A Study of the Expression and Function of Differentiation Inhibiting Activity and its Receptor in the Early Mouse Embryo

By Jennifer Nichols

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DECLARATION

Unless stated otherwise, all the work described in this thesis was carried out by J. Nichols.

ABSTRACT

In this thesis the maintenance of the pluripotential stem cells of the early mouse embryo is investigated. Differentiation Inhibiting Activity (DIA) is a cytokine able to prevent the differentiation of pluripotential embryonic stem (ES) cells *in vitro* when added to the medium in picomolar concentrations. In this study, the mRNA for DIA and the components of its receptor are shown, by *in situ* hybridisation, to be present in the murine blastocyst. DIA is expressed primarily in the trophectoderm, the first tissue to differentiate in the embryo, whereas the expression of its receptor is predominantly restricted to the inner cell mass (ICM). This suggests a paracrine interaction between the trophectoderm and the ICM such that the differentiated trophectoderm produces DIA which binds to the receptors on the cells of the ICM to maintain their pluripotential state. The effects of DIA on the developing embryo were investigated using ES cells which had been mutated to produce increased amounts of DIA and injected into blastocysts to generate chimaeras. The resulting abnormal phenotypes are consistent with perturbation of the stem cell pool, and indicate that the cells in the early developing embryo are responsive to DIA. These results are considered in the light of recent findings that homozygous DIA-deficient embryos are capable of development to term, but that homozygous females cannot support implantation.

Alternative factors which may play a role in stem cell maintenance have been investigated. Likely candidates are interleukin-6 (IL-6), oncostatin M (OSM) and ciliary neurotrophic factor (CNTF), since, in common with DIA, their receptors utilise the signal transducing subunit gp130. ES cell lines could be derived with high efficiency in medium supplemented with IL-6 or OSM, but less effectively with CNTF. However, no significant expression has been seen in early embryos by *in situ* hybridisation using probes for IL-6, CNTF or CNTF receptor. The possible existence of another factor(s) which utilises a separate signal transduction pathway is considered.

CONTENTS

CHAPTER 1

INTRODUCTION

Early Mammalian Embryology

The entire body of a mouse is formed by a series of divisions from a single cell. Some of these divisions are differentiative, producing lineage-restricted cells. As specific tissues are formed, some cells are maintained in a pluripotent state. This thesis is concerned with how these cells are prevented from undergoing spontaneous differentiation, so that a pool of stem cells is available for the growth of the embryo and the future formation of tissues.

All of the cells in embryos up to the 8 cell stage are totipotent, and can give rise to all tissues of the resulting animal when transferred to foster mothers alone or with the use of carrier blastomeres (Kelly, 1977). From the 16 cell stage the outermost cells of the morula begin to become committed to the trophectoderm lineage in response to their peripheral position (Tarkowski and Wroblewska, 1967). They acquire tight junctions as the embryo divides, and begin to pump fluid from the environment, leading to the formation of a cavity, the blastocoel, which characterises this stage as the blastocyst. A clump of undifferentiated cells remains attached to the trophectoderm at the polar region. These cells constitute the inner cell mass (ICM), and will give rise to the embryo proper and various extra-embryonic tissues, including the parietal and visceral endoderm, the visceral mesoderm, the amnion and the allantois over the next few days. Immediately before implantation a second extra-embryonic tissue, the primitive endoderm,

differentiates on the blastocoelic surface of the ICM. The undifferentiated cells in the embryonic region are now known as the primitive ectoderm or epiblast (fig. 1.1a).

Implantation of the embryo is characterised by the initiation of a decidual swelling, which is produced by the uterus. This can be induced artificially by trauma or the administration of air or mineral oil to the uterus of a hormonally-responsive female mouse (Parkes, 1929; Orsini, 1963; Finn, 1965). The deciduum persists beyond mid-gestation, but it becomes thinner as the conceptus grows. Between implantation and gastrulation the structure within the deciduum is known as an egg cylinder. The cells of the primitive ectoderm remain undifferentiated until they have passed through the primitive streak, when they transform into mesoderm. By the end of gastrulation at 8.5 days *post coitum* (dpc) the primordial germ cells are the only totipotent cells remaining in the embryo, although many tissues possess multipotent stem cells, which generate new cells in a more lineage-restricted manner (fig. 1.1b).

Prior to the completion of gastrulation the developing embryo possesses a considerable capacity for regulation. During preimplantation stages removal of blastomeres (Kelly, 1977), or the newly-differentiated trophectoderm (Handyside, 1978; Hogan and Tilly, 1978; Rossant and Tamura-Lis, 1979; Spindle, 1978; Nichols and Gardner, 1984) can still result in the development of a normal individual. A normal foetus may still develop even following ablation of more than 80% of the embryonic cells at the egg cylinder stage (Snow and Tam, 1979). The factors which govern the ability to regulate the overall size and maintain the appropriate proportions of

Fig. 1.1 Diagrammatic representation of early mouse embryology

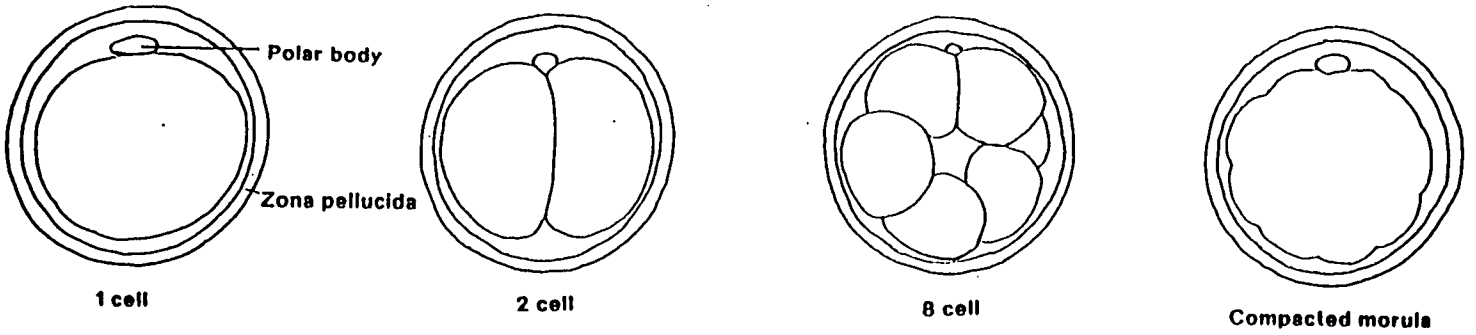
a. Preimplantation (sizes approximately proportional)

b. Postimplantation until completion of gastrulation

Pluripotential stem cells are shaded blue

Fig. 1.1a

PREIMPLANTATION DEVELOPMENT



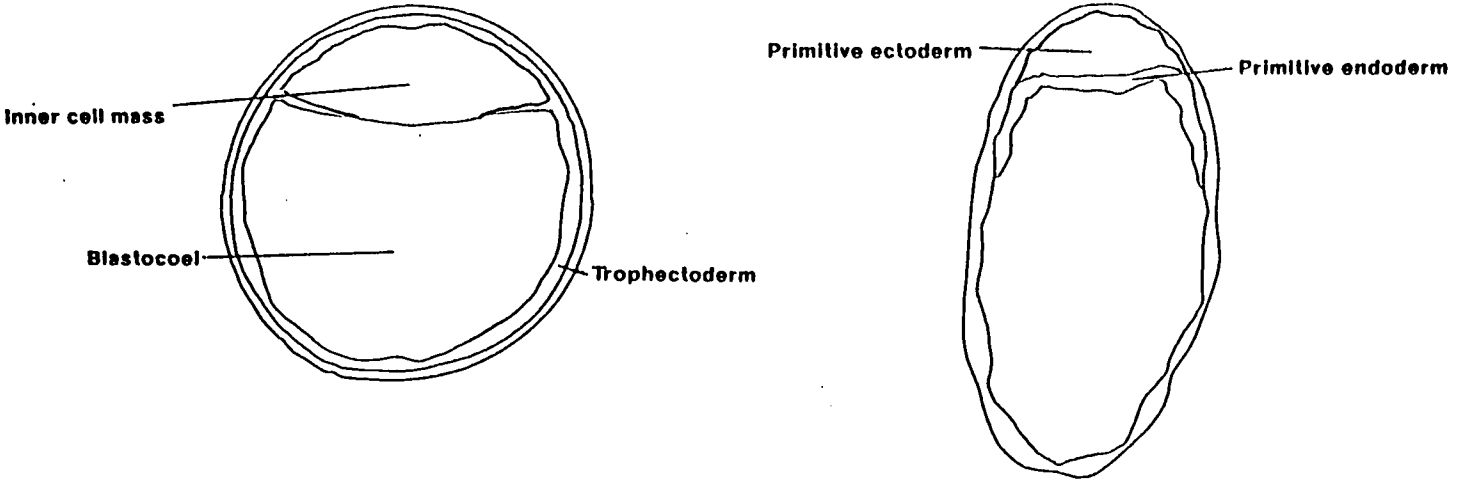
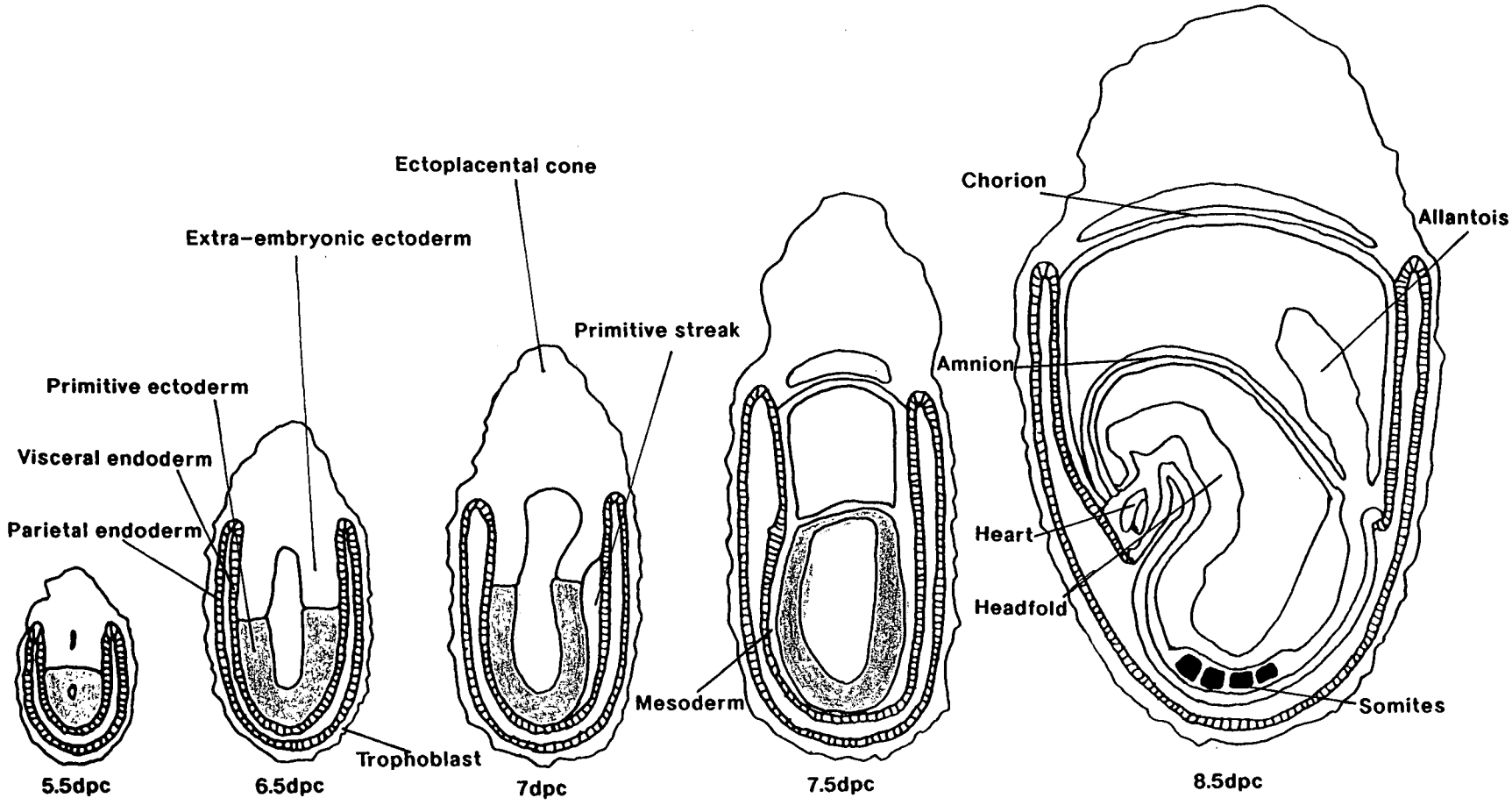


Fig. 1.1b

POSTIMPLANTATION DEVELOPMENT



each cell type in the embryo have not yet been identified. The regulation of the stem cell populations is likely to be an important aspect of this flexibility during embryonic development.

Grafting blastocysts, ICMs or the epiblast from egg cylinders under the testis or kidney capsule of adult mice can result in the growth of teratocarcinomas, which are tumours composed of various tissue types and undifferentiated malignant stem cells with an unlimited proliferative capacity and a tendency to metastasize (Stevens, 1970; Solter et al., 1970). The stem cells of these tumours can differentiate into a wide range of tissues when presented with appropriate conditions in culture (Martin et. al., 1977), or following injection into blastocysts (Evans, 1972; Bernstein et al., 1973; Brinster, 1974; Martin and Evans, 1975; Mintz and Illmensee, 1975; Papaioannou et al., 1975), or aggregation with morulae (Stewart, 1982; Fuji and Martin, 1983). Such embryonal carcinoma (EC) cells have been derived from most inbred strains of mouse, but greater success has been achieved when particular strains, notably the 129 inbred strain, are selected for the host (Damjanov et al., 1983). The reason for this is not entirely clear, but the increased ability to support teratocarcinoma formation may be connected with the high tendancy for 129 mice to develop spontaneous testicular tumours. EC cells are multipotential stem cells which will remain undifferentiated under certain conditions. There are several EC cell lines which have been used for various applications. The properties of the individual lines tend to vary; the most notable differences are the range of tissues to which each has the potential to give rise, and the ability or inability of each to propagate the stem cell phenotype

in the absence of a feeder cell layer (Martin and Evans, 1975). Although colonisation of most somatic tissues has been achieved with some EC cell lines in chimaeras, contribution to the germline has been reported rarely (Stewart and Mintz, 1981).

Embryonic Stem Cells

More recently, it has been possible to derive embryonic stem (ES) cells directly from blastocysts or ICMs in culture (Evans and Kaufman, 1981; Martin, 1981). ES cells are morphologically indistinguishable from EC cells in their undifferentiated state; they are small cells with a high nuclear to cytoplasmic ratio which grow in culture as compact colonies. ES cells are thought to represent the cells of the ICM (Beddington and Robertson, 1987) or the primitive ectoderm of early embryos (Smith, 1992). ES cells have provided an invaluable means of studying some of the physiological, biochemical and genetic aspects of early embryos. The equivalent material from embryos *in utero* is relatively inaccessible and available only in small quantities. Within the last few years it has become possible to manipulate the genome of ES cells. Specific alleles can be removed or inactivated by means of homologous recombination (Lin et al., 1985; Smithies et al., 1985; Thomas and Cappecchi, 1986; 1987) and the resulting mutant ES cells can be injected into blastocysts to generate chimaeras which can then be bred to homozygosity. In this way, the functions of genes may be investigated by association with any phenotype which may arise from the ablation of individual gene

activity. Multiple mutations can be created by crossing animals carrying null alleles for different loci, so that genes able to compensate for one another may be identified, and gene interactions investigated. Gene trapping has recently become extensively employed as a means of identifying the genes important for specific phenomena. This technique depends upon integration into random genes using a visibly detectable marker and screening for expression patterns and phenotypes in the ES cells and the resulting chimaeras or their offspring (Gossler et al., 1989; Friedrich and Soriano, 1991; Skarnes et al., 1992).

ES cells maintained under optimal conditions in culture have a cell cycle time of 18 to 24 hours (Robertson 1987). The cells in the ICM divide approximately every 12 hours, whereas in the epiblast the cells are estimated to cycle every 4 to 5 hours (Snow, 1976; 1977; Poelman, 1980). Presumably, for incorporation into chimaeras, ES cells decrease the duration of their cell cycles. Several characteristic markers, such as SSEA-1, alkaline phosphatase and Oct 4 are expressed by ICM, epiblast and ES cells (Solter and Knowles, 1978; Tam and Snow, 1981; Snow and Monk, 1983; Schöler et al., 1990). Like the cells of the ICM or epiblast, ES cells can give rise to all somatic tissues and germ cells in chimaeras when injected into blastocysts after many generations *in vitro* (Bradley et al., 1984). However, the ES cell lines in general use to date have been derived from only a few strains of mice, namely the 129 and C57Bl/6 strains. The reason why the stem cells of other strains have proved somewhat resistant to propagation in culture remains obscure.

Originally, to maintain ES cells in an undifferentiated state, it was necessary to grow them on a layer of feeder cells such as mitotically-inactivated fibroblasts (Martin and Evans, 1975). Subsequently, it was found that medium conditioned by Buffalo Rat Liver (BRL) cells obviated the requirement for the feeder layer (Smith and Hooper, 1987). From this conditioned medium a glycoprotein, differentiation inhibiting activity (DIA) was purified, and found to be capable of suppressing the differentiation of ES cells when added to the culture medium in picomolar concentrations (Smith et al, 1988).

Differentiation Inhibiting Activity

DIA is a glycoprotein of 40-45kDa, of which the core protein is approximately 21kDa. It is now known to be equivalent to the haemopoietic cytokine leukaemia inhibitory factor (LIF), a molecule able to induce macrophage differentiation in M1 murine myeloid leukaemic cells (Tomida et al., 1984). LIF has been used independently to maintain the pluripotential state of ES cells in culture (Williams et al, 1988). LIF was cloned from a T lymphocyte cDNA library (Gearing et al, 1987).

DIA has many other diverse actions. It is involved in induction of the acute phase response of hepatocytes (Baumann and Wong, 1989); it inhibits the differentiation of renal cells in kidney rudiments (Bard and Ross, 1991) and of preadipocytes (Mori et al, 1989). It maintains viability and proliferation of haematopoietic

progenitors (Leary et al., 1990; Fletcher et al., 1990) , primordial germ cells (De Felici and Dolci, 1991; Matsui et al., 1991) and sensory neurones (Murphy et al., 1991), while it induces the trans-differentiation of sympathetic neurones (Yamamori et al., 1989). It inhibits bone resorption in foetal long bones (Lorenzo, et al., 1990), but stimulates bone resorption in foetal calvaria (Abe et al., 1986). *In vivo* DIA is thought to play a role in the action of osteoblasts and in calcium metabolism. Irradiated mice engrafted with haematopoietic cells producing high levels of DIA exhibit increased amounts of new bone formation, calcification of the heart and skeletal muscle and abnormalities of the pancreas, thymus, adrenal cortex and ovaries and cachexia (Metcalf and Gearing, 1989). Injection of DIA into adult mice produced various effects, depending upon the dose and frequency of injection. At the most severe, loss of body fat, thymus atrophy and behavioural differences were observed. The haematopoietic system was affected in several ways. An increase in the numbers of various haematopoietic progenitor cells, including megakaryocyte and platelet precursors was observed (Metcalf et al., 1990). A cell line, SEKI, isolated from a human melanoma which was associated in the patient with severe cachexia, was found to produce a protein identified as DIA, which was responsible for the cachectic condition (Mori et al., 1989). Thus, DIA serves many diverse functions and can act on a variety of tissue types. The nature of the activity of DIA in a given context is likely to be dictated by the target cell. DIA is known to bind to specific receptors which are present on responsive cells (Smith et al., 1988;

Williams et al., 1988; Hilton et al., 1988; Allan et al., 1990; Hilton et al., 1991).

Involvement of DIA in Embryonic Development

A factor produced by cells derived from embryos which can maintain stem cells in culture may be considered as a candidate for stem cell maintenance in embryos *in vivo*. To address this question, the expression of mRNA for DIA has been investigated in egg cylinder stage embryos, when totipotent stem cells are known to be abundant (Rathjen et. al., 1990b). Embryos of 6.5 and 7.5dpc were, therefore, dissected into embryonic and extra-embryonic regions to ascertain the presence of DIA mRNA, and its approximate localisation. This was performed by RNase protection, which is a very sensitive technique, able to detect as few as 1-2 transcripts per cell (Robertson et al., 1993). Sensitivity is an essential prerequisite for this application, since DIA is active at such low concentrations, and therefore, unlikely to be very abundant. The requirement for relatively large amounts of tissue for this procedure would demand prohibitively high numbers of preimplantation embryos, and it is primarily for this reason that the analysis has not been carried out on blastocysts.

The expression of DIA mRNA has been detected in the extra-embryonic regions of 6.5 and 7.5dpc egg cylinders (Rathjen et. al. 1990b). There is a high proportion of differentiated tissue in the extra-embryonic region when compared with the embryonic part.

The level of expression of DIA mRNA decreases from 6.5 to 7.5dpc in the egg cylinder, and is negligible by 8.5dpc. This coincides with the depletion of the pluripotent stem cell pool which occurs in the embryo over this period. The observation that DIA is expressed in such tissues *in vivo* is somewhat reminiscent of previous reports on ES cells grown in the absence of DIA. The characteristic differentiated cells in culture produce relatively high levels of DIA, which results in foci of ES cells retaining the stem cell phenotype (Rathjen et al., 1990b). At low cell density, differentiation of the ES cells tends to proceed to completion without added DIA. The differentiated cells which arise from cultures of ES cells are not, however, morphologically similar to the cells of the extra-embryonic region of the egg cylinder.

In situ hybridisation is a technique which has been adopted for examining the expression of genes in various organisms, initially using *Drosophila* (Levine et. al., 1983; Akam, 1983). A labelled probe binds to that part of an organism in which the mRNA is being produced. The expression pattern of the gene thus revealed may aid in the elucidation of the function of the gene product. *In situ* hybridisation has been used widely in the detection of abundant transcripts, but to date it has been seldom applied to genes expressing mRNA in very small amounts. An exception to this is the putative signalling molecule Wnt-1, which is expressed initially at low levels in certain neural tissues in the presumptive brain region of embryos from 8.5dpc (Wilkinson et al., 1987). The levels of DIA transcript present in egg cylinders estimated by RNAse protections (Rathjen et al.1990b) are at the predicted limits of sensitivity for *in*

situ hybridisation. However, for specific localisation of gene expression and for the study of very small embryos, it is the most appropriate technique currently available. Here, the expression of mRNA for the components of the DIA system are investigated in early embryos. Much effort was devoted to optimising the conditions to create maximum visualisation of signal over the non-specific background. No attempt has been made here to examine the distribution of DIA protein on embryos. This is because sufficiently sensitive labelled antibodies to detect the anticipated small amounts of protein are not available.

Effects of Ectopic Expression of DIA

Over-production of DIA in the bone marrow, spleen and lymph nodes of adult mice has been brought about by injecting haematopoietic cells transfected with a retroviral construct containing DIA cDNA into irradiated or unirradiated mice (Metcalf and Gearing, 1989). As a result, these mice developed a fatal syndrome, the symptons of which included cachexia, excess bone formation, calcification in skeletal muscle and heart, and abnormalities of pancreas, thymus, adrenal cortex and ovarian corpora lutea.

Some indication of the action of DIA in the embryo may be obtained by causing the production of DIA at inappropriately high levels and/or in ectopic sites during the early stages of development. This may be brought about by the introduction of additional DIA to the developing embryo in the form of ES cells which have been modified to produce ubiquitously relatively high levels of the

cytokine. Injection of these ES cells into the embryo is not carried out until the blastocyst stage, so the host embryo is likely to remain unaffected until after implantation. This means that subtle and specific effects can be sought in later embryos which will possess a wide variety of tissue types. The diffusible form of DIA is unlikely to be cell-autonomous. The response of the cells of the host embryo to the increased level of DIA may, therefore, result in an abnormal phenotype. This reaction may represent an amplification of the normal effect of DIA in the developing embryo. The interpretation of the phenotype of chimaeras may, therefore, provide some insight into the action of DIA in the unmanipulated embryo.

Embryos Without DIA

The requirement for the addition of DIA to ES cells in culture to maintain their undifferentiated phenotype, and the finding that DIA is expressed in a temporal and spatial pattern in the embryo consistent with a role in stem cell maintenance suggests that the removal of DIA from the environs of the embryo could seriously disrupt its development. Inactivation of the DIA locus in embryos has been accomplished in ES cells by several groups (Stewart et al., 1991; Escary et el., 1993; Smith et al., unpublished). A replacement strategy was used in which homologous recombination in ES cells substituted exogenous DNA sequences for one of the wild type DIA alleles, such that a truncated form of the DIA protein was produced (Stewart et al., 1991), or the whole of the coding region was removed

(Escary et al., 1993; Smith et al., unpublished) Germline chimaeras were generated from these cells and the offspring bred to homozygosity. Surprisingly, the null homozygous embryos were viable, but only if they were allowed to develop in a wild type or heterozygous mother. In the published studies no significant deviation was observed from the expected proportion of homozygous null individuals (Stewart et al., 1991; Escary et al., 1993), but subsequent unpublished results exhibit a tendency for the number of liveborn homozygous null pups to be significantly fewer than anticipated (10/181 observed to date, where the expected ratio would be 45/181, Smith et al., unpublished) Homozygous null females were incapable of supporting implantation. The implication from these results is that embryonic expression of DIA is not absolutely essential for the development of the early embryo in an unchallenged environment. However, DIA may be necessary after implantation, when it may be supplied to the developing embryo by secretion from the uterine endometrial glands (chapter 3, and see below), or via transplacental delivery. The possible existence of another factor or factors able to maintain stem cells in the embryo, particularly when DIA is absent, must be considered.

The Involvement of DIA in Implantation

The highest expression levels of DIA detected so far are seen in the uterus of the adult mouse (Bhatt et al., 1991; Robertson et al., 1993). In one study using Northern hybridisation the level of expression

was undetectable in non-pregnant animals, but was found to reach a maximum in the uteri of pregnant females just before implantation (Bhatt et. al. 1991). However, significant expression has been detected in the uteri of non-oestrous virgin females using the optimised RNase protection protocol (Robertson et al., 1993). The mRNA has been localised by *in situ* hybridisation to the uterine endometrial glands (Bhatt et al., 1991; Smith et al., 1992). The function of these glands is unknown. Female mice homozygous for the disrupted DIA allele are unable to support implantation without administration of exogenous DIA (Stewart et al., 1992). DIA, therefore, must be essential for implantation. Transfer of wild type blastocysts to DIA negative mothers failed to initiate any decidual response in the uterus, whereas the removal of DIA negative blastocysts to wild type or heterozygous recipients resulted in the birth of viable pups, suggesting that the DIA required for implantation must come from the mother (Stewart et al., 1992). Whether the DIA produced by the endometrial glands is utilised by the uterus to facilitate the decidual response or by the implanting embryo remains unclear.

Cytokines in Development

Cytokines are proteins synthesised by various producer cells in response to inducing stimuli. They bind to the receptors on specific target cells. Signal transduction across the plasma membrane then enables the target cell to respond to the cytokine. Among the

numerous roles of cytokines are the control of cell proliferation, differentiation and the resultant phenotype, processes essential during development. There are several classes of cytokines, defined broadly according to the function for which the members were initially identified. These classes include growth factors, transforming growth factors, tumour necrosis factors, interferons and lymphokines. DIA has been assigned to the family of colony stimulating factors (Clemens, 1991). Members of this class function mainly to regulate proliferation and differentiation of various types of haematopoietic cell. DIA fits into this class because of its characterisation as leukaemia inhibitory factor (LIF), which induces macrophage differentiation in M1 murine myeloid leukaemic cells (Gearing et al., 1987) DIA has been found to affect the haematopoietic system of mice when administered to mice (Metcalf and Gearing, 1989; Metcalf et al., 1990). Like DIA, many other cytokines are now known functionally to span several of the categories of current nomenclature. The pleiotropic nature of these proteins is becoming better understood now that the molecular structure and signal transduction pathways of their receptors are being unravelled. It is primarily the action of cytokines upon stem cell populations that has stimulated the interest here in their possible involvement in the regulation of early embryonic development.

Cytokine Receptor Complexes

The receptor for DIA belongs to the class 1 cytokine receptors, also refered to as the haematopoietin receptors. The main characteristics of this class are :

1. They possess no intrinsic enzymatic activities, such as kinase or phoshatase activity.
2. They have a structure in the extracellular domain of 14 anti-parallel β−strands, about 200 amino acids long. These fold into 2 barrel-like structures similar to the fibronectin type III domain (Patthy, 1990).
3. There is a conserved pattern of 4 cysteine residues in the amino-terminal domain.
4. A characteristic motif of Trp-Ser-X-Trp-Ser (where X is any amino acid) is present in the carboxy-terminal domain between the fibronectin type III modules. Mutations in this region greatly reduce the binding capability of the receptor (Yawata et al., 1993).

Other members of this class include many of the interleukins, ciliary neurotrophic factor, oncostatin M, erythropoietin, granulocyte colony-stimulating factor, growth hormone and prolactin. The membrane-proximal region of these receptors is recognised by members of the Jak-Tyk tyrosine kinase family (Stahl et al., 1994). The association is thought to occur in the absence of ligand, but kinase activation depends upon cytokine stimulation.

DIA Receptor

A low affinity cell surface receptor for DIA (K_d=1-3 nM; Hilton et al., 1991) was first isolated by expression cloning of cDNA from a human placental expression library in Cos cells (Gearing et al., 1991). The structure of this receptor is represented in fig.1.2. Its protein sequence is somewhat homologous to that of the signal transducer gp130, previously identified as part of the receptor complex for interleukin-6 (IL-6; Taga et al., 1989; Hibi et al., 1991), from the Trp-Ser-X-Trp-Ser motif of the membrane-proximal haematopoietin receptor domain to the C terminus. The greatest region of homology (65%) is seen in the transmembrane domain (Gearing et al., 1991). They both also exhibit the fibronectin type III-like conformation in the extracellular domain. High affinity binding (K_d=10-200pM; Yamamoto-Yamaguchi et al., 1986; Hilton et al., 1988; Williams et al., 1988; Rodan et al., 1990; Tomida et al., 1990), however, is brought about by dimerisation of the DIA-specific component with gp130. A soluble form of the DIA specific subunit is known to exist which binds DIA with an affinity equivalent to that of the non-diffusible form (Gearing et al., 1991).

Gp130 is involved in signal transduction. It is an essential component of the receptors for DIA, IL6, ciliary neurotrophic factor (CNTF) and oncostatin M (OSM). Cytokines identified more recently, such as IL-11, are also associated with gp130 (Yang et al., 1992; Yin et al., 1993). Each of the cytokines was identified independently, on the basis of distinct actions, but the involvement of gp130 in their receptors is probably responsible for the many over-lapping actions

Fig. 1.2 Diagrammatic representation of some of the receptors utilising the signal transducer gp130 and the current understanding of the operational conformation with their ligands

DIA=differentiation inhibiting activity

IL-6=interleukin-6

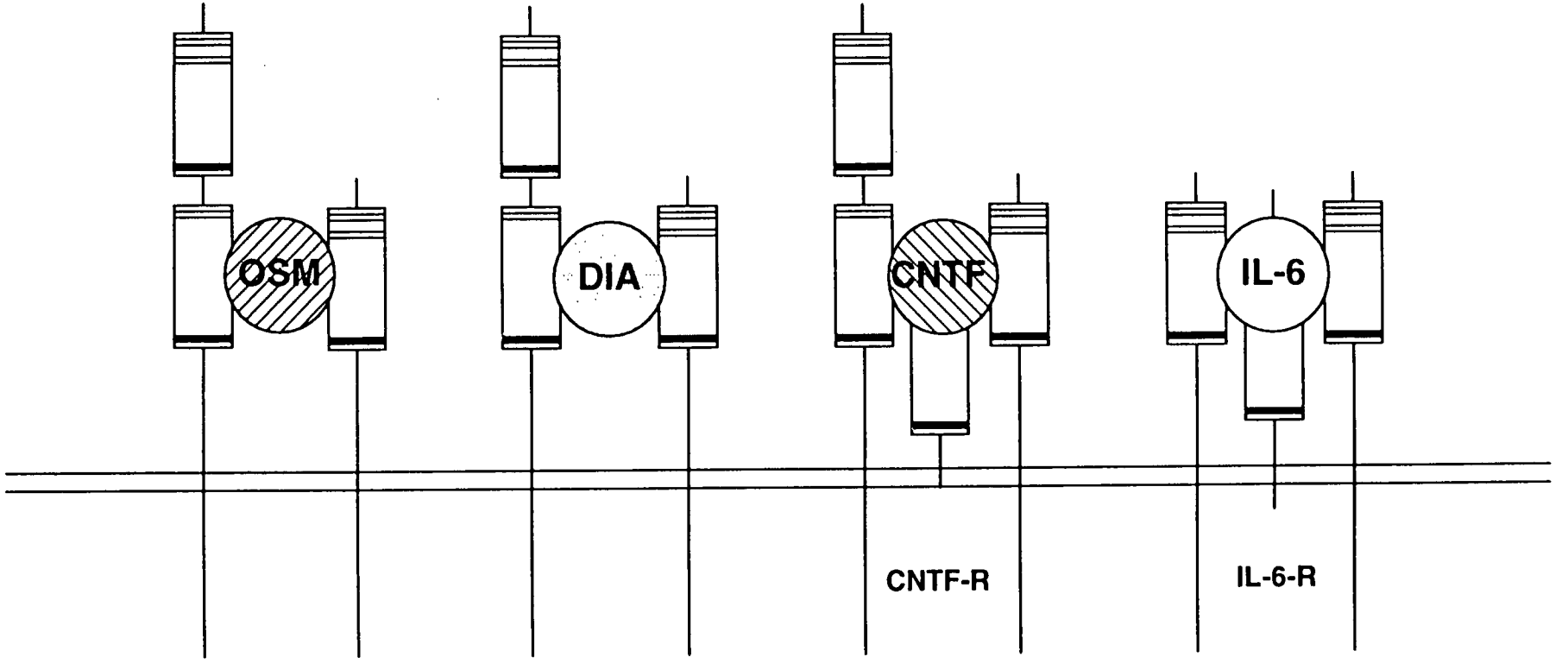
CNTF=ciliary neurotrophic factor

OSM=oncostatin M

Each rectangular box represents a fibronectin type III module. The thin parallel lines within each signify the conserved cysteine residues; the thick lines indicate the position of the Trp-Ser-X-Trp-Ser motif.

Fig.1.2

Composition of receptor complexes for DIA/LIF, IL-6, CNTF &OSM



of these factors. Of particular interest in the context of stem cell maintenance in the early embryo are the recent findings that IL-6, CNTF and OSM are able to substitute for DIA in the inhibition of differentiation of established ES cells in culture (Yoshida et al., 1994; Conover et al., 1994; Wolf et al., 1994; Rose et al., 1994).

Interleukin-6 and its Receptor

IL-6 was isolated initially as a factor which stimulates B cell differentiation (Hirano et al., 1986). It is now known to have many other biological functions, including T-cell activation (Takai et al., 1988; Uyttenhove et al., 1988), neuronal cell differentiation (Satoh et al., 1988; Hama et al., 1989), induction of acute phase response to injury and inflammation (Andus et al., 1987; Gauldie et al., 1987) and differentiation of haematopoietic cells of the myeloid lineage (Liebermann et al., 1982; Shabo et al., 1988; Ishibashi et al., 1989). As would be predicted from the wide variety of activities of IL-6, this cytokine is produced by many different cell types. These include T lymphocytes, monocytes/macrophages, fibroblasts and endothelial cells (Kishimoto, 1989). The action of IL-6 on its target cells is brought about by homodimerisation of gp130 in response to ligand binding to the IL-6-specific receptor component (Yamasaki et al., 1988; Taga et al., 1989; Hibi et al., 1990). This leads to the activation of a receptor-coupled tyrosine kinase (Murakami et al., 1993). A schematic representation of the IL-6 receptor complex is

shown in fig. 1.2. The expression patterns of the components of the IL-6 receptor have not yet been investigated.

Ciliary Neurotrophic Factor and its Receptor

CNTF was originally characterised because of its ability to support the survival of parasympathetic neurons from chick ciliary ganglia (Adler et al., 1979; Lin et al., 1989; Stöckli et al., 1989). It has been attributed more recently with involvement in the survival of a wide range of neurons (Skaper and Varon, 1986; Blottner et al., 1998; Ernsberger et al., 1989; Hoffman, 1988; Arakawa et al., 1990; Sendtner et al., 1990; Ip et al., 1991; Oppenheim et al., 1991; Martinou et al., 1992; Sendtner et al., 1992). High levels of CNTF have been demonstrated in adult rat sciatic nerve (Stöckli et al., 1989, 1991). More moderate expression levels of mRNA, detected by Northern hybridisation, were found in adult brain and muscle, and in the head region and skeletal muscle of 14dpc rat embryos; the earliest detectable CNTF mRNA is seen at 11dpc (Ip et al., 1993). One unusual feature of CNTF is the absence of a signal sequence, which means that the protein is confined to the cytoplasm, and is released only if the tissue becomes damaged. The receptor is believed to consist of the complex for the high affinity DIA receptor, with the addition of a CNTF-specific component (Ip et al., 1993; see fig.1.2). The CNTFRα subunit does not possess an intracytoplasmic region, but is bound to the cell membrane by means of a glycosyl-phosphatidylinositol linker, which suggests that it is not involved in signal transduction. Transcripts for this subunit are found by

Northern blotting of adult rat tissues to be restricted to the nervous system (Ip et al., 1993). *In situ* hybridisation to sections of rat embryos reveals that the expression of the CNTF-specific subunit is confined to presumptive neural tissues and derivatives of the neural crest (Ip et al., 1993).

Oncostatin M and its Receptor

Growth inhibition of human melanoma cells was the function which led to the initial identification of OSM (Zarling et al., 1986; Brown et al., 1987; Malik et al., 1989). Additional roles include macrophage differentiation of M1 cells (Horn et al., 1990) and induction of acute phase protein synthesis (Richards et al., 1992). So far, only a human form of OSM has been characterised. It is a 28kDa glycoprotein expressed in activated monocytic and lymphocytic cell lines and in normal adherent macrophages. Cloning of the murine form of OSM based on the available human sequence has proved difficult, suggesting that the homology between the two species is not great. Therefore, probes synthesised from the human form of OSM will not bind specifically to murine tissues. *In situ* hybridisation for OSM on mouse embryos will not be carried out until the murine form of OSM is available.

Human OSM has been found to bind with high affinity (K_d=10-1000pM) to the DIA receptor complex in certain murine culture systems such as hepatoma and haematopoietic cells (Linsley et al.,

1989; Gearing and Bruce, 1992; Baumann et al., 1993; Gearing et al., 1994). However, whereas DIA binds to the DIA-specific subunit, OSM is capable of binding only to gp130 (Lui et al., 1992; Gearing et al., 1992). The gene organisation and protein structure of OSM and DIA are similar. Interestingly, OSM has been found to map closely to DIA on human chromosome 22, suggesting evolutionary linkage of the two cytokines (Rose and Bruce, 1991; Giovannini et al., 1993). There is evidence for the existence of an OSM-specific receptor component, since certain cell lines have been found to bind OSM with high affinity, whereas the binding of DIA to these is undetectable (Linsley et al., 1989; Gearing and Bruce, 1992). This OSM-specific subunit is yet to be characterised.

ES Cells and Other Cytokines

The signal transducing component gp130, common to the receptor complexes of DIA, IL-6, CNTF, and OSM, is likely to be responsible for the ability of these cytokines to perform many of the same functions. ES cells can be maintained in culture in an undifferentiated state by the addition to the medium of concentrations of OSM or CNTF similar to those required for DIA (Conover et al., 1993). The IL-6-specific receptor subunit is available in a soluble form (sIL-6R), which is effective in IL-6 binding and induction of dimerisation of gp130 (Taga et al., 1989; Yasukawa et al., 1990). ES cells do not express the IL-6-specific receptor component (Saito et al., 1992). Therefore, sIL-6R must be added to the culture medium in addition to the ligand in order that the differentiation of ES cells in culture can be inhibited

(Yoshida et al., 1994). OSM efficiently maintains the undifferentiated state of ES cells in culture. This probably occurs via the DIA receptor complex, since this is present on ES cells. It is not known whether the OSM-specific receptor component is also used in this context.

In the viable homozygous DIA negative embryos recently described (Stewart et al., 1992) an alternative cytokine such as IL-6, CNTF or OSM may fulfil the role of differentiation suppression via the pathway normally invoked by DIA, since the receptor complex for each of these includes the signal transducer gp130. Inhibition of differentiation of the stem cells in the early embryo by these other cytokines, as in the derivation of ES cells from blastocysts *de novo*, would provide a means of demonstrating that the early mammalian embryo is able to respond to cytokines other than DIA. Also, it may be informative to show that the embryo is able to produce these cytokines and their receptors. In this study, these questions are addressed firstly by determination of the ability of each of these cytokines to substitute for DIA in the derivation of ES cell lines from blastocysts in culture, and secondly by using *in situ* hybridisation to investigate the presence in the early embryo of transcripts of mRNA for these proteins. The implication that other factors exist that are able to substitute for the action of DIA in stem cell maintenance in the mammalian embryo does not necessarily mean that this role is not normally fulfilled by DIA. A degree of duplication of function may be expected in a process so crucial for mammalian development.

Since the initiation of these studies, phenotypes for mice lacking IL-6 or CNTF have been established by homologous recombination in ES cells and breeding of the resulting chimaeras. In

neither case was an embryonic lethal phenotype observed. For the ablation of IL-6, the phenotype generated in one report is manifested only in female mice beyond the age of reproductive fertility, or following ovariectomy. An increase in the level of IL-6 in the circulation normally arises as a result of cessation of oestrogen production by the ovaries. This burst of IL-6 production is thought to be responsible for the bone loss associated with ovariectomy or the menopause. In IL-6-deficient mice no such hormonally-associated bone loss occurs (Poli et al., 1994). Another study revealed that the absence of IL-6 leads to impaired immune and acute phase responses on exposure of homozygous null individuals to infection or injury (Kopf et al., 1994). CNTF-deficient mice suffer progressive atrophy and loss of motor neurons when adult (Masu et al., 1993). The fact that viable animals can develop in the absence of either IL-6 or CNTF suggests that neither of these cytokines can be wholly responsible for stem cell maintenance in the developing embryo. If double mutants are generated which lack both DIA and one or both of these other factors, the resulting phenotype may be more informative. Alternatively, inactivation of the receptor complex, particularly the common gp130 subunit, may provide a means to ascertain the importance of the cytokines operating via this pathway in embryonic development. The possible existence of another means of stem cell maintenace operating in the early mouse embryo which utilises a different signal transduction mechanism may then be considered.

In summary, the aim of this project is to investigate the expression patterns of the genes for DIA and its receptor components

in the early mouse embryo to establish whether these are consistent with the possible involvement of DIA in stem cell maintenance and the regulation of early embryonic development. Attempts are also made to determine whether DIA exerts specific effects on the cells of the early embryo by exposing them to DIA at inapproriate levels and areas, and analysing the resulting phenotypes. Finally, the possible involvement of other cytokines which utilise the same signal transduction pathway as DIA in the maintenance of stem cells in the early embryo is investigated.

CHAPTER 2

MATERIALS AND METHODS

In Situ Hybridisation

SOURCE OF EMBRYOS FOR SECTIONS

Embryos to be used for *in situ* hybridisation were produced mostly from matings of random bred MF1 GPI-1a/a mice, and occasionally from inbred 129 Ola or C57Bl/6 strains. All mice were maintained on a 14 hour light / 10 hour dark regime and supplied with food and water *ad libitum.*

Stages of embryos are represented as number of days *post coitum* (dpc), assuming fertilisation to have occurred in the middle of the dark period preceding the discovery of the vaginal copulation plug. Post implantation embryos from 5.5 to 8.5dpc were retained in the uterus, which was dissected from the animal and rinsed in phosphate buffered saline (PBS; Sigma) prior to overnight fixation at 4°C in 4% paraformaldehyde (PFA; Fisons) in PBS. Pre-implantation embryos of up to 2.5dpc were flushed from the oviduct; 3.5dpc, 4.5dpc and implantation-delayed embryos from the uterus, with phosphate buffered medium 1 (PB1; Whittingham and Wales, 1969). For ease of handling, all pre-implantation embryos still retaining their zonae pellucidae were transferred into oviducts, which were then rinsed in PBS, and fixed in 4% PFA. Embryos which had hatched from their zonae (4.5dpc and implantation-delayed blastocysts) were instead embedded in blocks of agarose (Flowgen) because the absence of the zona tended to cause them to adhere to the oviduct

walls and become indistinguishable from the oviduct tissue in the sectioned material.

EMBEDDING AND SECTIONING

Following over night fixation in 4% PFA specimens were rinsed in PBS, then 0.85% NaCl (BDH) and dehydrated through a series of alcohols (AnalaR, BDH), cleared in Histoclear (National Diagnostics) and embedded in paraffin wax (Shandon). The duration of each step depended upon the size of the tissue being processed, but was generally between 10 minutes and 1 hour. Glass slides (Chance Propper) were cleaned by a brief immersion in 10% HCl (AnalaR, BDH) in 70% ethanol, and rinsed in water then 95% ethanol. They were heated to 150°C and allowed to cool before being immersed in 4% TESPA (3-aminopropyltrioxysilane; Sigma) in acetone (AnalaR, BDH). They were rinsed twice in acetone, then water and allowed to dry at 42°C. Following this treatment they could be stored dessicated in the fridge for up to a month. Sections were cut at 5 or 6μm, placed on slides flooded with distilled water at 40°C and allowed to expand, dried overnight at 25°C, and stored dessicated at 4°C until required for *in situ* hybridisation.

Recombinant DNA Manipulations

cDNA Synthesis

Total RNA prepared from adult mouse brain (kindly supplied by K. Lee) was used for the preparation of cDNA. First strand cDNA synthesis was performed by incubating 5µg of RNA with either 500µg of a specialised primer comprising a sequence containing a selection of restriction enzyme sites and a string of 17 thymidine residues for oligo dT cDNA (Oswel DNA Services), or 100pm/µl of random hexamers (Pharmacia) for random prime cDNA, in a total volume of 20µl. Each tube was incubated at 68°C for 5 minutes to denature the RNA, then cooled rapidly on ice for a further 5 minutes. To each tube was then added the following:

0.5µl RNase inhibitor (Boehringer)
4µl 10mM dNTPs
2µl Super RT (reverse transcriptase from the exon trapping system of Boehringer)
8µl 5xRT buffer (exon trapping system)
4µl 0.1M DTT (DL-dithiothreitol, Sigma)

These tubes were then incubated at 37°C for 80 minutes, with the addition of a further 2µl of Super RT halfway through the incubation. The temperature was then increased to 70°C for 10 minutes. 60µl of dH_2O was added, giving a final volume of 100µl. The cDNA was stored at -20°C.

Amplification of cDNA by Nested PCR

For the first round of PCR amplification a pair of oligonucleotide primers (A and B) were chosen to flank a stretch of at least 300 base pairs of the gene, to produce ultimately an optimum length of probe for *in situ* hybridisation. Each was selected to represent 18 nucleotides of DNA from regions of complete amino acid identity between the protein sequences of two different species (rat and human in the case of CNTFR), to have similar melting temperatures and to have the minimum possible amount of degeneracy (utilising stretches of amino acids coded for by the minimum number of alternative codons). The nucleotide sequence of one of these primers was converted to its complementary sequence so that DNA synthesis would be primed on both strands. For the second round of PCR amplification an additional 18-mer oligo primer (primer C) was designed to hybridise to a sequence within the first amplified product, but close to the 3' end of it. Second round amplification was carried out using primers B and C. Details of the exact sequences and the positions of the primers used for CNTFR are shown in chapter 4.

Random prime and oligo dT cDNA from rat brain was used as a control for the operation of the primers, this being the organism whose sequence was used for their design. The integrity of the mouse cDNA was tested by performing PCR using primers which had been used successfully to clone metabotropic glutamate receptors known to be expressed in the brain from murine cDNA (gift from B. Pickard). A 30 cycle PCR program was designed. The mixture was

heated to 96°C for 4 minutes to denature the template DNA, then cooled to 41°C for the addition of enzyme. Each tube for the reaction contained the following:

10µl	10 x reaction buffer (Promega)
5µl	25mM $MgCl_2$ (AnalaR, BDH)
2µl	10nM dNTPs (Pharmacia)
1µl (500µg)	each of oligos A and B (Oswel DNA Service)
5µl	cDNA
75.5µl	H_2O

After thorough mixing each reaction was covered with a layer of mineral oil (Sigma) and the program initiated. After the hot start, 0.5µl of Taq DNA polymerase (Pharmacia) was added, mixed in thoroughly and the cycle resumed. The mixture was incubated at 41°C for 30 seconds to allow the primers to anneal to the template DNA. Elongation of the sequence from the primer complementary to the DNA template then proceeded at 72°C for 5 minutes. After this time the newly synthesised sequence should have extended beyond the position of the other primer on the original complementary strand of template DNA. An increase in temperature to 93°C induced separation of the strands again, followed by a return to the primer annealing temperature, enabling the synthesis of further strands in the same manner. Thirty such cycles of PCR were performed to generate new DNA, which should consist predominantly of the desired sequence flanked by the two primer annealing sites. Upon

completion of the cycle aliquots of each reaction were run on a 2% agarose gel to visualise any fragments which had been amplified.

A second round of PCR amplification was performed as follows: 5µl of the first round PCR product was amplified with 500µg of oligos B and C under the same conditions as for the first round, until a sufficient amount of the desired fragment had been produced for purification by means of a Quiex kit (Quiegen).

The purified fragment was sub-cloned into pBluescript II KS (Stratagene) and its identity confirmed by sequencing. The sites chosen were Sal 1 and Bam H 1, which are located at the 5' and 3' ends of the fragment. Sall and Bam H 1 sites in the polylinker region of Bluescript enabled the insertion of the fragment in the desired orientation for the subsequent production of probe template.

PURIFICATION OF FRAGMENTS AND VECTORS

All of the fragments produced in this study, apart from DIA-R, were sub-cloned into pBluescript II KS (pBS). The reason for this is that pBS contains the promoters T3 and T7 located on either side of the multiple cloning site in opposite orientations to enable transcription initiation for either sense or antisense probe synthesis. The vector was prepared by excision of the insert from a pre-existing plasmid by digestion using restriction enzymes (Boehringer) which cut at specific sites in the polylinker region of the plasmid, corresponding to the sites present on the ends of the fragment. Purification of the vector was performed either by excising the desired band from an

agarose gel and extracting the DNA using a Quiex kit, or by running the appropriate fragment onto a piece of dialysis tubing (The Scientific Instrument Centre, Ltd) and recovering the DNA using the Elutip method (Schleicher and Schuell). For this 5-10μg of digested DNA were run on a 400ml 1% agarose gel at 15V overnight. A slot was then cut in the agarose immediately in front of the band of the required size and a piece of dialysis tubing, which had been prepared by boiling in 5mM EDTA (BDH), inserted into this. The gel was then run until the band adhered to the tubing. The tubing was carefully extracted and placed into 1-2ml of low salt buffer for Elutip purification. The Elutip was washed first with 1-2ml of high salt buffer, then 5ml of low salt buffer before the DNA in low salt buffer was passed through it. The Elutip was washed again with 2mls of low salt buffer, then 0.5ml of high salt buffer were run through and the solution collected in an Eppendorf tube (Sanstedt) and 1ml of ethanol added. Following ethanol precipitation for 30 minutes at -20°C, the DNA was pelleted by centrifugation for 10 minutes, washed and resuspended in 50μl of TE buffer. The concentration of the purified vector was calculated from the optical density reading taken by means of a spectrophotometer (Ultrospec III, Pharmacia), and from this the molarity could be determined. The purification of insert fragments was performed using the same principle.

LIGATIONS AND TRANSFORMATIONS

Ligation reactions were set up with an insert to vector ratio of 1:1 by molarity. Separate tubes were also set up in which there was a 10 fold excess of each. Reactions were carried out in 30µl volumes to include T4 DNA ligase (0.5µl; Boehringer) and 3µl of 10 x ligation buffer (Boehringer). Tubes were wrapped in paper tissues, placed into larger tubes and incubated in the fridge overnight. This served to reduce the temperature of the mixture gradually to enable the reaction to proceed, but deterioration of the product was prevented by maintaining it at the reduced temperature for the rest of the night which was ultimately reached after the reaction was complete. XL1 blue competent cells (Stratagene) were used for the transformations, which were performed according to the following procedure:

1. Rapidly thaw cells and place immediately on ice
2. Add 15µl of the ligation mixture to 100µl of cells
3. Place on ice for 10 to 30 minutes
4. Heat-shock for 45 seconds at 42°C
5. Return to ice for 2 minutes
6. Add 1ml of SOB medium
7. Shake at 37°C for 45 minutes
8. Remove 100µl from each tube and spread on to an Ampicillin plate with the addition of X gal (Sigma). Spread the remaining mixture on another plate

9. After drying the plates for a few minutes, incubate at 37°C overnight.

Growth of white colonies indicated an interruption of the Lac Z gene in the plasmid, which suggested the occurrence of a successful integration event. The appearance of blue colonies implied activity of the intact host gene. White colonies were, therefore, picked for the preparation of DNA.

SMALL SCALE PREPARATION OF PLASMID DNA

Miniprep DNA was prepared according to the protocol described:

1. Pick single colonies using a sterile Gilson tip and drop into 10mls of LB medium containing 1/1000 Ampicillin (Sigma)
2. Shake bottles over night at 37°C
3. Transfer 1.5ml to an Eppendorf tube and spin for 3 minutes
4. Remove the supernatant and resuspend the pellet in 200µl of TEN
5. Add 20µl of phenol (Gibco BRL)/chloroform (BDH) and vortex each tube for 2 seconds
6. Spin for 3 minutes
7. Transfer the upper phase to a new tube and precipitate with 2.5 volumes of ethanol
8. After spinning for 10 minutes discard the supernatant
9. Wash the pellet with 70% ethanol, dry and redissolve in 50µl TE buffer

Upon completion of this procedure digests using appropriate restriction enzymes (Boehringer) were performed to ascertain whether the correct insertion event had occurred. Two independent clones found to contain the appropriate insert were expanded by the following method to generate stock plasmids for future uses such as probe synthesis and sequencing.

LARGE SCALE PREPARATION OF PLASMID DNA

1. Dilute 2.5ml of the original culture with 500ml of LB medium supplemented with Ampicillin (1/1000) in a 2 litre flask and grow overnight at 37°C on an automatic shaker (New Brunswick Scientific Co. Inc.)
2. Divide the mixture into 250ml centrifuge bottles and spin at 6000rpm at 4°C
3. Discard the supernatant and resuspend the pellets in 25ml TGE
4. Add 5ml of 10mg/ml lyzozyme (Sigma) in TGE and allow the bottles to stand for 10 minutes
5. Add 60ml of a fresh 9:1 dilution of 0.22M NaOH (BDH)/10% SDS (Sigma) and leave the bottles on ice for 5 minutes
6. Incorporate 30ml of 5M potassium acetate (AnalaR, BDH), pH 5, and leave the bottles on ice for a further 15 minutes
7. Spin the bottles for 10 minutes at 6000rpm
8. Filter the supernatant through gauze into a 500ml GS3 bottle (Sorvall)

9. Add 0.6 volume of isopropanol (BDH) and pellet the DNA by spinning at 8000rpm and 4°C for 10 minutes

10. Remove the supernatant and take up the pellet in 8ml of TE

11. Titrate to neutrality with 3M Tris (Boehringer) and transfer to 30ml Corex tubes

12. Add an equal volume of Tris-saturated phenol

13. Spin at 8000 rpm and remove the aqueous phase to a fresh tube

14. Add an equal volume of chloroform and take the aqueous phase into a fresh tube

15. Add 1ml of 3M sodium acetate (AnalaR, BDH), pH 5 and 18ml ethanol to each tube and precipitate the DNA at -20°C for at least an hour

16. Spin at 8000 rpm for 5 minutes at 4°C and remove the supernatant

17. Wash the pellets twice with 70% ethanol,then dry and dissolve in 5ml TE

18. For each tube weigh out 10g of CsCl (Boehringer) and dissolve in 5ml TE

19. Mix the pellet and CsCl solution, add 1ml of 10mg/ml ethidium bromide (EtBr; Sigma) and spin down the precipitate

20. Transfer the supernatant to quick-seal tubes (Beckman), balance using CsCl and spin overnight at 50,000rpm (L7 Ultracentrifuge, Beckman), 20°C

21. Fluoresce the tubes under LW UV light and withdraw the lower fluorescent band using a syringe and needle

22. Add an equal volume of TE and extract the mixture with an equal volume of n-butanol (BDH) until all traces of EtBr are removed

23. Add an equal volume of sterile water and 2 volumes of ethanol, mix and centrifuge at 10K for 10 minutes at 4°C

24. Rinse the pellet in 70% ethanol, dry and resuspend in 500µl of TE

PROBE SYNTHESIS

^{35}S and ^{33}P (Dupont or Amersham) labelled RNA probes were prepared by *in vitro* transcription (Krieg and Melton, 1987; Rathjen et al., 1990a). Initially, ^{35}S was used for probes because it had been found by other workers to provide satisfactory results. Less was known about the merits of ^{33}P in the context of *in situ* hybridisation at that time. ^{33}P is more expensive, but it is required in smaller amounts for probe synthesis than is ^{35}S. Also, using ^{35}S DTT must be present throughout the reaction to prevent the formation of disulphide bonds which will interfere with hybridisation. This is not necessary when making probes using ^{33}P, which has the advantage of reducing the number of ingredients in the protocol and also reducing the cost to counter-act somewhat the higher price of ^{33}P compared with ^{35}S. However, the primary advantage of ^{33}P is the considerable reduction in exposure time in this context from 6 weeks for ^{35}S to 10 days. Both forms of radioactive label can be stored prior to use for 4 to 6 weeks. Probes were synthesised by mixing together the following ingredients at room temperature:

2ng	Template DNA
0.75µl	200mM DTT (for ^{35}S labelled probes)

0.75µl	2mg/ml bovine serum albumen (BSA; Sigma)
2.25µl	mixture of equal volumes of 0.5mM adenine, guanine and uridine for ^{35}S, or adenine, guanine and cytidine (Boehringer) for ^{33}P
0.5µl	RNase inhibitor (Boehringer)
6.25µl	CTP ^{35}S labelled (1000-1500 Ci/mM), or UTP ^{33}P labelled (2000 Ci/mM)
1.5µl	10 x transcription buffer
1µl	polymerase, either T7, T3 or SP6 (Boehringer)

The reaction was incubated at 37°C for 1 hour, after which 2µl of RNase-free DNase (Boehringer) were added and the incubation continued for a further 20 minutes. Probes were made up to 100µl by the addition of 85µl of dH_2O, phenol/chloroform extracted and separated from unincorporated nucleotides by centrifugation through a column of sephadex G-25 (Pharmacia) in a 1ml syringe. Incorporation of radioactivity was measured using a liquid scintillation analyzer (1600CA Tricarb, Canberra Packard), and the size distribution estimated by running on a 4-6% acrylamide (National Diagnostics) denaturing gel. Satisfactory probes were stored at -80°C for up to a month.

PREPARATION OF SECTIONS FOR HYBRIDISATION

Pretreatment of sections was carried out according to a modified form of the protocol devised by Wilkinson and Green, (1990). Slides

were passaged through histological staining dishes (Raymond Lamb) containing the following solutions at room temperature:

xylene (BDH)	2 X 10 minutes
100% ethanol	2 X 2 minutes
90, 80, 70, 50, 30% ethanol	2 minutes each
0.85% NaCl	5 minutes
PBS	5 minutes
4% PFA in PBS	20 minutes
PBS	2 x 5 minutes
20µg/ml proteinase K (Sigma) in 50mM Tris, 5mM EDTA (on slides laid horizontally)	7.5 minutes
PBS	5 minutes
4% PFA in PBS	5 minutes
sterile dH_2O	10 seconds
0.1M Triethanolamine (TEA; Sigma), pH 8	30 seconds
0.1M TEA + 625µl acetic anhydride (BDH)	10 minutes
PBS	5 minutes
0.85% NaCl	5 minutes
30, 50, 70, 80, 90% ethanol	1 minute each
100% ethanol	3 x 5 minutes

Slides were air dried for a few hours prior to the prehybridisation step.

Prehybridisation was carried out at 55°C in plastic copling jars (Raymond Lamb) for 2 hours. The prehybridisation mix contained the following:

25ml	deionised formamide (IBI)
0.5ml	100 x Denhardt's
1ml	1M tris HCl, pH 8
3ml	5M NaCl
2.5ml	0.1M EDTA
5ml	0.1M sodium phosphate (BDH)
2.5ml	10mg/ml Yeast RNA (Boehringer)
10ml	50% dextran sulphate (Pharmacia)

This step was introduced to reduce non-specific binding of the probe to the tissue. Probe was then added to 1ml of the hybridisation mix to an approximate concentration of 1 x 10^5 dpm/µl, DTT was added to 50mM in the case of ^{35}S labelled probes, and the mix applied to the slides from which the pre-hybridisation solution had been drained. Hybridisation was allowed to proceed overnight in sealed containers, humidified with 50% formamide at 55°C. The probe was retained on the sections by means of clean glass coverslips. After hybridisation, the coverslips were removed by a 15 to 20 minute incubation in 5 x SSC, (including 10mM DTT for ^{35}S labelled probes). Post-hybridisation washing was carried out:

50% formamide, 2 x SSC, (0.1M DTT)	1.5 hours	70°C
NTE buffer	3 x 10 minutes	37°C
NTE + 40ug/ml RNase	30 minutes	37°C
NTE	15 minutes	37°C
50% formamide, 2 x SSC, (0.1M DTT)	1 hour	70°C
2 x SSC	3 x 10 minutes	RT
0.1 x SSC	3 x 10 minutes	RT
30, 50, 70, 90% ethanol in 0.3M ammonium acetate (AnalaR, BDH)	1 minute each	RT
100% ethanol	2 x 5 minutes	RT

Air dry

(DTT is required only for ^{35}S labelled probes)

AUTORADIOGRAPHY

Procedures were carried out in a dark room under a safe light (Ilford 914). Approximately 10mls of emulsion (Ilford K5) were melted in a water bath at 40°C and mixed with an equal volume of water containing a drop of glycerol (AnalaR, BDH) to prevent shearing of the emulsion. Slides were coated by plunging them in pairs, back to back, into a slide dipper (custom-made by G. Robertson) containing the molten emulsion mix. They were allowed to drain before being placed into a light-tight box containing silica gel (BDH) at room

temperature to dry over night. They were then transferred to a sealed slide box with silica gel, wrapped in foil, and stored at 4°C for the desired exposure time, usually 6 weeks for ^{35}S or 10 days for ^{33}P. Slides were allowed to warm up to room temperature, developed by immersing in D19 developer (Kodak) for 4 minutes, rinsing in water and fixing in a 1:3 mix of Kodafix (Kodak) in distilled water for 4 minutes, all at room temperature. After extensive rinsing in water, the slides were stained with 0.1% methyl green (Gurr, BDH), rinsed, dried and mounted in DPX (Gurr, BDH). Observation and photography could then be carried out at leisure after the mountant had set.

OBSERVATION AND PHOTOGRAPHY OF RESULTS

A Vanox microscope (Olympus) was used for all detailed observation and photography of sections. For preimplantation embryos, a x60 oil immersion objective was employed. Other specimens were generally studied using x4, x10, x20 or x40 dry objectives.

ES Cell Culture Medium

UHP water	170ml
10 x GMEM (Gibco)	20ml
Sodium bicarbonate (7.5% stock, Gibco)	6.6ml
Non-essential Amino Acids (100 x stock, Gibco)	2ml
Glutamine+Sodium Pyruvate (200mM+100mM stock, Gibco)	4ml
Mercaptoethanol (Sigma)	200µl
Foetal Calf Serum (FCS; Gibco)	to 10%
Factor (eg DIA)	as required

ES Cell Derivation Medium

As for ES cell culture medium, but with 15% FCS

GPI Isomerase Electrophoresis

Tris Glycine Buffer (pH8.1-8.5)

Tris	3g
Glycine (Gibco)	14.4g
dH_2O	1 litre

Tris Citrate Buffer (pH8.0)

Tris	20.1g
Citric acid (Sigma)	8g
dH_2O	500ml

Stain

Fructose-6-phosphate (Sigma)	20mg
NADP (Nicotinamide adenine dinucleotide phosphate, Sigma)	2.7mg
NBT (Nitroblue Tetrazolium, Sigma)	2.7mg
Glucose-6-phosphate dehydrogenase (Sigma)	10µl
PMS (Phenazine methosulphate, Sigma)	30µl of 10mg/ml stock

Make up fresh, protect from light.

LB Medium

Tryptone (Difco)	10g/l
Yeast (Difco)	5g/l
NaCl	5g/l
$MgCl_2.6H_2O$ (BDH)	2g/l
dH_2O	1 litre

NTE Buffer (pH8.0)

NaCl	0.5M
TrisHCl	10mM
EDTA	5mM

PB1 Medium (pH7.0-7.2)

	g/104mls
NaCl	0.822
KCl	0.021
Na_2PO_4 (Sigma)	0.300
KH_2PO_4 (BDH)	0.020
Glucose (Sigma)	0.104
Sodium pyruvate (Gibco)	0.0045
Penicillin (Sigma)	0.0062
$CaCl_2$ (BDH)	14mg
$MgCl_2$ (Sigma)	10mg
1% phenol red (Sigma)	0.1ml
UTP H_2O	to 100mls

Foetal calf serum 10%

(For material to be subsequently injected or maintained in culture)

SOB Medium

Bacto-Tryptone (Difco)	20g/l
Yeast Extract (Difco)	5g/l

NaCl	0.5g/l

Autoclave

Before use, add 1ml of filter sterilised $MgCl_2/MgSO_4$ (BDH) solution (9.5g/12g in 100ml H_2O).

20xSSC (ph7)

NaCl	876.5g
tri-sodium citrate (BDH)	441g
dH_2O	to 5 litres

Solutions for Southerns

Solution A

0.25M HCl

Solution B

1.5M NaCl

0.5M NaOH

Solution C

1.5M NaCl

0.5M TrisHCl, pH 7.5

1mM EDTA, pH 8.0

TBE Buffer

Tris	15g
Boric acid (AnalaR, BDH)	2.5g
EDTA/Na	1g
dH_2O	1 litre

TE Buffer

TrisHCl, pH7.5	10mM
EDTA/Na, pH8	1mM

TEN Buffer

TrisHCl, pH8.0	0.1M
EDTA, pH8.0	0.01M
NaCl	1M

TGE Buffer

Glucose	50mM
TrisHCl, pH8	25mM
EDTA/Na, pH8	10mM
in dH_2O	

10 x Transcription Buffer

Tris, ph7.5	400mM
MgCl	60mM
Spermidine (Sigma)	20mM

Trypsin

NaCl	3.5g
Glucose	0.5g
Na_2HPO_4 (Sigma)	0.15g
Trizma base (Sigma)	1.5g
KCl	0.185g
KH_2PO_4	0.12g
EDTA	0.2g
Phenol red, 0.5%	0.5ml
Trypsin (Gibco)	1.25g
UHP water	500ml

X-Gal

Phosphate Buffer

Na_2HPO_4 (Sigma)	0.1M
NaH_2PO_4 (Sigma)	0.1M

Fixative

Gluteraldehyde (Sigma)	0.2%
Phosphate buffer (pH7.3)	0.1M
$MgCl_2$	2mM
EGTA (pH8)	5mM

Wash

Phosphate buffer, pH7.3	0.1M
$MgCl_2$	2mM
sodium desoxycholate (Sigma)	0.1%
NP40 (Nonidet 40, Sigma)	0.02%
BSA (bovine serum albumin, Sigma)	0.05%

Stain

X-Gal (Sigma)	25mg
dissolve in dimethyl formamide	0.5mls
Wash	25mls
$K_3Fe(CN)_6$ (Sigma)	41mg
$K_4Fe(CN)_6$ (Sigma)	52.5mg
0.085%NaCL	0.4mls

Filter

CHAPTER 3

IN SITU HYBRIDISATION ANALYSIS OF THE EXPRESSION OF DIA AND ITS RECEPTOR IN THE EARLY MOUSE EMBRYO

Introduction

DIA is a pleiotropic cytokine which is able to maintain embryonic stem (ES) cells in an undifferentiated state when added to the culture medium in picomolar concentrations (Smith et al., 1988). It can act on the stem cells in the blastocyst when put into culture, enabling the establishment of ES cell lines (Nichols et al., 1990; Pease et al., 1990). DIA may, therefore, be considered as a candidate molecule for maintenance of the stem cells required during early embryogenesis *in vivo*. Transcripts for DIA mRNA have been detected in mouse blastocysts by RT-PCR (Conquet and Brûlet, 1990, Murray et. al., 1990). Preliminary analysis by *in situ* hybridisation and RT-PCR has suggested that DIA is expressed in the egg cylinder, where there is known to be a residual core of pluripotential stem cells (Conquet and Brûlet, 1990). DIA mRNA has also been detected in the egg cylinder by RNase protection, particularly in the extra-embryonic region (Rathjen et. al., 1990b). The levels of transcript estimated by this technique are extremely low, representing less than 10 copies per cell.

There are known to be two forms of DIA (Rathjen et. al. 1990a). One is diffusible (DIA-D), the other is associated with the extracellular matrix (DIA-M). The two forms of transcript differ at the 5' end as a result of alternative promoter and first exon

usage (Fig. 3.1). This results in the transcript for DIA-D possessing a unique sequence of about 150 nucleotides at the 5' end, whereas the DIA-M-specific sequence is only about 50 nucleotides long. It has been possible by RNase protection to distinguish both forms of DIA, but for sufficient sensitivity using the *in situ* hybridisation technique, a sequence longer than 50 nucleotides is desirable. It is for this reason that a probe specific for only the D-form has been generated in the present case.

In situ hybridisation has been carried out here using a probe for DIA which will hybridise to both the D- and M- form, a probe specific for the D- form, and probes for the DIA receptor subunit (DIA-R) and the signal transducer gp130. The embryonic stages selected were morulae, blastocysts of 3.5dpc, peri- and delayed implantation, and postimplantation embryos of 5.5 to 8.5dpc. Pluripotential stem cells are abundant until 7.5dpc, but are gone by 8.5dpc, when gastrulation is complete. Because of the very low levels of transcript previously documented for DIA in the embryo, it is possible that *in situ* hybridisation may fail to detect areas of expression. Optimisation of the protocol has been carried out to achieve maximal signal over background, but this technique still remains less sensitive than RNase protection analysis. However, it provides a means of detecting more specifically the localisation of the signal and may be used to investigate the expression patterns of preimplantation embryos, where the small size restricts the availability of tissue. Because the low levels of mRNA are likely to result in translation of only small amounts of protein, detection of the DIA protein using

Fig. 3.1 Diagrammatic representation of the DIA gene showing the regions recognised by the probes used for *in situ* hybridisation.

UTR=untranslated region

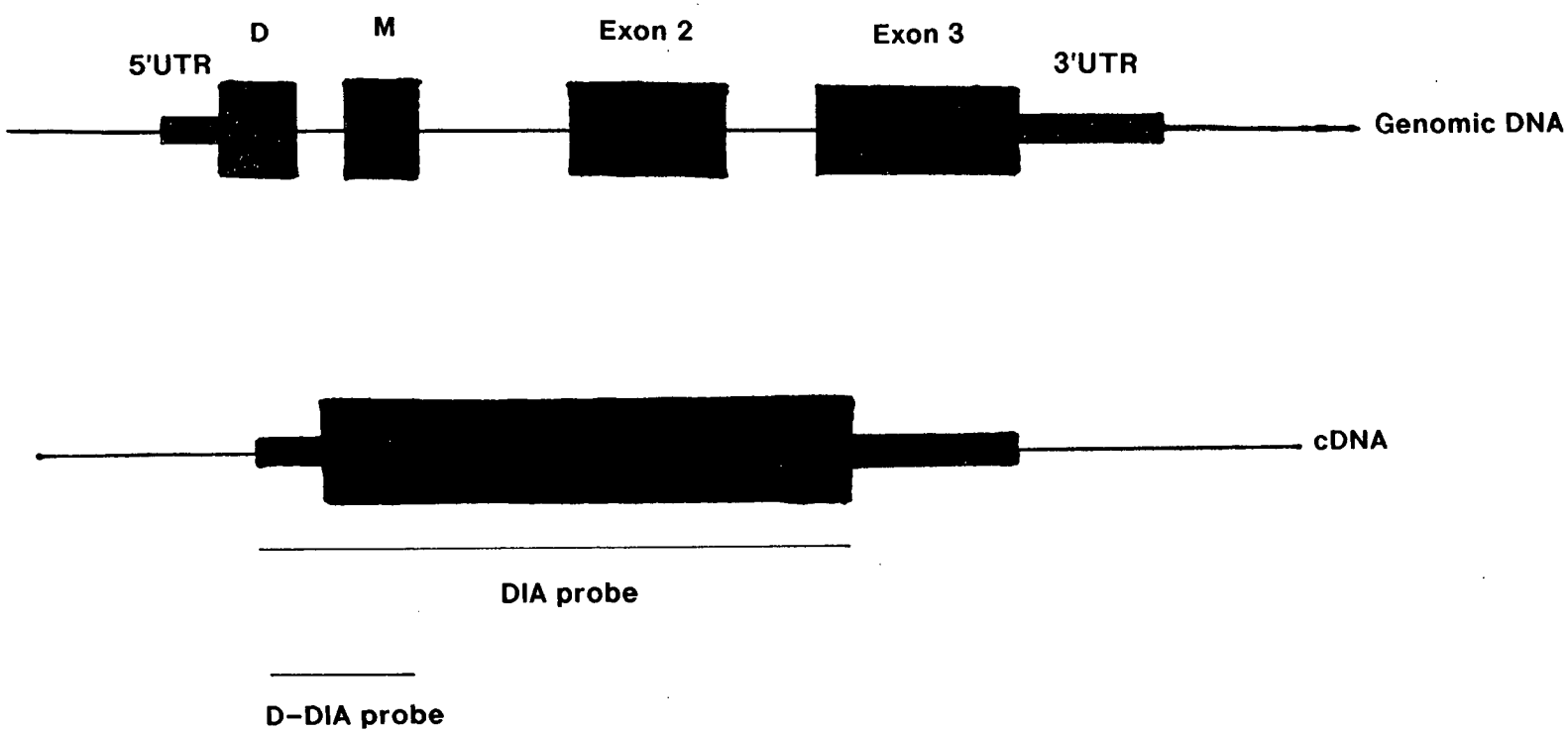
D=promoter and first exon specifying the diffusible form of DIA

M=alternative promoter and first exon specifying the matrix-associated form of DIA

DIA probe=650bp

D-DIA probe=163bp

Fig.3.1


D
M
Exon 2
Exon 3
5'UTR
3'UTR
Genomic DNA
cDNA
DIA probe
D–DIA probe

labelled antibodies is likely to be equivocal, unless such antibodies are very sensitive. Tracing the distribution of DIA and its receptor by this means has not been attempted here, since sufficiently sensitive antibodies were not available for this study. A series of *in situ* hybridisations has been compiled to determine the onset of expression of the mRNAs for DIA and its receptor, and to establish whether their patterns of expression are compatible with the proposal that DIA plays a role in the development of the early mammalian embryo.

Materials and Methods

Embryos and uterine samples were obtained generally from mice of the MF1 (albino) outbred strain, and occasionally from the inbred 129 or C57Bl/6 strains. They were processed as described in chapter 2, sectioned at 5 or 6μm and taken through the *in situ* hybridisation protocol of chapter 2. Probes were designed and constructed as follows:

DIA (both D- and M- forms)

The plasmid pDR1 contains a 650bp EcoR1 insert spanning the whole of the coding region of the murine DIA cDNA in pBluescript II KS (Rathjen et al., 1990a; fig 3.2a). A template for the anti-sense probe was made by linearising this plasmid by digestion with HinDIII; the sense control template was produced by digestion with BamH1. Radioactively-labelled RNA probes were synthesised as described in chapter 2. For the anti-sense probe transcription was initiated from the T7 promotor; synthesis from the T3 promoter was adopted for the sense control (fig 3.2a).

DIA-D (specific for the diffusible form)

The sequence specifying the alternative first exon which leads to the transcription of the mRNA specific for the diffusible form of DIA was excised from the above plasmid by digestion with Eae1

Fig. 3.2 Diagrammatic representation of the plasmids used for synthesis of radioactively-labelled probes for *in situ* hybridisation

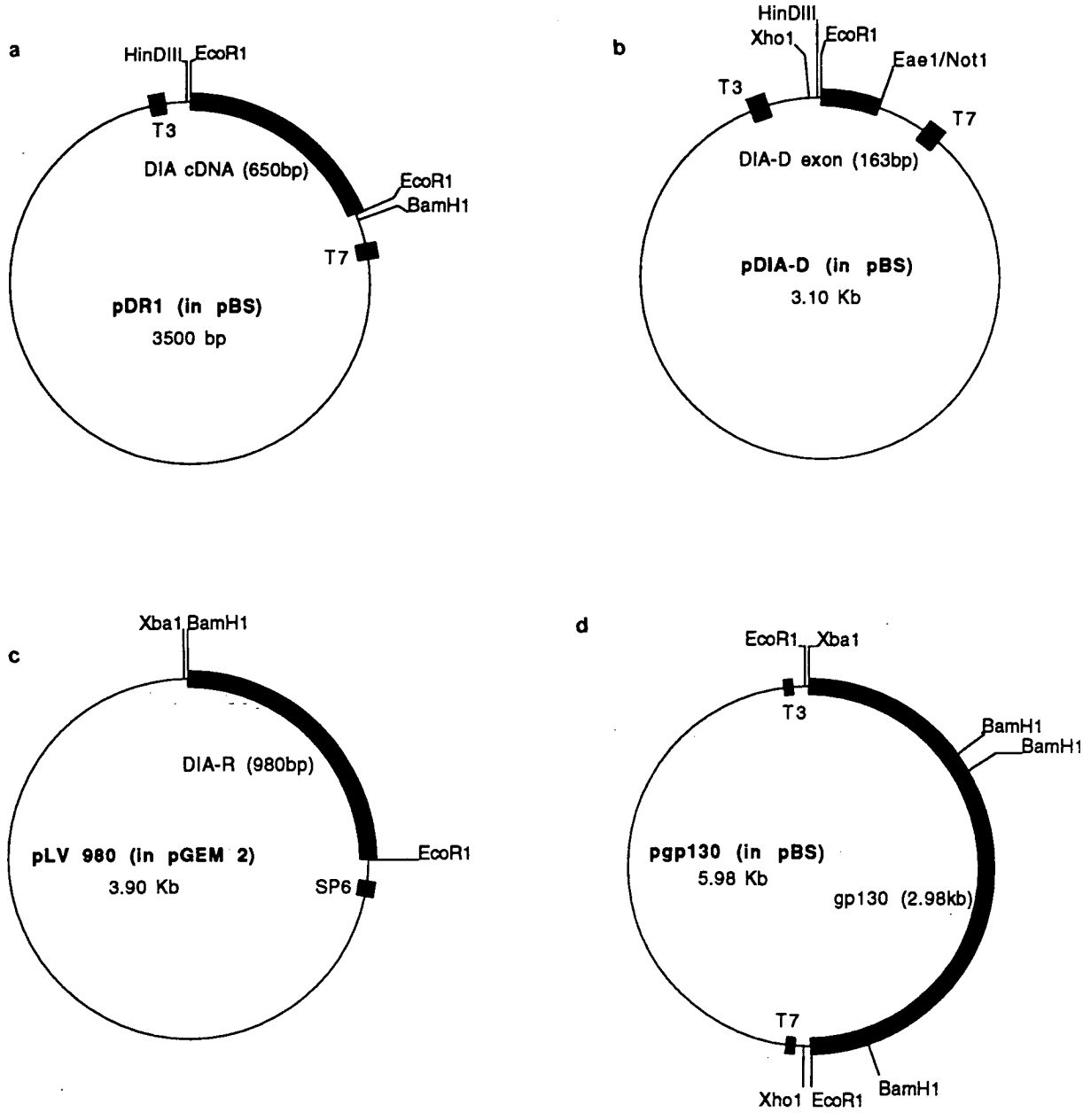
a. An anti-sense probe for the whole coding region of the DIA gene (650bp), recognising mRNA for both the diffusible and matrix-associated form of DIA is generated by linearisation with HinDIII and transcription from the T7 promoter; the sense control probe is initiated from T3 after linearisation with BamH1.

b. A probe to recognise only the diffusible form of DIA mRNA (163bp) is created by linearisation using HinDIII and transcription from the T7 promoter.

c. Linearisation using Xba1 and transcription from the SP6 promoter generates a probe of 980bp which recognises the extra-cellular domain of the DIA-specific component of DIA receptor (DIA-R, see fig. 1.2).

d. Two probes for gp130 have been used. A short probe (330bp) recognising the transmembrane domain (see fig. 1.2) is made by digestion of the plasmid using BamH1 and transcription from the T7 promoter. The longer probe (2980bp), recognising the whole of the coding region for gp130 arises from linearisation using Xba1 and transcription from the T7 promoter.

Fig. 3.2


a
HinDIII
EcoR1
T3
DIA cDNA (650bp)
EcoR1
BamH1
T7
pDR1 (in pBS)
3500 bp
b
HinDIII
Xho1
EcoR1
Eae1/Not1
T3
T7
DIA-D exon (163bp)
pDIA-D (in pBS)
3.10 Kb
c
Xba1
BamH1
DIA-R (980bp)
pLV 980 (in pGEM 2)
3.90 Kb
EcoR1
SP6
d
EcoR1
Xba1
T3
BamH1
BamH1
pgp130 (in pBS)
5.98 Kb
gp130 (2.98kb)
T7
Xho1
EcoR1
BamH1

and Xho1. Double digestion produced 6 fragments, from which the desired fragment of 163bp was excised from an agarose gel and purified by means of the Elutip method (see chapter 2). This was sub-cloned into pBluescript at sites in the polylinker produced by digestion with Xho1 and Not1 by the method described in chapter 2. This plasmid is now known as pDIA-D (fig. 3.2b). It was linearised using HinDIII and the labelled antisense probe synthesised as described using T7 RNA polymerase.

DIA Receptor

The probe for *in situ* hybridisation for the murine DIA receptor was prepared from plasmid pLV980 (see chapter 2). This plasmid contains a 980bp fragment of DIA-R cDNA cloned by PCR amplification of adult mouse liver cDNA, using oligonucleotide pairs to correspond to amino acids 196 to 201 and 509 to 514 of the published mouse soluble DIA-R protein sequence (Gearing et al., 1991; I. Chambers, unpublished). The fragment was cloned into pGEM-2. The probe for *in situ* hybridisation is directed at the region of the gene coding for the extracellular part of the protein, and should, therefore, detect transcripts encoding both the transmembrane and soluble forms of the receptor (I. Chambers, unpublished). The plasmid was linearised using Xba1, and synthesis initiated using SP6 RNA polymerase (fig. 3.2c).

Gp130

A murine gp130 cDNA was provided by T. Taga (Osaka). This is a 2.9kb EcoR1 fragment in pBluescript SK. Two probes were generated for *in situ* hybridisation. One was directed at a short region of the cytoplasmic domain. This probe was generated by digestion of the plasmid with BamH1 and gel purification of the 4kb band, representing the linearised plasmid from which 1.8kb of the gp130 sequence had been excised (fig. 3.2d). Transcription from the T7 promoter produces a sequence of 330bp; this was radioactively-labelled for use as a probe for *in situ* hybridisation (see chapter 2). The other probe included the full length coding region (2.9kb), and was made after linearising the plasmid with Xba1 and transcribing from the T7 promoter (fig. 3.2d). After labelling, the probe was hydrolysed to generate smaller lengths of RNA of around 200bp, which are thought to penetrate the tissue more efficiently (Wilkinson and Green, 1990). Hydrolysis was performed by incubating the probe for 75 minutes with equal volumes of 80mM $NaHCO_3$ and 120mM Na_2CO_3, followed by purification through a Sephadex G-50 column.

The specificity of the probes could be ascertained by testing their ability to hybridise specifically on a Southern or Northern blot from murine tissues to a band or bands which could be identified as transcripts from the gene under investigation. The absence of additional bands indicated that the probe would not cross-hybridise to other RNAs.

Results

Preimplantation stages

Specific deposits of silver grains can be detected by the blastocyst stage at 3.5dpc. The distribution of grains indicates differential expression of the mRNAs. Using the probe for DIA, the signal is apparently restricted to the trophectoderm (fig. 3.3a). Of 24 blastocysts observed during successful runs of the *in situ* protocol (when specific signal was detected on the endometrial gland controls, see below), 23 exhibited specific localisation of grains over the trophectoderm region. The probe for D-DIA exhibited a less dense distribution of silver grains when used on blastocysts, compared with that obtained using the full length probe for DIA. However, in experiments when D-DIA and full length DIA were used on blastocysts during the same run, the level of expression detected with the full length DIA probe was lower than had been observed on previous occasions. The reason for this variability between runs of *in situ* hybridisation is not clear, since volumes of probe were added to each hybridisation to provide equivalent numbers of counts per minute, and, as far as possible, the other conditions during the procedure were standardised. Localisation of signal to the trophectoderm region could be clearly seen in blastocysts of 4.5dpc and in delayed implantation using both the full length DIA probe and the probe specific for the diffusible form (figs. 3.4b and 3.5b).

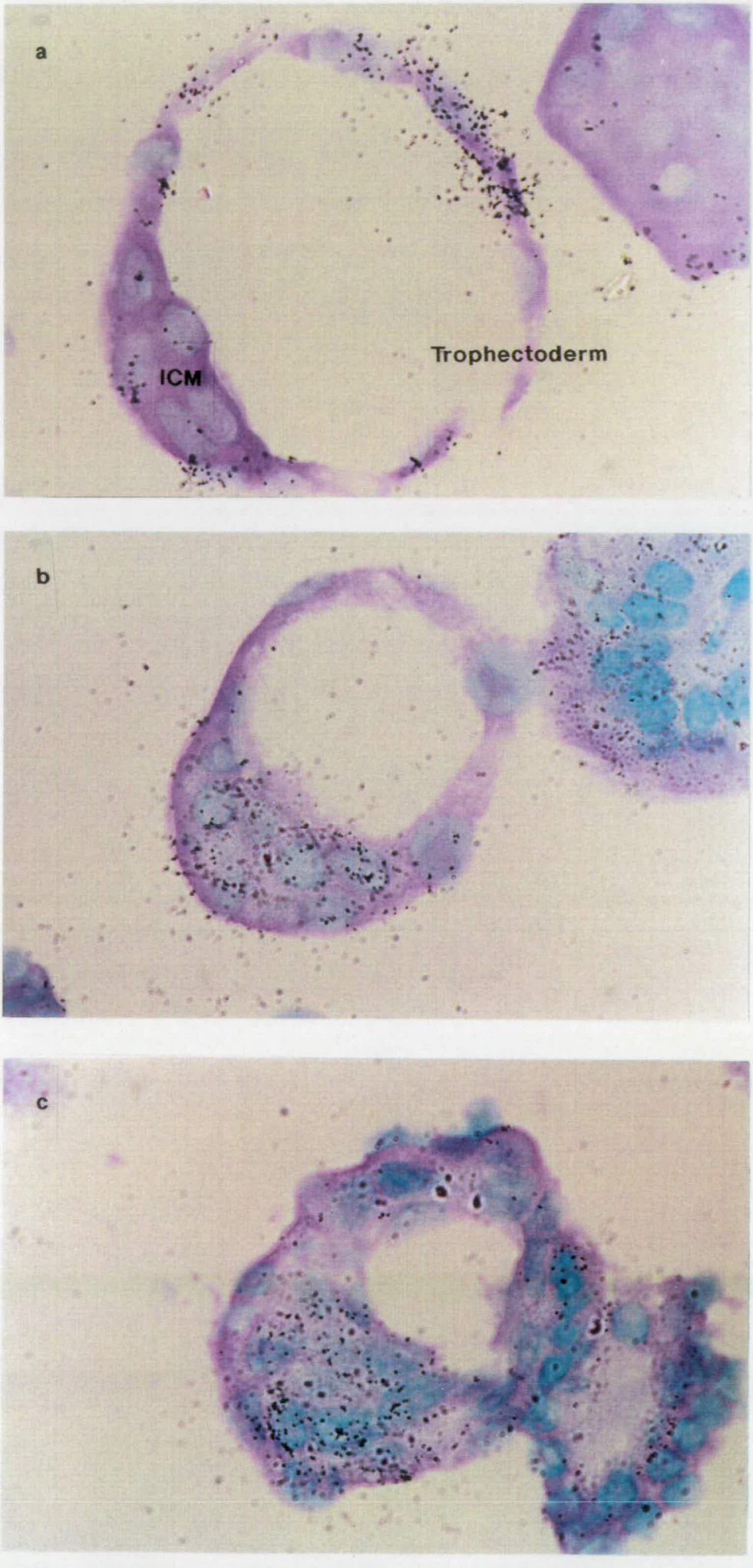
Fig. 3.3 *In situ* hybridisation on sections of 3.5dpc blastocysts (x60 oil immersion).

a. The probe for the whole of the coding region for the DIA gene can be seen to hybridise to cells of the trophectoderm.

b. Hybridisation using the DIA-R probe is predominantly confined to the inner cell mass (ICM).

c. The probe for gp130 can be seen to hybridise to the whole blastocyst, but a greater concentration of grains is seen over the ICM.

Fig. 3.3



On blastocysts exposed to probes for the DIA-specific receptor subunit (DIA-R) and gp130 the grains are predominantly located over the ICM (figs. 3.3b and c). In addition, there does appear to be some gp130 mRNA in the trophectoderm (fig. 3.3c). For DIA-R, 26 out of 30 blastocysts examined displayed a higher concentration of signal over the ICM. In the case of gp130, 30 out of 33 blastocysts bore deposits of silver grains, but because gp130 is probably ubiquitously expressed, specific signal is not always distinguishable from non-specific binding. However, such a concentration of grains over the tissue was not seen using sense control probes for DIA. Similar patterns of distribution are seen on both the 4.5dpc peri-implantation (figs. 3.5c and d) and implantation-delayed blastocysts (figs. 3.4c and d).

Implantation delayed blastocysts can be held at the same stage for many days (Bergstrom 1978). In this study, embryos were delayed for 4 days. To achieve embryonic diapause, the source of oestrogen is removed by ovariectomy. Hatching from the zona pellucida occurs, but giant cell transformation is prevented and differentiation of the primitive endoderm is reduced (McLaren, 1968; Van Blerkom et al., 1979; Given and Weitlauf, 1981). *In situ* hybridisation on delayed blastocysts using probes for DIA and its receptor complex has produced a similar picture to that seen with non-delayed blastocysts (fig. 3.5 vs fig. 3.3 and 3.4). This persistence of the expression patterns establishes that the transcription of DIA and its receptor complex is maintained during delay. Therefore, the expression of these

Fig. 3.4 *In situ* hybridisation on sections of blastocysts in implantation delay (x60 oil immersion).

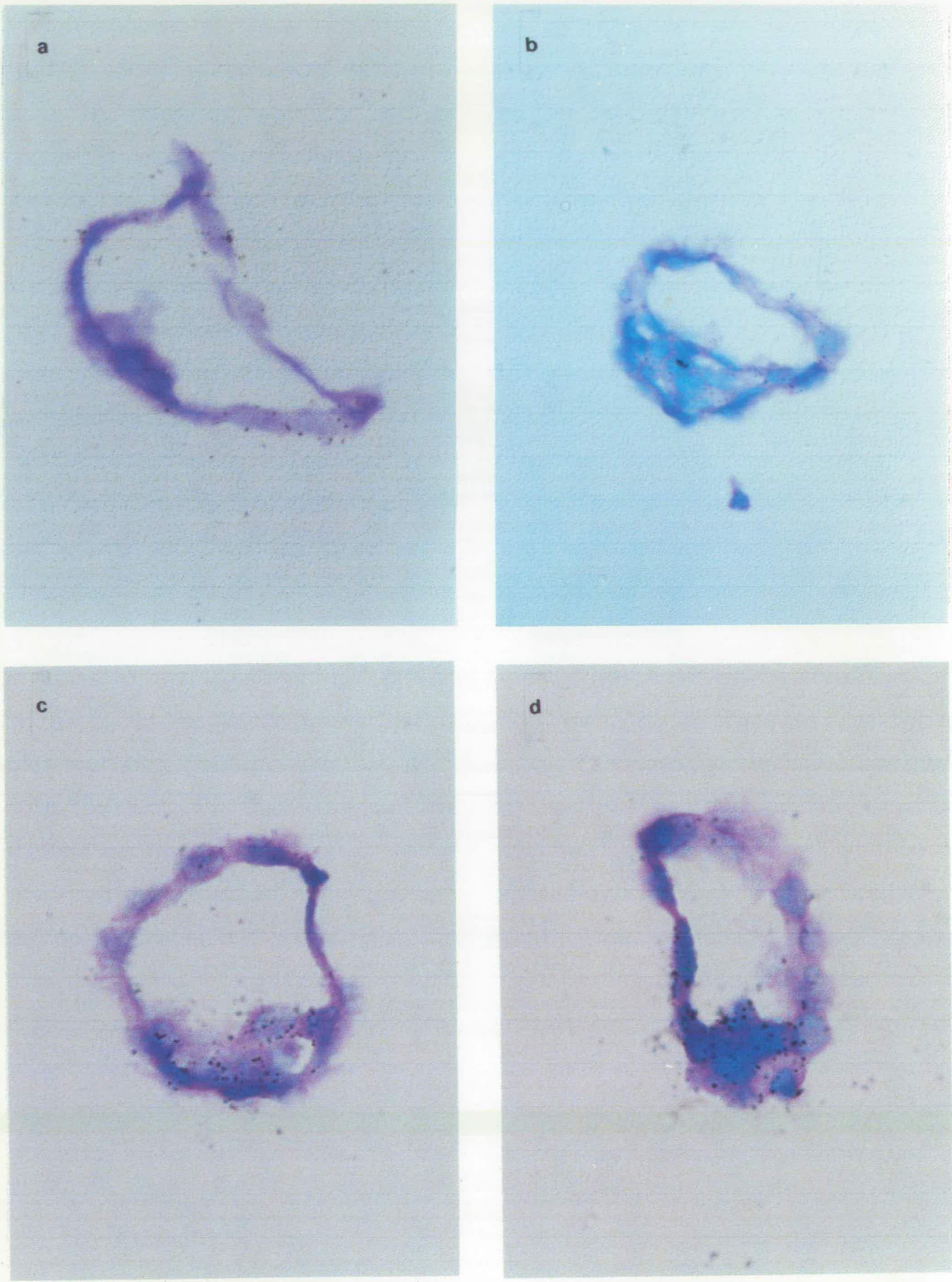
a. The probe for DIA hybridises to the trophectoderm.

b. mRNA for the diffusible form of DIA is produced predominantly in the trophectoderm.

c. DIA-R mRNA is predominantly localised to the ICM.

d. The probe for gp130 mRNA hybridises to the whole blastocyst, but mostly to the ICM.

Fig. 3.4



genes in the early embryo is unlikely to represent a simple response to the maternal hormone oestrogen.

Unequivocal deposits of silver grains have not been detected using probes for DIA, D-DIA, DIA-R or gp130 on morulae, although sections of morulae have been included in experiments when specific signals have been observed on older embryos. However, on all stages up to the morula a peculiar artifact has been observed. Accumulations of dark granules, which do not exactly resemble autoradiographic silver grains are often seen over the cytoplasm (fig. 3.6a). These were also detected in 1 and 2 cell embryos, even using the sense control probe for DIA. What these granules represent is not clear. They are unlikely to be melanin granules, since non-pigmented mice were used as donors for most of the specimens for *in situ* hybridisation. These accumulations made it difficult to detect any specific hybridisation, but authentic silver grains were rarely observed in areas not obliterated by the artifact at these early stages. *In situ* hybridisation has previously been performed on such early preimplantation stages to study the expression of the Oct-4 gene (Yeom et al., 1991). The very abundant transcripts for Oct-4 in these totipotent cells produce sufficient autoradiographic grains to obliterate completely any background artifact which would interfere with a less intense signal. The expression of mRNAs for FGF-4 and its receptor have been detected previously in the blastocyst (Niswander and Martin, 1992; Rappolee et al., 1994) by *in situ* hybridisation. FGF-4 mRNA has also been seen in morulae, but at very low levels (Niswander

Fig. 3.5 *In situ* hybridisation on sections of blastocysts at 4.5dpc immediately before implantation (x60 oil immersion).

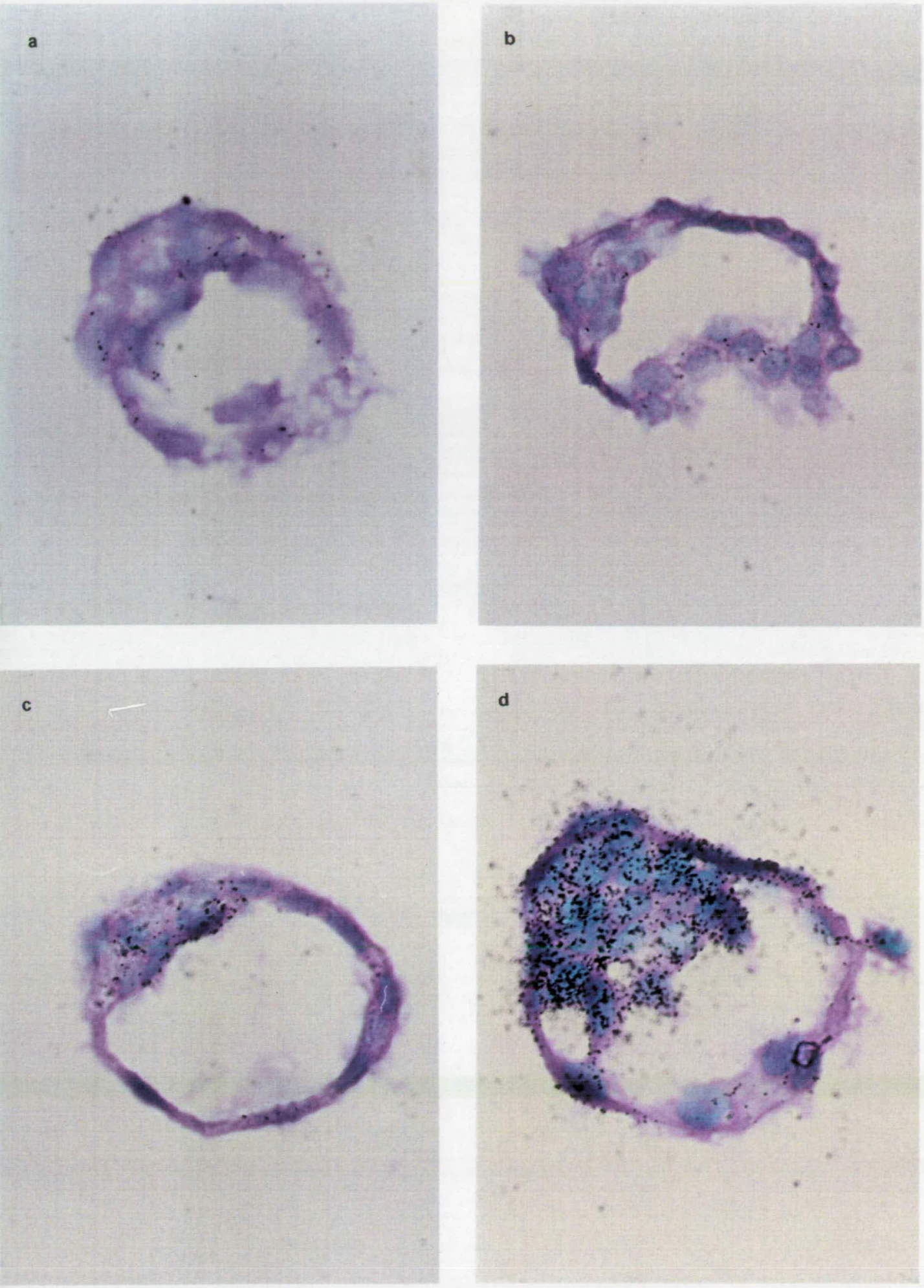
a. The probe for DIA hybridises to the trophectoderm, and also to a region over the ICM adjacent to the blastocoel.

b. Transcripts for mRNA for the diffusible form of DIA are located in the trophectoderm and in juxtacoelic regions of the ICM.

c. DIA-R mRNA is expressed mainly in the ICM.

d. The probe for gp130 hybridises predominantly to the ICM, but transcripts are also apparent in the trophectoderm.

Fig. 3.5



and Martin, 1992). The preimplantation artifact was apparently not a problem in that study.

Uterine expression

From 4.5dpc until 8.5dpc (all of the uterine stages studied), silver grains are seen in the endometrial glands using all 4 probes (fig. 3.7). The levels differ slightly with stage and probe, but this may reflect the variability inherent in the procedure. The strongest endometrial gland signal is seen with the probe for gp130 (fig. 3.7d). The level of signal over the rest of the uterus tended to be higher generally for gp130. Using the probe for DIA-R, a concentration of grains on the epithelium lining the uterine lumen was observed in addition to the distribution seen over the endometrial glands. This expression pattern seems to persist through all the stages studied, and is a phenomenon observed here only with the probe for DIA receptor (fig. 3.13).

Postimplantation stages

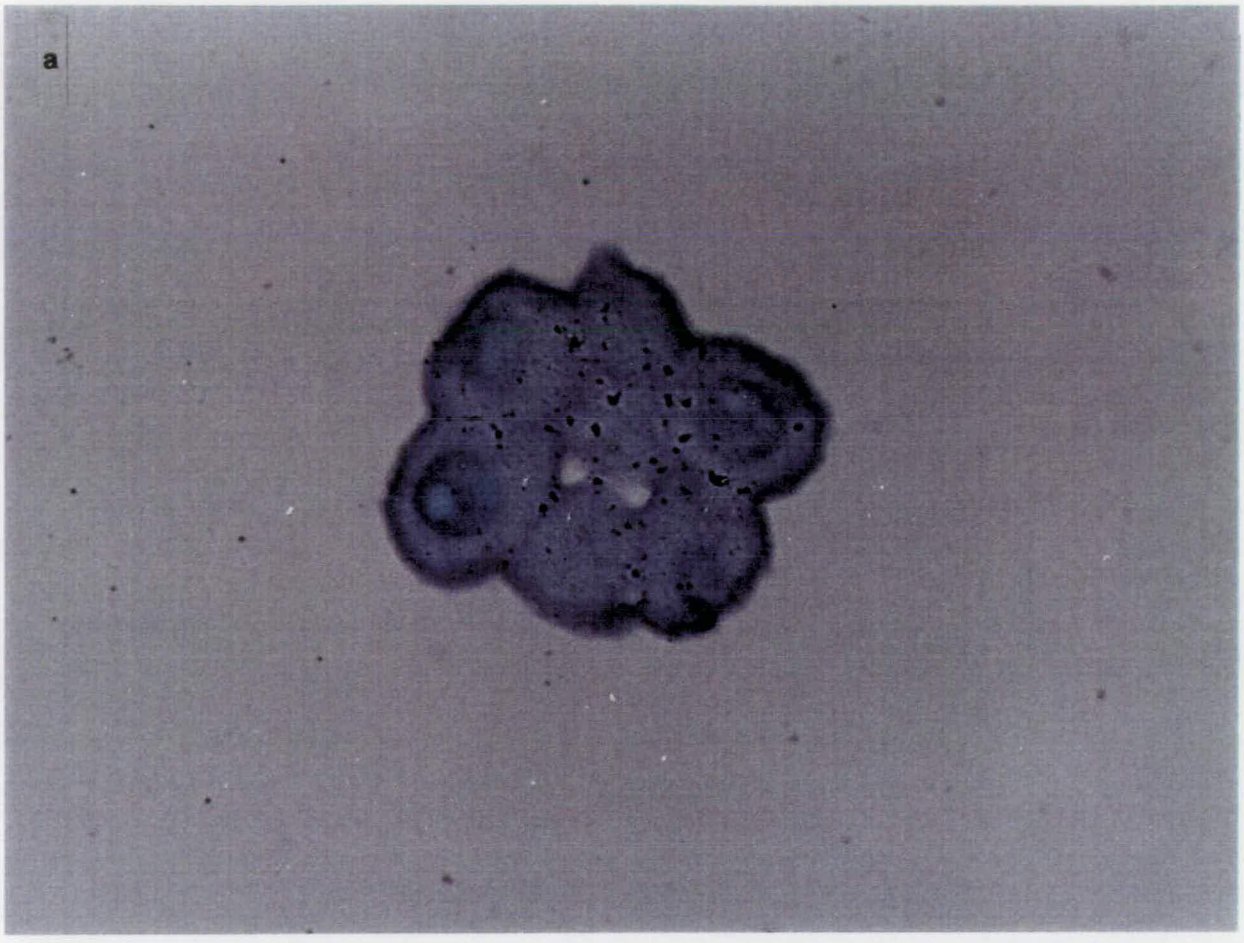
Specific transcripts could be detected in 5.5dpc postimplantation embryos using the full length DIA probe (fig. 3.6b). This signal was localised to the extraembryonic and ectoplacental cone region, consistent with the results on 6.5 and 7.5dpc embryos previously obtained using RNase protection (Rathjen et al., 1990b). In that

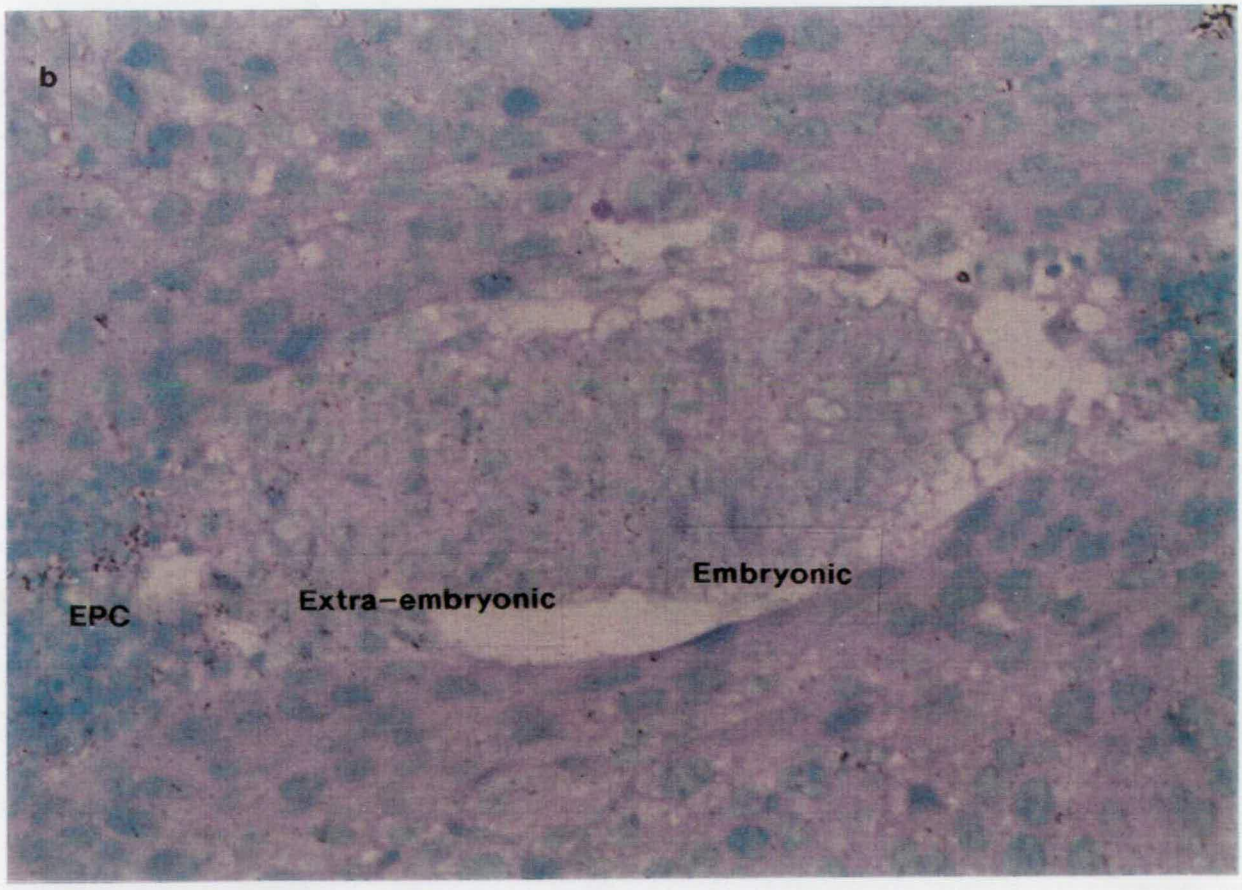
Fig. 3.6

a. *In situ* hybridisation on a section of a morula using a sense control probe for the DIA gene (x60 oil immersion). The black particles present in the cytoplasm do not represent autoradiographic grains.

b. *In situ* hybridisation of a section through a 5.5dpc embryo (x20 objective) using a probe for DIA. A few silver grains can be seen in the embryonic region (Emb) of the embryo, and a slightly higher concentration is present in the extra-embryonic (Ex emb) and ectoplacental cone (EPC) region.

Fig. 3.6





report, mRNA for the D-form, and less for the M-form of DIA was seen in the extra-embryonic part of the egg cylinder with only a trace being detectable in the embryonic region. Appart from the expression always seen in the endometrial glands of the uterus, no clear signal for DIA mRNA could be detected in postimplantation embryos or their decidua.

With the probe for DIA-R a significant concentration of grains could not be seen in any of the embryonic tissues at 5.5, 6.5 and 7.5dpc, although there was a high level of expression in the decidual tissue surrounding the embryos (figs. 3.8a and c and 3.9a), and a specific localisation of signal in the endometrial glands. The decidual expression was at its highest in proximal regions, and appeared to diminish with increasing age of the embryo. At 8.5dpc only a trace of the signal in the deciduum could be seen (fig.3.9c), but by this time other tissues such as the amnion, neural tube, somites and pre-somitic mesoderm were exhibiting significant deposits of silver grains (fig. 3.10).

Gp130 was also expressed in the decidual tissue surrounding the embryo, but in contrast to the expression patterns seen with DIA-R, the level of gp130 increased with the age of the embryo (figs.3.8b and d and 3.9b and d). Gp130 expression was also apparent in most tissues at 8.5dpc, particularly the mesenchyme, amnion and visceral yolk sac (fig. 3.11).

Fig. 3.7 *In situ* hybridisation on sections of uteri of 4.5 or 5.5dpc. In each case the autoradiographic grains localise to the endometrial glands (x20 objective).

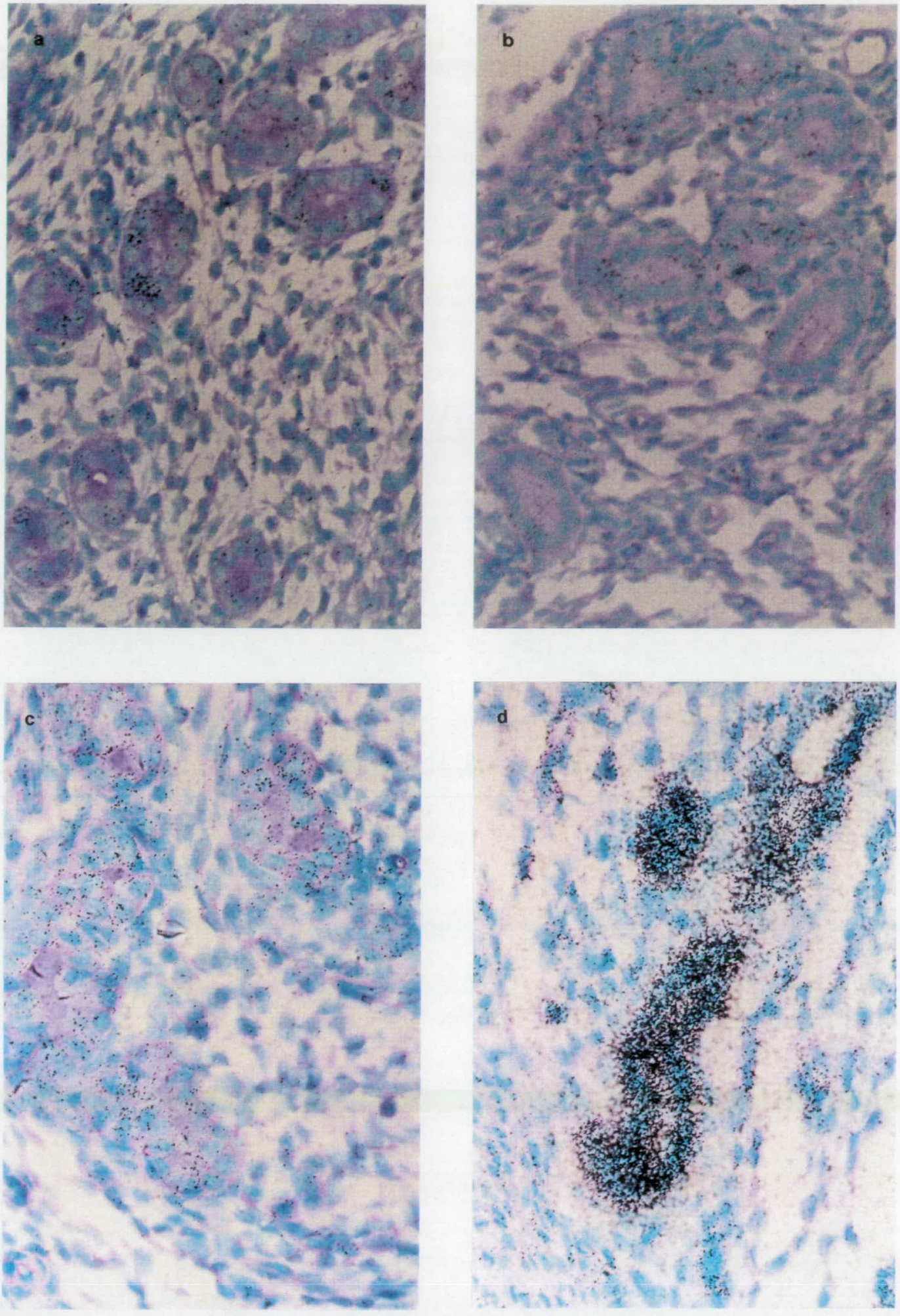
a. 5.5dpc uterus using a probe for the whole of the coding region for DIA

b. 5.5dpc uterus using a probe specific for the diffusible form of DIA

c. 5.5dpc uterus using the DIA-R probe

d. 4.5dpc uterus using the gp130 probe

Fig. 3.7



Discussion

The majority of previous reports of the expression of cytokines in the preimplantation mouse embryo have been based on the techniques of PCR and cDNA library screening (Rappolee et al., 1988; 1992; Conquet and Brûlet, 1990; Lee et al.,1990; Rothstein et al., 1992). The inferences drawn from the PCR studies are that certain cytokines, such as epidermal, transforming, platelet-derived and insulin-like growth factors (IGFs) are expressed in preimplantation embryos (Rappolee et al., 1988; 1992; Lee et al., 1990). Expression of the mRNA for the receptors for IGF-I, IGF-II and insulin has also been reported from RT-PCR studies (Rappolee et al., 1992). Screening of the cDNA libraries revealed transcripts for IL-1β, IL-6, and interferon-γ at the blastocyst stage (Rothstein et al., 1992). IL-6 and DIA have been reported by PCR to be expressed in blastocysts (Murray et al., 1990; Conquet and Brûlet, 1990). Inappropriate amplification of contaminating sequences is one of the hazards associated with the PCR technique, especially when the quantity of starting material is limited, as is the case with small numbers of preimplantation embryos (Conquet and Brûlet, 1990; Murray et al., 1990). Neither of the techniques used previously provides any information about the spatial distribution of the transcripts for the genes under investigation. Using *in situ* hybridisation the expression of a gene can be assigned unambiguously to specific tissues or cells. This technique has been used on preimplantation embryos in the study of FGF-4 (Niswander and Martin, 1992).

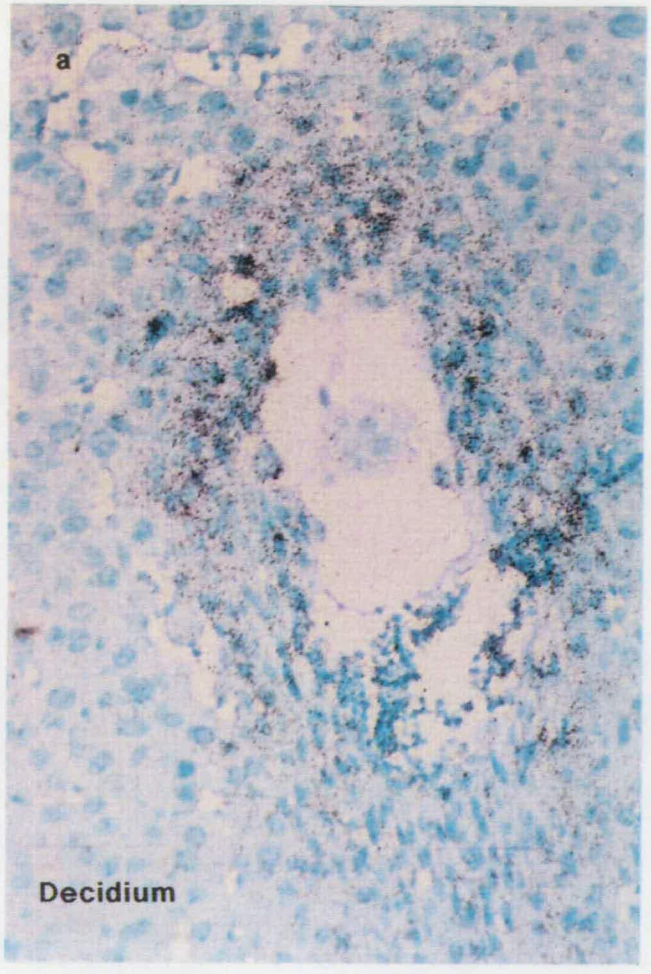
Fig. 3.8 *In situ* hybridisation on sections of postimplantation embryos (x20 objective) using probes for the components of the DIA receptor

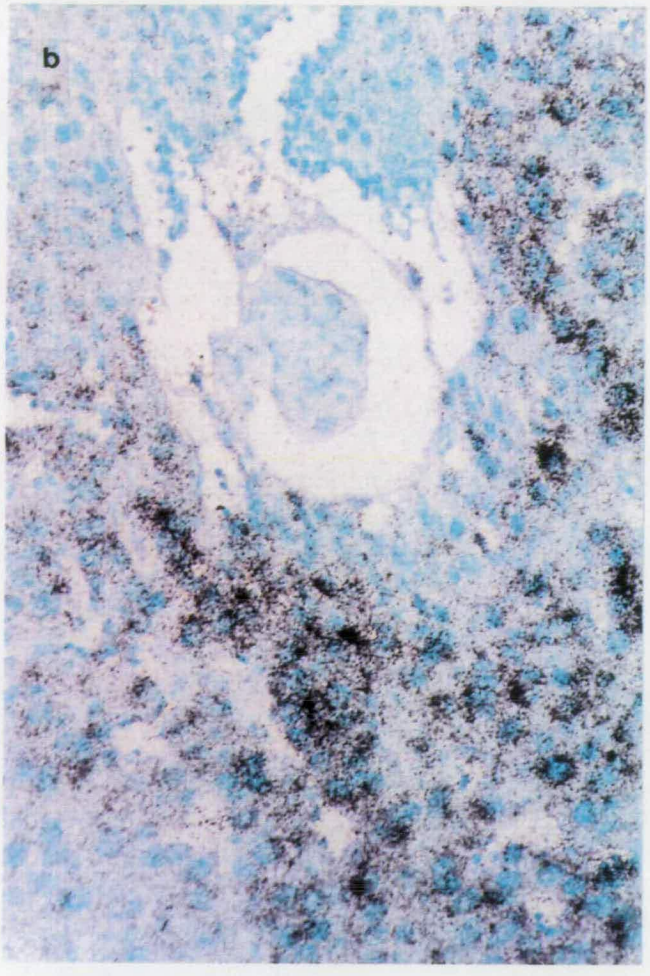
a. 5.5dpc using the DIA-R probe showing hybridisation to the decidual tissue surrounding the embryo

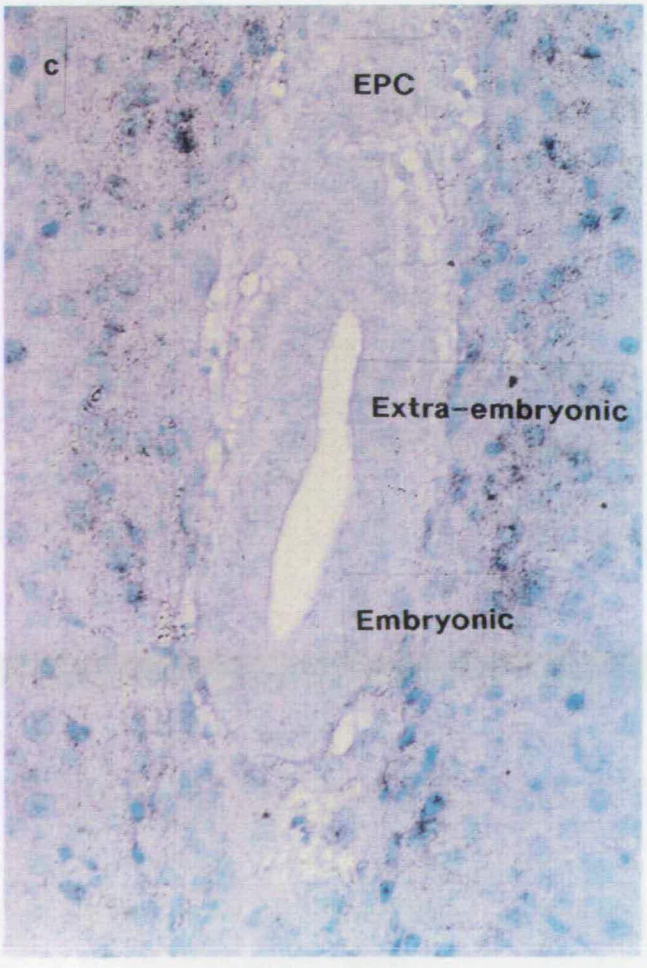
b. 5.5dpc using the probe for gp130, which hybridises most strongly to the decidual tissue around the embryo

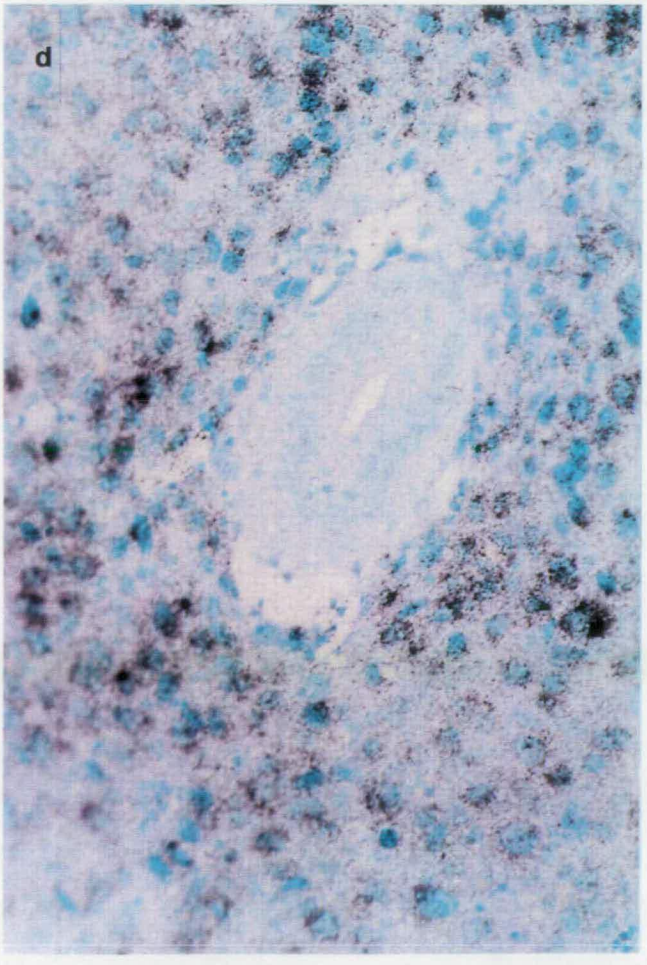
c. 6.5dpc showing transcripts for DIA-R mRNA in the decidual tissue around the embryo
EPC=ectoplacental cone
Ex Emb=extra-embryonic region of conceptus
Emb=embryonic region

d. 6.5dpc using the gp130 probe showing expression particularly in the decidual tissue surrounding the embryo


a
Decidium


b


c
EPC
Extra-embryonic
Embryonic


d

Clear and specific localisation of the grains is evident in the blastocysts investigated here using probes for DIA and the components of its receptor. DIA mRNA transcripts can be detected in the trophectoderm, but not significantly in the ICM (figs. 3.3a, 3.4a and b, 3.5a and b). In contrast, mRNAs for the DIA receptor components predominate in the ICM, with little expression of DIA-R in the trophectoderm (figs. 3.3b and c, 3.4c and d, 3.5c and d). For gp130, in addition to the significant expression seen in the ICM, there are more grains in the trophectoderm than encountered with DIA-R. This may reflect the more ubiquitous role of gp130 owing to its involvement in the receptor complexes of other cytokines such as interleukin-6 (IL-6), ciliary neurotrophic factor (CNTF) and oncostatin M (OSM; Hibi et al., 1990; Gearing et al., 1992; Ip et al., 1992b).

The expression patterns for DIA and the components of its receptor are suggestive of a paracrine interaction between the first tissue to differentiate, the trophectoderm, and the pluripotential ICM. The implication is that the cells of the trophectoderm produce DIA, which binds to the receptors on the ICM cells, enabling them to remain in an undifferentiated state.

The trophectoderm cells in the region between the ICM and the blastocoel produce processes that extend over the ICM cells, covering about 80% of their surface (Fleming et al., 1984). These trophectodermal processes are present from the onset of cavitation. Grains in blastocysts which were hybridised with a probe for DIA can occasionally be seen in regions which may represent these trophectoderm processes (fig. 3.12). As

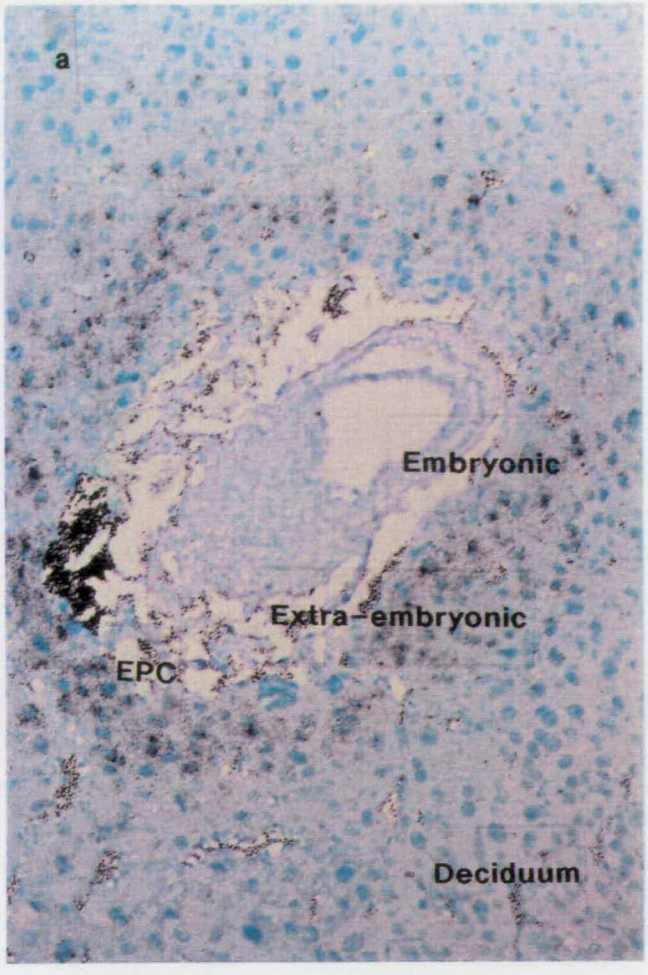
Fig. 3.9 *In situ* hybridisation on section of postimplantation embryos using probes for the componenets of the DIA receptor

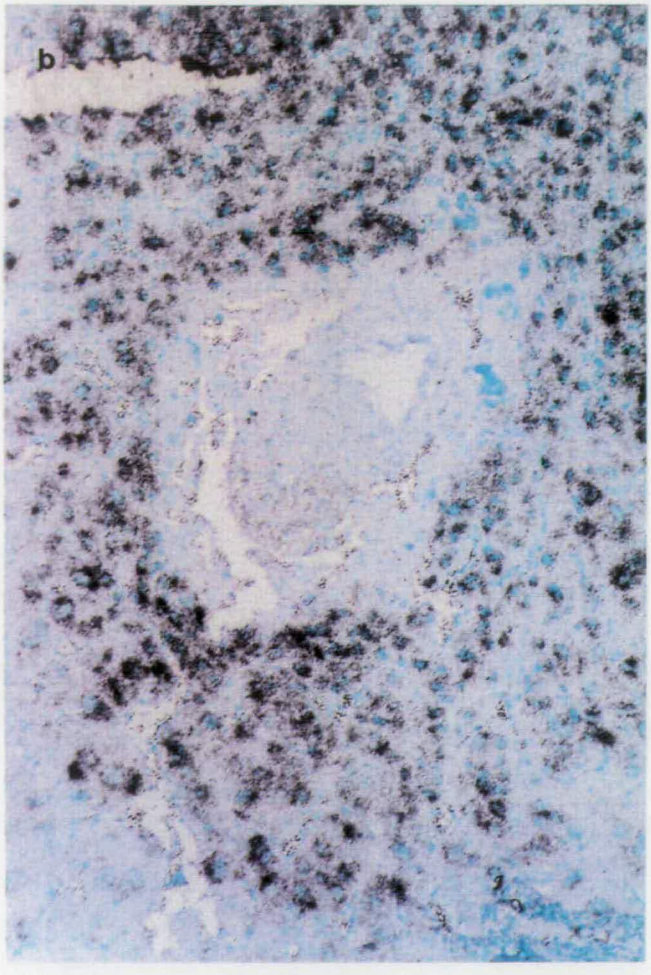
a. 7.5dpc (x10 objective) using the DIA-R probe, which hybridises to the deciduum close to the embryo
Emb=embryonic region
Ex Emb=extra-embryonic region
EPC=ectoplacental cone

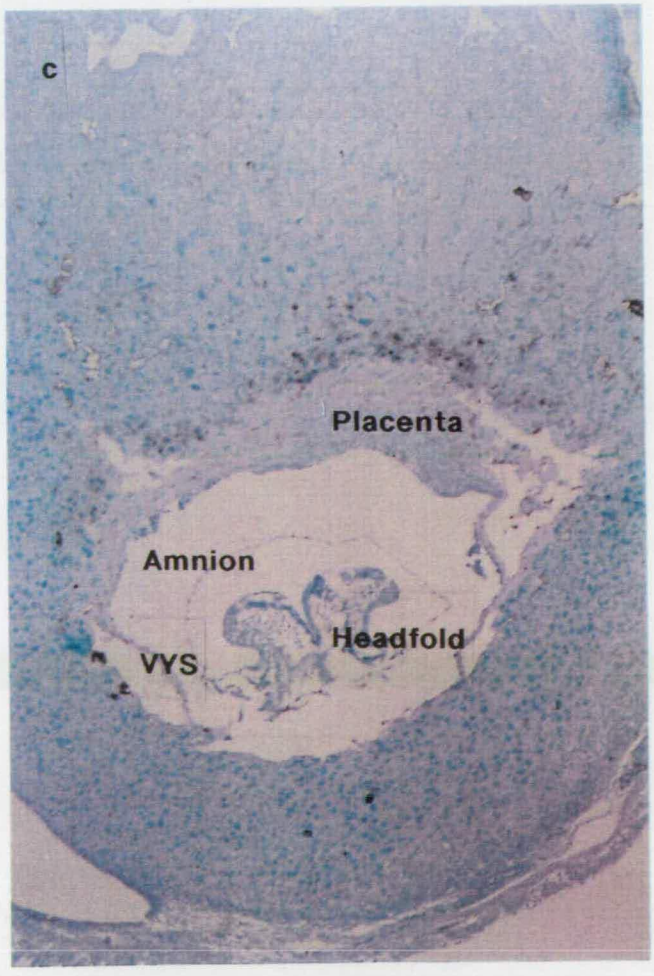
b. 7.5dpc (x10 objective) showing transcripts for gp130 in the decidual tissue around the embryo

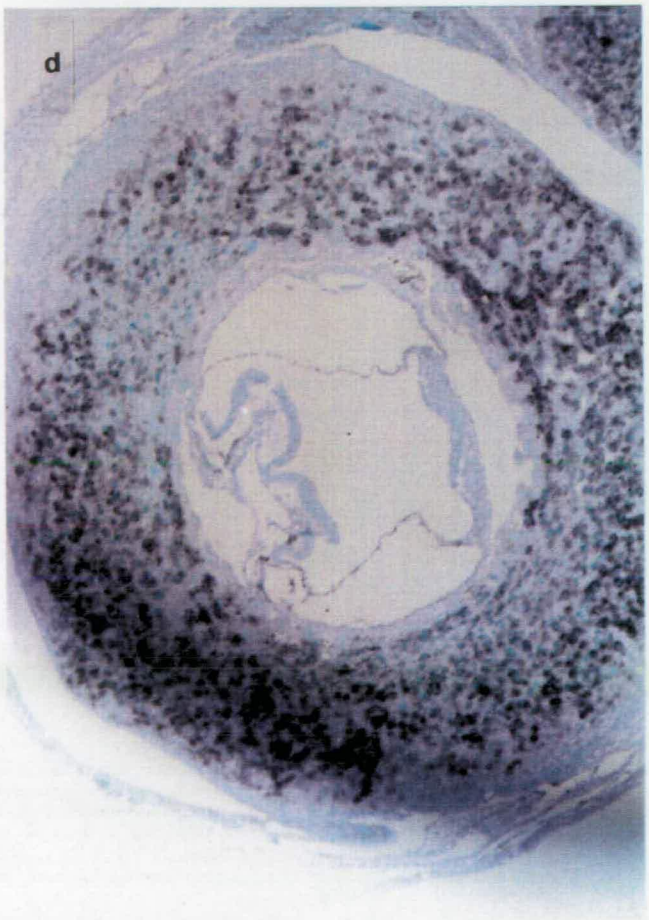
c. 8.5dpc (x4 objective) showing transcripts for DIA-R mRNA in a small area of the deciduum near the placenta
P=placenta
A=amnion
H=headfold
VYS=visceral yolk sac

d. 8.5dpc (x4 objective) showing transcripts for gp130 over most of the deciduum


a
Embryonic
Extra-embryonic
EPC
Deciduum


b


c
Placenta
Amnion
Headfold
VYS


d

development proceeds, these processes are gradually withdrawn, but traces persist even when the blastocyst is fully expanded. Blastocysts of 4.5dpc and in implantation delay may also possess the remains of such processes, so any grains detected in the juxtacoelic region of the ICM may represent signal from either the presumptive primitive endoderm or from the trophectoderm processes.

In the results presented here, the absence of significant numbers of detectable silver grains on morulae suggests either that DIA and the components of its receptor are not being expressed at this stage, or that they are being transcribed at a level beneath the sensitivity of detection by the *in situ* hybridisation technique. The small number of cells present at this stage (8-16), and the low nuclear to cytoplasmic ratio encountered during cleavage means that the amount of mRNA per embryo at these stages is likely to be very low. The apparent artifact of granular material in the blastomeres (fig. 3.6a), which seems to be restricted to early preimplantation embryos, also makes detection of autoradiographic grains very difficult. It has not, therefore, been possible to detect any signal in such early embryos in this study.

Since the onset of this work mice lacking functional DIA have been generated (Stewart et al., 1992; Escary et al., 1993; Smith et al., unpublished). Surprisingly, the embryos are viable up to the blastocyst stage, and can develop to adulthood, providing the host uterus is wild type or heterozygous. DIA is not, therefore, indispensible for development of the preimplantation

Fig. 3.10 *In situ* hybridisation on sections of 8.5dpc embryos (x20 objective) using a probe for DIA-R

a. Section through the trunk region showing expression of DIA-R over most tissues, especially the neural tube, somites and mesenchyme

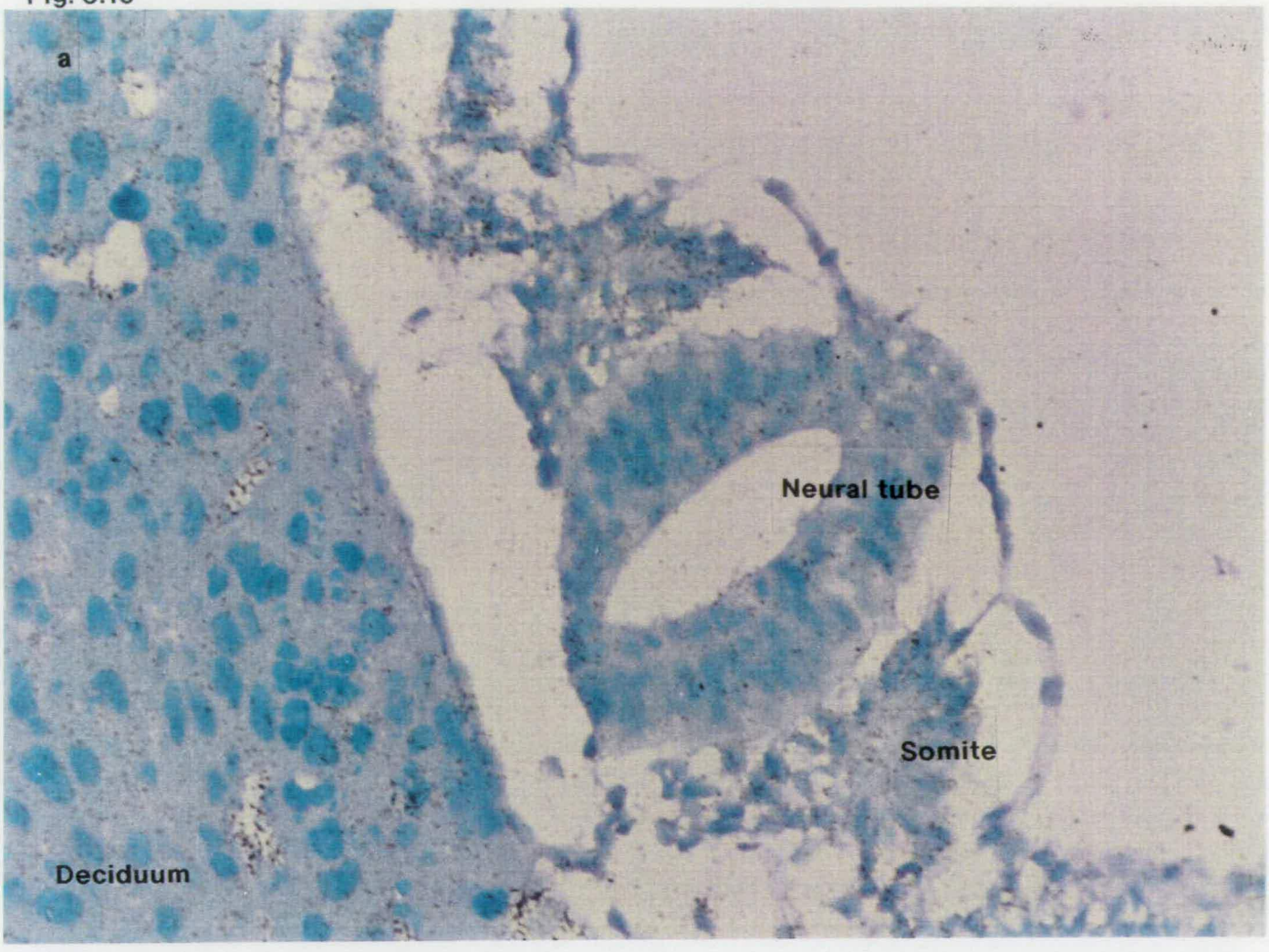
NT=neural tube

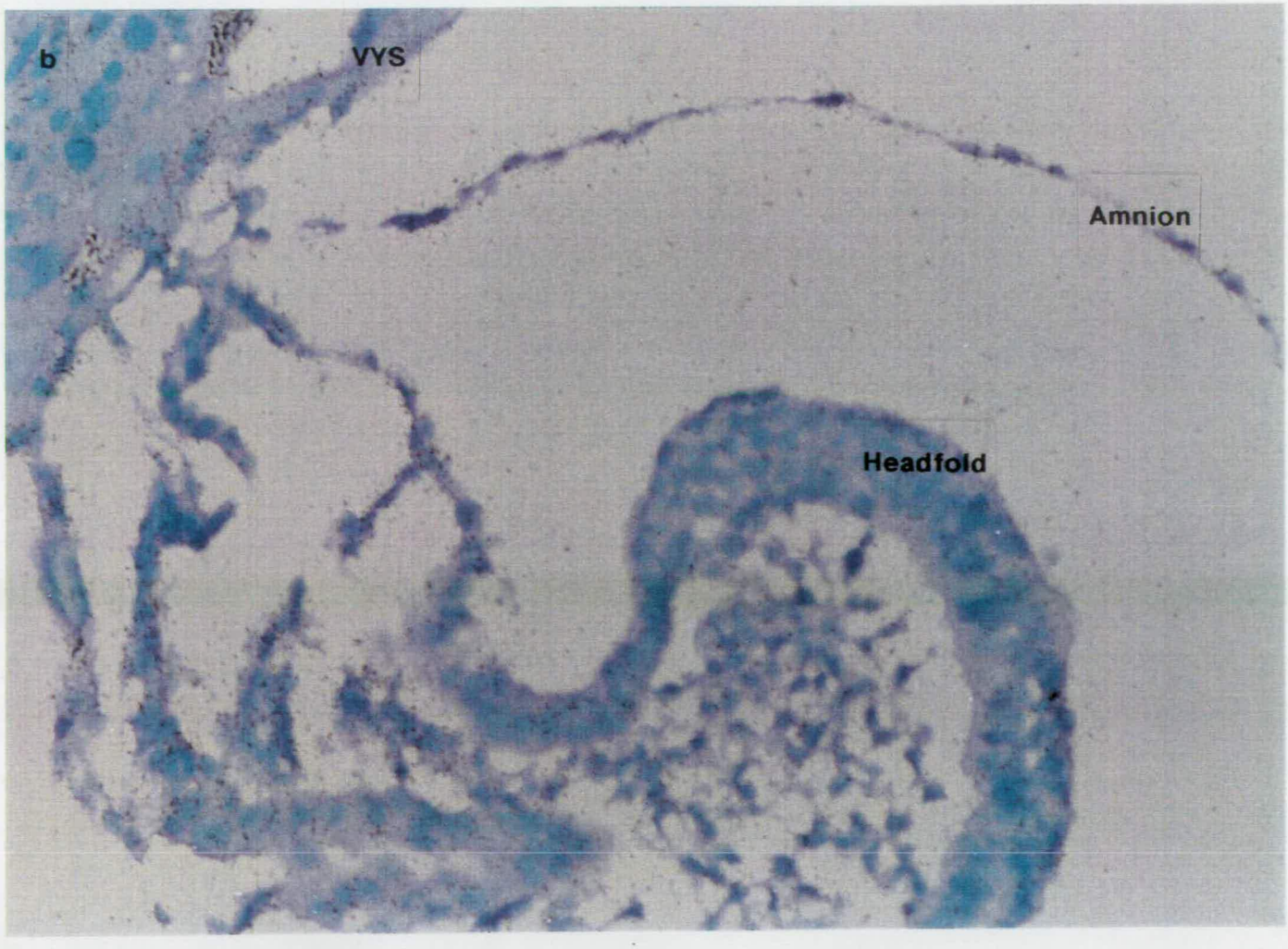
S=somite

b. Section through the anterior region showing transcripts for DIA-R in the visceral yolk sac (VYS), amnion (A), neural tissue and mesenchyme

H=headfold

Fig. 3.10





embryo. It is possible that DIA has no role in early development, but the specific expression patterns of the mRNAs for DIA and its receptor seen in blastocysts by *in situ* hybridisation suggest some function. The most likely role for DIA in this context, considering its ability to inhibit the differentiation of ES cells in culture, is in the maintenance of the stem cells in the ICM. An alternative means for achieving this may be available. However, the possiblity that DIA has no purpose in the early embryo, or that it is involved in some other function cannot be ruled out.

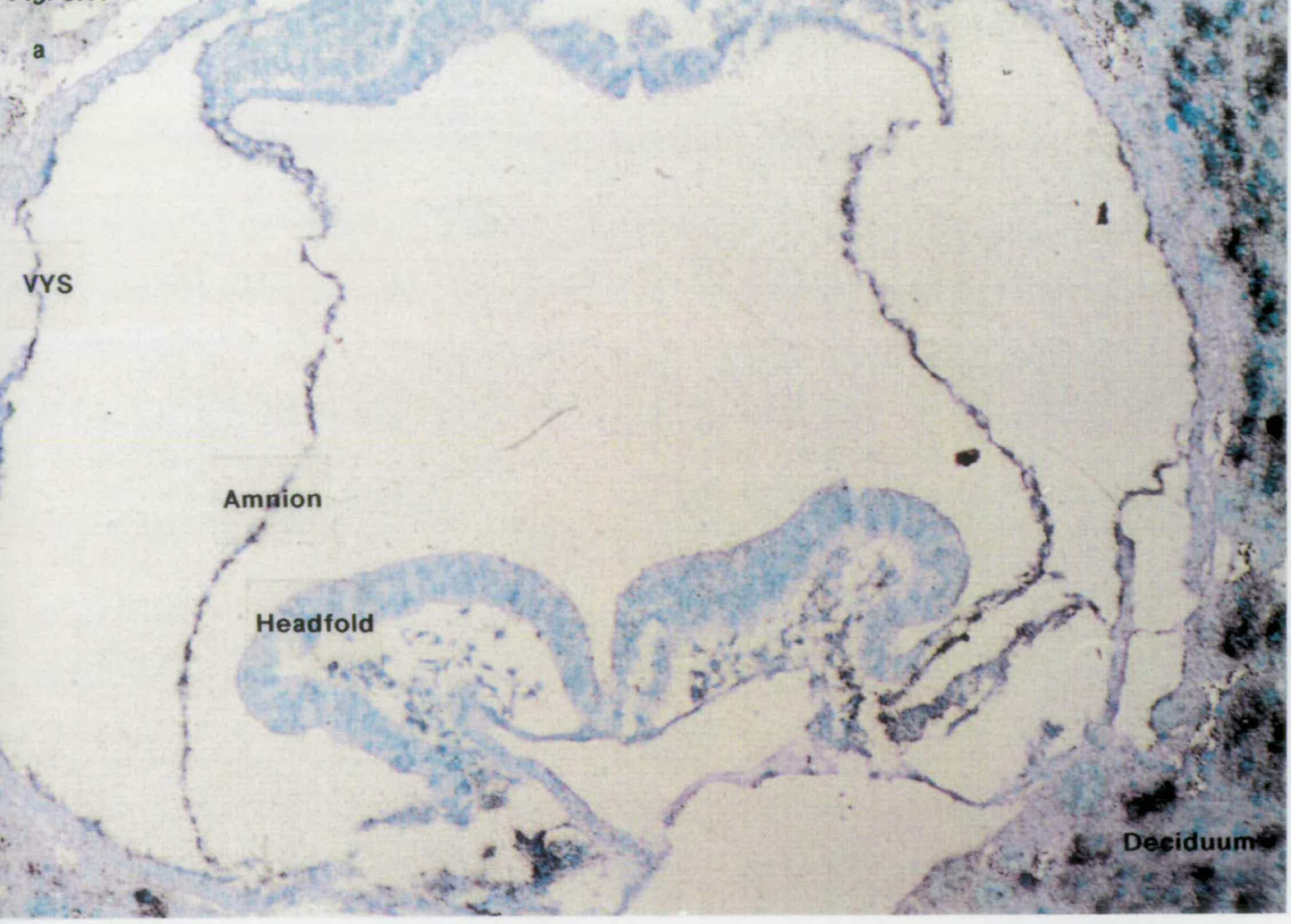
By the time the blastocyst is ready to implant at 4.5dpc, the mural trophectoderm cells have undergone giant cell transformation and the primitive endoderm has begun to differentiate on the blastocoelic surface of the ICM. The expression patterns of DIA and its receptor complex observed in the 3.5dpc blastocyst are maintained. The layer of cells on the blastocoelic surface of the ICM is able to overcome the inhibition of differentiation and become the primitve endoderm (see chapter 1, fig 1.1a). How this is achieved is not known, but it probably involves the action of additional differentiation factors. The remaining undifferentiated ICM tissue is now known as the primitive ectoderm or epiblast. The pluripotential state of this is maintained for several more days.

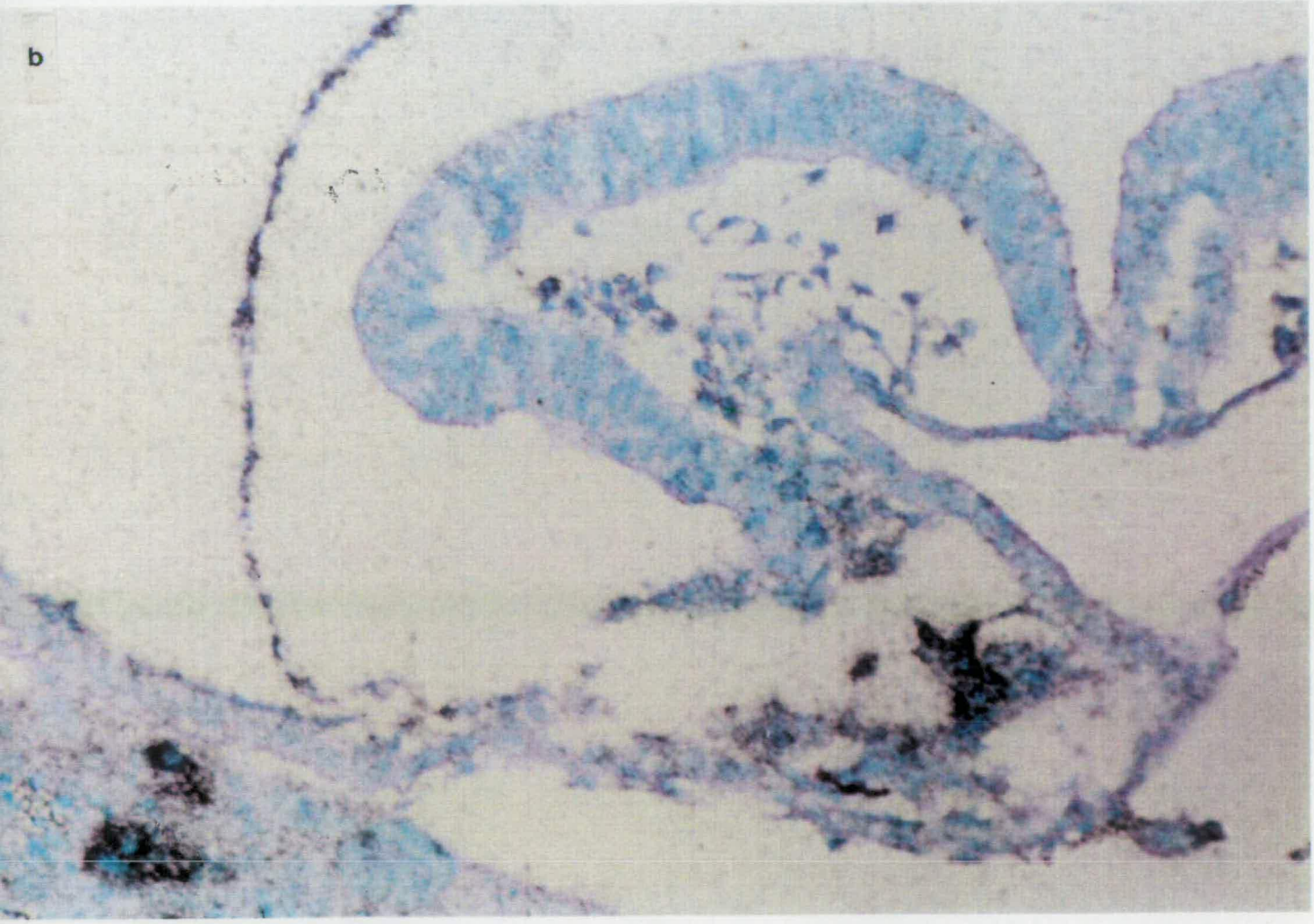
Transcripts for DIA have been detected in postimplantation embryos by RNase protection studies (Rathjen et. al., 1990b). By crude dissection of the egg cylinders into embryonic and extra-embryonic regions, expression has been localised to the extra-embryonic portion (Rathjen et. al. 1990b). This conforms with the

Fig. 3.11 *In situ* hybridisation on a section through the anterior region of an 8.5dpc embryo using a probe for gp130

a. Transcripts for gp130 are present on all tissues seen in this section, but are more apparent over the deciduum, amnion, visceral yolk sac (VYS) and mesenchyme (x10 objective)

b. x20 objective


a
VYS
Amnion
Headfold
Deciduum


b

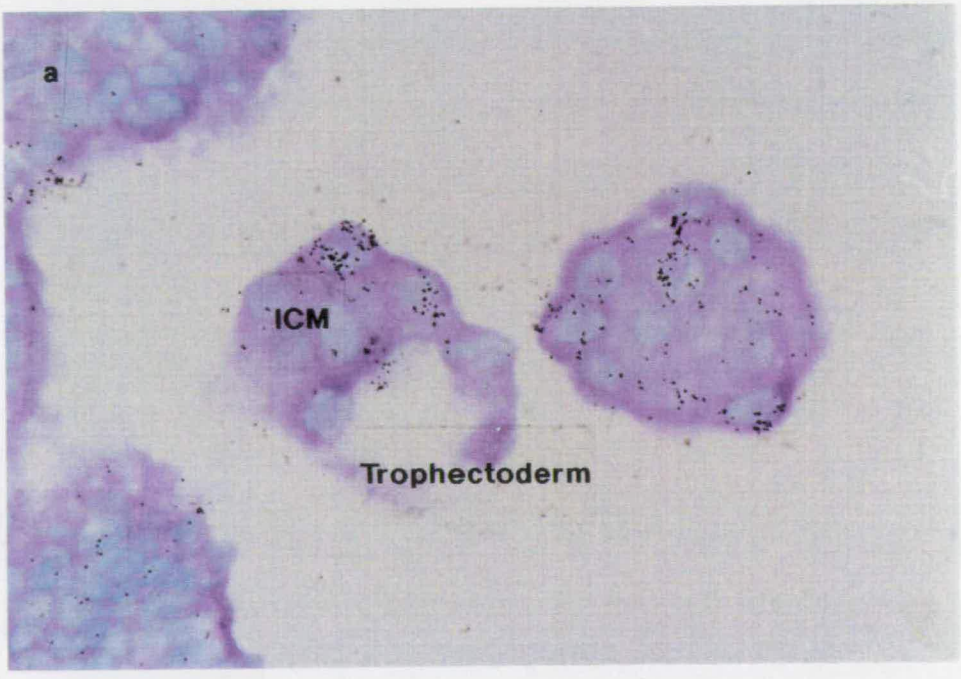
clearly understood, but they may play a role in implantation. The finding that mutant female mice which do not produce active DIA are unable to support implantation lends weight to this idea (Stewart et al., 1992). However, endometrial glands are present in the uterus of homozygous DIA negative female mice several days after fertilisation (fig. 7.2). The observation that both DIA-R and gp130 mRNAs are also concentrated in the uterine endometrial glands suggests an autocrine activity for DIA in this tissue. In addition to its expression in the endometrial glands, DIA-R mRNA is found in the epithelium lining the uterine lumen (fig. 3.13), which may also be somehow involved in implantation. Such an elevation of signal is not seen with gp130. The reason for this is not known, but it serves to demonstrate that the expression of DIA-R and gp130 are not always under the same regulatory control. It has not been possible to obtain a probe specific for the putative soluble form of DIA-R, but it seem likely that the expression of DIA-R seen in the epithelium lining the uterine lumen may represent the soluble form. The expression of this form is more likely to serve some function in the absence of coordinate expression of gp130.

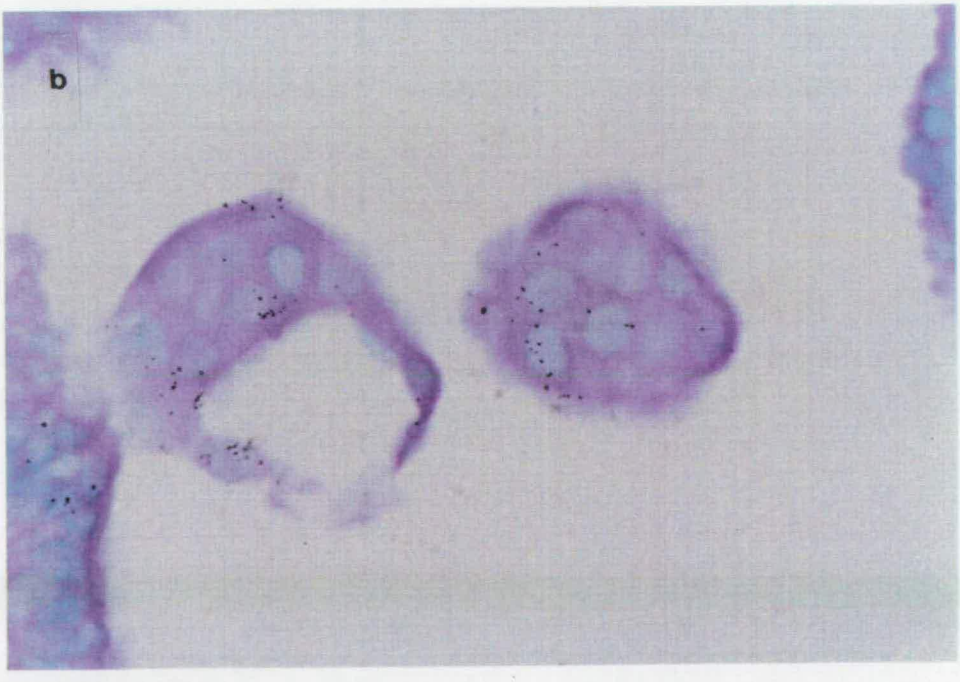
DIA-R and gp130 mRNAs are seen in the decidual tissue surrounding the embryo in all of the postimplantation stages studied (figs. 3.8 and 3.9). The level of DIA-R mRNA in the deciduum appears to decrease at 8.5dpc (fig. 3.9c), whereas that of gp130 increases (fig. 3.9d). This provides another example of the independent regulation of expression for DIA-R and gp130. At the egg cylinder stage the levels of transcripts for DIA-R mRNA

Fig. 3.12 *In situ* hybridisation on sections of blastocysts (x60 oil immersion objective) using a probe for DIA. Transcripts are apparent in the trophectoderm, but also in the regions of the inner cell mass (ICM) adjacent to the blastoceol, which may represent either presumptive primitive endoderm or trophectoderm processes over-lying the ICM

TE=trophectoderm

Fig. 3.12



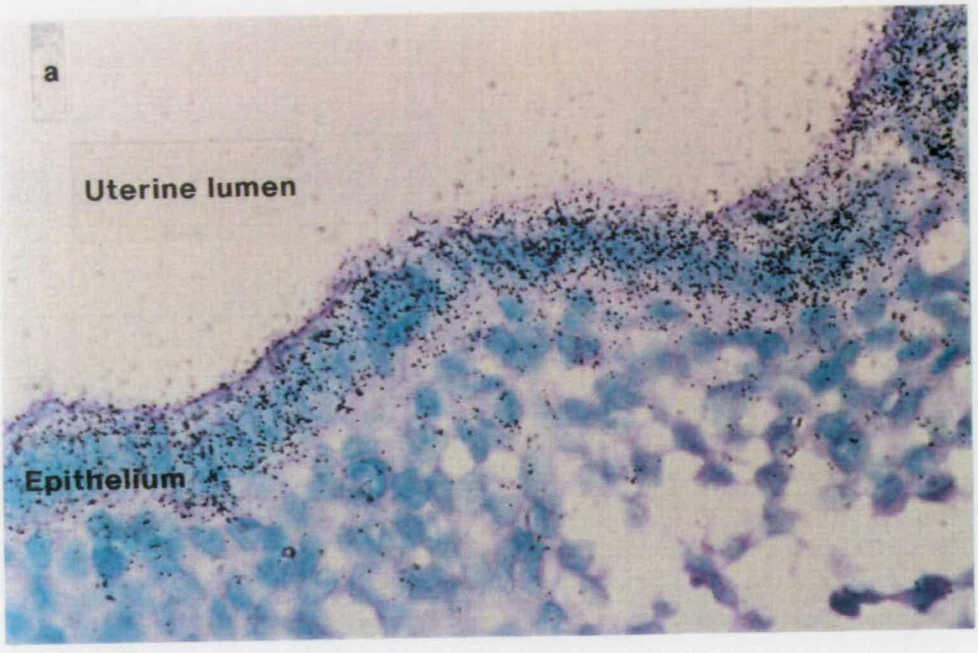


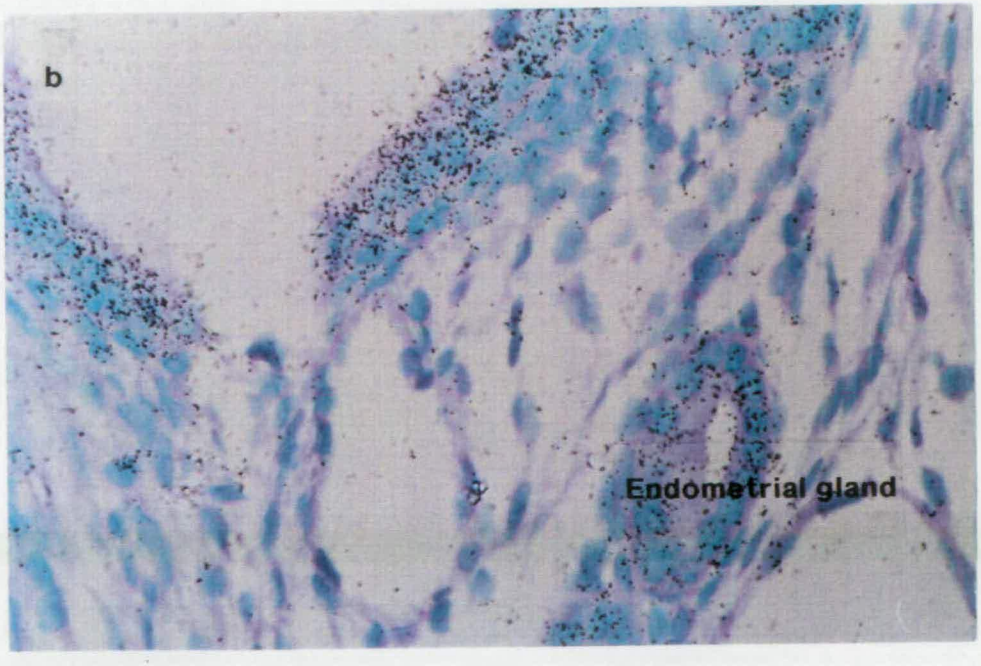
are not significantly above background in the embryo (fig. 3.8a and c, fig. 3.9a). This indicates a down-regulation in the production of DIA-R around the time of implantation. This is consistent with the gradual restriction in pluripotency that occurs during gastrulation, and suggests an alteration in cytokine dependence. Low level expression of gp130 is seen over most of the tissues at this stage (figs. 3.8b and d, fig. 3.9b). At 8.5dpc there is an obvious increase in expression of DIA-R in most tissues, including the neural tube, amnion, visceral yolk sac and pre-somitic mesoderm (fig. 3.10). Similar, but higher concentrations of grains are seen using the probe for gp130 (fig. 3.11). DIA-R and gp130 are components of the receptors for CNTF and OSM; the expression of these subunits may, therefore, reflect some function of either of these cytokines. Transcripts for DIA-R are evident in the neural plate/neural tube region of 8.5dpc embryos (fig. 3.10). As will be seen in chapter 4, this correlates with the detected expression of the CNTF-specific receptor component at the same stage.

There are several possible explanations for the abundance of transcripts for DIA-R and gp130 in decidua, some of which may be clarified by the availability of probes for the soluble form of DIA-R. The production of DIA by the endometrial glands may be detrimental to the embryo following implantation. Exposure of the developing embryo to elevated levels of DIA can cause abnormalities (Conquet et al., 1992; see chapter 6). Receptors on the decidual cells surrounding the embryo may serve to capture the DIA and prevent it from reaching the embryo. Alternatively,

Fig. 3.13 *In situ* hybridisation on sections through a uterus at 4.5dpc (x60 oil immersion objective) using a probe for DIA-R. Transcripts localise to the epithelium lining the uterine lumen aswell as to the endometrial glands

Fig. 3.13





the embryo may benefit from the DIA produced by the uterus, and the receptors in the deciduum are involved in delivery of DIA to the embryo. In this case, a soluble form of the receptor would be required. The presence of mRNA for the DIA receptor may, alternatively, suggest a role for DIA in decidual function. The deciduum is thought to be involved in nutrition of the embryo (Finn, 1969). The cells closest to the embryo possess large numbers of ribosomes and intracellular fibrils and an extensive endoplasmic reticulum. The localisation of mRNAs for the components of the DIA receptor to this region implies that these cells are targets for DIA. The inability of female mice lacking DIA to support implantation (Stewart et al., 1992; Escary et al., 1993; Smith et al., unpublished) may be related to the involvement of DIA in the action of the deciduum, particularly in the process of its initiation.

The differential expression patterns of both DIA and its receptor in the mouse blastocyst documented here provide strong evidence for the involvement of DIA in development of the early mouse embryo. The finding since the onset of this work, that embryos can develop until implantation in the absence of DIA (Stewart et al., 1992; Escary et al., 1993; Smith et al., unpublished), suggests that either DIA is not required for early development, or that some other molecule is able to fulfil the same role. In the following chapter, the expression patterns of other cytokines which operate via the same signal transduction pathway as DIA will be considered.

CHAPTER 4

Introduction

The finding that mutant embryos lacking DIA are able to survive up to the blastocyst stage without any additional administration of DIA, and can reach adulthood in non-mutant or heterozygous foster mothers implies that there is not an absolute requirement for DIA in early mouse development (Stewart et al., 1991). It is possible that the role of stem cell maintenance in the embryo can be achieved by DIA in the normal situation, but another means must exist. The receptor for DIA contains a signal transduction subunit, gp130, which is also involved in the receptor complexes of a number of other cytokines, including interleukin-6 (IL-6), ciliary neurotrophic factor (CNTF) and oncostatin M (OSM). Providing that the other components of the receptor complex are available, each of these cytokines is able to inhibit the differentiation of ES cells when added to the culture medium (Yoshida et al., 1994; Conover et al., 1993; Wolf et al., 1994; Rose et al., 1994). These cytokines may, therefore, be considered as possible candidates for maintaining the stem cells in the early embryo. A necessary criterion for the involvement of any of these cytokines in early development is their availability to the embryo *in utero*.

Maintenance of ES cells by IL-6 in culture requires the provision of the soluble IL-6-specific receptor component (sIL-6R; Yoshida et al., 1994). Since the sIL-6R component is absent from ES cells (Saito et al., 1992), it is unlikely to be produced by the cells of the ICM. Possibly, it may be produced and secreted by the cells of the trophectoderm. Blastocysts have been shown previously to

express IL-6 mRNA, and to secrete IL-6 protein into the medium during culture (Murray et al., 1990). What function the IL-6 serves at this stage is unknown. So far, there is no evidence that IL-6 can operate via an alternative signal transduction pathway to that initiated by gp130. In the ES cell system the maintenance of the undifferentiated state in culture is dependent upon the provision of the soluble receptor in addition to the ligand (Yoshida et al., 1994).

Although murine ES cells can be maintained in an undifferentiated state by the supplementation of the medium with human OSM, to date, attempts to clone the murine form of OSM have proved difficult. This is likely to reflect a dissimilarity in the genes for the two species. A human probe is, therefore, unlikely to bind very specifically to murine tissues during *in situ* hybridisation. No signal has been detected by Northern or Southern hybridisation on murine RNA or DNA using the probe for human OSM (C. Dani, unpublished). A study of the expression patterns of this cytokine in the developing mouse embryo has not yet been attempted.

The amino acid sequences for the CNTF-specific receptor component (CNTFR) of the rat, rabbit and human are extremely well conserved, having a homology of 94% (Ip et al., 1993). It seems likely that the mouse form will also be very similar. The procedure selected for cloning the murine CNTFR, therefore, was reverse transcription polymerase chain reaction (RT-PCR). This approach was also adopted for the CNTF ligand, although the amino acid sequence of this molecule is a little less well conserved between the species studied (Ip et al., 1993). The murine form of CNTFR was successfully cloned from mouse cDNA, as verified by sequencing (figs. 4.1-4.3), but the mouse form of CNTF could not be easily isolated using this method. *In situ* hybridisation was, therefore,

carried out on sections of embryos using probes generated from the mouse form of CNTFR and the rat form of CNTF. Previous Northern hybridisation analysis on adult rat tissues has revealed that transcripts for CNTFR are mainly restricted to the nervous system, in particular the olfactory bulb, thalamus, midbrain, hindbrain, spinal cord and peripheral nerves (Ip et al., 1993), and also to skeletal muscle (Davis et al., 1991). In the embryo CNTFR mRNA has been found by Northern analysis and *in situ* hybridisation to predominate in neural tissue and derivatives of the neural crest (Ip et al., 1993). Although the levels are significantly lower, CNTF mRNA has been shown by Northern blotting to have a similar distribution in both adult and embryonic rats (Ip et al., 1993). However, another study has failed to detect any CNTF protein in embryonic rats (M. Sendtner, unpublished).

Here, *in situ* hybridisation has been carried out on sections of blastocysts and 8.5dpc embryos using probes for mouse IL-6, rat CNTF and mouse CNTFR to investigate the potential involvement of any of these cytokine systems in development of the early mouse embryo.

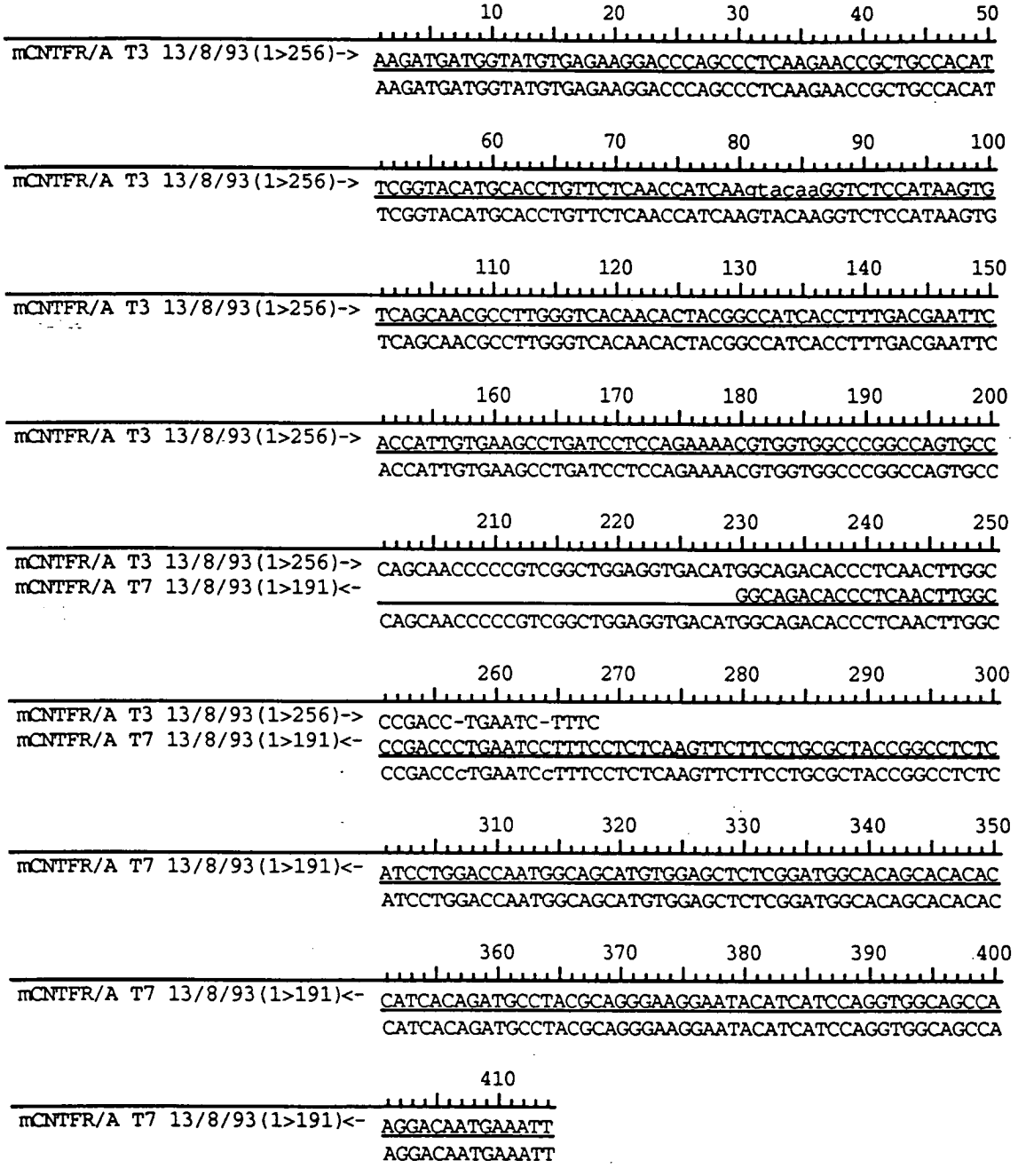
Materials and Methods

Mouse CNTFR

The mouse CNTFR gene was cloned using RT-PCR, as described in chapter 2. The oligonucleotide primers used for cloning the mouse CNTFR were predicted from the published rat and human CNTFR protein sequence (Ip et al., 1993). The first were from amino acids 121 to 126 (oligo A) and 282 to 287 (oligo B). These were selected because they are identical in the rat and human and have the same predicted melting temperatures (49°C). They flank a sequence of 498 base pairs (bp). A successful series of PCR amplification should, therefore, generate a fragment of about 498bp To increase the probability of amplifying the desired sequence from cDNA, another primer in the 5' region was selected to generate a shorter fragment of 411bp. This oligonucleotide (oligo C) had a predicted melting temperature of 49°C. A second round of PCR was performed upon the product generated by the reaction using the original primers, to obtain a new product of the appropriate size (411bp). This fragment was amplified and subcloned into pBluescript II KS (fig) and its authenticity verified by sequencing, as described in chapter 2. The template was linearised using SalI, and a radioactively labelled probe synthesised from the T7 promoter using either ^{35}S-labelled CTP or ^{33}P-labelled UTP (see chapter 2). *In situ* hybridisation was performed using the CNTFR probe on sections of mouse blastocysts (3.5dpc) and 8.5dpc embryos.

Fig. 4.1 DNA sequence as read from the sequencing gel for part of the murine form of the ciliary neurotrophic factor-specific receptor subunit (mCNTFR)

Fig. 4.1


10 20 30 40 50
mCNTFR/A T3 13/8/93(1>256)-> AAGATGATGGTATGTGAGAAGGACCCAGCCCTCAAGAACCGCTGCCACAT
AAGATGATGGTATGTGAGAAGGACCCAGCCCTCAAGAACCGCTGCCACAT
60 70 80 90 100
mCNTFR/A T3 13/8/93(1>256)-> TCGGTACATGCACCTGTTCTCAACCATCAAgtacaaGGTCTCCATAAGTG
TCGGTACATGCACCTGTTCTCAACCATCAAGTACAAGGTCTCCATAAGTG
110 120 130 140 150
mCNTFR/A T3 13/8/93(1>256)-> TCAGCAACGCCTTGGGTCACAACACTACGGCCATCACCTTTGACGAATTC
TCAGCAACGCCTTGGGTCACAACACTACGGCCATCACCTTTGACGAATTC
160 170 180 190 200
mCNTFR/A T3 13/8/93(1>256)-> ACCATTGTGAAGCCTGATCCTCCAGAAAACGTGGTGGCCCGGCCAGTGCC
ACCATTGTGAAGCCTGATCCTCCAGAAAACGTGGTGGCCCGGCCAGTGCC
210 220 230 240 250
mCNTFR/A T3 13/8/93(1>256)-> CAGCAACCCCCGTCGGCTGGAGGTGACATGGCAGACACCCTCAACTTGGC
mCNTFR/A T7 13/8/93(1>191)<- GGCAGACACCCTCAACTTGGC
CAGCAACCCCCGTCGGCTGGAGGTGACATGGCAGACACCCTCAACTTGGC
260 270 280 290 300
mCNTFR/A T3 13/8/93(1>256)-> CCGACC-TGAATC-TTTC
mCNTFR/A T7 13/8/93(1>191)<- CCGACCCTGAATCCTTTCCTCTCAAGTTCTTCCTGCGCTACCGGCCTCTC
CCGACCcTGAATCcTTTCCTCTCAAGTTCTTCCTGCGCTACCGGCCTCTC
310 320 330 340 350
mCNTFR/A T7 13/8/93(1>191)<- ATCCTGGACCAATGGCAGCATGTGGAGCTCTCGGATGGCACAGCACACAC
ATCCTGGACCAATGGCAGCATGTGGAGCTCTCGGATGGCACAGCACACAC
360 370 380 390 400
mCNTFR/A T7 13/8/93(1>191)<- CATCACAGATGCCTACGCAGGGAAGGAATACATCATCCAGGTGGCAGCCA
CATCACAGATGCCTACGCAGGGAAGGAATACATCATCCAGGTGGCAGCCA
410
mCNTFR/A T7 13/8/93(1>191)<- AGGACAATGAAATT
AGGACAATGAAATT

Rat CNTF

The published amino acid sequences for CNTF of the rat, human and rabbit show a reduced similarity compared with those of CNTFR (Stöckli et al., 1989; Ip et al., 1993). However, attempts were made to clone the mouse form of CNTF by the method described above for CNTFR, but as the desired band was not apparent on the gel following PCR, even using the nested primer technique, sub-cloning and probe synthesis was carried out using the rat form of cDNA. As the procedure adopted for this was very similar, and run in parallel with the preparation of the mouse CNTFR probe, it is not described separately in detail here. Oligonucleotide primers were predicted from the published protein sequences for rat and human CNTF to lie in regions of complete identity between the two species (Stöckli et al., 1989). For the first round of PCR the oligos used were from amino acid 38 to 43 and 137 to 142. From these, a fragment of 312bp should be amplified. Primer nesting was performed by replacing the latter oligo with one from amino acids 102 to 107 to generate a fragment of 207bp when PCR was performed on the product of the previous reaction. This fragment was gel purified and subcloned into pBluescript II KS (fig) using Sal1 and BamH1 restriction sites which had been included in the oligo pairs. Authenticity of the product as rat CNTF cDNA was established by sequencing. The plasmid was linearised with Sal1 and radioactively-labelled probe synthesised by transcription from the T7 promoter. *In situ* hybridisation was performed as described on sections of 3.5 and 8.5dpc embryos (see fig).

Fig. 4.2 Comparison of the DNA sequence of the murine form of CNTFR with that of the rat. The sequences differ in 6 base positions.

Fig. 4.2

Seq1(1>414) mCNTFR con	Seq2(1>1194) RAT CNTFR DNA SEQ	Similarity Index	Gap Number	Gap Length	Consensus Length
(4>162)	(531>689)	96.9	0	0	159

```
                        10        20        30        40        50        60        70        80
mCNTFR con          AAGATGATGGTATGTGAGAAGGACCCAGCCCTCAAGAACCGCTGCCACATTCGGTACATGCACCTGTTCTCAACCATCAA
                    AA ATGATGGT TGTGAGAAGGACCCAGCCCTCAAGAACCGCTG CACATTCGGTACATGCACCTGTTCTCAACCATCAA
RAT CNTFR DNA SEQ   AAAATGATGGTCTGTGAGAAGGACCCAGCCCTCAAGAACCGCTGTCACATTCGGTACATGCACCTGTTCTCAACCATCAA
                      530       540       550       560       570       580       590       600
                        90       100       110       120       130       140       150       160
mCNTFR con          GTACAAGGTCTCCATAAGTGTCAGCAACGCCTTGGGTCACAACACTACGGCCATCACCTTTGACGAATTCACCATTGTGA
                    GTACAAGGTCTCCATAAGTGTCAGCAACGCCTTGGGTCACAACAC ACGGC ATCACCTT GACGAATTCACCATTGTGA
RAT CNTFR DNA SEQ   GTACAAGGTCTCCATAAGTGTCAGCAACGCCTTGGGTCACAACACCACGGCTATCACCTTCGACGAATTCACCATTGTGA
                      610       620       630       640       650       660       670       680

mCNTFR con          AG
                    AG
RAT CNTFR DNA SEQ   AG
```

Mouse IL-6

The full length murine IL-6 fragment of 650bp subcloned into pUC was supplied by T. Taga. For probe synthesis, this fragment was released by digestion with Xba1 and BamH1 and subcloned into pBluescript II KS (fig). Linearisation was performed using EcoR1, and transcription to generate a radioactively-labelled probe initiated by means of the T7 promoter.

Fig. 4.3 Comparison of the protein sequence for mouse CNTFR with that of the rat. There is one amino acid at position 112 which is different between the two species.

Fig. 4.3

Seq1(1>138) M CNTFR PROTEIN	Seq2(1>372) RAT CNTFR PROTEIN	Similarity Index	Gap Number	Gap Length	Consensus Length
(1>138)	(150>287)	99.3	0	0	138

```
                          10        20        30        40        50        60        70        80
M CNTFR PROTEIN    KMMVCEKDPALKNRCHIRYMHLFSTIKYKVSISVSNALGHNTTAITFDEFTIVKPDPPENVVARPVPSNPRRLEVTWQTP
                   KMMVCEKDPALKNRCHIRYMHLFSTIKYKVSISVSNALGHNTTAITFDEFTIVKPDPPENVVARPVPSNPRRLEVTWQTP
RAT CNTFR PROTEIN  KMMVCEKDPALKNRCHIRYMHLFSTIKYKVSISVSNALGHNTTAITFDEFTIVKPDPPENVVARPVPSNPRRLEVTWQTP
                   150       160       170       180       190       200       210       220

                           90       100       110       120       130
M CNTFR PROTEIN    STWPDPESFPLKFFLRYRPLILDQWQHVELSDGTAHTITDAYAGKEYIIQVAAKDNEI
                   STWPDPESFPLKFFLRYRPLILDQWQHVELS:GTAHTITDAYAGKEYIIQVAAKDNEI
RAT CNTFR PROTEIN  STWPDPESFPLKFFLRYRPLILDQWQHVELSNGTAHTITDAYAGKEYIIQVAAKDNEI
                   230       240       250       260       270       280
```

Results

The resultant amplified portions of mouse CNTFR and rat CNTF cDNAs were successfully cloned into pBluescript KS II. The identity of the clones was established by sequencing (Fig) and Southern hybridisation to rat cDNA from the PCR reaction. Although several bands were present on the agarose gel in addition to the one of the expected size, only one band was obtained following Southern blotting. The single band obtained indicated that neither probe cross-reacted with any other gene, and that there were no alternative transcripts for CNTF or CNTFR (data not shown). Radioactively-labelled probes were synthesised for *in situ* hybridisation (chapter 2).

Using the mouse CNTFR probe, grains localised to the neural plate/neural tube region and also to the neural crest on sections of 8.5dpc embryos (figs). A few grains could be distinguished in the ICM region of 3.5dpc blastocysts (figs), but the intensity of the signal was significantly lower than that observed in the neural regions of the 8.5dpc embryos. A higher signal was seen using a probe for the DIA receptor on sections of blastocysts (chapter 3, fig), although this experiment was performed on a separate occasion, and variability between runs can be a problem with *in situ* hybridisation. However, both probes have been used in several experiments, and the characteristic intensity of expression was maintained. The DIA-R probe (980bp; see chapter 3) is more than twice the length of the CNTFR probe (411bp). Previous studies have indicated that longer probes yield weaker signals; the suggested explanation for this is that the longer probe is less well

Fig. 4.4 Diagrammatic representation of the plasmids used to generate the probes for *in situ* hybridisation to investigate the expression of some of the components of cytokine systems which utilise gp130 as part of the receptor

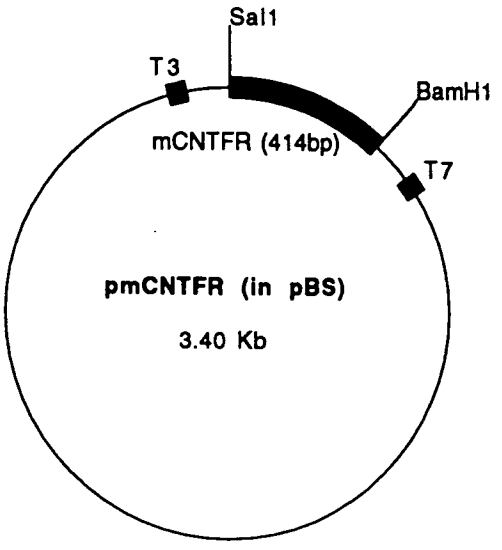
a. A probe for murine ciliary neurotrophic factor receptor (mCNTFR, 414bp) is synthesised by linearisation of the plasmid using Sal1 and transcription from the T7 promoter

b. A probe for the rat form of CNTF (rCNTF, 220bp) can be made by linearisation of the plasmid using Sal1 and transcribing from the T7 promoter

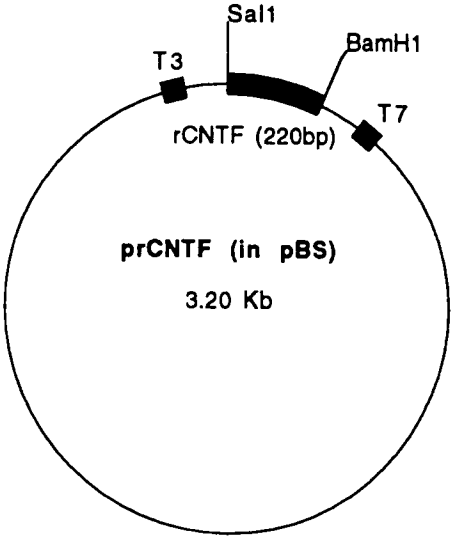
c. An interleukin-6 (IL-6, 600bp) probe is created by linearisation of the plasmid with EcoR1 and transcription from the T7 promoter

Fig. 4.4

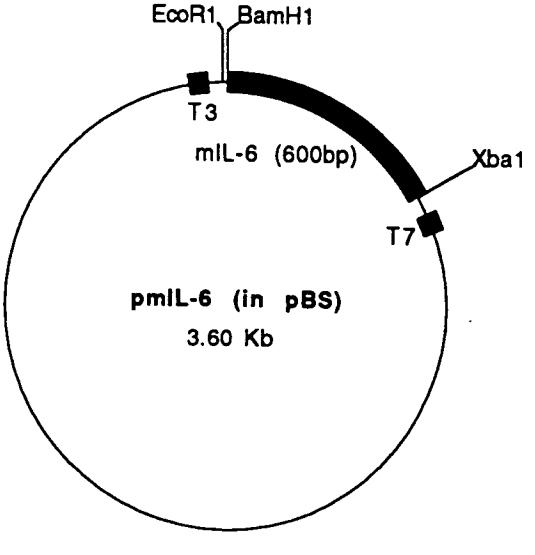
a


Sal1
T3
BamH1
mCNTFR (414bp)
T7
pmCNTFR (in pBS)
3.40 Kb

b


Sal1
BamH1
T3
T7
rCNTF (220bp)
prCNTF (in pBS)
3.20 Kb

c


EcoR1
BamH1
T3
mIL-6 (600bp)
Xba1
T7
pmIL-6 (in pBS)
3.60 Kb

///

able to penetrate into the cross-linked tissue (Wilkinson and Green, 1990). Therefore, in the light of the significant signal observed on sections of 8.5dpc embryos probed for CNTFR, the few grains seen on the ICMs of the blastocysts using the same probe probably represent either background or lower expression levels of the CNTF receptor gene compared to that of the DIA receptor.

No specific signal could be seen on sections of either 3.5 or 8.5dpc mouse embryos using the probe for rat CNTF. Previous studies have failed to detect CNTF mRNA in rat embryos by *in situ* hybridisation (Stöckli et al., 1989; 1991), and transcripts are barely detectable by Northern blotting in the 14dpc rat embryo (Ip et al., 1993).

There was no obvious hybridisation on sections using the probe for IL-6, either in blastocysts or in 8.5dpc embryos. The expression patterns of IL-6 mRNA in embryos prior to the onset of haematopoiesis has not been documented, although the presence of IL-6 mRNA in blastocysts and secretion of the protein from these has been reported (Murray et al., 1990). A positive control for the probe in this series of *in situs* was not available, and for this reason, it is difficult to draw conclusions from this data about the expression patterns of IL-6 in the early embryo.

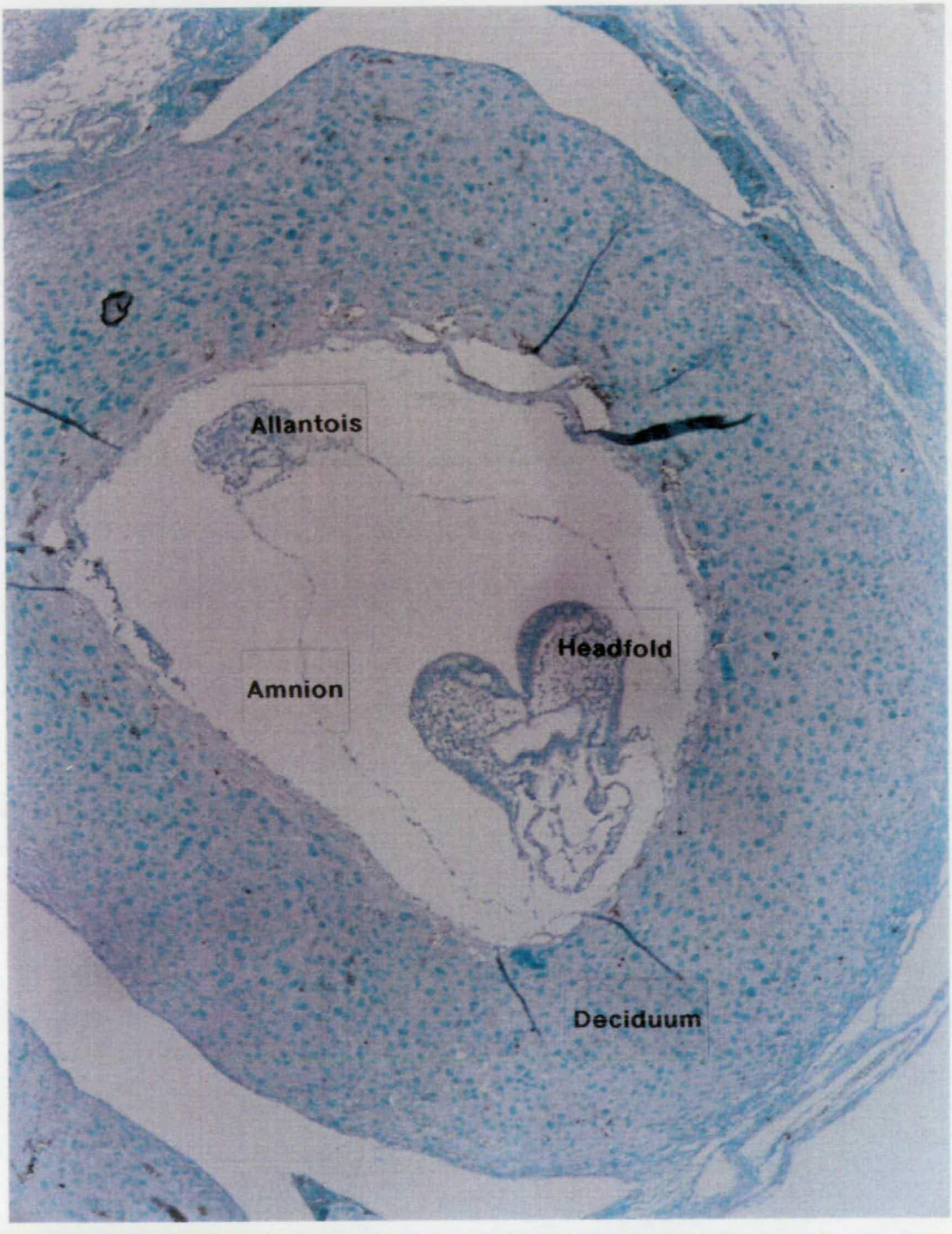
Fig. 4.5 *In situ* hybridisation on a section through an 8.5dpc embryo using a probe for mCNTFR (x4 objective). No grains are visible at this magnification; the figure was included for the purpose of orientation for subsequent figures

Al=allantois

Am=amnion

H=headfold

Fig. 4.5



Discussion

Transcripts for CNTFR mRNA are clearly detectable by *in situ* hybridisation in the 8.5dpc embryo and are localised to the neural plate/neural tube and neural crest (figs. 4.5-4.7). These are expected targets for a cytokine playing a role in neural development (Adler et al., 1979; Lin et al., 1989; Stöckli et al., 1989), and are consistent with the expression patterns previously observed in later rat embryos (Ip et al., 1993). A few autoradiographic grains can be seen in the ICMs of 3.5dpc blastocysts, but these are at rather equivocal levels (fig. 4.8). However, this is the region in which expression of CNTFR would be anticipated if CNTF is able to act on the ICM to maintain the cells in their undifferentiated state.

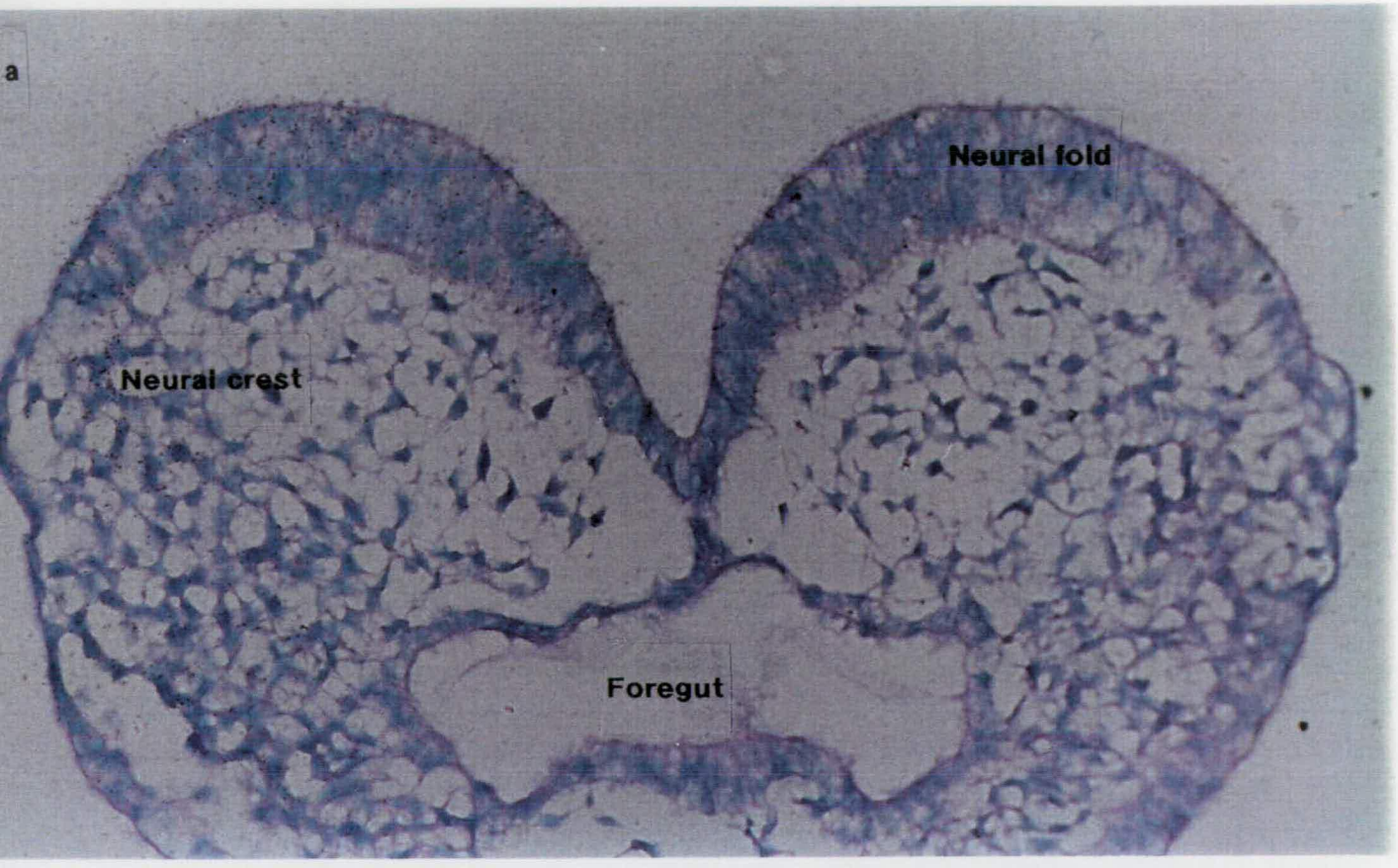
The absense of detectable CNTF mRNA in these embryonic stages suggests that CNTF is unlikely to be involved in early development, although the possibility that the protein is provided by the mother cannot be ruled out. There was no significant concentration of silver grains on any of the sections probed here with CNTF. This is probably because CNTF mRNA is not produced during any of the embryonic stages used in this study. Although CNTF is abundantly expressed in peripheral nerves, myelinating Schwann cells and reactivating astrocytes (Williams et al., 1984; Manthorpe et al., 1986; Rende et al., 1992; Sendtner et al., 1992; Friedman et al., 1992; Ip et al., 1992), cytokines are generally active at low concentrations. It is possible that CNTF is actually transcribed at the embryonic stages studied, but at a level that is undetectable by *in situ* hybridisation. However, a feature of CNTF

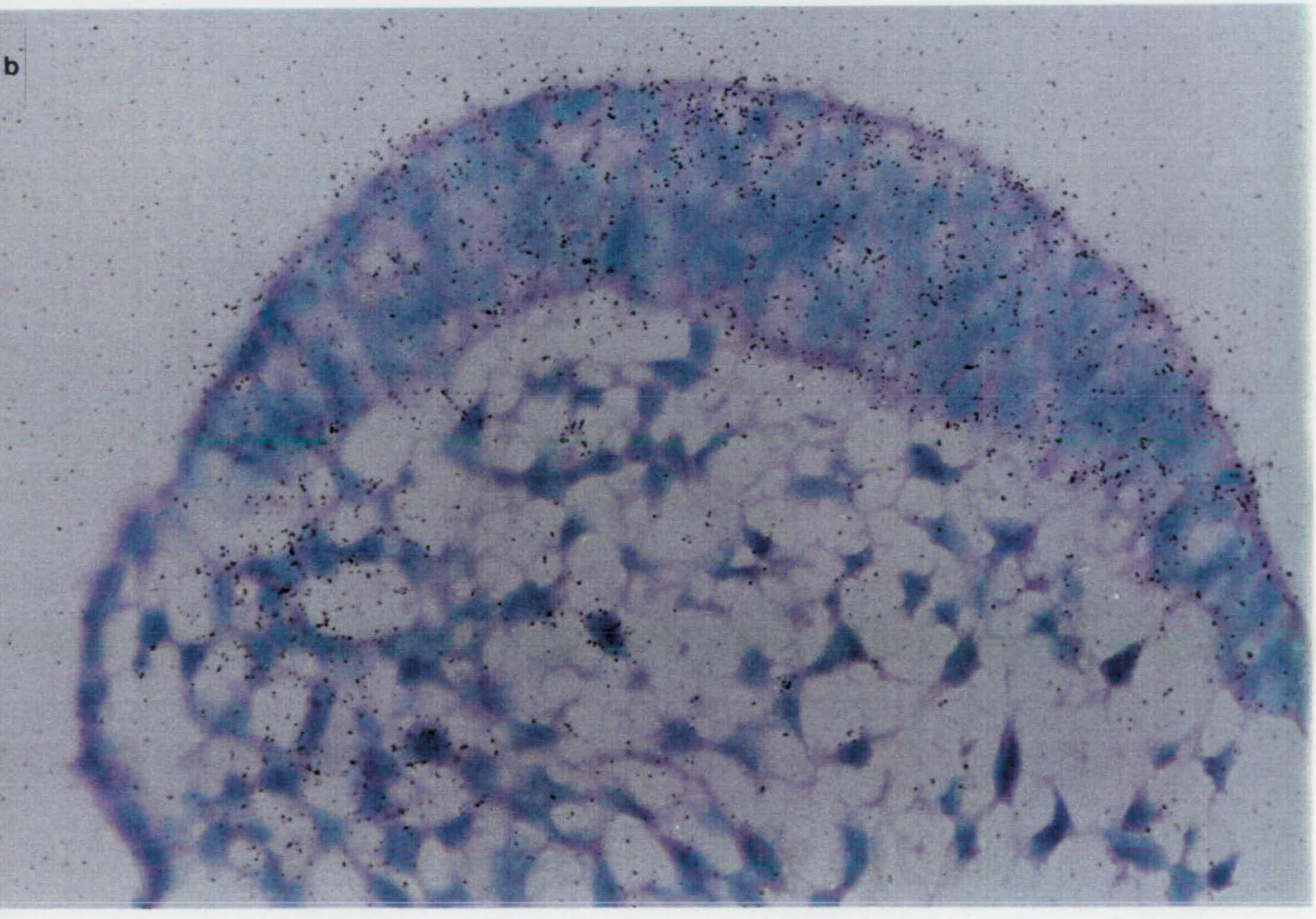
Fig. 4.6 *In situ* hybridisation on a section through an 8.5bn embryo in a region similar to that of fig. 4.5 using a probe for mCNTFR

a. Transcripts for mCNTFR mRNA can be seen in the neural tissue and neural crest of the headfold (x20 objective)

b. A higher magnification of fig. 4.6a (x60 oil immersion objective)

Fig. 4.6





which may reduce its potential efficiency as a signalling molecule in the early embryo is the absence of any hypertrophic leader sequence, causing it to be retained in the cytoplasm (Dittrich et al., 1994). Restricion of the cytokine to the cytoplasm of the cell by which the molecule is synthesised may render CNTF an unlikely candidate for paracrine interactions.

In the case of DIA, transcripts are known to be present in the extra-embryonic region of the 6.5dpc embryo from RNase protection analysis (Rathjen et al., 1990b), but are not obvious by *in situ* hybridisation. The CNTF probe administered to the sections was made using the rat form, which may be less sensitive when applied to a heterologous species such as the mouse. Previous reports of *in situ* hybridisation using a rat CNTF probe on rat embryonic tissues at 15dpc have failed to detect any transcripts (Ip et al., 1993; Stöckli et al., 1989, 1991), although CNTF mRNA has been observed in this embryonic stage by Northern blotting (Ip et al., 1993). It is possible that CNTF is present in the blastocyst at levels undetectable by *in situ* hybridisation.

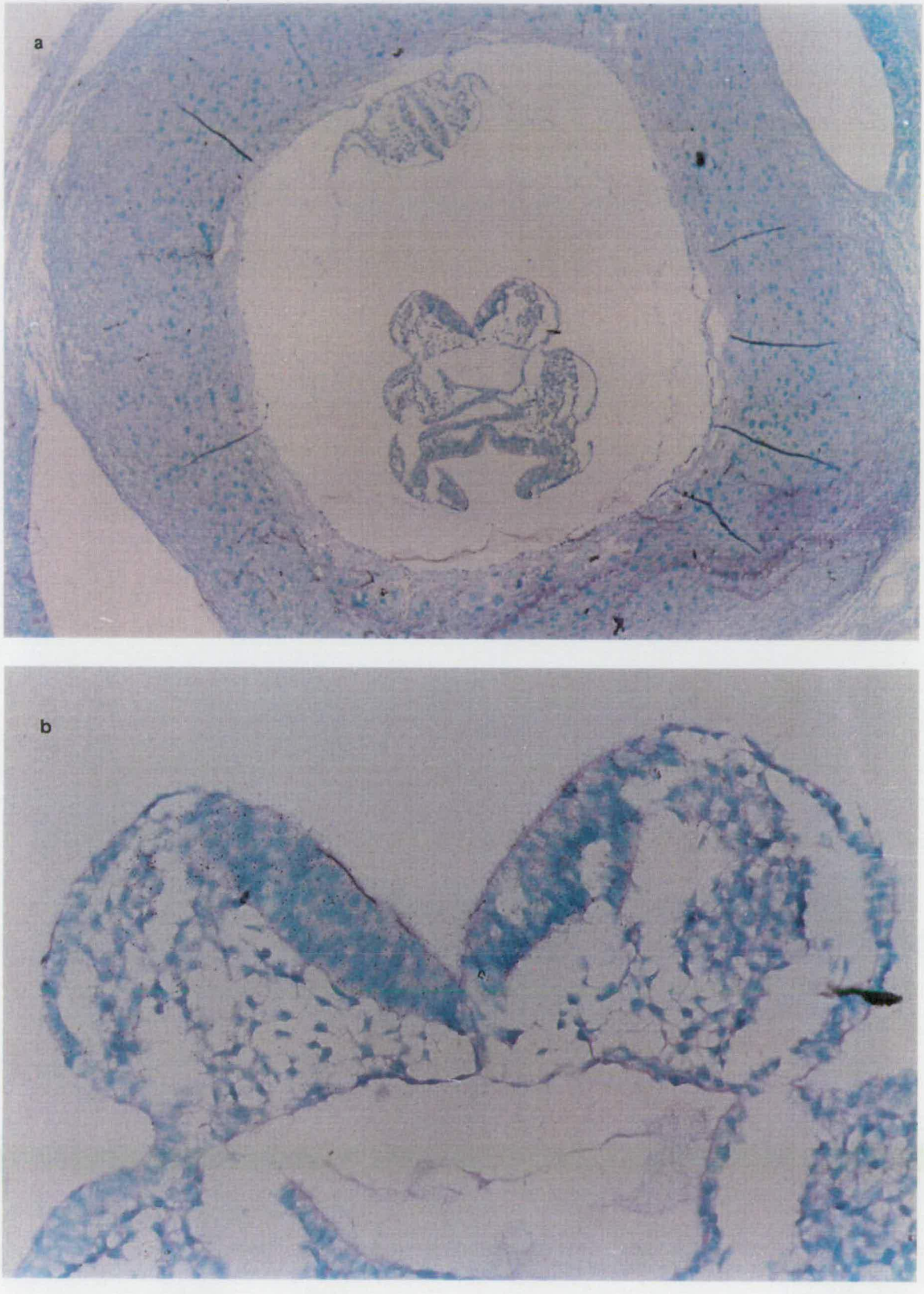
One of the properties of DIA which led to the suggestion that it may be involved in stem cell proliferation in the early embryo is its ability to support ES cells and to enable their derivation *de novo* from blastocysts in culture. The addition of CNTF to culture medium has been shown to inhibit the differentiation of established ES cells (Conover et al., 1993; Wolf et al., 1994), indicating that ES cells probably possess the relevant receptors for the action of CNTF. However, experiments in which BAF-B03 cells (which express neither gp130, nor the specific receptor components for DIA and CNTF) were transfected with the DIA-specific receptor component and gp130 resulted in

Fig. 4.7 *In situ* hybridisation on a section through a more posterior region of an 8.5dpc embryo than that of fig. 4.6 using the mCNTFR probe

a. A low magnification section for orientation purposes (x4 objective)

b. Silver grains can be seen in the neural folds and neural crest (x20 objective)

Fig. 4.7



proliferation of the cells upon exposure to CNTF (Gearing et al., 1994). This occured even in the absence of the CNTF-specific receptor component, although addition of this subunit did enhance the activity. This suggests that, contrary to previous understanding (Ip et al., 1993), CNTF may be able to elicit a response in the absence of its specific receptor subunit.

Attempts to isolate ES cells from blastocysts in medium supplemented with CNTF have yielded a significantly lower proportion of lines compared with the relatively high number obtained with DIA, OSM or IL-6 +sIL-6R (see chapter 5). One possible interpretation of this phenomen is that the receptor for CNTF is not expressed in the blastocyst at sufficient levels to facilitate adequate binding of CNTF for the effective initiation of the signal transduction pathway in this system. During the expansion of ES cell lines, the expression of the CNTF receptor may be up-regulated, enabling the preservation of established ES cells by the supplementation of the medium with CNTF (Conover et al., 1993). Preliminary analysis by RNase protection indicates that mRNA transcripts for the CNTF-specific receptor subunit are present on ES cells; the expression levels increase as the ES cells differentiate (M. Robertson, unpublished).

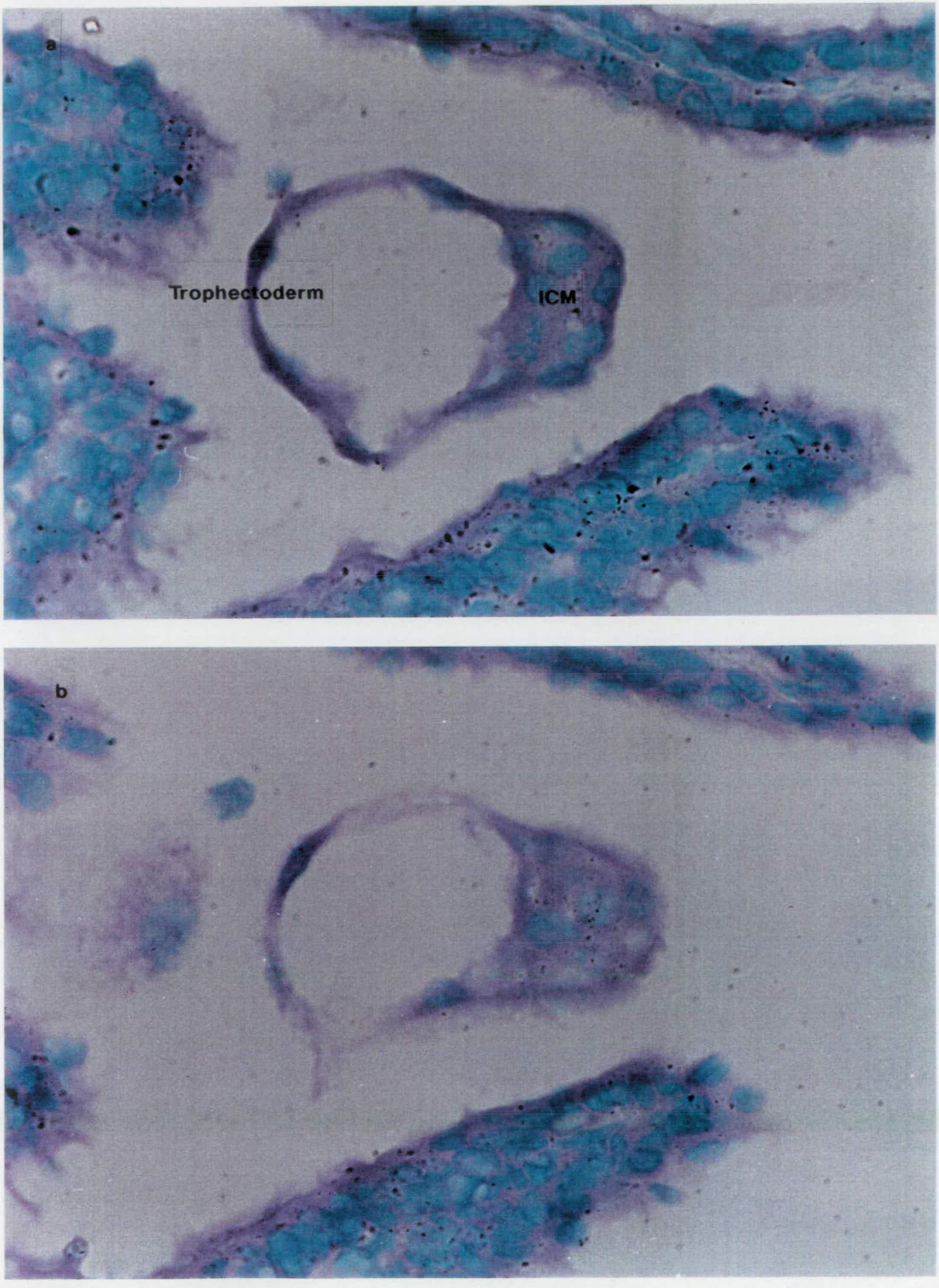
Reports have recently emerged in which mice lacking IL-6 (Poli et al., 1994; Kopf et al., 1994) and CNTF (Masu et al., 1993) have been generated. For neither cytokine was the phenotype lethal during embryonic stages, although adult animals were affected in certain tissues. The conclusion from these results is that neither IL-6 nor CNTF is essential during development.

Since OSM can act on target cells via the DIA receptor complex (Gearing et al., 1992; 1994), this may be considered as a

Fig. 4.8 *In situ* hybridisation on a section through a 3.5dpc blastocyst (x60 oil immersion objective) using the probe for mCNTFR. Very few grains can be seen over the inner cell mass (ICM) region

TE=trophectoderm

Fig. 4.8



more likely alternative candidate for embryonic stem cell maintenance. This question will remain unanswered until the murine form of OSM has been cloned and suitable probes for *in situ* hybridisation to sections of mouse embryos can be generated.

CHAPTER 5

ESTABLISHMENT OF PLURIPOTENTIAL ES CELL LINES FROM BLASTOCYSTS IN CULTURE USING MEDIUM SUPPLEMENTED WITH ALTERNATIVE CYTOKINES

Introduction

One of the features of DIA supporting the suggestion that it may be involved in the maintenance of the stem cells in the developing embryo is its ability to act directly on mouse blastocysts in culture, resulting in the generation*de novo* of pluripotential ES cell lines (Nichols et al., 1990; Pease et al., 1990). Any other molecule considered as a candidate for the inhibition of differentiation in the early embryo would also be expected to fulfil such a role on blastocysts in culture, thus illustrating that the pluripotential cells in the early embryo are able to respond to such a factor. The cytokines which, in common with DIA, have recently been found to operate via receptors which utilise the signal transducing subunit gp130, (Gearing et al., 1992; Murakami et al., 1992; Ip et al., 1992), have been considered as likely alternatives to DIA in this context. Oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and interleukin-6 (IL-6), supplemented with the soluble form of its specific receptor component (sIL-6R) have been shown to inhibit the differentiation of pre-existing ES cells when added to the culture medium in very low concentrations (Rose et al., 1994; Conover et al., 1993; Wolf et al., 1994; Yoshida et al., 1994). In this chapter, blastocysts are cultured in the presence of such factors with the aim of establishing germline-competent ES cell lines.

Materials and Methods

Implantation-delayed blastocysts were flushed from mice of the 129 OLA strain, homozygous for chinchilla ($c^{ch}c^{ch}$) 4 days after ovariectomy, which was performed on the third day of pregnancy. These embryos were then cultured as described in chapter 2. Medium was supplemented with either 10ng/ml of DIA/LIF (murine, obtained by transfection of COS cells with), 500ng/ml of IL-6 and 5000ng/ml of sIL-6R (human; gift from T. Taga, Osaka), 50ng/ml OSM (human, Genzyme), or 10ng/ml CNTF (rat, Genzyme). After 5 days, disaggregation was performed as described (chapter 2) and resultant ES cell colonies appearing in the cultures over the next 14 days were expanded and injected into blastocysts from C57Bl/6 inbred mice. Chimaeras were tested for their ability to transmit ES cell-derived germ cells by test breeding with MF1 albino mice. The appearance of grey offspring implied successful germline transmission of the ES cell genotype, whereas agouti mice were indicative of procreation from the C57Bl/6 host blastocyst.

Results

The frequency of isolation of ES cell lines *de novo* from blastocysts in culture is summarised in Table 1. Six ES cell lines were obtained from a total of 20 blastocysts cultured in medium supplemented with IL-6 and sIL-6R which exhibited the morphology and growth characteristic of previously derived ES cells (Nichols et al., 1990). In the absence of IL-6 and sIL-6R such cells differentiate, but they can also be maintained in an undifferentiated state in the presence of DIA. Chromosome counts on each clone revealed a high proportion of cells exhibiting the normal number of 40 chromosomes. As summarised in table 2, all of these produced chimaeras following injection into host blastocysts, the chimaerism being extensive as judged by coat colour contribution (fig. 5.1a and b), and comparing favourably with those produced by injection of one of the clones derived simultaneously in DIA. Germline transmission has been obtained from all of these clones (table 2).

From 12 blastocysts cultured in medium supplemented with OSM, 5 ES cell lines were derived (table 1). Each of these was injected into blastocysts and produced chimaeras with substantial coat colour contribution (fig. 5.1c). Test breeding proved 4 out of these 5 lines were able to pass through the germline (table 2).

Using medium supplemented with CNTF, only one ES cell line was generated from 11 blastocysts. Several subsequent attempts were made following the first experiment in which a fresh batch of the cytokine was employed, and the concentration in the medium increased, but none of these produced stable ES cell lines The one established line gave rise to a single male chimaera when injected into blastocysts. Although by coat colour he appeared to possess an adequate

Table 5.1 A summary of the number of ES cell lines obtained using the cytokines interleukin-6 (IL-6), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and differentiation inhibiting activity (DIA/LIF), whose receptors utilise the signal transducer gp130

Table 5.1

Factor	Source	Concentration	No. blastocysts	No. cell lines
IL-6+sIL-6R	Human	500+5000ng/ml	20	6
OSM	Human	50ng/ml	12	5
CNTF	Rat	10ng/ml	11	1
DIA/LIF	Human	10ng/ml	14	5

contribution from the ES cells, he proved to be sterile, and was unable to transmit the ES cell genotype through his germline (fig. 5.1d).

When transferred to the kidney or testis of a suitable recipient the embryonic ectoderm cells of the 6.5dpc egg cylinder proliferate to form teratocarcinomas (Stevens, 1970; Solter et al., 1970), which can then be expanded in culture as embryonic carcinoma (EC) cells (Evans, 1972). As part of this study, several attempts have been made to capture the pluripotential stem cells of early egg cylinders in culture directly. No success has been achieved, even using alternative cytokines, such as IL-6 with its soluble receptor, added to the culture medium. To date, all ES cell lines in use have been derived from either the 129 or the C57Bl/6 strain of mouse. The ability to derive cell lines from other strains of mice may aid in the elucidation of the mechanism by which the stem cells are maintained *in vivo*. Attempts to derive ES cell lines from blastocysts of other strains have proved unsuccessful using DIA, or IL-6 +sIL-6R. The reason for this is unclear. It has been suggested that the variation in the rates of commitment and development for different strains of mice necessitates an alteration in the timing of the procedures used for the derivation of ES cells (A. Bradley, personal communication). However, using a range of time points for the disaggregation of cultured blastocysts of the CBA inbred strain, it has not yet been possible to derive ES cell lines (J. Nichols, unpublished). Alternatively, the propagation of stem cells from early embryos of the non-129 or C57Bl/6 strains *in vitro* may require another factor, which acts alone or in combination with DIA. The nature of this possible alternative activity remains unknown.

Table 5.2 Summary of the chimaeras obtained following injection into blastocysts of ES cells derived using the cytokines interleukin-6, differentiation inhibiting activity, oncostatin M or ciliary neurotrophic factor

Table 5.2

Cell line	No. blastocysts injected	No. born	No. chimaeras % male	No. germline
IL6 A	27	9	8 (100)	6
IL6 B	20	11	8 (87.5)	5
IL6 C	20	11	5 (60)	3
IL6 D	20	5	3 (0)	1
IL6 E	20	9	6 (83.3)	5
IL6 F	20	8	4 (25)	1
DIA 3	20	12	4 (50)	1
OSM 1	22	9	7 (57)	4
OSM 2	22	16	10 (60)	2
OSM 3	22	10	1 (0)	0
OSM 4	22	15	5 (100)	4
OSM 5	20	10	4 (25)	2
CNTF 1	20	8	1(100)	0

Discussion

The generation of germline-transmitting chimaeras in medium supplemented with IL-6 + sIL-6R and OSM establishes that these cytokines can substitute for DIA in the derivation and maintenance of ES cells from mouse blastocysts. All of the ligands so far shown to inhibit ES cell differentiation require gp130 as a transmembrane component in their receptor complexes. IL-6 can associate with gp130 by means of a specific receptor component which lacks a transmembrane domain. This subunit is not expressed on ES cells (Saito et al.,1992), so must be added to the medium in soluble form, along with the IL-6 ligand, when applied to ES cells. In the case of IL-6, the maintenance of pluripotential ES cells apparently occurs without the involvement of the DIA-specific receptor subunit, since the IL-6-specific receptor component induces homodimerisation of gp130 (Yoshida et al., 1994). The generation of 6 germline-competent ES cell lines from 20 blastocysts cultured in the presence of IL-6 + sIL-6R (table 1) indicates that the DIA-specific receptor component is not essential for the maintenance of stem cells in the ICM, and that, in this context, signalling through gp130 alone is sufficient.

The number of ES cell lines derived from blastocysts cultured in medium supplemented with OSM (5 out of 12), and the proportion exhibiting transmission through the germline (4 out of 5) compares favourably with those derived in the presence of DIA (table 1). OSM has been found to bind with high affinity to the receptor complex utilised by DIA (Gearing et al., 1992), although it does also interact with another specific subunit (Gearing and Bruce, 1992; Liu et al., 1992). The inability so far to demonstrate the existence of a murine form of OSM

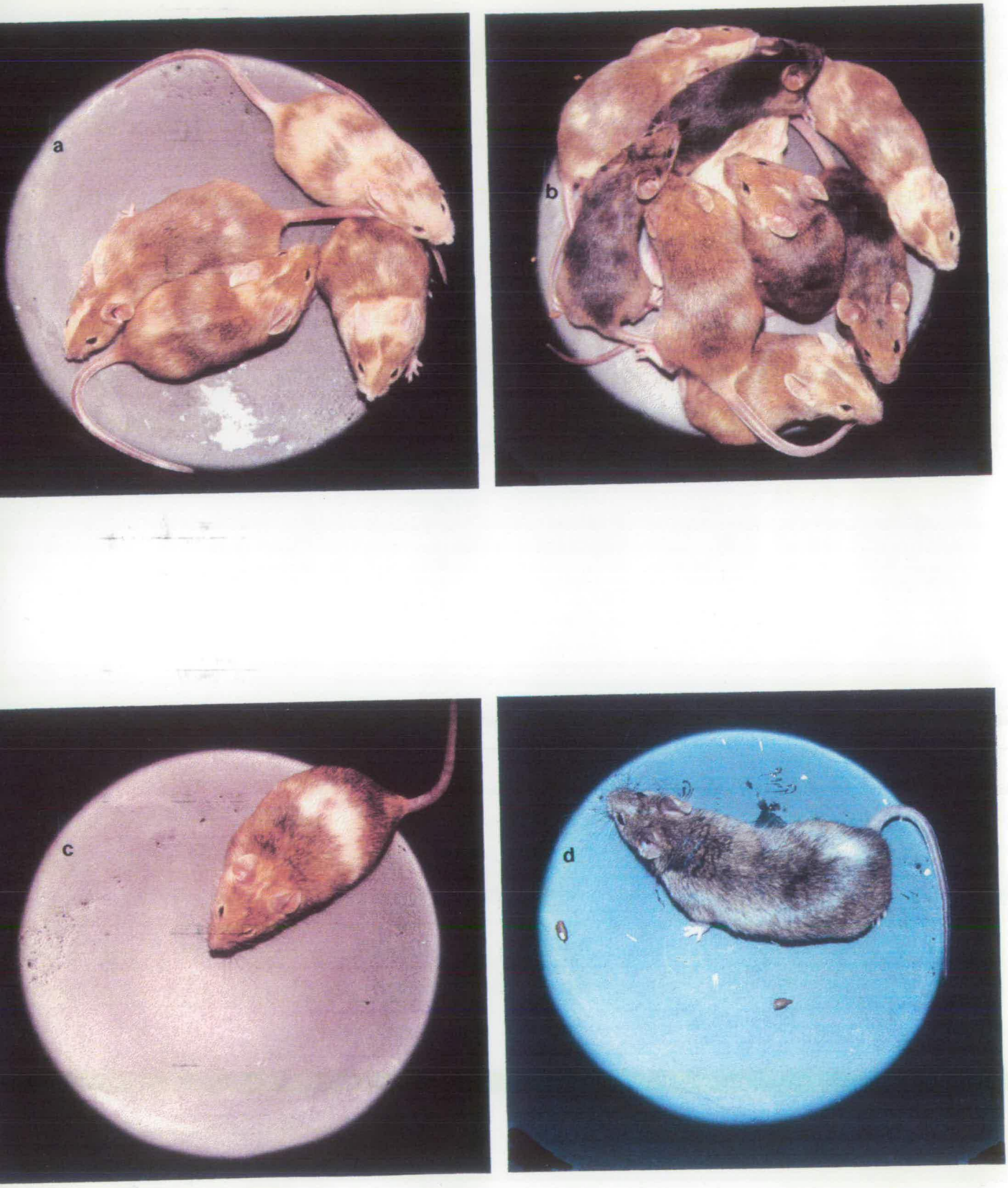
Fig. 5.1 Chimaeric mice produced by injection of blastocysts with ES cells derived in medium supplemented with factors other than DIA

a and b. Chimaeras produced with ES cells derived using oncostatin M (OSM)

c. Example of a typical chimaera produced using ES cells derived in medium supplemented with interleukin-6 and the soluble form of its receptor (IL-6+sIL-6R)

d. The single sterile male chimaera produced from ES cells derived in medium supplemented with ciliary nuerotrophic factor (CNTF)

Fig. 5.1



does, however, hinder further attempts to substantiate the suggestion that OSM may be involved in aspects of early mouse development.

Only one ES cell line was obtained from the blastocysts grown in medium supplemented with CNTF, despite several repeated attempts to generate more. Why this is the case is not clear. Established ES cells can be inhibited from differentiation by the addition to the medium of CNTF (Conover et al., 1993). This may imply that they express the necessary receptor components for the action of CNTF. Preliminary analysis by RNase protection suggests that ES cells do express mRNA for the CNTF-specific receptor component, and that the level of expression increases as the ES cells differentiate (M. Robertson, unpublished). It is known that serum contains the soluble form of the CNTF-specific receptor component (Davis et al., 1993; Dittrich et al., 1994), and this may be utilised by the ES cells. The reason why the stem cells of blastocysts cannot be maintained in culture may be that the expression of the CNTF-specific receptor subunit becomes up-regulated during the establishment of ES cells. Alternatively, they may require a higher concentration of the soluble subunit than is present in the serum in the culture medium, or that they are unable to utilise it. The *in situ* hybridisation experiments described in chapter 4, in which the presence of CNTFR mRNA was sought in blastocysts, seem to suggest that there are very few, if any, CNTFR transcripts in the pluripotential ICM cells. By 8.5dpc CNTFR autoradiographic grains are readily apparent in the developing neural tissue and neural crest, demonstrating that the probe binds specifically to tissues known to produce CNTFR (Ip et al., 1993). *In situ* hybridisation using the CNTFR probe on ES cells and blastocysts in parallel may help to establish whether there is a higher level of expression of CNTFR in ES cells. In spite of the apparent lack of CNTFR transcripts in the normal blastocyst, it is possible that expression of this

receptor component is up-regulated in embryos which are unable to synthesise DIA, but which are viable. If this is the case, CNTF may be able to maintain stem cells in the early embryo when DIA is absent. *In situ* hybridisation to compare the expression of CNTFR in DIA-negative blastocysts with wild type embryos may provide some insight into this question.

Since the outset of this work, null mutant mice for both IL-6 and CNTF have been produced by homologous recombination in ES cells (Poli et al., 1994; Kopf et al., 1994; Masu et al., 1993), and in neither case is the phenotype lethal in the homozygous condition. The removal of IL-6 was reported to result in impairment of the immune and acute-phase response of adult animals when challenged with tissue damage or infection (Kopf et al., 1994), and to protection of female mice from the bone loss normally associated with depletion of oestrogen (Poli et al., 1994). Absence of CNTF results in progressive atrophy of motor neurons in adult mice (Masu et al., 1993). While these data suggest that each of these cytokines is dispensible during early development, they may be able to fulfil the role of stem cell maintenance in the absence of an alternative mechanism. However, the involvement of an alternative signal transduction pathway to that initiated by gp130 is a possibility.

CHAPTER 6

THE EFFECT OF ELEVATED LEVELS OF DIA ON THE DEVELOPING EMBRYO *IN UTERO*

Introduction

The expression patterns for the mRNAs of DIA and its receptor in the blastocyst suggest that DIA plays a role in early development (see chapter 3). The effect of DIA on the pluripotential cells of blastocysts in culture is to allow proliferation, resulting in the establishment of ES cell lines (Nichols et al., 1990; Pease et al., 1990). Alteration of the level and location of DIA expression in the developing embryo may, therefore, be expected to disrupt the stem cell pool. The availability of elevated concentrations of DIA over certain regions of the embryo may lead to an increase in the size of the stem cell population. The consequences of this for development cannot be predicted reliably in advance, but such phenomena as teratocarcinoma formation and the disruption of gastrulation may be anticipated.

Administration of ectopic and additional DIA to the developing embryo by pronuclear injection of an over-expression vector at the one cell stage was not attempted for several reasons. Exposure of the embryo to elevated levels of DIA is predicted to cause disruption of development. The establishment of a stable transgenic line will not, therefore, be possible, and all investigations must be carried out on transgenic individuals generated *de novo*. Incorporation of the transgene after pronuclear injection is random. Of the embryos that survive the injection procedure, the DNA may fail to integrate in some and in others it may insert into a position from which it is not

expressed. Random insertion into active genes will result in a variety of expression levels and timings for the onset and termination of transgene activity, dictated by the promoters of the interrupted genes. Any phenotypes obtained will, therefore, probably vary, making comparisons impossible to draw. Also, phenotypes obtained from a disruption within the genome may be complicated because of secondary gene expression effects. Allowing the developing embryonic cells to be exposed to external sources of the gene product avoids such complications.

One method of presenting controllable quantities to the embryo is to use ES cells in which the required gene has been manipulated so as to alter the level and region of expression in the resulting differentiated products (Williams et al., 1988; Boulter et al., 1991; Wang et al., 1991). These ES cells can be characterised and the expression levels measured prior to injection into blastocysts, where they can, potentially, colonise all developing tissues (Robertson, 1986), and the consequences observed in the resulting chimaeras. Various degrees of chimaerism may then provide a range of severity, so that a possible dosage effect of the gene product may be observed. If the ES cells are marked in some way, the effects of the availability of the gene product to different areas of the embryo can be monitored. Thus, in principle, the use of ES cells as a means of delivering DIA to the developing embryo has several advantages. The level of the gene product available to the embryo can be controlled, to a certain extent, by the selection of an appropriate promoter and by the number of cells injected into each blastocyst, and the effects on specific regions of the embryo can be correlated

with the different patterns of ES cell colonisation (Rashbass et al., 1991). The effects of the diffusible form of DIA are unlikely to be cell-autonomous. Any phenotypes observed in chimaeras formed by injection of ES cells over-expressing the diffusible form of DIA, particularly those exhibiting small contributions from the ES cells, are likely, therefore, to be attributable to responses from the host cells, rather than to aberrant proliferation of the injected cells.

To increase the amount of DIA available to the embryo, a construct was generated in which expression of the DIA gene is controlled by the murine PGK-1 promoter (Adra et al., 1987). This is considered to be a ubiquitous promotor, so the expression of DIA, and, hence, its potential action, will not be confined to particular parts of the embryo. The expression of genes driven by the PGK promoter is believed to proceed, regardless of the differentiative state of the cell. A continuous exposure of the tissues to DIA may, therefore, be achieved. Thus, a comprehensive screen for the effect of DIA on all developing tissues is potentially available. The manufacture of chimaeras using stably transfected cells should result in the ectopic production of DIA over a range of areas of the embryo and at varying levels, dictated by the ultimate position and number of donor cells. In this case, the injected cells carry a genetic variant of glucose phosphate isomerase (GPI) so that they can be distinguished from the host embryo. Chimaeras can be analysed at a selected time point during gestation by dissection, observation and isozyme electrophoresis to estimate the effects and level of contribution of the manipulated ES cells.

Materials and Methods

The generation of constructs, transfection into ES cells and analysis of DIA RNA and protein production were all performed by Annette Düwel. Briefly, the plasmid giving rise to the results discussed in this chapter consisted of a 650bp fragment of human DIA cDNA under the control of the PGK promoter in pMC1 NEO poly A (fig. 6.1). The neo resistance gene is separated from the DIA gene by means of DHFR (dihydrofolate reductase), which has been found previously to improve the stability of the plasmid (Kaufman et al., 1989; Whyatt et al., 1993). Two clones which produced elevated levels of DIA were selected for injection into blastocysts. Blastocyst injection was performed as described in chapter 2, and the resulting conceptuses analysed at 8.5 or 9.5dpc. Abnormalities were assessed by examination under a dissecting microscope. Levels of chimaerism were estimated by GPI electrophoresis, as described in chapter 2. Using this technique, embryos can be divided into small pieces to test for chimaerism in a tissue-specific manner, but precise *in situ* localisation is not possible.

Fig. 6.1 Diagrammatic representation of the plasmid used for the production of ES cells over-expressing DIA

PGK=promoter for the phosphoglycerate kinase gene

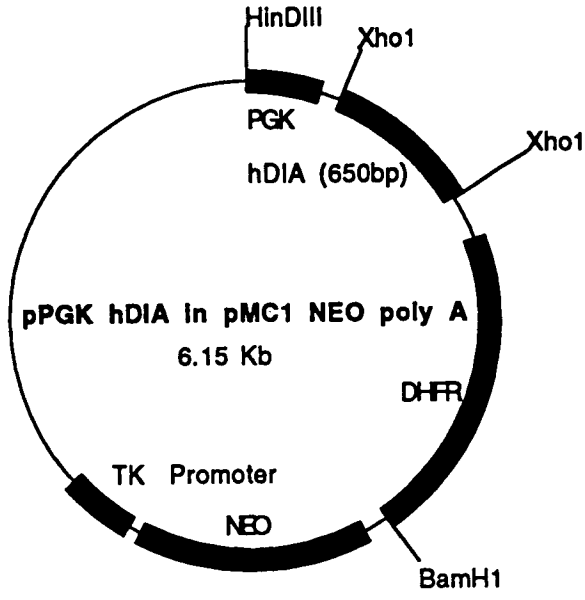
hDIA=human differentiation inhibiting activity

DHFR= murine dihydrofolate reductase gene

TK promoter=promoter for the thymidine kinase gene

NEO=Neomycin phosphotransferase (to confer resistance to the drug G418 upon cells incorporating the plasmid)

Fig.6.1


HinDIII
Xho1
PGK
Xho1
hDIA (650bp)
pPGK hDIA in pMC1 NEO poly A
6.15 Kb
DHFR
TK Promoter
NEO
BamH1

Results

Two clones, D1 and D7, were selected which produced significant amounts of DIA in both differentiated and undifferentiated ES cells. This elevation in the production of DIA is known to originate from the transgene, since the DIA cDNA used for the construct is derived from the human gene. This can be distinguished from the murine form by means of antibodies. Both clones behaved in culture in a manner reminiscent of the parent cell line. Upon removal of DIA from the culture medium, they both underwent differentiation into flattened, fibroblast-like cells, typical of those seen following withdrawal of DIA from the medium of other ES cells. This suggests that, following injection into blastocysts, these cells will be able to differentiate and contribute to developing tissues. Inability to differentiate and integrate with the host embryonic tissues may result in the formation of teratocarcinomas from the injected cells, the effects of which cannot be attributed purely to a response of the host embryo to the elevated amounts of DIA. Confirmation that differentiation had occured in the transfected clones was provided by the absence of alkaline phosphatase staining in the cells which had been grown at low density in the absence of DIA for several days, whereas those which had been constantly in the presence of DIA exhibited staining characteristic of undifferentiated ES cells. However, both lines were inclined to maintain foci of stem cells more abundant than those observed with the parent cell line when grown at high density and in the absence of exogenous DIA.

Control injections were performed using ES cells of the parental cell line EFC, which had not been transfected with the over-expression construct. The only abnormalities observed in the embryos dissected following injection of the control cells were retardation or resorption (Table 1). The numbers of abnormal embryos obtained from each experiment performed using D1 compared with control injections are summarised on table 2. Various abnormalities could be seen in embryos injected with the clone producing the most DIA (D1) when they were dissected at 8.5 or 9.5dpc. Some of the same phenotypes were observed using D7. The results obtained using D7 are probably not so severe because this line produces less DIA than does D1. The phenotypes observed using both lines are categorised in Table 1. The most commonly observed abnormality is described in the table as 'disorganised'. These structures were contained within comparatively small decidua and bore apparently normal ectoplacental cones. Each was surrounded by Reichert's membrane, but other tissues were not easy to distinguish. They tended to be multi-lobed structures, each lobe consisting of two layers, superficially reminiscent of the primitive ectoderm and visceral endoderm of an early egg cylinder (figs. 6.2b, 6.4a). The second most abundant manifestation was the appearance of many uniform small protuberances (blebs) in the visceral yolk sac (VYS; fig. 6.2c and d). Apart from this, the embryo generally looked normal. Several embryos were obtained in which the body axis had been duplicated, but both axes were within the same visceral yolk sac (fig. 6.4b). Another deformation could sometimes be seen in the head region. The two neural folds tended to gape open and either or

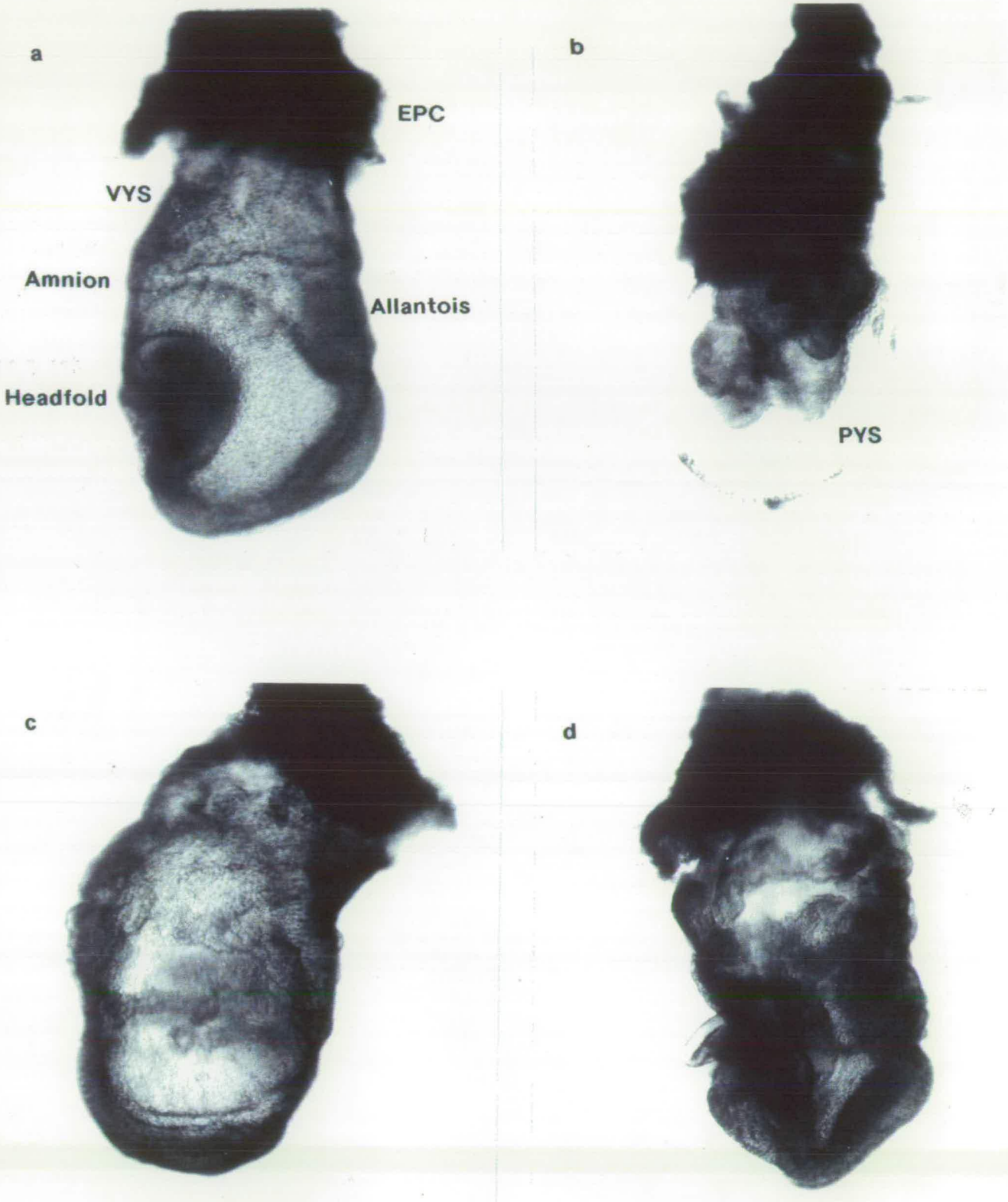
Fig. 6.2 Embryos dissected at 8.5dpc following injection of D1 ES cells into blastocysts (photographs taken using an Olympus dissecting microscope)

a. A morphologically normal 8.5dpc embryo. EPC=ectoplacental cone, VYS=visceral yolk sac

b. A 'disorganised' conceptus consisting of an apparently normal ectoplacental cone (EPC) and parietal yolk sac (PYS), but having multiple lobes in the place of the embryonic structure

c and d. Conceptuses exhibiting fairly normal embryonic structure (c is a little retarded), but showing 'blebs' on the visceral yolk sac

Fig. 6.2



both were distorted (figs. 6.3a and b, 6.4c and d). Additional outgrowths resembling allantoises were seen in a significant number of cases (fig. 6.3a). The region in the embryo from which these protruded was not constant. Slightly less commonly, the definitive allantois was deformed (figs. 6.3a and b, 6.4c and d), being large, spherical and vascularised. A couple of specimens exhibited additional extra-embryonic membranes, which resembled amnions or yolk sacs. The least frequently observed phenotype was a kinking of the body axis (fig. 6.3b). In both examples, the phenomenon was accompanied by an abnormal allantois, suggesting that the deformity may have been brought about by mechanical distortion as the embryo attempted to turn.

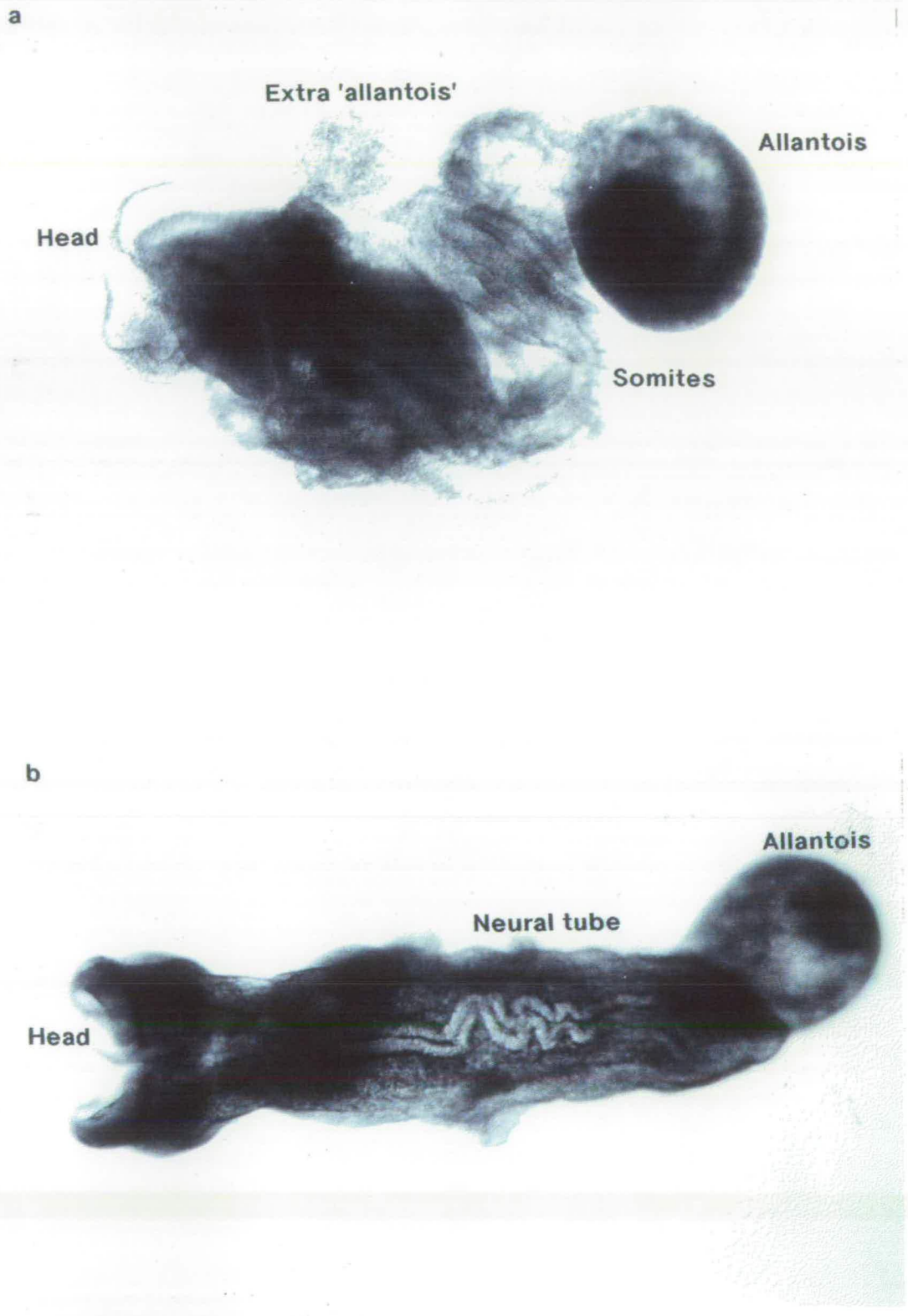
Several embryos exhibited more than one of the abnormalities listed in the table; consequently, the total number of implantation sites is lower than the sum of the listed phenotypes. The level of chimaerism was investigated in many of the conceptuses by GPI electrophoresis (table 3). The estimated percentage of the tissue exhibiting the donor ES cell form of the enzyme (GPI 1aa) was classified as more than 40%, between 5 and 40%, or undetectable. GPI values are not available for all of the specimens studied; some were reserved for wholemount *in situ* hybridisation using a probe for human DIA, but no specific signal above background could be detected.

Fig. 6.3 Abnormal embryos produced from blastocysts injected with D1 ES cells dissected at 8.5dpc (photographed using an Olympus dissecting microscope)

a. An embryo showing an abnormal head structure and allantois, and an additional outgrowth from the anterior region resembling an allantois

b. An embryo exhibiting an abnormal head and allantois and a kinked neural tube

Fig. 6.3


a
Extra 'allantois'
Allantois
Head
Somites
b
Allantois
Neural tube
Head

Discussion

A study was previously published in which chimaeras have been made from ES cells over-expressing either the diffusible (D-DIA) or matrix-associated (M-DIA) form of DIA (Conquet et al., 1992). According to that report, and in contrast to the results described here, no effect was seen when embryos were exposed to high levels of D-DIA, whereas the use of M-DIA produced abnormalities proportional to the level of chimaerism. The interpretation of these results by the authors is that elevated levels of the diffusible form of DIA do not affect the early development of mouse embryos, whereas an increase in the exposure of the embryo to the matrix-associated form of DIA results in the inhibition of gastrulation and abnormal proliferation of the embryonic ectoderm. No mesodermal structures could be discerned by histological analysis, and *in situ* hybridisation using a probe for the mesodermal marker *Brachyury* could not detect any specific expression. Alternatively, these abnormalities in high level chimaeras may actually reflect a restriction in the ability of the injected cells to differentiate. Indeed, no evidence is presented that these cells were even capable of differentiation; clones over-expressing DIA which were to be used for the injections were grown in the absence of feeder cells or exogenous DIA, and failed to differentiate *in vivo*, even after several passages. Chimaeras obtained following injection of the M-DIA over-expressing cells were reported to exhibit an abnormal phenotype when the level of chimaerism, as estimated by GPI electrophoresis, was above 50%.

Fig. 6.4 Abnormal structures generated by injection of D1 ES cells into blastocysts dissected at 8.5dpc

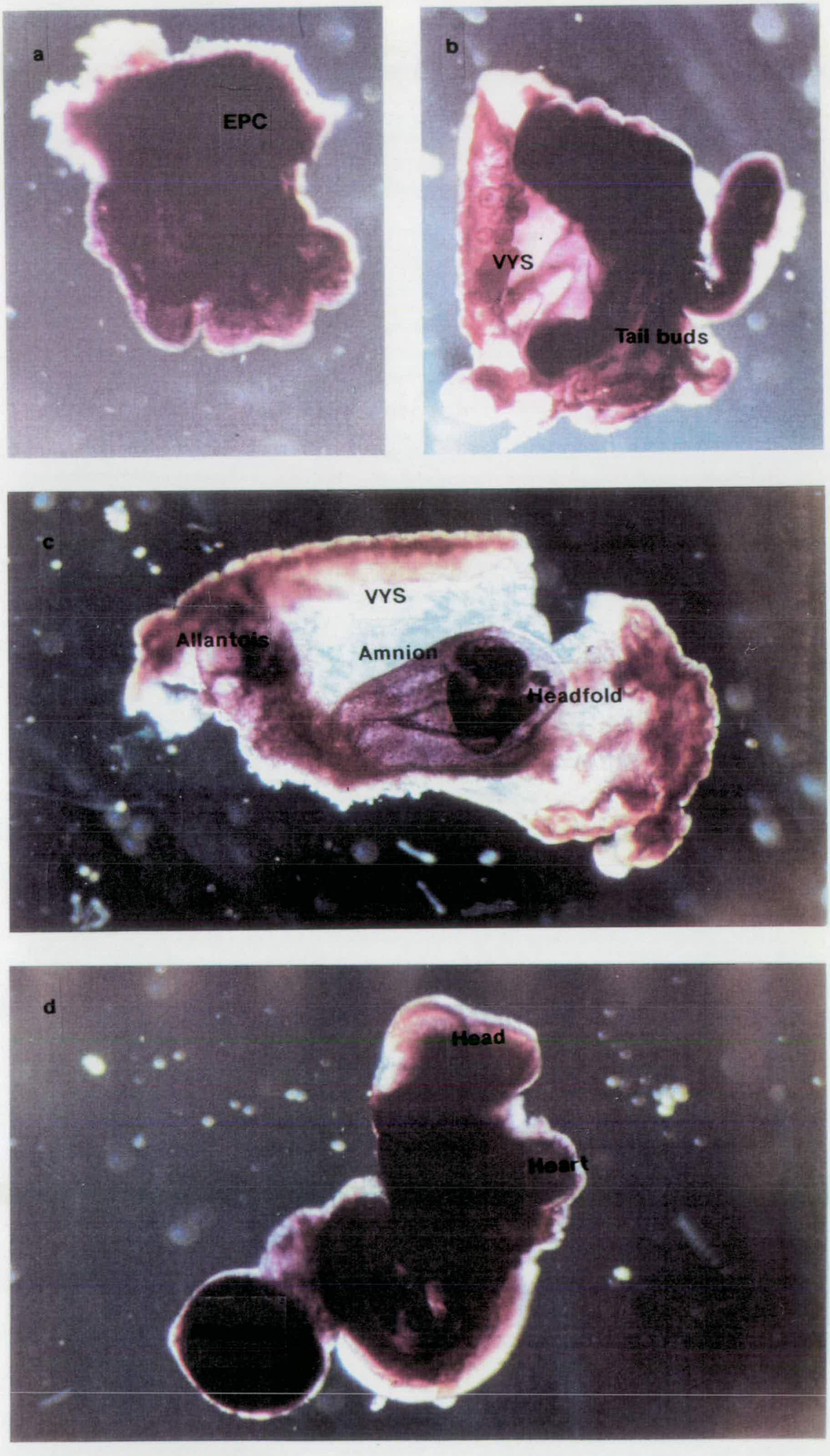
a. A 'disorganised' multiple-lobed structure
EPC=ectoplacental cone

b. An embryo exhibiting a duplication of the body axis, such that two tail buds and a double head can be seen in the photograph
VYS=visceral yolk sac

c. An embryo showing deformities in the allantois and head region, including an additional outgrowth from the extreme anterior
VYS=visceral yolk sac
Al=allantois
Am=amnion
H=headfold

d. An embryo showing an abnormal allantois and head region

Fig. 6.4


a
EPC
b
VYS
Tail buds
c
VYS
Allantois
Amnion
Headfold
d
Head
Heart

Few host cells were, therefore, available in the developing foetus to respond to the effects of the injected cells. These abnormal embryos were composed of two layers, morphologically resembling an epithelium and visceral endoderm. No mesodermal tissues could be detected. Consistent with the argument that the phenotypes obtained resulted from abnormal growth of the injected cells rather than from an effect of excessive DIA on the developing embryo, such abnormalities were not obtained when ES cells over-expressing D-DIA were injected. The matrix-bound form of DIA is less likely to be available to parts of the embryo which are physically separate from the injected cells than is the diffusible form. Chimaeras were made by injection into host embryos at the morula stage. The use of this technique was previously shown to result in a significant number of abnormal embryos, even when putative normal ES cells were used (Lallemand and Brulet, 1990). The study by Conquet et al., (1992) may not, therefore, represent true effects of DIA on the cells of the developing embryo.

The findings documented here demonstrate that injection into blastocysts of ES cells expressing abnormally high levels of the diffusible form of DIA can perturb the development of the embryo. Chimaeras generated by injection of the parental cell line did not produce abnormalities, other than a few retarded or resorbed foetuses. The phenotype and degree of abnormality obtained from injection of the cells over-expressing DIA varied, although not in strict proportion to the level of chimaerism. However, they could be grouped into categories which were specific, and have rarely been encountered previously. The most commonly observed abnormality

Table 6.1 Summary of the abnormalities observed in embryos obtained following injection into blastocysts of ES cells over-expressing DIA (D1 and D7) compared with control ES cells

Table 6.1

ABNORMALITY	INCIDENCE		
	EFC(control)	D1	D7
Disorganised	0	26	0
Blebby visceral yolk sac	0	15	0
Additional allantois-like structure	0	8	0
Abnormal allantois	0	7	4
Abnormal head	0	8	4
Duplicated axis	0	8	0
Extra membranes	0	2	0
Kinked	0	2	4
Resorbed	4	5	7
Retarded	1	9	2
Normal	67	75	37

was the disorganised, multi-lobed structure (figs. 6.2b, 6.4a). As far as could be seen by gross observation without sectioning, each lobe seemed to be composed of two layers of cells similar to the primitive ectoderm and visceral endoderm of an early egg cylinder. One of the anticipated effects of excess DIA on the stem cells in such a system is the prolonged preservation of the undifferentiated state. The resultant larger pool of stem cells might be expected to give rise to a giant egg cylinder. Such a structure was never seen in these experiments. However, the disorganised phenotype described as the most commonly observed abnormality may be imagined to resemble a collection of egg cylinders, which may be the result of differentiation from several foci. No histology was performed on these structures to investigate this idea, since they had to be disrupted for GPI analysis. Gastrulation in the mouse embryo is thought to occur when the egg cylinder attains a specific size (P. Tam, unpublished). If this is the case, the putative multiple egg cylinders obtained here may represent the outcome from an enlarged stem cell pool caused by the presence in the embryo of elevated levels of DIA.

Embryos were also obtained which exhibited multiple axes with recognisable body structures (fig. 6.4b). Most of the members of this category were twins, but some embryos with three axes were also observed. These may represent an alternative form of the multiple egg cylinders described above, in which the individual axes were able to attain a more advanced developmental state.

On several occasions, individuals with structures protruding from various parts of the embryo, superficially resembling

Table 6.2 Summary of the number of abnormal embryos obtained from each experiment of injection into blastocysts of ES cells over-expressing DIA (D1) compared with control ES cells (EFC)

Table 6.2

EXPRT	INJECTED	IMPLANTED	NORMAL	RETARDED	RESORBED	ABNORMAL
1	24	19	10	2	4	3
2	41	28	10	1	3	14
3	7	7	4	1	0	2
4	10	8	5	0	0	3
5	12	5	3	0	0	2
6	26	13	2	1	0	10
7	19	19	11	0	0	8
8	46	42	26	7	0	9
TOTAL (D1)	185	141	71	12	7	51(36%)
CONTROL (EFC)	125	85	74	5	2	4(5%)

supernumerary allantoises were observed (fig. 6.3a). In normal development, the remaining core of stem cells (the primitive streak) is restricted to the posterior region of the developing foetus at the time of the appearance of the allantois. The allantois is derived at gastrulation from the cells which migrate posteriorly from the primitive streak as the extra-embryonic mesoderm. The persistence of stem cells in ectopic positions along the embryo caused by local synthesis of DIA may result in the ectopic differentiation of extraembryonic mesoderm, and subsequent outgrowth of a supernumery allantois. Alternatively, it may represent an undefined overgrowth of stem cells. If this is the case, this category may be considered to have arisen in much the same way as the disorganised and duplicated axis classes. It is not possible to distinguish between these derivations purely from morphology.

Another phenotype observed occasionally was a comparatively normal embryo with a large, bulbous and generally vascularised allantois (figs. 6.3a and b, 6.4c and d). On two occasions the trunk region of the embryo, particularly the neural tube, was kinked. This probably represents mechanical distortion due to obstruction by the inflated allantois rather than a direct effect of the injected cells upon the body axis. Why the allantois should grow in such a way is not clear, but it could be a result of a proliferative signal coming from the regional elevation in the concentration of DIA arising from the injected cells. However, the phenomenon of such a deformed allantois has been observed previously in chimaeras made using high passage number ES cells of the parental line (data not shown), which

Table 6.3 Summary of the levels of chimaerism by glucose phosphate isomerase analysis (GPI) observed in the normal and abnormal embryos obtained following injection into blastocysts of ES cells over-expressing DIA (EFC D1)

Table 6.3

Cell line	Phenotype	Total	Level of chimaerism		
			>40%	5-40%	Undetectable
EFC D1	Normal	75	5	16	26
EFC (control)	"	67	21	19	21
EFC D1	Disorganised	26	1	9	8
"	Blebby VYS	15	2	4	5
"	Retarded	9	1	4	4
EFC (control)	"	1	0	0	1
EFC D1	Duplicated axis	8	1	3	0
"	Abnormal head	8	1	4	0
"	Extra 'allantois'	8	2	3	0
"	Resorbed	5			
EFC (control)	"	4			
EFC D1	Extra membrane	2	0	1	1
"	Kinked	2	1	1	0

Total number of implantations	
EFC D1	149
EFC (control)	72

The levels of chimaerism were compared between mild and severe abnormalities for D1 abnormal embryos using χ^2 analysis.

Comparing all three columns (>40%, 5-40% and undetectable chimaerism; 7:17:10 (mild) versus 2:12:8 (severe)), $\chi^2=1.35$ 2df P=0.51. This suggests that there is no significant difference in the level of chimaerism between the mildly and severely abnormal groups of embryos.

Comparing detectable versus undetectable chimaerism in the mild versus severe categories (24:10 versus 14:18), $\chi^2=0.296$ P=0.59, confirming the previous observation.

High level (>40%) chimaeras were compared with low level (5-40%) chimaeras in the mild versus severe categories of D1 embryos (7:17 versus 2:12). Using Fisher's exact test, no significant difference was detected.

suggests that it may not be an effect specific to the over- expression of DIA.

Some embryos appeared normal, apart from a distribution of blebs on the visceral yolk sac (fig. 6.2c and d). These may be a result of slight over-growth of the visceral endoderm or mesoderm during development, or they may represent undifferentiated stem cells which have been trapped inside the yolk sac. Without the option of being able to perform histology on these specimens such alternatives cannot be distinguished. The abnormal heads occasionally obtained are also difficult to explain (figs. 6.3a and b, 6.4c and d). The cells of the neural plate are likely to be responsive to DIA, since significant levels of DIA-R mRNA have been detected in this tissue (see chapter 3). Failure of the neural tube in the anterior region to close properly could be caused mechanically by a thickening of the tissue in that region, caused by over-proliferation of the cells, which would provide resistance against the curvature required for tube formation. Alternatively, there may have been some reduction in the adhesive nature of the neural folds which normally holds them together once they have become associated.

The fact that some of the embryos exhibiting gross abnormalities had an almost negligible contribution from the injected ES cells (table 3) raises some concern that the phenotypes observed in this experiment are, in fact, an artifact of the manipulation. However, some relatively high level chimaeras appeared to be unaffected, and the control injections did not generate these abnormalities. Both of the experimental cell lines produced similar abnormal phenotypes, although those arising from the injections

using D7 were notably less severe. This is probably a reflection of the reduced expression of DIA by D7 when compared with D1.

The degree of abnormality does not correlate well with the level of chimaerism. There may be several explanations for this. Firstly, a contribution of less than 5% from the donor cells is not clearly discernable by GPI electrophoresis, so the abnormal embryos in which no contribution from the ES cells has been recorded may be *bona fida* chimaeras, but of a level beneath detection. Secondly, the injected over-expressing cells may be selected against during development so that they are removed to a large extent after they have initiated an effect, and consequently are not detected by GPI electrophoresis on the embryos when they reach the equivalent of 8.5 or 9.5 days of development. The abnormalities obtained in some conceptuses may depend on the injected cells occupying a critical position in the embryo. In the low or negligible level chimaeras exhibiting abnormalities, the host embryo cells must be responding to the injection, since their is an insufficient contribution from the injected cells to explain the gross phenotype obtained. The abnormalities seen are not, therefore, caused merely by inappropriate proliferation of the injected cells (table 1).

The fact that some of the normal embryos exhibit significant chimaerism suggests that the injected cells are not producing enough DIA to affect the embryo detrimentally in these instances. This may result from the transgene becoming inactive, either globally or in some of the donor cells of these apparently unaffected embryos.

To distinguish between these possible explanations attempts have been made in collaboration with Annette Düwel to detect over-

expressing cells by means of wholemount *in situ* hybridisation using a digoxygenin-labelled probe for DIA. The wholemount *in situ* hybridisation technique proved to be insufficiently sensitive to detect the increased expression of DIA above background. Certainly, by this method, it would be impossible to detect at a cellular level a slight increase in the amount of DIA mRNA which may be responsible for the abnormalities obtained in the experiment. Consequently, it has not been possible to map the critical positions at which DIA may act to influence the maintenance of stem cells in the developing embryo.

An alternative approach also adopted was to try to produce chimaeras using cells over-expressing DIA but also carrying the Lac Z gene as an *in situ* marker for the injected cells. The Lac Z gene in association with neomycin-resistance was incorporated into the plasmid together with DIA and the picornaviral internal ribosome-entry site (IRES), which enables cap-independant translation of Lac Z from a fusion transcript. Thus, the production of DIA from successfully transfected ES cells should be accompanied by expression of β gal, which can be visualised by staining with X-gal (see chapter 2). Different intensities of staining should reflect different levels of production of DIA. Unfortunately, upon removal of the stably transfected ES cells from selection with G418, the level of production of DIA decreased. Presumably, the DIA activity had diminished when these cells were injected into blastocysts so that only morphologically normal embryos were recovered. Some of these were highly chimaeric, as estimated by the degree of staining.

The range of phenotypes which have been reported in this chapter, and the low level of contribution by injected ES cells to some of the very abnormal embryos provides strong evidence that DIA is able to influence the development of the host cells in the early embryo and elicit specific responses which may be attributable to increases in the stem cell pool. Clearly, the exposure of certain early embryonic cells to increased levels of DIA does influence the subsequent development of the embryo, and the effects seen here serve to illustrate that cells in the embryo are responsive to DIA.

CHAPTER 7

DISCUSSION

In the mammalian embryo the pluripotential cells become progressively committed to specific lineages. Cell division is not synchronous and the cell cycle times vary. There is much flexibility in the system at the early stages; removal or ablation of large proportions of the embryo up to the egg cylinder stage can be overcome by the recruitment of other cells from the existing stem cell pools, resulting in normal embryogenesis (Snow and Tam, 1979). The absence of rigid pre-determination of the specific tissues in the early mouse embryo, and the ability of the embryo to regulate its overall size and the proportions of the component cell types necessitates a mechanism for modification. Factors produced by the constitutive cells may be envisaged as a means of signal; the output from a particular cell type may be required to reach a threshold level before an inhibitory mechanism is brought into play to restrict the ultimate size of the tissue. Cytokines and their receptors are probably involved in the operation of such a regulatory system.

When removed from the *in vivo* environment the cells in the early embryo tend to differentiate in the absence of any exogenous stem cell maintenance factor. The investigation of the action and distribution of candidate molecules for the prevention of differentiation of the stem cells in the early embryo has been the subject of this study. The questions addressed have included the ability of the stem cells in the early embryo to respond to candidate molecules in culture, the availability of such molecules to the embryo *in vivo*, and the response of early embryonic cells

in developing embryos to exposure to elevated levels of one of these molecules.

The first candidate molecule for the maintenance of the stem cells in the early embryo is the cytokine differentiation inhibitory activity (DIA). This was selected because of its ability to inhibit the differentiation of ES cells in culture (Smith et al., 1990). The data presented in this study are consistent with the suggestion that DIA plays a role in the preservation of the pluripotential stem cell pool in the early murine embryo.

The extra-embryonic regions of early egg cylinders have been shown previously, by RNase protection, to express DIA mRNA (Rathjen et al.,1990b). Using *in situ* hybridisation it has not been possible consistently to detect transcripts for DIA in egg cylinders after 6dpc. This is almost certainly because of the decrease in sensitivity of the *in situ* hybridisation technique compared with RNase protection. The *in situ* data, however, indicate that there is a down-regulation in the expression of DIA after the blastocyst stage. In later embryogenesis and post-natally, transcription is detectable in certain tissues, generally at very low levels (Robertson et al., 1993).

In situ hybridisation has been used here to demonstrate that transcripts for DIA mRNA are restricted to the first cells to differentiate in the preimplantation embryo, namely the trophectoderm (fig. 3.3a). The undifferentiated cells of the inner cell mass (ICM), which are the anticipated target for DIA in this context, have been shown to express the components of the DIA receptor. The DIA-specific subunit appears to be restricted to the ICM (fig. 3.3b), while gp130, the signal transducing subunit, which is also a component of the receptor complexes of several other

cytokines, is expressed predominantly in the ICM, but transcripts are also apparent in the trophectoderm (fig. 3.3c). Thus, a paracrine interaction is implied between the trophectoderm and the ICM, where the differentiated cells secrete DIA, which binds to the high affinity receptors located on the surface of the cells of the ICM, protecting them from precocious differentiation.

Although transcripts for DIA mRNA have been detected by RNase protection analysis in the extra-embryonic region of the egg cylinder (Rathjen et al., 1990b), they are not evident by *in situ* hybridisation (chapter 3). Unequivocal expression of DIA is seen by this method in blastocysts. This implies a decrease in the level of transcription of DIA as the embryo approaches and undergoes gastrulation. Indeed, by RNase protection there is a distinct decrease in the detectable expression of DIA in embryos from 6.5 to 7.5dpc (Rathjen et al., 1990b). Administration of elevated levels of DIA to developing embryos can lead to the formation of multiple-lobed ('disorganised') structures or duplications of the body axis (chapter 6). It is envisaged that the multiple lobed structures may be composed of several egg cylinders. If this is the case, a role for DIA in the regulation of the size of the embryo and the timing of egg cylinder formation is implied. The down-regulation of DIA mRNA expression in the embryo following implantation may be important for restriction in the size of the stem cell pool.

As seen by *in situ* hybridisation, the level of DIA receptor mRNA is lower in egg cylinders compared with ICMs, which suggests that the receptor becomes down-regulated prior to the onset of overt differentiation in the embryonic ectoderm (chapter 3). This is possibly connected with the reduction of DIA mRNA

expression over the same period, and may also be involved in the reduction in the pluripotentiality of the stem cell pool prior to gastrulation.

By 8.5dpc significant expression of DIA-R mRNA is re-activated in most tissues of the embryo (figs. 3.8a and c, 3.9a and c). This may be because DIA-R contributes to the receptors for other cytokines, such as ciliary neurotrophic factor (CNTF), which is thought to be involved in neuronal development (Ip et al., 1993). The expression of gp130 mRNA in egg cylinders is detectable, but significantly lower than in the surrounding decidual tissue (figs. 3.8b and d, 3.9b and d, 3.10). As with DIA-R mRNA, the level of gp130 mRNA increases in embryonic tissues at 8.5dpc (fig. 3.11).

In the decidual tissue surrounding the embryo following implantation transcripts for DIA-R and gp130 mRNA are abundant (figs. 3.8 and 3.9). By 8.5dpc the levels of DIA-R have decreased significantly, whereas the expression of gp130 has undergone a significant increase. The reason for this reciprocal regulation of expression is not understood. There are several possible explanations for the production of the components of the DIA receptor in the deciduum of the early postimplantation embryo. The adult tissue in which the highest levels of DIA expression have been detected is the uterus (Robertson et al., 1993). During all the stages of pregnancy studied here, relatively abundant transcripts have been localised to the endometrial glands. The function of these glands is not known, but they may be involved in implantation. The inability of female homozygous null mutants for DIA to support implantation is consistent with such a role for these glands to which the DIA mRNA in the uterus has been

localised (Smith et al., 1992; Stewart et al., 1992; chapter 3). Also, the level of expression of DIA mRNA in the uterus is reported to undergo a dramatic increase just before implantation (Bhatt et al., 1992). Transcripts for the diffusible form of DIA have been detected in the pregnant uterus (Robertson et al., 1993; chapter 3), suggesting that DIA may be secreted into the uterine lumen. In addition to the decidual expression, both DIA-R and gp130 mRNA are present in the endometrial glands. The DIA receptor probably, therefore, binds the secreted DIA, but whether the purpose of this interaction is to present the DIA to the developing embryo, or to immobilise it in the deciduum is not clear. As demonstrated in chapter 6 and reported by Conquet et al., (1992), exposure of the embryo to elevated levels of DIA is detrimental to development. Fine control to ensure that an appropriate amount of DIA is available to the developing embryo is likely to be important. Therefore, the function of the DIA receptor in the deciduum may be to protect the developing embryo from the DIA being secreted by the endometrial glands.

At the outset of this study, nothing was known about the phenotype of embryos lacking DIA. Subsequently, several groups have created null mutations (Stewart et al., 1992; Escary et al., 1993; Smith et al., unpublished). In these studies females lacking DIA are unable to support implantation, but homozygous null blastocysts can implant and undergo normal development if transferred to the uterus of a wild type or heterozygous foster mother. This result indicates that DIA is not indispensable for the protection of undifferentiated stem cells in the early embryo. The DIA mRNA detected in the uterus has been shown by *in situ* hybridisation to localise to the endometrial glands (chapter 3;

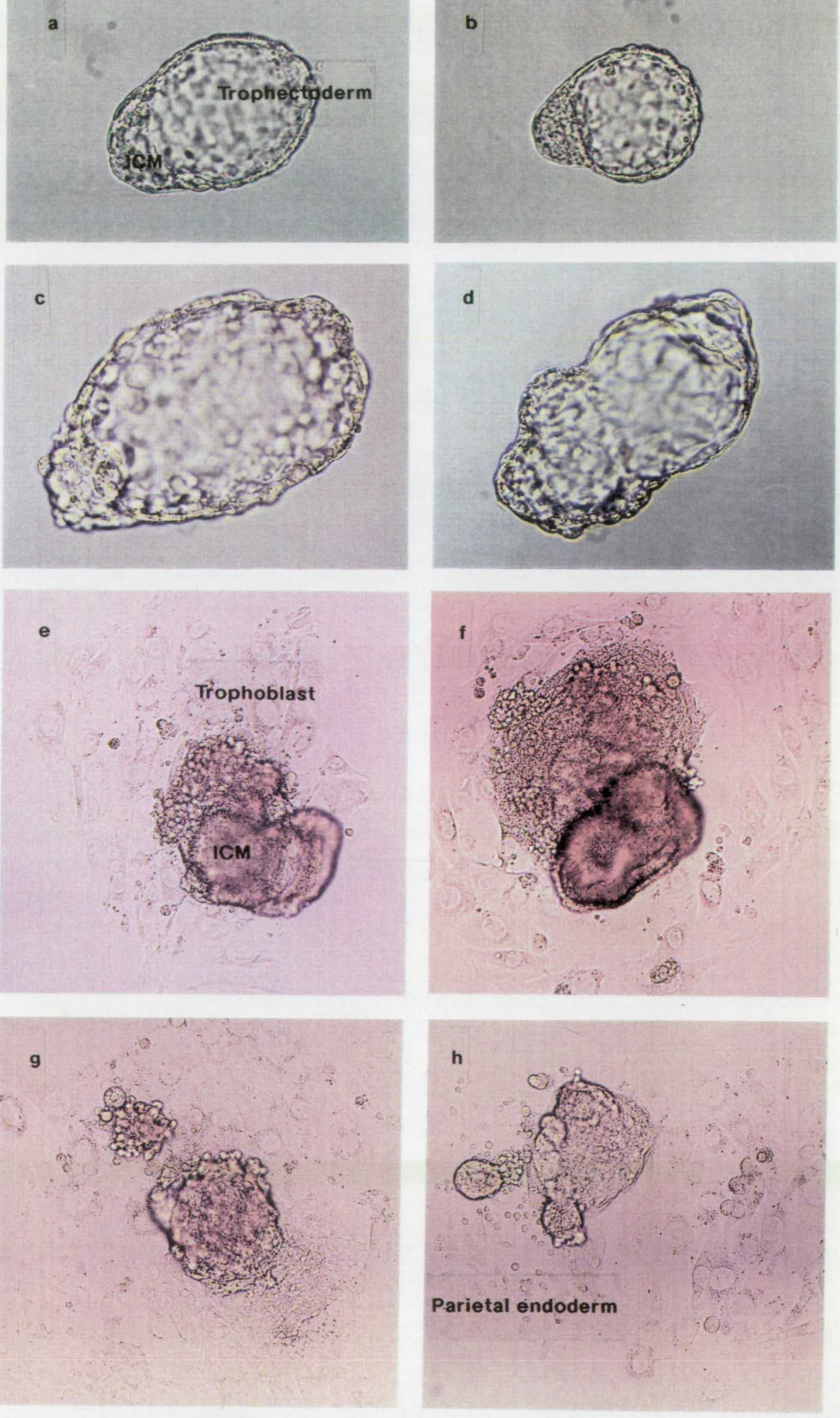
Bhatt et al., 1992; Smith et al., 1992). The inability of null mothers to support implantation implies a role for these glands in this process. However, endometrial glands are present in null mothers several days after fertilisation (fig. 7.2). Embryos flushed from homozygous DIA-negative mothers at 6.5dpc closely resemble the blastocysts from wild type mice which have been held in delayed implantation for several days, both morphologically (fig. 7.1), and in their behaviour in culture. Null embryos are able to attach to the surface of a tissue culture plate and grow in a manner indistinguishable from that of wild type delayed blastocysts (fig. 7.1). This provides evidence that the inability of embryos to implant in a null mother is unlikely to result from an inadequacy in the invasive potential of the trophectoderm of the null blastocyst, and strengthens the case for the involvement of DIA in preparing the uterus for implantation. It also implies that the blastocyst itself does not need DIA, either embryonic or maternal.

Recently, characterisation of the high affinity DIA receptor has identified a signal transducing subunit, gp130, which was initially discovered as a component of the receptor for interleukin-6 (IL-6; Taga et al., 1989; Hibi et al., 1990). Ciliary neurotrophic factor (CNTF) and oncostatin M (OSM) are now known also to operate via receptors that utilise gp130 (Gearing and Bruce, 1992; Gearing et al., 1992; Ip et al., 1992; Murakami et al., 1991). The ability of these cytokines to mimic the effect of DIA in the context of differentiation inhibition in pluripotential stem cells has been tested by addition of each to the culture medium for ES cells in the place of DIA (Yoshida et al., 1994; Conover et al., 1993; Wolf et al., 1994; Rose et al., 1994). Because

Fig. 7.1 A comparison of embryos homozygous for the absence of DIA flushed from null mothers at 7.5dpc (left hand side) with wild-type implantation-delayed embryos flushed from mothers 4 days after ovariectomy (right hand side)
Photographs taken using a x20 objective on an Olympus IMT2 microscope

a and b. Embryos immediately after flushing
ICM=inner cell mass
TE=trophectoderm
c and d. Embryos attaching to the tissue culture dish 2 days after flushing
e to h. Embryos having been allowed to attach and outgrow in culture for 5 to 6 days
ICM=inner cell mass
T=trophoblast
PE=parietal endoderm

g. 7.1

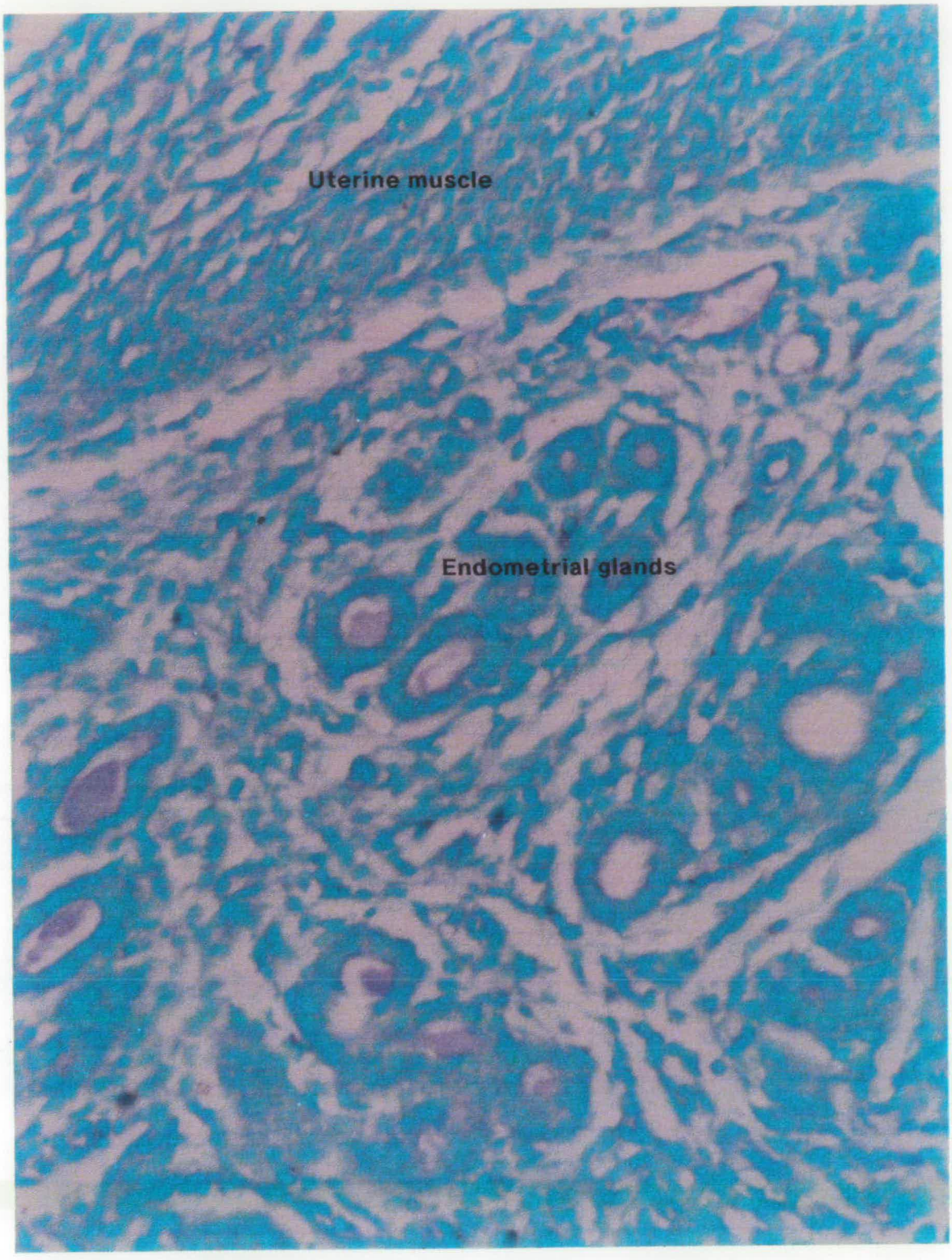

a
Trophectoderm
ICM
b
c
d
e
Trophoblast
ICM
f
g
h
Parietal endoderm

ES cells are known not to express the IL-6-specific receptor component (sIL-6R; Saito et al., 1992), a soluble form of this is required in addition to the IL-6 ligand. In each case, the alternative cytokine has been shown to inhibit the differentiation of ES cells in culture in a manner indistinguishable from that achieved using DIA. Following injection into blastocysts, the lines maintained for several generations in the presence of each cytokine have been able to contribute to the germline of resulting chimaeras.

Results presented here have demonstrated that CNTF, OSM and IL-6+sIL-6R are able to act directly on the pluripotential stem cells of the ICM, resulting in generation *de novo* of pluripotential ES cell lines (chapter 5). The proportion of blastocysts giving rise to germline-competent ES cells in the presence of OSM or IL-6+sIL-6R is comparable to the efficiency achieved with DIA in this context. The number of ES cell lines generated using CNTF was very low, and no germline transmission has yet occured. It is possible that the CNTF-specific receptor component is not sufficiently abundant in the ICM for efficient response to the CNTF ligand. The *in situ* hybridisation studies described in chapter 4 using a probe directed at CNTFR suggest that there is little or no expression of the gene in the mouse blastocyst. A soluble form of CNTFR is present in serum (Davis et al., 1993; Dittrich et al., 1994). This may be utilised by established ES cells, whereas the cells of the ICM may be less well able to harness the component from serum. Preliminary results using RNase protection analysis suggest that ES cells do produce transcripts of mRNA for CNTFR, the level of expression increasing as the ES cells undergo differentiation (M. Robertson, unpublished). In the embryo

Fig. 7.2 Section through the uterus of a female mouse homozygous for the absence of DIA 7.5dpc stained with methyl green to show the presence of endometrial glands (x20 objective)

Fig. 7.2



detectable levels of CNTFR are seen by *in situ* hybridisation in the neural tube and neural crest at 8.5dpc, representing a significant increase of expression accompanying differentiation *in vivo* (chapter 4).

The *in situ* hybridisation data for CNTF, CNTFR and IL-6 reported in chapter 4 suggest that the mRNA for neither of these cytokine systems is being produced by the blastocyst in detectable quantities. As discussed in chapter 4, investigation of the expression of OSM in the mouse embryo is not yet possible. The ability of OSM to operate via the DIA receptor (Gearing et al, 1992), suggests that this cytokine may be the most likely substitute for DIA.

IL-6 and CNTF have been removed from mice independently by means of homologous recombination in ES cells (Kopf et al., 1994; Poli et al., 1994; Masu et al., 1993). Although in each case specific effects were reported, the phenotypes became apparent only when the homozygous mutants reached adulthood. Neither of these cytokines is, therefore, essential for development of the early embryo. Double mutants were created by crossing CNTF null mice with DIA null mice. In these animals the characteristics observed in CNTF-/- individuals were exascerbated, but they were able to reach adulthood (Sendtner et al., in preparation). The generation of complex mutants lacking functional genes for all the cytokines which utilise the gp130 signal transduction pathway may be a means to determine whether this is the only pathway involved in the maintenance of the stem cells in the early mouse embryo. However, targeting experiments for the receptor components may be more informative.

Recently, other cytokines, such as IL-11 have been found to bind to receptors which utilise gp130. However, the existence of an alternative signal transduction pathway for this purpose is now being considered. The generation of mice lacking the DIA receptor has recently been achieved (Meng Li, in preparation). Homozygous null individuals are apparently able to undergo embryogenesis. This strongly implies that an alternative route to the DIA receptor complex is available for stem cell preservation in early embryos. Research in progress suggests that removal of gp130 from mice results in a phenotype that is lethal in the homozygous condition, but not until the later stages of gestation (Taga et al., in preparation). If such individuals are genuinely devoid of gp130, an alternative signal transduction pathway for the suppression of differentiation in the early embryo must exist. Until such a pathway has been identified, characterised and ablated it is not possible to ascertain which system is likely to be the predominant controller of stem cell regulation. If inhibition of the putative alternative system results in lethality at an early embryonic stage, it must be assumed that the DIA system is dispensible for early embryonic events other than implantation.

Differentiated ES cells in which both DIA alleles have been removed by homologous recombination have been found to produce a factor which can inhibit the differentiation of normal established ES cells in culture (C. Dani et al., in preparation). The identity of this factor, and its mode of operation have yet to be determined. However, it has been found to be active in the presence of blocking antibodies against gp130.

The removal of each of the components of the DIA system from the mouse embryo does not interfere with early

development. The implication from such observations is that DIA is not essential for the initial stages of embryogenesis. The data presented in this study, however, suggest that DIA is expressed in the blastocyst in a temporal and spatial pattern consistent with a role in stem cell regulation. Also, application of DIA to embryonic stem cells in culture and *in utero* affects their subsequent behaviour. From these results it seems probable that DIA is normally involved in stem cell maintenance in the early mouse embryo, but that an alternative mechanism exists which is able to fulfil the same role, and can compensate for failure of the DIA system in compromised laboratory animals.

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