies half of the cell cycle and spans the time in development during which compaction occurs. It is therefore not clear from these experiments whether the observed changes in phosphoprotein profile result directly from the longer labelling period or from changes associated with compaction. In order to differentiate changes in phosphoprotein band pattern resulting from the prolonged presence of ^{32}P from those due to arrival at a particular stage of development, groups of timed 8-cell embryos (0h, 3h, 6h and 9h post-division) were pulse-labelled and either harvested immediately or cultured for a series of 2h and 5h chase periods, such that the fourth cell cycle was covered (sampling protocol illustrated

5.4

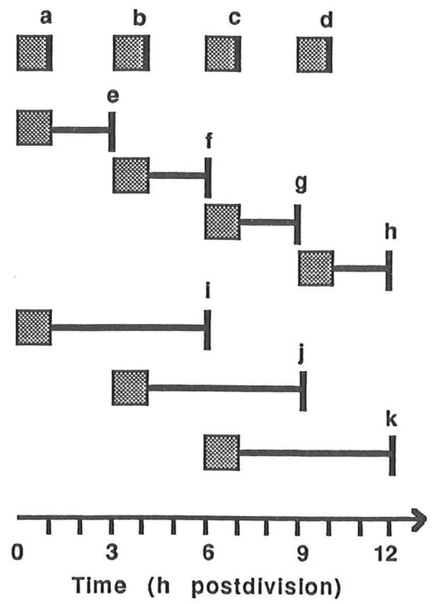


Figure 5.4
Diagram to illustrate the protocol of an experiment to distinguish phosphoprotein bands that appear with increasing chase duration form those that appear at a particular time of passage through the 8-cell stage. The horizontal axis represents time in hours post-division. Boxes indicate the 1h labelling period and horizontal bars indicate the duration of the chase period, if any. Letters and vertical bars indicate the time of harvesting and correspond to lanes in Figure 5.5.

in Fig. 5.4).

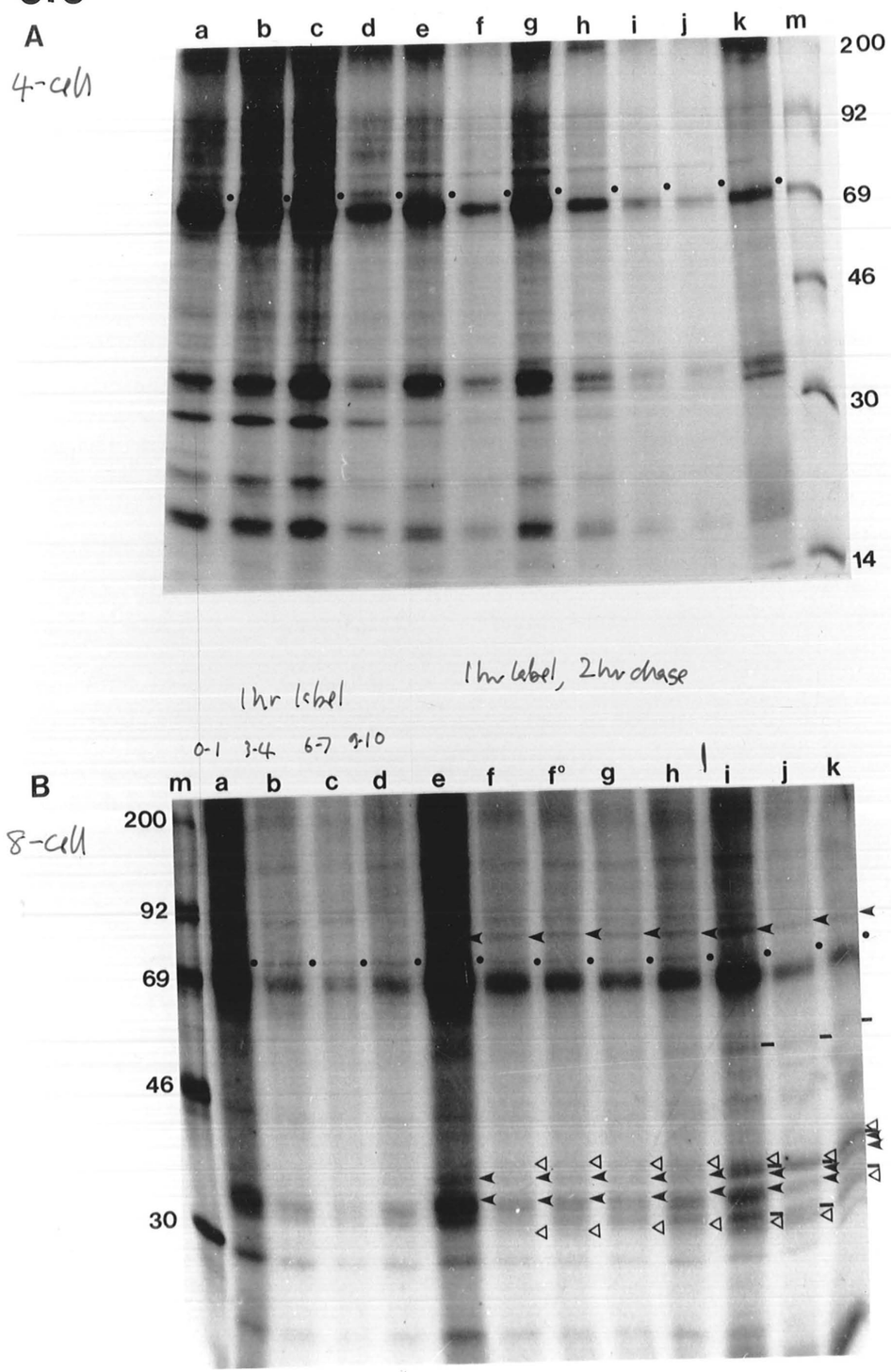
As an additional control, the changing pattern of phosphoprotein bands during the 4-cell stage was mapped according to a similar schedule. Comparison of the 4-cell and 8-cell stages should allow phosphoprotein profile changes associated specifically with compaction to be distinguished from those occurring with passage through each cell cycle. The labelled phosphoproteins obtained from this experimental protocol were then separated in one dimension (Fig. 5.5) for preliminary analysis. Selected samples of similarly timed embryos were then further analysed by two-dimensional SDS/PAGE (see below, Fig. 5.6).

5.4.2 The behaviour of some phosphoprotein bands after pulse-labelling or pulse-chase is similar in 4-cell and 8-cell embryos

As described for 8-cell embryos above (Fig. 5.3), a phosphoprotein band M_r 76K (marked with dots, Fig. 5.5) also decreases in relative intensity in pulse-chased samples compared to pulse-labelled samples in 4-cell embryos (Fig. 5.5A, 4-cells and Fig. 5.5B, 8-cells, compare lanes e-k with lanes a-d). However, the majority of phosphoprotein bands detected in 4-cells did not alter in relative intensity after 1h pulse plus 2h chase (Fig. 5.5A, lanes e-h) or 1h pulse plus 5h chase (Fig. 5.5A, lanes i-k) compared to 1h pulse-labelled samples (Fig. 5.5A, lanes a-d).

5.4.3 Several phosphoprotein bands are detected only in 8-cells after pulse-chase

Most of the phosphoprotein bands that appear or increase in relative intensity with a chase period compared to pulse-labelling are detected only in 8-cell embryos. In Figure 5.5B, solid arrowheads (M_r 35K, 37K, 84K) indicate phosphoprotein bands detected after 1h pulse plus 2h or 5h chase, but only faintly, or not at all after 1h pulse alone (compare lanes a-d with lanes e-k). In addition, bars (M_r 32K, 39K, 54K) indicate the position of phosphoprotein bands that appear or increase in relative intensity only after 1h pulse plus 5h chase (compare lanes i-k with lanes a-h). By contrast to these observations for 8-cell embryos, no marked increase in intensity of corresponding phosphoprotein bands is detectable in 4-cell embryos (Fig. 5.5A).



5.4.4 Some phosphoprotein bands are detected only in compact 8-cells

In addition to those phosphoprotein bands detected at the 8-cell stage that vary in intensity with chase duration, two phosphoprotein bands are detectable that only appear in pulse-chased samples more than 3h into the fourth cell cycle, independent of chase duration (Fig. 5.5B, compare lanes f-k with lanes a-e; open arrowheads, M_r 31K, 40K). There are no bands that behave in a corresponding way in the third cell cycle (Fig. 5.5A).

Figure 5.5 (facing page)

One-dimensional SDS-PAGE separation of [^{32}P]orthophosphate-labelled polypeptides synthesised by (A) 4-cell embryos and (B) 8-cell embryos after pulse-labelling or pulse-chase according to the protocol illustrated in Figure 5.4. Groups of embryos 0h, 3h, 6h or 9h post-division were incubated for 1h in ^{32}P-medium and immediately harvested: (a) 0-1h; (b) 3-4h, 8-cell embryos partially flattened; (c) 6-7h; (d) 9-10h post-division. Additional groups were further incubated for 2h in a non-labelled chase: (e) labelled 0-1h, harvested 3h, 8-cell embryos partially flattened; (f) labelled 3-4h, harvested 6h, 8-cell embryos partially flattened; (f°) labelled 3-4h, harvested 6h, 8-cell embryos fully flattened; (g) labelled 6-7h, harvested 9h (h) labelled 9-10h, harvested 12h post-division. Some embryos were pulse-labelled for 1h followed by 5h in a non-labelled chase: (i) labelled 0-1h, harvested 6h (j) labelled 3-4h, harvested 9h (k) labelled 5-6h, harvested 12h post-division. Embryos taken as 12h post-division to 4-cells or 8-cells had divided to 8-cells and 16-cells respectively. Dots indicate the position of a phosphoprotein band M_r 76K that decreases in relative intensity with increasing chase duration (arrow, Fig. 5.3); closed arrowheads indicate bands that appear or increase in intensity with 2h chase, M_r 35K, 37K, 84K; bars indicate bands that appear or increase in intensity with 5h chase, M_r 32K, 39K, 54K; open arrowheads indicate bands appearing only in 8-cell embryos more than 3h post-division, M_r 31K, 40K. Relative molecular mass markers (m) were run with each gel as in Fig. 5.1.

5.4.5 Two-dimensional separation reveals additional phosphoproteins associated with the 8-cell stage and pulse-chase

Most of the phosphoproteins that showed differences between labelling protocols or between different stages of development migrate in the same region of a one-dimensional SDS-polyacrylamide gel (M_r 30-40K) and are therefore difficult to resolve with confidence. Selected groups of 8-cell embryos from similar labelling regimes were therefore separated by two dimensional electrophoresis (Fig. 5.6)

Two phosphoprotein bands are described above as increasing in relative intensity with passage through development (31K) or with chase duration (32K). These may correspond to a single phosphoprotein spot on a two-dimensional gel (M_r 32K, pI` approximately 5.75, marked with a large arrowhead in Fig. 5.6). This spot appears to increase in relative intensity and to shift to a slightly more acidic pI` both with increasing chase duration and with passage through the 8-cell stage. This observation is consistent with the phosphoprotein being more heavily phosphorylated under these two conditions.

There are no spots the behaviour of which correspond clearly to that of the bands of 35K and 37K described after one-dimensional separation, increasing in relative intensity with increasing chase duration. Their appearance on one-dimensional gels may correspond to the increase in relative intensity of the chain of phosphoprotein spots M_r 35K indicated with large arrowheads in Figure 5.2. Such an additional modification is not easily detectable on two-dimensional gels but could cause an apparent shift in M_r on one-dimensional SDS gels. Alternatively, the phosphoproteins (M_r 35K and 37K) seen after one-dimensional separation may be migrating beyond the range of this IEF system.

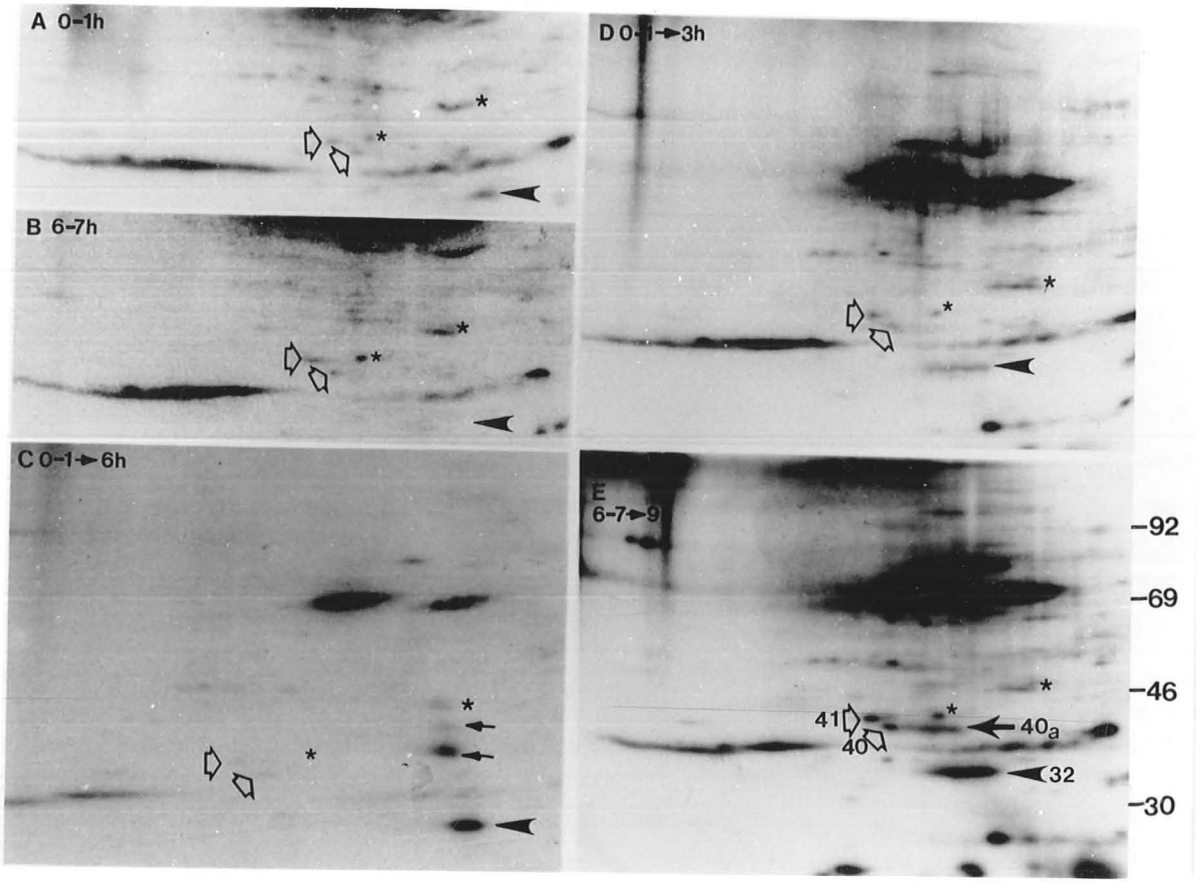
In addition to the bands that increase in relative intensity described after one-dimensional separation, two phosphoprotein spots M_r 42K and 44K are apparent only after 1h pulse and 5h chase (arrows, Fig. 5.6C). Two phosphoprotein spots M_r 40K and 41K, pI` approximately 6.0 appear more heavily labelled in late 8-cells than early 8-cells after 1h pulse and 2h chase (open arrows, Fig. 5.6, compare D & E). These two spots are detectable but at reduced relative intensity in pulse-labelled early and late 8-cells (open arrows, Fig. 5.6A, B); the spot M_r 40K is not detectable in pulse-labelled

4-cells (small arrowhead, Fig. 5.2). This phosphoprotein migrates very close to, but is more basic than, the diffuse 40K phosphoprotein spot (pI` approximately 5.85, large arrow, Fig. 5.6E, marked as "40_a") that appears only in 8-cells more than 6h post-division. This group of phosphoprotein spots may therefore be detectable for the first time in early 8-cell embryos and be increasingly phosphorylated or further modified with passage through the 8-cell stage.

Figure 5.6 (facing page)

Two-dimensional IEF/SDS-PAGE separation of [^{32}P]orthophosphate-labelled polypeptides reproducibly detected in 8-cell embryos, labelled (A) pulse 0-1h post-division, as for Fig. 5.5 lane a; (B) pulse 6-7h post-division as for Fig. 5.5B lane c; (C) pulse 0-1h, chase to 6h post-division as for Fig. 5.5B lane i; (D) pulse 0-1h, chase to 3h post-division, embryos partially flattened, as for Fig. 5.5 lane e; (E) pulse 6-7h, chase to 9h post-division as for Fig. 5.5B lane g. Only part of each gel (approx. M_r 30K-70K) is shown in (A) and (B) for comparison as the entire gels are shown in Figure 5.2 (C) and (D) respectively. The large arrowhead (M_r 32K) shows the position of a phosphoprotein spot that may correspond to phosphoprotein bands, M_r 31K and 32K, marked with an arrowhead and a bar in Fig. 5.5B, increasing in intensity with chase duration (see text). Small arrows show the position of phosphoprotein spots M_r 42K & 44K that appear after 1h pulse plus 5h chase that were not detected on one-dimensional gels. Open arrows show the position of phosphoprotein spots (M_r 40K & 41K) that increase in intensity in late 8-cells compared to early 8-cells after 2h chase, and which may correspond to a phosphoprotein band marked with an open arrowhead in Fig. 5.5B. A large arrow (E) marks a diffuse spot (M_r 40K; "40_a") that appears only in 8-cell embryos more than 6h post-division. Asterisks mark reference phosphoprotein spots that do not appear to alter in intensity (as in Fig. 5.2). The gel shown in (C) has been exposed for less time than those in (A), (B), (E) and (D). Timing of embryos and markers (m) as for Fig. 5.1

5.6



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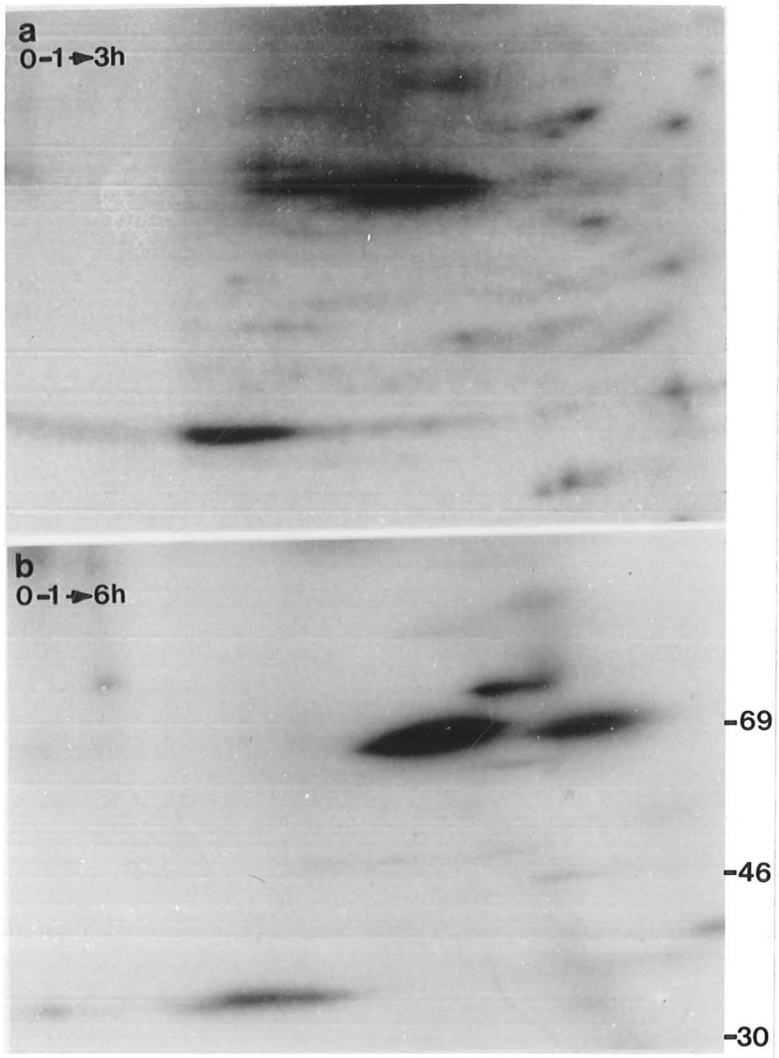


Unlike the situation in 8-cell embryos, when 4-cell embryos were pulse-labelled or pulse-chased and the phosphoproteins separated in two dimensions, no additional spots appeared reproducibly different either between pulse-labelled and pulse-chased samples or those chased for different periods (Fig. 5.7, compare with Fig. 5.2A and B), or between samples taken early and late in the cell cycle (data not shown).

Figure 5.7 (facing page)

Two-dimensional IEF/SDS-PAGE separation of [^{32}P]orthophosphate-labelled polypeptides detectable in 4-cell embryos after (A) 1h pulse and 2h chase at 0h post-division, as Fig. 5.5A lane e; (b) 1h pulse and 5h chase at 0h post division, as Fig. 5.5A lane i. Unlike the situation for 8-cell embryos, no novel phosphoprotein spots are reproducibly detectable after pulse-chase that are not also detectable after 1h pulse-labelling (compare with Fig. 5.2A & B). The IEF gradient of the gel shown in (a) is shifted to the left relative to that shown in (b); markers as in Fig. 5.1.

5.5 Discussion

This chapter describes the changes in phosphoprotein profile that accompany compaction. Blastomeres of 8-cell mouse embryos differ from those of 4-cell embryos by their ability to flatten onto each other and to polarise their contents and microvilli along an axis determined by the positions of cell contacts (see 1.4). These events seem to be controlled post-translationally, using proteins present in the embryo from at least the 4-cell stage onwards (Kidder & McLachlin, 1985; Levy *et al.*, 1986). By studying the changes in protein phosphorylation that occur during this key time in development, it may be possible to elucidate further the molecular events underlying the morphological changes that occur at compaction.

The only previous study of phosphoproteins at these stages is that by Lopo and Calarco (1982) who pulse-labelled large groups of relatively heterogeneous preimplantation embryos with [^{32}P]orthophosphate. In the present study, smaller groups of embryos have been synchronised more precisely and exposed to ^{32}P for varying periods and combinations of pulse and chase at intervals through the third and fourth cell cycles. Whilst no difference was observed in the pattern of polypeptides synthesised by embryos (as assessed by pulse-labelling with [^{35}S]methionine, Fig. 5.1), pulse-labelling 4-cell and 8-cell embryos with [^{32}P]orthophosphate revealed clear differences in phosphoproteins within and between cell cycles. These differences may represent the novel phosphorylation of proteins or increased phosphorylation of existing phosphoproteins to a level detectable by autoradiography.

Different profiles of phosphoproteins were detected in samples taken 1h after the addition of ^{32}P to the medium compared to samples taken some time later. As particular protein kinases use phosphate from different sources of nucleotide triphosphate, this observation may reflect different rates at which nucleotide triphosphate pools equilibrate with ^{32}P (Cooper *et al.*, 1983). Different rates of turnover of phosphate on different phosphoproteins have also been noted, in particular between those containing phosphotyrosine and those containing phosphoserine or phosphothreonine (Hunter *et al.*, 1980). It is also possible that proteins are cleaved after phosphorylation, or are further post-translationally modified (Krebs, 1986).

It is important to distinguish both those proteins described here whose

phosphorylation state varies with duration of pulse and chase (M_r 32K, 35K and 84K, Figs. 5.3-5.6) and those whose phosphorylation state varies with passage through each cell cycle (such as those of M_r 35K that have been described previously; Howlett, 1986), from phosphoproteins unique to a particular cell cycle. Most of the phosphoproteins restricted to the fourth cell cycle were only detectable after pulse-labelling embryos for a prolonged period of time (which decreases embryo viability) or after pulse-labelling for a short period followed by a longer "chase" in non-radioactive medium (which does not affect viability). Chase periods of increasing length were associated with increased labelling of certain phosphoproteins and with the detection of novel phosphoproteins in 8-cell embryos (M_r 32K, 37K and 84K after 2h or 5h chase; M_r 40K, 42K, 44K, 54K in 8-cells only after 5h chase; Figs. 5.5, 5.6). These results suggest that specific pathways of protein phosphate metabolism may be used at the 8-cell stage. This might be of significance for the events of compaction occurring at this time in development. Additionally, some novel phosphoproteins were only detectable during the second half of the fourth cell cycle (M_r 31K, 40K, Figs. 5.5, 5.6). Such phosphoproteins might be involved in the late events of cell flattening and polarisation such as stabilisation of the polar phenotype (see 1.4), or in processes occurring after, or as a consequence of, compaction.

It is not unreasonable to suppose that changes in protein phosphorylation might mediate cellular events such as cell flattening and polarisation since it has been apparent for some time that changes in the phosphorylation state of an enzyme can alter its activity fundamentally (Krebs, 1985). Many extracellular signals such as hormones and growth factors bind to receptors in the plasma membrane and produce changes in protein kinase activity, directly or indirectly, which are presumed to mediate some or all of their physiological effects (reviewed by Hunter & Cooper, 1985; Krebs, 1985; Sibley *et al.*, 1987). Changing patterns of cell-cell contact at the 8-cell stage of preimplantation development might produce alterations in protein phosphorylation by similar mechanisms.

Of particular interest is the observation that phosphorylation of many cytoskeletal proteins alters their function significantly (reviewed by Backman, 1988).

Thus, phosphorylation of actin binding proteins (Stossel *et al.*, 1985; Pollard & Cooper, 1986), myosin (Citi & Kendrick-Jones, 1987), tubulin (Hargreaves *et al.*, 1986), vimentin (Inagaki *et al.*, 1987; Geiger, 1987) and nuclear lamins (Ottaviano & Gerace, 1985) are all associated with changes in function and / or intracellular localisation. Less direct evidence for the role of phosphorylation in changing cell shape and cytoskeletal organisation comes from cells transformed with oncogenes (Jove & Hanafusa, 1987; Kellie, 1988) or using drugs such as phorbol esters to stimulate specifically protein kinase C (see 4.5).

It seems feasible to propose that cell-cell contact causes cell polarisation and flattening via the extracellular activation of protein kinases which are either themselves localised at cell contacts, or are locally activated or act on localised substrates. The experiments described in the next chapter aim to assess how closely changes in phosphoprotein profile are linked to compaction by examining the changes to the phosphoprotein profile that occur if the events of compaction are manipulated experimentally.

CHAPTER SIX

EXPERIMENTAL MANIPULATION OF COMPACTION ALTERS THE PATTERN OF PROTEIN PHOSPHORYLATION

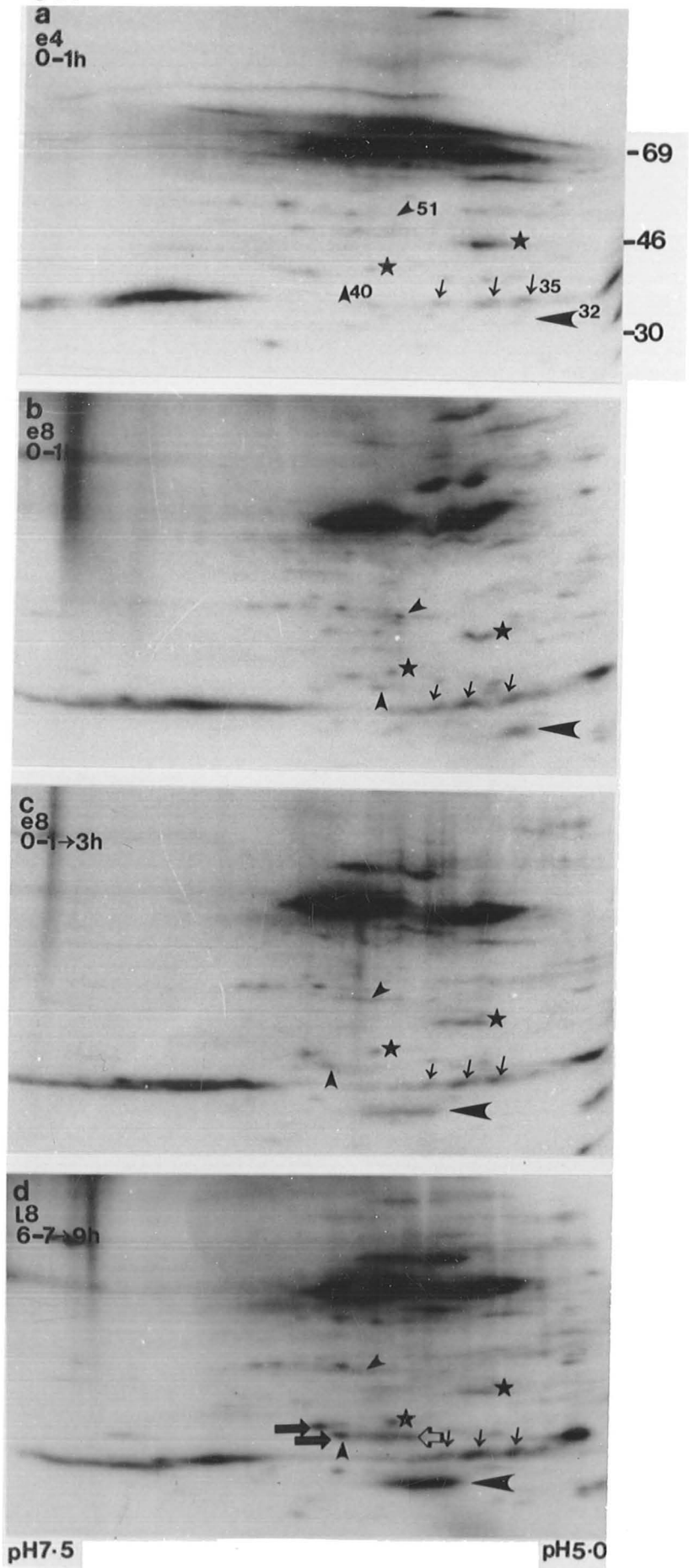
CHAPTER SIX EXPERIMENTAL MANIPULATION OF COMPACTION ALTERS THE PATTERN OF PROTEIN PHOSPHORYLATION

6.1 Introduction

The changes in phosphoprotein profile that occur as mouse embryos pass through the 8-cell stage of development were described in the previous chapter. In this chapter, four treatments that affect features of compaction have been used to assess the link between these changes in phosphoprotein profile and the events of compaction.

The first treatment used was the incubation of embryos in DMAP, a putative inhibitor of phosphorylation (Neant & Guerrier, 1988) that causes intercellular flattening to occur in 4-cell embryos by a mechanism that seems to be independent of uvomorulin (see 3.3). DMAP can also affect levels of protein synthesis, but does not do so if the drug has been incubated at 37°C for several hours before the addition of embryos (see 3.4). In this chapter, it is found that DMAP can affect the global level of protein phosphorylation but that this effect is also abolished if the drug is pre-incubated at 37°C for several hours. However, in the absence of any global effect on protein phosphorylation, DMAP can still cause premature flattening in 4-cell and 8-cell embryos and this flattening is found to be associated with the increased relative intensity of ^{32}P-labelling of a single phosphoprotein spot. The second treatment used was the incubation of embryos in protein synthesis inhibitors, which causes premature intercellular flattening in 4-cell embryos (Levy *et al.*, 1986; see 1.4 & 3.1) but is found to be associated with very few changes to the phosphoprotein profile. Other groups of 4-cell and 8-cell embryos were incubated in medium containing phorbol ester which reverses flattening and perturbs the cytoskeletal organisation of 8-cell embryos, by means which may correspond to an extreme version of compaction (see 4.7; Fig. 4.10). Finally, embryos were incubated in Ca^{2+}-free medium which prevents cell flattening and delays polarisation of 8-cells (Ducibella & Anderson, 1975; Fleming *et al.*, 1989; see 1.4). In each of the latter two cases, a similar sub-set of the phosphoproteins characteristic of untreated 8-cell embryos were affected. The incidence of these phosphoproteins may therefore be linked to compaction.

6.1



6.2 Summary of the differences in phosphoprotein profile between 4-cell and 8-cell embryos after pulse-labelling and pulse-chase

The difference in phosphoprotein profile between 4-cell and 8-cell embryos after pulse-labelling and pulse-chase that were described in detail in Chapter Five are summarised below and in Figure 6.1. A standardised system for labelling similar phosphoprotein spots is used throughout this chapter to facilitate comparison between gels.

After 1h pulse-labelling, phosphoprotein spots M_r 32K, 40K and 51K (small

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Figure 6.1 (facing page)

Differences in phosphoproteins detected in 4-cell and early and late 8-cell embryos after pulse-labelling and pulse-chase, summarised from Figs. 5.2 & 5.6.

Two-dimensional IEF/SDS-PAGE separation of [^{32}P]orthophosphate-labelled polypeptides from (a) early 4-cell embryos (0h post-division), 1h pulse-labelled; (b) early 8-cell embryos (0h post-division), 1h pulse-labelled; (c) early 8-cell embryos (0h post-division), 1h pulse + 2h chase; (d) late 8-cell embryos (6h post-division), 1h pulse + 2h chase. IEF is from left (≈pH 7.5) to right (≈pH 5.0) and the migration of M_r markers (m) 69, 46, 30 x 10^3 is indicated. Small arrowheads indicate the position of phosphoprotein spots (M_r 40K & 51K) that appear in 8-cell embryos (b-d) but not in 4-cells (a). Small arrows indicate the position of a group of phosphoprotein spots (M_r 35K) that increase in relative intensity in 8-cells (b-d) compared to 4-cells (a). A large arrowhead shows the position of a phosphoprotein spot (M_r 32K) that increases in size and relative intensity with arrival at the 8-cell stage (b), with chase (c) and with passage through the 8-cell stage (d). The apparent shift in pI` of this spot in (b) was not detected in replicate gels. Large arrows (d) indicate phosphoprotein spots (M_r 40K, 41K) that increase in relative intensity in late 8-cells (d) compared to early 8-cells (c). An open arrow indicates the position of a phosphoprotein spot (M_r 40K; referred to as "40K$_a$") that appears only in late 8-cells. Stars are beside reference phosphoprotein spots, the intensity of which does not appear to change.

arrowheads, Fig. 6.1a & b) and a group of spots, M_r 35K (small arrows, Fig. 6.1a & b), are reproducibly detected at greater intensity in 8-cell embryos (Fig. 6.1b-d) than 4-cells (Fig. 6.1a; compare intensity to that of reference phosphoproteins marked with stars).

In addition, where 1h pulse-labelling was followed by a 2h cold chase, a phosphoprotein spot M_r 32K increases in relative intensity, and apparent size, in early 8-cells following pulse-chase (large arrowhead, compare Fig. 6.1b & c) and increases further in intensity (and size) in late 8-cells compared to early 8-cells after pulse-chase (large arrowhead, compare Fig. 6.1c & d). Two phosphoproteins, M_r 40K and 41K, increase in relative labelling intensity in late 8-cells after pulse-chase compared to early 8-cells after pulse-chase (large solid arrows, Fig. 6.1d). One of these co-migrates with the 40K phosphoprotein spot that is detected in 8-cells but not in 4-cells after pulse-labelling (small arrowhead). A further, diffuse phosphoprotein spot, M_r 40K but with slightly more acidic pI` than the 40K spot described above, appears only in late 8-cells after pulse-chase (referred to as "$40K_a$"; open arrow, Fig 6.1d). This data is summarised in Table 6.2 (p102).

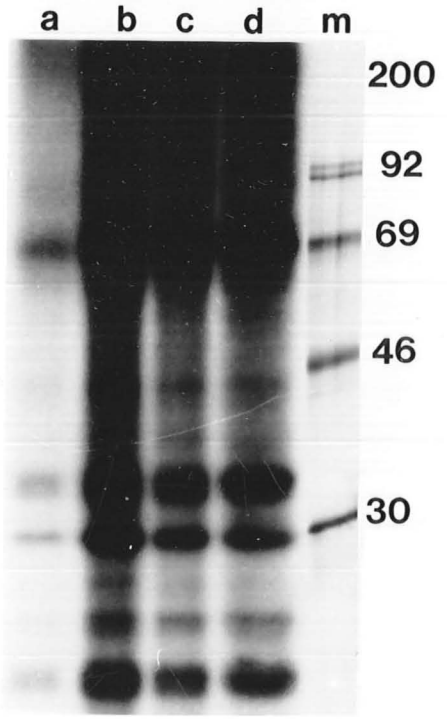
In order to detect potential changes in phosphoprotein profile in the experiments described below, embryos were either pulse-labelled for 1h in medium containing 1mCi/ml [^{32}P]orthophosphate before harvest ("pulse-labelled"), or pulse-labelled for 1h followed by a 2h chase period in non-labelled medium ("pulse-chased").

6.3 DMAP has two distinct effects on protein phosphorylation

6.3.1 DMAP can affect global levels of protein phosphorylation

Neant & Guerrier (1988) have reported that DMAP can inhibit all protein phosphorylation in starfish oocytes while having no effect on protein synthesis. As described in 3.4, DMAP can affect protein synthesis levels in mouse embryos but does not do so if the drug has been incubated at 37°C overnight before use. Groups of 4-cell and 8-cell embryos (0h post-division) were therefore pre-incubated for 1h and pulse-labelled for 1h with [^{32}P]orthophosphate in control medium or in medium containing

6.2



4mM DMAP. The medium containing DMAP had previously been incubated either at 4°C for 16h or at 37°C for 1h or for 16h. As shown in Figure 6.2, only the medium containing DMAP that had previously been incubated at 37°C for just 1h had a significant effect on the level of protein phosphorylation (Fig. 6.2, compare intensity of a-d). The reduction in ^{32}P-labelling appears non-specific, affecting all phosphoprotein bands equally (compare bands in a-d).

As described in 3.4, incubation of embryos in 4mM DMAP can result in a similar global decrease in the incorporation of [^{35}S]methionine into protein under similar incubation conditions to those that result in a decrease in ^{32}P-labelling. It has not proved possible to separate the inhibition of protein synthesis from the inhibition of protein phosphorylation in mouse 8-cell embryos. A similar range of effects of DMAP on [^{35}S]methionine incorporation and ^{32}P-labelling have also been found in oocytes and 1-cell embryos (J. McConnell, unpublished observations).

However, the morphological effect of DMAP, resulting in premature flattening in 4-cell and 8-cell embryos, is still apparent in embryos incubated in DMAP that has previously been incubated for 16h or more at 37°C (see 3.5 and below, Table 6.1). It is therefore possible to achieve this morphological effect while having no apparent effect

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Figure 6.2 (facing page)

The effects of DMAP on the intensity of phosphoprotein bands obtained after SDS-PAGE varies with prior incubation conditions.

One-dimensional SDS-PAGE of ^{32}P -labelled polypeptides obtained by 1h pulse-labelling with [^{32}]orthophosphate of (a) 4-cell embryos pre-incubated for 1h and labelled in 4mM DMAP that had previously been at 37°C for 1h; (b) 4-cell embryos pre-incubated for 1h and labelled in 4mM DMAP that had previously been at 4°C for 16h; (c) 4-cell embryos pre-incubated for 1h and labelled in 4mM DMAP that had previously been at 37°C for 16h; (d) control 4-cell embryos, 1h post-division; (m) markers as in Fig. 5.1. Compare with effects of DMAP on [^{35}S]methionine incorporation, Fig. 3.8.

on global levels of ^{32}P-labelling or [^{35}S]methionine incorporation. In the experiments described below, DMAP was, therefore, always incubated at 37°C for 16h before use.

6.3.2 The morphological effect of DMAP correlates with the increased ^{32}P-labelling of a single phosphoprotein spot

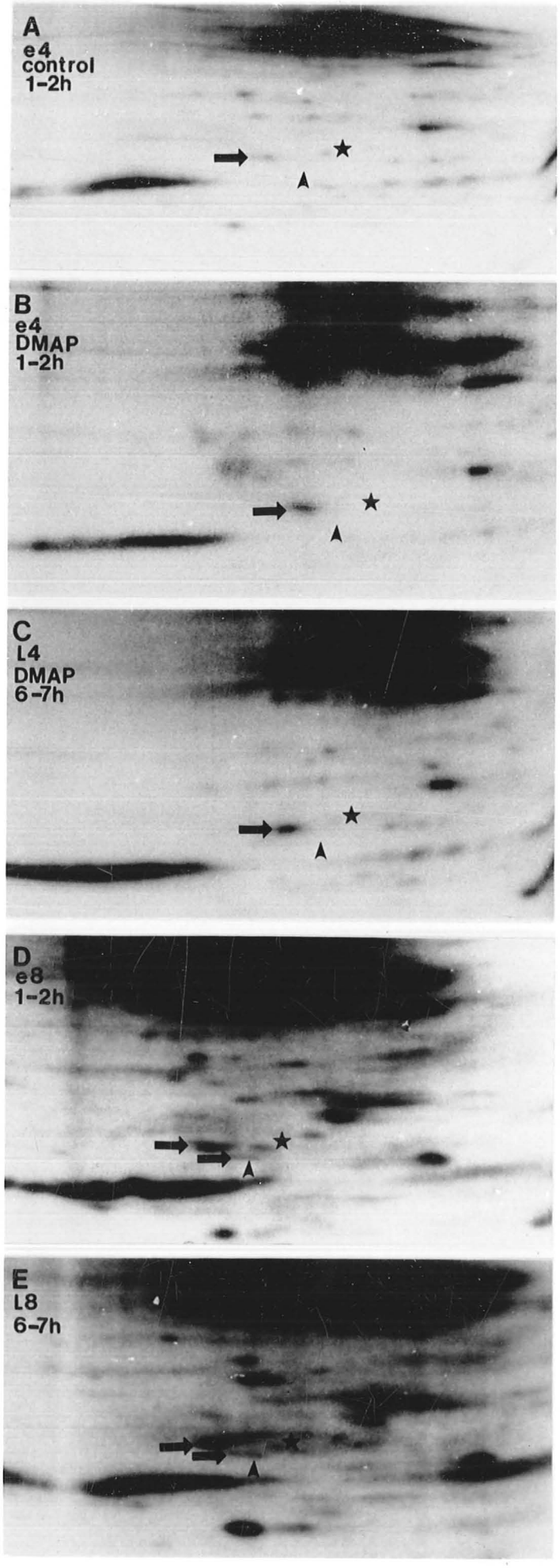
When 4-cell embryos were pre-incubated for 1h and then labelled with [^{32}P]orthophosphate for 1h in 4mM DMAP that had previously been incubated at 37°C for 16h, the pattern of phosphoprotein bands obtained after one-dimensional SDS-PAGE was not detectably different from that of controls of the same age (compare Fig. 6.2c & d). Groups of 4-cell and 8-cell embryos (0h and 5h post-division) were therefore pre-incubated in 4mM DMAP (16h prior incubation at 37°C) for 1h and then pulse-labelled in 4mM DMAP (16h prior incubation at 37°C) containing 1mCi/ml [^{32}P]orthophosphate for 1h. Flattening scores for such experiments are shown in Table 6.1. After separation of the ^{32}P-labelled polypeptides obtained in two dimensions, a single phosphoprotein spot, M_r 41K, was reproducibly increased in relative intensity in all DMAP-treated groups compared to controls (large arrow, Fig. 6.3B-E compared to 6.3A and Fig. 6.1). This phosphoprotein spot appears to co-migrate with a spot M_r 41K that is usually detected in 4-cell and 8-cell embryos but which increases in relative intensity in pulse-chased late 8-cells compared to early 8-cells or 4-cells (large arrow, Fig. 6.3 & Fig. 6.1d). These data are summarised in Table 6.2 (p102).

Table 6.1

Flattening scores of 4-cell and 8-cell embryos pre-incubated for 1h and then pulse-labelled for 1h in 4mM DMAP that had previously been incubated at 37°C for 16h

Developmental stage	n (embryos)	% flattening at end of pulse-labelling (2h in DMAP)	
		controls	DMAP-treated
early 4-cell (0-2h)	150	0	54
late 4-cell (6-8h)	156	0	62
early 8-cell (0-2h)	148	47	76
late 8-cell (6-8h)	152	89	92

6.3



6.4 Protein synthesis inhibition alters the pattern of phosphoproteins detected in both 4-cell and 8-cell embryos

Inhibition of protein synthesis early in the third cell cycle causes uvomorulin-mediated cellular flattening to occur prematurely, during the ensuing few hours (Levy *et al.*, 1986). The impact of this treatment and resulting premature flattening on the phosphoprotein profile of 4-cell embryos was therefore examined.

6.4.1 Inhibition at the time of labelling with [^{32}P]orthophosphate

Four-cell and 8-cell embryos were incubated for 3h immediately post-division in either 10μM anisomycin or 400μM cycloheximide, each of which was sufficient to prevent, within 1h, all detectable incorporation of [^{35}S]methionine into protein (assessed by SDS-PAGE; data not shown). Embryos were then pulse-labelled

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Figure 6.3 (facing page)

The effects of DMAP on phosphoprotein profile.

Two-dimensional IEF/SDS-PAGE separation of polypeptides labelled by 4-cell and 8-cell embryos during 1h pulse in medium containing 4mM DMAP (previously incubated at 37°C for 16h) and 1mCi/ml [^{32}P]orthophosphate (A) early 4-cell embryos, 1h post-division; (B) late 4-cell embryos, 6h post-division; (C) early 8-cell embryos, 1h post-division; (D) late 8-cell embryos, 6h post-division. All embryos were pre-incubated for 1h in 4mM DMAP before labelling. A large arrow in each frame indicates a phosphoprotein spot, M_r 41K, that increases in relative intensity in all DMAP-treated embryos (B-D) compared to controls (A & Fig. 6.1; compare intensity to that of reference spot marked with a star in each). This 40K spot appears to co-migrate with a 40K spot that increases in relative intensity in pulse-chased late 8-cells (Fig. 6.1d) compared to early 8-cells (Fig. 6.1c), and is marked similarly in Fig. 6.1 The second arrow in D & E marks a phosphoprotein spot M_r 40K that behaves similarly to the 40K spot in untreated embryos but does not appear to alter in intensity after exposure to DMAP. IEF and relative molecular mass markers (m) as Fig. 6.1.

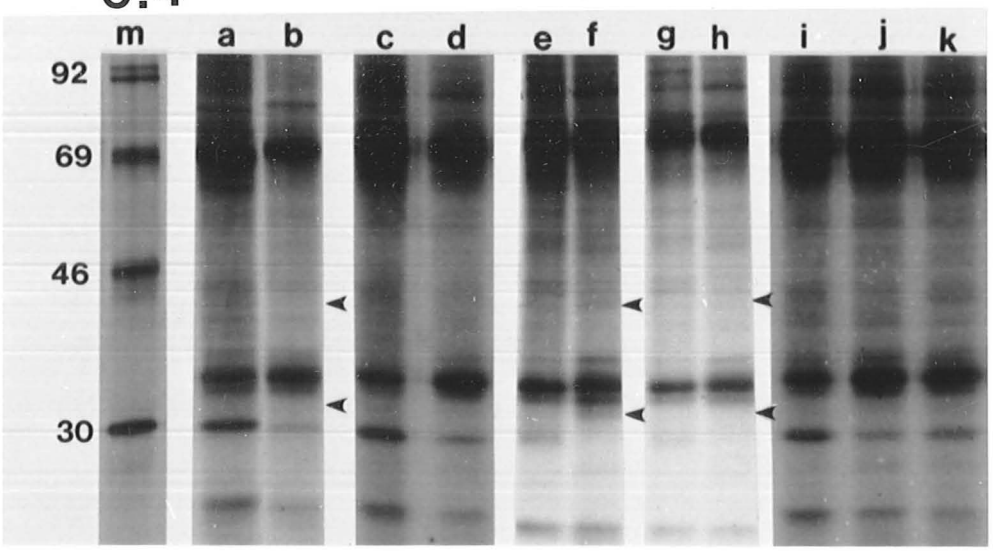
with [^{32}P]orthophosphate in the continued presence of protein synthesis inhibitor and some groups of embryos were "chased" in medium also containing the inhibitor. Pulse-labelling or pulse-chase in the presence of protein synthesis inhibitor had little apparent effect on the phosphoprotein bands detectable in 4-cell embryos after separation by one-dimensional SDS-PAGE (Fig. 6.4a & c pulse-labelled; b & d, pulse-chased). Similarly, pulse-labelling of 8-cell embryos revealed similar phosphoprotein bands in the presence or absence of protein synthesis inhibitor (Fig. 6.4e & g). These results indicate that most of the phosphoprotein bands detected on gels represent the phosphorylation of pre-existing protein species.

However, by contrast, when pulse-chased 8-cell embryos were examined for the appearance of novel phosphoproteins, bands M_r 32K and 40K were no longer detectable if the medium used for pulse and chase included either of the protein synthesis inhibitors (arrowheads, Fig. 6.4f & h). These phosphoprotein bands are apparent in controls at the 8-cell stage after pulse-chase but not at the 4-cell stage (arrowheads Fig. 6.4; compare f, 8-cell, & b, 4-cell). They probably correspond to phosphoprotein spots M_r 32K and 40K that appear or increase in relative intensity in late 8-cells after pulse chase (shown with a large arrowhead and a large or open arrow, respectively, in Fig. 6.1d, see Table 6.2). Inhibition of protein synthesis during pulse-chase may therefore reduce or prevent, selectively, the development of certain 8-cell-specific, pulse-chase-specific, phosphoproteins.

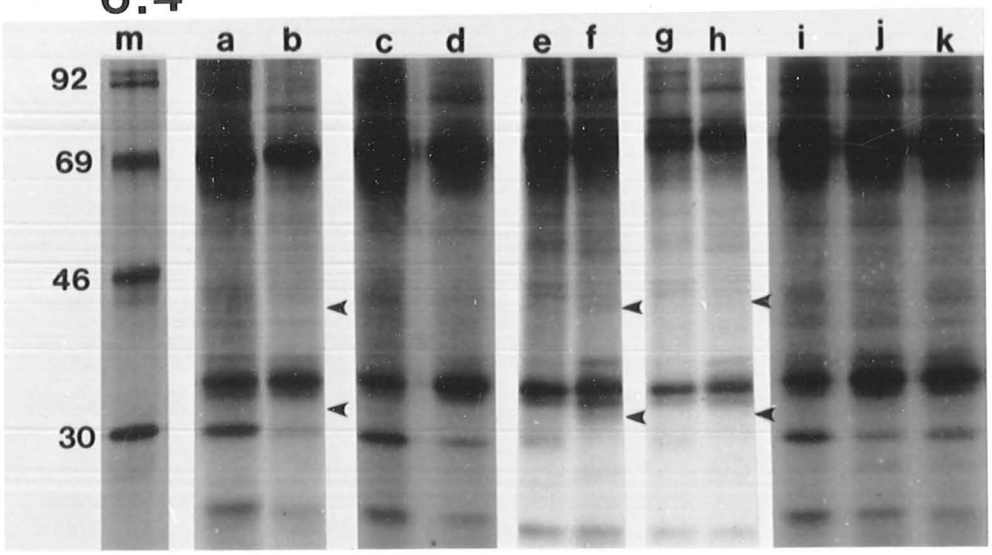
6.4.2 Inhibition prior to labelling with [^{32}P]orthophosphate

Since the incidence of at least some phosphoproteins is susceptible to inhibition of protein synthesis, it is difficult to relate changes in compaction unambiguously to changes in phosphoprotein profile as long as the protein synthesis inhibitor remains present. However, Levy *et al.* (1986) showed that a short period of exposure to protein synthesis inhibitor at the early 4-cell stage was sufficient to generate premature cell flattening by the late 4-cell stage. In confirmation of this result, when groups of 4-cell embryos were incubated for 3h in medium containing 10μM anisomycin immediately post-division, washed and cultured for a further 3h before assessment of intercellular flattening, scores of 59-64% flattening (n= 50 in each of 5 experiments) were

6.4



6.4



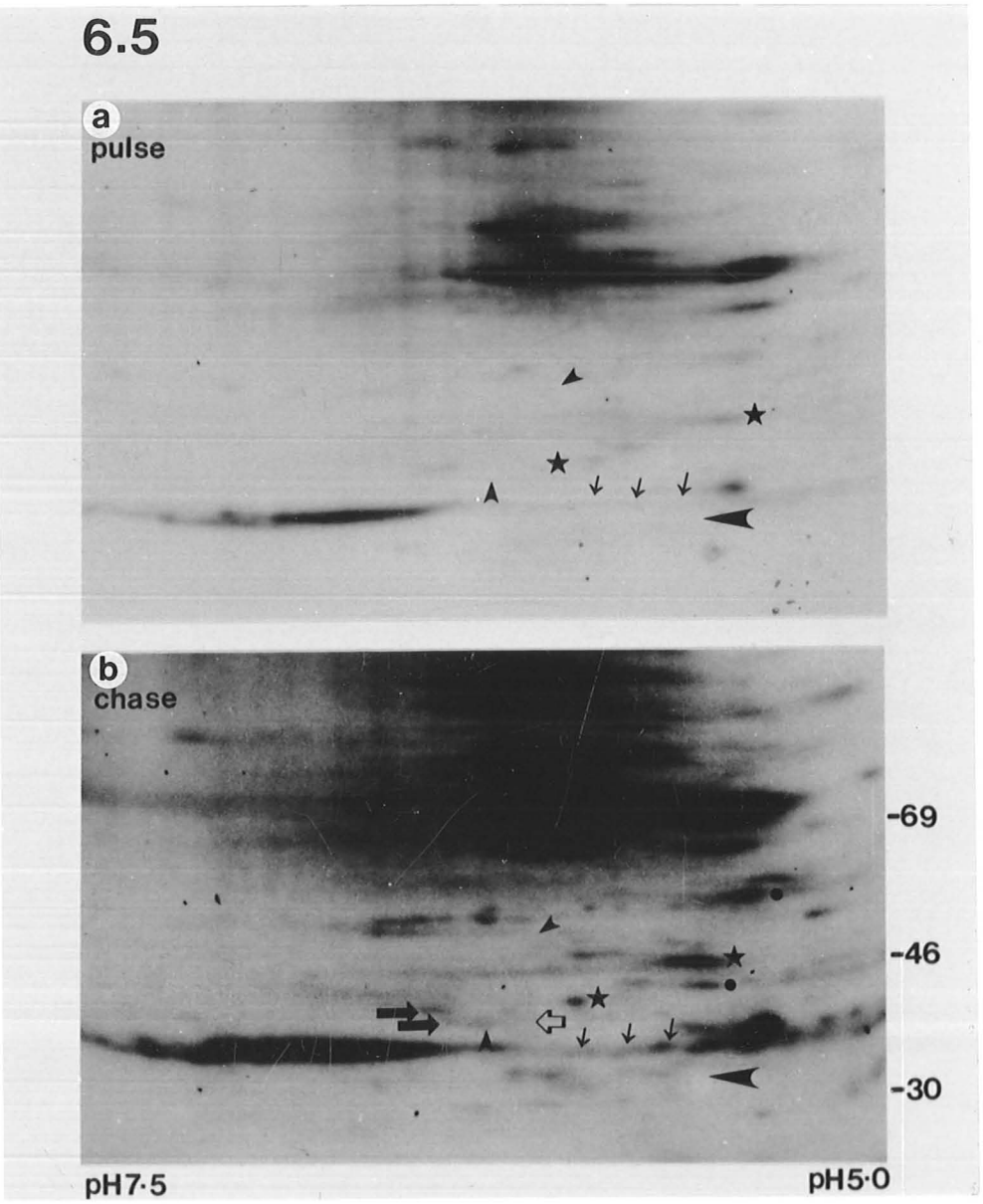
obtained, compared to 0% flattening in untreated 4-cell embryos 6h post-division. The effect of prior inhibition of protein synthesis on the phosphoprotein profile of 4-cell embryos was therefore examined.

Figure 6.4 (facing page)

The effects of protein synthesis inhibition on the phosphoprotein bands detected in 4-cell and 8-cell embryos.

One-dimensional SDS-PAGE separation of [^{32}P]orthophosphate-labelled polypeptides from (a) early 4-cell embryos (3h post-division), 1h pulse-labelled; (b) early 4-cell embryos (3h post-division), 1h pulse + 2h chase; (c) early 4-cell embryos (3h post-division), incubated from time of division in 10μM anisomycin, 1h pulse-labelled in anisomycin; (d) early 4-cell embryos (3h post-division), incubated from time of division in anisomycin, 1h pulse + 2h chase in anisomycin; (e) early 8-cell embryos (3h post-division), 1h pulse-labelled; (f) early 8-cell embryos (3h post-division), 1h pulse + 2h chase; (g) early 8-cell embryos (3h post-division), incubated from time of division in 400μM cycloheximide, 1h pulse-labelled in cycloheximide; (h) early 8-cell embryos (3h post-division), incubated from time of division in cycloheximide, 1h pulse + 2h chase in cycloheximide; (i) 4-cell embryos (6h post-division), incubated for 3h from time of division in anisomycin followed by 3h in untreated medium, 1h pulse-labelled in untreated medium; (j) 4-cell embryos (6h post-division), incubated for 3h from time of division in anisomycin followed by 3h in untreated medium, 1h pulse + 2h chase in untreated medium; (k) 4-cell embryos (6h post-division), incubated for 3h from time of division in anisomycin followed by 3h in untreated medium, 1h pulse + 1h chase in untreated medium followed by final 1h of chase in 25 ng/ml PMA. Arrowheads indicate phosphoprotein bands (M_r 32K & 40K) detected in control pulse-chased 8-cells but not in 8-cells pulsed and chased in the presence of 400μM cycloheximide, nor in 4-cell embryos. Lanes a-d, i-k & m are taken from a single gel and lanes e-h from a second gel. Relative molecular mass markers (m) as for Fig. 5.1.

6.5



Groups of partially-flattened 4-cell embryos, resulting from a 3h exposure to anisomycin followed by 3h in control medium, were pulse-labelled in control medium with [^{32}P]orthophosphate at 6h post-division. This procedure revealed no phosphoproteins separable in one or two dimensions different from those of control 4-cells (compare Fig. 6.4i & a; Fig. 6.5a compared to Fig. 6.1a, arrows, arrowheads and stars mark similar positions in each). After pulse-chase (flattening 72-75% by the end of the chase period), most of the phosphoprotein profile also remained characteristic of 4-cell embryos (Fig. 6.4j and Fig. 6.5b). In particular, most of the phosphoproteins characteristic of late 8-cell embryos after pulse-chase (M_r 32K, 35K, 41K, 51K; see Fig. 6.1 and Table 6.2) were not apparent in prematurely flattened late 4-cells after pulse-chase (arrows and arrowheads mark approximate positions in 6.5b).

Some novel phosphoprotein spots did appear in pulse-chased, prematurely

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Figure 6.5 (facing page)

The effects of protein synthesis inhibition on phosphoprotein profile.

Two-dimensional IEF/SDS-PAGE separation of [^{32}P]orthophosphate-labelled polypeptides from (a) 4-cell embryos (6h post-division), incubated for 3h from time of division in anisomycin followed by 3h in untreated medium, 1h pulse-labelled in untreated medium, as Fig. 6.4i; (b) 4-cell embryos (6h post-division), incubated for 3h from time of division in anisomycin, then 3h in untreated medium followed by a 1h pulse + 2h chase in untreated medium, as Fig. 6.4j. Arrows and arrowheads indicate similar positions to those on corresponding gels in Fig. 6.1b &d. Dots indicate phosphoprotein spots that appeared non-reproducibly in 4-cells after anisomycin pre-treatment. A phosphoprotein spot, M_r 40K, reproducible faintly detected in pulse-chased (b) but not pulse-labelled (a) anisomycin pre-treated 4-cells and not in untreated 4-cells (Figs. 5.7 & 6.1a) is marked with a small arrowhead and large arrow and appears to co-migrate with a spot marked similarly in Fig. 6.1; see also Table 6.2. The autoradiograph in (b) was exposed for longer than that in (a). IEF and M_r markers as in Fig. 6.1

flattened late 4-cells, but in a non-reproducible manner (examples M_r approx. 44K and 56K are marked with dots on Fig. 6.5b). A single phosphoprotein spot M_r 40K was reproducibly faintly detected that was not detectable in untreated 4-cells (Fig. 6.5b, small arrowhead and large solid arrow). This phosphoprotein was only apparent after 2-dimensional separation (Fig. 6.4b and j indistinguishable) and appears to co-migrate with a phosphoprotein spot normally detectable in 8-cell embryos but not in 4-cells (small arrowhead, M_r 40K, Fig. 6.1a & b). However, a correlation between this phosphoprotein spot and the 8-cell-specific spot, M_r 40K, with which it appears to co-migrate, must remain tentative due to the low intensity of the spot observed and the appearance of other novel phosphoprotein spots following anisomycin pre-treatment.

Unlike the situation for 4-cell embryos, incubation of 8-cell embryos in anisomycin for 3h immediately post-division followed by washing into control medium for a further 3h had no detectable effect on the rate or extent of intercellular flattening. When such embryos were pulse-labelled or pulse-chased in control medium at 6h post-division, the phosphoproteins obtained were not detectably different from those of controls of the same age (data not shown).

In summary, the appearance of phosphoproteins M_r 32K and 40K in 8-cells following pulse-chase is dependent on continuing protein synthesis at the time of labelling. Most of the phosphoproteins of 4-cell embryos are unaffected either by the presence of protein synthesis inhibitor during labelling or by premature flattening following an earlier exposure to protein synthesis inhibitor. A faint 40K phosphoprotein spot occurs in 4-cell embryos that have been exposed previously to protein synthesis inhibitor and are pulse-chased after the restoration of protein synthesis; this appears to migrate in a similar position to a 40K phosphoprotein that normally occurs only in 8-cells.

6.5 Effects of phorbol ester on phosphoproteins

Phorbol esters such as PMA are believed to exert their effects on cells by the stimulation of the activity of protein kinase C (see 4.1 and 4.7). Incubation of 8-cell embryos in phorbol ester (PMA, 25ng/ml) for 1h prevents and reverses intercellular flattening (see 4.3, Figs. 4.2, 4.3). In addition, the actin cytoskeleton is disrupted very severely in late 8-cell embryos but much less severely affected in earlier embryos or oocytes, after exposure to PMA (see 4.4). To assess the effects of incubation in PMA on the phosphoprotein profile, embryos were pulse-labelled with [^{32}P]orthophosphate in untreated medium or medium containing 25ng/ml PMA. After separation in one dimension, the phosphoproteins obtained from 2-cell and early and late 4-cell embryos were apparently identical after the presence or absence of PMA in the labelling or chase medium (Fig. 6.6, compare a & b, late 2-cell; e & f, early 4-cell; i & j, late 4-cell; the

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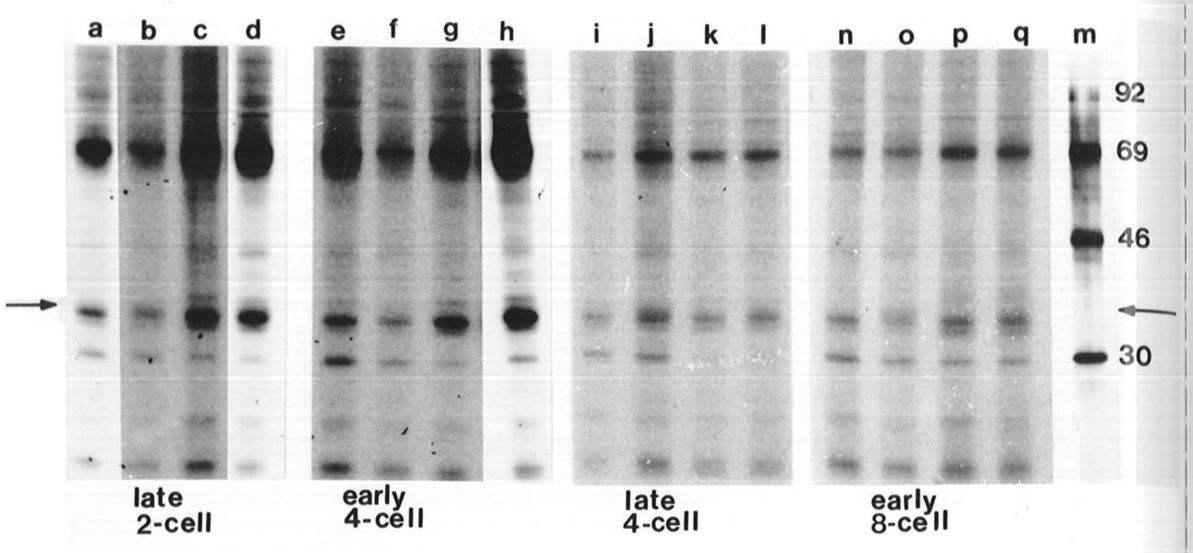
Figure 6.6 (facing page)

The effects of PMA on phosphoproteins after pulse-labelling and pulse-chase

One-dimensional SDS-PAGE separation of [^{32}P]orthophosphate-labelled polypeptides of embryos of different stages, either pulse-labelled for 1h without (a, e, i, n) or with (b, f, j, o) 25ng/ml PMA in the labelling medium; or pulse-labelled for 1h and chased for 2h without (c, g, k, p) or with (d, h, l, q) 25ng/ml PMA in the medium used for the final 1h of the chase period. Timing of embryos: (a-d) late 2-cells, 48h post-hCG; (e-h) early 4-cells, 0h post-division; (i-l) late 4-cells, 6h post-division; (n-q) early 8-cells, 0h post-division; (m) M_r markers, as in Fig. 5.1. Arrows mark the position of a phosphoprotein band M_r 35K that is detectable only in 8-cells labelled or chased in the presence of PMA; see also Fig. 6.7. Lanes have been exposed for different periods of time to compensate for differences in loading between samples. Data for oocytes and 1-cell embryos is not shown as these stages labelled far less intensely with [^{32}P]orthophosphate than later stages and so are not comparable. However, no differences in the pattern of phosphoprotein bands of oocytes or 1-cells were detectable following pulse-labelling or pulse-chase with or without PMA.

6.6



phosphoproteins obtained from oocytes and 1-cell embryos were also apparently unaffected by PMA, data not shown). In 8-cell embryos, a novel phosphoprotein band M_r 35K was apparent after incubation in PMA (arrow, Fig. 6.6, compare n & o).

Similar groups of embryos were pulse-labelled and then either chased in untreated medium for 2h or chased in untreated medium for 1h and in medium containing 25 ng/ml PMA for the final 1h of the chase. This protocol was used for two reasons. Firstly, using PMA in pulse-chase experiments allowed examination of the effects of PMA on phosphoproteins that are detected after pulse-chase but not after pulse-labelling. Secondly, using PMA in the final hour of the chase period allowed comparison of the effects of PMA on phosphoproteins with the effects on the cytoskeleton and cell surface that were described in Chapter 4, in which embryos were exposed to 25ng/ml PMA for 1h immediately before harvesting.

Preliminary analysis of the phosphoprotein bands obtained by separation in one dimension revealed that the phosphoproteins obtained from oocytes and embryos up to the late 4-cell stage were apparently identical whether or not PMA was present in the chase medium (Fig. 6.6, compare c & d, late 2-cell; g & h, early 4-cell; k & l, late 4-cell). In 8-cell embryos, a novel phosphoprotein band M_r 35K appeared after chase in PMA (arrow Fig. 6.6, compare p & q; see also Fig. 6.7). This band appears to co-migrate with that found in 8-cell embryos pulse-labelled in the presence of PMA (Fig. 6.6n & o; see also Fig. 6.7). In order to establish more precisely when, during development, this novel phosphoprotein band appeared in response to PMA, closely-timed groups of late 4-cell and 8-cell embryos were pulse-labelled and pulse-chased in the presence or absence of PMA. The 35K band was not detectable in 4-cells that had been incubated previously for 3h in anisomycin and showed premature flattening, even though this flattening was reversed by PMA (Fig. 6.4k). It was also not detectable in late 4-cells (9h post-division) that were pulse-labelled and chased such that they were early 8-cells by the time of incubation in PMA (Fig. 6.7 compare c & d). The 35K band was detectable in both early 8-cells (Fig. 6.7f) and late 8-cells (Fig. 6.7h).

In order to establish whether the phosphoprotein band M_r 35K that increased in intensity after exposure to PMA was related to the phosphoproteins of similar M_r that increased in relative intensity with passage from the 4-cell to the 8-cell stage, the

phosphoproteins of groups of PMA-treated embryos were separated in two dimensions. The changes due to PMA after one-dimensional separation can be correlated with the increase in relative intensity of a group of phosphoprotein spots M_r 35K (small arrows Fig. 6.7j & k; compare intensity with that of the reference phosphoprotein spots marked stars). Such an increase in labelling intensity is consistent with an increased level of phosphorylation. The increase in intensity of these phosphoproteins M_r 35K is not seen in 4-cells that were pulse-labelled and then chased in the presence of PMA (Fig. 6.7i). The increase in intensity appears slightly more extreme in late 8-cells than early 8-cells (compare Fig. 6.7j & k). The most acidic members of this group of phosphoprotein spots migrate close to the bottom of the IEF gels used here so their behaviour cannot be described with confidence. The group of M_r 35K that are affected by exposure to PMA co-migrate with those that are more intensely labelled in control 8-cells than 4-cells (small arrows, Fig. 6.1).

In addition, phosphoprotein spots M_r 32K, 46K and 50K are more intensely labelled in PMA-treated embryos than in controls (large arrowhead and wide arrows, Fig. 6.7C). The phosphoprotein spot M_r 32K co-migrates with the spot that increases in intensity with developmental age in untreated embryos (large arrowhead, Fig. 6.1). The phosphoprotein spot M_r 46K that appears after PMA-treatment of 8-cells may correspond to a phosphoprotein spot with similar M_r and pI` that occurs in untreated 8-cells after 1h pulse and 5h chase (arrow, Fig. 5.6C). The phosphoprotein spot M_r 50K that appears after PMA-treatment does not seem to correlate with any spot detected under other circumstances. The remainder of the 8-cell-specific phosphoproteins (M_r 40K, 41K, 51K) appeared in PMA-treated 8-cells as in controls (Fig. 6.7 compared to Fig. 6.1). These results are summarised in Table 6.2 (p102).

The effects of PMA on the cytoskeleton and surface of 8-cell blastomeres can be interpreted as an extreme, but spatially disorganised version of the process that causes cell flattening and polarisation to occur at the 8-cell stage (Chapter 4). PMA seems to cause the increased ^{32}P-labelling of phosphoproteins that are naturally more heavily labelled at the 8-cell stage than the 4-cell stage. The behaviour of the phosphoproteins affected, M_r 32K and 35K, may therefore correlate with the morphological effects of PMA.

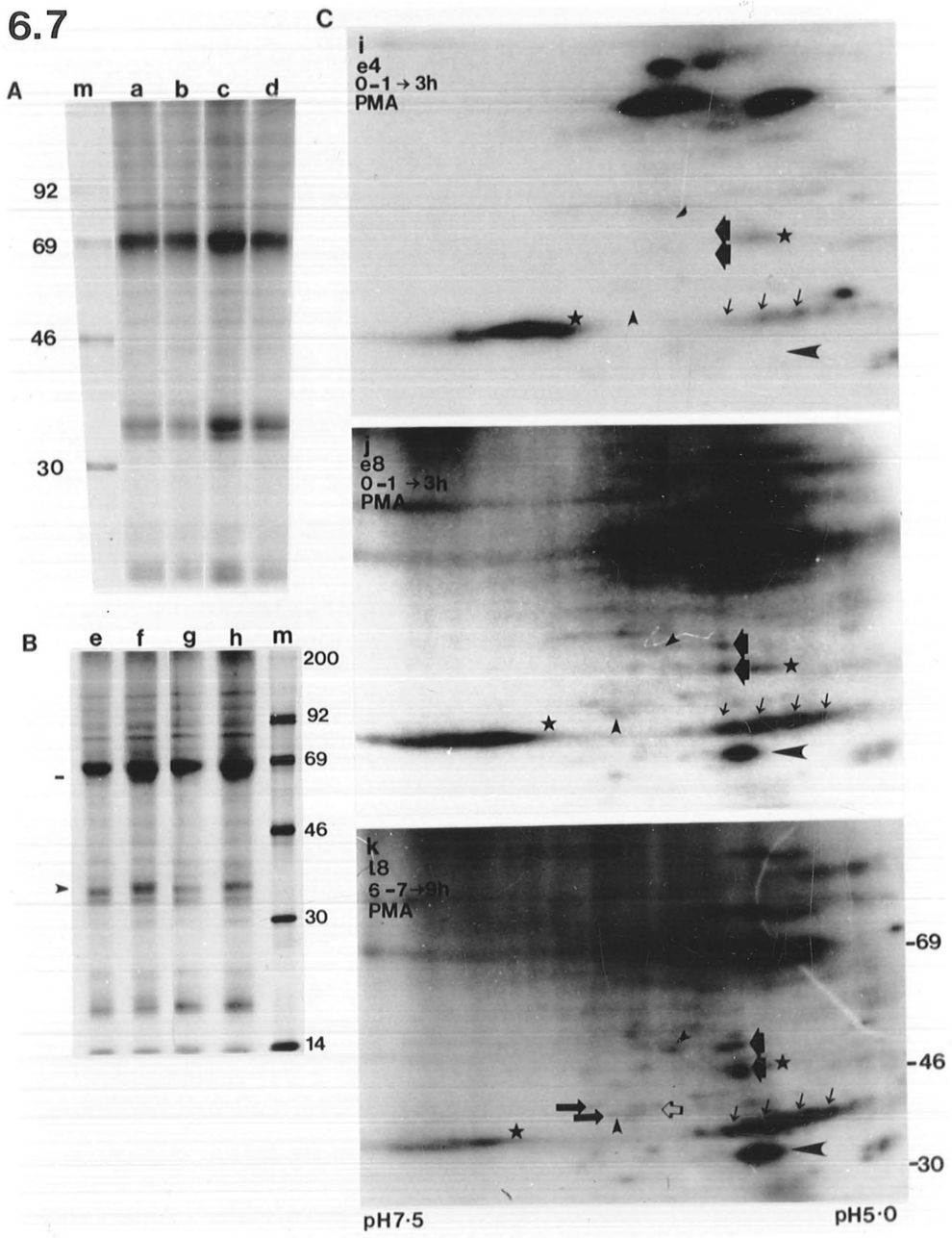


Figure 6.7 (facing page)

The effects of PMA on the phosphoprotein profile of 4-cell and 8-cell embryos.

A. One-dimensional SDS-PAGE separation of [^{32}P]orthophosphate-labelled polypeptides from (a) 4-cell embryos (6h post-division), 1h pulse + 2h chase, as Fig. 6.6g; (b) 4-cell embryos (6h post-division), 1h pulse + 2h chase, final 1h of chase in 25 ng/ml PMA, as Fig. 6.6h; (c) late 4-cell embryos (9h post-division), 1h pulse + 2h chase, 8-cells at time of harvest; (d) late 4-cell embryos (9h post-division), 1h pulse + 2h chase, 8-cells at time of harvest, final 1h of chase in PMA; (m) relative molecular mass markers as in Fig. 5.1.

B. One-dimensional SDS-PAGE separation of [^{32}P]orthophosphate-labelled polypeptides from (e) early 8-cell embryos (0h post-division), 1h pulse + 2h chase, as Fig. 6.6p; (f) early 8-cell embryos (0h post-division), 1h pulse + 2h chase, final 1h of chase in 25 ng/ml PMA, as Fig. 6.6q; (g) late 8-cell embryos (6h post-division), 1h pulse + 2h chase; (h) late 8-cell embryos (6h post-division), 1h pulse + 2h chase, final 1h of chase in PMA. A bar indicates the position of BSA that was not adequately washed from PMA-treated samples, causing distortion of phosphoprotein bands in this region of the gel. An arrowhead indicates the phosphoprotein band M_r 35K that is detected in PMA-treated 8-cells but not in controls; see also Fig 6.6.

C. Two-dimensional IEF/SDS-PAGE separation of [^{32}P]orthophosphate-labelled polypeptides from (i) early 4-cell embryos (0h post-division), 1h pulse + 2h chase, final 1h in 25ng/ml PMA, as Fig. 6.6h; (j) early 8-cell embryos (0h post-division), 1h pulse + 2h chase, final 1h in 25ng/ml PMA, as f, above; (k) late 8-cell embryos (6h post-division), 1h pulse + 2h chase, final 1h in 25ng/ml PMA, as h, above. Small arrows (M_r 35K) and a large arrowhead (M_r 32K) indicate phosphoprotein spots that increase in relative intensity after incubation of 8-cells in PMA; these co-migrate with spots marked similarly in Fig. 6.1. Large wide arrows indicate phosphoprotein spots (M_r 46K, 50K) newly detectable after incubation of 8-cells in PMA; stars mark reference phosphoprotein spots that do not seem to alter in intensity. Other arrows and arrowheads mark similar positions to those shown in Fig. 6.1. IEF and M_r markers (m) as in Fig. 6.1. The data from this Figure are summarised in Table 6.2 (p102).

6.6 Incubation in Ca^{2+}-free medium affects the pattern of phosphoproteins detected in 8-cell embryos

6.6.1 Incubation in Ca^{2+}-free medium causes several changes to the phosphoproteins of 8-cell embryos

Incubation of early 8-cell embryos in medium containing no added Ca^{2+} prevents the development of intercellular flattening (Ducibella & Anderson, 1975) and delays blastomere polarisation (Fleming *et al.,* 1989). Four-cell and 8-cell embryos immediately post-division were incubated in Ca^{2+}-free medium for 6h and then pulse-labelled with [^{32}P]orthophosphate in Ca^{2+}-free medium. There were no reproducible differences detected in the phosphoprotein profile of 4-cell embryos compared to untreated controls (compare Figs. 6.8a and 6.1a). By contrast, when 8-cell embryos immediately post-division were incubated similarly for 6h in Ca^{2+}-free medium (by which time intercellular flattening had occurred in control embryos) and were then pulse-labelled with [^{32}P]orthophosphate in Ca^{2+}-free medium with or without a further chase in Ca^{2+}-free medium, changes to the phosphoprotein profile were evident.

As shown in Figure 6.8b, after pulse-labelling the pattern of phosphoprotein spots seen differs from that of control 8-cell embryos by the absence of the phosphoprotein spot M_r 32K (large arrowhead). This phosphoprotein spot characteristically appears in pulse-labelled 8-cells but not 4-cells (compare Fig. 6.8b with Fig 6.1b) and co-migrates with the phosphoprotein spot, M_r 32K, that is more intensely labelled after incubation in PMA (Fig. 6.7j & k). In addition, the group of phosphoprotein spots M_r 35K, that would normally increase in labelling intensity in 8-cells compared to 4-cells (small arrows, Fig. 6.1), do not do so after Ca^{2+}-free incubation, appearing more characteristic of control 4-cells (small arrows, Fig. 6.8b; compare with Fig. 6.1a & b). These co-migrate with the group of phosphoprotein spots M_r 35K that increase in relative intensity in PMA-treated 8-cells compared to controls (Fig. 6.7j, k). However, incubation in Ca^{2+}-free medium does not make the entire 8-cell phosphoprotein profile resemble that of 4-cells; the phosphoprotein spot, M_r 40K, characteristic of pulse-labelled 8-cells but not 4-cells <u>is</u> detectable after incubation of 8-cells in Ca^{2+}-free medium (small arrowhead, Fig. 6.8b).

6.8

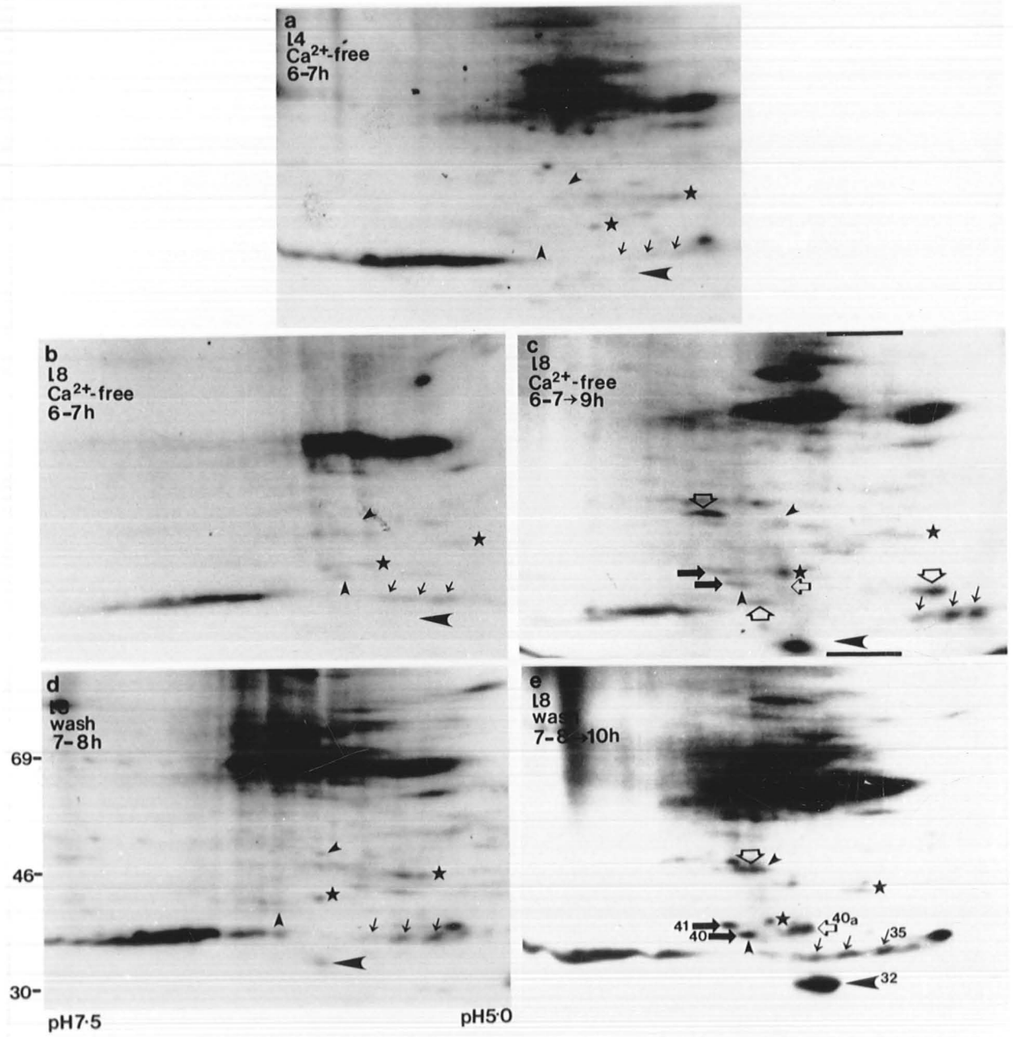


Figure 6.8 (facing page)

The effects of pre-incubation in Ca^{2+}-free medium on the phosphoprotein profiles of 4-cell and 8-cell embryos.

Two-dimensional IEF/SDS-PAGE separation of ^{32}P-labelled polypeptides from (a) 4-cell embryos (6h post-division), incubated from time of division in Ca^{2+}-free medium, 1h pulse-labelled in Ca^{2+}-free medium; (b) 8-cell embryos (6h post-division), incubated from time of division in Ca^{2+}-free medium, 1h pulse-labelled in Ca^{2+}-free medium; (c) 8-cell embryos (6h post-division), incubated from time of division in Ca^{2+}-free medium, 1h pulse + 2h chase in Ca^{2+}-free medium; (d) 8-cell embryos (6h post-division), incubated from time of division in Ca^{2+}-free medium, washed for 1h in complete medium, 1h pulse-labelled in complete medium; (e) 8-cell embryos (6h post-division), incubated from time of division in Ca^{2+}-free medium, washed for 1h in complete medium, 1h pulse + 2h chase in complete medium. A large arrowhead indicates a phosphoprotein spot M_r 32K that decreases in intensity compared to controls in pulse-labelled samples both after incubation in Ca^{2+}-free medium and after washing from Ca^{2+}-free medium. However, this spot increases in intensity in pulse-chased compared to pulse-labelled samples after incubation in in Ca^{2+}-free medium, as in pulse-labelled and pulse-chased controls, and increases further in intensity, to a similar level to controls, in pulse-chased samples following washing from Ca^{2+}-free medium. Small arrows indicate phosphoprotein spots (M_r 35K) that decrease in size and relative intensity compared to control 8-cells after incubation in in Ca^{2+}-free medium (b, c). Small arrowheads indicate phosphoprotein spots (M_r 40K, 51K) that appear in 8-cells but not in 4-cells, after incubation in Ca^{2+}-free medium and in controls. Large solid arrows indicate phosphoprotein spots (M_r 40K, 41K) that fail to increase in relative intensity in late 8-cells pulse-labelled and chased in Ca^{2+}-free medium, compared to controls. After washing from Ca^{2+}-free medium, M_r 40K & 41K (large arrows) appear like controls but M_r 35K (small arrows) does not (d, e). An open arrow indicates a diffuse phosphoprotein spot M_r 40K ("$40K_a$") present in pulse-chased control late 8-cells but absent from pulse-chased late 8-cells after incubation in Ca^{2+}-free medium. This spot appears after pulse-chase following washing from Ca^{2+}-free medium as in controls (e). Open wide arrows are above novel phosphoprotein spots and shifts in pI` of existing phosphoprotein spots seen only after pulse-chase following incubation in Ca^{2+}-free medium. Stars are to the right of reference phosphoprotein spots that do not appear to change in intensity. Note that the gel shown in (c) appears stretched in the region shown by bars above and below the frame; compensation must be made for this in comparing (c) with other gels. IEF and M_r markers as in Fig. 6.1. These data are summarised in Table 6.2.

If, after 6h pre-incubation and pulse-labelling with [^{32}P]orthophosphate in Ca^{2+}-free medium, embryos were further chased in Ca^{2+}-free medium, certain chase-dependent features of the late 8-cell pulse-chase phosphoprotein profile were affected. The phosphoprotein spot M_r 32K increased in intensity compared to embryos that had been pulse-labelled with [^{32}P]orthophosphate in Ca^{2+}-free medium (large arrowhead, compare Fig. 6.8b & c) , but did not increase to the level of control pulse-chased late 8-cells (compare Fig. 6.8c with Fig. 6.1d; compare intensity to reference polypeptides marked with stars in each). The group of phosphoprotein spots M_r 35K also increased in intensity after pulse-chase compared to pulse-labelling, but this group seemed to focus at a slightly more acidic pI than in controls (small arrows, Fig. 6.8b & c; compare intensity and position with reference polypeptide marked with a star).

In addition, those changes in phosphoprotein profile that are specifically characteristic of pulse-chased late 8-cells failed to occur after pulse-chase of late 8-cells in Ca^{2+}-free medium: the phosphoprotein spots M_r 40K and 41K that increase in intensity in late 8-cells after pulse-chase (large solid arrows, Fig. 6.8c) and the more acidic, diffuse phosphoprotein spot M_r 40K that appears only in late 8-cells after pulse-chase (open arrow, Fig. 6.8c) failed to do so after pulse-chase in Ca^{2+}-free medium (compare Fig. 6.8c with Fig. 6.1d). Some new phosphoprotein spots and shifts in pI of other phosphoprotein spots are also detectable after pulse-chase following incubation in Ca^{2+}-free medium (marked with open wide arrows in Fig. 6.8c). These results are summarised in Table 6.2 (p102).

6.6.2 The effects of incubation in Ca^{2+}-free medium are reversible

To establish that incubation in Ca^{2+}-free medium had not had any long-term deleterious effect on embryos, groups of 8-cell embryos were incubated for 6h in Ca^{2+}-free medium (flattening score 0) and then washed and incubated for a further 1h in complete medium before pulse-labelling, with or without a chase, also in complete medium. After 1h wash in complete medium, some intercellular flattening had occurred in most embryos (flattening scores 13%-21% in 6 separate experiments, n=50 in each). Further flattening occurred during the 1h pulse-labelling period (flattening score 40-44% in 6 separate experiments, n=50 in each) and during the chase

(flattening score 63-86%, 4 experiments, n=50 in each).

Following washing from Ca^{2+}-free medium and pulse-labelling, the phosphoprotein spot, M_r 32K, that was not detectable in pulse-labelled 8-cells in Ca^{2+}-free medium was now faintly detected (large arrowhead, Fig. 6.8d). This 32K phosphoprotein spot appeared at a similar intensity to controls following an additional 2h chase in complete medium (large arrowhead, Fig. 6.8e). The group of phosphoprotein spots, M_r 35K, that decreased in relative intensity with Ca^{2+}-free incubation, remained at lower relative intensities than in controls after pulse-labelling or pulse-chase in complete medium (small arrows, Fig. 6.8d & e compared to Fig. 6.1c & d; compare intensity to that of reference spots marked with stars in each). However, the remaining phosphoprotein spots characteristic of pulse-chased late 8-cells (M_r 40K and 41K) did appear after pulse-chase following washing from Ca^{2+}-free medium (large solid arrows M_r 40K & 41K and open arrow M_r $40K_a$, Fig. 6.8e compared to 6.8c and Fig. 6.1d). These results are summarised in Table 6.2 (p102). It is not possible to distinguish whether the recovery of late 8-cell phosphoprotein profile seen with pulse-chase but not with pulse-labelling is due to increased time after restoration of Ca^{2+} to the medium or due directly to the differences between the two labelling protocols.

In summary, incubation in the absence of Ca^{2+}, which prevents intercellular flattening and delays polarisation, is associated with the absence of several phosphoproteins characteristic of early and late 8-cell embryos. Restoration of Ca^{2+} to the medium, which rapidly restores cell flattening, is associated with the reappearance of phosphoproteins M_r 32K, 40K and 41K characteristic of late 8-cell embryos. Polarisation is restored less rapidly after the replacement of Ca^{2+} in the medium (Fleming *et al.,* 1989). The group of phosphoproteins, M_r 35K, also do not revert so rapidly to control levels of ^{32}P-labelling after the restoration of Ca^{2+} to the medium.

6.7 Discussion

Protein phosphorylation has long been implicated in the control of protein function (see Greengard, 1978; Krebs, 1985), and is central to the regulation of cellular processes such as mitosis and transformation (Hunter, 1987). In this chapter, the possible link between protein phosphorylation and compaction has been investigated. Changes in protein phosphorylation that accompany compaction at the 8-cell stage were described in the previous chapter. In this chapter, four methods of disrupting compaction have been used, and the effects on phosphoproteins of synchronised groups of 4-cell and 8-cell embryos have been compared. This approach allows comparison of protein phosphorylation between the two cell cycles and the elimination of changes known to occur with passage through each cell cycle (Howlett, 1986, McConnell & Lee, 1989). Both pulse-labelling and pulse-chase regimes have been used to detect differences in phosphoprotein profile.

DMAP causes rapid flattening of 4-cell and 8-cell embryos that does not seem to be mediated by uvomorulin, is not accompanied by cell polarisation and so does not seem to be related to that occurring naturally at compaction (see Chapter 3). When 4-cell or 8-cell embryos were incubated in DMAP, two distinct effects on protein phosphorylation were apparent. The results obtained depended on the conditions in which the drug had been incubated prior to the addition of embryos. If DMAP was added to warmed culture medium from a freshly-made stock only 1h before the addition of embryos and labelling with [^{32}P]orthophosphate, a global reduction in the level of labelling of proteins with ^{32}P was apparent (Fig. 6.2). By contrast, if DMAP was incubated at 37°C overnight before addition of embryos and labelling with [^{32}P]orthophosphate, little effect was apparent either on the global level of ^{32}P-labelling or on the pattern of phosphoprotein bands obtained by one-dimensional separation (Fig. 6.2). It is noteworthy that DMAP can also inhibit the incorporation of [^{35}S]methionine into protein and that this effect is also abolished by incubation of the drug overnight at 37°C (see 3.4). In a series of experiments in which the range of doses over which DMAP inhibited ^{32}P-labelling or [^{35}S]methionine incorporation were assessed in parallel groups of oocytes or 1-cell embryos, DMAP was found always to

inhibit [^{35}S]methionine incorporation at doses at which ^{32}P-labelling is reduced (J. McConnell, unpublished observations). It is therefore not possible to interpret unambiguously any experiment on mouse embryos in which DMAP is used as a general inhibitor of protein phosphorylation.

However, in the absence of any effect on global levels of ^{32}P-labelling or [^{35}S]methionine incorporation, DMAP can still cause rapid flattening of 4-cell and 8-cell embryos (Table 6.1). Under these conditions, the phosphoprotein profile of pulse-labelled 4-cell and 8-cell embryos was apparently altered by the increased labelling intensity of only a single phosphoprotein spot, M_r 41K (Fig. 6.3; Table 6.2). This spot co-migrates with a spot that is detected in untreated 4-cell and 8-cell embryos both after pulse-labelling and pulse-chase, but which increases in intensity specifically after pulse-chase at the late 8-cell stage (Fig. 6.1; Table 6.2). Incubation in DMAP therefore seems to have both advanced the appearance of this spot in developmental time and reduced the time necessary for it to become labelled with ^{32}P.

As the phosphoprotein spot M_r 41K is normally intensely-labelled only at the late 8-cell stage, DMAP may have advanced the developmental program that controls the increased labelling of this spot. The characteristics of the cellular flattening caused by DMAP have led to the suggestion that it may be related to the flattening that normally occurs in 16- to 32-cell morulae, rather than the initial, uvomorulin-mediated flattening of 8-cells (see 3.5). It is therefore possible to speculate that the enhanced labelling of the 41K phosphoprotein in 4-cells and 8-cells exposed to DMAP, resembling that of untreated embryos of later stages, corresponds to the enhanced labelling of the 41K phosphoprotein spot that is also characteristic of later embryos.

However, it is also necessary to explain the altered labelling kinetics of this spot following incubation in DMAP. DMAP causes the 41K phosphoprotein spot to be intensely labelled by a 1h pulse, whereas the spot with which it co-migrates is only intensely labelled in late 8-cells after pulse-chase (Figs. 6.3 and 6.1; Table 6.2). Phosphoprotein spots that increase in relative intensity in samples in which ^{32}P has been present for 3h (pulse-chased) compared to those pulse-labelled for 1h are presumed to be phosphorylated by different kinases or using phosphate from different sources than those which label maximally after 1h exposure to ^{32}P (see 5.5). It is

therefore possible that the primary effect of DMAP has been on a particular cellular phosphate source or the activity of a particular protein kinase, which is usually only used from the late 8-cell stage, to result in the increased pulse-labelling of this phosphoprotein spot that is normally only intensely-labelled after pulse-chase. This effect might be related to, or quite separate from, the effect of DMAP on flattening.

The second method used to effect compaction was the incubation of embryos in inhibitors of protein synthesis. When 8-cell embryos were labelled in [^{32}P]orthophosphate in the presence of protein synthesis inhibitors, certain phosphoprotein bands that normally occur after pulse-chase were no longer detected (Figure 6.4). New protein synthesis, therefore, seems to be needed for these 8-cell specific, chase-dependent phosphoproteins to become labelled with ^{32}P, although it is not needed for cell flattening and cell surface polarisation to occur in 8-cells (Fleming *et al.,* 1989). It is not established whether the phosphoproteins affected are synthesised *de novo* or are pre-synthesised but dependent on the synthesis of some other protein for their phosphorylation. It should be noted that no [^{35}S]methionine-labelled, newly-synthesised polypeptides were detectable at the 8-cell stage that were not also present at the 4-cell stage (Fig. 5.1).

Incubation of 4-cell embryos in protein synthesis inhibitor for a short period early in the cell cycle is sufficient to cause premature flattening even if protein synthesis is subsequently restored (Levy *et al.,* 1986). It was therefore possible to compare the phosphoprotein profile of prematurely-flattened 4-cells once protein synthesis had been restored with the profile of untreated, non-flattened 4-cells or untreated, flattened 8-cells. Although some novel phosphoprotein spots are apparent (non-reproducibly) in prematurely-flattened 4-cells, the majority of the phosphoproteins are indistinguishable from those of untreated 4-cells. Thus, the premature flattening of 4-cell embryos is not associated with the appearance of those phosphoprotein spots that normally occur in 8-cells at the time of cell flattening. A faint phosphoprotein 40K spot that appears to co-migrate with a spot usually found only at the 8-cell stage is detected in prematurely-flattened 4-cells and may possibly be

related to the premature intercellular flattening (Fig. 6.5, Table 6.2) .

Brief exposure to protein synthesis inhibitor causes premature cell flattening but the effect on the timing and incidence of cell polarisation has not been established (Levy *et al.,* 1986). It is, however, possible to predict that cells are unlikely to have polarised to any significant extent by the time of sampling used in the experiments described here. Continuous exposure to protein synthesis inhibitor causes premature flattening of cells and also does advance cell surface polarisation. However, polarisation does not occur as rapidly as cell flattening, only approximately one third of 4-cells having polarised by the time that controls divide to 8-cells and begin to polarise naturally (Levy *et al.,* 1986). The presumed low level of cell polarisation (if any) may be reflected in the absence of the majority of "8-cell specific" phosphoproteins following the advancement of cell flattening with protein synthesis inhibitor.

The third method used to disrupt compaction was the incubation of embryos in medium containing PMA which has severe effects on the cytoskeleton and surface morphology of 8-cell embryos which can be interpreted as an extreme version of compaction (See 4.7). When the phosphoproteins of PMA-treated 8-cell embryos were examined, certain phosphoprotein spots which are usually more intensely labelled in 8-cell embryos than in 4-cells now appeared even more intensely labelled than in control 8-cells (Fig. 6.7, Table 6.2). The phosphoprotein profiles of oocytes and embryos earlier than the 8-cell stage did not seem to be affected by incubation in PMA (Figs. 6.6 & 6.7). The exaggerated increase in ^{32}P-labelling of certain phosphoproteins after treatment with PMA may possibly be related to the extreme effects on the cytoskeleton caused by PMA.

By contrast, when a fourth method for disrupting compaction was used, namely the incubation of embryos in medium depleted of Ca^{2+}, most of these same phosphoprotein spots that normally increase in intensity specifically in 8-cell embryos, failed to appear (Fig. 6.8, Table 6.2). The absence of Ca^{2+} prevents uvomorulin-mediated intercellular flattening at the 8-cell stage (Ducibella & Anderson, 1975; Hyafil *et al.,* 1980; Johnson *et al.,* 1979, 1986b) and delays and disorients

cell polarisation (Fleming *et al.*, 1989; see 1.4). Restoration of Ca^{2+} to the medium restores cell flattening and, more slowly, polarisation. Much of the effect on the phosphoprotein profile of 8-cells was also reversible if Ca^{2+} was added back to the medium, but only following pulse-chase. Like incubation in PMA, incubation in Ca^{2+}-free medium had no detectable effect on the phosphoprotein profile of 4-cell embryos.

The appearance of selected phosphoprotein spots, using the different experimental strategies described above, is summarised in Table 6.2 (overleaf). There are several caveats to any attempt to correlate the behaviour of particular phosphoproteins with particular features of compaction. Firstly, cell flattening and polarisation are partially interdependent, as the prevention of flattening delays and disorients polarisation. In addition, each of the treatments used here to affect compaction may also have other, less specific effects. Finally, the detection of spots on gels and alterations in labelling intensity can not be used as reliable indicators of the extent of phosphorylation of particular proteins. However, it is possible to draw limited conclusions about the behaviour of particular phosphoproteins from these experiments.

Each of the treatments used has a different effect on intercellular flattening. Incubation in DMAP causes cell flattening that does not seem to be related to that occurring normally in 8-cell embryos; the possible link between the flattening that does occur and the labelling intensity of a phosphoprotein spot, M_r 41K, have been discussed above. A period of protein synthesis inhibition early in the cell cycle causes some uvomorulin-dependent flattening to occur prematurely in 4-cell embryos. This is associated with a phosphoprotein profile that is very similar to that of untreated 4-cells, suggesting that the appearance of most 8-cell specific phosphoproteins is not related simply to the process of cell flattening. A single novel phosphoprotein spot is reproducibly detected faintly in prematurely-flattened 4-cells that co-migrates with a spot that occurs naturally only in 8-cell embryos (Table 6.2). However, incubation in Ca^{2+}-free medium prevents the development of cell flattening and, in this case, the 40K phosphoprotein spot <u>is</u> detected, as in control 8-cells, although it is no longer more heavily labelled with ^{32}P at the late 8-cell stage than at the early 8-cell stage. It therefore seems unlikely that the incidence of this phosphoprotein *per se* is associated

Table 6.2 Comparison of the appearance of selected phosphoprotein spots under different experimental conditions

Developmental Stage	Labelling Protocol	Treatment	Source of Data	Phosphoprotein M_r 32K	35K	40K	40K$_a$	41K
4-cell	pulse	CONTROL	Figs. 5.2 5.4 5.7 (6.1)	-	+	-	-	+
early 8-cell				+	+ +	+	-	+
late 8-cell				+	+ +	+	-	+
4-cell	pulse-chase			-	+	-	-	+
early 8-cell				+ +	+ +	+	-	+
late 8-cell				+ + +	+ +	+ +	+	+ +
4-cell	pulse	DMAP	Fig. 6.3	-	+	-	-	[+ +]
early 8-cell				+	+ +	+	-	[+ +]
late 8-cell				+	+ +	+	-	[+ +]
4-cell	pulse	ANISOMYCIN PRE-TREATMENT	Fig. 6.5	-	+	-	-	+
4-cell	pulse-chase			-	+	[±]	-	+
4-cell	pulse-chase	PHORBOL ESTER	Fig. 6.7	-	+	-	-	+
early 8-cell				[+ + +]	[+ + +]	+	-	+
late 8-cell				[+ + + +]	[+ + + +]	+	+	+ +
4-cell	pulse	Ca^{2+}-FREE MEDIUM	Fig. 6.8	-	+	-	-	+
late 8-cell				[-]	[+]	+	-	+
late 8-cell	pulse-chase			[+ +]	+ +[1]	[+]	[-]	[+]
late 8-cell	pulse	WASHED FROM CALCIUM-FREE MEDIUM	Fig. 6.8	[±]	[+]	+	-	+
late 8-cell	pulse-chase			+ +	[+]	+	+	+ +

- indicates spot not detected;
+ indicates spot detected;
± indicates faint spot detected;
++ and +++ denote increased relative intensity of a particular spot; intensity is not intended to be comparable between spots.

[ ] Differs in intensity from controls

40K$_a$ Acidic, diffuse 40K phosphoprotein spot (open arrow, Figs. 1-4)

[1] Shifted slightly in position relative to controls

specifically with occurrence of cell flattening. It is possible that the detection of this phosphoprotein corresponds rather to the "competence" of cells to flatten either in 4-cells following protein synthesis inhibition or in 8-cells in the absence of Ca^{2+}. Restoration of Ca^{2+} to the medium restores both flattening and the late 8-cell appearance of this phosphoprotein spot (Table 6.2). The effects of PMA on cell flattening are more difficult to define. Cells do round up after PMA-treatment, but this may be indirect, due to effects on the actin cytoskeleton, the integrity of which is needed for the cell flattening process (Pratt *et al.*, 1981, Houliston *et al.*, 1989). The phosphoprotein spot M_r 40K described above appeared in PMA-treated 8-cells as in controls.

In summary, the appearance of most 8-cell-specific phosphoproteins does not seem to be related to cell flattening. It is possible to speculate that the appearance of the spot M_r 40K followed by its increased phosphorylation might reflect competence of cells to flatten followed by the attainment of a flattened state.

Can the behaviour of particular phosphoprotein spots be related to cell polarisation ? DMAP does not seem to affect the polarisation of embryos (see 3.3) and protein synthesis inhibition is also unlikely to have affected polarisation to a significant extent in these experiments (see above). The morphological effects of PMA can be interpreted as an extreme version of cell polarisation in which the cortex of the whole cell resembles the basal region of a polarised late 8-cell, being relatively depleted of assembled microfilaments and microvilli (and, in certain circumstances, microtubules). By contrast, Ca^{2+}-free treatment may also make the cortex equivalent around the whole cell, but in this case assembled microfilaments, microvilli and microtubules are present around the entire cell surface. The cortex, following Ca^{2+}-free treatment may therefore be related more closely to that of the apical region of a polarised, late 8-cell blastomere.

It may therefore be significant that many of the same phosphoproteins seem to be affected by PMA as by Ca^{2+}-free treatment. These are the phosphoprotein spots, M_r 32K and 35K, that are naturally more intensely labelled with ^{32}P in 8-cells than 4-

cells (Fig. 6.1; Table 6.2). They tend to increase in relative labelling intensity in the presence of PMA (Fig. 6.7; Table 6.2) and decrease in relative labelling intensity after incubation in Ca^{2+}-free medium, compared to controls (Fig. 6.8; Table 6.2). It is therefore be possible to correlate the behaviour of this group of phosphoproteins with the incidence of cell polarisation, rather than cell flattening. Thus, passage from 4-cell to late 8-cell is associated with increasing cellular polarisation and the appearance of a clear basal domain adjacent to regions of cell contact (Fig. 1.3) and also with increased intensity of these phosphoprotein spots. PMA may cause the entire blastomere cortex to resemble basal regions (Fig. 4.10) and is associated with further increased intensity of apparently similar spots. By contrast, incubation in Ca^{2+}-free medium is associated with decreased polarisation and thus a reduction in the extent of the basal cortical domain and is associated with decreased labelling intensity of these spots. Finally, removal of calcium at the late 8-cell stage, which reverses flattening, does not seem to affect the labelling intensity of these phosphoprotein spots (unpublished observations), nor does it affect the polarity of cells (Johnson & Ziomek, 1981a; Fleming *et al.*, 1989).

Taken together, these results suggest that these phosphoproteins (M_r 32K and 35K) may possibly be involved in the establishment asymmetry in the cell cortex that occurs at polarisation, rather than reflecting the capacity of cells to polarise or arising as a consequence of cell polarisation. However, the inhibition of protein synthesis at the 8-cell stage, which has little effect on the establishment of cell flattening or polarisation (Fleming *et al.*, 1989) appears to prevent the increased phosphorylation of the 32K phosphoprotein that normally occurs in the late 8-cell following pulse-chase (detectable after one-dimensional separation, Fig. 6.4). This observation suggests that the 32K phosphoprotein may also be involved in an event that is dependent on protein synthesis and occurs later than the initial establishment of cell polarity.

Many workers have investigated the role of protein phosphorylation in major cellular changes. Studies have tended to be of three types. In the first type, the phosphorylation of a particular, identified protein or group of proteins is investigated. This approach has revealed useful information about the behaviour of, for example,

cytoskeletal proteins such as actin-binding proteins (Stossel *et al*, 1985; Pollard & Cooper, 1986; Citi & Kendrick-Jones, 1987), tubulin (Hargreaves *et al.*, 1986), vimentin (Inagaki *et al.*, 1987; Geiger, 1987) and nuclear lamins (Ottaviano & Gerace, 1985) as well as many enzymes (Krebs, 1985).

In the second type of study, the possible role of a particular protein kinase has been investigated. Such studies using protein kinase C are of particular relevance to the experiments reported here. Protein kinase C is capable of phosphorylating a wide range of proteins *in vitro* (reviewed by Takai *et al.*, 1985). However, these may bear little relationship to the preferred substrates of protein kinase C *in vivo*. Several workers have stimulated a variety of cell types with phorbol esters or with natural agonists and have found, in each case, that the same or similar proteins were phosphorylated following exposure to phorbol ester or the natural agonist; for example, insulin and somatomedin receptors are phosphorylated in IM-9 cells stimulated with phorbol ester or with insulin (Jacobs *et al.*, 1983) and epidermal growth factor receptor is phosphorylated in A431 cells stimulated with phorbol ester or with epidermal growth factor (Iwashita & Fox, 1984). In addition, several unidentified proteins are phosphorylated in different cell types, for example M_r 66K, 37K, 31K and 25K in murine peritoneal macrophages stimulated with phorbol ester or with immune complexes (O'Brian *et al.*, 1984); M_r 70K, 56K and 35K in hepatocytes stimulated with phorbol ester or with vasopressin (Garrison *et al.*, 1984); M_r 82K, 80K 59K, 60K in GH_3 pituitary cells stimulated with phorbol ester or with thyrotropin-releasing hormone (Drust & Martin, 1984). It is evident from this list that, when different types of cells are compared for the types of proteins phosphorylated in each case, little cross-cell type similarity exists (except for the proposed autophosphorylation of protein kinase C itself, $M_r \approx$ 80K). The pattern of proteins phosphorylated *in vivo* on stimulation of protein kinase C seems to reflect the substrates available in particular differentiated cell types.

In the third type of study of protein phosphorylation, the global pattern of protein phosphorylation accompanying passage through the cell cycle (Endo *et al.*, 1986; Howlett, 1986; Karsenti *et al.*, 1987; McConnell & Lee, 1989), cell transformation (Cooper & Hunter, 1981, 1983; Jove & Hanafusa, 1987; Kellie, 1988)

or early development (Lopo & Calarco, 1982; Endo *et al.*, 1986) has been examined. In these cases it is harder to correlate the behaviour of a particular phosphoprotein with a particular event. Additionally, the phosphoproteins most easily detected may not be those that are the most biologically important.

In this study, the changes in protein phosphorylation associated with compaction have been manipulated experimentally. The data presented therefore provide indirect correlations of cell state with protein phosphorylation. However, as the same small number of phosphoproteins are affected in each case, it may prove possible to identify and locate these molecules and assess their function, if any, in cell flattening and polarisation.

CHAPTER SEVEN

DISCUSSION

CHAPTER SEVEN DISCUSSION

The work described in this thesis has addressed the question of how blastomeres of the 8-cell mouse embryo become polarised and flattened at compaction. The data obtained from two different types of experiments suggest that protein phosphorylation may be involved in the process of compaction. This Discussion will be divided into two parts; the first will summarise the results obtained and attempt to assess their implications. More detailed discussion of particular points can be found at the end of each chapter. In the second part, a comparison will be made between compaction and some of the variety of processes involved in making cells asymmetrical in other organisms and at other stages of development.

7.1 Summary of results

The first type of experiment used to investigate the mechanism of compaction involved the exposure of embryos to drugs that affect pathways of intracellular signalling and post-translational modification and assessment of the impact on cell morphology. DMAP (6-dimethylaminopurine) is an adenine analogue and putative phosphorylation inhibitor that is found to inhibit protein phosphorylation in mouse embryos but only under conditions in which the rate of protein synthesis is also depressed. However, under conditions in which the global rates of protein synthesis and phosphorylation are unaffected, DMAP still causes premature flattening of 4-cell and 8-cell embryos. This flattening, unlike the flattening that occurs naturally at compaction, seems not to be mediated by uvomorulin nor is it accompanied by cell polarisation. DMAP-induced flattening may resemble the more stable flattening that normally occurs in 16-cell embryos (see Chapter 3).

PMA (Phorbol myristate acetate), by contrast, tends to promote protein phosphorylation and is presumed to act primarily *via* stimulation of protein kinase C (PKC; Nishizuka, 1984; Bell, 1986). When 8-cell embryos are incubated in medium containing PMA, cell flattening is prevented and a widespread disassembly of the

cytoskeleton occurs. Disassembly does not occur to the same extent in embryos of other stages when exposed to PMA (Chapter 4). These results suggest that PMA might be promoting cytoskeletal disassembly by mechanisms related to those that cause spatially restricted cytoskeletal disassembly in 8-cell blastomeres at compaction (in the basolateral domain: see Fig. 4.10).

This hypothesis predicts a role for PKC activity, and hence protein phosphorylation, in the process by which cell asymmetry is generated at compaction. It remains to be shown whether the effects of PMA arise solely by stimulation of PKC, that PKC is activated naturally at the time of compaction or that it is activated *via* phosphoinositide breakdown (Kester *et al.*, 1987; Rossoff *et al.*, 1988). In addition, the effects of PMA at the 8-cell stage seem to reflect the fundamental change in cell physiology which initiates compaction but shed no light on the nature of this trigger; it seems that PKC (if involved) acts "downstream" of the trigger to initiate compaction. The possibility that the phosphorylation of proteins by PKC might be involved in the progression and/or stabilisation of compaction has led to a search for possible substrate proteins, by an examination of the phosphoproteins present in embryos before and during compaction.

The second experimental approach used was, therefore, an examination of phosphoproteins labelled *in vivo* with [^{32}P]orthophosphate, both during the natural progression from the 4-cell to the 8-cell stage and following the experimental modulation of compaction. While many of the phosphoproteins detected are similar at each stage examined, some reproducible differences between the phosphoprotein profiles of 4-cell and 8-cell embryos were found. In addition, the appearance of several novel phosphoproteins at the 8-cell stage was found to depend on the prolonged presence of ^{32}P (summarised in Fig. 6.1 and Table 6.2). This result suggests that both novel substrates for phosphorylation and novel pathways of protein phosphate metabolism operate at the 8-cell stage (Chapter 5). It may also be possible that novel protein kinases, or kinase isotypes, are activated at the 8-cell stage, with different kinetics and/or different substrate specificities from those operating earlier in development (Carpenter *et al.*, 1987; Hunter, 1987; Nishizuka, 1988). This possibility is not addressed directly by the experiments described here.

Although novel phosphoproteins can be detected at the time of compaction, no differences are apparent in the pattern of proteins synthesised during the 4-cell and 8-cell stages (Fig. 5.1). This corresponds to the evidence obtained by other workers that compaction is controlled post-translationally, using proteins that are present in the embryo from at least the 4-cell stage (Kidder & McLachlin, 1985; Levy *et al.,* 1986). The detection of novel phosphoproteins is therefore presumed to represent the phosphorylation of existing proteins. It is interesting to note that the ^{32}P-labelling of some 8-cell specific phosphoproteins depends on continuing protein synthesis (Fig. 6.4). This dependence on new protein synthesis might be indirect, reflecting the need to continue synthesis of a pre-requisite of the phosphorylation process.

In contrast to the results reported here for the third and fourth cell cycles, there are major changes in protein synthetic patterns apparent both earlier and later in the preimplantation development of the mouse embryo. During the first cell cycle after fertilisation, many changes occur in the post-translational modification, particularly phosphorylation, of a group of proteins whose behaviour parallels that of the cell cycle (Pratt *et al.,* 1983; Howlett & Bolton, 1985; Howlett, 1986; Endo *et al.,* 1986). During the second cell cycle there are more marked changes in protein synthesis as the embryonic genome is transcribed for the first time (Van Blerkom, 1981; Flach *et al,* 1982; Pratt *et al,* 1983). Several studies have also shown changes in polypeptide synthetic rates (Epstein & Smith, 1973) and patterns (Van Blerkom *et al.,* 1976; Levinson *et al,* 1978; Martin *et al,*1978; Handyside & Johnson, 1978; Braude, 1979; Johnson, 1979) accompanying development from the compacted morula to blastocyst stages. The data presented here, using precisely timed groups of embryos, detect no polypeptides being synthesised as late as 6h into the fourth cell cycle which are not also made during the third cell cycle. The changes in synthetic patterns described by other authors must therefore be presumed to occur later than the events of compaction. Such changes might reflect early differences between the inner, apolar cells of the morula and the outer, polar cells which tend to give rise to inner cell mass and trophectoderm of the blastocyst, respectively (see 1.3).

In the final group of experiments described in this thesis, several treatments have been used to affect the process of compaction and assess the effects on the

phosphoprotein profiles of 4-cell and 8-cell embryos. The rapid, uvomorulin-independent cell flattening caused by incubating embryos in DMAP was found to be associated with the increased ^{32}P-labelling of a single phosphoprotein spot. This spot co-migrates with one that is naturally detected in both 4-cell and 8-cell embryos but is more intensely labelled in late 8-cells (Fig. 6.3, Table 6.2). The premature appearance of a late 8-cell-specific phosphoprotein in DMAP-treated 4-cell and 8-cell embryos may be associated with the premature flattening that occurs, which shares some characteristics with the natural flattening of embryos of later stages. Although cell flattening that is dependent on uvomorulin could be advanced by incubating 4-cell embryos in protein synthesis inhibitors, this had little effect on the pattern of phosphoproteins detected, suggesting that few, if any, of the 8-cell specific phosphoproteins reflect simply the process of uvomorulin-mediated cell flattening (Chapter 6).

Embryos were also incubated in two conditions that seem to affect cell polarisation. In each case most of the phosphoprotein profile appeared unchanged but the ^{32}P-labelling of a small group of 8-cell-specific phosphoproteins was affected (Table 6.2). Incubation in medium containing PMA affects cell morphology in ways that can be interpreted as an extreme version of the process that leads to cell polarisation. A group of 8-cell-specific phosphoproteins, that were more intensely labelled in 8-cell embryos than in 4-cells, were also more intensely labelled after exposure to PMA. Incubation in medium depleted of calcium ions prevents cell flattening and also delays cell polarisation; in this case these 8-cell-specific phosphoproteins were less intensely labelled with ^{32}P than in untreated embryos. The level of ^{32}P-labelling of these phosphoproteins is presumed to reflect their level of phosphorylation; it seems to reflect the extent to which basolateral cytoskeletal disassembly had occurred in each instance. Thus, increasing phosphorylation of this group of phosphoproteins correlates with increased basolateral disassembly (Chapter 6).

It should be noted that no treatment caused either the premature appearance or the total removal of all of the 8-cell specific phosphoproteins. This observation suggests that the appearance of (at least) some 8-cell-specific phosphoproteins may reflect simply the passage of developmental time. The appearance of such phosphoproteins can

be described in two different ways. The appearance of these phosphoproteins might be tightly associated to passage through development and arrival at the 8-cell stage (see 1.2, 1.4). If this is the case, the factors controlling their appearance might also be linked to the fundamental changes that occur at the 8-cell stage and which permit the initiation of cell polarisation. Alternatively, the appearance of these phosphoproteins may be associated more directly with the initiation of cell polarisation, which has not been advanced to occur before the 8-cell stage by any of the conditions used. In this case, those 8-cell-specific phosphoproteins that appear to reflect the state of polarisation (varying in labelling intensity on exposure to PMA and Ca^{2+}-free medium) can be considered to be "downstream" of the polarisation process whereas the " truly 8-cell specific" phosphoproteins may be "upstream" of the process, involved in the facilitation and / or initiation of polarisation. It remains to be established when, during development, the 8-cell-specific phosphoproteins are first synthesised, as distinct from the time of their phosphorylation. These phosphoproteins may be translated from maternal gene transcripts or might be amongst the earliest products of transcription from the embryonic genome (Flach *et al.*, 1982; Johnson *et al.*, 1984)

Neither of the approaches used in this thesis provides definitive proof that the phosphorylation of particular proteins is central to compaction. Nevertheless, two strands of evidence have been presented that, firstly, suggest that protein phosphorylation may be involved and, secondly, highlight a correlation between the incidence of certain phosphoproteins and particular components of compaction. How might the phosphorylation of particular proteins be involved in the mechanisms underlying compaction ? As described in Chapter 1, the cell adhesion molecule uvomorulin seems to be involved in generating a change in the cytocortex of 8-cell blastomeres that results in flattened cells, each with a stabilised asymmetrical distribution of cytoskeletal elements and organelles (Fig. 1.3). It seems feasible to propose that the phosphorylation of certain 8-cell specific phosphoproteins is part of the fundamental mechanism underlying the propagation of the basolateral cytoskeletal disassembly from points of cell-cell contact, and may also be involved in the stabilisation of a polar phenotype. If this proves to be the case, the proteins concerned might be expected to be localised in the cell cortex, and might be associated, directly or

indirectly, with uvomorulin (see 1.4). However, it remains equally possible that novel protein phosphorylation is simply an early manifestation of the newly-acquired polar phenotype and is not related to the mechanisms that allow the progression or stabilisation of compaction, in any way other than by temporal coincidence. In this case, an examination of the mechanisms controlling novel protein phosphorylation may, in turn, lead back towards an elucidation of the underlying controls. The changes in phosphoprotein profile that have been presented constitute a carefully synchronised description, at the molecular level, of changes occurring before and during compaction. This description will facilitate further molecular examination of this fundamental process of mammalian development.

7.2 The relationship between compaction and other processes by which cellular asymmetry is established

To what extent is an understanding of the process of compaction relevant to the elucidation of the processes used by other organisms, and at other stages in development, to establish asymmetry within and between cells? As discussed in the Introduction, compaction may be analogous to the processes by which asymmetry is generated in the eggs and early embryos of many species, but occurs at a slightly different developmental time. In each case, intracellular asymmetry is first generated and stabilised and then translated *via* cell division into patterns of differential gene expression in different cell lineages. At compaction, an initially labile asymmetry of organisation is established and stabilised in the cytocortex. When asymmetrically organised 8-cell blastomeres cleave, they can give rise to both apolar, inner and polar, outer progeny. There is evidence that these two cell types constitute the precursors of the inner cell mass and the trophectoderm of the blastocyst respectively (see 1.3 and 1.4).

It remains unclear how an asymmetry of organisation of, for example, the cell adhesion molecule uvomorulin, or other cytoskeletal-associated proteins, can give rise to an asymmetry of factors that will determine cell fates. Evidence obtained from other embryonic systems suggests that the cytoplasmic localisation of mRNAs encoding either transcriptional regulators or cell-cell signalling molecules (or the localisation of their protein products), may be sufficient to generate differential gene expression in progeny cells which inherit different parts of the cytoplasm (reviewed by Akam, 1986; Nusslein-Volhard *et al.*, 1987; Lawrence, 1988). The cytoskeleton seems central to the determination of cell shape and the organisation of the cytoplasm in most cells (Kirschner & Mitchison, 1986). It therefore seems feasible to propose, on available evidence, that regulatory proteins or mRNAs may become localised in the egg or embryo *via* interactions with the cytoskeleton, either directly or indirectly (Jeffery, 1985). A scheme can be envisioned in which an asymmetrically-organised cytoskeleton is involved in the cytoplasmic localisation of the mRNA or protein products of regulatory genes that, in turn, regulate the expression of other genes. Controlled planes of cell division, partitioning the regulatory gene products between daughter cells, would then

be sufficient to generate differences in gene expression between the daughter cells. A brief review of some of the evidence for this hypothesis follows.

It has been apparent for many years that the eggs and embryos of many species contain identifiable regions or components of the cytoplasm that are reproducibly inherited by cells of particular lineages (for example the yellow pigment granules of the myoplasm of ascidians that will contribute to the muscle cell lineage of the larva, Whittaker, 1979; the *p* granules that partition exclusively into to the germ cell lineage of *Caenorhabditis,* Strome & Wood, 1982, 1983; reviewed by Davidson, 1986). More recently, descriptions have been made, at a molecular level, of the cytoplasmic localisation of specific mRNAs and protein products (for example, the mRNAs and protein products of many genes of *Drosophila* such as *bicoid*, that seems central to the determination of antero-posterior polarity; reviewed by Nusslein-Volhard *et al.,* 1987; Lawrence, 1988). Most of the genes of *Drosophila* that encode products that are regionally localised within the egg or early embryo have been identified, using genetic mutations, as genes involved in pattern formation (Anderson & Nusslein-Volhard, 1984).

In organisms other than *Drosophila*, the composition and function of localised "determinants" is less well established. It seems likely that the granules, identified as determinants, within the cytoplasm of many species include mRNAs encoding regulatory proteins, but this remains to be shown. It has proved possible to identify differentially localised mRNAs that are candidates for functional determinants; for example, mRNAs enriched in the myoplasm of ascidians (Jeffery *et al.,* 1983) and mRNAs differentially localised between the animal and vegetal halves of *Xenopus* eggs (Rebagliati *et al.,* 1985) Indeed, in *Xenopus,* as in *Drosophila,* it has been possible to identify a particular gene product, *Vg1,* with a known distribution and predicted function (*Vg1* of *Xenopus,* Weeks & Melton, 1987; compared to *bicoid* of *Drosophila,* Nusslein-Volhard *et al.,* 1987). Both the *Vg1* and *bicoid* gene products are localised in the egg cytoplasm and encode putative regulatory proteins, *Vg1* having homology to cell surface signalling molecules and *bicoid* to transcription factors. It is therefore becoming possible to explain how partitioning the egg cytoplasm may be sufficient to achieve differential gene expression in daughter cells if different progeny cells inherit or do not inherit these

"determinants".

In some species, an initial asymmetry of organisation in the egg is superceded by a re-organisation in the first cell cycle (for example, ascidians, Whittaker, 1973; *Xenopus*, Gerhart *et al.*, 1981; *C. elegans*, Strome & Hill, 1988). Compaction of the mouse embryo may be more analogous to the cell-autonomous re-organisation processes in the embryos of such species than to the mechanisms by which asymmetry is first generated while the egg is in the ovary. The cytoplasmic re-organisations occurring after fertilisation have been perturbed using cytoskeletal-disrupting drugs (in *Xenopus* by Elinson, 1983; in *C. elegans* by Hill & Strome, 1988, Strome & Hill, 1988) or centrifugation (in *Xenopus* by Gerhart *et al.*, 1981). Such experiments have shown, indirectly, a role for the cytoskeleton in the re-organisation process. A different type of evidence for the role of the cytoskeleton in the cytoplasmic localisation of "determinants" has come from the work of Jeffery and colleagues, using ascidian embryos. Differential extraction techniques have been used to show that mRNA can be co-localised selectively with elements of the actin cytoskeleton (reviewed by Jeffery, 1985). These data suggest that mechanisms of cytoplasmic localisation of mRNA may involve the cytoskeleton. Such fundamental cellular mechanisms might be expected to be conserved between species. In future, students of mRNA localisation and the control of gene expression may find common ground with those examining the structure and function of the cytoskeleton.

As the mechanisms by which axes of asymmetry are established in eggs and early embryos are beginning to be understood, so studies of the stabilisation and maintenance of cellular asymmetry are also bearing fruit. Many workers have observed current fluxes in asymmetrical cells and postulated a role for ion currents in the establishment or maintenance of cell asymmetry (reviewed by Jaffe, 1979). In the eggs of the seaweeds *Fucus* and *Pelvetia*, it seems that ion fluxes may indeed be involved in primary axis formation (Jaffe, 1968; Nucitelli & Jaffe, 1974; Nucitelli, 1978). However, in most other systems it is not clear that ion fluxes precede other manifestations of cellular asymmetry. Rather, a primary asymmetry may become translated into an asymmetry of ion pump and channels proteins in the cell membrane that, in turn, produces currents that may be involved in the re-enforcement and

stabilisation of the primary axis (Rodriguez-Boulan & Nelson, 1989). The mechanisms by which a polarised cell phenotype is re-established and maintained following disruption has been extensively studied in cultured kidney cell lines (for example MDCK cells, reviewed by Rodriguez-Boulan & Nelson, 1989). It is evident that in this system, amongst the earliest molecules polarised in their distribution are spectrin and ankyrin, proteins believed to be involved in forming links between the plasma membrane and the cytoskeleton (Backman, 1988), and which may form complexes with uvomorulin. The re-distribution of ion channels and pumps seems to follow an initial asymmetry in the cytoskeleton. (Rodriguez-Boulan & Nelson, 1989). However, it is not clear to what extent this process is related to that by which intracellular asymmetry is generated *de novo,* in previously non-polarised eggs and embryos (reviewed by Fleming & Johnson, 1988).

The various types of study described above have shed light on the mechanisms both of asymmetrical distribution of material in eggs and embryos and of the maintenance of a stable polarised cell phenotype later in development. However, the missing links between these two types of processes remain. How does a gradient in the distribution of, for example cytoskeletal elements, initially form and subsequently lead to a graded distribution of mRNAs that may dictate cell fate ? Studying the process of cellular polarisation at compaction in mouse embryos, which takes place in large, yolk-free cells with a leisurely pace of development, may provide results that are of general relevance to an understanding of the processes of cell diversification at the molecular level.

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