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Connexin Gene Expression And The Control Of Gap Junction Assembly In Preimplantation Development

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CONNEXIN GENE EXPRESSION AND THE CONTROL OF GAP JUNCTION ASSEMBLY IN PREIMPLANTATION DEVELOPMENT

by

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ABSTRACT

Gap junctional communication in preimplantation mouse embryos was explored by investigating: (1) the expression pattern of the connexin (Cx; gap junction protein) multigene family in maturing mouse oocytes and preimplantation embryos; (2) the relative role of Cx43 in gap junctional communication in embryos; and (3) the control of the acquisition of gap junctional coupling.

Both Cx43 and Cx32 transcripts were detected in maturing oocytes but these transcripts declined to undetectable levels shortly after ovulation. Cx43 transcripts were detected again at the 4-cell stage and accumulated steadily thereafter to reach a maximum in blastocysts. Cx26 and Cx46 transcripts could not be detected in embryos. By Western blotting Cx43 was detected in 1-cell embryos, the levels decreased by the 2-cell stage, and then increased gradually from the 4-cell stage to the blastocyst stage. Oogenetic expression of Cx32 and 43 is therefore replaced by exclusive Cx43 expression in the embryo and it is probably Cx43 that is the main contributor to the assembly of gap junctions at the 8-cell stage. To attempt to inhibit Cx43 gene expression, embryos were either microinjected with Cx43 antisense deoxyoligonucleotides, or cultured in their presence. Dye coupling and Cx43 mRNA levels were not noticeably affected by the presence of the antisense reagents.

Treatment of 4-cell embryos with an activator of protein kinase C triggered premature compaction without an accompanying establishment of gap junctional communication. On the other hand, when coupled, 8-cell embryos were treated with the

activator, intercellular coupling was diminished. Western blot analysis revealed that Cx43 becomes phosphorylated between the late 4-cell stage and the late morula stage. Aphidicolin experiments showed that the third and fourth rounds of DNA synthesis are not required for the normal acquisition of gap junctional communication. However, embryos in which the second round of DNA synthesis was delayed by 10 hours failed to initiate gap junctional coupling. Immunofluorescence localization of Cx43 in these communication-deficient embryos revealed extensive cytoplasmic Cx43 staining but punctate interblastomeric staining, indicative of gap junction plaques, was absent.

These results are consistent with a model in which the regulated step in the onset of gap junction assembly involves the phosphorylation of cytoplasmic Cx43.

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AMV	.....	Avian myeloblastosis virus
BSA	.....	Bovine serum albumin
cDNA	.....	Copy DNA
COC	.....	Cumulus-oocyte-complex
Cx	.....	Connexin
DAG	.....	Diacylglycerol
DEPC	.....	Diethylpyrocarbonate
DMSO	.....	Dimethylsulfoxide
FM	.....	Flushing medium
hCG	.....	Human chorionic gonadotropin
ICM	.....	Inner cell mass
MBT	.....	Midblastula transition
ODN	.....	Oligodeoxynucleotide
PAGE	.....	Polyacrylamide gel electrophoresis
PCR	.....	Polymerase chain reaction
PKA	.....	Protein kinase A
PKC	.....	Protein kinase C
PMSF	.....	Pregnant mare's serum gonadotropin
RT-PCR	.....	Reverse transcriptase coupled PCR
SDS	.....	Sodium dodecyl sulfate

S-ODN	.....	Phosphorothioate ODN
SECM	.....	Standard egg culture medium
TBS	.....	Tris buffered saline
TTBS	.....	Tween TBS
TE	.....	Trophectoderm

CHAPTER 1

GENERAL INTRODUCTION

1.1 The Discovery of Intercellular Communications and the Gap Junction

Gap junctions are collections of intercellular channels which allow cells to share small molecules such as inorganic ions, amino acids, nucleotides and secondary messengers. The first evidence for such a phenomenon was the discovery of a low resistance electrical pathway (electrotonic or ionic coupling) between the pre- and post-synaptic cells at the crayfish giant motor synapse (Furshpan and Potter, 1959). Subsequently ionic coupling was documented in a variety of excitable tissues (Hagiwara and Morita, 1962; Bennett et al., 1963; Furshpan, 1964), but it was not until Loewenstein and Kanno (1964) reported ionic and dye coupling in non-excitatory Drosophila salivary glands that the generality of low resistance intercellular communications was established. With the aid of fluorescent marker molecules of varying size it was established that the main determinant of permeability is size, with the upper size limit for vertebrate channels at about 1000 daltons (Simpson et al., 1977; Flagg-Newton et al., 1979; Schwartzmann et al., 1981).

The gap junction was not recognized as a distinct morphological entity until Revel and Karnovsky (1967), by combining uranium staining and lanthanum tracing with thin section electron microscopy, showed that what had hitherto been considered a tight junction was actually a new type of junction. In cross sections of this new junction the

two unit membranes of the contacting cells came into very close apposition, but a narrow 2-4 nm "gap" continous with the extracellular space, remained between the two membranes. Also, tangential sections through gap junctions in lanthanum treated tissues showed a lattice of hexagonally arranged particles that were not connected with the extracellular space. Similar lattices or plaques of hexagonally arranged particles were seen in freeze-fracture and freeze-fracture deep-etch replicas of both native and split gap junctions (Chalcroft and Bullivant, 1970; Goodenough and Revel, 1970; Goodenough and Gilula, 1974; Hirokawa and Heuser, 1982). The particles in these images had a diameter of about 8-9 nm with a central 1-2 nm pore and a center-to-center spacing of about 9-10 nm, leaving approximately 1-2 nm of extracellular space between particles. Furthermore, each particle appeared to span the lipid bilayer of the plasma membrane and particles in the apposed membranes appeared to line up end-to-end across the 2-4 nm extracellular gap. That the gap junction was the morphological equivalent of the low resistance pathway was suggested by studies which correlated the presence of gap junctions with physiological and metabolic measures of intercellular communications (Payton et al., 1969; Gilula et al., 1972). Further support for this suggestion came from studies in which experimental disruption of ionic coupling led to a loss of gap junction ultrastructural integrity (Barr et al., 1968; Pappas et et., 1971; Peracchia, 1977). On the basis of these findings it was postulated that the particles, termed connexons by Goodenough (1975), seen in freeze-fracture replicas and outlined by lanthanum in thin sections were the functional units for intercellular communications and that the central

pore was continous with the cytoplasms of both contacting cells (Chalcroft and Bullivant, 1970; Duguid and Revel, 1975).

Further fine structural analyses have confirmed and extended the basic concept of gap junctions as outlined above. By correlating optical and X-ray diffraction data from isolated murine liver gap junctions, Caspar et al. (1977) and Makowski et al. (1977) showed that the connexon was composed of six protein subunits that were arranged around a central aqueous pore. Electron microscopy with three dimensional reconstructions based on Fourier transformations showed that although the connexon subunits were symmetrically arranged around the channel they were also tilted with respect to it (Unwin and Zampighi, 1980). Furthermore, this study suggested that the pore diameter was narrower at the cytoplasmic end of the connexon and that the pore could be closed at this end by a simple rotation and sliding of the subunits relative to each other. Further support for this model was provided by the freeze-fracture studies of Hirokawa and Heuser (1982). Recently, using the novel atomic force microscopy technology on isolated hepatic gap junctions, the basic model of the gap junction as outlined above was confirmed (Hoh et al., 1991). The recent application of high resolution X-ray diffraction methods (Tibbitts et al., 1990) and circular dichroism spectroscopy (Cascio et al., 1990) to isolated hepatic gap junctions has revealed that a major portion of the connexon subunits spanning the lipid bilayer is α-helical.

1.2 Biochemistry and Molecular Biology of Gap Junction Proteins

The initial step in the biochemical characterization of gap junction proteins was the development of methods for the isolation of relatively pure gap junctions from mouse livers (Goodenough and Stoeckenius, 1972; Duguid and Revel, 1975; Goodenough, 1974, 1975). When the gap junctions isolated by these methods were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) the number of bands and the relative molecular mass (M_r) of these bands varied considerably, although the junctions were morphologically pure and uniform in appearance. The term connexin was coined by Goodenough (1974) as a generic name for these gap junction proteins. As methods improved, contamination by non-junctional proteins was reduced as was proteolysis of the gap junction protein(s), and the consensus became that there was one major gap junction protein in liver with a M_r of approximately 28,000 (Duguid and Revel, 1975; Henderson et al., 1979; Nicholson et al., 1981; Revel et al., 1985). However, a M_r 21,000 protein was sometimes also present in these preparations (Henderson et al., 1979; Nicholson et al., 1981). The availability of purified M_r 28,000 protein made the production of antisera against this protein possible. Although the results of surveys of different rodent tissues and of various vertebrate species with these antisera were not unequivocal, they led to the conclusion that a protein homologous, if not identical to the major gap junction protein from liver was widely distributed among different tissues and vertebrates (Dermietzel et al., 1984; Hertzberg and Skibbens, 1984). It also became apparent from these studies that some tissues, e.g. heart, lens and ovarian

granulosa cells, known to possess gap junctions by physiological and morphological criteria did not contain a protein that was sufficiently related to the M_r 28,000 protein from liver to react with all antisera raised against it. Peptide mapping and limited protein sequencing also suggested that the M_r 28,000 and 21,000 gap junction proteins from liver as well as gap junction proteins from heart and lens were related but distinct (Nicholson et al., 1981, 1987; Gros et al., 1983; Revel et al., 1985; Manjunath et al., 1987; Kistler et al., 1988).

To address the questions of how many gap junction proteins there are and how closely they are related to one another required the application of recombinant DNA methods. From these efforts it now appears that gap junction proteins are encoded by a multigene family with at least a dozen members in rodents. A nomenclature system for these proteins has been adopted that uses the generic term connexin (Cx), proposed by Goodenough (1974), followed by the predicted M_r of the protein, based on the nucleotide sequence of its cDNA (Beyer et al., 1987). The first gap junction cDNA to be cloned was for the hepatocyte M_r 28,000 protein from rat (Heynkes et al., 1986; Kumar and Gilula, 1986; Paul, 1986) and human (Kumar and Gilula, 1986). Based on the nucleotide sequence of this cDNA the predicted M_r of the protein was about 32,000; it therefore became known as connexin32 (Cx32). The difference between the M_r determined from SDS-PAGE of purified junctions and the M_r based on the cDNA has been shown to be artifactual and is dependent on gel concentration and the amount of protein loaded onto the gel (Green et al., 1988). Subsequently, rat cDNAs were isolated for the major gap junction protein from heart (Cx43) (Beyer et al., 1987), the M_r 21,000

protein from liver (Cx26) (Zhang and Nicholson, 1989) and a lens fiber gap junction protein (Cx46) (Beyer et al., 1988; Paul et al., 1991). More recently a number of new rodent connexins have been cloned: Cx30.3 (Hennemann et al., 1992c), Cx31 (Hoh et al., 1991; Hennemann et al., 1992a), Cx31.1 (Hennemann et al., 1992c; Haefliger et al., 1992), Cx33 (Haefliger et al., 1992), Cx37 (Willecke et al., 1991a; Haefliger et al., 1992), Cx40 (Beyer et al., 1992; Haefliger et al., 1992; Hennemann et al., 1992b), Cx45 (Hennemann et al., 1992a) and Cx50 (White et al., 1992). Table 1.1 summarizes information on the connexins that have been cloned from rodents. Connexins have also been cloned from non-mammalian vertebrates including chicken [Cx42 (Beyer, 1990), Cx43 (Musil et al., 1990) and Cx45 (Beyer, 1990)] and Xenopus [Cx30 (Gimlich et al., 1988), Cx38 (Ebihara et al., 1989; Gimlich et al., 1990) and Cx43 (Gimlich et al., 1990)].

Comparisons of the primary sequences of the different connexins show a considerable amount of sequence similarity, although the degree of similarity varies depending on region. Hydropathy analyses of the connexins also predict a common membrane topology: four transmembrane regions, two extracellular loops, and a cytoplasmic location for a central loop and the N- and C-termini (Fig. 1.1). The transmembrane regions and especially, the extracellular loops show a high degree of sequence identity, whereas the regions facing the cytoplasm are divergent both in sequence and in length (Bennett et al., 1991; Willecke et al., 1991b). Direct experimental evidence for this topological model is available for Cx32 (Zimmer et al., 1987; Goodenough et al., 1988; Milks et al., 1988) and Cx43 (Beyer et al., 1989;

Table 1.1 Summary of cloned connexins.

Connexin	M_r (X 10^{-3})	Size of mRNA (kb)	Type [cDNA (C), genomic (G)] and species origin [mouse (m), rat (r)] of clones	Examples of tissue distributions *
Cx26	21-25	2.5	mG, rC	li,k,st,b
Cx30.3	~30	1.9;3.2	mG	sk
Cx31	?	1.7-1.9;2.3	mC, rG	sk
Cx31.1	~30	1.6	mC, mG, rG	sk
Cx32	26-28	1.6	mG, rG, rC	li,st,b,k
Cx33	?	2.3	mG	t
Cx37	?	1.5-1.7	mC, mG, rG	lu,b,h,t,o
Cx40	?	3.3-3.5	mG, rG, rC	lu,h,k,sk,,o
Cx43	43-47	3.0	mG, rC	h,o,u,b,k
Cx45	?	2.2	mC	lu,k,
Cx46	46-56	2.8	rC, mG,	le,h,
Cx50	63-65	8.5	mG	le

* liver (li), lung (lu), lens (le), heart (h), skin (sk), stomach (st), brain (b), kidney (k), testes (t), ovary (o), and uterus (u).

Figure 1.1 A model depicting the membrane topology of the connexins. Both the NH_1 and COOH termini, as well as a central loop, are cytoplasmically located. There are also four transmembrane regions and two extracellular loops. Most of the sequence divergence between the connexins is located in the central cytoplasmic loop and the COOH tail. Size differences between the connexins are largely due to differing lengths of the COOH tails. (Adapted from Zimmer et al., 1987).

Extracellular Gap
Plasma Membrane
Cytoplasm
NH_2
COOH

Yancey et al., 1989; Laird and Revel, 1990; Yeager and Gilula, 1992), and comes from experiments using limited proteolysis of isolated gap junctions in conjunction with site-specific antisera.

The expression patterns of the different connexin genes are highly variable. Some, such as Cx32 (Paul, 1986) and Cx43 (Beyer et al., 1987), have wide, but distinct, tissue distributions, whereas others are expressed almost exclusively in only one tissue, e.g. Cx30.3, Cx31, and Cx31.1 in skin (Hoh et al., 1991; Willecke et al., 1991b; Hennemann et al., 1992a; Heaefliger et al., 1992), Cx33 in testis (Haefliger et al., 1992), and Cx46 and Cx50 in lens fiber cells (Paul et al., 1991; White et al., 1992). From the overlapping tissue distribution patterns of the connexins it is clear that a given tissue can contain different connexins and this raises the exciting possibility that single cells might contain gap junctions composed of more than one connexin. That this can be the case has been shown in mouse liver where Cx26 and Cx32 have been immunolocalized to the same gap junction plaques (Nicholson et al., 1987; Traub et al., 1989), and in F9 embryonal carcinoma cells (Hennemann et al., 1992a,c) and mouse keratinocytes (Brissette et al., 1991) where eight and five different connexin transcripts have been detected respectively. In addition to homotypic channels, where the two hemichannels are made up of a single connexin type, it is known that functional heterotypic channels can form, in which each half of the channel is made up of a different connexin. This was suggested initially when cells of different origin formed functional gap junctions when brought into contact by coculture (Epstein and Gilula, 1977), and more recently from experiments where Xenopus oocytes made to express a

single connexin type can form functional channels with oocytes expressing exclusively another type (Swenson et al., 1989; Werner et al., 1989; Barrio et al., 1991). In cells in which gap junction plaques contain more than one connexin it is not yet clear whether the connexons are made up of a single connexin type (homomeric) or whether connexons can form with more than one connexin type (heteromeric). However, a recent study of the biophysical properties of Cx26 and Cx32 in the Xenopus oocyte expression system indicates that functional heteromeric channels can form (Barrio et al., 1991). In cells expressing two or more connexins the potential number of combinatorial associations is therefore much greater than the number of connexins. The significance of this is that each connexin seems to form channels with distinct biophysical properties, e.g. unitary conductances, sensitivities to transjunctional voltages and secondary messengers, etc.(Dahl et al., 1987; Swenson et al., 1989; Eghbali et al., 1990; Barrio et al., 1991; Paul et al 1991; Willecke et al., 1991a; Hennemann et al., 1992b); the possibility thus exists that cells can exquisitely regulate their gap junctional intercellular communications by altering the combination of connexin genes transcribed.

1.3 Modulation of Gap Junctional Communications

As one might anticipate based on the foregoing discussion, regulation of gap junctional communication is a complex phenomenon. Numerous agents, some physiological and others non-physiological, are known to affect coupling (reviewed by Spray and Bennett, 1985, and Bennett et al., 1991). Since it is mainly the effects of

protein kinases that are relevant to this thesis the following discussion will be limited to these modulators.

In principle gap junctional communications can be modulated at two levels. On one level gene expression can be modulated at several points between transcription and the mature functional protein. One might expect modulation at this level to be on a slow time scale. At the other level, modulation can be achieved by changes in the permeability or gating of individual channels. Modulation at this level might be expected to be on a more rapid time scale. The two levels are not mutually exclusive; the same agent may have an immediate influence on gating and a longer-term effect on gene expression. For example, elevation of cAMP in primary cultures of hepatocytes leads to increased junctional conductance within minutes (Saez et al., 1986), apparently via serine phosphorylation of Cx32 by a cAMP-dependent protein kinase (PKA)(Saez et al., 1990). On the other hand when these cells are initially plated they become coupled but this is followed by a dramatic loss of coupling and Cx32, 5-8 hours after plating. This loss of Cx32 and coupling is delayed by treatment with cAMP analogs, evidently by extending the half life of the Cx32 mRNA (Saez et al., 1989). There is also the possibility that cAMP could regulate transcription of the Cx32 gene because two cAMP response elements have been identified in its promoter region (Miller et al., 1988). No reports of this have appeared to date. PKA modulation of gap junctional communications is not limited to Cx32. Cx43 junctions can also respond to PKA; in cardiac myocytes it increases conductance, whereas in myometrial and Sertoli cells it decreases conductance (Bennett et al., 1991).

It has been recognized for some time that viral transformation of cells often leads to reduced gap junctional communications (Musil and Goodenough, 1990)(this will be explored in more detail later). In fibroblasts, the oncogene v-src induces down-regulation of coupling. This process is dependent on activation of the protein tyrosine kinase encoded by v-src (Azarnia et al., 1988) and is correlated with tyrosine phosphorylation of Cx43 (Crow et al., 1990; Filson et al., 1990). Furthermore, expression of v-src with Cx43 in the Xenopus oocyte system blocks coupling and induces tyrosine phosphorylation of Cx43. However, similar experiments show a very small effect of v-src on Cx32 coupling and phosphorylation (Swenson et al., 1990). It is not yet clear whether modulation of gap junctional communications by tyrosine protein kinases occurs during normal development, but if it does it may be connexin specific. Not all cases of viral transformation induced down-regulation of coupling are due to Cx43 tyrosine phosphorylation. For example, down-regulation of coupling in mouse keratinocytes by the oncogene ras is associated with serine phosphorylation of Cx43 (Brissette et al., 1991).

Activation of calcium- and phospholipid-dependent protein kinase (PKC) is correlated with rapid inhibition of gap junctional communications in several cell types (Enomoto and Ymasaki, 1985; Gainer and Murray, 1985; Somogyi et al., 1989; Berthoud et al., 1992; Enkvist and McCarthy, 1992; Reynhout et al., 1992). In bovine lens epithelial cells (Reynhout et al., 1992) and Madin Darby Canine Kidney (MDCK) cells (Berthoud et al., 1992) the down-regulation of coupling by PKC is correlated with phosphorylation of Cx43. Recently a role for Cx43 phosphorylation in gap junction

assembly has been demonstrated (Musil et al., 1990a,b; Musil and Goodenough, 1991). It appears that, at least in some cells, newly synthesized Cx43 is transported to the plasma membrane in an unphosphorylated form. At the plasma membrane the Cx43 becomes phosphorylated and this is associated with its incorporation into gap junction plaques. The phosphorylation is on serine residues but the identity of the kinase responsible is not known.

1.4 The Role of Gap Junctions

Numerous roles have been proposed for gap junctions that stem from the ability of these channels to allow for the relatively free exchange of small molecules between coupled cells while restricting the flow of macromolecules. The evidence for these proposed functions is correlative in most cases and in only a few cases is strong direct evidence available. The strongest case is for the role gap junctions play in the spread of action potentials in electrically excitable tissues such as the crayfish giant synapse (Furshpan and Potter, 1959), in uterine smooth muscle during parturition (Garfield et al., 1977) and in cardiac myocytes during contraction (Barr et al., 1965; Severs, 1990).

Another frequently cited function is metabolic cooperation. The main evidence for this comes from in vitro experiments in which cells unable to grow because of a deficiency in a metabolic pathway can grow when they form gap junctions with normal cells (Gilula et al., 1972; Sheridan et al., 1979). In vivo, a situation like this could dampen the effects of somatic mutations. Other in vivo situations in which gap junctions

are likely to serve metabolic functions are those in which cells do not have direct access to the vascular system, e.g. lens fiber cells (Goodenough et al., 1980) and the growing oocyte (Heller et al., 1981; Haghighat and Van Winkel, 1990).

A role for gap junctions in the spread of cell-to-cell signaling molecules has long been purported. For instance, when co-cultured cardiac myocytes and ovarian follicle cells form gap junctions they can be cross-stimulated by cell-type specific hormones (Lawrence et al., 1978). The meiotic maturation of mammalian oocytes is controlled by gonadotropins. The receptors for these ligands are not located on the oocytes themselves but on the follicle cells which surround the oocytes. The secondary messengers generated by the activation of these receptors are transmitted to the oocytes via gap junctions (Eppig, 1991; Fagbohun and Downs, 1991). In Xenopus follicles gap junctions mediate the transfer of a calcium mobilization factor from the follicle cells to the oocyte in response to the binding of angiotensin II to its receptor on the follicle cells (Sandberg et al., 1990). The spread of calcium waves through astrocyte networks also appears to depend on patent gap junctions for the transmission of secondary messengers (Cornell-Bell et al., 1990; Charles et al., 1991, 1992; Enkvist and McCarthy, 1992).

One of the most frequently cited functions for gap junctional communications is in the transfer of growth regulatory molecules between cells. This area has received enormous attention because of the implications it has to cancer research. Initially this idea came from the observation that many transformed cell lines have reduced if not absent gap junctional communications (Azarnia and Loewenstein, 1971; Loewenstein, 1979). Subsequently, it has been shown that the growth rate of many transformed cells

is correlated with the level of gap junctional communications (Mehta et al., 1986, 1989; Rivedal and Sanner, 1992). More direct evidence has been obtained by transfecting communication-deficient tumor cells with cDNAs for Cx43 (Zhu et al., 1991; Naus et al., 1992) or Cx32 (Eghbali et al., 1991). Low levels of gap junctional communication in the parent cell lines was correlated with uncontrolled growth whereas experimentally increased gap junctional communication, reduced growth rates. Despite all the attention given to this problem, the precise role gap junctional communications play in the transformation process is not yet clear.

Finally, ever since ionic coupling was observed between cells of the squid embryo (Potter et al., 1966) gap junctions have been thought to play an important role in development, specifically in the transmission of developmentally significant molecules. In general, gap junctional communications first become established relatively early in embryonic development, interconnecting all cells in the embryo. The timing of this event is species-specific. This is followed by a restriction or loss of gap junctional communications between different parts of the organism at a later stage, thereby leading to the formation of communication compartments (Weir and Lo, 1982). The observation that the establishment and restriction of gap junctional communications is very often spatially and temporally correlated with major developmental events has formed a cornerstone in the argument for a developmental role for gap junctions. For example, in embryos of Patella, a gastropod mollusc, gap junctions and ionic coupling are present from the 2-cell stage (Dorresteijn et al., 1982; Serras and Van den Biggelaar, 1989) but dye coupling does not become established until the 32-cell stage when all the blastomeres

become coupled (de Laat et al., 1980; Dorresteijn et al., 1983). Electron microscopy has shown that gap junctions increase in size and number at this stage but it is not clear whether there is an increase in the permeability of existing channels as well (Dorresteijn et al., 1982, 1983). In Patella, the 32-cell stage is when, through contact mediated cell interactions, one of the blastomeres becomes commited to form the mesoderm stem cell and the symmetry of the embryo changes from radial to bilateral (Dorresteijn et al., 1983). However, beginning at the 72-cell stage clones of cells can be identified with reduced dye-coupling but unimpaired ionic coupling. Later these cells lose their dye coupling altogether and the ionic coupling becomes attenuated. This occurs at about the same time that these already commited cells begin to differentiate to form a part of the prototroch, a larval structure (Serras et al., 1990). Later, in the trochophore larva three main regions with different developmental fates are recognized. The cells within each of these regions are coupled to each other whereas no coupling is observed between regions (Serras et al., 1985, 1990).

The larval insect epidermis is arranged into a metameric pattern of segments in which each segment is a developmental compartment (Locke, 1959). Developmental compartments form, when during development the organism becomes progressively subdivided into polyclonal groups of cells (compartments) with a common fate that is different from cells in other compartments. The compartments develop autonomously and are separated by distinct borders that cells cannot cross (Crick and Lawrence, 1975). In the insect segmental epidermis it turns out that the segmental border separating developmental compartments is also a communication compartment border. Epidermal

cells within each segment are highly dye coupled, but dye spread across the segmental border is restricted or absent despite the presence of ionic coupling (Warner and Lawrence, 1982; Blennerhassett and Caveney, 1984). The border cells resemble intrasegmental cells in the number and size of their gap junctions (Lawrence and Green, 1975), suggesting that these cells regulate the permeability of their gap junctions, perhaps in order to restrict the distribution of a morphogen. Similar restrictions in gap junctional intercellular communications at developmental compartment boundaries have also been reported in Drosophila imaginal discs (Weir and Lo, 1982, 1984).

Although dye injection experiments on early cleavage stage Xenopus embryos have failed to reveal communication compartments, they have revealed a pattern of gap junctional communications that is stage- and position-specific (Guthrie, 1984; Guthrie et al., 1988). Between the 16- and 64-cell stages, blastomeres in the future dorsal region of the embryo tend to be more highly coupled than blastomeres in the future ventral region. This is significant because it is at these stages that important events relating to the patterning of the dorsoventral axis of the embryo take place (Guthrie et al., 1988). Treatment of embryos with lithium or UV irradiation are known to cause abnormalities in the dorsoventral axis of the embryo. When the effects of these agents on gap junctional communications at the 32-cell stage were investigated it became apparent that the asymmetry normally seen in dye coupling between the future dorsal and ventral regions was abolished (Nagajski et al., 1989).

During preimplantation mouse development all the blastomeres become coupled via gap junctions for the first time at the 8-cell stage, coincident with the major

morphogenetic event of compaction (reviewed by Kidder, 1987; and Gilula et al., 1992). As gap junctions in preimplantation mouse development are the subject of this thesis and will be discussed at length later, this topic will not be treated in detail here. Shortly after implantation, the two primary cell lineages generated during preimplantion development [the inner cell mass (ICM) cells and trophectoderm (TE) cells] become uncoupled from one another, and later on dye spread within the ICM is even further subdivided (Lo and Gilula, 1979b). Analysis of dye coupling during gastrulation reveals similar features i.e. the spatial coincidence of developmental and communication compartments, in that dye spreads between cells within a germ layer but not between cells that belong to different germ layers (Kalimi and Lo, 1988). The extraembryonic membranes appear to form separate communication compartments as well (Kalimi and Lo, 1989).

As noted earlier, the results from correlative studies linking gap junctions and development, such as those cited above, have served as the main evidence for a developmental role for gap junctions. Although numerous agents are available that strongly affect gap junctional conductance [e.g. protons, calcium ions, voltage, long chain alcohols and anesthetics (Spray and Bennett, 1985)], the absence of more direct evidence stems from a lack of reagents that could specifically disrupt gap junctional communications. Recently more direct evidence has become available, chiefly from the use of antibodies raised against purified Cx32 from liver to inhibit coupling in developmental systems. When these antibodies were microinjected into one blastomere of the 8-cell Xenopus embryo the progeny of the injected blastomere were not coupled to their neighbours at the 32-cell stage whereas in control embryos injected with

preimmune serum these blastomeres were fully coupled. These experimental embryos, when allowed to develop to the tadpole stage, showed gross abnormalities in structures derived from the antibody-injected cell (Warner et al., 1984). Consider also the coelenterate Hydra, an animal with a simple body plan consisting of a head on one end, a foot on the other, and a cylindrical body column in between. A piece of tissue removed from the body column can regenerate the entire animal if cultured on its own, however if it is grafted into the body column of a host animal the tissue is resorbed. When the host's cells were loaded prior to grafting with the same antibody as that used in the Xenopus studies outlined above, the incidence of secondary body axes increased significantly, presumably because the gap junction mediated transfer of a so called head inhibitory substance from the head region was suppressed (Fraser et al., 1987). This same antibody has also been used to investigate the role of gap junctional communications in the establishment of the anterior-posterior axis of the chick limb bud. Limb bud cells are coupled in a graded fashion, coupling being most extensive in the posterior region and diminishing in the anterior direction (Coelho and Kosher, 1991). A group of cells at the posterior margin of the limb bud play a crucial role in the patterning process; grafting of these cells to the anterior margin leads to mirror image duplication of digits. Loading of the posterior cells with antibody prior to grafting reduces the incidence of duplications significantly, suggesting a fundamental role for gap junctions in the patterning process, presumably in the transfer of a morphogen (Allen et al., 1990). Finally, this same antibody has been used to inhibit gap junctional communications in preimplantation mouse embryos. Such inhibition leads to

decompaction, and failure of the affected cells to participate in the formation of the blastocyst (Lee et al., 1987; for a more thorough discussion of this study see later sections). Similar results have been obtained from the mouse embryo using a different direct approach to the specific inhibition of gap junctional communications. In this study, the microinjection of Cx32 antisense RNA led to loss of coupling, and decompaction and extrusion of the affected cells (Bevilacqua et al., 1989; this study will be discussed in more detail later).

1.5 Preimplantation Mouse Development and Gap Junctions

Our knowledge of preimplantation mammalian development is for the most part derived from the mouse. The reasons for the popularity of the mouse as a model for preimplantation development are several, including: relatively low cost; the ability to generate embryos with an uniform genetic background by using inbred strains of mice; the ability to generate large number of embryos; and the ability to culture embryos through the entire preimplantation period in simple, chemically defined, media. The preimplantation period in the mouse spans about 4.5 to 5 days, culminating in implantation of the approximately 128-cell embryo in the uterus (Pratt, 1987).

Two major morphogenetic events take place during preimplantation development. The first, termed compaction, occurs early in the 8-cell stage and includes at least two components: cell flattening and blastomere polarization. Cell flattening is mediated by the Ca-dependent cell adhesion molecule uvomorulin (also known as E-cadherin and L-

CAM) and can be prevented by the administration of agents that interfere with this adhesion system (Hyafil et al., 1980; Shirayoshi et al., 1983; Johnson et al., 1986). Blastomere polarization is of major developmental significance being the initial step in the establishment of the first two embryonic cell lineages, the ICM and the TE. As with cell flattening, intercellular interactions mediated by uvomorulin play a major role in polarization. Although 8-cell blastomeres have an intrinsic ability to become polarized, fewer than normal do so if cell contact is denied. Moreover, the pattern of intercellular contacts determines the axis of polarity (Ziomek and Johnson, 1980; Johnson et al., 1986; Houliston et al., 1989; Pierce and Calarco, 1990). Manifestations of polarization are evident both at the cell surface and intracellularly. The plasma membrane develops a free apical domain, and a basolateral domain in contact with adjacent blastomeres, each domain being characterized by a set of distinct proteins and organelles. Intracellularly, a number of cytoplasmic components become asymmetrically distributed into apical and basolateral domains as well (Ziomek and Johnson, 1980; Reeve and Ziomek, 1981; Fleming and Pickering, 1985; Pierce and Calarco, 1990). It is this asymmetric distribution of cellular components that is crucial to the formation of the two cell lineages. When the 8-cell blastomeres divide, in some cases the cleavage plane is perpendicular to the axis of polarity. The two daughter cells are therefore not equal; one inherits most of the apical domain and will remain polar whereas the other one inherits most of the basolateral domain and will be apolar. At the 16- to 32-cell stages apolar blastomeres become located on the inside of the embryo and later form the ICM of the blastocyst, whereas the polar blastomeres become located on the outside and later form

the TE (Ziomek and Johnson, 1981, 1982; Kimber et al., 1982; Sutherland et al., 1990). Following implantation the ICM gives rise to the embryo proper whereas the TE contributes to the extraembryonic tissues (Gardner et al., 1973).

The second major morphogenetic event, cavitation, is initiated at the 32-cell stage when small fluid filled cavities begin to appear between the blastomeres. It concludes with the formation of the blastocyst, containing a large fluid filled cavity the blastocoel, as well as the ICM and the TE (reviewed by Fleming and Johnson 1988).

All animal embryos, including the mouse, depend on RNA and protein synthesized during oogenesis to carry out some of the early developmental processes. Mouse embryos differ from most other species investigated, however, in that the period of exclusive dependance on maternal macromolecules is relatively short, with several lines of evidence indicating that at the 2-cell stage the control of development switches from the maternal to the embryonic genome. The ovulated oocyte contains a substantial store of mRNA (Bachvarova and De Leon, 1980; Pikó and Clegg, 1982) which is only minimally augmented by new synthesis during the first cell cycle (Clegg and Pikó, 1983a,b). About two thirds of this mRNA has been degraded by the late 2-cell stage (Pikó and Clegg, 1982; Clegg and Pikó, 1983a) when transcription of new mRNA increases sharply indicating activation of the embryonic genome (Levey et al., 1978; Clegg and Pikó, 1983a,b). The studies cited above measured bulk mRNA. Measurements of specific transcripts, e.g. ß-actin (Giebelhaus et al., 1983, 1985) and histone (Giebelhaus et al., 1983; Graves et al., 1985), have yielded results consistent with the bulk measurements; i.e., the amount of transcripts declines dramatically from

the ovulated oocyte to the 2-cell stage when it begins to increase again to reach high levels in the blastocyst. Not all transcripts follow this pattern. For example, Taylor and Pikó (1987) have quantitated transcripts corresponding to 69 random cDNA clones isolated from a late 2-cell library. Transcripts corresponding to only about half of these clones were also present in 1-cell embryos, indicating a major qualitative difference between maternal and embryonic transcription. The RNA data are reinforced by data showing a major shift in protein synthetic profiles at the 2-cell stage (van Blerkom and Brockway, 1975; Levinson et al., 1978) as well as the detection of protein products encoded for by paternally inherited genes for the first time at the 2-cell stage (Sawicki et al., 1982). Finally, development beyond the 2-cell stage is sensitive to the transcriptional inhibitor α-aminitin (Flach et al., 1982; Bolton et al., 1984), suggesting a requirement for embryonic transcription for development to proceed beyond this stage. Treatment of embryos with α-amanitin has also shown that compaction and cavitation are dependent on embryonic transcription (Kidder and McLachlin, 1985).

Thin section electron microscopy (Ducibella et al., 1975; Ducibella and Anderson, 1975) and freeze-fracture methods (Magnuson et al., 1977) reveal gap junctions in preimplantation mouse embryos beginning at the 8-cell stage and an increase in the size and number of these junctions to the blastocyst stage. As mentioned previously, embryo-wide ionic (Lo and Gilula, 1979; McLachlin et al., 1983; Goodall and Johnson, 1984) and dye coupling (Lo and Gilula, 1979; Goodall and Johnson, 1982, 1984; McLachlin et al., 1983) are also detectable for the first time at the 8-cell stage, and are coincident with compaction. In agreement with the morphological data, the rate

of dye spread increases as development proceeds from the early 8-cell stage (McLachlin and Kidder, 1986). The onset of coupling at the 8-cell stage is a precisely timed event, beginning two to five hours following the third cleavage (Goodall and Johnson, 1982, 1984). Attempts to induce coupling prior to this stage have failed (Goodall and Johnson, 1982; McLachlin et al., 1983) despite that studies utilizing transcriptional and translational inhibitors (McLachlin et al., 1983; McLachlin and Kidder, 1986) have indicated that by the 4-cell stage there is already sufficient gap junction mRNA and protein present for the establishment of coupling. Although these transcriptionally and translationally blocked embryos become coupled at the same time as controls the extent of coupling is reduced, demonstrating a requirement for continued gap junction gene expression for embryos to become fully coupled (McLachlin and Kidder, 1986). In addition to gene expression, microtubules, microfilaments, cell flattening (see below), and cytokinesis have been shown to not control the timing of gap junction assembly (Kidder et al., 1987). The temporal relationship between the onset of coupling and compaction suggests a causal link but this, it turns out, is not the case. The evidence disproving such a link includes the fact that: (1) as a population of embryos undergoes compaction not all partially compacted embryos are coupled (McLachlin and Kidder, 1986); (2) gap junctions can form when cell flattening is inhibited by agents that interfere with the uvomorulin cell adhesion system (Goodall, 1986); (3) inhibition of cell flattening through treatment of 8-cell embryos with cytochalasin B does not interfere with the establishment or maintenance of coupling (Kidder et al., 1987); (4) in aggregation experiments, isolated 2- and 4-cell blastomeres can induce polarization of 8-cell isolated

blastomeres despite the absence of coupling (Goodall and Johnson, 1982, 1984); and (5) inhibition of coupling does not necessarily inhibit the establishment of blastomere polarization (Goodall, 1986). Apparently then, the establishment of compaction and coupling are controlled independently. Maintenance of compaction, on the other hand, seems to depend on functional gap junctions. As outlined previously, experimental perturbation of gap junctional communications with antibody (Lee et al., 1987) and antisense RNA (Bevilacqua et al., 1989) probes leads to decompaction and failure to cavitate. Similar conclusions have been drawn from experiments with embryos derived from crosses between female DDK mice and males from a different strain (Buehr et al., 1987). When these embryos are at the 16-cell stage blastomeres begin to decompact spontaneously and many of these are eventually extruded. Very few of these embryos form blastocysts. Investigation into the underlying cause of this effect revealed reduced coupling at the 8-cell stage compared to controls. Treatment of the embryos with methylamine, an agent known to increase gap junctional permeability, was able to elevate coupling to control levels and increase the number of embryos reaching the blastocyst stage. Gap junctional communications seem, therefore, to be required for the maintenance of the compacted state and by extension for the formation of the blastocyst and implantation.

1.6 General Objectives

Several important questions arise from the preceding discussion. For instance, based on studies discussed above, it is highly likely that gap junctions are essential for successful preimplantation development. Yet next to nothing is known about how the junctions function and what precise role they play in preimplantation embryos. A necessary first step in addressing these questions is to determine a) the molecular components involved in intercellular communication in preimplantation embryos, and b) how they are regulated in time and space. Chapter 2 documents experiments aimed at characterizing connexin gene expression in preimplantation embryos as an initial step in such a determination.

The experiments presented in Chapter 3 stem directly from results obtained in Chapter 2. The results from Chapter 2 indicated that Cx43 is the main, if not the only connexin gene transcribed in the embryo. Chapter 3 describes experiments with antisense oligonucleotides that had the dual purpose of confirming these findings as well as determining the requirement for Cx43 gap junctional communications in preimplantation development.

The transcriptional and translational inhibitor studies reviewed above suggest that gap junction mRNA and protein begin to accumulate at least by the 4-cell stage, well in advance of gap junction assembly. This raises the question of what triggers the initiation of gap junction assembly at the 8-cell stage or what inhibits the initiation prior to the 8-cell stage. As outlined above, several potential control factors have been eliminated as

being involved in this process, but we do not yet know the answer to this question. Experiments aimed at solving this problem form the basis of Chapter 4.

CHAPTER 2

CONNEXIN GENE EXPRESSION DURING PREIMPLANTATION DEVELOPMENT AND OOGENESIS

2.1 Introduction

As has been noted in the GENERAL INTRODUCTION, there is a temporal association between the initiation of gap junction assembly and other morphogenetic events of great developmental significance in the preimplantation mouse embryo. The initial onset of gap junctional communication occurs abruptly, against a background of zero coupling, and furthermore, there is an absolute requirement for coupling for successful preimplantation development. Elucidation of the molecular components involved in gap junctional communication in the embryo is therefore an ideal stepping stone in gaining an overall understanding of the genetic program directing preimplantation mouse development. For these reasons I began my thesis research by characterizing the expression patterns of members of the connexin multigene family in preimplantation development. It had been shown previously by Western blotting that Cx32 is present throughout preimplantation development, whereas its mRNA could not be detected at any stage (Barron et al., 1989). This study also reported the presence of Cx43 transcripts in a sample of late morulae and early blastocysts. My first objective was therefore to determine the preimplantation developmental profile of the Cx43 mRNA and its protein product. The second objective was to determine if the Cx26 and Cx46

genes were expressed during preimplantation development by screening embryos for Cx26 and Cx46 transcripts (these were the only other connexins known when I began my research).

To explain the results documenting the presence of Cx32 in the absence of its mRNA, Barron et al. (1989) hypothesized that Cx32 is an oogenetic product that persists through preimplantation development with no augmentation from zygotic gene expression. A necessary requisite for this hypothesis to hold true is that Cx32 mRNA be present in oocytes during oogenesis. The third objective was to address this hypothesis by assaying for Cx32 transcripts in maturing oocytes.

The involvement of gap junctions in early mouse development is not limited to cleavage stage embryos but is also integral to oogenesis. In fact, the cumulus-oocyte complex (COC) provides one of the clearest examples of the involvement of gap junctional intercellular coupling in a developmental process (reviewed by Larsen and Wert, 1988, and Eppig, 1991). The mural and cumulus granulosa cells of the mature follicle are functionally interconnected by gap junctions. Furthermore, threadlike extensions of the cumulus granulosa cells traverse the zona pellucida and make close contacts with the oocyte plasma membrane; gap junctions at these sites of contact complete the unification of the entire cumulus-granulosa cell complex into a functional syncytium. In addition to providing a pathway for metabolic cooperation between the oocyte and its surrounding somatic cells during follicular development, the cumulus-oocyte gap junctions transmit an as yet unidentified signal which triggers meiotic maturation (Fagbohun and Downs, 1991). In light of the well-characterized involvement

of gap junctional coupling during oocyte growth and maturation, the fourth objective was to characterize the connexins which contribute to gap junctions in the COC, by assaying for Cx32 and Cx43 in maturing oocytes and cumulus granulosa cells.

2.2 Materials and Methods

2.2.1 Embryo and oocyte collection

CF1 female mice (Charles River Canada Ltd., St. Constant, Quebec) and $CB6F_1/J$ male mice (The Jackson Laboratory, Bar Harbor, ME) were used throughout this research. The females were superovulated by intraperitoneal injections of 5 international units (IU) of pregnant mare's serum gonadotropin (PMSG; Sigma Chemical Co., St Louis, MO) followed 46-48 hours later by 5 IU of human chorionic gonadotropin (hCG; Sigma) and caged with males for 9-15 hours. Successful matings were confirmed by the presence of vaginal plugs. To recover ovulated oocytes and embryos, mice were anaesthetized with carbon dioxide and euthanized by cervical dislocation at appropriate time points following hCG injection (see below). Embryos and ovulated oocytes were flushed from the reproductive tract with either flushing medium I (FM-I; oocytes and embryos up to the late morula stage) or flushing medium II (FM-II; blastocysts)(for media formulations see Spindle, 1980). Embryos were washed by passage through at least three drops of the flushing medium followed by five drops of phosphate buffered saline containing 3 mg/ml polyvinylpyrrolidone (PBS-PVP). They were then transferred

to an Eppendorf tube in 5 μl or less of PBS-PVP, and quick-frozen in a dry ice-methanol bath. A comparable volume of the last wash from which the embryos had been taken was also frozen. The frozen embryos and companion wash samples were stored at -80°C until sufficient numbers had been collected for an experiment.

Unfertilized oocytes were collected from females that had been superovulated but not mated. These oocytes were passed through several drops of medium containing 800 U/ml hyaluronidase (Sigma) to remove the cumulus cells prior to the PBS-PVP washing steps. The effectiveness of the hyaluronidase treatment was monitored by viewing a small sample under a Zeiss Photomicroscope I using Nomarski optics. In some cases an aliquot of dissociated cumulus granulosa cells was collected, washed, and frozen in the same manner as the oocytes.

The staging of oocytes or embryos and its relation to hours post-hCG was as follows: oocytes, 16-18 hours; 1-cell zygotes, 18 hours; 2-cell, 48 hours; 4-cell, 60 hours; 8-cell, 71 hours; late morulae, 82 hours; blastocysts, 94 hours. The oocytes or embryos were exposed to culture conditions for no more than 1-2 hours prior to freezing.

For the isolation of COCs, females were primed with PMSG as above and then sacrificed either before or after an hCG injection. The ovaries were dissected out and placed in FM-I. Following removal of fat and connective tissue, mature follicles were punctured with a sterile 26 gauge needle to release COCs. The released COCs were transferred to a holding drop of fresh FM-I. Following the addition of collagenase (Type 1A, Sigma) or pronase (Type XXV, Sigma) to a final concentration of 100 U/ml or

0.1%, respectively, the ovaries were teased apart with fine forceps and any released COCs and immature oocytes were collected and added to the holding drop. The oocytes were stripped of cumulus cells as described above and then washed free of enzymes and somatic cells by serially transferring them through five drops of PBS-PVP. Ovarian oocytes were frozen and stored as described for ovulated oocytes and embryos. Aliquots of ovarian somatic cells collected from the bottom of the dishes in which the ovaries had been teased apart were similarly washed and frozen.

2.2.2 RNA isolation

For Northern blots, total RNA was extracted from frozen oocytes or embryos (1200-1500 per extraction) using a modification of the single step method of Chomczynski and Sacchi (1987). Embryos or oocytes were lysed, without prior thawing, in 100 μl of solution D (4 M guanidine thiocyanate, 0.5% Sarkosyl, 100 mM 2-mercaptoethanol, 25 mM sodium citrate, pH 7.0) with 20 μg carrier E. coli tRNA (Sigma) added. After thorough mixing, 10 μl of 2 M sodium acetate, pH 4.0, was added and the sample was phenol-chloroform extracted once. The phases were separated by centrifugation at 10,000 X g at 4°C for 20 minutes after which the aqueous phase was precipitated overnight at -20°C with an equal volume of isopropanol. The following morning the RNA precipitate was pelleted by centrifugation at 10,000 X g at 4°C for 20 minutes, washed once with 70% ethanol, and air dried. As positive controls, total RNA was also extracted from adult mouse brain, kidney, liver, and heart in the same fashion.

For the reverse transcription coupled polymerase chain reaction (RT-PCR), oocyte and ovarian somatic cell RNA was isolated based on the method of Rappolee et al. (1988) with some modifications. Oocytes or somatic cell aliquots were solubilized by the addition of 100 μl of solution D at room temperature to which 20 μg E. coli rRNA (Boehringer-Mannheim, Montreal, Quebec) was added as a carrier. The lysate was layered over a 100 μl cushion of 5.7 M CsCl in 0.1 m EDTA, pH 7.5, in a polyallomer ultracentrifuge tube. The samples were then spun for 4 hours at 80,000 rpm and 20°C in a TLA-100 rotor in a Beckman TL-100 tabletop ultracentrifuge. The RNA pellets were dissolved in 100 μl of 2.5 M ammonium acetate and ethanol precipitated overnight at -20°C. The following day the RNA was pelleted, washed twice with 70% ethanol, and air dried.

Total RNA from ovary and liver for RT-PCR was isolated by a modification of the method of Chirgwin et al. (1979). In short, the tissues were homogenized in solution D. The RNA was then pelleted through a 5.7 M CsCl cushion in a 10 ml Beckman open conical tube and spun in a SW 41 Ti rotor at 30,000 rpm for 14 hours at 20°C. The RNA pellets were dissolved in 10 mM Tris-HCl (pH 7.4), 5 mm EDTA, 1% SDS, and this solution was extracted once with phenol-chloroform, followed by a chloroform extraction. After overnight ethanol precipitation the RNA was pelleted, washed two times with 70% ethanol and air dried. The RNA pellets were redissolved in diethylpyrocarbonate (DEPC)-treated water and the concentration was determined spectrophotometrically by reading the absorbance at 260 nm, and assuming 1 A_{260} = 40 μg/ml (Sambrook et al., 1989). The purity of the RNA was checked

spectrophotometrically as well by also measuring the absorbance at 280 nm and calculating the A_{260}/A_{280}. This ratio was 1.9 for the RNA used for experiments.

2.2.3 Northern blots

The dry RNA pellets were dissolved in 4.5 μl of sterile, DEPC-treated water, after which deionized formamide was added to 50%, formaldehyde to 6.5%, and phosphate buffer to 5 mM, pH 7.0, in a total volume of 20 μl. Following denaturation at 55°C for 15 minutes, 4 μl of 6X RNA gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol in DEPC-treated water; Sambrook et al., 1989) and 1 μl ethidium bromide (1 mg/ml) were added and the samples were separated, along with RNA ladder (Gibco-BRL, Burlington, Ont.), by electrophoresis in 1% denaturing formaldehyde-agarose gels containing 660 mM formaldehyde and 10 mM phosphate buffer, pH 7.0. Electrophoresis was carried out in 10 mM phosphate buffer, pH 7.0, for 6-8 hours at 50 volts. After electrophoresis the RNA was transferred by capillary action to a Hybond-N blotting membrane (Amersham, Oakville, Ont.) for 16-18 hours in 10X SSC (1X SSC = 150 mM NaCl, 15 mM sodium citrate; Sambrook et al., 1989). The blots were probed with cDNAs labeled with ^{32}P by the random primer method (Feinberg and Vogelstein, 1983) to a specific activity of 1-2 x 10^9 cpm/μg using a Random Primers DNA Labeling System (Gibco-BRL). The pre-hybridization and hybridization solutions consisted of 5X SSPE (1X SSPE = 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.0, and 1 mM EDTA; Sambrook et al., 1989), 50% formamide, 5X

Denhardt's solution (50X Denhardt's = 1% Ficoll, 1% PVP, and 1% bovine serum albumin; Sambrook et al., 1989), 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS), and 100 μg/ml sheared, denatured salmon sperm DNA. The blots were pre-hybridized in a plastic bag for 4 hours at 42°C and then hybridized in a HybridEase chamber (Hoefer Scientific Instruments, San Francisco, CA) at 42°C for 40-44 hr at a probe concentration of 1-3 ng/ml. Post-hybridization washes were to moderate stringency (0.5X SSC, 0.5% SDS, 65°C) after which the blots were exposed at -70°C to Kodak XRP X-ray film using DuPont Cronex intensifying screens. Autoradiograms were scanned using an LKB Ultrascan XL laser densitometer.

2.2.4 Western blots

For each embryonic stage analyzed, about 600 frozen embryos were lysed in 20 μl of 2X Laemmli sample buffer (1X Laemmli buffer = 62.5 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.05% bromophenol blue; Laemmli, 1970) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). As a positive control for connexin43, mouse heart plasma membrane preparations enriched for intercalated discs were isolated according to the procedure described by Green and Severs (1983). Four mouse hearts were homogenized in 1 mM sodium bicarbonate (BIC). The homogenate was allowed to stand for 10 minutes and was then filtered. The filtrate was centrifuged at 750 X g for 5 minutes, the supernatant was discarded, and the pellet was resuspended in BIC. The centrifugation and resuspension steps were repeated but after the second

centrifugation step the pellet was resuspended in BIC with 0.6 M KCl and 8% sucrose. This solution was stirred for 4 hours and then centrifuged at 1,000 X g for 5 minutes. The supernatant was collected and centrifuged at 12,000 X g for 5 minutes; the resultant pellets were resuspended in BIC, aliquoted and frozen at -70°C. Prior to electrophoresis, an equal volume of 2X Laemmli buffer was added. To avoid potential aggregation of gap junction protein (Green et al., 1988) neither the embryo samples nor the intercalated disc samples were boiled before being loaded onto the gels.

Polyacrylamide gel electrophoresis was carried out in the SDS-discontinous system of Laemmli (1970) using a Bio-Rad minigel apparatus (Bio-Rad Laboratories (Canada), Mississauga, Ont.). The gels consisted of a 4% stacking gel (4% total acrylamide, 2.7% N,N'-methylene-bis-acrylamide as crosslinker (i.e. 4%T, 2.7%C); 125.0 mM Tris-HCl, pH 6.8, 0.1% SDS) and a 12% resolving gel (12%T, 2.7%C; 375 mM Tris-HCl, pH 8.8, 0.1% SDS). The tank buffer was 25 mM Tris-HCl, pH 8.3, 192 mM glycine and 0.1% SDS. The gels were run at a constant current of 20 mA/gel until the tracking dye reached the bottom of the gel.

The separated proteins were electrophoretically transferred to nitrocellulose (Bio-Rad) in 25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol and 0.1% SDS (Towbin et al., 1979). Transfers were carried out in a Transfor TE 42 cell (Hoefer Scientific Instruments) at 40 volts for 80 minutes. The blots were blocked for 1-4 hours in 5% Carnation® skim milk powder in TBS (2 mM Tris, pH 7.5, 500 mM NaCl), washed twice in TTBS (0.01% Tween-20 in TBS) and incubated overnight with the primary antiserum in 1% skim milk powder in TTBS. The blot was stripped of unbound primary antiserum

by three 10 minute washes in TTBS and placed in alkaline phosphatase-conjugated secondary antibody in 1% skim milk powder in TTBS for 3-4 hr. Following three 10 minute washes in TTBS and a single 10 minute TBS wash the bound antibody was localized by developing the blot with 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and p-nitro blue tetrazolium (NBT) as substrates. The secondary antibodies and color development reagents (Bio-Rad, or Promega Corp., Madison, WI) were used as suggested by the suppliers.

The M_r was estimated by comparing the distance a protein migrated in the gel with a standard curve. The standard curve was constructed by plotting the distance migrated by a series of proteins of known M_r against the $\log_{10}$ of their M_r. The proteins used as standards and their M_r were as follows: BSA 66,000, ovalbumin 45,000, glyceraldehyde-3-phosphate dehydrogenase 36,000, carbonic anhydrase 29,000, trypsinogen 24,000, trypsin inhibitor 20,100, and α-lactalbumin 14,200 (SDS-7 kit; Sigma). A lane of prestained M_r markers (α_2-macroglobulin 180,000, ß-galactosidase 116,000, fructose-6-phosphate kinase 84,000, pyruvate kinase 58,000, fumarase 48,500, lactic dehydrogenase 36,500, and triosephosphate isomerase 26,600; Sigma) was included on each gel to monitor the progress of the electrophoresis, and the completeness of the transfer, and to approximate M_r.

2.2.5 Antisera and cDNAs

The Cx43 specific antiserum used in this study (gift from Dr. D. Paul, Harvard Medical School, Boston, MA) was raised in a rabbit immunized with a synthetic peptide corresponding to a unique region (amino acids 252-271) of the predicted connexin43 polypeptide (Beyer et al., 1989). This antiserum was used at 1:1000 dilution and the pre-immune serum at the same dilution served as a control.

The four connexin cDNAs used in this study [Cx26 (gift from Dr. B. Nicholson, SUNY, Buffalo, NY), Cx32 and Cx46 (gift from Dr. D. Paul), and Cx43 (gift from Dr. E. Beyer, Washington University, St. Louis, MO)] were isolated by screening rat cDNA libraries and each represents the entire coding sequence of the respective mRNA (Paul, 1986; Beyer et al., 1987, 1988; Zhang and Nicholson, 1989). The Cx32 cDNA was isolated from a liver library and hybridizes to 1.6 kb mRNA in liver and several other adult tissues (Paul, 1986). The Cx26 cDNA was isolated from a liver library as well and detects a 2.5 kb RNA in preparations from several adult tissues (Zhang and Nicholson, 1989). The Cx43 cDNA was isolated from a heart library and hybridizes to a 3.0 kb RNA present in several adult tissues (Beyer et al., 1987) and in mouse blastocysts (Barron et al., 1989). The Cx46 cDNA was obtained from a lens fiber library and detects a 2.8 kb RNA abundant in lens fibers and present at low levels in heart (Beyer et al., 1988; Paul et al., 1991).

As an internal positive control we also hybridized each Northern blot with a histone H3.2 clone. This clone contains a genomic fragment from the histone H3 gene

(Giebelhaus et al., 1983). The thoroughly characterized profile of this message through preimplantation development (Giebelhaus et al., 1983; Graves et al., 1985; Barron et al., 1989) and its small size (0.6-0.8 kb) make it ideal for this purpose.

2.2.6 RT-PCR

Total RNA was reverse transcribed into cDNA in a volume of 20 μl containing 2 units/μl recombinant RNasin (Promega), 20 ng/μl oligo dT primer (Boehringer-Mannheim), 1 mM each dATP, dCTP, dGTP, and dTTP (Boehringer-Mannheim), 1.5 U/μl avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer-Mannheim) in 50mM Tris, pH 8.3, 60 mM KCl, 3 mM $MgCl_2$ and 10 mM dithiothreitol. For oocytes and ovarian somatic cells the RNA pellet was dissolved in DEPC-treated water and all of the solution was reverse transcribed, whereas 4 μg of liver and whole ovary RNA was transcribed in each reaction. The reaction, which was carried out at 42°C for 1.5 hours, was stopped by boiling for 10 minutes, followed by quenching on ice. The reaction was diluted with sterile double distilled water such that 10 μl of the diluted reaction used for each PCR reaction contained 10-15 oocyte equivalents; the companion samples of ovarian somatic cells were diluted to the same final volume. The liver and total ovarian cDNAs were diluted to 400 μl.

PCR (Saiki et al., 1988) was carried out in a total volume of 50 μl essentially as described by Rappolee et al. (1988). Ten μl of cDNA were combined with 1-2 units Taq polymerase (Gibco-BRL) in a solution containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl,

3.7 ng/μl DNase-free BSA (Pharmacia LKB Biotechnology AB, Uppsala, Sweden), 200 μM each dATP, dCTP, dGTP, dTTP (Boehringer-Mannheim), 1-3 mM $MgCl_2$ and 1 μM each sequence specific primer. The mixture was overlaid with paraffin oil and amplified in a Perkin Elmer-Cetus DNA thermal cycler for 30 cycles where each cycle included 1 minute denaturation at 94°C, 1 minute annealing at 60°C, and 2 minute extension at 72°C. Fifteen μl of the PCR products were combined with 3 μl of loading buffer (0.25% bromophenol blue and 40% sucrose in water; Sambrook et al., 1989) and run on a 3% agarose gel (3:1 low melting point agarose and regular agarose) containing 0.38 μg/ml ethidium bromide. The gels were run at 100 volts for 2-3 hours in 1X TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA; Sambrook et al., 1989) and the gel was photographed and Southern blotted.

In a few preliminary experiments total RNA from ovary and isolated Cx32 cDNA insert were subjected to RT-PCR as described above. To confirm the identity of the PCR products in these experiments they were digested with the the restriction enzyme Ava I prior to electrophoresis. PCR products were combined with 10X One-Phor-All buffer (final concentration 2X; Pharmacia) and Ava I (final concentration approximately 0.15 units/μl; Boehringer-Mannheim) and incubated at 37°C for 1 hour. The DNA was precipitated at -20°C overnight by the addition of ammonium acetate to 2.2 M and two volumes of 100% ethanol. The DNA precipitate was pelleted by centrifugation at 10,000 X g at 4°C for 20 minutes, washed twice with 70% ethanol and air dried. The pellet was dissolved in 20 μl of water and 4 μl of loading buffer were added before electrophoresis.

2.2.7 PCR primers

All primers were synthesized by Dr. G. Mackie, Department of Biochemistry, University of Western Ontario. Each RT preparation was amplified at least once with a primer set based on the mouse β-actin cDNA sequence (Tokunaga et al., 1986). Amplification from cDNA with these primers yields a 243 bp fragment, but since the primers flank an intron, amplification from genomic DNA will yield a 330 bp fragment (Nudel et al., 1983). Any genomic DNA contamination of the RNA is therefore readily apparent. Secondly, because these primers are located approximately 2000 bp from the 3' end of the cDNA, they also provide an indication of the efficiency of the reverse transcription. The 5' primer was 5'-CGTGGGCCGCCCTAGGCACCA-3' and the 3' primer was 3'-GGGGGGACTTGGGATTCCGGTT. The Cx32 primer set was based on the sequence of the rat cDNA (Kumar and Gilula, 1986; Paul 1986). The 5' primer was 5'-CTGCTCTACCCGGGCTATGCC-3' and the 3' primer was 3'-TCGCTGGCTACGAGTCGGACG-5' and the expected size of the amplified fragment was 387 bp (nucleotides 494-880; corresponding to the amino acid sequence between the second extracellular loop and the carboxy terminal). The Cx43 primer set was constructed from a rat cDNA as well (Beyer et al., 1987). The 5' primer was 5'-TACCACGCCACCACCGGCCCA-3' and the 3' primer was 3'-AAGGGACTGCTGTCGGTTTTACGG-5' and the expected size of the amplified fragment was 294 bp (nucleotides 940-1233; corresponding to the cytoplasmic, carboxy terminal).

2.2.8 Southern blots

To confirm the identity of the PCR products the DNA was capillary blotted to Hybond-N membrane (Amersham) according to Sambrook et al. (1989) and probed with the appropriate connexin cDNA sequence. Briefly, the DNA was denatured by soaking the gel in 1.5 M NaCl, 0.5 N NaOH for 45 minutes. After rinsing the gel in distilled water it was neutralized in 1 M Tris, 1.5 M NaCl, pH 7.4 and then transferred in the same fashion as described for Northern blots. cDNAs were ^{32}P labeled by the random primer method (Feinberg and Vogelstein, 1983) to a specific activity of 1-2 X 10^9 cpm/μg using a Megaprime® kit (Amersham). Prehybridizations and hybridizations as well as posthybridization washes were as described for Northern blots, except that they were performed in a Robbins Hybridization Incubator (model 310) and hybridizations were carried out overnight.

2.3 Results

2.3.1 Northern blot analysis of connexin gene expression in preimplantation embryos

To determine the developmental profile of Cx43 mRNA, a Northern blot containing total RNA from 1500 embryos per lane and total RNA from brain, liver, kidney, and heart was probed simultaneously with the Cx43 and histone probes. When hybridized separately at moderate stringency, each of these two probes produces a single

band (Barron et al., 1989). Figure 2.1 shows an autoradiogram of this blot exposed for three days. The Cx43 cDNA hybridizes to a 3 kb RNA in both embryo and tissue samples. This RNA is present in the zygote (ZY), becomes undetectable at the 2-cell stage (2C), and then gradually increases in abundance from the 4-cell (4C) to the blastocyst stage (EB). A subsequent two week exposure of this blot failed to reveal a signal at the 2-cell stage. The presence of Cx43 mRNA in brain (B), kidney (K), and heart (H) but not liver (L) conforms with the known tissue distribution of this message (Beyer et al., 1987). To determine if the signal in zygotes could have resulted from residual cumulus cell contamination, RNA was extracted from 1400 oocytes that were subjected to several successive hyaluronidase treatments and then were verified microscopically to be free from cumulus cells. As shown in Figure 2.2, removal of the cumulus cells resulted in loss of the 3 kb band. Densitometric scans of various exposures of the autoradiogram in Figure 2.1 revealed an exponential increase in the abundance of both Cx43 and histone H3 mRNA following activation of the embryonic genome at the 2-cell stage (Fig. 2.3). Comparison of the accumulation profile of histone mRNA with published data (Giebelhaus et al., 1983; Graves et al., 1985) indicates that the RNA extraction procedure resulted in proportional recovery, i.e. approximately the same proportion of the total RNA was recovered at each embryonic stage.

The Northern blot in Figure 2.1 was reprobed with Cx26 cDNA along with the histone DNA after the previous signals had decayed. No Cx26 signal was detected in the embryo RNAs, even on autoradiograms exposed for two weeks, whereas the previous histone mRNA profile was reproduced (Fig. 2.4). The presence of a 2.5 kb Cx26

Figure 2.1 Cx43 mRNA is present in preimplantation embryos. A Northern blot containing 5 μg total RNA from brain (B), liver (L), kidney (K), about 2 μg from heart (H), and total RNA from 1500 embryos each of zygotes (ZY), 2-cell (2C), 4-cell (4C), 8-cell (8C), late morulae (LM), and early blastocysts (EB) was simultaneously probed with connexin43 cDNA and the histone H3.2 clone. The Cx43 cDNA hybridized to a single 3.0 kb band in brain, kidney and heart and in all the embryo stages except the 2-cell stage. The histone probe hybridized to a single 0.6-0.7 kb band. The autoradiogram was exposed for three days. Signal intensities were quantified by laser densitometry and are shown in Fig. 2.3

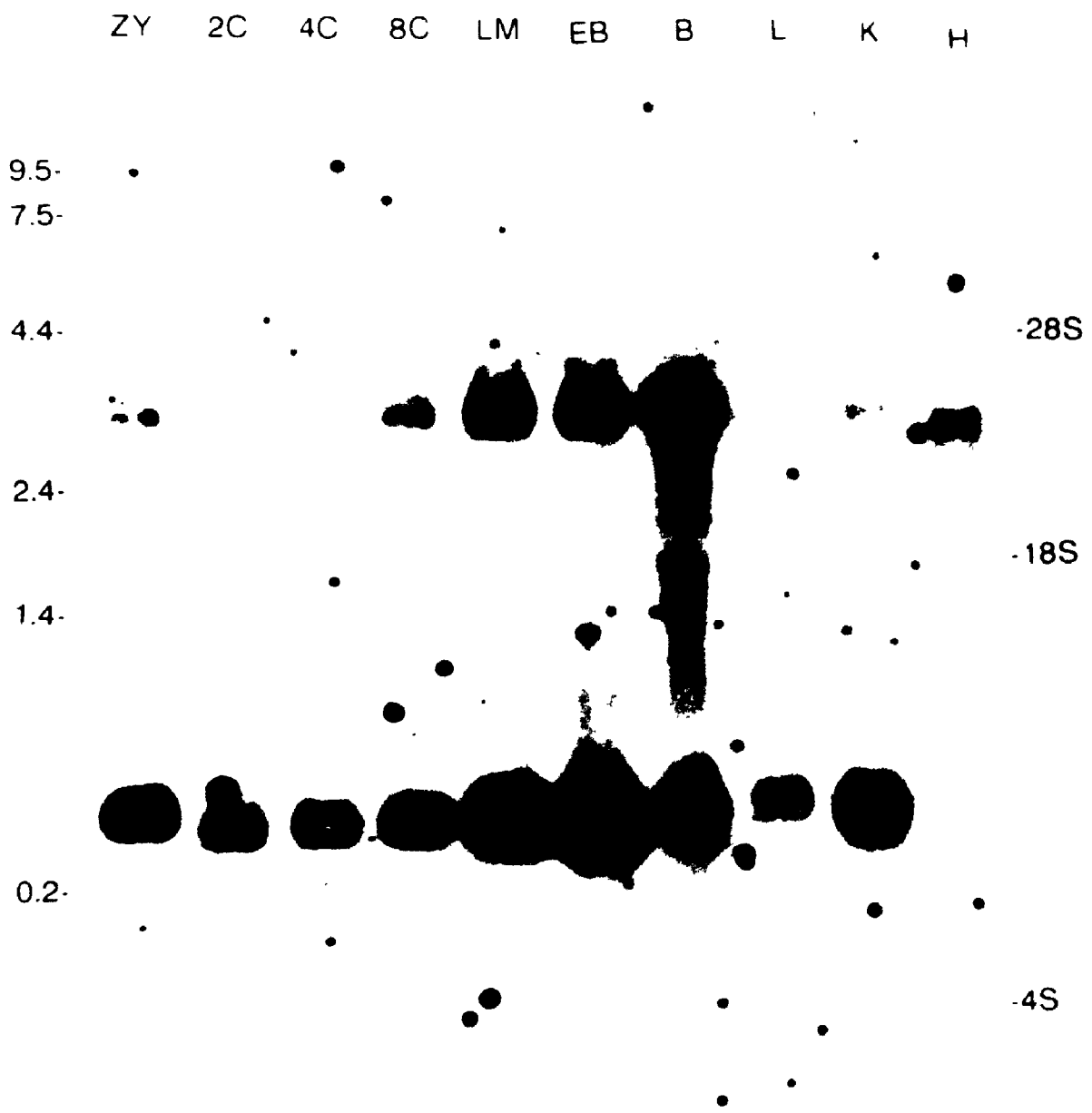

ZY
2C
4C
8C
LM
EB
B
L
K
H
9.5
7.5
4.4
2.4
1.4
0.2
28S
18S
4S

Figure 2.2 Cx43 mRNA is not present in 1-cell zygotes after complete removal of cumulus cells. This Northern blot contained total RNA from 1200 unfertilized oocytes (O) and from the cumulus cells (C) that had been removed from them by extensive hyaluronidase treatments. This resulted in loss of the 3 kb Cx43 band (arrow) seen in Fig. 2.1, while a portion of the histone signal (arrowhead) remained. The cumulus cells retained all of the Cx43 mRNA and a large portion of the histone mRNA. The autoradiogram was exposed for three days. A subsequent two week exposure failed to reveal a 3 kb band in the oocyte lane.

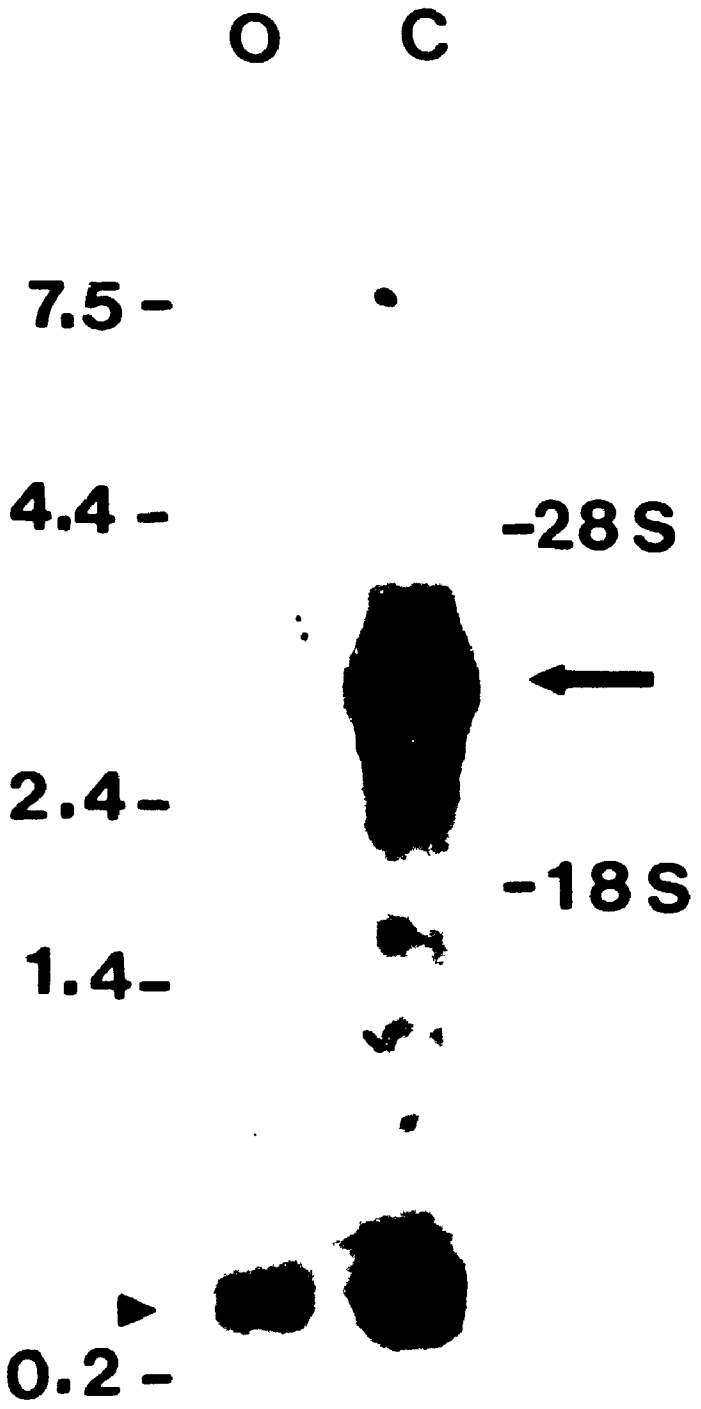

O
C
7.5 -
4.4 -
-28S
2.4 -
-18S
1.4 -
0.2 -

Figure 2.3 Relative abundance of Cx43 and histone H3 transcripts during preimplantation development. The Northern blot from Fig. 2.1 was scanned with a laser densitometer to quantify transcript levels. The transcript abundance is expressed as a percentage of the blastocyst level. The Cx43 profile is derived from the autoradiogram shown in Fig. 2.1 (3 day exposure) whereas the histone profile is derived from an autoradiogram exposed for 24 hours. Time points are shown for 2-cell embryos (48 hours post-hCG, 4-cell embryos (60 hours post-hCG), 8-cell embryos (71 hours post-hCG), late morulae (82 hours post-hCG), and blastocysts (94 hours post-hCG).

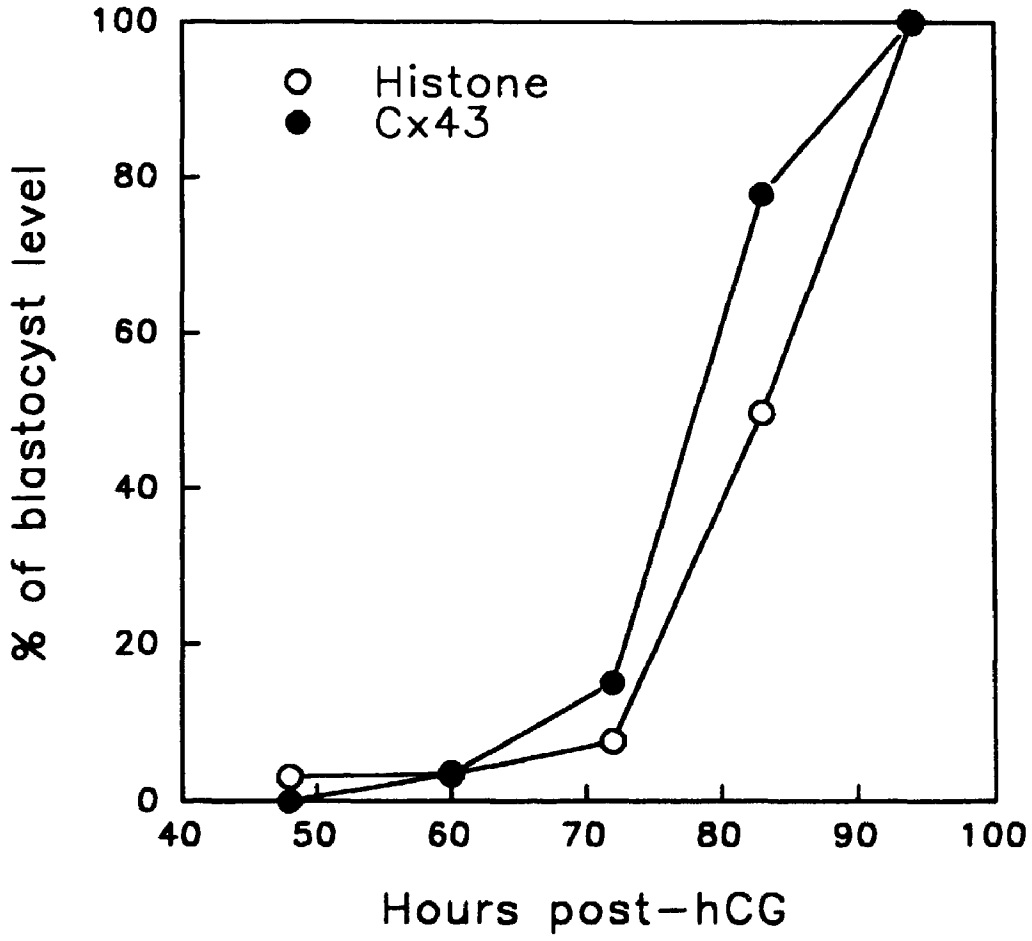
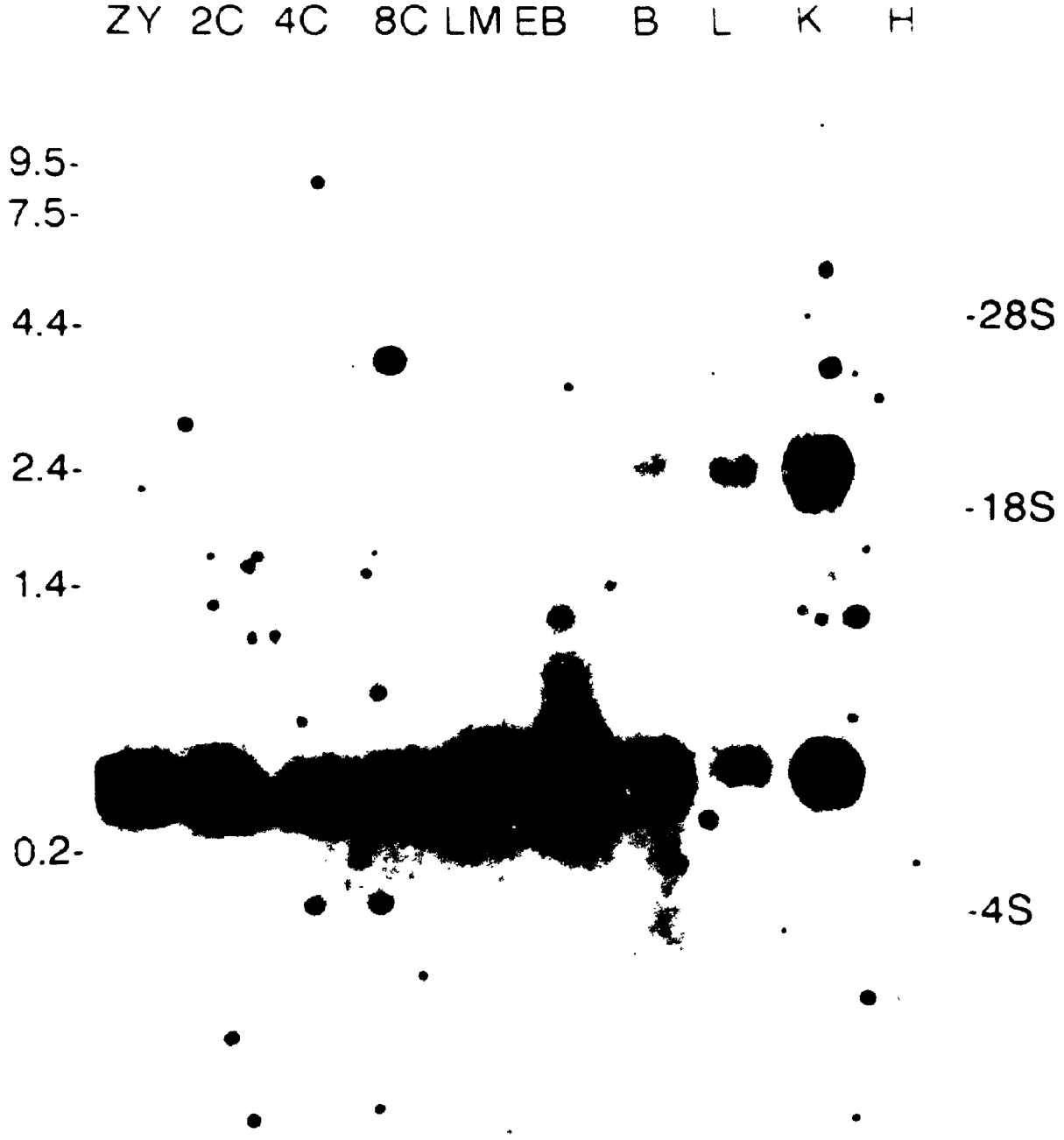

100
80
60
40
20
0
% of blastocyst level
Histone
Cx43
40
50
60
70
80
90
100
Hours post-hCG

Figure 2.4 Cx26 mRNA was not detected in preimplantation embryos. The same Northern blot as shown in Fig. 2.1 was reprobed simultaneously with the Cx26 and histone H3.2 probes. Lane designations are the same as in Fig. 2.1. The Cx26 cDNA hybridized to a single 2.5 kb band in brain, liver and kidney RNA which was not detected in heart or embryo RNA. The histone profile from Fig. 2.1 was reproduced, indicating retention of the RNA. The autoradiogram was exposed for 14 days.


ZY 2C 4C 8C LM EB B L K H
9.5-
7.5-
4.4-
2.4-
1.4-
0.2-
-28S
-18S
-4S

mRNA in liver and kidney is in agreement with published data (Zhang and Nicholson, 1989) but its presence in brain has not been reported previously. This same blot was similarly reprobed with the Cx46 and histone probes. The same histone profile was reproduced again, but no Cx46 signal was detected in embryo or tissue RNA (Fig. 2.5).

2.3.2 Western blot analysis of Cx43 in preimplantation embryos

Since only Cx43 transcripts were detected in preimplantation embryos, an investigation of the developmental profile of this protein was performed using Western blot analysis. Figure 2.6 shows one such blot, in which anti-connexin43 antiserum was used to probe embryo lysates from various stages of preimplantation development (600 embryos/lane). A polypeptide of M_r 43,000-48,000 was detected in all of the embryo samples. In general, the level of this polypeptide is high in the zygote (but see Discussion), decreases sharply at the two cell stage, and then increases gradually to the blastocyst stage. The particular blot displayed here has a very low signal at the 4-cell stage (lane 3). This is not, however, a consistent feature since other blots have exhibited a 4-cell signal similar in strength to that seen at the 8-cell stage (Fig. 2.6, lane 8). The apparent doublet nature of the connexin43 band in the embryo lanes is also not a consistent feature; on other blots only a single band was visible. The 100 kD band present in all the embryo stages is due to an endogenous alkaline phosphatase and appears even when one or both of the antibodies are omitted from the procedure. To ascertain the specificity of the antiserum an embryo lysate from 800-900 blastocysts was probed

Figure 2.5 Cx46 mRNA was not detected in preimplantation embryos. The same Northern blot as shown in Fig. 2.1 was reprobed simultaneously with the Cx46 and histone H3.2 probes. Lane designations are the same as in Fig. 2.1. No Cx46 hybridization was detected in embryo or tissue RNA. The histone profile from Fig. 2.1 was reproduced, indicating retention of the RNA. The autoradiogram was exposed for three days.

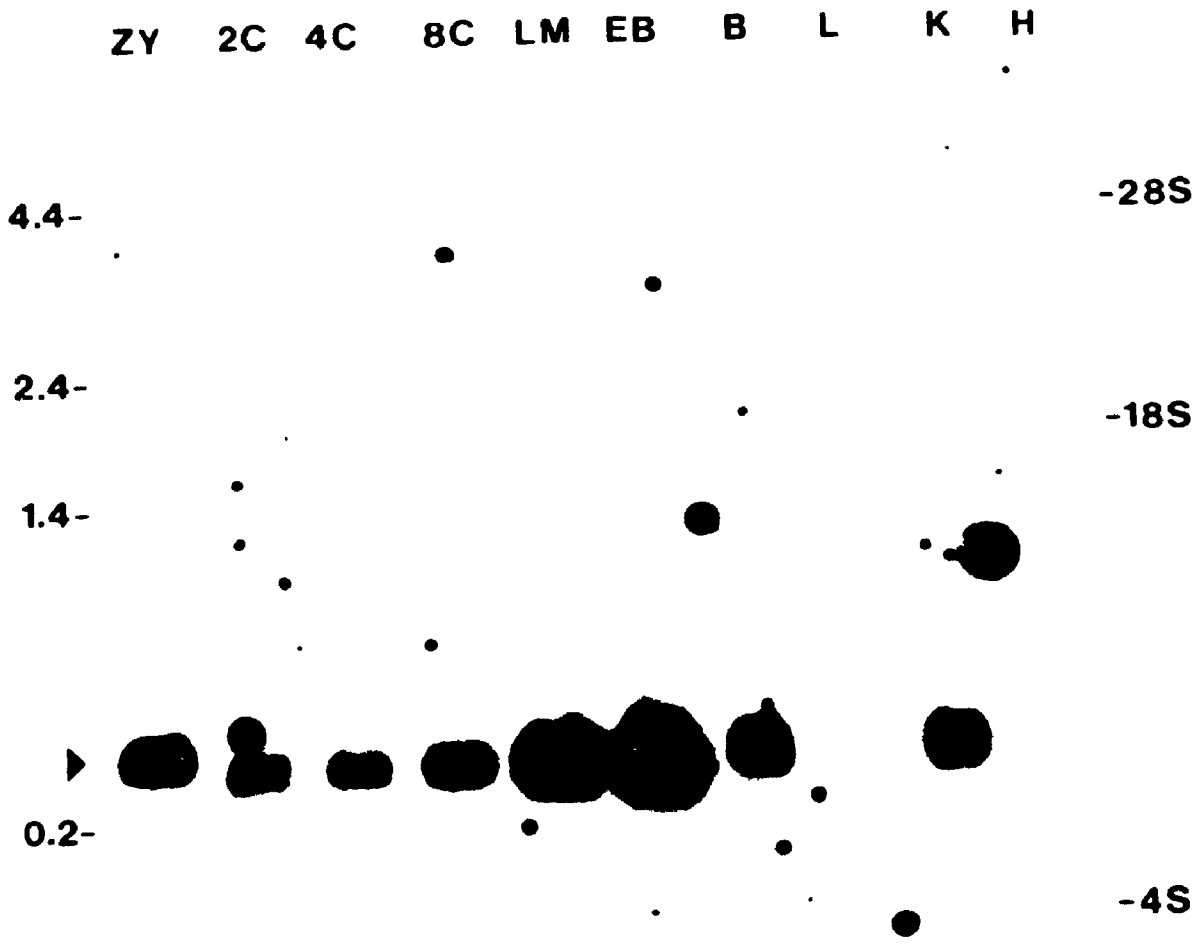
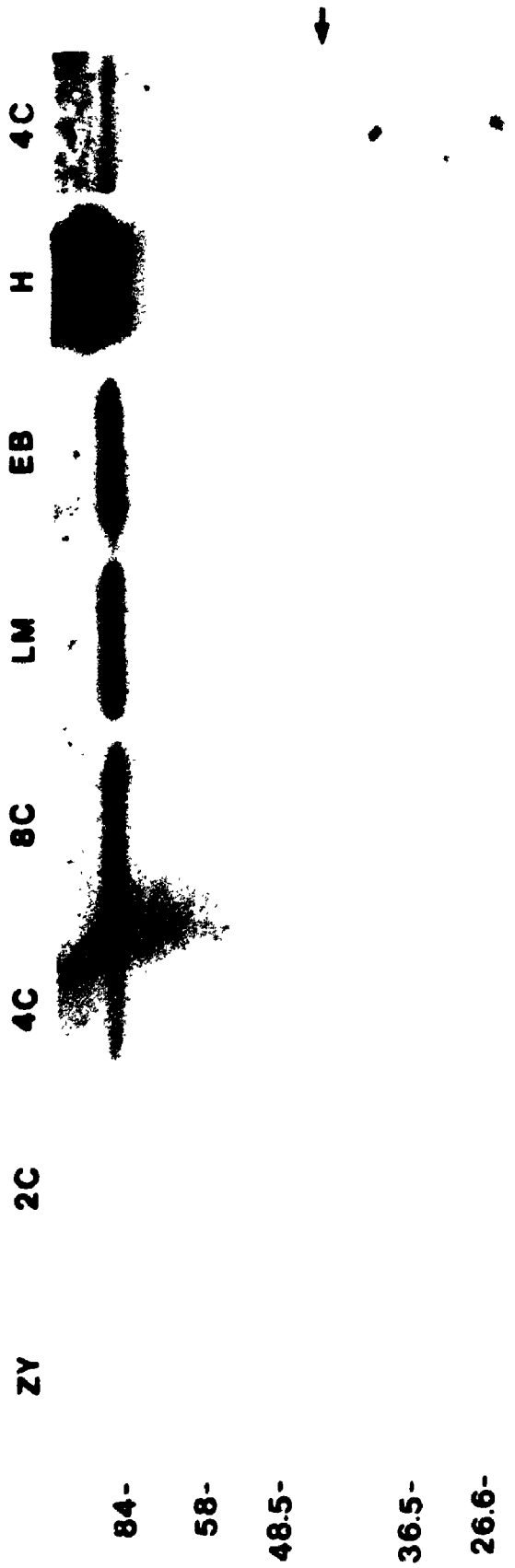

ZY
2C
4C
8C
LM
EB
B
L
K
H
4.4-
2.4-
1.4-
0.2-
-28S
-18S
-4S

Figure 2.6 Cx43 is present throughout preimplantion development. A Western blot containing zygote (ZY), 2-cell (2C), 4-cell (4C), 8-cell (8C), late morula (LM), and early blastocyst (EB) lysates (600 embryos each) and an intercalated disc-enriched membrane preparation from mouse heart (H) was probed with a Cx43 specific antiserum. A doublet with M_r of 43-48,000 was detected in all but the 4-cell stage while a single band was detected in the heart preparation. The panel on the extreme right shows a 4-cell lane from another Western blot probed with the same antiserum. It was included to show that faint Cx43 bands (arrow) could be detected in some embryo batches at that stage. The M_r X 10^{-3} of marker proteins are indicated on the left.


ZY
2C
4C
8C
LM
EB
H
4C
84-
58-
48.5-
36.5-
26.6-

with the pre-immune serum. No bands other than the alkaline phosphatase band were detected (Fig. 2.7).

2.3.3 RT-PCR analysis of connexin gene expression in COCs

To address the hypothesis that the Cx32 detected in preimplantation embryos is an oogenetic product, the very sensitive technique of RT-PCR was utilized to assay for Cx32 transcripts in maturing oocytes. Preliminary experiments (Fig. 2.8) showed that the Cx32 specific primers could amplify a 387 bp segment from total ovarian RNA (lane 2) which upon digestion with Ava I was cut into 225 and 153 bp fragments diagnostic of Cx32 (lane 3). Purified Cx32 cDNA insert was used as a positive control. Amplification of this template also resulted in a 387 bp amplification product (lane 6) that was cut by Ava I to yield the 225 and 153 bp diagnostic restriction fragments (lane 5). To determine if the Cx32 mRNA present in total ovarian RNA was derived from the germ cell component of the ovary, total RNA was isolated from oocytes collected at three different time points relative to hormonal injections. All animals were PMSG primed. One group was collected 46-48 hours post PMSG with no hCG injection (ovarian oocytes), another group 2-6 hours post hCG (ovarian oocytes) and the third group 18 hrs post hCG (ovulated oocytes). The RNA was then subjected to RT-PCR using Cx32 sequence specific primers. Figure 2.9 shows a Southern blot from one such experiment. Only one band, the predicted 387 bp Cx32 band, was detected in any of the

Figure 2.7 The Cx43 pre-immune serum does not detect Cx43. A Western blot containing an aliquot of the last wash solution (lane 1), 850 early blastocysts (lane 2), and an intercalated disc-enriched membrane preparation from mouse heart (lane 3) was probed with the Cx43 pre-immune serum. Only the endogenous alkaline phosphatase band was detected in the blastocyst sample. The M_r X 10^{-3} of marker proteins are indicated on the left.

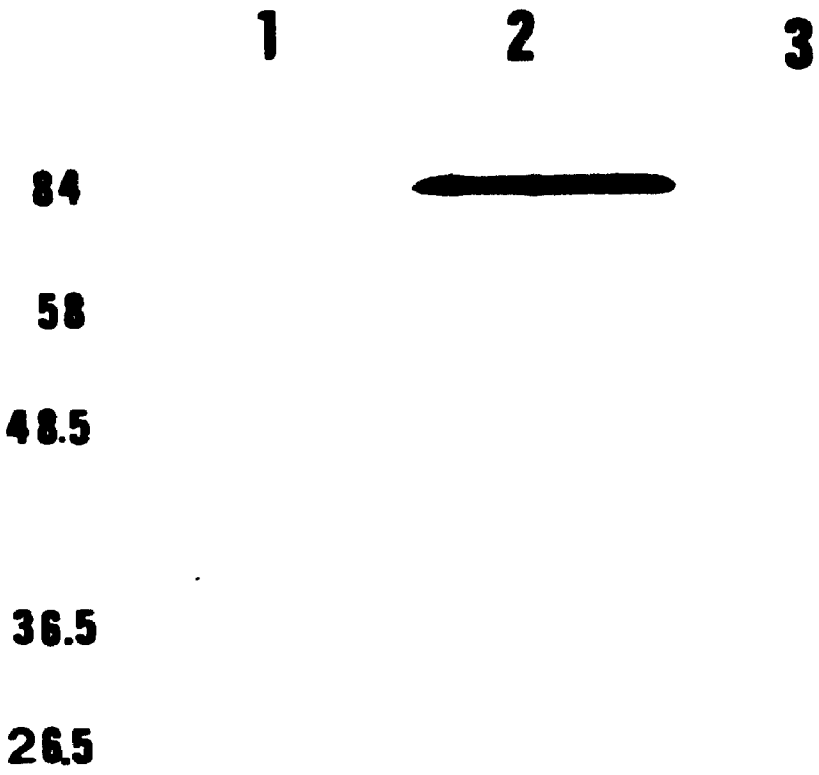

1
2
3
84
58
48.5
36.5
26.5

Figure 2.8 Cx32 mRNA is present in the ovary. Total RNA from ovary (lanes 2 and 3) was reverse transcribed and PCR amplified with Cx32 specific primers. Cx32 cDNA insert (lanes 5 and 6) served as a positive control and water (lane 4) served as a negative control. Two separate reactions were run on each type of RNA; the products of one of the reactions was loaded directly on the gel (lanes 2 and 6) whereas the other reaction was digested with Ava I prior to electrophoresis (lanes 3 and 5). A single 387 bp Cx32 band was detected in the undigested reaction products. Digestion of the reaction products with AvaI yields restriction fragments of 225 and 153 bp which are diagnostic for the 387 bp Cx32 amplification product. Lane 1 was loaded with molecular size markers (1 kb ladder; Gibco-BRL) the sizes of which (in bp) are noted on the left.

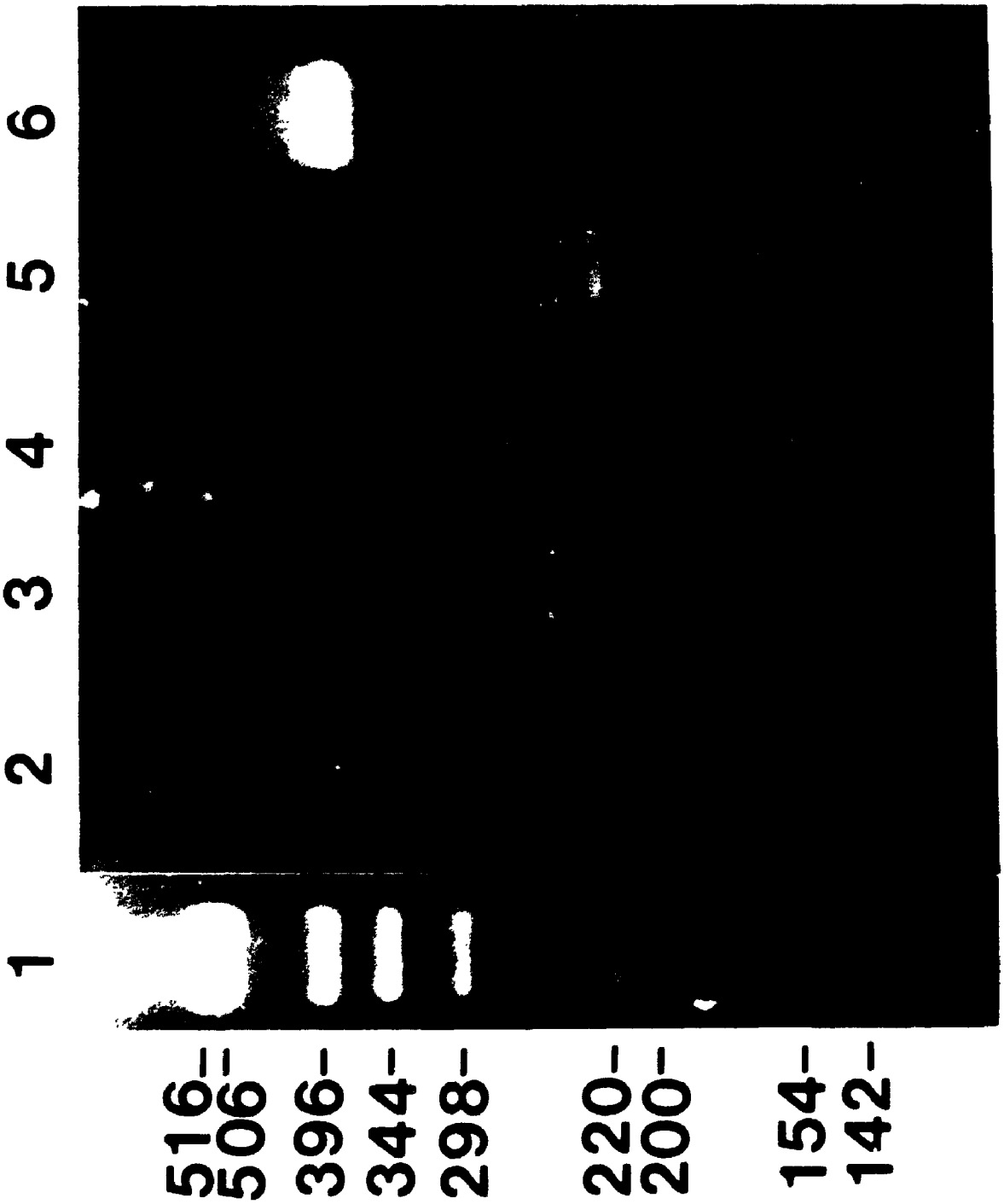
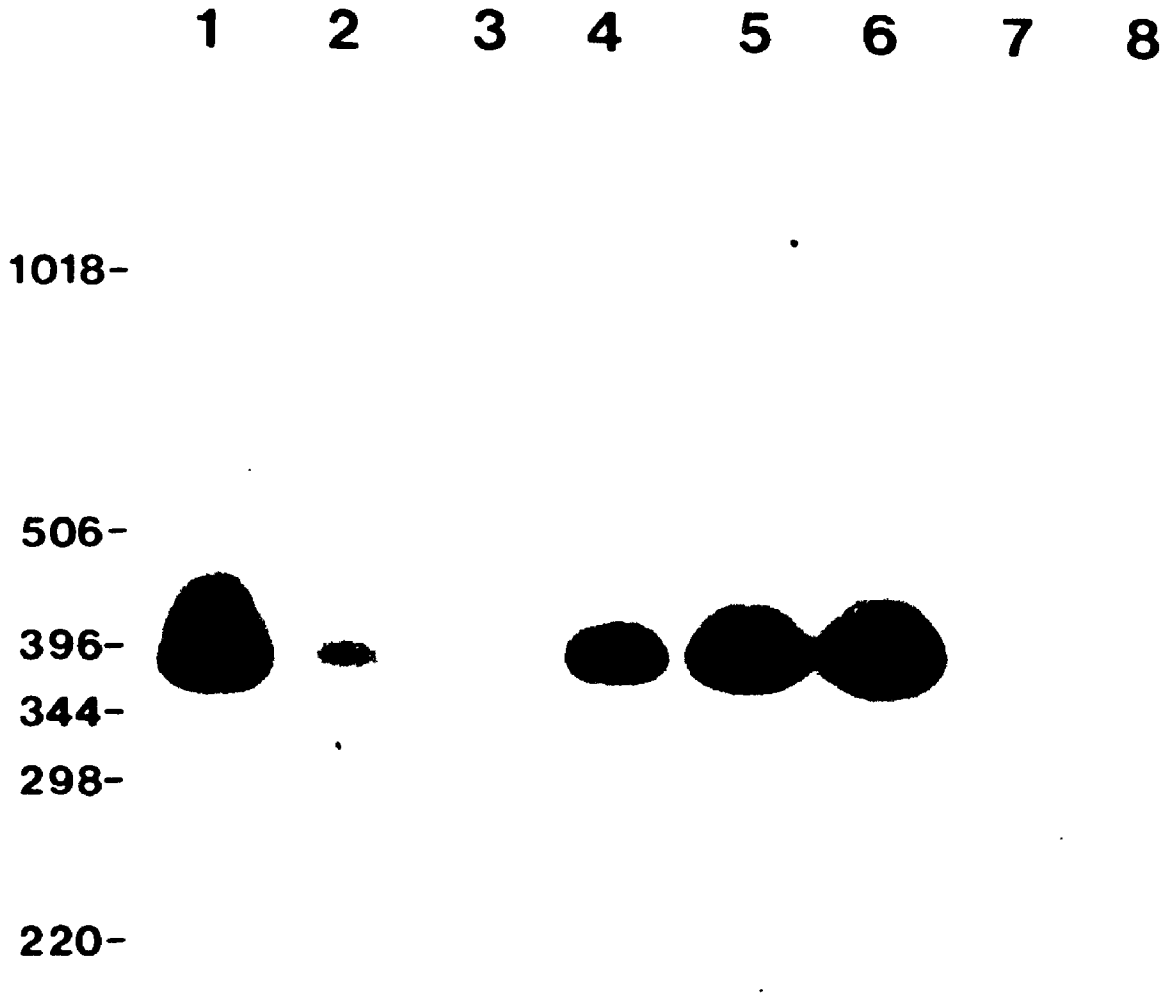

1
2
3
4
5
6
516–
506–
396–
344–
298–
220–
200–
154–
142–

Figure 2.9 Cx32 mRNA is present in oocytes and its levels are hormonally modulated. RNA from ovarian oocytes not exposed to hCG (lane 1), ovarian oocytes 2-6 hours following hCG injection (lane 2), ovulated oocytes 18 hours after hCG injection (lane 3), cumulus granulosa cells (lane 4), and ovarian somatic cells (lane 5) was reverse transcribed and PCR amplified with Cx32 specific primers. Liver RNA (lane 6) was used as a positive control, while E. coli rRNA (lane 7) and water (lane 8) served as negative controls. A portion of the reaction products (30%) was run on an agorose gel, Southern blotted and probed with a labeled Cx32 cDNA. A single 387 bp Cx32 band was detected in all of the samples that contained eukaryotic RNA and the level declined sharply in oocytes following exposure to hCG.


1 2 3 4 5 6 7 8
1018-
506-
396-
344-
298-
220-

samples that contained eukaryotic RNA as starting material, demonstrating the specificity of the amplification procedure. A high level of Cx32 transcripts was detected in ovarian oocytes that were not exposed to hCG (lane 1). The level of Cx32 transcripts declined sharply following hCG administration (lane 2) to reach a barely detectable level a few hours following ovulation (lane 3). The number of oocyte equivalents amplified was 10 for lanes 1 and 2, and 15 for lane 3; 30% of each reaction was loaded on the gel. A strong Cx32 signal was also detected in cumulus cells (lane 4) and ovarian somatic cells (lane 5). No quantitative inferences should be drawn from these last two samples since the starting amount of RNA was not known. Furthermore, the proportion of the various ovarian cell types in the ovarian somatic cell sample was not known. The majority of the cells in this sample were follicular in origin, but thecal and stromal cells were also present. These same results were obtained in seven separate PCR amplifications with cDNA from two separate RNA isolations. Liver RNA (lane 6), which has previously been shown to contain an abundance of Cx32 mRNA (Kumar and Gilula, 1986; Paul, 1986), was included as a positive control.

Because of the extreme sensitivity of RT-PCR, false positive signals have to be carefully controlled for. Each experiment therefore included two negative controls. One (lane 7) had E. coli rRNA as starting material. The amount of RNA in mouse oocytes is very small; to maximize the yield of oocyte RNA E. coli rRNA was employed as a carrier in the RNA isolation protocols. The rRNA control is a sample that was processed in parallel with the oocyte samples from the beginning of RNA isolation to PCR amplification, but with only E. coli rRNA added. The absence of an amplification

product in this lane therefore indicates that the reagents and samples did not become contaminated with DNA at any stage of the procedure. For the second negative control (lane 8), sterile distilled water was added in place of DNA template for the PCR amplification, thereby controlling for contamination of the PCR components.

Although the relative strength of the oocyte Cx32 signals varied somewhat between experiments, the general trend in the oocyte Cx32 signal described above was reproduced, as judged from ethidium bromide stained gels, in seven separate PCR amplifications with cDNA from two separate RNA isolations. To demonstrate the constancy of this trend further, densitometric scans were made of the autoradiogram from Fig. 2.9 and an autoradiogram from a separate set of PCR reactions and the relative changes in oocyte Cx32 signal intensity were compared. According to this analysis, in Fig. 2.9 there is an approximately four and 10 fold decrease in signal intensity between lanes 1 and 2, and lanes 1 and 3, respectively. Scans of the second autoradiogram revealed 10 and 28 fold decreases in the corresponding comparisons.

All of these samples were also amplified with the ß-actin primers. Only the 243 bp fragment was detected (same samples as lanes 1-6) indicating absence of genomic DNA contamination (Fig. 2.10).

To determine if Cx43 transcripts could be detected in ovarian oocytes, the same RNA samples that Cx32 was amplified from were used for RT-PCR with Cx43 specific primers. Figure 2.11 shows that a weak Cx43 signal of the expected size (294 bp) was detected in ovarian oocytes, both before (lane 1) and shortly after hCG injection (lane 2). No change in the level of Cx43 transcripts was detected in response to hCG

Figure 2.10 The Cx32 amplification product detected in oocytes is derived from cDNA, not genomic DNA. The cDNA preparations from ovarian oocytes 4-6 hours post-hCG injection (lane 1), ovarian somatic cells (lane 2), ovulated oocytes 18 hours after hCG injection (lane 3), and cumulus granulosa cells (lane 4) were subjected to PCR amplification with ß-actin specific primers. E. coli rRNA (lane 5) and water (lane 7) served as negative controls. Liver cDNA (lane 6) served as a positive control (co-amplified with the Cx32 specific primers, hence the presence of a 387 bp fragment). A portion of the reaction products (30%) was run on an agarose gel and photographed. No 330 bp fragment was detected, indicating the absence of genomic DNA contamination.

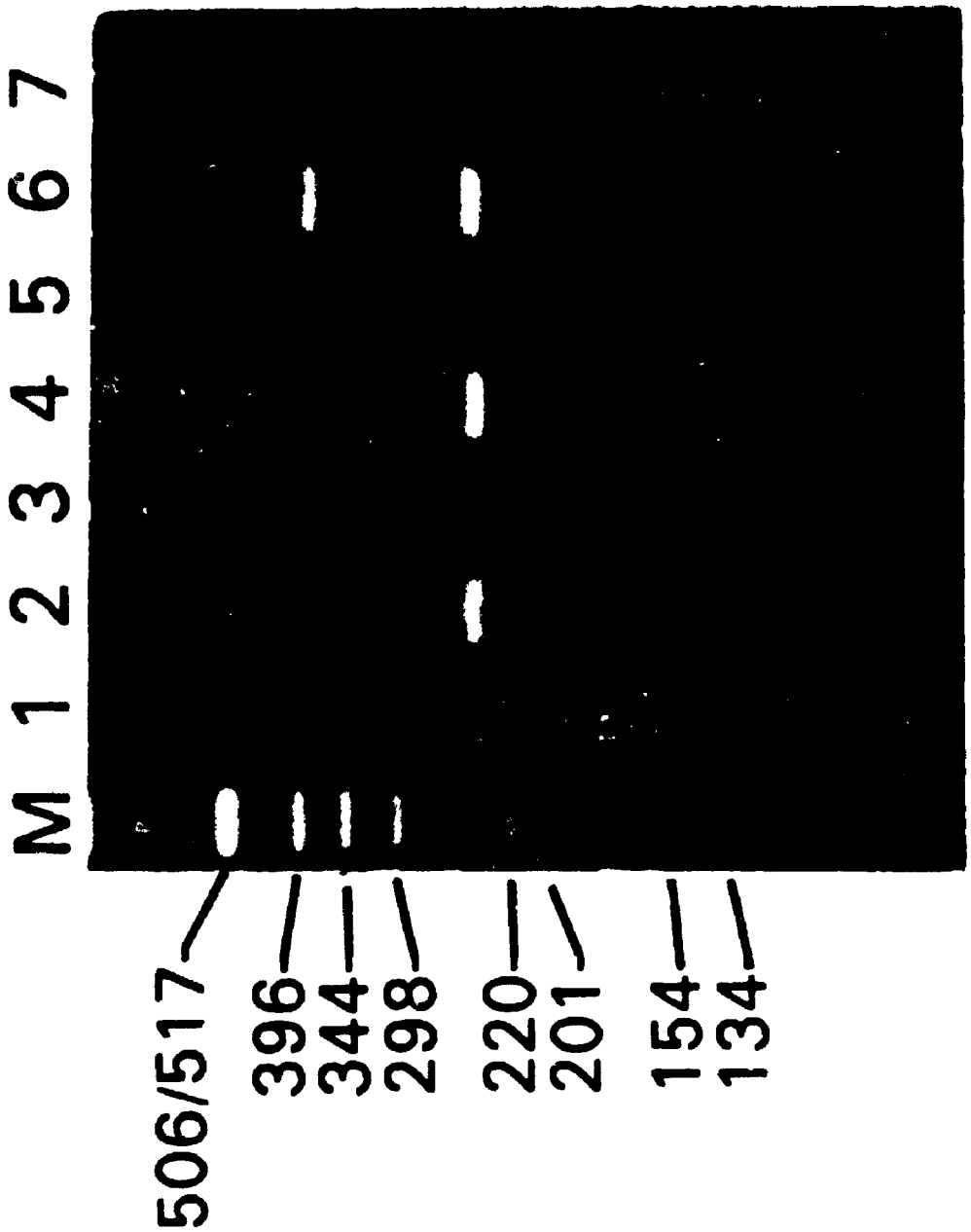
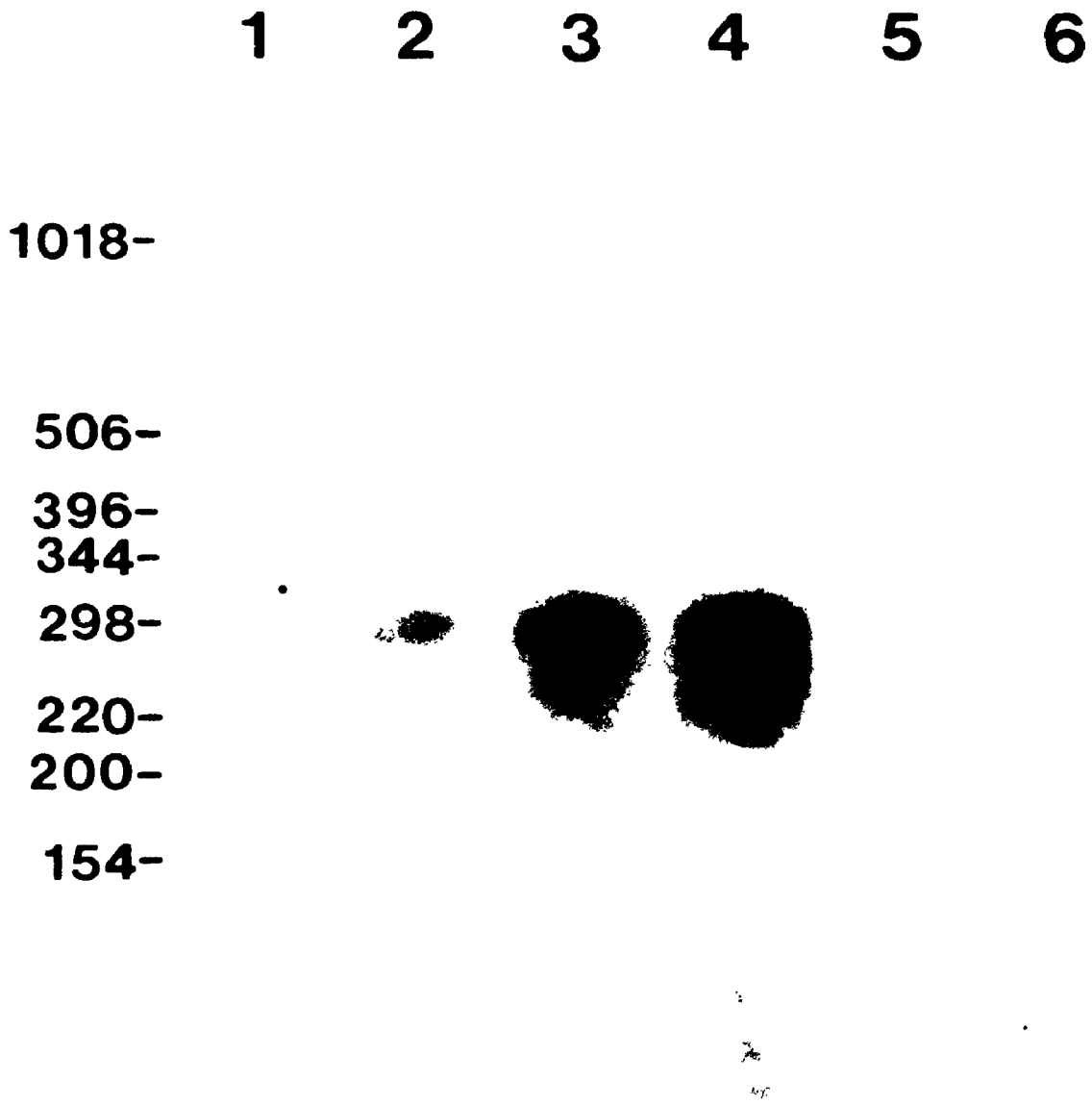

M 1 2 3 4 5 6 7
506/517
396
344
298
220
201
154
134

Figure 2.11 Cx43 mRNA is present in ovarian oocytes. A Southern blot of an experiment similar to the one shown in Fig. 1 except that Cx43 specific primers were used for PCR amplification and the blot was probed with labeled Cx43 cDNA. Low levels of Cx43 mRNA are present in ovarian oocytes; the amount remains relatively constant from before hCG exposure (lane 1) to 2-6 hours following hCG injection (lane 2). Ovarian somatic cell RNA (lane 3) and whole ovary RNA (lane 4) provided positive controls, whereas E. coli rRNA (lane 5) and water (lane 6) provided negative controls.


1
2
3
4
5
6
1018-
506-
396-
344-
298-
220-
200-
154-

administration. A strong Cx43 signal was detected in RNA from the ovarian somatic cell fraction (lane 3), composed mostly of cumulus cells, and total ovarian RNA (lane 4). No Cx43 amplification products were detected in the negative controls [E. coli rRNA (lane 5) and sterile distilled water (lane 6)].

2.4 Discussion

2.4.1 Connexin gene expression in preimplantation embryos

The results presented here demonstrate that a protein with antigenic and structural similarity to Cx43, a known gap junction protein from rat organs, accumulates during preimplantation development in the mouse. This protein is present along with another which, according to the same criteria, is a homolog of rat Cx32 (Barron et al., 1989). However, the Northern blot results taken together with the previous failure to detect any Cx32 mRNA in preimplantation embryos (Barron et al., 1989) strongly suggest that the Cx43 gene is the predominant, if not the only, known member of the rodent connexin gene family transcribed during this early period of embryogenesis. Essentially identical results, obtained with RT-PCR, have been published recently (Nishi et al., 1991). While it is most likely that it was the mouse equivalents of rat Cx43 and its mRNA that were detected, the possibility that the products of other related genes were detected instead cannot be totally discounted. However, this seems unlikely given the established specificity of the antiserum (Beyer et al., 1989), the stringency of the washes for the

Northern blots, and the fact that the sizes of the bands on both Northern and Western blots conformed closely with those found in rat organs.

That a doublet of polypeptides reacts with the Cx43 antiserum is intriguing, especially since only a single polypeptide was recognized in heart. One possible explanation of this finding is that Cx43 undergoes postranslational modification, such as proteolysis or phosphorylation, during preimplantation development. Multiple polypeptides reactive with Cx43 antibodies have been noted in other cell types, and in some cases this has been demonstrated to result from phosphorylation (Crow et al., 1990; Musil et al., 1990a,b; Musil and Goodenough, 1991). The variable ratio between upper and lower bands on the blots in the present study could be explained by the action of phosphatases released during the cell lysis procedure, or it might be developmentally significant. This subject will be addressed further in Chapter 4.

At present, it is difficult to interpret the detection of Cx43 in 1-cell zygotes and 2-cell embryos by immunoblotting. The Cx43 transcript in zygotes is clearly a contaminant contributed by the remaining cumulus cells, which are rich in Cx43 junctions (Beyer et al., 1989; Risek et al., 1990). The Cx43 gene is also transcribed during oogenesis, but its transcripts must decay by the time of ovulation (see section 2.4.2). The protein itself, however, may be a product of the oocytes that persists beyond fertilization. On the other hand, even carefully denuded oocytes or zygotes might be expected to retain Cx43 of cumulus cell origin since the gap junctions which couple the oocyte to the cumulus prior to ovulation are located on the tips of cumulus cell projections which penetrate through the zona and into the oocyte (Anderson and Albertini,

1976; Gilula et al., 1978). It is not known if these junctions are retained by the oocyte or withdrawn during cumulus expansion (Larsen and Wert, 1988). Immunocytochemistry at the electron microscope level will be required to resolve this uncertainty and to identify the connexins that contribute to the oocyte-cumulus gap junctions.

The gradual increase in Cx43 and its mRNA from the 2-cell stage onwards is consistent with the increase in both size and number of gap junction plaques between the 8-cell and morula stages (Magnuson et al., 1977), and with data from transcriptional and translational inhibition experiments, which showed that in the 4-cell stage there is already a sufficient stockpile of gap junctional precursors to establish coupling in the 8-cell stage (McLachlin et al., 1983; McLachlin and Kidder, 1986). The developmental profile of Cx43 mRNA indicates that transcription of the connexin43 gene is initiated as part of the general activation of the zygotic genome which takes place in the 2-cell stage (reviewed by Schultz, 1986). Moreover, the profile of accumulation of this message is similar to that of actin, histone, and Na^+,K^+-ATPase α-subunit mRNAs as well as most of the unidentified transcripts which first appear following genomic activation (Giebelhaus et al., 1983; Watson et al., 1990; Taylor and Pikó, 1987). The fact that Cx43 transcripts were not detected in 2-cell embryos in the present study may simply mean that the abundance of the message had not yet reached the detection limit of the Northern blot assay. In fact, Cx43 transcripts have now been detected in 2-cell embryos using RT-PCR (Kidder, 1993). Such low abundance could be due to a low transcription rate or to a high turnover rate for this message.

Polypeptides bearing the epitope recognized by the antiserum used for the Western blots in the present study, become incorporated into plaque-like foci in opposed plasma membrane regions during compaction, indicating that the immunoreactive material is a gap junction component (Valdimarsson et al., 1991). Prior to this, cloud-like foci of Cx43 immunoreactivity are seen in the cytoplasm; some of these persist after gap junction assembly has begun. That this staining pattern represents the true distribution of Cx43 is further supported by the fact that two other antisera, recognizing different epitopes of Cx43, produce essentially the same staining pattern (Nishi et al., 1991; De Sousa et al., 1993).

Do both Cx32 and Cx43 contribute to the establishment of gap junctional coupling at the time of compaction? This is certainly a possibility, since co-localization of two different connexins in the same gap junction plaque has been demonstrated in hepatocytes (Nicholson et al., 1987). These, and other results (Nishi et al., 1991; Valdimarsson et al., 1991; De Sousa et al., 1993), make it very likely that Cx43 plays a role in gap junction assembly, and two other recent reports have been interpreted as demonstrating a functional role for Cx32 as well.

First, Lee et al. (1987) injected affinity-purified polyclonal antibodies, raised against Cx32 from rat liver, into 8-cell compacted mouse blastomeres. The antibodies caused the injected blastomeres to uncouple from their neighbors and eventually to decompact. The same antibodies stained the surface membranes of morula-stage embryos in a punctate pattern. The most straightforward explanation for these results is that the antibodies were binding to Cx32 localized in functional gap junctions. On the other

hand, the antibodies used by Lee et al. (1987) are known to recognize at least one conformational epitope that may be conserved among the connexins (Milks et al., 1988), and it has therefore been argued that the disruption caused by these antibodies is due to their binding to Cx43 gap junctions (Gilula et al., 1992). However, an examination of their reactivity towards gap junctions from Cx43-containing organs such as heart has not been published and, furthermore, on immunoblots these antibodies react with a M_r 54,000 polypeptide in embryo lysates (Lee et al., 1987). This is consistent with the antibodies recognizing the dimer of Cx32 (Green et al., 1988) but not Cx43.

In the second report, Bevilacqua et al. (1989) described microinjection experiments with Cx32 antisense RNA which gave results very similar to those of Lee et al. (1987), i.e. the antisense RNA caused uncoupling and decompaction. The apparent absence of Cx32 mRNA in preimplantation embryos makes it likely, however, that the antisense RNA in that experiment was cross-hybridizing with Cx43 message. While the mechanisms involved in antisense RNA inhibition are not well understood, in many respects the parameters of nucleic acid hybridization in solution seem to apply; specificity depends on the prevailing stringency conditions as well as the degree of sequence complementarity (Harland and Weintraub, 1985; Izant and Weintraub, 1985; Freier et al., 1992). The extensive sequence similarity between Cx32 and Cx43 mRNA makes the interpretation presented above plausible (Paul, 1986; Beyer et al., 1987). It seems then that the involvement of Cx32 in the establishment of coupling during compaction has not yet been conclusively demonstrated, and seems unlikely in light of the fact that immunofluorescence studies have failed to localize Cx32 in structures resembling gap

junction plaques in preimplantation embryos (Nishi et al., 1991; Valdimarsson et al., 1993).

2.4.2 Connexin gene expression in COCs

The results reported here demonstrate that mRNA for both Cx32 and Cx43 can be detected in ovarian oocytes, and that the abundance of Cx32 transcripts in oocytes is hormonally modulated. The results showing the presence of Cx32 transcripts in ovarian oocytes directly support the hypothesis that the Cx32 found in preimplantation embryos has an oogenetic origin. There is, however, an apparent disagreement between these results and studies reporting the absence of Cx32 transcripts in ovulated oocytes (Nishi et al., 1991) and whole ovary (Risek et al., 1990). The discrepancy between the present results and those of Risek et al. (1990) are almost certainly due to a difference in sensitivity of the methods used; the RT-PCR method employed in the present study is much more sensitive than the Northern blotting used by Risek et al. (1990). The reason Nishi et al. (1991) failed to detect Cx32 transcripts in ovulated oocytes is not clear since they also used RT-PCR, but it may stem from the age of the oocytes used. Compared to ovarian oocytes, the results presented here show only a very weak and declining signal in ovulated oocytes at 18 hours post hCG. Nishi et al. (1991), in contrast, assayed their ovulated oocytes at 22 hours post hCG. It is possible that the abundance of Cx32 transcripts had declined to below the threshold of detection by this stage. This may have been further accentuated by the use of different mouse strains in the two studies.

A second finding of interest is the hormonal control of Cx32 mRNA levels in maturing oocytes. Compared to non-hCG stimulated oocytes, the level of Cx32 transcripts was consistently much lower in hCG exposed oocytes, even as early as 2-6 hours following hCG injection. These results suggest that it is the ovulatory surge in lutenizing hormone that triggers downregulation of Cx32 transcripts. This is consistent witn experiments showing that hCG causes a dramatic reduction in coupling and loss of gap junctions within the COC, as well as meiotic resumption and ovulation (Larsen and Wert, 1988). A similar decline in oocyte c-mos transcripts occurs in response to the ovulatory signal (Mutter et al., 1988), suggesting a general mechanism whereby the ovulatory signal triggers downregulation of maternal mRNAs. From these results it is not possible to say whether the downregulation in Cx32 mRNA levels occurs as a consequence of a shortened half life for the message or because of reduced transcription rates. The latter possibility is made plausible by the fact that two cAMP response element-like sequences reside in the promoter region of the Cx32 gene (Miller et al., 1988) and that the oocytes' source of cAMP (mural granulosa cells)(Eppig, 1989) becomes attenuated by the ovulatory signal due to cumulus cell gap junction degradation (Dekel et al., 1981; Larsen et al., 1986, 1987; Wert and Larsen, 1990).

The results showing low levels of Cx43 transcripts in ovarian oocytes, combined with the Northern blot results showing the absence of Cx43 transcripts in ovulated oocytes, as well as published results to the same effect (Nishi et al., 1991) suggest that oocyte Cx43 mRNA is hormonally modulated as well. This is supported by a report showing that Cx43 mRNA levels fluctuate in a hormonally dependent manner in the

ovary during pregnancy (Risek et al., 1990) and by numerous studies showing loss of gap junctions in the COC in response to the ovulatory stimulus (Wert and Larsen, 1990; for reviews see Larsen and Wert, 1988; and Gilula et al., 1992). In the previous section (2.4.1) two explanations were offered as to the possible origin for the Cx43 detected in 1-cell zygotes by immunoblotting. The protein could be of oogenetic origin, or it might be of cumulus cell origin inherited from cumulus-oocyte gap junctions that the oocyte retained following cumulus expansion. The detection of Cx43 transcripts in ovarian oocytes reported here is consistent with the first explanation, although is does not exclude the second one.

The Northern blots (section 2.3.1) show that cumulus granulosa cells have a large store of Cx43 mRNA. This has been confirmed by RT-PCR (Nishi et al., 1991) and immunofluorescence studies indicate that these cells are rich in Cx43 containing gap junctions (Beyer et al., 1989; Risek et al., 1990; Valdimarsson et al., 1993). The present demonstration of Cx32 mRNA in cumulus granulosa cells and the immunofluorescent localization of Cx32 in cumulus-cumulus gap junctions (Valdimarsson et al., 1993) indicates that these cells have the potential of establishing complex modes of intercellular communication. Since the oocyte expresses both Cx32 and Cx43 as well, the possibility exists that the functional coupling of the entire COC (Eppig, 1991) is accomplished via at least three types of junctions: Cx32-Cx32, Cx32-Cx43, and Cx43-Cx43. This is assuming homomeric, heterotypic channels can form in this system; if heteromeric connexons can form the number of possible channel types increases considerably.

2.4.3 Conclusions

Based on the foregoing discussion on connexin gene expression in early mouse development, the following scenario can be constructed. Cx32 and Cx43 are expressed by maturing oocytes both at the mRNA and protein level. Both transcripts decline sharply during ovulation and become undetectable by the late 1-cell stage. The oogenetic Cx32 persists throughout preimplantation development but is not augmented by zygotic gene expression and probably does not contribute to gap junctional communication in preimplantation embryos. The oogenetic Cx43 on the other hand declines substantially by the 2-cell stage when the Cx43 gene is reactivated. This is followed by a gradual increase in Cx43 mRNA and its protein product and it is probably Cx43 that is the major, if not the exclusive, contributor to the de novo assembly of gap junctions at compaction. Several questions remain unanswered. For example, is the oogenetic Cx32 and Cx43 inherited by the early embryo derived from cumulus-oocyte junctions that were internalized by the oocyte or does it derive from stores of unassembled connexins stored by the oocyte ? Where is the oogenetic Cx32 in the embryo located, in what form is it (i.e. post-translational modifications, assembly status, etc.), and how is its unusually long half-life effected ?

Recent developments suggest that the hypothesis that Cx43 is the only contributor to gap junctional communication in the preimplantation embryo may have to be modified. Preliminary screening of blastocyst RNA with RT-PCR suggests that Cx31.1, Cx37, and Cx40 transcripts are also present in the embryo (Dr. G.M. Kidder, personal

communication). Furthermore, preliminary immunofluorescence experiments indicate that blastocysts contain Cx40. It was not clear, however, if this protein is localized in gap junction plaques (P. De Sousa, personal communication). Further study is required to determine the significance of these results.

CHAPTER 3

ANTISENSE OLIGODEOXYRIBONUCLEOTIDES AS INHIBITORS OF CX43 GENE EXPRESSION IN PREIMPLANTATION MOUSE EMBRYOS

3.1 Introduction

The results presented in CHAPTER 2, along with other evidence (Nishi et al., 1991; Valdimarsson et al., 1991; De Sousa et al., 1993), led to the conclusion that the Cx43 gene is the main, if not the only, connexin gene contributing to gap junctional communication in the preimplantation mouse embryo. This conclusion was based solely on descriptive data. The purpose of the experiments described in CHAPTER 3 was to explore the function of the Cx43 expressed in preimplantation mouse embryos.

Until very recently, questions regarding the biological role of a cloned gene were difficult to address meaningfully, especially in vertebrates where mutational analysis is generally not practicable due to long generation times and large genome sizes. However, in the last 5-10 years a technology has been developing which promises to make these types of questions amenable to experimentation. The technology, as it is most frequently practiced, is based on the premise that the expression of any gene can be reduced or abolished by introducing into the cell a nucleotide sequence that is complementary to the nucleotide sequence of the mRNA transcribed from the gene of interest. Clearly, the successful application of this technology requires knowledge of at least a portion of the nucleotide sequence of the target mRNA.

The current popularity of this technology stems from the exquisite specificity it promises. It is thought that by appropriately selecting the complementary sequence, only the target mRNA will be affected, leaving all the other thousands of mRNAs in the cell unaffected. Theoretical calculations indicate that a sequence 13 nucleotides long (13-mer) is sufficient to define a unique sequence (Woolf, 1992; Woolf et al., 1992). This is based on the assumption that there are 10,000 different transcripts in the cell and that the average length of the transcripts is 2,000 nucleotides (sequence complexity = 20×10^6). A random 13 nucleotide sequence would have sequence complexity in excess of this ($4^{13} \approx 67 \times 10^6$). This experimental approach has become known as antisense technology or antisense strategy, where the nucleotide sequence of the target mRNA is the sense sequence and the complementary nucleotide sequence is the antisense sequence.

Several approaches can be used in antisense experiments. Both RNA and synthetic oligodeoxynucleotides (ODN) antisense sequences have been used effectively. Antisense RNA can be produced in vivo by transfecting cells with plasmids containing a cDNA for the target mRNA in an inverted orientation relative to the promoter (Izant and Weintraub, 1985; Ao et al., 1988). Alternatively, the antisense RNA can be transcribed in vitro from similarly constructed vectors and microinjected into cells (Harland and Weintraub, 1985; Bevilacqua et al., 1988, 1989; Strickland et al., 1988). ODNs can also be delivered by microinjection (O'Keefe et al., 1989; Paules et al., 1989; Dagle et al., 1990), but more conveniently, in the case of cultured cells and embryos, they can be administered by simply adding them to the culture medium (Loke et al., 1989; Murphy et al., 1992; Rappolee et al., 1992).

Antisense inhibition of gene expression is in all cases dependent on the specific hybridization, by Watson-Crick base pairing, of the antisense reagent to its target mRNA (Akhtar and Juliano, 1992; Freier et al., 1992). In general, the mechanisms of antisense inhibition are not well understood but several different possibilities have been suggested and it is probable that circumstances, such as method of delivery and nature of the antisense reagent, will influence the mode of action. For instance, processing of the pre-mRNA and transport of the processed message from the nucleus to the cytoplasm can both be inhibited by antisense reagents (Strickland et al., 1988; Cirullo et al., 1992) but both require the presence of the antisense reagent within the nucleus. This requirement can be met by ODNs and by cells stably transfected with antisense vectors. Antisense inhibition can also arise because the hybridized mRNA is sterically hindered from being translated (Cirullo et al., 1992; Liebhaber et al., 1992); this mechanism is probably common to all antisense reagents. Finally, stability of the mRNA target can be affected by antisense reagents. For example, the mRNA can become the target of RNase H, a nuclease which cleaves the RNA component of an RNA-DNA hybrid (Dagle et al., 1990; Izant, 1992).

Although the majority of the published antisense studies have utilized cultured cell systems, a number of reports have also appeared on the application of antisense technology to developmentally relevant questions. First, microinjection of krüppel antisense RNA into Drosophila wild type embryos has been found to cause patterning defects that are identical to the krüppel mutation (Rosenberg et al., 1985). Second, one-cell Xenopus embryos microinjected with antisense ODNs for the α-subunit of the

mitochondrial ATPase (An2) are known to exhibit delayed gastrulation and become developmentally arrested before neurulation (Dagle et al., 1990). Third, by microinjecting a series of short antisense RNAs, complementary to different regions of the tissue plasminogen activator transcript, Strickland et al. (1988) demonstrated a critical role for the 3' noncoding region in the translational activation of this mRNA during oocyte meiotic maturation in the mouse. Finally, microinjection of c-mos antisense ODNs into germinal vesicle stage mouse oocytes has been shown to block meiotic maturation (O'Keefe et al., 1989; Paules et al., 1989) demonstrating a role for the c-mos protooncogene in this process.

The feasibility of using antisense strategy on mouse embryos was first demonstrated in two studies published in 1988. Ao et al. (1988) were able to abolish the expression of an exogenously supplied gene construct by simultaneously injecting the embryos with the antisense gene construct. Bevilacqua et al. (1988) were able to reduce the levels of ß-glucuronidase by 75% in blastocysts by microinjecting all four blastomeres of 4-cell embryos with antisense ß-glucuronidase RNA. This reduction was not accompanied by an apparent developmental defect. This latter group subsequently reported that the injection of antisense Cx32 RNA led to reduced dye coupling and compaction failure (Bevilacqua et al., 1989) [this study has been discussed in detail in CHAPTER 2, section 2.4.1]. Recently, Rappolee et al. (1992) have demonstrated that insulin-like growth factor II (IGF-II) antisense ODNs added to the culture medium caused a reduction in the expression of this gene and a reduction in developmental rates.

This chapter describes ODN antisense experiments aimed at answering the following two questions arising from CHAPTER 2: (1) Does the Cx43 present in preimplantation embryos contribute to functional gap junction channels and, if so, to what extent is coupling dependent on Cx43? (2) To what extent is preimplantation development dependent on Cx43 and what are the developmental consequences of reduced Cx43 levels?

3.2 Materials and Methods

3.2.1 Embryo collection and culture

Methods of embryo collection were as outlined in the MATERIALS AND METHODS section of CHAPTER 2, with the following exception. After the embryos were flushed from the reproductive tract they were passed through three drops of FM-I to remove reproductive tract debris and then placed in standard egg culture medium (SECM; Spindle, 1980) in a Falcon 3037 organ tissue culture dish (Becton Dickinson Labware, Lincoln Park, NJ). All embryo culturing was carried out in SECM in incubators set at 37°C, 5% CO_2 in air, and 100% relative humidity. The culture medium was pre-equilibrated in the incubators for at least 2 hours prior to embryo addition.

3.2.2 ODNs

All ODNs were obtained from Dr. G. Mackie, Department of Biochemistry, University of Western Ontario. ODNs were purified by ethanol precipitation at -20°C with ammonium acetate added to 4 M. The ODNs were pelleted by centrifugation at 10,000 X g for 20 minutes at 4°C. The pellet was washed once in 70% ethanol, air dried and dissolved in double distilled, sterile water. The concentration of ODNs was determined spectrophotometrically (Beckman DU-8 Spectrophotometer) by measuring the absorbance at 260 nm and assuming 1 A_{260} = 33μg/ml (Sambrook et al., 1989). The concentration of ODNs administered varied between experiments and will be reported in the RESULTS section. The ODNs were coded blind before use and the identity was not revealed to the experimenter until the experiment was completed. For microinjection experiments the ODNs were mixed with a 10% stock solution of rhodamine dextran (M_r 10,000; Molecular Probes, Eugene, OR) to give a final rhodamine dextran concentration of 2.5%.

Three different Cx43 specific antisense ODNs were used in this study. The ODN was 18 bases long in each case and the complementary sense sequence served as a negative control. The first antisense ODN (antisense 1; see sequence below) was based on the rat Cx43 cDNA sequence (Beyer et al., 1987) and was targeted against the extreme 5' end of the coding sequence including the ATG start codon (nucleotides 1-18). The second antisense ODN (antisense 2; see sequence below) was based on the mouse sequence (Beyer and Steinberg, 1991; Hennemann et al., 1992b) and was targeted

against a region 3' to antisense 1 (nucleotides 28-45). Both antisense 1 and 2 and the corresponding sense ODNs were synthesized as unmodified ODNs. The sequence of the third antisense ODN (antisense 3) was identical to antisense 2 but this ODN and its corresponding sense ODN were synthesized as phosphorothioate ODNs (S-ODNs). In these ODNs each phosphodiester bond has been modified by the replacement of one of the oxygen atoms by a sulfur atom. This modification has been reported to increase the stability of ODNs (Akhtar et al., 1992; Cohen, 1992).

ODN sequences:

Antisense ODN 1: 3'-TACCCACTGACCTCACGG-5'

Sense ODN 1: 5'-ATGGGTGACTGGAGTGCC-3'

Antisense ODN 2: 3'-GACGACCTGTTCCAGGTT-5'

Sense ODN 2: 5'-CTGCTGGACAAGGTCCTT-3'

3.2.3 ODN microinjections

Pipettes were pulled on a Flaming-Brown horizontal micropipette puller (Model P-80/PC; Sutter Instruments, San Rafael, CA) from borosilicate glass tubing. The injection pipettes were pulled from glass tubing containing an internal filament (GC100TF-15; Clark Biomedical Instruments, Reading, UK) and were filled by back-loading by capillary action. Delivery of material out of the injection pipette was controlled by an Eppendorf pneumatic microinjector (Model 4242) that was connected

to the back of the injection pipette via Tygon tubing. Holding pipettes were pulled from glass tubing without an internal filament (GC100-15; Clark Biomedical Instruments) and were shaped on a Narishige MF-79 microforge (Carsen Medical Scientific, Markham, Ont.) so that the outer diameter was 60-80 μm and the inner diameter 20-40 μm. The holding pipette was controlled by a Gilmont syringe connected to the back of the holding pipette via paraffin oil-filled Tygon tubing. Microinjections were carried out on a Zeiss ICM 405 inverted microscope fitted with phase-epifluorescence optics and Leitz micromanipulators

Embryos were injected in FM-I in a Falcon 1006 petri dish (50 X 9 mm; Becton Dickinson) placed on the microscope stage. The dish was modified by cutting a round piece out of the bottom and gluing a round glass coverslip in its place. Only 4-cell embryos were injected. However, the actual age of the embryos at the time of injection (in hours post-hCG) varied between experiments and will be noted in the RESULTS. Embryos were manipulated into place under visible light and then injected in ultraviolet (UV) light with a rhodamine filter set placed in the light path. This way the progress of the injection could be monitored visually and estimates made of the volume of injected material (see Appendix 1 for calculations). Approximately 113 femtoliters (fl) was injected into one blastomere of each 4-cell embryo. Following injection, the embryos were placed individually in 15 μl drops of SECM under paraffin oil in petri dishes and cultured until uninjected controls had formed compacted, 8-16 cell morulae. At this time the gross morphological features of the embryos were scored as was the presence and location of rhodamine fluorescence. Only embryos containing a rhodamine signal were

included in further analyses. In some cases this was followed by tests for dye coupling or the embryos were frozen for later RT-PCR analysis. The procedure used to freeze embryos was the same as that described in CHAPTER 2.

3.2.4 Embryo culture in the presence of ODNs

Culturing in the presence of ODNs was carried out in SECM in humidified chambers without an oil overlay. The chambers were constructed by placing the culture drops (30 μl) in 50 X 9 mm petri dishes which in turn were placed inside larger 85 X 14 mm petri dishes along with filter paper wetted with sterile distilled water. Only S-ODNs were used for these experiments. Four-cell embryos (beginning at 56-61 hours post-hCG; about 30 embryos/drop) were cultured until control embryos, cultured in SECM without any ODNs, formed 8-16 cell morulae (78-81 hours post-hCG). At the end of the culture period the gross morphological features of the embryos were noted and in some cases this was followed by tests of dye coupling or the embryos were frozen for later RT-PCR analysis. The procedure for embryo freezing was the same as that described in CHAPTER 2.

3.2.5 Dye injections

To test the effects of the antisense ODNs on intercellular communication, embryos were microinjected with fluorescent dyes at a time when control embryos,

unexposed to ODNs, had become extensively dye coupled. Embryos that had been injected with ODNs were tested for dye coupling using the same equipment and procedure used for ODN injections. A 5% solution of Lucifer yellow CH (M_r 457; Polysciences Inc., Warrington, PA) in sterile distilled water was injected with the fluorescein filter set in the UV light path. When the injected blastomere had become intensively fluorescent the injection was terminated but the injection pipette was left in place. The fate of the injected dye was then followed by intermittent observations for up to a maximum of 30 minutes. An embryo was scored as being coupled if dye had spread beyond the injected blastomere and into at least two other blastomeres during the 30 minute observation period. The spread of Lucifer yellow in relation to the location of the rhodamine-containing blastomeres was noted.

Dye coupling in embryos cultured in the presence of ODNs was tested with 10 mM 6-carboxyfluorescein (M_r 376; Eastman Kodak, Rochester, NY) in distilled water. The dye was injected iontophoretically using a continous train of hyperpolarizing current pulses of approximately 10 nA (200 msecond duration, one pulse per second) until the injected blastomere became very bright. Observations were made, with the injection pipette in place, as described above. The holding pipettes were prepared as described above but the injection pipettes were pulled on a Narishige vertical micropipette puller from borosilicate glass tubing with an internal filament (Kwikfil; W-P Instruments, New Haven, CT). The injection pipettes were back-filled by capillary action, mounted onto a DeFonbrune pneumatic micromanipulator (Beaudouin, Paris) and connected by chloride silver wire holders to an electrometer (M707; W-P Instruments). The bathing medium

(FM-I) was grounded through a KCl/agar bridge. The holding pipette was mounted onto a DeFonbrune micromanipulator fitted with a pipette holder with a suction port (W-P Instruments), and connected with Tygon tubing to a 20 ml plastic syringe (Becton Dickinson). Injections and observations were performed on a Zeiss Axiovert 35 inverted microscope fitted with phase-epifluorescence optics and a fluorescein filter set.

3.2.6 RT-PCR and Southern blots

The procedures for RT-PCR and Southern blotting were as described in CHAPTER 2 with the following exceptions. To facilitate comparisons of Cx43 mRNA signal strength between samples, each PCR reaction was amplified simultaneously with ß-actin primers and Cx43 primers. All four primers were used at a concentration of 1 μM. The ß-actin primer set was the same one described in CHAPTER 2, whereas the Cx43 primer set used here was based on the mouse Cx43 sequence (Beyer and Steinberg, 1991; Hennemann et al., 1992b), and amplified a 332 bp segment corresponding to nucleotides 807-1138. The 5' Cx43 primer was 5'-TGGCTGCTCCTCACCAACGGC-3' and the 3' primer was 3'-CGTCTGGAGCCGGACTACTGG-5'. PCR amplification was carried out for 40 cycles. The Southern blots were probed simultaneously with the same Cx43 probe described previously and a full length chicken ß-actin cDNA probe (gift from Dr. B. Atkinson, Department of Zoology, University of Western Ontario).

3.3 Results

3.3.1 Effects of Cx43 antisense ODN microinjections on morphology and dye coupling

In an initial set of experiments late 4-cell embryos (61-69 hours post-hCG) were injected with sense and antisense ODN 1, at a concentration of 0.13-0.26 $\mu g/\mu l$. The effects of these injections were assessed at 80-85 hours post-hCG, i.e approximately 20 hours after the ODN injection. An embryo was scored as having an abnormal morphology if it contained decompacted or degenerating blastomeres, or if it appeared to contain fewer blastomeres than control embryos. Only embryos possessing rhodamine fluorescence were scored; since the embryos were injected late in the 4-cell stage when cytoplasmic bridges have closed (Kidder et al., 1988), this generally meant that about one quarter of the blastomeres in rhodamine-positive embryos contained rhodamine. Although a relatively low proportion of the embryos injected with the antisense ODN had normal morphology the proportion was similarly low for the embryos injected with the control, sense ODN (Table 3.1). To assess the possibility that the antisense ODN was having an effect on coupling without affecting gross morphology, embryos injected with the antisense ODN and showing normal morphology at 80-85 hours post-hCG were tested for dye coupling. Six out of six embryos tested were fully dye coupled (Table 3.1), i.e. the injected Lucifer yellow spread throughout the embryo within 30 minutes, in most cases within 10 minutes. The spread of Lucifer yellow was not impaired by blastomeres containing rhodamine; the tracer moved into and out of these blastomeres at apparently

Table 3.1
Effects on embryo morphology and dye coupling of microinjecting 0.13-0.26 μg/μl Cx43 antisense ODN 1. Injections were performed 61-69 hours post-hCG and observations made 80-85 hours post-hCG.

ODN injected	Normal morphology (%)	Abnormal morphology (%)	Dye coupling* (%)
Sense 1 (n = 31)	19 (61.3)	12 (38.7)	ND**
Antisense 1 (n = 48)	32 (66.7)	16 (33.3)	6/6 (100)

* Number dye coupled/number tested
** Not determined

the same rate as it moved into and out of non-rhodamine blastomeres. These results show that the injection of antisense ODN 1, at 0.13-0.25 $\mu g/\mu l$, did not cause a noticeable reduction in dye coupling, suggesting that Cx43 levels were not dramatically reduced by this antisense ODN when used as described here.

At the time this set of experiments was initiated, a mouse Cx43 cDNA sequence was unavailable. For this reason, the nucleotide sequence of antisense ODN 1 was complementary to the rat Cx43 cDNA sequence (Beyer et al., 1987). However, the mouse Cx43 cDNA sequence did become available shortly thereafter. In light of the lack of success in the first set of experiments, a second set of experiments was undertaken for which a new antisense ODN (antisense ODN 2), complementary to the mouse sequence, was constructed. In this set of experiments, early 4-cell embryos (51-58 hours post-hCG) were injected with antisense ODN 2 and sense ODN 1 at a concentration of 0.8 $\mu g/\mu l$. The effectiveness of these injections was assessed at 77-79 hours post-hCG, i.e. about 20-25 hours after the ODN injection. The fact that early 4-cell embryos, still possessing open cytoplasmic bridges (Kidder et al., 1988), were injected meant that in most embryos the rhodamine dextran spread to a second, sister blastomere. When rhodamine-positive embryos were scored, about half of the blastomeres therefore displayed rhodamine fluorescence.

In contrast to the first set of experiments, a considerably higher proportion of both sense and antisense ODN injected embryos had normal morphology in the second set of experiments (Table 3.2). However, approximately the same proportion of sense and antisense ODN injected embryos displayed normal morphology, suggesting that the

Table 3.2
Effects on embryo morphology and dye coupling of microinjecting 0.8 $\mu g/\mu l$ Cx43 antisense ODN 2. Injections were performed 51-58 hours post-hCG and observations were made 77-79 hours post-hCG.

ODN injected	Normal morphology (%)	Abnormal morphology (%)	Dye coupling* (%)
Sense 1 (n = 27)	25 (92.6)	2 (7.4)	6/8 (75.0)
Antisense 2 (n = 27)	23 (85.2)	4 (14.8)	4/5 (80.0)

* Number dye coupled/number tested

antisense ODN 2 was not grossly interfering with intercellular coupling. Figure 3.1 shows examples of sense (A,B) and antisense ODN (C,D) injected embryos; both are normal, compacted morulae, despite the presence of antisense ODN 2 in about half of the blastomeres of one of them (C,D).

When, in the second set of experiments, sense and antisense ODN injected embryos with normal morphology were tested for dye coupling, a high proportion of the embryos were extensively dye coupled, irrespective of which ODN had been injected (Table 3.2). Thus, despite the change in protocol, the results were essentially the same in both the first and second sets of experiments, i.e. no noticable reduction in dye coupling occurred, suggesting that Cx43 levels were not reduced substantially by either antisense ODN.

In a final set of microinjection experiments, the experimental protocol was essentially the same as in the second set of experiments, except that 1.5 μg/μl S-ODNs were used. The results were also essentially the same. Most of both sense and antisense ODN injected embryos had normal morphology and were fully dye coupled (Table 3.3). There was no apparent difference in these two parameters between the two embryo populations.

3.3.2 Effects on morphology and dye coupling of culturing embryos in the presence of Cx43 antisense ODNs

Embryos were cultured from the mid 4-cell stage (beginning at 56-61 hours post-

Figure 3.1 Microinjection of Cx43 antisense ODNs does not affect the gross morphology of embryos. Early 4-cell embryos were injected with either sense (A,B) or antisense (C,D) ODNs and cultured until uninjected controls were compacted morulae. Phase-contrast images are on the left and rhodamine fluorescence images of the corresponding embryos on the right. Based on rhodamine fluorescence (B,D), the injected ODNs are present in about half of the blastomeres, yet the antisense ODN injected embryo possesses normal morphology (C,D). Scale bar = 25 μm.

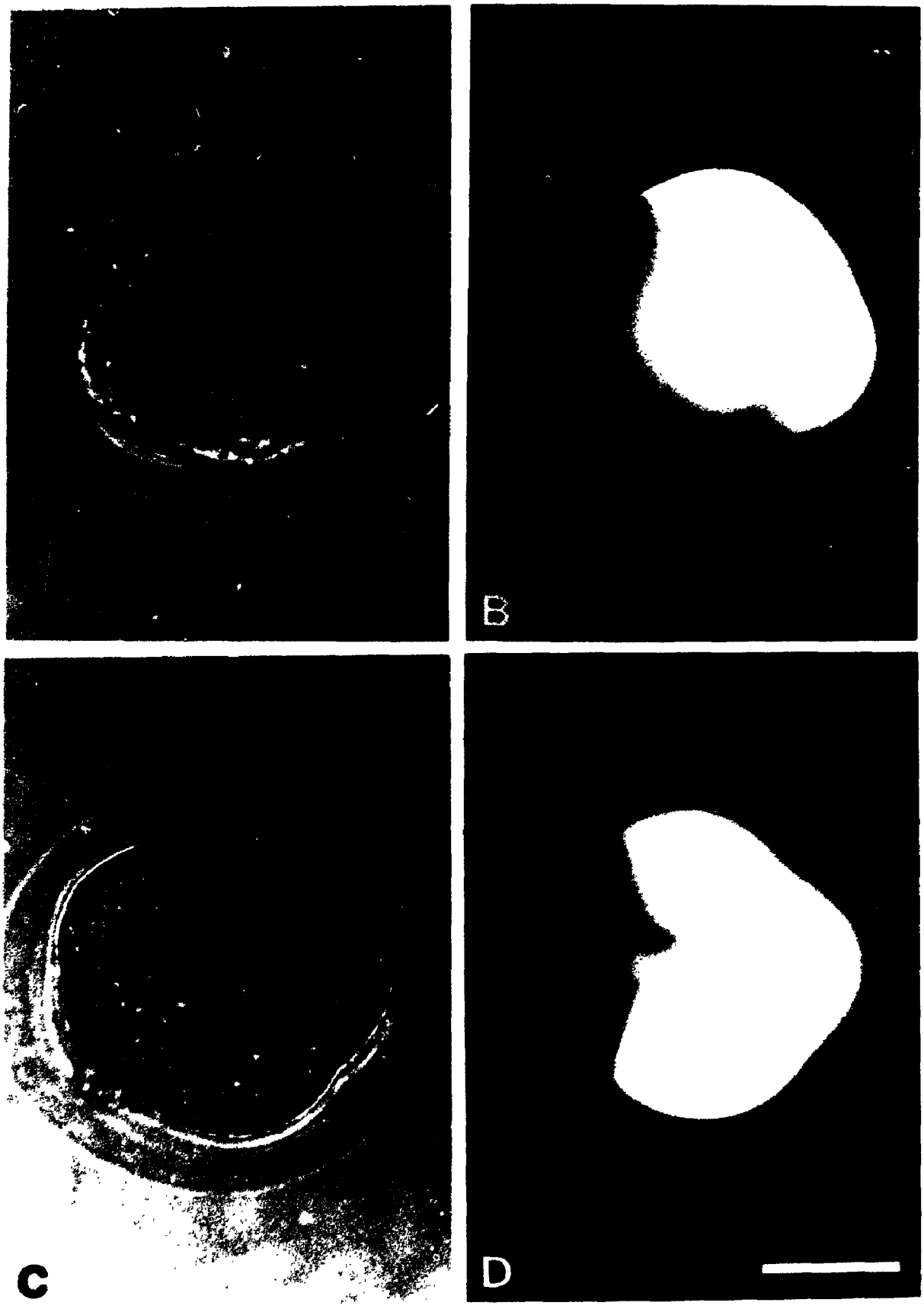

B
C
D

Table 3.3
Effects on embryo morphology and dye coupling of microinjecting 1.5 μg/μl Cx43 antisense S-ODN 3. Injections were performed 53-57 hours post-hCG and observations were made at 77 hours post-hCG.

ODN injected	Normal morphology (%)	Abnormal morphology (%)	Dye coupling* (%)
Sense 3 (n = 12)	11 (91.7)	1 (8.3)	5/7 (71.4)
Antisense 3 (n = 16)	15 (93.8)	1 (6.2)	4/5 (80.0)

* Number of embryos dye coupled/number tested

hCG) to the late morula stage (78-81 hours post-hCG) under one of three conditions: in SECM alone, in SECM + 150 μM sense S-ODN, or in SECM + 150 μM antisense S-ODN. A S-ODN concentration of 150 μM was chosen because preliminary experiments had shown that concentrations of 18, 36, and 72 μM were ineffective. A higher concentration was not used because S-ODNs are generally toxic at concentrations above 100-200 μM (Cohen, 1992). Approximately 30 embryos were allocated to each condition, in each of three separate experiments, such that about 90 embryos in total were assessed for each condition. The presence of the S-ODNs in the culture medium had no discernable effects on the gross morphology or developmental rate of the embryos; all three groups were morphologically identical at the end of the culture period, i.e. fully compacted late morulae. This point is demonstrated in Figure 3.2, which shows random samples of embryos cultured in the presence of sense S-ODN (A) and antisense S-ODN (B).

Dye coupling tests on embryos cultured in the presence of sense and antisense S-ODNs revealed that the majority of the embryos were fully and equally dye coupled (Table 3.4). This suggests that Cx43 levels were not substantially reduced by culturing embryos in 150 μM antisense S-ODN.

3.3.3 Effects of antisense ODNs on Cx43 mRNA levels

An RT-PCR analysis was carried out to assess whether the antisense ODNs affected Cx43 transcript levels. Total RNA was extracted from 20 embryos injected with

Figure 3.2 Culturing embryos in the presence of Cx43 antisense S-ODNs did not affect the gross morphology of the embryos. Embryos were cultured in the presence of 150 μM sense (A) and antisense (B) S-ODNs from the mid 4-cell stage to the late morula stage These phase-contrast images show both embryos populations to be normal. Scale bar = 25 μm.

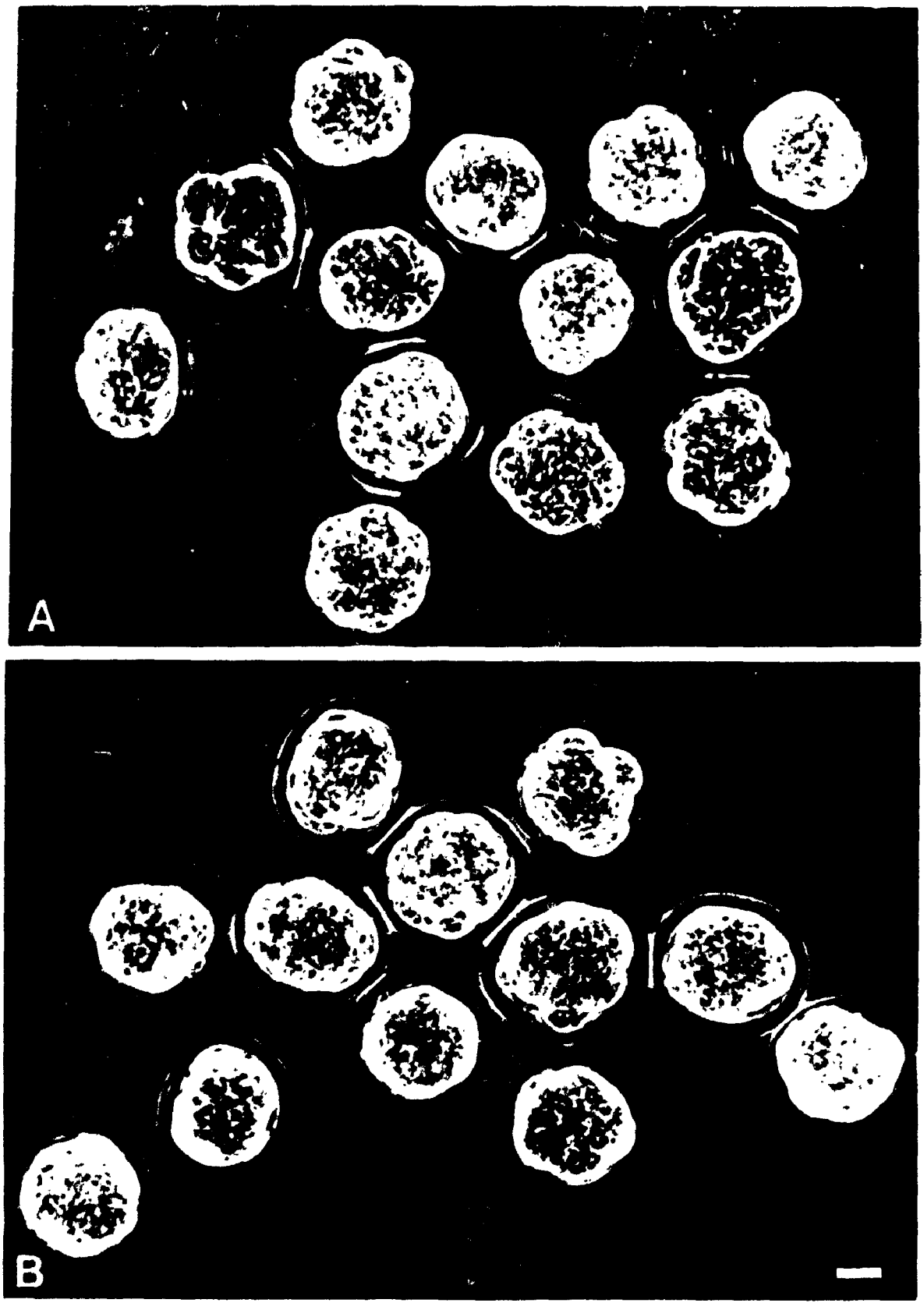

A
B

Table 3.4
Effects on dye coupling of culturing embryos in the presence of 150 μM Cx43 antisense S-ODN 3 from the 4-cell stage (56-61 hours post-hCG) to the late morula stage (78-81 hours post-hCG).

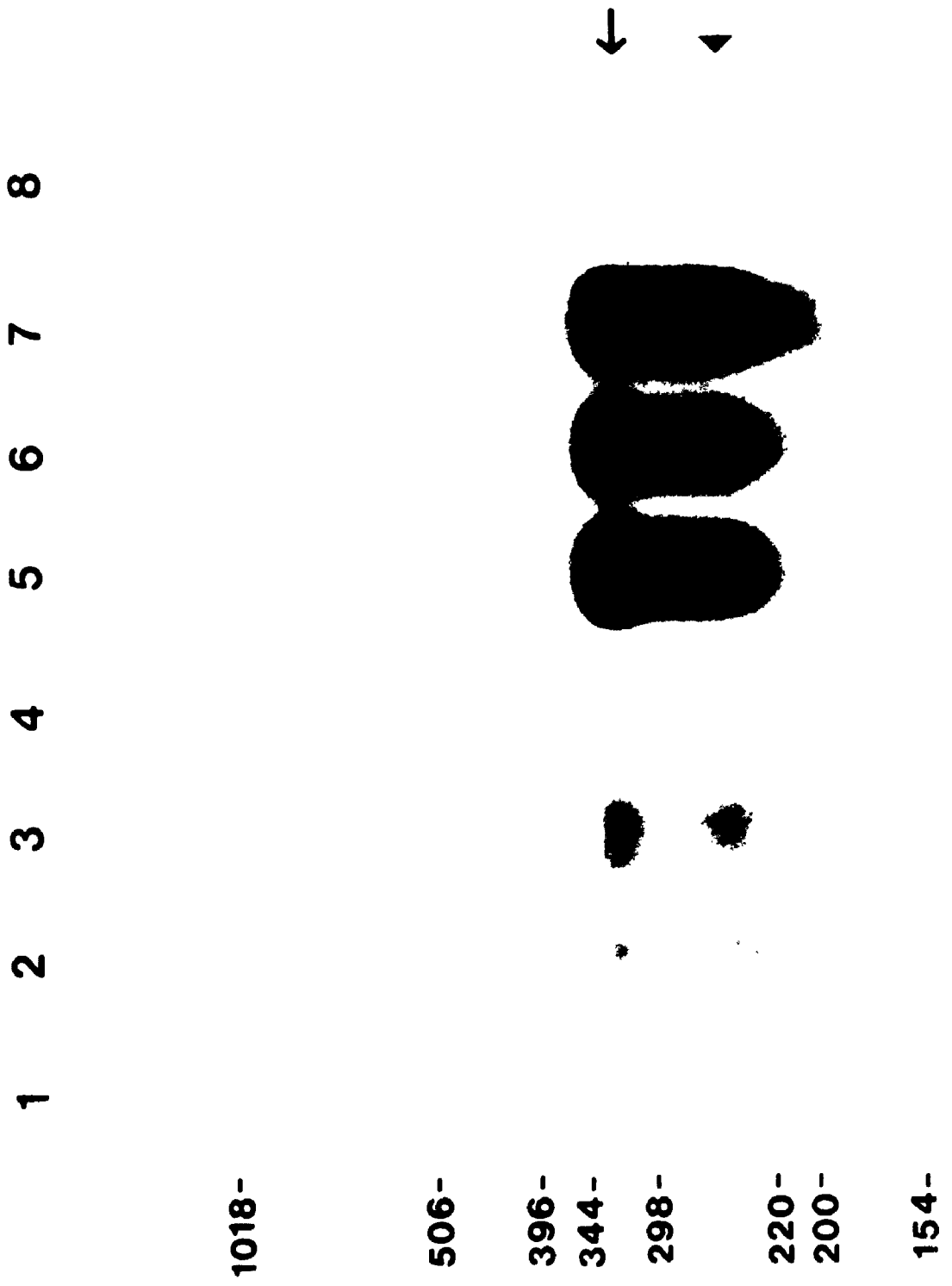
ODN	Dye coupled (%)	Not dye coupled (%)
Sense 3 (n = 11)	10 (90.9)	1 (9.1)
Antisense 3 (n = 14)	10 (71.4)	4 (28.6)

sense ODN 1 and from 20 embryos injected with antisense ODN 2 at 0.8 $\mu g/\mu l$ (from the second set of microinjection experiments). Total RNA was also extracted from embryos cultured in the presence of ODNs; 30 embryos were extracted for each treatment condition. This RNA was subjected to RT-PCR with Cx43 and ß-actin sequence-specific primers such that 10 embryo equivalents of reverse transcribed product were amplified in each case. The same ovarian cDNA described in CHAPTER 2, section 2.2.6 was used as a positive control.

A Southern blot showing the results of this analysis is presented in Figure 3.3. Lanes 1-3 are derived from the culture experiment whereas lanes 5 and 6 are derived from the microinjection experiment. In each case both the Cx43 specific 332 bp fragment and the ß-actin specific 243 bp fragment were amplified. Since the ß-actin signal would be expected to remain unaffected by the Cx43 antisense ODNs, the intensity of Cx43 signal relative to the ß-actin signal can be used to gauge Cx43 antisense ODN effectiveness. Visual inspection indicated that the intensity of the Cx43 signal relative to the ß-actin signal was not substantially different between samples from embryos cultured in the presence of sense S-ODN (lane 2) and from embryos cultured in the presence of antisense S-ODN (lane 3). Similarly, the intensity of the Cx43 signal relative to ß-actin signal was not substantially different between samples from embryos injected with sense ODN (lane 5) and antisense ODN (lane 6). The constancy of the relative signal intensities indicates that the antisense ODNs were ineffective in causing a reduction in Cx43 mRNA levels.

The signals from the sample of embryos cultured in SECM without any ODNs

Figure 3.3 Cx43 antisense ODNs do not cause an obvious reduction in Cx43 mRNA abundance. RNA was extracted from embryos cultured in SECM (lane 1), in SECM + 150 μM sense S-ODNs (lane 2), in SECM + 150 μM antisense S-ODNs (lane 3), from embryos microinjected with sense ODNs (lane 5), and from embryos microinjected with antisense ODNs (lane 6) and subjected to RT-PCR with Cx43 and ß-actin specific primers. A portion (30%) of the reaction product was run on a 3% agarose gel, Southern blotted, and hybridized with labelled Cx43 and ß-actin cDNAs. A 332 bp Cx43 specific fragment (arrow) and a 243 bp ß-actin specific fragment (arrowhead) was amplified from each sample, but the intensity of these signals, relative to one another, remained relatively constant between sense and antisense treated embryos. Ovarian RNA (lane 7) served as a positive control; rRNA (lane 4) and water (lane 8) served as negative controls.


1
2
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6
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1018-
506-
396-
344-
298-
220-
200-
154-

were very weak, for reasons that are not clear. Conversely, the signal strength was much higher for both fragments in the samples from the microinjection experiment. This may have been due to inefficiencies in the RNA extraction and/or reverse transcription of the RNA from cultured embryos. (RNA was extracted and reverse transcribed from the ODN microinjected and ODN cultured embryos on separate occasions; PCR amplification, on the other hand, was carried out on all of the samples at the same time, using a common mixture of reagents). The positive control, ovarian RNA (lane 7) gave a signal pattern very similar to the samples from microinjected embryos. Both the E. coli rRNA (lane 4) and water (lane 8) negative controls were devoid of signals.

3.4 Discussion

The effectiveness of the antisense ODNs in inhibiting Cx43 gene expression was assessed by three criteria: morphology, dye coupling, and Cx43 mRNA levels. The rationale for using morphology as a measure of connexin antisense efficacy was that previous studies had shown that blockage of gap junctional communication leads to decompaction and/or exclusion of the blocked blastomere(s) from the remainder of the embryo (Buehr et al., 1987; Lee et al., 1987; Bevilacqua et al., 1989). Thus a simple morphological observation would indicate whether the antisense ODNs were inhibiting intercellular communication. Dye coupling was used as a more stringent criterion of intercellular communication. Since dye coupling tests are time consuming, they were used mainly to confirm the morphological observations, with only a limited number of

embryos tested in each experiment. In any antisense experiment, it is important to assess the target mRNA and/or protein levels to ascertain the specificity of the phenotypic effect. In experiments such as the present one, in which no phenotypic effect was observed, this can also give indications as to the reasons for the lack of effectiveness.

ODNs, rather than RNA, were used in these experiments for the following reasons. (1) Stability. ODNs are more stable than in vitro transcribed RNA, which is very susceptible to degradation by ubiquitous RNases (Izant, 1992). S-ODNs have been reported to be considerably more stable than unmodified ODNs (Akhtar et al., 1992; Cohen, 1992) (2) Simplicity. The use of in vivo transcribed RNA requires the construction of expression vectors containing either an inducible promoter or a constituitive promoter active in mouse embryos (Ao et al., 1988). This would be a substantial undertaking in and by itself. Furthermore, the level of transcription from such vectors is highly variable, potentially complicating comparisons between embryos (Chrisey, 1990; Munir et al., 1992). (3) Potency. RNA-RNA hybridization is reversible and the RNA-RNA duplex is not a substrate for RNase H. ODN-RNA hybrids, on the other hand, can be irreversible and the RNA in such hybrids is a target for RNase H (Chrisey, 1990). (4) Specificity. When these experiments were begun the existence of at least four different connexin genes had been established. Contrasting the Northern blot results in CHAPTER 2 (showing only Cx43 mRNA in preimplantation embryos), with the results of microinjecting antisense Cx32 RNA into embryos (Bevilacqua et al., 1989; for a more thorough consideration of this study see CHAPTER 2, section 2.4.1) suggested that the use of antisense RNA in this system did not yield

sufficiently specific results to differentiate between the connexins. By designing the ODNs in such a way that they were complementary to unique regions of Cx43, this potential problem was eliminated.

The antisense Cx43 ODNs used in the present study did not cause a large scale degradation of the Cx43 mRNA, downregulation of intercellular communication, or gross morphological deformities. Thus, by the three criteria outlined above, these ODNs failed to elicit an antisense effect. The 20-30% increase in the proportion of both sense and antisense injected embryos displaying normal morphology between the experiments reported in Table 3.1 and those reported in Tables 3.2 and 3.3 is attributable to an increase over time in the experimenter's ability to control the volume of injectate. Great care had to be taken not to inject too large a volume of ODNs since it was found that this could result in embryos with decompacted and/or excluded blastomeres. This is consistent with Colman's (1992) observation that "...it is sometimes easy to generate apparently specific developmental lesions by the antisense approach". He suggests that this "...probably reflects the acute sensitivity of certain developmental processes to the slightest perturbation". The inability of the antisense ODNs to mediate large scale destruction of the Cx43 mRNA pool appears to be the root cause of the failure of these experiments. Interpretation of these results is complicated by the fact that the predicted Cx43 amplification product is similar in size (332 bp) to the predicted β-actin amplification product from genomic DNA (330 bp). Genomic DNA contamination would therefore obscure a reduction in the Cx43 signal. Although this possibility cannot be totally discounted consideration of two factors makes it seem unlikely that this was the

case. First, pelleting RNA through a CsCl gradient is generally considered the method of choice to separate RNA from DNA (Sambrook et al. 1989). Second, testing of numerous samples isolated in the same fashion with the β-actin primers alone have confirmed the absence of genomic contamination. Genomic DNA contamination of the RNA samples in this experiment therefore seems unlikely. Although the exact reasons for the lack of Cx43 mRNA degradation can not be determined from the data, consideration of the experimental protocol and the literature which follows offers potential explanations.

One potential problem lies in the time scale of these experiments. The experimental protocol used dictated an approximately 24 hour incubation period between the administration of antisense ODNs and the assessment of effect. This time frame was chosen for several reasons. Based on the Northern blot results (CHAPTER 2; section 2.3.1), Cx43 transcripts begin to accumulate at the 4-cell stage. It was reasoned that by beginning treatment at this stage one might be able to swamp the relatively small pool of Cx43 mRNA already present and neutralize any new mRNAs as they were transcribed, thereby maximizing the likelihood of preventing gap junction assembly at the early 8-cell stage. In most of the microinjection experiments early 4-cell embryos were injected. Therefore, in most cases half of the blastomeres received antisense ODNs. (Had the injections been performed at the mid to late 4-cell stage, when cytoplasmic bridges between sister blastomeres had closed, only one quarter would have received antisense ODNs). This should have facilitated detection of antisense effects since more blastomeres would have been affected. A 24 hour incubation period was

chosen because the initial experiments indicated that this length of time was required for most of the sense ODN injected embryos to become tightly compacted and highly dye coupled. Clear, well defined end-points would make any subtle differences between sense and antisense treated embryos more easily detectable. In essence, then, the time frame of these experiments was dictated, and constrained, by the biological material used and the problem under study.

Comparative information on both the length of time required to achieve maximal antisense inhibition and the length of time antisense inhibition is maintained by a single application is scarce but it appears that these parameters can vary considerably. For instance, Izant (1992) stated that ODN suppression of gene expression is generally transient, although no data to this effect were presented. Dagle et al. (1990) showed an almost complete loss of An2 mRNA within 2-4 hours of injecting antisense An2 ODNs into Xenopus oocytes and 1-cell embryos. As mentioned previously, these embryos underwent delayed gastrulation and became developmentally blocked before neurulation (about 12 hours post injection; Colman, 1992). Whether An2 transcript levels are still substantially reduced at these time points was not addressed, nor was the question of protein levels. On the other hand, Selinfreund et al. (1990) reported that treatment of rat C6 glioma cells with antisense ODNs against the S100ß calcium binding protein mRNA did not lead to reduced levels of this protein until 24-48 hours after the inititiation of ODN treatment, and an effect on growth rate was not observed before the 48 hour mark.

There have been only two studies published in which antisense ODNs were used to inhibit gene expression in mouse embryos. In the first of these Rosner et al. (1991) reported that microinjection of Oct-3 antisense ODNs into 1-cell embryos blocked development. Indeed, this apparently successful application of the antisense technology to mouse embryos provided much of the impetus for the experiments reported in the present Chapter. However, after the present experiments were completed it was made public that the evidence presented by Rosner et al. (1991) had in fact been fabricated (Rosner et al., 1992). The only other published study that is directly comparable to the present study is that of Rappolee et al. (1992) on the expression of IGF-II in preimplantation mouse embryos. In this experiment it was shown that embryos cultured for 24 hours in the presence of ODNs (30 μM), beginning at the 8-cell stage, had reached steady state levels of about 10^6 ODN molecules per embryo at the end of the culture period. A substantial decrease in IGF-II and IGF-II mRNA in response to antisense ODNs was observed after 72 hour and 48 hour culture periods, respectively. These were single time point determinations, however, and it is therefore difficult to come to any conclusions regarding the time course of inhibition. What is clear though, is that antisense ODN inhibition in mouse preimplantation embryos can be long-lasting. Whether the time frame of the present experiments was a factor in the absence of observable antisense effects is difficult to assess, but on balance it seems unlikely that there was a transient antisense effect that reversed itself within the 24 hour incubation period. On the other hand, some of the studies cited above would indicate that it is possible that inhibition had not yet developed by 24 hours.

There are two main, interdependent factors to consider in understanding why the time frame of an antisense experiment might influence its outcome. One is the stability of the antisense reagent and the other is the half-life of the target protein. The longer the half-life of the target protein, the more stable the antisense reagent needs to be. Clearly, if the antisense reagent is degraded rapidly it is unlikely that an antisense effect will be observed. Although, as mentioned previously, ODNs are generally considered more stable than RNA, this is only marginally so for unmodified ODNs. Antisense reagents are vulnerable to degradation in both the extracellular and intracellular compartments, but extracellular degradation is unlikely to have been a significant factor in the present study. First, by using microinjection delivery, exposure to the extracellular compartment was avoided. Second, only the more stable S-ODNs (Akhtar et al., 1992; Cohen, 1992) were used for the culture experiments. Third, it has been shown that the main cause of ODN degradation in culture experiments is exonuclease cleavage; the primary source of this activity being the serum added to most culture media (Akhtar et al., 1992; Tidd, 1992; Wickstrom, 1992). The SECM culture medium used here does not contain any serum. Finally, in the Rappolee et al. (1992) study cited above, antisense effects were observed, even with less stable unmodified ODNs in media similar to SECM.

While extracellular degradation was probably not a factor, intracellular degradation is a factor in all antisense studies. The speed with which it occurs is variable, depending on the particular ODN and cell system under study. Unmodified ODNs are particularly vulnerable to degradation. For example, Dagle et al. (1990)

showed that radiolabeled, unmodified ODNs injected into Xenopus oocytes and embryos were cleaved by both exo- and endonucleases to the extent that they became undetectable within 10 minutes. Conversely, Wickstrom (1992) reported that about 25% of the unmodified c-myc antisense ODNs taken up by HL-60 cells in culture remain intact even after 24 hours. It has also been reported that when the intracellular stabilities of unmodified ODNs and S-ODNs are compared by incubation with cytoplasmic and nuclear extracts, the S-ODNs are found to be considerably more stable in both extracts (Akhtar et al., 1992). In short, the literature suggests that ODN instability might have been a contributing factor in the lack of antisense effect. However, the fact that S-ODNs were also ineffective argues against this.

The literature on connexin half-life is scant in general, and in particular with respect to Cx43. The half-life of Cx32 in liver has been reported to be in the 3-10 hour range (Traub et al., 1983; Revel et al., 1984) and in cultured hepatocytes in the 1.5-3 hour range (Traub et al., 1987, 1989). A half-life of 5 hours and 1.3-2 hours has been reported for Cx26 in liver and cultured hepatocytes, respectively (Fallon and Goodenough, 1981;Traub et al., 1989). Bevilacqua et al. (1989), using Cx32 antisense RNA, estimated a turnover time of 30-60 minutes for Cx32 in preimplantation mouse embryos. However, given that both the present experiments (CHAPTER 2) and those carried out by Nishi et al. (1991) failed to detect any Cx32 mRNA in preimplantation embryos it seems likely that these latter estimates are incorrect. If, however, the Cx32 antisense RNA cross-hybridized to Cx43 mRNA as previously suggested, then the estimates provided by Bevilacqua et al. (1989) could be taken to represent the turnover

times for Cx43. At present, there is no direct experimental evidence for or against this interpretation (but see below). A half-life of 2-2.5 hours has been reported for Cx43 in primary cultures of chick lens epithelial cells (Musil et al., 1990) and a number of cultured cell lines (Musil et al., 1990; Musil and Goodenough, 1991).

The very limited direct data available on the half-life of Cx43 in mouse preimplantation embryos makes it difficult to decide if this factor could have influenced the outcome of these experiments. However, results from experiments with the protein synthesis inhibitor cycloheximide offer some clues. Embryos treated with cycloheximide for up to 17 hours, beginning at the 4-cell stage, formed a limited number of functional gap junction channels, allowing them to become very weakly dye coupled, at the same time as controls (McLachlin et al., 1983; McLachlin and Kidder, 1986). This would suggest that some gap junction protein (probably Cx43) is stable for at least 17 hours. This is supported by an experiment in which compacted morulae treated with cycloheximide for 4 hours contained as much immunofluorescently detectable Cx43 in gap junction-like plaques as did controls (De Sousa et al., 1993). These experiments therefore suggest that the Cx43 in gap junctions of preimplantation embryos is stable for at least 4 hours and perhaps for as long as 17 hours. These estimates predict a considerably slower turnover rate for Cx43 in preimplantation embryos than most estimates of connexin stability would indicate. However, preimplantation mouse embryos may be unusual in this respect, a suggestion which is supported by the fact that Cx32 synthesized during oogenesis is still present at the blastocyst stage (CHAPTER 2; Barron et al., 1989). Although the variability in the half-life estimates outlined above is

considerable, the half-lives are in all cases less than 24 hours. It therefore seems unlikely that the 24 hour incubation period used in the present study was insufficiently long for Cx43 levels in gap junctions to drop significantly, in the absence of new synthesis.

One important factor not considered so far is the question of dose of antisense reagent necessary for eliciting a specific, observable antisense effect. In the case of culture experiments, ODN concentrations in the range of 5 μM (Murphy et al., 1992) to 100 μM (Rappolee et al., 1992) have been reported to elicit a specific antisense response; unmodified ODNs generally requiring higher concentrations than S-ODNs. In the study by Rappolee et al. (1992) on IGF-II in preimplantation mouse embryos, the peak antisense response with unmodified antisense ODNs was seen at 30 μM, with no nonspecific toxicity observed below 100-200 μM. Microinjection of ODNs into mouse embryos has not been reported, but in two studies on the role of c-mos in meiotic maturation in mouse oocytes an antisense effect was observed after injecting 10 pg of either unmodified ODNs (O'Keefe et al., 1989) or S-ODNs (Paules et al., 1989). Considerations of antisense doses such as these, however, are not very meaningful since they do not take into account the amount of target. Theoretically, a much more useful measure of dose is the molar ratio of antisense reagent to target mRNA. Unfortunately, from the relatively few cases in which this ratio is reported, it appears that it is highly variable as well. At one extreme, it required 10^6 X molar excess of sal antisense RNA to produce sal phenocopies in Drosophila embryos (Schuh and Jäckle, 1989), while at the other extreme 3-4 X molar excess of ribosomal protein rp49 antisense RNA was

sufficient to phenocopy the Minute mutation in Drosophila embryos, albeit at a relatively low frequency (Patel and Jacobs-Lorena, 1992). Most reports fall in the 100-2,000 X range. In the present study, estimates of molar excess in the microinjection experiments are in the 230-1360 X range, whereas the molar excess in the culture experiments was estimated to be about 35 X (Appendix 1). Although these values fall within the range of published values, the fact that the published values vary so widely makes it difficult to evaluate the adequacy of the doses used.

As outlined in the INTRODUCTION, there are several potential points in the life of a mRNA at which antisense inhibition might take place. The argument has been made that for antisense ODNs targeted against the coding region of a mRNA, such as the ones used in this study, the most likely mode of action is mRNA degradation via RNase H cleavage (Toulmé, 1992). RNase H mediated antisense effect has been documented in Xenopus oocytes and embryos (Dash et al., 1987; Dagle et al., 1990), and experiments from mouse oocytes (O'Keefe et al., 1989) and embryos (Rappolee et al., 1992) suggest the presence of RNase H in these systems. In fact, the RT-PCR experiments in this chapter were predicated on the assumption that RNase H activity is present in the embryos. However, there has been no direct demonstration of RNase H activity in mouse preimplantation embryos and it is therefore possible that the failure of the experiments reported here stemmed from a lack of RNase H. On the other hand, since Cx43 levels were not assessed directly, the possibility exists that the antisense ODNs caused an undetected reduction in the level of this protein, perhaps by sterically blocking translation. This possibility cannot be totally discounted because of lack of direct

evidence to the contrary, but consideration of two pieces of circumstantial evidence suggests that this scenario is unlikely. First, connexins other than Cx43 would have to be able to maintain coupling in the antisense treated embryos at a level equivalent to controls. In addition to Cx43, only Cx32 has been detected in preimplantation embryos (Barron et al., 1989; Nishi et al., 1991; CHAPTER 2) where it does not appear to form gap junctions (Nishi et al., 1991; Valdimarsson et al., 1993), making this possibility seem remote. As mentioned earlier, preliminary evidence suggests that other connexins may be expressed in the embryo (G.M. Kidder and P. De Sousa, personal communications). If this turns out to be the case, then the results from this study will need to be reevaluated. The second piece of circumstantial evidence is that RNase H is found in a wide variety of mammalian cells (Wagner and Nishikura, 1988) and in the design of antisense ODN experiments it is generally assumed to be present (Murray and Crockett, 1992; Toulmé, 1992).

The most straightforward and likely explanation for the absence of an antisense effect in the present experiments is that the antisense ODNs simply did not hybridize to the Cx43 mRNA. Although ODNs targeted against the region around the ATG start codon have generally enjoyed more success that ODNs targeted against other regions, this is by no means unequivocal (Cohen, 1992; Murray and Crockett, 1992). There are two main reasons why an antisense ODN might not hybridize to its complementary sequence. The first is that the target sequence might be involved in secondary or tertiary structures and the second is that the target sequence might be masked by proteins. In either case the target sequence is not available for hybridization with the antisense ODN.

Computer models have been used to choose target regions predicted not to be involved in higher order structures, but this has met with only very limited success (Stull et al., 1992; Colman, 1992).

The preceding discussion makes it clear that the antisense technology is still largely empirically driven and that it is only now that general principles are beginning to emerge. This makes it impossible to pinpoint with any certainty the reason(s) for the lack of antisense effect in the experiments reported here, and elsewhere. In fact, it turns out that the application of the antisense technology, and the interpretation of results, has generally proved to be less straightforward than was initially hoped (Dolnick, 1990; Murray and Crockett, 1992). Indeed, "...it is a common experience within the scientific community to be *unable* to achieve an antisense effect" (Dolnick, 1990).

In conclusion, Cx43 antisense ODNs affected neither intercellular coupling nor Cx43 mRNA levels in preimplantation mouse embryos. Several potential reasons for this ineffectiveness have been discussed but it is not at all clear which one, if any, is responsible. Due to the negative outcome of these experiments the questions posed in the INTRODUCTION remain unanswered.

CHAPTER 4

TEMPORAL CONTROL OF GAP JUNCTION ASSEMBLY IN PREIMPLANTATION MOUSE EMBRYOS

4.1 Introduction

One of the most striking features of embryonic development is the precise timing and orderly sequence of morphological and molecular events. Numerous mechanisms have been suggested to account for this phenomenon, including: time elapsed since fertilization, absolute number of cells, number of mitoses, number of DNA replication cycles, gene expression, critical nucleocytoplasmic ratios, and endogenous cytoplasmic "clocks." In a few instances the time-keeping mechanism responsible for the timing of a developmental event has been elucidated. In amphibians, for example, an event termed midblastula transition (MBT) is associated with transcriptional activation of the embryonic genome, the first appearance of G_1 and G_2 phases of the cell cycle, and initiation of both asynchronous cell divisions and cell motility (Kirschner et al., 1985; Satoh, 1985). The timing of the MBT is independent of the number of mitoses, number of DNA replication cycles, and the time elapsed since fertilization, but is critically dependent on the nucleocytoplasmic ratio (Kobayakawa and Kubota, 1981; Newport and Kirschner, 1982a,b). The nucleocytoplasmic ratio is thought to exert its effect through the titration, by the DNA, of a finite quantity of a maternally supplied cytoplasmic factor (Newport and Kirschner, 1982b). It has recently been suggested that the MBT

concept is an oversimplification of the known facts, and that rather than a single transition, several independent transitions take place, at not exactly the same time (Yasuda and Schubiger, 1992). Unlike MBT, gastrulation in amphibians is not timed by the nucleocytoplasmic ratio, but rather seems to be timed by an autonomous cytoplasmic "clock" (Kobayakawa and Kubota, 1981; Satoh, 1985; Kirschner et al., 1985; Cooke and Smith, 1990). This illustrates an important point in that all developmental events within an embryo are not necessarily controlled by a single time-keeping mechanism, but rather, there may be several time-keeping mechanisms operating simultaneously and independently within an embryo. A MTB-like event also takes place during Drosophila embryogenesis, the timing of which appears to be controlled by the nucleocytoplasmic ratio as well (Edgar et al., 1986; Cooke and Smith, 1990). The available evidence on the time of appearance of three different histospecific enzyme systems in ascidian embryos indicates that they are all regulated by the absolute number of rounds of DNA replication, but that the actual number of rounds required differs for each enzyme system (Satoh and Ikegami, 1981a,b; Satoh, 1982).

The temporal control of developmental events in mouse embryos has also received considerable attention, although in most cases more is known about mechanisms that are not involved in a particular process than those that are involved. For example, transcriptional activation of the zygotic genome at the 2-cell stage is not dependent on cytokinesis or the second round of DNA replication (Bolton et al., 1984), although a dependence on the first round of DNA synthesis has been documented (Howlett, 1986; Petzoldt, 1984). Also, the timing of the appearance of several stage specific proteins has

been shown to be independent of both cytokinesis (Petzoldt, 1986) and the nucleocytoplasmic ratio (Petzoldt and Muggleton-Harris, 1987). The timing of compaction has been demonstrated to be independent of immediate transcription or translation (Kidder and McLachlin, 1985; Levy et al., 1986) and of DNA replication in the third and fourth cell cycles (Smith and Johnson, 1985). However, embryos that had the second round of DNA synthesis delayed by 10 hours failed to compact (Smith and Johnson, 1985), demonstrating that not only is there a simple requirement for the second DNA replication cycle to take place but that it must take place within a specific window of time.

As outlined in section 1.5, the initiation of gap junctional communication in preimplantion mouse embryos is also a precisely timed event. Gene expression, cell contact, microtubules, microfilaments, cell flattening, and cytokinesis have all been eliminated as being involved in the time-keeping mechanism controlling this event (Goodall and Johnson, 1982, 1984; McLachlin et al., 1983; McLachlin and Kidder, 1986; Goodall, 1986; Kidder et al., 1987). In fact, all attempts so far at either inducing premature gap junction assembly or preventing gap junction assembly at the normal time have failed. The question of what controls the timing of gap junction assembly is therefore still unanswered. This chapter describes experiments aimed at solving this problem.

Recently it was reported that treatment of 4-cell mouse embryos with activators of PKC, such as phorbol esters or diacylglycerol (DAG) analogs, can cause premature compaction (Winkel et al., 1990). Although, as was outlined in section 1.5, the initiation

of compaction and gap junction assembly are not obligatorily linked, the fact that gap junctional coupling is required for the maintenance of compaction (Buehr et al., 1987; Lee et al., 1987; Bevilacqua et al., 1989) suggested that perhaps the assembly of gap junctions might also be triggered by a mechanism involving PKC. Furthermore, Cx43 contains a PKC phosphorylation consensus site (Lau et al., 1992). PKC has been shown to phosphorylate Cx43 on serine residues (Brisette et al., 1991; Berthoud et al., 1992; Reynhout et al., 1992), and serine phosphorylation of Cx43 has been shown to be associated with its assembly into gap junction plaques (Musil et al., 1990a,b; Musil and Goodenough, 1991). These findings raise several interesting questions, two of which will be addressed here: 1) Is the premature compaction elicited by activators of PKC accompanied by premature assembly of gap junctions? 2) Does Cx43 become phosphorylated at the time of gap junction assembly during normal development? As a side project connected with question 1), the effect of PKC activation on dye coupling in 8-cell compacted embryos was also investigated.

As outlined above, inhibition of the third and fourth rounds of DNA replication with aphidicolin, an inhibitor of DNA polymerases α and δ (Sheaff et al., 1991; Sorscher and Cordeiro-Stone, 1991), did not influence the timing of compaction, but delaying the second round by 10 hours completely inhibited this event (Smith and Johnson, 1985). Given this, and the fact that gap junctional communication seems to be required for the maintenance of the compacted state, it is of obvious interest to investigate gap junctional coupling in aphidicolin-treated embryos. For this reason, the final studies included in this chapter addressed the question of whether or not the timing

of gap junction assembly in the mouse preimplantation embryo is controlled by DNA replication cycles. The experiments of Smith and Johnson (1985) were therefore repeated in the studies presented here, but in this case the embryos were scored for dye coupling rather than compaction. The spatial distribution of Cx43 in aphidicolin-treated 2-cell embryos was also assessed.

4.2 Materials and Methods

4.2.1 Embryo collection and culture

Superovulation and embryo collection were as described in section 2.2.1. The culture media varied between experiments as indicated below. All culturing was done in Falcon 1006 petri dishes (Becton Dickinson) at 37°C, 5% CO_2 in air, and 100% relative humidity unless otherwise noted. The culture media were pre-equilibrated for at least 2 hours prior to embryo addition.

4.2.2 diC_8 treatment

The DAG analog diC_8 (1,2 dioctanoyl-rac-glycerol; Sigma) was made up as 100 mM stock in dimethylsulfoxide (DMSO) and was added to SECM to a final concentration of 100-200 μM. DMSO at 0.1-0.2% served as control. The lipophilic nature of diC_8

required that culturing be performed in humidified chambers, without oil, as described in section 3.2.4.

Four-cell embryos (54-55 hours post-hCG) or compacted morulae (74.5-76.5 hours post-hCG) were initially cultured in SECM in an organ culture dish at 8-10% CO_2 in air for a minimum of 1 hour. The embryos were then transferred to diC_8 or DMSO containing drops (50 μl) in the humidified chambers and cultured for an additional 0.5-2 hour at 8-10% CO_2 before being tested for dye coupling. Elevated CO_2 concentration is required for diC_8-stimulated compaction of 4-cell embryos (Winkel et al., 1990).

4.2.3 Aphidicolin treatment

Aphidicolin (Sigma) was made up as a 2 mg/ml stock in DMSO and added to the culture medium to a final concentration of 2-4 μg/ml. Control cultures contained DMSO at 0.1-0.2%. Some of these experiments required that embryos be cultured through the 2-cell stage. The CF1 X $CB6F_1/J$ embryos used in most of my experiments are frequently affected by the "2-cell block" phenomenon (Goddard and Pratt, 1983) when they are cultured in SECM. This problem was overcome by using CZB culture medium (Chatot et al., 1989) in all aphidicolin experiments.

Two distinct experimental protocols were used in this study, both based on Smith and Johnson (1985). In the first protocol, 1-cell embryos were flushed at 29 hours post-hCG and then washed three times with FM-I followed by two CZB washes. The embryos were placed in CZB in an organ tissue culture dish (Becton Dickinson) for about

0.5 hour and then transferred in groups of about 30, to 30μl drops of CZB under paraffin oil. Observations were made at 1 hour intervals, over a 4-5 hour period, and all 2-cell embryos and 1-cell embryos showing signs of cell division were removed. The embryos that were removed at each time point were split equally between CZB drops (30μl) containing aphidicolin or DMSO, under paraffin oil, and cultured for 10 hours. At the end of this culture period the embryos were washed separately through five drops of pre-warmed CZB to remove the drug, placed separately in drops of fresh CZB (30μl) under oil, and cultured until they were either tested for dye coupling (beginning at 80-83 hours post-hCG) or fixed for immunofluorescence (85 hours post-hCG). At 74 hours post-hCG, 2 μl of 100 mg/ml glucose in CZB were added to each culture drop.

In the second protocol 2-cell embryos were flushed at 50 hours post-hCG and handled in essentially the same fashion as described above. At hourly intervals 4-cell and 3-cell embryos, and 2-cell embryos showing signs of division to 3-cells, were removed from the CZB drops and transferred to aphidicolin and control drops. The embryos were cultured continously in these drops until testing for dye coupling was begun at 79.5-81 hours post-hCG. Glucose was added as described above. In some of these experiments the cell number of the embryos was determined by counting DAPI stained nuclei. The embryos were fixed overnight in 4% paraformaldehyde in calcium- and magnesium-free PBS-PVP (CMF-PBS-PVP), stained for 1 hour in 5 μg/ml DAPI (Polysciences) in CMF-PBS-PVP, washed three times in CMF-PBS-PVP and mounted onto a microscope slide in 9:1 glycerol:10 X TBS. The embryos were examined and the nuclei counted on a Zeiss Photomicroscope I fitted with epifluorescence optics.

4.2.4 Dye injections

Embryos were tested for dye coupling by iontophoretic injection of 10 mM 6-carboxyfluorescein as described in section 3.2.5. The only change in protocol was that in the diC_8 experiments the embryos were injected in FM-I containing diC_8 at the same concentration as was used during culture.

4.2.5 Immunofluorescence microscopy

The immunofluorescence procedure used was as described in Valdimarsson et al. (1991) and De Sousa et al. (1993). Embryos were washed four times at room temperature with CMF-PBS-PVP and once with PHEM buffer (PHEM = 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 1 mM $MgCl_2$, pH 6.9) before being fixed in 1% paraformaldehyde in PHEM for 1 hour at 4°C. All subsequent steps of the procedure were also carried out at 4°C. The embryos were freed of fixative by four washes in PHEM and then permeabilized by two washes in 0.1% Tween-20 in PHEM, the second wash being of 20 minute duration. This was followed by two washes in CMF-PBS-PVP and a further 20 minute permeabilization in 0.1% Tween-20 in CMF-PBS-PVP. Next, non-specific binding sites were blocked by incubating the embryos for 1-2 hours in CMF-PBS-PVP containing 1% bovine serum albumin (BSA) and 0.1% Tween-20 (block solution). The embryos were treated overnight with the primary antiserum (see below)

in block solution and then washed six times with 0.1% Tween-20 in CMF-PBS-PVP, the last wash lasting at least 5 hours. This was followed by a 1 hour incubation with secondary antibody (see below) in block solution, and then six washes in 0.1% Tween-20 in CMF-PBS-PVP, the last wash lasting overnight. The embryos were mounted onto glass microscope slides in about 15 μl of FITC-guard mounting medium (Testog, Chicago, Il), covered with an 18 X 18 mm number 1 coverslip and analyzed on a Bio-Rad MRC-600 Confocal Laser Scanning Microscope (Bio-Rad Microscience, Hertfordshire, UK).

To facilitate comparisons between samples, all parameters were kept constant during image collection (black level = 5; gain = 10; laser neutral density filter = 3; pin hole = 1) except that for embryos treated with the peptide absorbed antiserum the pin hole setting had to be increased to 4 to make the embryos visible at all. A series of images was collected by focusing through each embryo and collecting images at 0.5 μm intervals. For presentation, three such images were stacked on top of one another, thus creating an approximately 1 μm optical section, which was then edge enhanced with filter C9A.

Immunolabeling was performed with an affinity-purified rabbit antibody raised against a synthetic peptide corresponding to amino acids 302-319 of Cx43 (gift form Dr. B. Nicholson, SUNY, Buffalo, NY; Garcia, White, Willecke, and Nicholson, manuscript in preparation; De Sousa et al., 1993; Naus et al., 1992). The crude antiserum, absorbed three times with the 302-319 peptide served as a control. Both the affinity-purified antibody and the peptide absorbed serum were used at a concentration of 6

μg/ml. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (ICN Biomedicals Canada Ltd., St. Laurent, Québec) at a 1:50 dilution was used to localize bound primary antibody.

4.2.6 Western blots

Western blot analysis was carried out as described in section 2.2.4, with the following exceptions. To assess the native phosphorylation status of Cx43, embryos were washed three times in PBS-PVP and then two times in PBS-PVP with the phosphatase inhibitors $NaVO_4$ and NaF added to 100 μM and 50 μM respectively. The embryos were transferred in 5 μl or less of the last wash solution to a 1.5 ml microfuge tube to which was added 15 μl of 2X Laemmli sample buffer, containing phosphatase inhibitors as above and protease inhibitors (2 mM N-ethyl maleimide, 2 mM phenylmethyl-sulfonyl flouride, 2 mM ortho-phenanthrolene). The samples were boiled for 5 minutes and frozen at -80°C.

To obtain dephosphorylated protein, embryos in 5 μl or less of PBS-PVP were lysed by the addition of 10 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1% NP-40, 1% SDS, and protease inhibitors as above). Two μl of 10X dephosphorylation buffer (0.5 M Tris-HCl, 1 mM EDTA, pH 8.5) and 4 μl of calf intestinal alkaline phosphatase (1 unit/μl; Boehringer-Mannheim) were added and the lysate was incubated at 37°C for 8-10 hours. This was followed by the addition of 15

μl of 2X Laemmli sample buffer and boiling for 5 minutes before the sample was frozen at -80°C.

Ovarian protein was used as a positive control for the experiments on the phosphorylation state of Cx43. Native phosphorylated ovarian protein was prepared at 4°C as follows. Four mouse ovaries were placed into PBS, sliced with a razor blade, washed three times with PBS, and centrifuged at 3000 X g for 5 minutes in a 1.5 ml microfuge tube. The pellet was homogenized with a hand-held teflon pestle in 5 volumes of lysis buffer, containing both protease and phosphatase inhibitors as described above. An equal volume of 2X Laemmli sample buffer was added, the sample was vortexed, and then boiled for 10 minutes. This was followed by 30 second sonication and centrifugation at 10,000 X g for 10 minutes at room temperature. The supernatant was used for experiments.

Dephosphorylated total ovarian protein was obtained by preparing an ovarian homogenate as described above, except that phosphatase inhibitors were omitted from the lysis buffer. The homogenate was spun at 1,000 X g for 5 minutes to remove large debris. Five μl of the supernatant were combined with 4 μl of 10X dephosphorylation buffer, 1 μl alkaline phosphatase (1 unit/μl; Boehringer-Mannheim), and 30 μl sterile, double distilled water and incubated at 37°C for 8 hours. Fifty μl of 2X Laemmli sample buffer was added, and then the sample was boiled for 10 minutes and stored at -80°C.

To obtain a better separation of phosphorylated forms of Cx43, a low bis-acrylamide gel system was used (Brisette et al., 1991), with a 12.6% resolving gel

(12.6%T, 0.1%C) and a 4% stacking gel (4%T, 0.03%C). The prestained M_r markers used and their M_r were as follows: myosin, 200,000; phosphorylase b, 97,400; BSA, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; trypsin inhibitor, 21,500; and lysozyme, 14,300 (Rainbow Protein Molecular Weight Markers; Amersham). The electrophoretically separated proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA) and Cx43 was detected with the same antibody used for immunofluorescence, but used at a concentration of 0.25 μg/ml. Three times peptide absorbed antiserum at 4.5 μg/ml was used as a control. Bound primary antibody was visualized with a horseradish peroxidase-linked goat anti-rabbit IgG (1:2,500 dilution; Promega) in conjunction with IBI High Sensitivity Enzygraphic Webs (Intersciences, Markham, Ont.)

4.3 Results

4.3.1 Effects of diC_8 on dye coupling in 4- and 8-cell embryos

In agreement with Winkel et al. (1990), the majority of 4-cell embryos (54-55 hours post-hCG) treated with 100 μM diC_8 displayed extensive cell flattening within 1 hour (Fig. 4.1). Twenty of these apparently fully compacted embryos were tested for dye coupling to determine if the analog also caused premature onset of coupling. In no case did the dye spread beyond the injected blastomere, even if the observation period was extended to 45 minutes (Fig. 4.2). These results indicate that diC_8 did not trigger

Figure 4.1 Four-cell embryos treated with diC_8 become prematurely compacted. Four cell embryos (54-55 hours post-hCG) were treated with 100 μM diC_8 (B) or 0.1% DMSO (A) for 1 hour. The DMSO-treated control embryos remained uncompacted whereas diC_8 treated embryos became highly compacted. Scale bar = 50 μm.

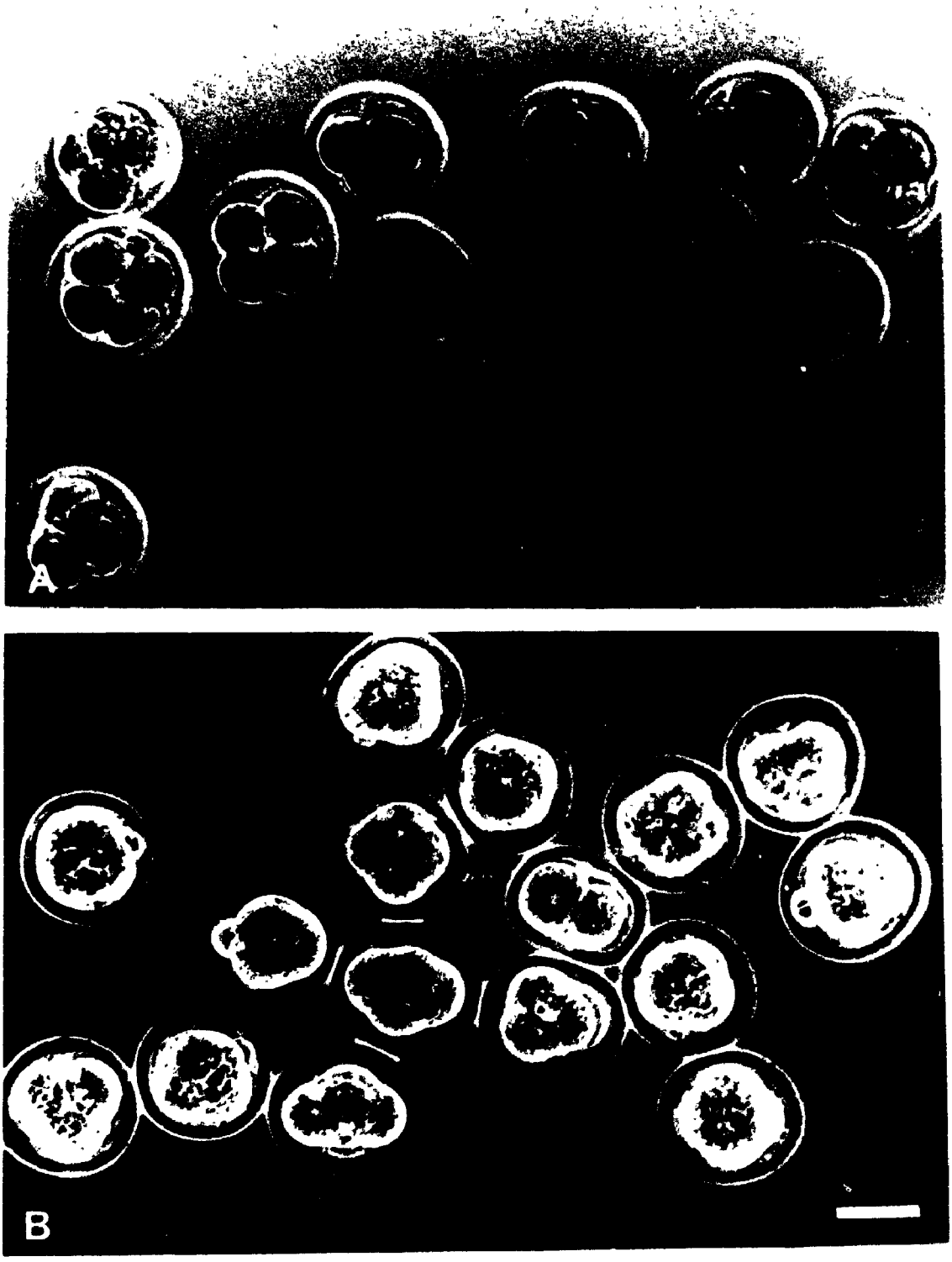

A
B

Figure 4.2 Premature compaction of 4-cell embryos by diC_8 is not associated with premature onset of dye coupling. A single blastomere of a 4-cell embryo (55 hours post-hCG) treated with diC_8 for 1 hour was microinjected with carboxyfluorescein and photographed at 15 (B), 30 (C), and 45 (D) minutes after the injection. No dye ever spread out of the injected blastomere, even 45 minutes after the initial injection (D). As can be seen from the phase-contrast image in (A), the apparent enlargement, over time, in the area occupied by dye is due to decompaction of the impaled blastomere. Scale bar = 25 μm.

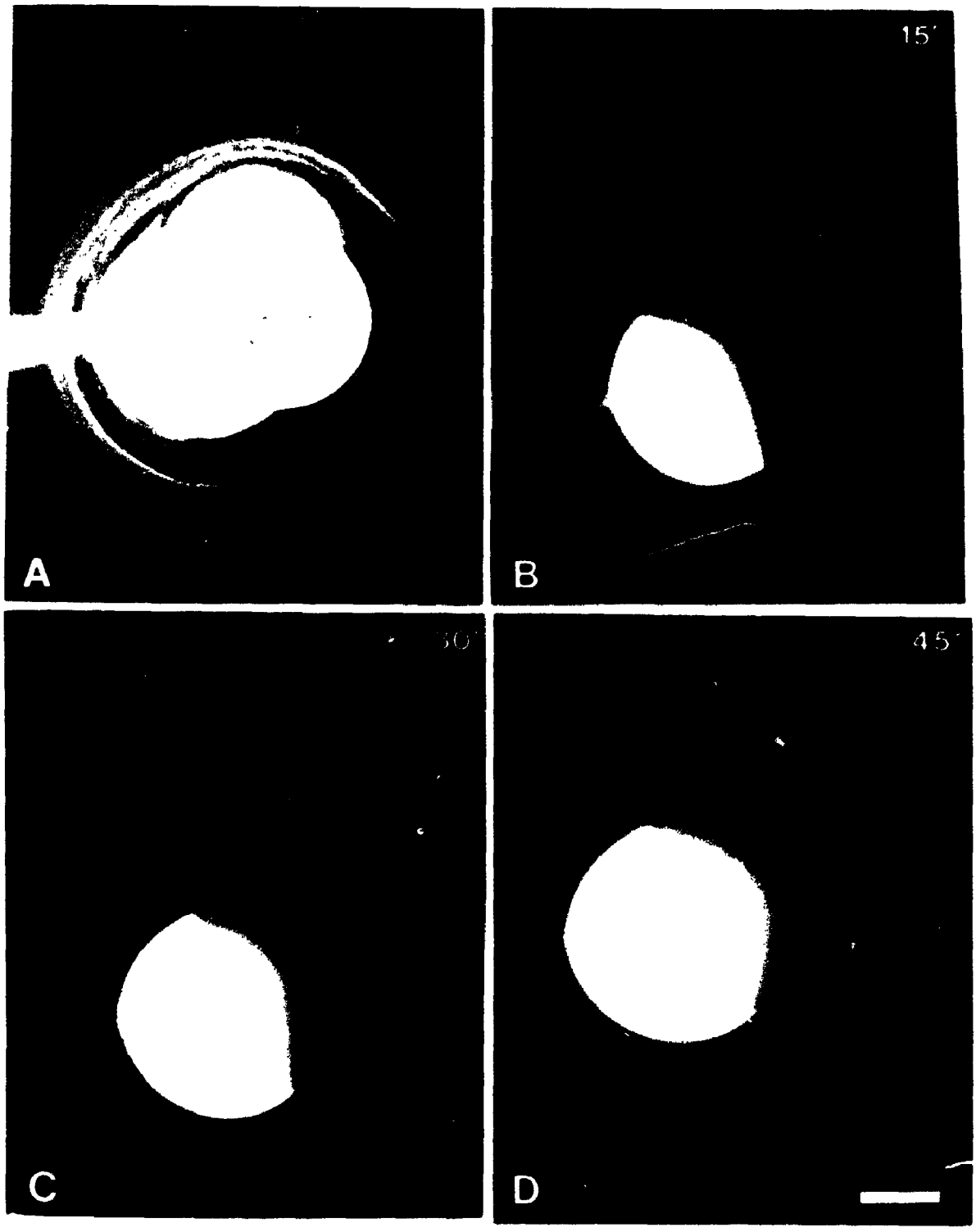
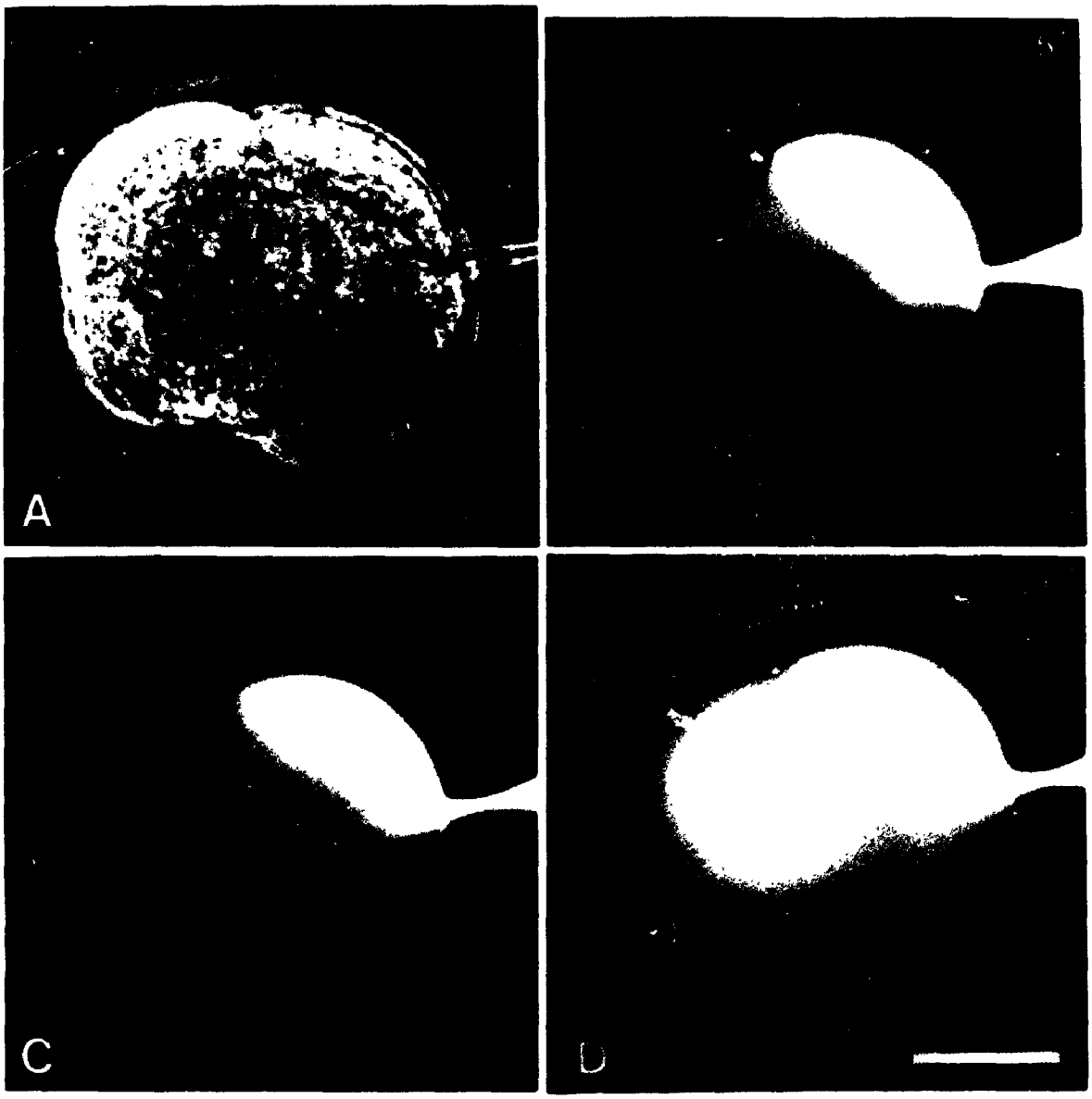

A
B
15'
C
30'
D
45'

Figure 4.3 Eight-cell compacted embryos are dye coupled. As a control, a single blastomere of each 8-cell compacted embryo was microinjected with carboxyfluorescein and the embryos were then observed over a 20 minute period. (A) is a phase-contrast image and (B,C,and D) are fluorescence images. The dye spread to all the blastomeres within 10 minutes of the injection and the dye became nearly equilibrated by 20 minutes, indicating a high degree of coupling. Scale bar = 25 μm.


A
C
D

the premature assembly of gap junctions. In control, 8-cell embryos (Fig. 4.3) the dye was generally detectable in all blastomeres within 5 minutes of injection and it frequently reached approximately equal intensity in all the blastomeres within 20 minutes. This description of dye spread in control embryos is applicable to all the dye coupling experiments that follow. Between 90 and 100% of the control embryos were dye coupled.

To determine if diC_8 had an effect on existing gap junctions, 8-cell compacted embryos (74.5-76.5 hours post-hCG) were treated with the analog at a concentration of 100 or 200 μM and tested for dye coupling. Compared to untreated controls, treatment with 100 μM diC_8 led to a moderate reduction in the number of dye coupled embryos, whereas treatment with 200 μM led to a very considerable decrease in the number of dye coupled embryos (Table 4.1). Although the rate of dye spread was not quantified, it was noticably slower in the analog-treated embryos than in controls, especially in the group treated with 200 μM diC_8. These data suggest that diC_8 caused existing gap junctional communication to be diminished.

4.3.2 Phosphorylation status of Cx43 in pre- and post-compaction embryos

The phosphorylation status of Cx43 in pre- and post-compaction embryos was evaluated by Western blotting. This approach was based of the premise that the SDS-PAGE mobility of Cx43 predictably reflects its level of phosphorylation (Crow et al., 1990; Musil et al., 1990a,b). This experiment was performed once and figure 4.4

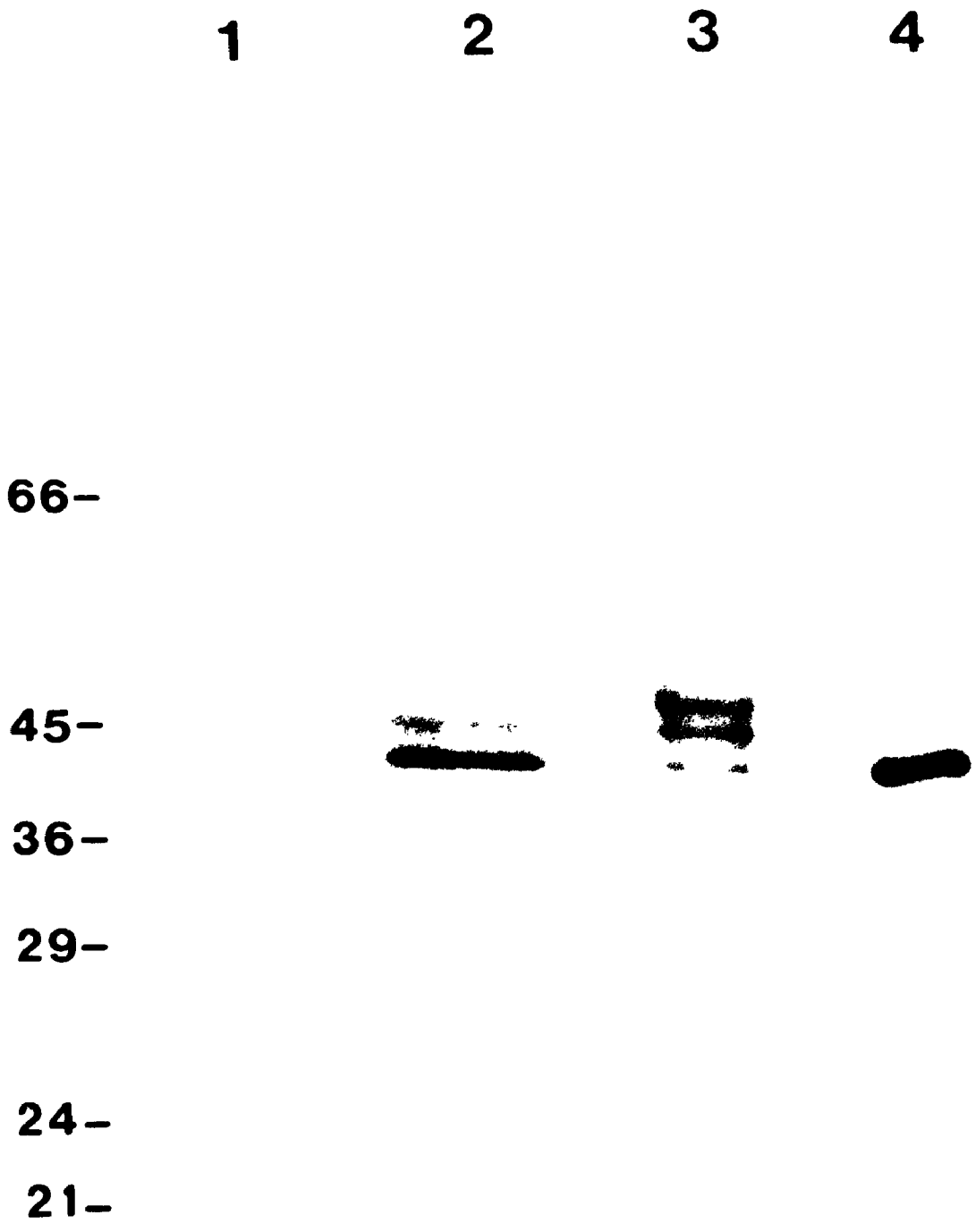
Table 4.1
Incidence of dye coupling in 8-cell compacted embryos (76-79.5 hours post-hCG) treated with diC_8

Treatment	Dye coupled (%)	Not dye coupled (%)
Control (n = 12)	11 (91.7)	1 (8.3)
diC_8 100 μM (n = 20)*	14 (70.0)	6 (30.0)
diC_8 200 μM (n = 22)**	9 (40.9)	13 (59.1)

* Data in this treatment condition were collected in four separate test sessions. A small number of uncoupled embryos was observed in each session.

** Data in this treatment condition were collected in three separate test sessions. Over 50% of embryos tested in each session were not dye coupled.

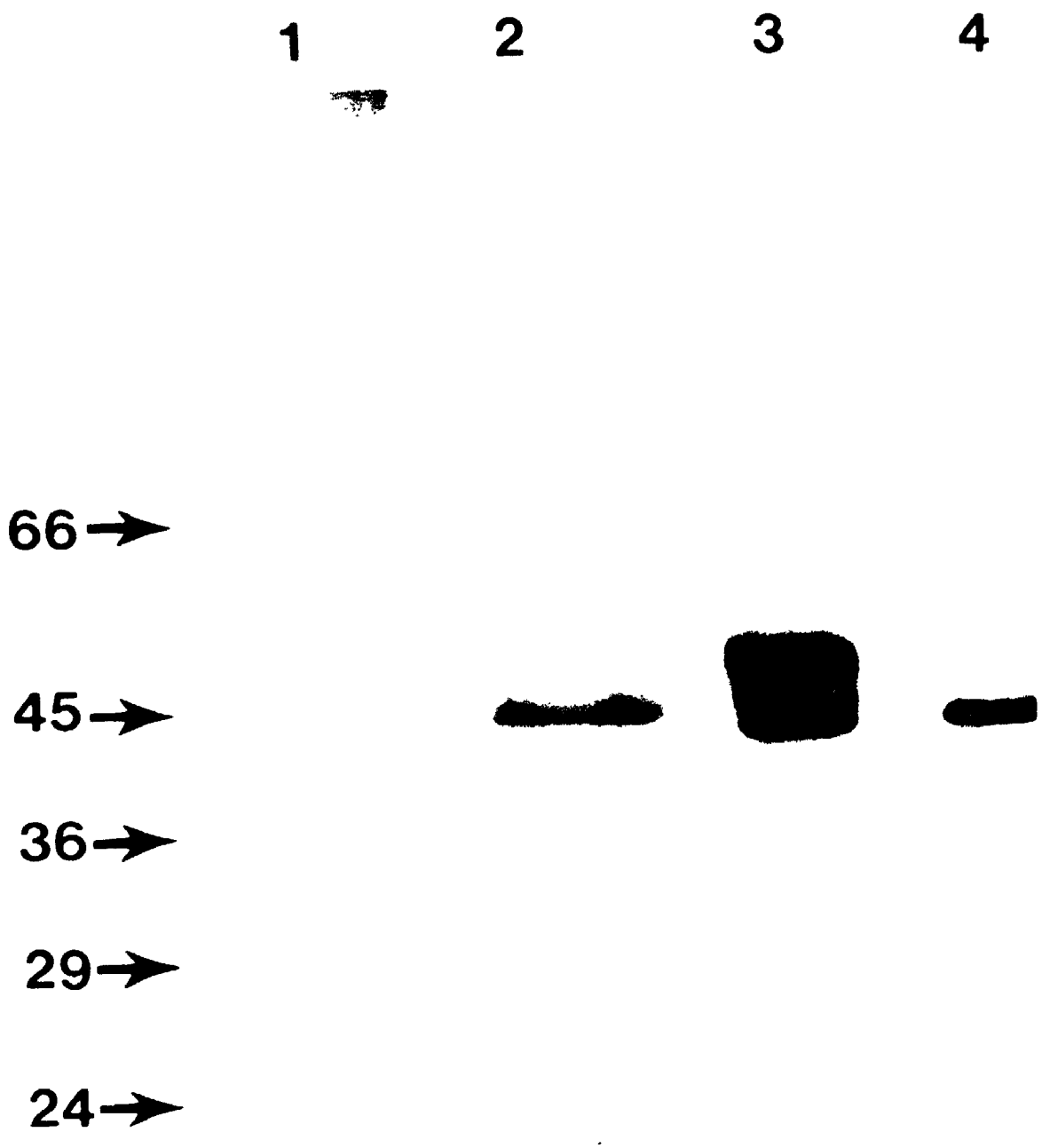
Figure 4.4 The PAGE mobility of Cx43 changes between the 4-cell and late morula stages. A Western blot containing lysates from 4-cell embryos (350 embryos; lane 1), late morulae (530 embryos; lane 2), and whole ovary (lanes 3 and 4) was probed with a Cx43 specific antibody. The lysates in lanes 1,2, and 3 were prepared in the presence of phosphatase inhibitors whereas the lysate in lane 4 was dephosphorylated with alkaline phosphatase prior to electrophoresis. A single M_r 43,000 band was detected in the 4-cell lysate and the dephosphorylated ovarian protein sample. In both the late morulae lysate and the ovarian lysate prepared in the presence of phosphatase inhibitors three bands of M_r 43,000, 45,000, and 47,000 were detected, although the M_r 47,000 band in the late morulae lysate was very faint. The M_r X 10^{-3} of marker proteins are indicated on the left.


1
2
3
4
66-
45-
36-
29-
24-
21-

shows the result from this experiment. A sample of dephosphorylated whole ovarian protein and samples of late 4-cell embryos (61 hours post-hCG; 350 embryos), late morulae (85 hours post-hCG; 530 embryos), and whole ovary prepared in the presence of phosphatase inhibitors were probed with the Cx43 specific antibody. In the dephosphorylated ovarian protein sample (lane 4) and the 4-cell embryo lysate (lane 1) only one major band, of approximately M_r 43,000, was detected. In the late morulae sample (lane 2) a major band of M_r 43,000 was detected, as well as a less intense M_r 45,000 band and a minor M_r 47,000 band. In the ovarian protein sample prepared in the presence of phosphatase inhibitors (lane 3) three bands of M_r 43,000, 45,000, and 47,000 were also detected. As a control, a blot was prepared with ovarian protein prepared in the presence of phosphatase inhibitors (four times the amount used in Fig. 4.4) and then probed with the three times peptide absorbed antiserum, at a protein concentration that was 18 X higher than that used with the specific antibody. No bands were detected in this case, thus demonstrating the specificity of the antibody.

To determine if the M_r 45,000 and 47,000 bands in the late morulae sample were due to phosphorylation of Cx43, samples of 600 late morulae and 670 4-cell embryos were lysed and incubated with alkaline phosphatase. Figure 4.5 shows that this treatment did not alter the mobility of the single M_r 43,000 band in the 4-cell sample (lane 1) suggesting that it represents unphosphorylated Cx43. The phosphatase treatment of the late morulae lysate resulted in a loss of both the M_r 45,000 and 47,000 bands but retention of the M_r 43,000 band (lane 2) indicating that the upper two bands represent phosphorylated forms of Cx43. As before, a single M_r 43,000 band was also detected

Figure 4.5 The change in Cx43 mobility is due to phosphorylation. A Western blot containing lysates from 4-cell embryos (670; lane 1), late morulae (600 embryos; lane 2), and whole ovary (lanes 3 and 4) were probed with a Cx43 specific antibody. The lysate in lane 3 was prepared in the presence of phosphatase inhibitors but the lysates in lanes 1, 2, and 4 were dephosphorylated with alkaline phosphatase prior to electrophoresis. A single M_r 43,000 band was detected in all three dephosphorylated lysates. Several bands in the M_r range of approximately 42,000 to 49,000 were detected in the ovarian lysate prepared in the presence of phosphatase inhibitors, but because this lane was overloaded the M_r of individual bands cannot be estimated accurately. The M_r X 10^{-3} of marker proteins are indicated on the left.


1
2
3
4
66
45
36
29
24

in the dephosphorylated ovarian protein sample (lane 4) and several bands can be seen in the ovarian protein sample prepared in the presence of phosphatase inhibitors (lane 3). In this case, accurate M_r estimates of the various bands in lane 3 could not be made because of protein overloading. According to a nomenclature scheme proposed by Musil et al. (1990b) the M_r 43,000, 45,000, and 47,000 bands will be referred to as Cx43-NP, Cx43-P_1, and Cx43-P_2, respectively. These results indicate that Cx43 becomes phosphorylated between the 4-cell stage and the late morula stage.

4.3.3 Effects on dye coupling of inhibiting both the third and fourth rounds of DNA replication

As part of the protocol for these experiments it was necessary to inhibit both the third and fourth rounds of DNA replication. It was therefore important to begin aphidicolin treatment before the initiation of DNA synthesis at the 4-cell stage. Two features of the cell cycles in early mouse embryos made this difficult. First, there is considerable asynchrony in the timing of developmental events within a population of embryos. Second, the G_1-phase of the third cell cycle lasts only about 1 hour (Smith and Johnson, 1986; Pratt, 1987). If one initiated drug treatment too early in an attempt to target this phase, complications could arise because of inhibition of the preceding round of DNA synthesis in some embryos. The experimental protocol was designed to try to avoid these potential problems. First, by initiating drug treatment only on embryos that were already divided or dividing, one could be confident that the preceding S-phase was

complete. Second, by selecting embryos at hourly intervals one could be fairly certain that new DNA synthesis had not been initiated yet.

Both control- and aphidicolin-treated embryos were tested for dye coupling at a time when age-matched, control embryos had become fully compacted (79.5-86.5 hours post-hCG). Since both drug-treated and control embryos were tightly compacted, it was difficult to determine the cell number. This problem was solved by DAPI staining the embryos. Counting of DAPI stained nuclei showed that the DMSO-treated controls were mostly 8-cell embryos (mean number of nuclei = 8.7, $\underline{n}$ = 17) whereas the aphidicolin-treated embryos remained as 4-cell embryos (mean number of nuclei = 4.3, $\underline{n}$ = 28). This is in agreement with Smith and Johnson (1985) and shows that 4-cell embryos cultured continuously in aphidicolin do not undergo further cell divisions, but do become compacted. When the drug-treated, 4-cell compacted, embryos were tested for dye coupling, most were found to be fully dye coupled (Fig. 4.6; Table 4.2) and no difference in dye coupling was observed between control and drug-treated embryos (Table 4.2). Thus inhibiting both the third and fourth rounds of DNA replication did not prevent gap junctions from assembling at the normal time.

4.3.4 Effects on dye coupling of delaying the second round of DNA replication by 10 hours

The protocol for the initiation of drug treatment used in these experiments was adopted for essentially the same reasons that were outlined for the previous experiment

Figure 4.6 Inhibition of both the third and fourth rounds of DNA replication does not affect the initiation of gap junction assembly. Embryos were tested for dye coupling after being treated continuously with aphidicolin from the early 4-cell stage (53-56 hours post-hCG) until age-matched controls formed compacted morulae (79.5-86.5 hours post-hCG). The embryos remained as 4-cell embryos. These embryos became compacted as can be seen in the phase-contrast image in (A) and they also became dye coupled as the fluorescence images in (B,C, and D) show. Scale bar = 25 μm.

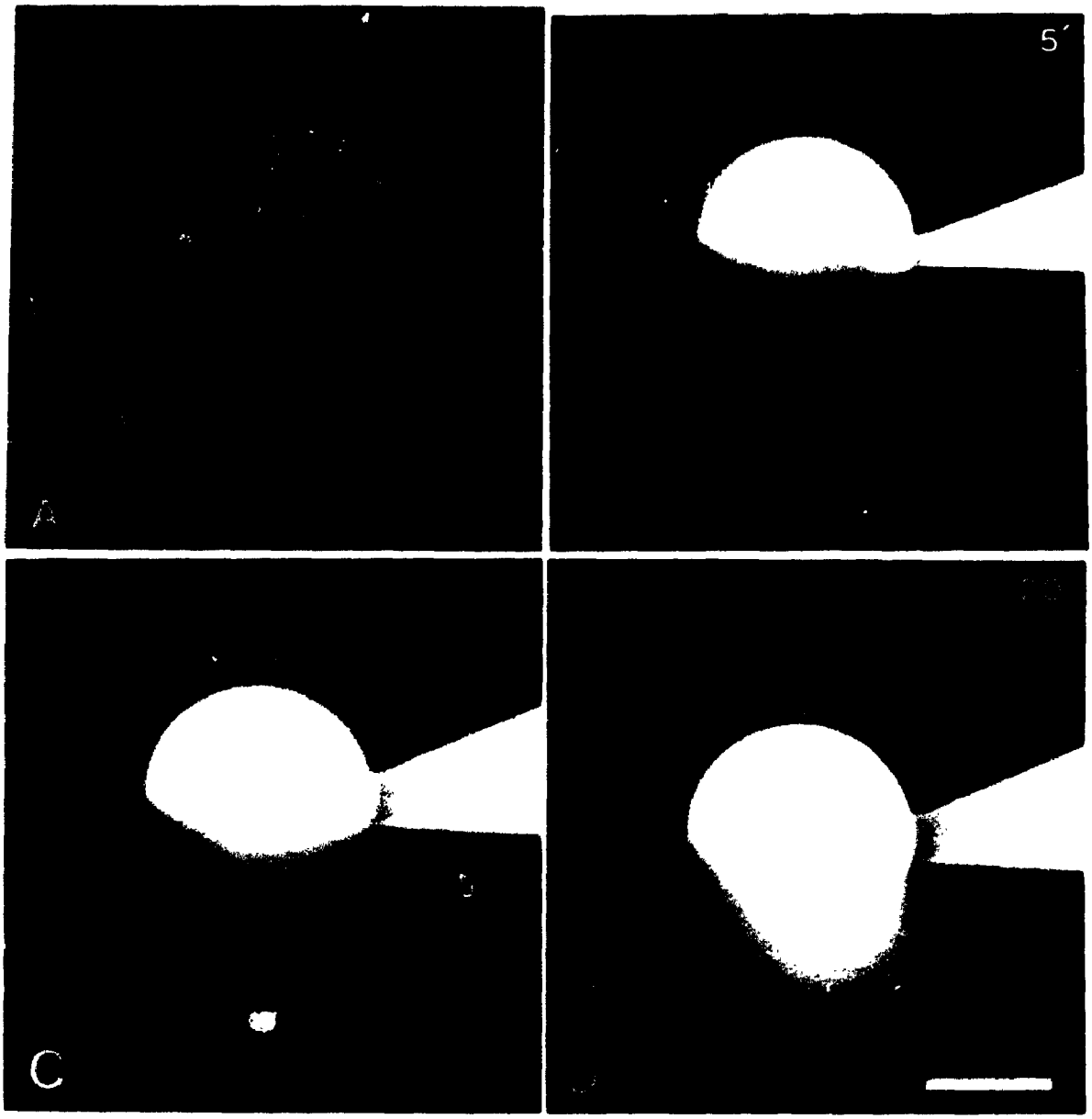

A
5′
C

Table 4.2
Incidence of dye coupling in embryos cultured continuously in aphidicolin from the early 4-cell stage (53-56 hours post-hCG) to the compacted 8-cell stage (79.5-86.5 hours post-hCG).

Treatment	Dye coupled (%)	Not dye coupled (%)
Control (n = 14)	13 (92.9)	1 (7.1)
Aphidicolin (n = 16)*	14 (87.5)	2 (12.5)

* Data in this treatment condition were collected in three separate test sessions. The percentage of dye coupled embryos exceeded 83% in each session.

(section 4.3.3). The G_1-phase of the second cell cycle is also of about 1 hour duration (Pratt, 1987).

Embryos treated with aphidicolin for 10 hours, beginning at the early 2-cell stage, remained as 2-cell embryos at the end of the culture period (after about 40 hours in drug-free medium). At this time most control embryos had formed compacted morulae, whereas about half of the aphidicolin-treated 2-cell embryos were compacted (Fig. 4.7A) and the other half were uncompacted (Fig. 4.8A).

Dye coupling tests revealed that the spread of dye was severely restricted in aphidicolin-treated 2-cell embryos (approx. 20% coupled) compared to controls (over 90% coupled; Table 4.3). Delaying the second round of DNA replication by 10 hours is therefore sufficient to inhibit the establishment of gap junctional coupling. More detailed examination of the data (Table 4.3) revealed that the few aphidicolin-treated embryos that were dye coupled were also compacted (Fig. 4.7). However, not all compacted, aphidicolin-treated 2-cell embryos were dye coupled, in fact only about one half of them were (Table 4.3). Conversely, dye coupling was never observed in aphidicolin-treated 2-cell embryos that did not compact (Table 4.3; Fig. 4.8).

4.3.5 Effects on Cx43 distribution of delaying the second round of DNA replication by 10 hours

To assess whether delaying the second round of DNA replication by 10 hours had an effect on the spatial distribution of Cx43, aphidicolin-treated 2-cell embryos were

Figure 4.7 Delaying the second round of DNA replication does not always inhibit compaction and a few embryos can even become dye coupled. Embryos were treated with aphidicolin for 10 hours during the early 2-cell stage (beginning 30.5-34.5 hours post-hCG). The embryos were then transferred into drug-free medium and cultured until age-matched controls formed compacted morulae (79-88 hours post-hCG), at which time both were tested for dye coupling. The drug-treated embryos remained as 2-cell embryos. About half of the embryos compacted (A; phase-contrast) and about half of those that compacted were also dye coupled (B, C, and D; fluorescence). Scale bar = 25 μm.

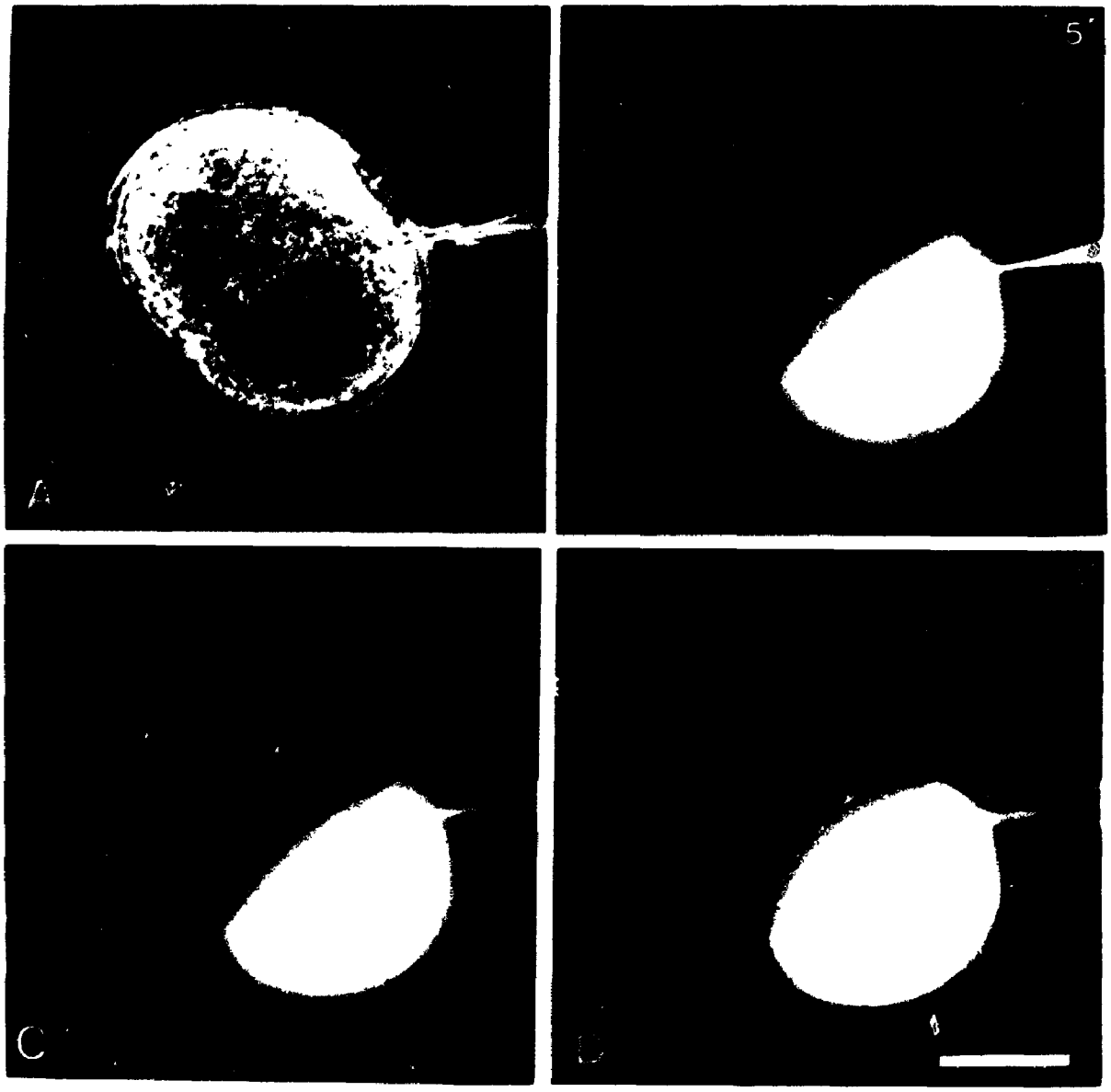

A
B
C
D

Figure 4.8 Delaying the second round of DNA replication inhibits compaction and the initiation of gap junctional communication. Embryos were treated with aphidicolin for 10 hours during the early 2-cell stage (beginning 30.5-34.5 hours post-hCG). The embryos were then transferred into drug-free medium and cultured until age matched-controls formed compacted morulae (79-88 hours post-hCG), at which time both were tested for dye coupling. The drug-treated embryos remained as 2-cell embryos. About half of the embryos did not compact (A; phase-contrast) and most of the embryos were not dye coupled (B, C, and D; fluorescence). Scale bar = 25 μm.

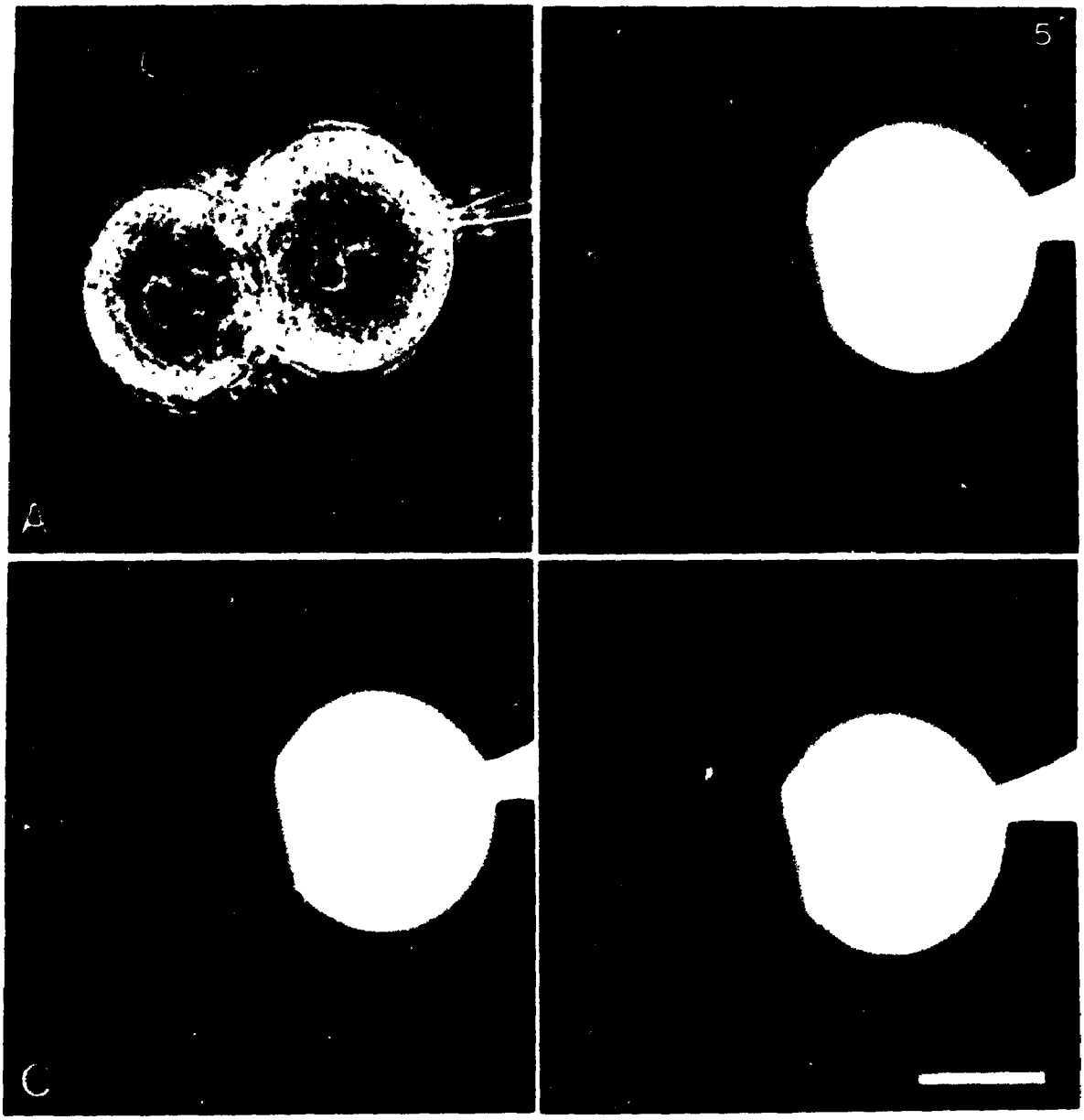

A
5'
C

Table 4.3
Incidence of dye coupling in embryos treated with aphidicolin for 10 hours at the 2-cell stage (beginning at 30.5-34.5 hours post-hCG). Dye coupling tests were done at 79-88 hours post-hCG.

Treatment	Dye coupled (%)	Not dye coupled (%)
Control (n = 28)	27 (96.4)	1 (3.6)
Aphidicolin (n = 26)*	5 (19.2)	21 (80.8)
Compacted (n = 12)	5 (41.7)	7 (58.3)
Non-compacted (n = 14)	0 (0.0)	14 (100.0)

* Data in this treatment condition were collected in five separate test sessions. A single dye coupled embryo was observed in each session.

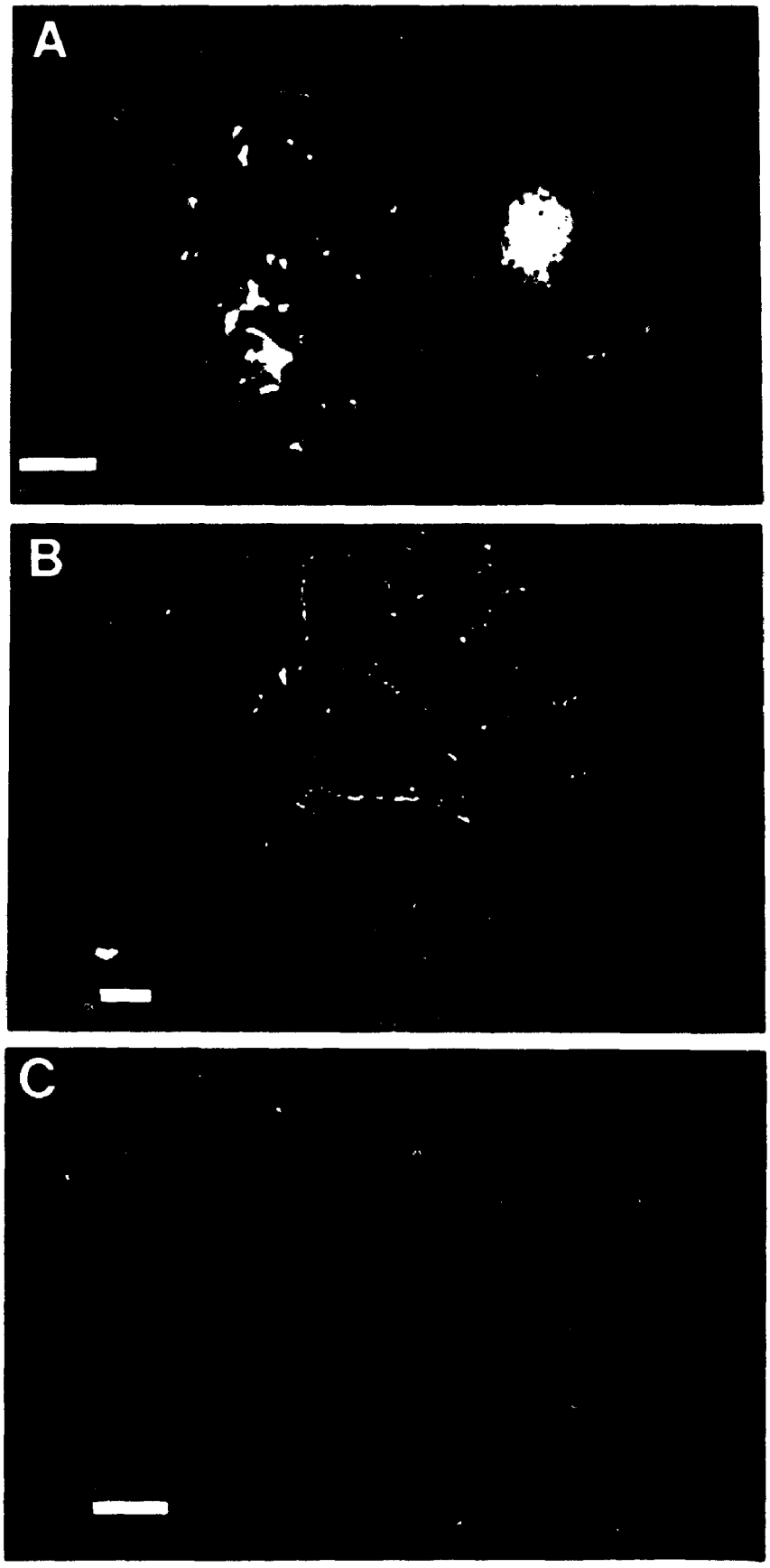
immunofluorescently labeled with an antibody specific for Cx43. Embryos were scored for the presence of both cytoplasmic and interblastomeric staining. The staining pattern seen in the control embryos was essentially as described by Nishi et al. (1991), Valdimarsson et al. (1991), and De Sousa et al. (1993); i.e. most embryos displayed both extensive cytoplasmic staining, and punctate interblastomeric staining suggestive of gap junction plaques (Table 4.4; Fig. 4.9B). On the other hand, while cytoplasmic staining was detected in the majority of the aphidicolin-treated 2-cell embryos as well, interblastomeric staining was very rare in these embryos (Table 4.4; Fig. 4.9A). No difference was observed in the staining pattern between compacted and non-compacted aphidicolin-treated 2-cell embryos (Table 4.4). In many, but not all, cases the cytoplasmic staining in drug-treated embryos was qualitatively different from that observed in controls. In control embryos, cytoplasmic immunofluorescence was generally localized to numerous small, diffuse foci (Fig. 4.9B), whereas in some of the aphidicolin-treated embryos a portion of the immunoreactivity was seen in large, cloud-like areas (Fig. 4.9A). None of the staining patterns described were ever seen when peptide-absorbed antiserum was used in place of the affinity-purified antibody (Fig. 4.9C).

Table 4.4
Pattern of Cx43 immunoreactivity in embryos treated with aphidicolin for 10 hours at the 2-cell stage (beginning at 30.5-34.5 hours post-hCG). The embryos were fixed at 85 hours post-hCG.

Treatment	Cytoplasmic staining (%)	Intercellular plaque staining (%)
Control (n = 31)	27 (87.1)	28 (90.3)
Aphidicolin (n = 27)*	21 (77.8)	3 (11.1)
Compacted (n = 14)	10 (71.4)	2 (14.3)
Uncompacted (n = 13)	11 (84.6)	1 (7.7)

* Data in this treatment condition were collected in three separate experiments. Two or fewer embryos showed intercellular plaque-like staining in each experiment.

Figure 4.9 The effect on the Cx43 staining pattern of delaying the second round of DNA replication by 10 hours. Embryos were treated with aphidicolin for 10 hours during the early 2-cell stage (beginning 30.5-34.5 hours post-hCG). The embryos were then transferred into drug-free medium and cultured until age matched controls formed compacted morulae (85 hours post-hCG), at which time both were fixed, labeled with a Cx43 specific antibody, and viewed on the confocal microscope. The drug-treated embryos remained as 2-cell embryos and contained considerable cytoplasmic Cx43 immunoreactivity (A). Punctate interblastomeric staining indicative of gap junction plaques was rarely seen. Control, DMSO-treated, embryos displayed both extensive cytoplasmic and punctate interblastomeric staining (B). No staining was observed in control embryos stained with 3X peptide absorbed antibody (C). Scale bar = 10 μm.


A
B
C

4.4 Discussion

4.4.1 The role of PKC in gap junctional communication in mouse embryos

The fact that activation of PKC at the 4-cell stage induced premature compaction but not gap junction assembly reinforces once again the idea that the initiation of these two events is independently regulated. Although these results clearly indicate that PKC activation is not the immediate trigger for gap junction assembly, they do not necessarily rule out a role for this kinase in gap junction assembly at the 8-cell stage. It should be pointed out, however, that presently there are no direct indications of such a role. Hypothetically, if PKC does play a role in the initiation of normal gap junction assembly, then there are at least two potential reasons why its activation in the present study failed to trigger assembly. First, the normal substrate for PKC phosphorylation may not be available prior to the 8-cell stage. This is based on the assumption that the initiation of gap junction assembly at the 8-cell stage is controlled by a cascade of regulatory events, one step of which is regulated by PKC protein phosphorylation. It may simply be that the prerequisite steps for PKC phophorylation in this hypothetical cascade have not yet taken place at the 4-cell stage. Second, the PKC substrate may be present but physically unavailable for phosphorylation, either because it is sequestered within a cellular compartment not accessible to PKC or because its conformation is such that the phosphorylation site is inaccessible. In fact, there is some experimental support for this latter possibility. Results from immunological studies with three different, peptide

specific, Cx43 antibodies indicate that the Cx43 molecule undergoes a conformational change at the time of gap junction assembly in 8-cell mouse embryos (De Sousa et al., 1993). Hence, it is conceivable that Cx43 itself might be a substrate for PKC but that, prior to compaction, its conformation is such that phosphorylation is blocked. This idea could be tested by treating early 8-cell embryos with PKC inhibitors, followed by dye coupling tests. This scheme, however, does not answer the question of what the time-keeping mechanism is, since it still requires an explanation for the timing of the Cx43 conformational change.

These results also bring into question the notion that functional gap junctions are required for the maintenance of compaction. Winkel et al. (1990) observed that the compaction induced by diC_8 in 4-cell embryos was maintained for at least 2.5 hours in the presence of the analog. In the present study, dye coupling was found to be absent for up to 1.5 hours after diC_8 induced compaction had become manifest. Admittedly, compaction in this case may be qualitatively different from the compaction that normally takes place at the 8-cell stage, but these results suggest that compaction can be maintained in the absence of coupling, at least in the short term (i.e. 1.5 hours). Alternatively, it is possible that during normal development PKC is a link between gap junctional coupling and compaction; when PKC is artificially activated, gap junctional coupling is no longer required for the maintenance of compaction.

Treatment of 8-cell compacted embryos with diC_8 led to a dose-dependent decrease in coupling, suggesting that once gap junctional communication is established, it can be downregulated by PKC phosphorylation. A similar dose-dependent decrease

in intercellular coupling has been observed with other PKC activators in cultured cells (Enomoto and Yamasaki, 1985; Gainer and Murray, 1985; Brissette et al., 1991; Berthoud et al., 1992; Reynhout et al., 1992). A complicating factor in the interpretation of these results is the lack of data on the time course of downregulation. Dye coupling measurements were made 0.5-2.0 hours after initiating treatment, but data were not collected on the length of treatment for individual embryos. If, relative to the testing period, downregulation was rapid and transient, or developed slowly, then it is possible that the number of embryos scored as non-coupled did not truly reflect the actual level of inhibition achieved. This may have been a contributing factor in that full inhibition of coupling was not observed. That this could be the case is suggested by a study by Gainer and Murray (1985) in which they tested dye coupling in cultured epidermal cells at several time points after the administration of a DAG analog at two different concentrations. At the lower concentration, inhibition was about 50% at 20 minutes and was completely relieved by 1.5 hours. At the higher concentration, the inhibition was over 90% at 20 minutes but at 2 hours it was down to about 20%. If, in the present study, the response of embryos to diC_8 is similar to this, then that could explain why the level of inhibition achieved was not more pronounced. On the other hand, Brissette et al. (1991) found that activation of PKC in keratinocytes with a phorbol ester led to phosphorylation of Cx43 and downregulation of dye coupling; the extent of phosphorylation, the length of time Cx43 phosphorylation was maintained, and the level of dye coupling inhibition were all concentration- and time-dependent. In this case, maximal inhibition was not observed until after 2 hours of treatment; at 1 hour only 60-

65% inhibition was observed, which is a similar level of inhibition to that seen with 200 μM diC_8 in the present study. This type of situation could therefore also explain the present results.

Another factor that may have influenced the results is the presence of multiple connexins. Preliminary results suggest that connexins other than Cx43 are present in preimplantation embryos (G.M. Kidder and P.A. De Sousa, personal communications). It is not known whether these other connexins are modulated by PKC. If they form gap junctions and are not downregulated by PKC then one would expect a portion of the dye coupling in these embryos to remain refractory to diC_8 treatment.

A general problem with experiments utilizing agents such as DAG analogs is the lack of specificity in the response. DAG analogs activate PKC specifically, but PKC is a modulator of numerous cellular processes which makes it very difficult to draw firm conclusions as to the level at which an observed PKC regulation is exerted and its specificity. What the results from the current experiments do indicate is that activation of PKC at the 4-cell stage is not sufficient for the initiation of gap junction assembly but that downregulation of coupling, once established, may be mediated by a process involving PKC.

4.4.2 Does Cx43 phosphorylation play a role in mouse embryo gap junction assembly?

The Western blot results suggest that Cx43 is phosphorylated in mouse preimplantation embryos and that this phosphorylation occurs between the late 4-cell

stage and the late morula stage. The identification of Cx43 as a phosphoprotein in the current study , based on the following criteria: 1) The antibody used in the Western analysis is specific for Cx43 as demonstrated in this study and by Naus et al. (1992) and De Sousa et al. (1993); all three bands are therefore detected because of specific binding of the antibody to bona fide Cx43. 2) Dephosphorylation removes both Cx43-P_1 and P_2, but does not affect Cx43-NP. 3) The estimated M_r of the three Cx43 bands correspond to published estimates in which Cx43-NP has been shown to be a non-phosphorylated form of Cx43, and Cx43-P_1 and -P_2 have been shown to be phosphorylated forms of Cx43 (Crow et al., 1990; Musil et al., 1990a,b; Musil and Goodenough, 1991; Laird et al., 1991; Brissette et al., 1991; Berthoud et al., 1992).

Although it might be argued that the inability to detect a Cx43-P_1 band in the 4-cell sample on the Western blot shown in figure 4.4 is due to the fact that less protein was loaded onto this lane than the late morulae lane, examination of the Western blot shown in figure 2.6 suggests that this is not the case. Lysates from an equal number of embryos were loaded onto this blot and while two bands are visible in the post-compaction samples only a single band, corresponding to the lower band in the post-compaction samples, was visible in the 4-cell sample.

The change in phosphorylation status of Cx43 between the non-coupled 4-cell stage and the coupled late morula stage is reminiscent of the situation described by Musil and her colleagues (Musil et al., 1990a,b; Musil and Goodenough, 1991), in which the phosphorylation status of Cx43 correlates with its location in the biosynthetic pathway. Their research indicated that in communication competent cells, Cx43 is synthesized and

transported to the plasma membrane as Cx43-NP. At the plasma membrane Cx43-NP is converted to the Cx43-P_1 and P_2 forms by phosphorylation and this conversion is associated with assembly into functional gap junction plaques. It has not been determined if Cx43-P_1 has a function separate from Cx43-P_2 or if it is simply an intermediary in the phosphorylation of Cx43-NP to Cx43-P_2. Interestingly, they found that in at least some communication-deficient cells, Cx43 is synthesized at levels comparable to those seen in the communication-competent cells, and that it is transported to the plasma membrane. However, in these cells the Cx43 is not phosphorylated (it remains as Cx43-NP) and is not assembled into gap junction plaques. While the Western blot results from the present study are consistent with this scheme, several lines of evidence suggest that the situation in mouse embryos is somewhat different. First, immunofluorescence studies with four different Cx43 antibodies have failed to detect Cx43 in the plasma membrane of embryos prior to compaction (Nishi et al., 1991; Valdimarsson et al., 1991; De Sousa et al., 1993). Second, 4-cell embryos or their isolated blastomeres are unable to form gap junctions when they are paired with communication-competent embryos (Goodall and Johnson, 1982; McLachlin et al., 1983). Third, experiments with protein trafficking inhibitors suggest that the assembly of gap junctions at compaction is dependent on one or more intracellular trafficking steps (De Sousa et al., 1993). Based on this, one can hypothesize that during the 4-cell and early 8-cell stages, newly synthesized Cx43 accumulates in an intracellular trafficking compartment, as Cx43-NP. At the time of compaction the Cx43-NP becomes phosphorylated to Cx43-P_1 and -P_2 and is transported to the plasma membrane where it is assembled into functional gap junction plaques in

areas of membrane contact. To test this hypothesis, the timing of the phosphorylation event would need to be narrowed down and the spatial distribution of the various forms of Cx43 would need to be determined.

Recent evidence suggests that the cell adhesion molecule uvomorulin behaves in a manner that is in some aspects analogous to the behavior of Cx43 as outlined in the hypothesis described above. Prior to compaction, uvomorulin is distributed uniformly in the plasma membrane surrounding each blastomere but it becomes redistributed to areas of membrane contact during both normal compaction (Johnson et al., 1986; Vestweber et al., 1987) and premature compaction induced with PKC activators (Winkel et al., 1990). This redistribution of uvomorulin at compaction has been shown to be temporally correlated with its phosphorylation (Sefton et al., 1992). Furthermore, several other, unidentified proteins have been shown to become phosphorylated at the time of compaction (Bloom and McConnell, 1990). Thus there is considerable evidence for a global change in the activity of protein kinase(s) around the time of compaction. These studies indicate that the aforementioned hypothesis on the temporal control of gap junction assembly in 8-cell embryos, is at least plausible.

The present results demonstrating that the Cx43 in embryos is phosphorylated strongly suggest that the doublet of polypeptides detected on the Western blot in CHAPTER 1 (Fig. 2.6, section 2.3.2) arose because of phosphorylation. On that blot the band detected in the intercalated disc preparation migrated more slowly than either of the bands detected in the embryo samples. This is consistent with the finding that Cx43 in adult heart gap junction plaques is highly phosphorylated, with most of the

protein in the Cx43-P_2 form (Laird and Revel, 1990; Laird et al., 1991). The size and relative position of the two bands in the embryo samples indicates that they correspond to Cx43-NP and -P_1, which is consistent with the results presented here (Fig. 4.4). The faint Cx43-P_2 band detected in the late morulae sample in the present chapter was probably not detected in previous embryo samples because of partial dephosphorylation. This partial dephosphorylation may have been due to endogenous phosphatases which, in the absence of added phosphatase inhibitors, were active during embryo lysis.

4.4.3 The role of DNA replication in the temporal control of mouse embryo gap junction assembly

The present results demonstrate that the third and fourth rounds of DNA replication are not required for the initiation of gap junctional communication in 8-cell embryos. Conversely, a 10 hour delay in DNA replication at the 2-cell stage leads to a failure to initiate gap junctional communication when these embryos reach the chronological age of 8-cell compacted embryos. Because the immunofluorescence results indicate that Cx43 levels are similar in these and normal 8-cell compacted embryos the failure to initiate gap junctional communication must arise at the post-translational level. Furthermore, the lack of punctate interblastomeric staining suggests that the underlying cause of the communication failure is an inability of Cx43 to assemble into gap junction plaques, perhaps because it is not transported to the plasma membrane.

These results parallel the findings of Smith and Johnson (1985) on the effects of DNA replication on compaction. They found that inhibition of the third and fourth rounds of DNA synthesis had no effects on compaction but that a 10 hour delay of the second round inhibited compaction. It thus appears that it is critical that the second round of DNA replication take place within a specified window of time for both compaction and gap junction assembly (and perhaps other developmentally significant events) to be initiated two cell cycles later. This type of time-keeping mechanism may not be restricted to mouse embryos because a similar mechanism has been shown to operate in the nematode C. elegans. In C. elegans embryos, the development of two biochemical markers (carboxylesterase and gut granules) in cells belonging to the gut cell lineage is dependent on DNA synthesis during the first cell cycle (8-cell stage) after the gut cell lineage is clonally established (Edgar and McGhee, 1988). The transcription necessary for the development of these markers takes place one to three cell cycles after the critical DNA replication cycle, and the markers are not expressed until the embryos contain 50-150 cells.

One discrepancy between the current results and those reported by Smith and Johnson (1985) is that, while they observed a nearly complete inhibition of compaction in embryos in which the second round of DNA replication had been delayed by 10 hours, the current results show that about half of the embryos in this group compacted. One can only speculate on the possible reasons for this difference. Perhaps these embryos managed to escape the inhibitory effect of aphidicolin, at least to a degree sufficient for them to become compacted and for dye coupling to develop in some of them. How this

escape might have occurred is difficult to say, but it is conceivable for instance, that the G_1-phase of the second cell cycle is shorter in the mouse strain used in the present study (CF_1 X $CB6F_1/J$) compared to the strain (F_1LAC X HC-CFLP) used by Smith and Johnson (1985). If this were the case then it is possible that some of the embryos had already entered S-phase when aphidicolin treatment was commenced and partial DNA replication could have taken place to an extent that was sufficient for compaction to develop. That there are strain differences in the length of cell cycle-related events has been suggested in the literature (Molls et al, 1983; Smith and Johnson, 1986). It is also suggested by the observation that, relative to hCG injection, first cleavage occurs about 1 hour earlier in CD_1 X CBF_1/J embryos than in CF_1 X CBF_1/J embryos (unpublished observations by the author). Moreover, the fact that the drug did inhibit cleavage in all of the embryos demonstrates that the failure to inhibit compaction is not simply due to inactivation of the aphidicolin.

The results presented here make it possible to exclude two more potential time-keeping mechanisms as being responsible for the timing of gap junction assembly. The fact that embryos treated with aphidicolin during the first 10 hours of the second cell cycle undergo a catch-up round of DNA synthesis when they are released from the drug and then arrest DNA synthesis permanently (Smith and Johnson, 1985) suggests that neither the absolute number of DNA replication cycles nor a critical nucleocytoplasmic ratio are required for the proper timing of gap junction assembly. Because of the catch-up DNA replication these embryos have undergone the same absolute number of DNA replication cycles (2) and therefore have the same nucleocytoplasmic ratio as have the

embryos that were treated continuously with the drug from the early 4-cell stage. Since this latter group of embryos initiated gap junctional communication normally, one can conclude that neither the absolute number of DNA replication cycles nor the nucleocytoplasmic ratio are important determinants of the temporal control of gap junction assembly.

The inhibition of gap junction assembly observed in the aphidicolin-treated 2-cell embryos could be due to such non-specific effects as: an inability of 2-cell or non-compacted embryos to assemble gap junctions; inhibition of the activation of the zygotic genome; or non-specific toxicity of the drug. These non-specific effects are unlikely however, since: 1) inhibition of compaction has been shown not to interfere with the initiation of gap junction assembly (Goodall, 1986; Kidder et al., 1987); 2) experiments with cytochalasin show that inhibition of cytokinesis from the 1-cell stage onward does not alter the timing of gap junction assembly (Kidder et al., 1987); 3) aphidicolin inhibition of DNA synthesis at the 2-cell stage does not affect the activation of the embryonic genome (Bolton et al., 1984); and 4) 4-cell embryos treated continuously for about 25 hours with aphidicolin initiated gap junctional communication at the normal time, whereas 10 hour treatment of 2-cell embryos led to inhibition of same. It is thus clear that the initiation of gap junction assembly at the 8-cell stage is dependent on the timing of the second round of DNA replication, in a specific manner.

How does the timing of the second round of DNA replication control the initiation of gap junction assembly, two cell cycles later? One can only speculate on the mechanism responsible because, in inhibitor studies such as the present one, the data do

not allow firm conclusions to be drawn about events beyond the immediate target of the inhibitor (which in this case was DNA replication). However, the immunofluorescence data indicate that, at least in relation to Cx43, the control is exerted post-translationally, perhaps at the level of transport to the plasma membrane or assembly into plaques. Edgar and McGhee (1988) came to a similar conclusion regarding the development of the two gut cell lineage markers in C. elegans embryos, i.e. the control of marker appearance by DNA replication was not found to be exerted directly at the level of expression of the marker structural genes. What the present results suggest is that an interaction of some kind between the cytoplasm and the DNA must take place at the 2-cell stage, and that this interaction requires DNA replication to take place at the normal time. If a mismatch is introduced between the DNA and the cytoplasm, e.g. by delaying DNA replication, a window of opportunity is missed in which the interaction could take place and the initiation of subsequent developmental events is compromised. Perhaps, a cytoplasmic "clock" that is already running interacts with the replicating DNA in such a way as to set in motion a regulatory cascade that does not require further DNA synthesis to operate, and that takes two cell cycles to run down, culminating in the assembly of gap junctions. Indeed, an autonomous cytoplasmic or zygotic "clock" has been reported to operate in 1- and 2-cell mouse embryos (Waksmundzka et al., 1984; Poueymirou and Schultz, 1987, 1989; Manjewala et al., 1991; Wiekowski et al., 1991). Alternatively, there may be a cytoplasmic factor, presumably maternally supplied, which carries out the same function as the hypothetical cytoplasmic "clock". The nature of the interaction between this putative cytoplasmic "clock" or factor and the DNA might

involve some sort of gene-modifying activity that can only occur during the major chromatin reorganization that takes place at the 2-cell stage (McGrath and Solter, 1984; Howlett et al., 1987; Wiekowski et al., 1991), in preparation for activation of the embryonic genome. The steps in the proposed regulatory cascade are unclear but presumably the penultimate step would be activation of a protein kinase leading to the final step of Cx43 phosphorylation and gap junction assembly. It is interesting to note in this regard that the antibody used in the present study does not normally detect cytoplasmic Cx43 in pre-compaction embryos, presumably because the epitope it recognizes is unavailable for binding (De Sousa et al., 1993). The "maturation" of Cx43 that normally takes place at compaction is therefore not dependent on the second round of DNA synthesis and is not sufficient for gap junction assembly to occur.

4.4.4 Conclusions

The results presented in this chapter demonstrate that activation of PKC is not sufficient for the induction of premature gap junction assembly. However, PKC may be involved in the gating of gap junctions once they are formed at the 8-cell stage. The initiation of gap junction assembly appears to be associated with phosphorylation of Cx43 and its timing is linked with DNA replication at the 2-cell stage. While it is not clear how the second round of DNA replication is involved in the initiation of gap junction assembly, the control point seems to be post-translational, perhaps involving phosphorylation of Cx43.

CHAPTER 5

GENERAL DISCUSSION

5.1 Summary and Conclusions

In this thesis a series of studies was undertaken to explore gap junctional intercellular communication in early mouse development. This involved studies of connexin gene expression during oogenesis and preimplantation embryogenesis (CHAPTER 2), studies aimed at elucidating the role of Cx43 gene expression in gap junctional communication in embryos (CHAPTER 3), and studies into the mechanisms controlling the assembly of connexins into gap junction plaques (CHAPTER 4).

The experiments described in CHAPTER 2 demonstrated that both Cx32 and Cx43 are transcribed during oogenesis but that shortly after ovulation, both transcripts become undetectable. Cx43 mRNA was detected again at the 4-cell stage and the abundance of this transcript then increased exponentially to the blastocyst stage. In contrast, Cx26 and Cx46 transcripts were not detected in preimplantation embryos. On the protein level, Cx43 was found to be present throughout the preimplantation period; its levels decreased sharply between the 1-and 2-cell stages and then gradually increased again to the blastocyst stage.

Based on these results and other results discussed in CHAPTER 2 (Barron et al., 1989; Nishi et al., 1991; Valdimarsson et al., 1991; De Sousa et al., 1993; Kidder, 1993; Valdimarsson et al., 1993) the following model of connexin gene expression

during early mouse development was constructed (model 1). The Cx32 and Cx43 mRNAs transcribed during oogenesis are translated in the oocyte and the protein products may participate in the formation of gap junctions with the cumulus granulosa cells which surround the oocyte. Shortly after ovulation, the two transcripts have been destroyed, but the proteins remain, perhaps in the form of internalized cumulus-oocyte gap junctions. Most of this Cx43 is lost by the two cell stage but the Cx32 persists at least to the late morula stage. Concomitant with the general activation of the zygotic genome at the 2-cell stage Cx43 transcription is reinitiated, and the abundance of Cx43 mRNA increases thereafter at an approximately constant rate on a per cell basis. Cx43 begins to accumulate again by the early 4-cell stage and is assembled into gap junction plaques beginning at compaction. Because the persistent oogenetic Cx32 does not contribute to gap junctions, the gap junctions in the preimplantation mouse embryo must be composed primarily of Cx43 (or other connexins not yet investigated).

In CHAPTER 3 the hypothesis that Cx43 is the connexin primarily responsible for gap junctional communication in the embryo was tested in experiments designed to selectively inhibit Cx43 gene expression. For these experiments the novel antisense technology was employed and embryos were either microinjected with the Cx43 antisense ODNs or cultured in their presence. However, the results proved inconclusive as the Cx43 ODNs did not appear to affect gap junctional coupling or Cx43 mRNA levels.

The experiments in CHAPTER 4 showed that activation of PKC in 4-cell embryos does not lead to premature initiation of gap junctional communication, despite its acceleration of cell flattening. However, activation of PKC in 8-cell compacted embryos

led to downregulation of gap junctional communication. Inhibition of both the third and fourth rounds of DNA replication had no major effect on the timing or extent of intercellular coupling. However, a 10 hour delay in the second round of DNA replication blocked the acquisition of gap junctional communication in most embryos. This interference with the onset of coupling was not associated with a noticable inhibition of Cx43 accumulation but the assembly of Cx43 into gap junction plaques appeared to be impaired. In addition, Cx43 was found to become phosphorylated, in vivo, between the late 4-cell stage and the late morula stage.

If the assumption that gap junctions in preimplantation embryos are composed mainly of Cx43 is correct, the results described in CHAPTER 4, together with observations from other laboratories (see sections 4.1.1, 4.4.2, and 4.4.3), would be consistent with the following model concerning the control of gap junction assembly (model 2). During S-phase of the second cell cycle, an interaction takes place between a cytoplasmic "clock" and the replicating DNA. This interaction sets in motion a regulatory cascade or a second cytoplasmic "clock" which counts down to the early 8-cell stage. Also, during the 2-cell stage Cx43 gene expression is reinitiated as a part of the general activation of the embryonic genome. Cx43, in the form of Cx43-NP, accumulates continuously during the 4- and early 8-cell stages, probably in intracellular trafficking organelles. At the early 8-cell stage the regulatory cascade or cytoplasmic "clock" activates a protein kinase, which in turn phosphorylates Cx43-NP to the Cx43-P_1 and -P_2 forms. This is the trigger for the protein to be transported to the plasma

membrane, where it then spontaneously assembles into gap junction plaques allowing gap junctional communication to be initiated.

5.2 Questions for Future Research

The results on connexin gene expression and the temporal control of gap junction assembly presented in this thesis extend and clarify the current understanding of these features of early mouse development. The results and the models derived from them are also important in that they suggest future directions for research in this area of preimplantation mouse embryology. For instance, it is clearly necessary to establish firmly whether connexins other than Cx43 are expressed in the embryo. Initially, this could be done relatively quickly using RT-PCR to screen for connexin transcripts. However, as exemplified by Cx32, the absence of a particular mRNA does not necessarily mean that its protein product is absent as well. Conversely, the presence of a mRNA does not necessarily mean that its protein product is present, i.e. there may be tight translational regulation. It will also therefore be important, eventually, to screen for the connexin proteins, but since immunological probes are not yet available for all the known connexins this has to be considered a long term project.

If connexin genes other than Cx43 are found to be expressed in preimplantation embryos it would be valuable to determine if these connexins contribute to functional gap junctions (this still needs to be ascertained for Cx43), and to establish the relative contribution each makes to intercellular coupling. These questions are not easily

answered and, despite the inconclusive outcome of the antisense experiments described in this thesis, the antisense approach still provides the most promise for attacking questions of this nature. To increase the probability of success in such experiments it might be advisable to use a series of overlapping ODNs, spanning most of the sequence of the mRNA. Using this approach, one could possibly "find" a region in the target mRNA that is available for hybridization. The probability of success might also be enhanced by using some of the newer designs for ODNs. For example, Dagle et al. (1990) reported that ODNs that had three to four internucleotide linkages on each end modified as phosphoramidate linkages, but that retained the internal linkages as phosphodiester bonds, were much less vulnerable to degradation than unmodified or S-ODNs but were still very effective mediators of RNase H cleavage of the target mRNA.

Another area in need of further study involves the interactions between the cytoplasm and the replicating DNA at the 2-cell stage. For example, is the cytoplasmic "clock" reported to operate in 1- and 2-cell embryos (Waksmundzka et al., 1984; Poueymirou and Schultz, 1987, 1989; Manjewala et al., 1981; Wiekowski et al., 1991) the same cytoplasmic "clock" proposed in model 2, above? What is the nature of the "clock"? What is the nature of the interaction between the "clock" and the replicating DNA and what exactly is the outcome of this interaction? Few clues are available for answering these questions except perhaps that the "clock" responsible for the general activation of the embryonic genome at the 2-cell stage requires protein phosphorylation by PKA (Poueymirou and Schultz, 1987, 1989; Manjewala et al., 1991).

The final steps in the assembly of gap junctions around the time of compaction also need to be examined in more detail. For instance the exact timing of the initial Cx43 phosphorylation event has yet to be determined, i.e. does it coincide with compaction and the initiation of gap junctional communication. Whether the Cx43 becomes phosphorylated while still in an intracellular compartment or after it reaches the plasma membrane remains to be experimentally demonstrated. What is the identity of the protein kinase responsible for the phosphorylation, and how is its activity regulated? Finally, and most importantly, does phosphorylation of Cx43 trigger its assembly into gap junctions? These questions will provide fertile ground for research for years to come.

Appendix I

Estimates of ODN dose

A) Sample calculations for second set of microinjection experiments (CHAPTER 3, section 3.3.1):

1) From a graph in Kidder (1993), depicting the abundance of various transcripts through preimplantation development, it can be determined that a late 8-cell embryo (approximately 76 hours post hCG) contains approximately 30,000 Cx43 transcripts. Since the embryos in this set of experiments were injected at the early 4-cell stage, one half of the 8-cell blastomeres will contain ODNs. There are therefore approximately 15,000 potential target mRNAs.

2) What volume of 0.8 μg/μl ODN needs to be microinjected to match the 15,000 target mRNAs?

a) 1 μg of antisense ODN 2 = 188 pmol

b) The molarity of the injection solution is therefore:

188 X 10^{-12} moles/μg X 0.8 μg/μl $\approx$ 1.5 X 10^{-10} moles/μl.

c) To obtain the number of ODN molecules per μl, multiply b) by Avogadro's number:

$1.5 \times 10^{-10} \times 6.022 \times 10^{23} \approx 9 \times 10^{13}$ ODN molecules/μl

d) Volume of c) needed to inject 15,000 molecules of Cx43 antisense ODN 2:

15,000 molecules $\div 9 \times 10^{13}$ molecules/μl $= 1.67 \times 10^{-10}$ μl $= 0.167$ fl.

3) Estimate of actual volume injected. During injections the injection solution frequently formed a bubble at the pipette tip, presumably due to surface tension or because a membranous compartment had been entered. These bubbles did not disperse until the injection needle was withdrawn or until the table top was gently tapped. This allowed measurements of the diameter of these bubbles to be taken with the aid of an ocular micrometer. By making the assumption that these bubbles were perfect spheres, estimates of volume injected could be made.

a) Average bubble radius (r) was 3.0 μm

b) Average bubble volume (V) according to the formula $V = 4/3\pi(r)^3$:

$4/3\pi(3)^3 \approx 113$ $\mu m^3 = 113$ fl.

The conversion from μm^3 to fl is based on the following:

$1\ cm^3 = 1 \times 10^{12}$ μm^3 and $1\ cm^3 = 1 \times 10^{-3}$ l

4) Excess volume injected (equal to molar excess)

113 fl $\div$ 0.167 fl $\approx$ 680 X

These particular estimates pertain to the second set of microinjection experiments. These estimates can be used to further estimate the range of doses used.

Example 1. Low end of range from first set of experiments. Concentration of ODNs was approximately 6 X lower than in the second set of experiments (0.13 μg/μl vs. 0.8 μg/μl) whereas the number of target molecules was one half (because these embryos were injected at the late 4-cell stage). The excess volume injected in this case is therefore 680 X 2/6 $\approx$ 230 X

Example 2. High end of range from third set of experiments. Concentration of ODNs was approximately 2 X higher than in the second set of experiments whereas the number of target molecules was the same. The excess volume injected in this case is therefore 680 X 2 = 1360 X

B) Culture experiments.

Assuming that the uptake kinetics for the S-ODNs were similar to those observed by Rappolee et al. (1992) then each embryo contains approximately 10^6 antisense molecules at the end of a 24 hour culture period. With 30,000 Cx43 mRNA molecules per embryo the molar excess of ODNs is $10^6 \div 30{,}000 \approx 35$ X

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