Gene Expression During Mouse Development and Teratocarcinoma Differentiation

A thesis presented by

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To my late father, my mother, and Belinda

for their love and support.

ABSTRACT

The studies described in this thesis concern the identification and further characterisation of transcripts that are preferentially expressed in the parietal endoderm of the mouse embryo. At 13.5 days post-coitum the 2.2Kb transcripts of the c-fos and Sparc genes were found to be more abundant in the parietal endoderm than in other extra-embryonic tissues and the embryo proper. The expression of both genes was further studied using both DNA probes and antisera directed against the predicted C-termini of the proteins.

Analysis of c-fos mRNA levels in murine extra-embryonic tissues revealed a 10-fold increase in the amnion during late gestation. The c-fos anti-peptide serum specifically recovered proteins of Mr 46,000 and 39,000 from extracts of parietal endoderm and amnion cells labelled for 15 minutes with ^{35}S-methionine. On sodium-dodecyl-sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) these proteins co-migrated with proteins immunoprecipitated using serum from rats innoculated with FBJ-MSV. Pulse-chase and ^{32}P-labelling analyses revealed that the Mr 46,000 protein is rapidly converted to higher molecular weight derivatives.

Differential screening of a cDNA library and Northern analysis identified SPARC as an abundant transcript of parietal endoderm cells at 13.5 days post-coitum. Sequence obtained from SPARC cDNAs was used to predict the amino acid sequence of the SPARC protein. This allowed an antipeptide serum to be raised against SPARC and revealed homology to bovine osteonectin and 43K. Metabolic labelling of parietal endoderm cells, followed by immunoprecipitation from the culture medium and SDS-PAGE, specifically recovered an Mr 43,000 secreted

glycoprotein.

Levels of both SPARC mRNA and protein increased 10- to 20-fold when F9 cells were differentiated to parietal endoderm. This was accompanied by an increase in the transcription rate of the gene.

These and other studies concerning the expression and function of c-fos and SPARC are discussed.

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CHAPTER 1

Extra-embryonic cell lineages of the mouse embryo - a system in which to study mammalian developmental gene regulation

1.1 Introduction

Probably the single most interesting problem which currently confronts the developmental biologist concerns an understanding of the molecular mechanisms that underlie determination and differentiation. What are the identities of the molecules that specify the developmental fate of a particular cell? Through what mechanisms do these "signal molecules" specify the changes in gene expression that are manifest as differentiation? Which genes are activated, repressed, or otherwise modulated during commitment and phenotypic maturation? At what level and by what mechanisms are these genes regulated? It is clear that progress towards an answer for certain of these questions requires a detailed examination of the regulation of individual genes and their products. The research that is described in this thesis concerns the identification and characterisation of two genes that may contribute to the molecular analysis of a particular differentiation event of murine embryogenesis.

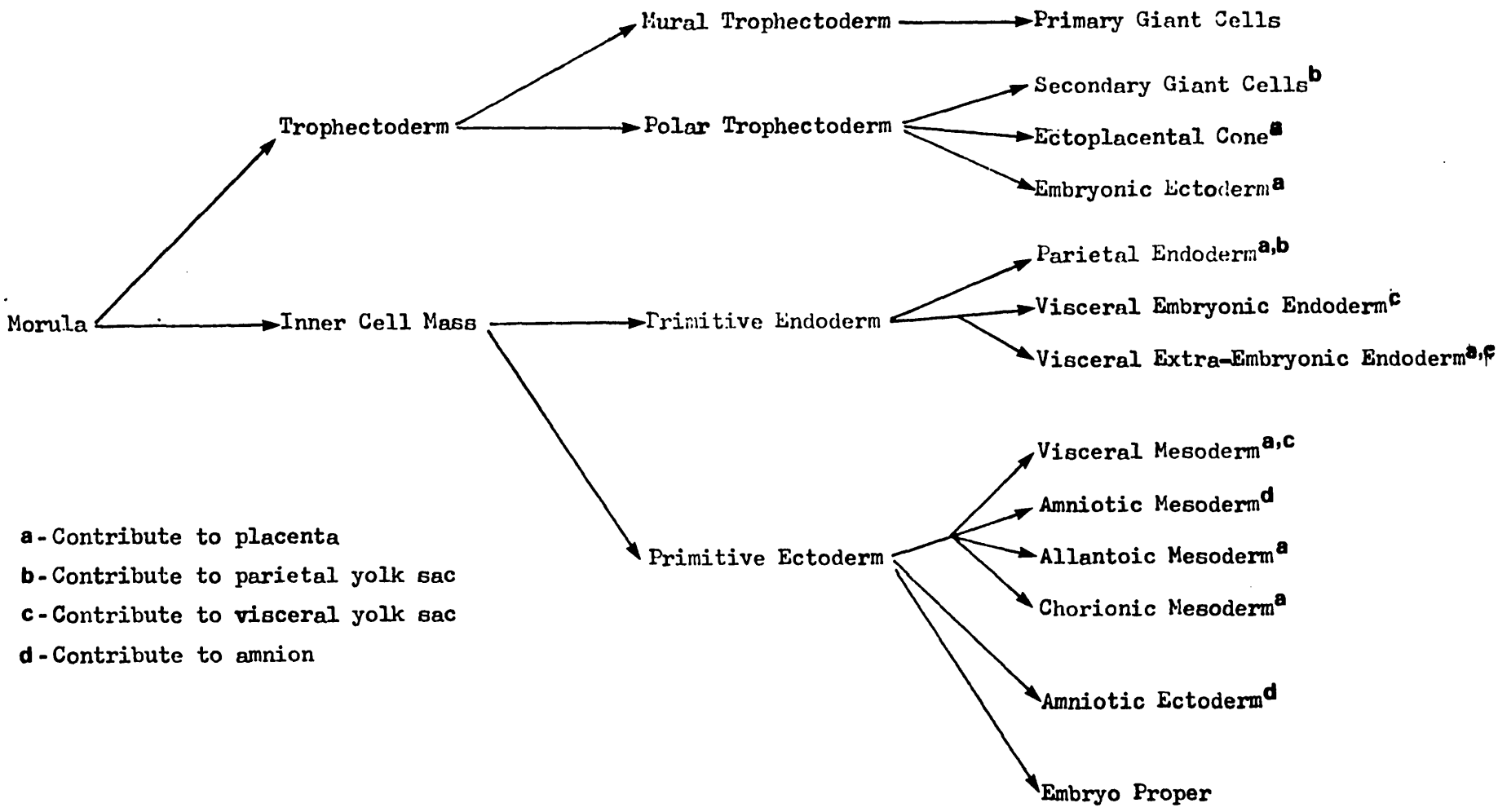
The study of mammalian development has proceeded more slowly than that of certain non-mammalian species, but is probably most advanced in the mouse; the small size and relatively short generation time of this mammal favour its use in the laboratory. The study of the molecular basis of development in Drosophila and Caenorhabditis

elegans has benefited from the identification and characterisation of numerous mutations that perturb the normal developmental programme. A similar wealth of developmental mutants is not available in the mouse, and its large genome size has, in most instances, precluded the molecular cloning of the altered genes which cause the murine developmental lesions that are known. Moreover, since mammals, with few exceptions, are viviparous, the normal development of more accessible vertebrate embryos (amphibians and birds) has been most extensively studied by embryologists.

The most detailed knowledge of murine development is derived from those events which can be reproduced in culture. Currently, in vitro techniques have been developed which will allow embryogenesis to procede to the somite stage (day 8 p.c.; Hsu et al., 1980). The events which occur prior to this stage mainly concern the formation of extra-embryonic tissues and the determination and differentiation of extra-embryonic cell lineages (Figure 1.1). The extra-embryonic tissues of the mid-gestation mouse embryo are the parietal yolk sac, visceral yolk sac, amnion, and placenta. They play an important role in the maintenance and protection of the foetus and offer a number of advantages in the study of mammalian developmental gene regulation. First, they can be dissected, with relative ease, free of contamination by other embryonic or extra-embryonic components. Second, the extra-embryonic membranes (parietal yolk sac, visceral yolk sac, and amnion) contain only two or very few different cell types and certain of these (parietal endoderm and visceral endoderm) can be isolated from the membranes as largely homogeneous populations.

The parietal endoderm and visceral endoderm are derived from a common stem cell population, and provide an opportunity to study a

Figure 1.1 Extra-Embryonic Lineages of the Mouse



a - Contribute to placenta
b - Contribute to parietal yolk sac
c - Contribute to visceral yolk sac
d - Contribute to amnion

binary developmental decision. This constitutes one of the earliest differentiation events during murine development. It appears to be modulated by relatively simple cell-cell interactions and involves cell migration, and changes in gene expression, cell shape, and intercellular associations - themes which pervade embryogenesis. Moreover, these processes can be mimicked *in vitro* by certain embryonal carcinoma cell lines following relatively simple manipulations of their culture conditions.

The studies described in this thesis concern the cloning and characterisation of genes which are preferentially expressed in parietal endoderm, with a view to their eventual use in the molecular dissection of the processes which underlie the differentiation of this tissue.

1.2 The development of the mouse - derivation and properties of the extra-embryonic tissues

A detailed description of mouse development is beyond the scope of this thesis, but may be found in Snell and Stevens (1968) and Hogan *et al.* (1986). The following account reviews those aspects of murine embryogenesis that are relevant to the formation of the extra-embryonic tissues and to the studies presented in this thesis.

1.2a The formation of the blastocyst

During the earliest stages of development, post-transcriptional mechanisms appear to be the most important means of gene regulation. Between fertilization and the first cleavage division of the zygote *in vitro*, RNA polymerase II activity (Moore, 1975) and the incorporation of labelled precursors into heterogeneous RNA (Knowland

and Graham, 1972) are undetectable. Protein synthesis is directed by maternally-derived mRNA (Braude et al., 1979; Bachovara and de Leon, 1980; Harper and Monk, 1983) and the selective translation of certain transcripts is dependent upon, or accelerated by, fertilization (Cascio and Wasserman, 1982; Howlett and Bolton, 1985).

Within 24 hours of fertilization, the zygote divides to produce two blastomeres of approximately equivalent size. At this 2-cell stage, a loss of maternally-encoded proteins and the synthesis of many novel peptides has been revealed by metabolic labelling followed by SDS-PAGE (Van Blerkom and Brockway, 1975; Bensaude et al., 1983). Inhibition with alpha-amanitin has revealed that this is due to the activation of RNA polymerase II transcription of the embryonic genome, which occurs in two phases at 18-21 and 26-29 hours post-fertilization (Flach et al., 1982). The activation of the embryonic genome is accompanied by the degradation of existing maternal RNA (Piko and Clegg, 1982; Clegg and Piko, 1983; Giebelhaus et al., 1983), and recent evidence suggests that the turnover rates of certain maternal proteins are also increased (Howlett and Bolton, 1985).

The second and third cleavages, which are not entirely synchronous (Kelly et al., 1978), occur at approximately 12 hour intervals to produce the 8 cell embryo. During this period few changes in the pattern of protein synthesis are detected by metabolic labelling followed by two-dimensional polyacrylamide gel electrophoresis (Levinson et al., 1978) and there is evidence that all of the blastomeres are equivalent and totipotent until after the 8 cell stage. Isolated blastomeres from 2-, 4- or 8-cell stage embryos can give rise to complete blastocysts when cultured in vitro, although there is a tendency for cell numbers in the inner cell mass to be

reduced (Tarkowski, 1959; Tarkowski and Wroblewska, 1967). These data are complemented by the studies of Kelly (1967) which demonstrate that all 8 blastomeres of the 8-cell stage can contribute to both the inner cell mass and trophectoderm-derivatives when combined with genetically-marked morulae to give chimaeric embryos.

At the 8-cell stage, the blastomeres of the pre-implantation mouse embryo undergo a dramatic change in shape and intercellular interactions. This process is known as "compaction" and is considered to be essential for the future segregation of presumptive cell types. There are three major features of this event:

I. Change in shape of the embryo.

II. The polarisation of individual blastomeres.

III. The formation of apical intercellular tight junctions.

The light microscope reveals a dramatic change in the gross morphology of the embryo during compaction; from an aggregate of 8 separate, spherical blastomeres to a smooth ball in which individual cells cannot be distinguished. This marked alteration is a consequence of the blastomeres flattening against each other - maximising cell-cell contact - and apparently arises from increased intercellular adhesion (Calarco and Epstein, 1973; Lehtonen, 1980). In common with many other adhesion processes, the flattening of compaction is calcium-dependent (Ducibella and Anderson, 1975). A second similarity with cell aggregation in other systems is the inhibition of compaction by tunicamycin, an antibiotic which specifically inhibits N-linked glycosylation of proteins (Surani et al., 1981). Qualitative analysis by SDS-PAGE revealed that the glycosylation of at least two cell-surface proteins, of Mr <68,000 and Mr >165,000 respectively, were inhibited in these studies, but these proteins were not further characterised.

The gross morphological changes of compaction can also be inhibited by whole antiserum or IgG Fab fragments raised against EC cells (Kemler et al., 1977; Johnson et al., 1979) and Hyafil et al. (1980, 1981) have determined that one of the antigens recognised by anti-EC sera is a surface glycoprotein, uvomorulin. The involvement of this protein in compaction has been demonstrated by inhibition of the flattening process with an uvomorulin-specific antibody. Furthermore, calcium alters both the antigenicity and proteolytic cleavage products of uvomorulin, suggesting that there is a calcium-dependent configurational change in the molecule. These observations raised the possibility that uvomorulin, which is identical to "cadherin" of F9 EC cells (Yoshida-Noro et al., 1984) and may be the same as CAM 120/80 (Damsky et al., 1983) and L-CAM (Gallin et al., 1983), might mediate compaction. This hypothesis is, however, difficult to reconcile with the observations that uvomorulin is also present on pre-compaction embryos and that extra-cellular calcium levels (about 1mM) are always well above those which induce decompaction and conformational changes in uvomorulin (0.04-0.06mM; Ducibella and Anderson, 1979; Hyafil et al., 1981). It is possible, however, that the cells are only competent to respond to uvomorulin for a limited period and that other components of the compaction mechanism are temporally regulated.

The flattening process of compaction is preceded by the polarisation of individual cells (Reeve and Ziomek, 1981). Scanning electron microscope and transmission electron microscope studies demonstrate that microvilli are evenly distributed across the surface of early 8-cell stage blastomeres, but that during compaction they become localised at the apical regions of the cells (Calarco and Epstein, 1973; Ducibella et al., 1977). This concentrates a large

surface area of cell membrane at one end of the cell and results in an increased density of binding of certain ligands, e.g. concanavalin A, in this area (Reeve and Ziomek, 1981). Cytoplasmic polarisation is also observed; nuclei migrate basally, a column of organelles is located between the nucleus and the villous pole, and microtubules become aligned parallel to areas of cell contact (Ducibella et al., 1977; Reeve, 1981). The mechanism by which polarisation is induced remains unclear, but there is evidence to suggest that it is the result of an asymetrically-perceived external signal arising from cell-cell interactions. Newly-formed 8-cell stage blastomeres, cultured individually, only rarely become polarised, although they do divide to form two 16-cell stage blastomeres at the appropriate time (Ziomek and Johnson, 1980). If each cell is aggregated, however, to a companion, newly-formed blastomere, both cells develop a polarised surface phenotype. Moreover, the axis of polarity appears to be determined by the point of intercellular contact. If contacts are established with several blastomeres the villous pole develops at the surface furthest from all points of contact (Johnson and Ziomek, 1981a). Experimental manipulations which inhibit the flattening processes of compaction (e.g. treatment with cytochalasin B, tunicamycin, anti-EC cell sera or concanavalin A, and culture in calcium-depleted medium) do not affect polarisation, suggesting that the two processes are separate (Johnson et al., 1984). Polarity can be induced in an 8-cell stage blastomere by contact with blastomeres of 2-cell to 16-cell stage embryos, but not by contact with unfertilized or fertilized eggs (Johnson and Ziomek, 1981a). This suggests that one component of the mechanism may be an early, embryo-encoded protein product.

Subsequent division of these polarised cells is asymetric. Six or

seven peripherally-located blastomeres divide such that one daughter inherits the villous apical pole and the other daughter inherits the avillous basal pole. The remaining cells divide parallel to the axis of polarisation, generating two equal cells (Handyside, 1981; Johnson and Ziomek, 1981b). Decompaction of 16-cell stage morulae in calcium-depleted medium reveals that two morphologically-distinct cell types are present - peripherally-located, villous, polarised cells and internally located, smaller, avillous, apolar cells (Johnson and Ziomek, 1982). However, cells at this stage are not irreversibly committed to different developmental fates since aggregates of all villous or all avillous cells of the 16-cell stage will still form complete blastocysts in vitro (Ziomek et al., 1982).

In the subsequent cleavage of the 16-cell stage embryo polarity is again retained; the majority of polar cells generating one polar and one apolar daughter cell (Johnson and Ziomek, 1982).

The third feature of compaction is the formation of specialised junctions between the blastomeres. Dye transfer and ionic coupling techniques have established that gap junctions are present between the 8-cell stage blastomeres prior to compaction and that these are retained throughout the compaction process (Lo and Gilula, 1979; Goodall and Johnson, 1982). However, in the compacted morula apical tight junctions are formed and these may establish a permeability seal between the external cells, isolating internal cells from the maternal environment (Ducibella et al., 1975; Magnuson et al., 1978).

During the 32-cell stage, about 3.5 days post-fertilization, an eccentrically-located, fluid-filled cavity appears in the morula. This cavity rapidly expands to form the blastocoel of the fully-expanded blastocyst. The blastocyst is composed of two cell types, an outer layer of trophectoderm which surrounds both the blastocoel and

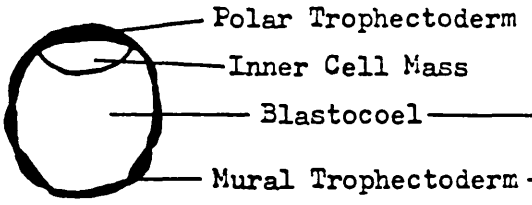
an inner clump of adherent cells, the inner cell mass, which projects into the blastocoel (Figure 1.2A). These two cell types are the product of the first differentiation event of murine development and the fate of a cell in the blastocyst appears to be related to the position of that cell in the morula. Peripheral, polarised cells tend to give rise to trophectoderm (also known as the trophoblast) and centrally-located, apolar cells tend to become inner cell mass.

The exact way in which the difference in position is converted into a difference of cell state is not understood, but two contrasting models have been proposed to account for this phenomenon. These are outlined below and are discussed in detail in Johnson et al. (1981) and Gardner (1983).

The "polarisation hypothesis" suggests that gap-junctional communication between the compacting and polarising blastomeres allows a radial gradient of cytoplasmic information to be set up throughout the morula. Subsequent cleavage is horizontal to the plane of this gradient and asymetrically partitions cytoplasmic or membrane-bound factors between the daughter cells. These molecules are believed to be responsible for specifying the developmental potential of the inner and outer cells. This is somewhat reminiscent of the localisation of cytoplasmic determinants in other embryos, for example the partitioning of pole plasm in Drosophila embryos (Illmensee and Mahowald, 1974) or mesoderm-specifying factors in amphibian eggs (Gurdon et al., 1985). Evidence that cytoplasmic regulatory molecules can influence both determination and differentiation has been obtained from both somatic cell hybrids and nuclear transplantation experiments. A human amnion nucleus will express muscle-specific genes in an amnion-myoblast heterokaryon (Blau et al., 1983) and the nucleus of an amphibian keratinised skin

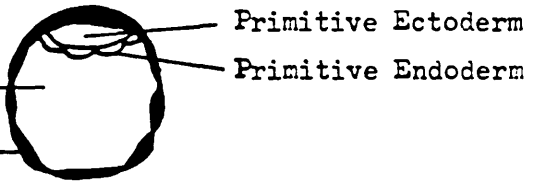
A Blastocyst

4 days p.c.



B Blastocyst

4.5 days p.c.

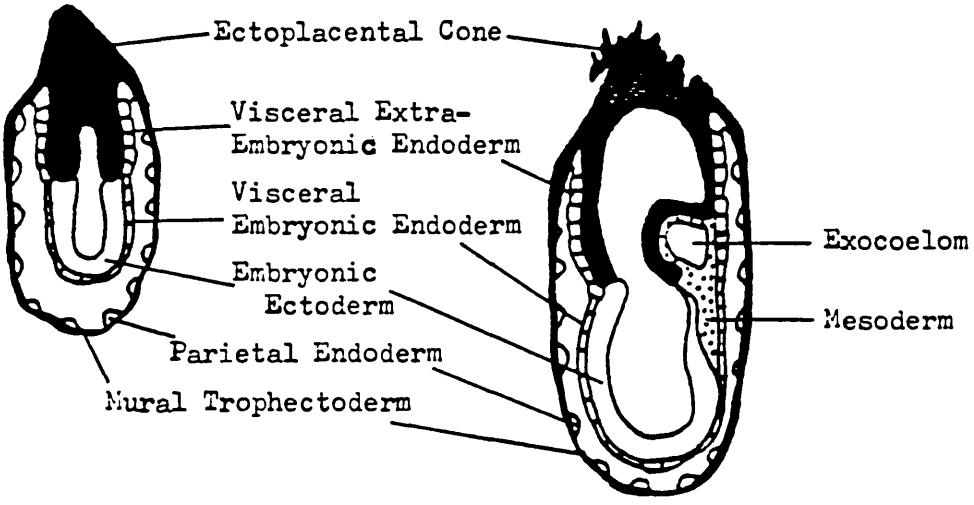


C Egg Cylinder

6 days p.c.

D Egg Cylinder

6.5 days p.c.

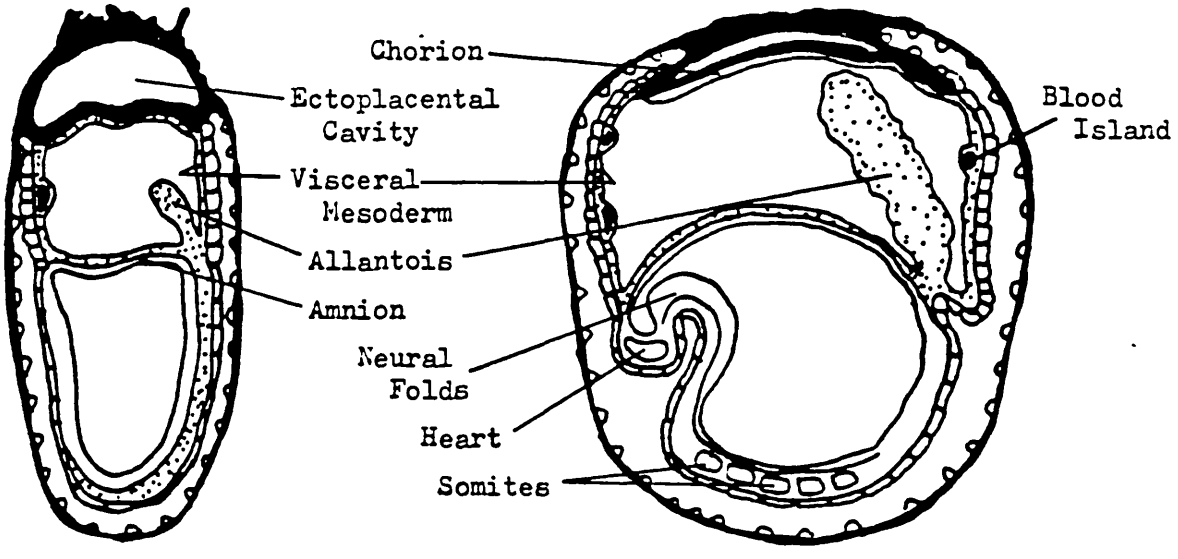


E Late Primitive Streak

7.5 days p.c.

F Somite Stage

8.5 days p.c.



G Mouse Embryo and Extra-embryonic Tissues at 13.5 days p.c.

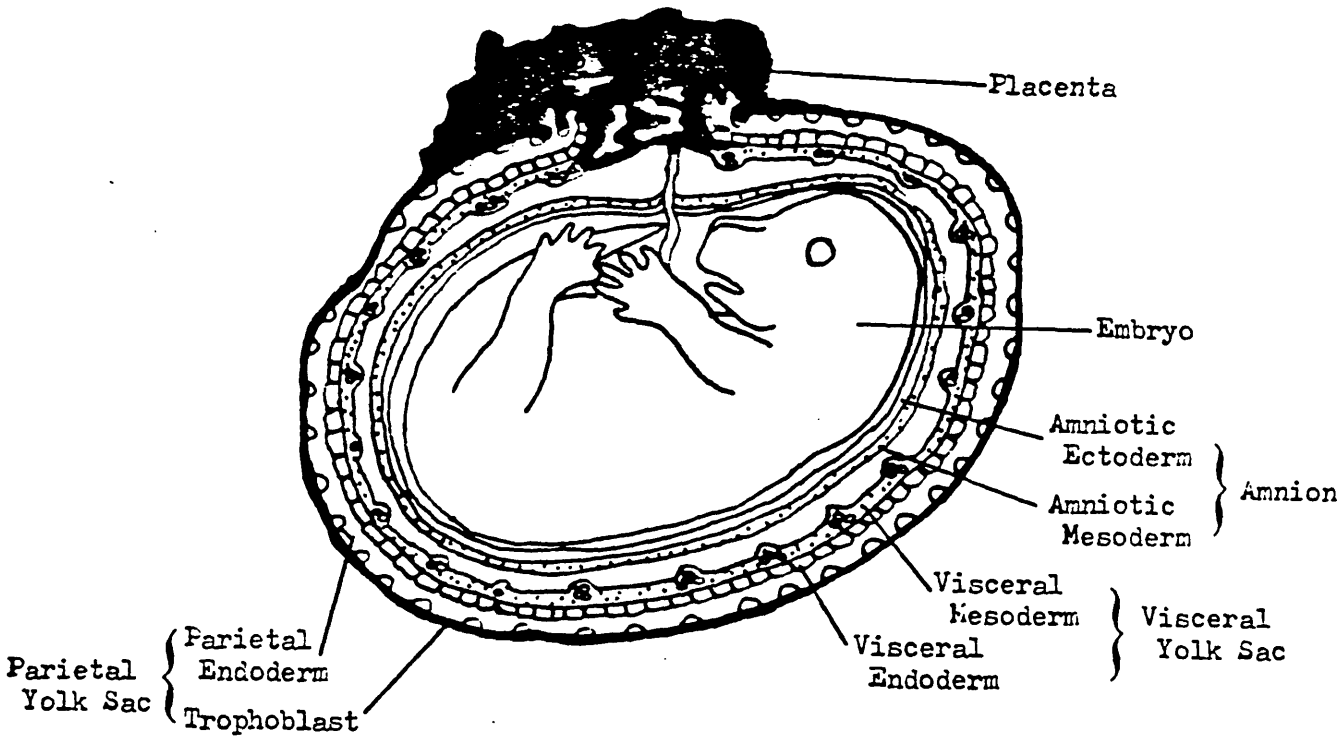


Figure 1.2 Diagrammatic Representation of Murine Development

cell, when transplanted into an egg, can support development at least as far as the swimming tadpole stage (Gurdon et al., 1975).

According to the alternative "microenvironment hypothesis", determination and differentiation are the result of the tight junction permeability seal which creates distinct internal and external environments in the compacted morula. This asymmetry in external influences is believed to be responsible for specifying the different fates of internal and external cells. Presumably, this process must also be mediated by cytoplasmic regulatory molecules.

The nuclear transplantation experiments of Illmensee and Hoppe (1981) suggested that inner cell mass nuclei of the day 4 p.c. mouse blastocyst are still totipotent since a small number supported the development of viable offspring when injected into enucleated, fertilised eggs. In contrast, trophectoderm nuclei did not support development beyond early cleavage. McGrath and Solter (1984a), however, have recently reported that nuclei from cleavage stages prior to blastocyst formation are unable to support development beyond the blastocyst stage.

In the fully expanded blastocyst of the 64-cell stage embryo about 15-20 cells are present in the inner cell mass, and the remainder constitute the trophectoderm. At about this time, the blastocyst hatches from the zona pellucida and implants in the uterine epithelium. Following implantation, the embryo, which remains constant in size throughout cleavage and compaction, begins to grow rapidly and the uterine wall is invaded by cells derived from the trophectoderm.

The trophectoderm cells of the early embryo have distinct apical and basal surfaces. Morphologically, they differ from the inner cell mass in their more flattened shape, paler cytoplasmic staining,

apicolateral junctional complexes, and basal position of secondary lysosomes (Fleming et al., 1984). At a molecular level, two-dimensional SDS-PAGE reveals a number of protein differences between the trophectoderm and inner cell mass (Van Blerkom et al., 1976). Following implantation the trophectoderm gives rise to two distinct and spatially-separate cell populations. The cells surrounding the blastocoel cavity, the primary mural trophectoderm (Figure 1.2A and B), cease cell division but continue DNA synthesis and become enlarged and polyploid. In contrast, trophectoderm cells in contact with the inner cell mass remain diploid and are known as polar trophectoderm. These cells proliferate rapidly and some migrate around the embryo replacing the primary mural trophectoderm cells and becoming polyploid, while other trophectoderm cells invade the maternal endometrium to form the placenta. As development proceeds the polar trophectoderm increases in size and projects into the blastocoel cavity to form the extra-embryonic ectoderm of the egg cylinder. This tissue later recedes towards the placenta to form the chorion (Figure 1.2F).

The second developmental decision occurs between day 4 and 4.5 p.c. and involves the delamination of a layer of primitive endoderm cells upon the blastocoel surface of the inner cell mass (Figure 1.2B). The remaining inner cell mass cells constitute the primitive ectoderm.

1.2b The primitive endoderm - a bipotential stem cell population

The founding population of primitive endoderm contains only about 20 cells (M.Snow cited in Hogan et al., 1983), and the small number means that there is very little hard information about them. They have an extensive rough endoplasmic reticulum (Enders et al., 1978)

and indirect immunofluorescence techniques reveal that they probably secrete laminin, type IV collagen and fibronectin (Adamson and Ayers, 1979; Wartiovaara et al., 1979; Leivo et al., 1980). The early primitive endoderm cells do not form a well-polarised epithelium and are associated with "patchy" deposits of fibronectin. It is possible that more complete polarisation requires the formation of a continuous layer of fibronectin beneath the basal surface.

The mechanism by which the primitive endoderm cells are specified is not understood, although Hogan et al. (1983) have speculated that it may have features in common with the polarisation hypothesis of compaction which was described earlier. Certainly, gap junctions do exist between inner cell mass cells (Lo and Gilula, 1979), but it is not known whether communication via gap junctions between inner cell mass and primitive endoderm or inner cell mass and trophectoderm cells is lost prior to primitive endoderm formation.

Injection of primitive endoderm cells, genetically-marked by a glucose phosphate isomerase isozyme or the Mod 1^{+}/Mod 1^{n} marker system, has revealed that primitive endoderm cells subsequently contribute to only two cell populations - the visceral endoderm and the parietal endoderm - and to no other embryonic or extra-embryonic lineages (Gardner, 1982, 1983, 1984).

As the primitive and extra-embryonic ectoderm layers grow into the blastocoel cavity and form the core of the egg cylinder, primitive endoderm cells which remain in contact with them differentiate into visceral endoderm. Cells which are in contact with the extra-embryonic ectoderm form the visceral extra-embryonic endoderm and cells which remain in contact with the embryonic ectoderm become visceral embryonic endoderm (Figure 1.2C). These two cell populations within the early visceral endoderm are morphologically and

biochemically distinct (see later). Other primitive endoderm cells migrate onto the surface of the trophectoderm and differentiate into parietal endoderm (Figure 1.2C). Parietal and visceral endoderm cells can be distinguished not only by their spatial separation in the embryo (Figure 1.2) but also on the basis of their very different morphology (Figure 1.3; Enders et al., 1978; Hogan, 1980; Hogan et al., 1983) and biochemical properties (Table 1.1; Hogan et al., 1983, 1986).

1.2c Parietal endoderm

The parietal endoderm cells form one layer of the bilayered parietal yolk sac (Figure 1.2C and G). Although there is some variation in morphology, these are mostly rounded cells with a large nucleus (Figure 1.3). They are usually separate, not forming extensive intercellular contacts, and studies using the transmission electron microscope have revealed a lack of tight junctions and desmosomes (Minor et al., 1976; Enders et al., 1978). Time-lapse cinematographic studies have revealed that parietal endoderm cells are motile and can move into available space (R.Gardner and D.Cockroft, cited in Hogan et al., 1983, 1986). In view of their lack of intercellular contacts, it is somewhat surprising that parietal endoderm cells express cytokeratins which are usually associated with epithelial cells (Lane et al., 1983).

The rate of cell division in the parietal endoderm has not been analysed in detail, but few mitoses are observed in sections of embryonic material (Hogan et al., 1983) and in vitro only 10% of the cells become labelled with ^{3}H-thymidine over 24 hours (Minor et al., 1976). How do new parietal endoderm cells arise during the expansion of the parietal yolk sac as development proceeds? While the

experiments of Gardner provide convincing evidence for the existence of bipotential primitive endoderm cells in the 4.5-6.5 day p.c. mouse embryo, it is not known whether these cells persist into later stages. It remains formally possible that the increase in the parietal and visceral endoderm populations during development is due to division of existing, differentiated cells. However, the evidence presented above suggests that the division rate of the parietal endoderm cells is too low to account for the rapid expansion of the parietal yolk sac (see later). Therefore, it would seem that new parietal endoderm cells would have to be derived from either the visceral endoderm or a primitive endoderm stem cell population which persists into later stages of development. Currently, there is evidence to support both hypotheses. It is possible that a primitive endoderm population remains at the junction of the parietal and visceral endoderm during development. Scanning electron microscope studies of 7.5-8.5 day p.c. embryos have detected endoderm cells with a morphology which may be intermediate between visceral and parietal endoderm in a band near this junction (Hogan and Newman, 1984). Whether these are endoderm stem cells and persist during later development, however, has not been ascertained. Visceral endoderm cells retain their properties when cultured in isolation, but, if they are co-cultured in contact with extra-embryonic ectoderm cells which are becoming polyploid, they differentiate into parietal endoderm cells (Hogan and Tilly, 1981). This may be due to transdifferentiation of the visceral endoderm cells into parietal endoderm or to the persistence of a primitive endoderm population within the visceral endoderm.

Parietal endoderm cells, migrating onto the trophectoderm, lay down a thick basement membrane known as Reichert's membrane (Figure

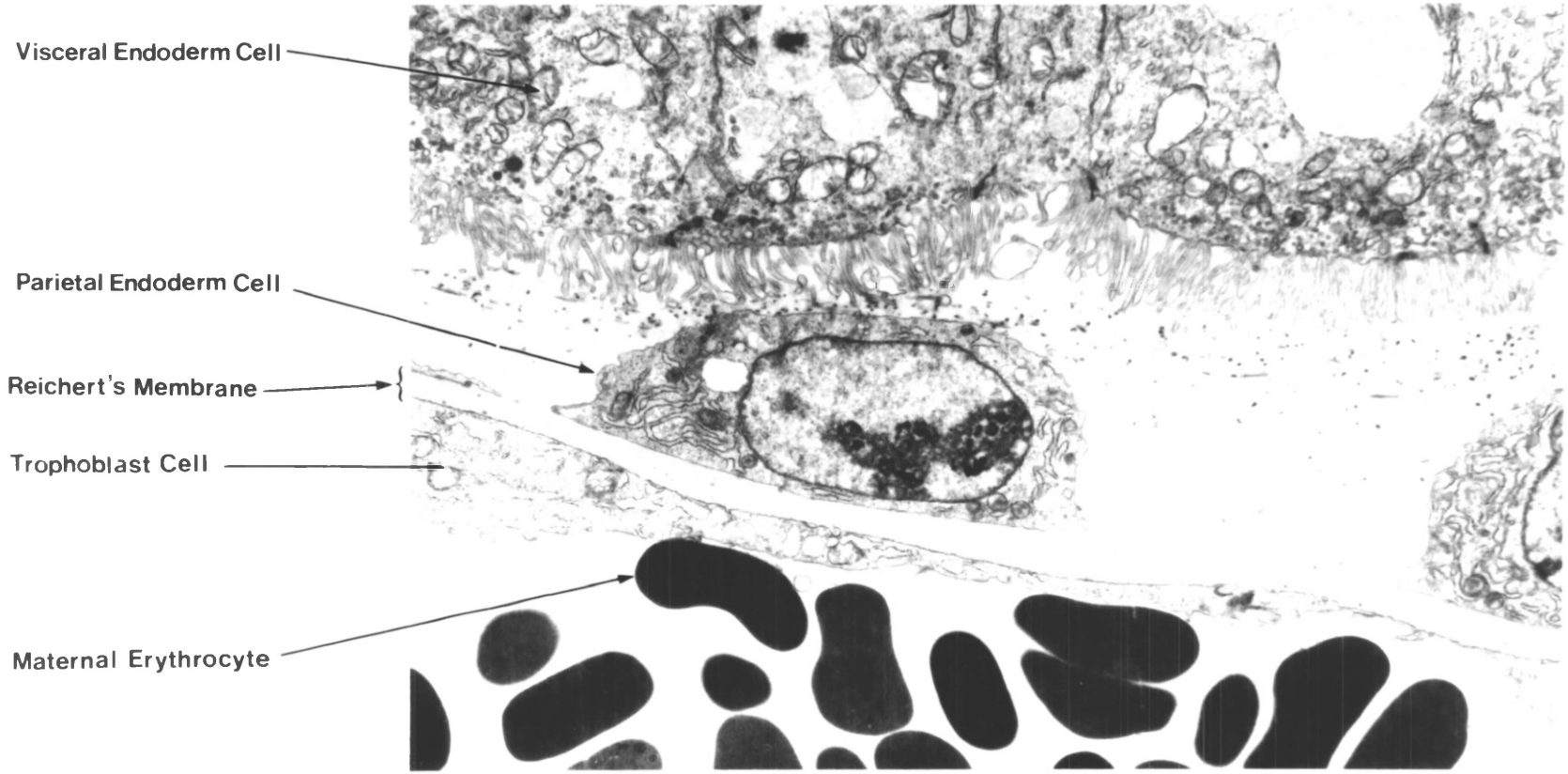

Visceral Endoderm Cell
Parietal Endoderm Cell
Reichert's Membrane
Trophoblast Cell
Maternal Erythrocyte

Figure 1.3 **Transmission electron micrograph of the parietal and visceral endoderm of the 7.5 day p.c. mouse embryo.**

Photograph provided by R.Tilly and B.Hogan.

Table 1.1 **Biochemical differences between the parietal and visceral endoderm of the 10-14 day p.c. mouse embryo.**

	Synthesis by	
Marker	**Parietal Endoderm**	**Visceral Endoderm**
Fibronectin	-	+
Laminin	+++	+
Type IV collagen	+++	+ or -
Type I collagen	-	+
Desmosomes	-	+
AFP	-	+++
Transferrin	-	+
Alpha-1 antitrypsin	-	+
Apolipoprotein A1	-	+
Tissue-type plasminogen activator	+++	-
Urokinase	-	+
Vimentin	+	-

Table adapted from Hogan *et al.* (1986).

- not detectable

+ expressed

+++ expressed at high levels

1.3) which is continuous over the entire trophectoderm surface by day 6 p.c. During development, Reichert's membrane undergoes a large increase in size which involves extensive remodelling of the membrane and the synthesis of new basement membrane components by the parietal endoderm cells. This accounts, at least in part, for the rich rough endoplasmic reticulum of these cells which is distended with secretory glycoproteins, at least some of which are basement membrane components (Martinez-Hernandez et al., 1974; Jensh et al., 1977). In the rat embryo, during mid-gestation (day 15 p.c.) Reichert's membrane reaches a thickness of about 11μm and between 11.5 and 17.5 days p.c., it expands ten-fold in surface area and increases twenty-fold in total protein content. The thickness of Reichert's membrane starts to decline towards the end of mid-gestation and it finally ruptures by day 18 p.c. in the rat (Clark et al., 1975) and day 16 p.c. in the mouse (Dickson, 1979; there is some variation in the exact timing between strains). These properties have made the parietal endoderm a favoured system for the study of the biosynthesis of basement membranes and the characterisation of basement membrane components e.g. laminin, type IV collagen, entactin and heparan sulphate proteoglycan (see for example Hogan, 1980; Cooper et al., 1981; Hogan et al., 1982a, 1982b; Semoff et al., 1982; Kurkinen et al., 1982, 1983b, 1983c; Barlow et al., 1984).

The function of Reichert's membrane is unclear. It is exposed to both maternal blood sinuses and to fluid between the parietal and visceral yolk sacs (Figure 1.3). It may act as a passive filter for molecules passing between the mother and foetus - permeability studies have revealed that it has rather a large pore size, allowing the passage of ferritin (diameter 6nm) but excluding thorotrast (diameter 15nm)(Jollie, 1968). However, it is possible that the

chemical composition of Reichert's membrane may influence those molecules which can diffuse freely across it.

Although human and primate embryos do not have a parietal yolk sac, an endoderm layer is present on the surface of the embryonic ectoderm (epiblast) at about 4.5 days of gestation (Luckett, 1978). Cells apparently migrate out from this layer to form a fine network of squamous cells within the blastocoel cavity. These cells may be analogous to parietal endoderm, however, they do not secrete a thick basement membrane (J.Enders pers.comm.).

1.2d Visceral endoderm

As described above, the early visceral endoderm consists of two distinct populations. The visceral embryonic endoderm cells are squamous and synthesise alphafoetoprotein (AFP), while the visceral extra-embryonic endoderm cells are more cuboidal and do not synthesise detectable amounts of AFP (Dziadek and Adamson, 1978; Dziadek and Andrews, 1983). However, it seems likely that these properties are determined by the underlying tissues. Visceral extra-embryonic endoderm and visceral embryonic endoderm both synthesise AFP when cultured in isolation or in contact with embryonic ectoderm, but do not synthesise AFP when cultured in contact with extra-embryonic ectoderm (Dziadek, 1978).

Later in development, a layer of mesoderm underlies the visceral endoderm and the visceral endoderm forms a homogeneous population of cells in the visceral yolk sac, which expands to surround the developing embryo (Figure 1.2E-1.2G). This mature visceral endoderm population forms a continuous polarised epithelium with numerous apical microvilli and desmosomal junctions. The cells contain many vacuoles, coated pits, and lysosomes (Figure 1.3; Hogan et al., 1983,

1986). These features have suggested that the major functions of the visceral endoderm are the absorption of maternally-derived material which filters through Reichert's membrane and secretion into foetal blood vessels which are present in the adjacent mesoderm layer.

Many of the proteins synthesised by the visceral endoderm are also produced by the foetal liver; these include AFP, transferrin, apolipoprotein A1 and alpha-1 antitrypsin. Products of the post-natal liver, e.g. major urinary proteins (MUPs) and alpha-1 antichymotrypsin, are not synthesised by the visceral endoderm (Tilghman and Belayew, 1982; Meehan et al., 1984). These observations have prompted speculation that the visceral endoderm and foetal liver perform similar functions during development.

The most well-characterised marker of visceral endoderm is AFP. This is a Mr 68,000 glycoprotein which is only synthesised by visceral endoderm, foetal or regenerating liver, and foetal gut (Ruoslahti and Seppala, 1979; Tilghman and Belayew, 1982; Krumlauf et al., 1985). AFP represents about 25% of the total protein synthesis at 15.5 days p.c. and 20% of the total poly(A)+ RNA (Janzen et al., 1982; Andrews et al., 1982a). Its precise function is not known, but AFP is the major alpha-globulin of the foetal circulation and, hence, may have the same role as albumin in adult blood. Indeed, the two genes are closely related and are adjacent in the genome, suggesting that they may have arisen from a common ancestral gene by duplication and divergence (Gorin and Tilghman, 1980; Eiferman et al., 1981; Kioussis et al., 1981).

The human embryo does have a visceral yolk sac with both endoderm and mesoderm layers, but it is relatively small and does not expand to surround the embryo. However, in common with the murine visceral yolk sac it does synthesise and secrete AFP, transferrin and

apolipoproteins (Shi et al., 1985).

1.2e Inactivation of the paternal X-chromosome and hypomethylation of the genome

Two features which appear to be peculiar to the earliest tissues to differentiate in the mouse embryo - the trophectoderm, parietal endoderm and visceral endoderm - are preferential inactivation of the paternal X-chromosome and undermethylation of the genome. The former may provide a useful model for the coordinate inactivation of a group of genes and also an insight into differences between the maternal and paternal genomes, which Solter and his co-workers have found to be developmentally non-equivalent (McGrath and Solter, 1984b; Solter et al., 1985). The latter observation, that the genome is hypomethylated in these lineages, may be exploited in studies to determine the function of DNA methylation both in development and in gene regulation.

In female mammals developmental regulation of X-chromosome activity results in dosage compensation of X-linked proteins. Numerous studies have suggested that in most mammals one entire X-chromosome is active in any given cell, while the other is inactive. An exception to this general rule appears to be certain genes on the short arm of the human X-chromosome, in which both alleles are expressed (Martin, 1982).

The inactive X-chromosome is heterochromatic and may be visible as a Barr body. In most tissues the replication of this chromosome occurs later in S phase than the replication of the autosomes or the active X-chromosome. These data indicate that a physical change in the inactive X-chromosome is either a cause or a consequence of its repression. The same chromosome will be inactivated in the daughter

cells of any somatic cell division, indicating that these changes are also heritable.

In early female embryos of placental mammals both X-chromosomes are believed to be active (Epstein et al., 1978; Monk and Harper, 1978). However, preferential inactivation of the paternal X-chromosome occurs in the trophectoderm and extra-embryonic endoderm cell lineages (Tagaki and Sasaki, 1975; West et al., 1977; Harper et al., 1982). Following the segregation of the primitive endoderm (about 4.5 days p.c.), both X-chromosomes remain active in the embryonic ectoderm for about 36 hours. But, by 6.5 days p.c., X-inactivation has occured in the majority of these cells (Monk and Harper, 1979) and appears to take place without preference for either the maternally- or paternally-derived chromosome (Monk, 1981). In contrast, preferential inactivation of the paternal X-chromosme appears to occur in all marsupial tissues.

There are certain observations which suggest that paternal X-chromosome inactivation in the trophectoderm and extra-embryonic endoderm of eutherian mammals may involve different mechanisms to the random inactivation in other tissues. The replication of the inactive X-chromosome appears to precede the replication of the autosomes and inactive X-chromosome, although in certain of these lineages it subsequently becomes late-replicating (Martin, 1982). Moreover, Kratzer et al. (1983), using an assay to detect electrophoretic variants of the X-linked hprt gene, have demonstrated that DNA from the inactive paternal chromosome of the murine yolk sac endoderm is active in DNA-mediated gene transfer. In contrast, DNA from the inactive X-chromosome of somatic tissues does not efficiently express HPRT in transformants.

As mentioned previously, a second striking feature of the

trophectoderm and primitive endoderm lineages of the mouse embryo is the global undermethylation of the genome in these tissues. Methylation is the only common base modification in vertebrates and occurs principally, if not entirely, at the 5 position on the cytosine ring of the dinucleotide CpG (Razin and Riggs, 1980; Ehrlich and Wang, 1981). Between 60% and 90% of these dinucleotides are methylated in the genome. Methylation of DNA has frequently been found to correlate with changes in gene activity (reviewed in Doerfler, 1983). A causative relationship between gene inactivity and hypermethylation has been established for certain genes by experiments in which methylated or unmethylated DNA has been introduced into cultured cells by microinjection or transfection (reviewed by Jaenisch and Jahner, 1984). However, a number of genes are expressed despite being hypermethylated, for example the Xenopus vitellogenin genes or the delta-crystalline gene (Burch and Weintraub, 1983; Grainger et al., 1983). Moreover, an increased expression of other genes (e.g glucose-6-phosphate dehydrogenase; Battistuzzi et al., 1985) is accompanied by methylation of certain cytosine residues. Busslinger et al. (1983) have demonstrated that repression of transcription of the human gamma globin gene only occurs when cytosine residues close to the initiation site of transcription are methylated. This observation has been developed by Bird (1986) who has recently reported that hypomethylated, CpG-rich regions or "islands" are located upstream of many genes and has suggested that these define areas available to transcription factors. The relevance of these studies can only be ascertained by investigating the methylation patterns of genes and their modulation during differentiation in vivo.

Studies on the trophectoderm and extra-embryonic endoderm cell

lineages of the mouse embryo, using the methylation-sensitive isoschizomeric restriction endonucleases MspI or HpaII, have revealed that centromeric satellite sequences, dispersed repetitive elements and single or low copy number sequences (MUP, AFP or albumin) are hypomethylated in these tissues when compared to other embryonic lineages (e.g. mesoderm or ectoderm)(Chapman et al., 1984; Sanford et al., 1985). Although these experiments also suggested that in the case of MUP (major urinary protein) or AFP methylation does not appear to correlate with gene activity, only a sub-set of CpG residues were analysed. Studies on the overall level of methylation of the rabbit trophoblast (Manes and Menzel, 1981) and the methylation of the human globin locus in the placenta (van der Ploeg and Flavell, 1980) provide preliminary evidence that hypomethylation is a general feature of the eutherian trophectoderm lineage. It is not known whether this relative hypomethylation is a consequence of de novo methylation in the embryonic ectoderm following segregation of the trophoblast and primitive endoderm or if the latter tissues undergo demethylation.

The function of hypomethylation in the extra-embryonic lineages is currently open to speculation. Sanford et al. (1985) have suggested that undermethylation may provide a flexibility in gene expression which allows the extra-embryonic lineages to perform functions associated with many different cell types later in development or in the adult. There may also be a correlation between hypomethylation and the segregation of the maternal and paternal genomes which is manifest in these lineages through preferential inactivation of the paternal X-chromosome. Hypomethylation of regions of the genomic contribution of one parent might heritably mark one set of chromosomes and would result in a decrease in the overall level of

genomic methylation.

1.2f Visceral mesoderm, amnion and placenta

In contrast to the extra-embryonic cell lineages described above, much less detailed information is available concerning the origins and properties of the other lineages which contribute to the mesodermal part of the visceral yolk sac, and to the amnion and placenta.

At about 6.5 days p.c., the process of gastrulation gives rise to mesodermal cells between the primitive ectoderm and visceral endoderm (Figure 1.2D). These cells express vimentin but not cytokeratins, whereas the ectoderm cells express cytokeratins but not vimentin (Franke et al., 1982). Those mesodermal cells generated close to the junction of the primitive ectoderm and extra-embryonic ectoderm actively migrate and contribute to the amnion, allantois, chorion and visceral yolk sac. The mesoderm of the visceral yolk sac gives rise to the first haematopoeitic tissues of the embryo - the mesodermal blood islands. These produce only primitive erythrocytes which synthesise embryonic haemoglobins (Barker, 1968; Gilman and Smithies, 1968). The relative contribution of these cells to the foetal erythrocyte pool declines during mid-gestation and correlates with the development of haematopoeitic cells in the foetal liver (Boussios et al., 1985). Thus, the mesodermal layer of the visceral yolk sac contains both haematopoietic cells, capillaries and mesodermal cells.

The mesoderm, together with cells derived from the embryonic ectoderm also form the third bilayered extra-embryonic membrane, the amnion (Figure 1.2E). This becomes continuous over the embryo and, following the "turning" of the embryo, forms a yolk sac around it (Figure 1.2G). Mesodermal cells also contribute to the chorion and

allantois (Figure 1.2F), both of which subsequently become incorporated into the placenta.

The placenta of the mid-gestation mouse embryo is a complex tissue which contains both foetal- and maternally-derived cells. In the mouse, but not the human, maternal blood vessels break down in the placenta to form the blood sinuses with the circulating blood cells in direct contact with foetal tissues. The foetal cells present in the placenta include trophectoderm, parietal endoderm, visceral endoderm and material from the allantois and chorion. The precise function of each contributing tissue is not known, but, in addition to its role in the transfer of nutrients and metabolites between mother and foetus, the placenta is an important endocrine organ (see Chapter 3).

1.3 Embryonal carcinoma - an in vitro system in which to model murine developmental gene regulation

1.3a Teratocarcinoma and EC cells in vivo

Teratocarcinomas are malignant tumours composed of multipotential stem cells (embryonal carcinoma or EC cells) and they may also contain a range of differentiated cell types. Spontaneous teratocarcinomas arise in the testis, from the abnormal proliferation of primordial germ cells, and in the ovary, probably as a result of the parthenogenetic activation of an oocyte (Stevens, 1983). Differentiation of cells within the tumour may give rise to cells representative of all three primary germ layers; mesoderm (e.g. bone), ectoderm (e.g. neural tissue or skin) and endoderm (e.g. gut). Additionally, extra-embryonic cell types, such as trophectoderm and visceral endoderm, may also be found (Martin, 1975). The

differentiated derivatives of EC cells *in vivo* are non-malignant and tumours which come to be composed solely of these cells are benign and known as teratomas. In humans such tumours are rare in males, but when they do occur are usually malignant teratocarcinomas, whereas in females benign teratomas are more common (dermoid cysts of the ovary), and teratocarcinomas are very rare. Certain mouse strains are especially prone to teratocarcinomas; one third of all males of the 129/JterSV strain have spontaneous congenital testicular teratocarcinomas or teratomas (Stevens, 1973) and half of the females of the LT strain have ovarian teratocarcinomas or teratomas (Stevens and Varnum, 1974).

EC cells can be maintained *in vivo* either as solid tumours or as ascites tumours in the peritoneal cavity. In the latter case, the tumour cells often grow in suspension as aggregates which have been called embryoid bodies due to their gross morphological similarity to pre-implantation embryos (Stevens, 1959). Two types of embryoid body can be obtained. Simple embryoid bodies, which consist of an outer layer of endoderm cells and a solid core of EC cells, and cystic embryoid bodies, which apparently arise from simple embryoid bodies and contain a fluid-filled cyst and a variety of differentiated cell types (Martin, 1975). EC cells in the embryoid bodies retain their multipotency and the injection of a single cell into a syngeneic host can give rise to a tumour containing several differentiated cell types (Kleinsmith and Pierce, 1964).

The observation that EC cells can differentiate *in vivo* into a wide range of cell types suggested that EC cells are analogous to multipotential cells of the mouse embryo. This hypothesis is supported by several other lines of evidence. First, teratocarcinomas may be experimentally induced by the transplantation of embryos

between day 2 and day 6.5 p.c. to ectopic sites, usually the testis or kidney capsule, of a syngeneic adult (Stevens, 1970a; Solter *et al.*, 1970). Teratocarcinomas are, however, not produced by embryonic material between day 4 and day 6.5 p.c. unless embryonic ectoderm is present (Solter and Damjanov, 1973). Teratocarcinomas can also be induced in certain mouse strains by grafting male genital ridges from 12.5 day p.c. embryos into the testes of adults (Stevens, 1970b). Second, Evans and Kaufman (1981) and G.R.Martin (1981) have demonstrated that undifferentiated pluripotential cell lines can be established *in vitro* directly from mouse blastocysts. These cells have the ability to differentiate both *in vivo* and *in vitro*. Third, EC cells can become non-malignant and contribute to normal development when placed in the embryonic environment. Mintz and Illmensee (1975) and Illmensee and Mintz (1976) have demonstrated that genetically-marked (coat colour or GPI isozyme) EC cells from simple embryoid bodies injected into the blastocoel cavities of normal blastocysts can contribute to a wide range of tissues, including germ cells (Illmensee, 1978), in the offspring. This was even possible using EC cells which had been passaged *in vivo* for several years (see Papaioannou and Rossant, 1983 for a review).

1.3b EC cell lines *in vitro*

EC cell lines have generally been established in culture from embryoid bodies or dissociated solid tumours. After plating, some of the cells attach, multiply and form nests or colonies; these often contain both differentiated cells and EC cells. EC cell lines and differentiated cell lines have been cloned to homogeneity from such colonies (Damjanov and Solter, 1974; Martin, 1975). A large number of EC cell lines have been established in this manner and these

frequently differ both in their culture requirements, for example the need for feeder cells, and in their potential for differentiation (Nicolas *et al.*, 1976).

Martin (1975) has documented certain morphological properties that are common to many EC cell lines. EC cells are generally small, bipolar or slightly rounded and adhere strongly to each other causing them to grow in colonies. They frequently attach only poorly to the substratum and have indistinct cell boundaries when viewed through the phase contrast microscope. On an ultrastructural level, these cells are very similar to EC cells *in vivo* and are generally highly tumorigenic when injected into a suitable recipient (Nicolas *et al.*, 1976). Papaioannou *et al.* (1975) have demonstrated that,in common with EC cells cultured *in vivo* as embryoid bodies, EC cells cultured *in vitro* can participate in normal development when introduced into blastocysts.

Clonal lines of EC cells, derived from murine teratocarcinomas, differ widely in their ability to differentiate *in vitro*. Pluripotent lines can often only be maintained as homogeneous populations with frequent subculturing, since they will start to differentiate when cultures become locally dense (Nicolas *et al.*, 1976). These cells also differentiate when small aggregates are transferred to bacteriological petri dishes to which they cannot adhere (Martin, 1980). Under the latter conditions, differentiation leads to the formation of embryoid bodies which are similar to those obtained by culturing EC cells *in vivo* as ascitic tumours. Simple embryoid bodies *in vitro* also consist of an outer epithelial layer of endoderm cells surrounding an inner core of EC cells (Martin and Evans, 1975a) and in the more complex cystic embryoid bodies a central cavity develops and the inner cells can differentiate into a range of ectodermal or

mesodermal cell types including muscle, neural tissue, cartilage, keratinocytes and pigmented cells (Martin and Evans, 1975b).

At the other end of the spectrum, nullipotent EC cells do not differentiate at all, even after prolonged culture in suspension. Thus the classification of these cells as EC cells is based solely on properties which they share with bone fide EC cells, for example common antigenic determinants and the ability to form tumours in syngeneic mice (Martin, 1975).

1.3c EC cells as in vitro models of development

The preceding discussions have described the analogy between EC cells and multipotential stem cells of the early mouse embryo, and the ability of pluripotential EC cell lines to differentiate into a range of cell types both in vivo and in vitro. Consequently, cell lines and simple manipulations of their culture conditions have been sought which will allow a homogeneous starting population of pluripotent EC cells, that normally exhibit only a low rate of spontaneous differentiation, to differentiate reproducibly in vitro to a single, defined cell type. A range of such cell lines are now available that can be used to model different developmental differentiation events and to investigate the concomitant changes in gene expression. Certain cell lines can differentiate into neuronal cells following aggregation (PCC7-S; Pfeiffer et al., 1981) or growth in serum-free medium (1003; Darmon et al., 1982). P19 cells can be differentiated into muscle in the presence of dimethyl sulphoxide (McBurney et al., 1982), or neuronal cells by treatment with retinoic acid (Jones-Villeneuve et al., 1982). In addition, a number of EC lines are available which can differentiate into cells with properties of extra-embryonic endoderm (Silver et al., 1983). These

include the F9 cell line which is discussed in more detail elsewhere in this chapter. Like their counterparts *in vivo*, the differentiated derivatives of these cell lines *in vitro* are non-tumorigenic, providing a system in which properties of the neoplastic state can be investigated (Strickland, 1981).

There are, however, certain reservations concerning the ability of EC cells to accurately model developmental processes *in vitro*. The derivation of these cells from tumours and their culture *in vivo* and, subsequently, *in vitro* may select for abnormal cells. Certainly, EC cells frequently possess karyotypic abnormalities, although, in less extreme cases, it does not prevent their participation in the normal developmental programme when used to produce chimaeric mice. However, only karyotypically normal EC cells can contribute to the germ line (Papaioannou and Rossant, 1983). More significantly, determination and differentiation during development appear to require positional values which are assigned to cells by interactions within the three-dimensional framework of the embryo. There must be some doubt concerning the ability of the simple addition of a chemical inducing agent to a monolayer of EC cells to precisely mimic these effects. But these disadvantages only restrict the usefulness of EC cells and require that conclusions derived from their use are confirmed *in vivo*. Certainly, such limitations do not preclude the use of EC cells as convenient systems in which to model and investigate the molecular mechanisms involved in coordinate gene regulation during development.

Experiments utilising EC cells have contributed towards the evidence for a role of hypermethylation in the inactivation of genes. Moloney murine leukaemia virus was found to integrate into the genome of both EC cells and pre-implantation embryos, but was not transcribed in either circumstance. This lack of expression

correlated with de novo methylation of the integrated viral DNA (Jahner et al., 1982; Stewart et al., 1982; Niwa et al., 1983; Gautsch and Wilson, 1983). Conversely, expression of integrated retroviral genomes in differentiated EC cells, post-implantation embryos or newborn mice correlated with hypomethylation of the viral genome (Stuhlmann et al., 1981; Jahner et al., 1982; Stewart et al., 1982; Gautsch and Wilson, 1983). EC cells thus provide a system in which to investigate the role of DNA methylation in the regulation of gene activity and also the regulation of mammalian methylase activity during development.

On a more macroscopic level, the use of female teratocarcinoma cells has potential for the study of X-chromosome inactivation. As previously described, the paternal X-chromosome is preferentially inactivated in the trophectoderm and extra-embryonic endoderm lineages. On the basis of X-linked enzyme activity, certain female EC cell lines have been found to have two active X-chromosomes, one of which is inactivated when they differentiate (Martin et al., 1978a; Martin, 1980). Furthermore, fusion of XO EC cells with female mouse thymocytes or bone marrow cells can re-activate genes on the inactive chromosome in the latter cell types (Tagaki et al., 1983). This phenomenon has not been detected in fusion experiments between adult cells, and suggests that the EC cells contain factors which can reverse the inactivation process, which was previously believed to be a property confined to germ cells. McBurney and Strutt (1980) reported that levels of the X-linked enzyme - phosphoglycerate kinase - decrease upon the differentiation of P10 EC cells to a range of phenotypes, including endoderm. This line is heterozygous for electrophoretic variants of the enzyme, a property which allowed the authors to demonstrate that the paternal chromosome was not

preferentially inactivated during endoderm formation, although the authors question whether the cells are truely analogous to extra-embryonic endoderm. However, this result suggests that the use of EC cells may be restricted to the study of the general phenomenon of random X-inactivation. The recent studies of Paterno *et al.* (1985) using the EC cell line, C86S1, have suggested that exposure to demethylating agents (5-azacytidine) can reactivate genes on an inactive X chromosome, providing evidence for a role of methylation in X-inactivation.

The use of EC cells in the study of development raises the question of which embryonic cell type they most resemble. The differentiation of certain EC cell lines into a primitive endoderm cell type (see Chapter 1.3d) has suggested that they may be analogous to cells of the inner cell mass. Several comparisons have been made on the basis of the electrophoretic separation of proteins synthesised by EC cells and embryonic tissues. Certain studies have detected differences between EC cells cultured as embryoid bodies *in vitro* and cultured inner cell masses, which also form embryoid bodies (Dewey *et al.*, 1978; Evans *et al.*, 1979; Lovell-Badge and Evans, 1980). However, this discrepency may be a consequence of the experimental manipulations. The qualitative two-dimensional gel electrophoresis experiments of Dewey *et al.* (1978) demonstrate that inner cell masses cultured in isolation synthesise several proteins which are not detected in intact blastocysts. Similarly, differences are detected between isolated EC cores and simple embryoid bodies derived from EC cells cultured *in vivo* as ascites tumours. A second criticism of these experiments is that the inner cell mass may contain transcripts from earlier developmental stages, even maternal mRNA, which would not be present in the EC cells. Consequently, it

could be argued that the protein profile of the cells is irrelevant to their developmental potential and may obscure their embryonic affiliations. Furthermore, since EC cells can arise from the ectopic grafting of adult germ cells, pre-implantation and post-implantation embryos, it is possible that different EC lines are most closely homologous to different embryonic cell types. Alternatively, it may be the case that, under culture appropriate conditions, EC cells may be phenotypically identical to cells of each of the progenitor populations. Nevertheless, on the basis of their protein profile using SDS-PAGE in a single dimension, EC cells appear to be most closely related to ectoderm cells of the 5.5 day p.c. egg cylinder (Martin et al., 1978b), but the question as to whether the diversity of EC cell lines represents a wide range of determined states present in the 5.5 day embryonic ectoderm remains open.

1.3d F9 EC cells can model the differentiation of murine extra-embryonic endoderm

The F9 EC cell line was established from a subline of the transplantable tumour OTT6050 (Bernstine et al., 1973), which was itself derived from a 6-day old male embryo transplanted to the testis of an F_1 hybrid A/He x 129 host (Stevens, 1970a). These cells are cultured on gelatinised dishes and they grow as tightly packed, homogeneous colonies.

F9 cells were originally believed to be nullipotent because they did not appear to differentiate in vitro or in vivo, although in the latter situation they are highly tumorigenic. However, Sherman and Miller (1978) have subsequently demonstrated that F9 EC cells do undergo a low level of spontaneous differentiation to endoderm-like cells both in vivo and in vitro. The rate of differentiation in vitro

can be increased by high density culture or by growth as aggregates in suspension.

Strickland and Madhavi (1978) reported that a much higher level of differentiation can be induced in F9 cultures by treatment with retinoic acid. Monolayers of F9 cells exposed to retinoic acid become flattened, more triangular and develop prominent cytoplasmic granules. The overall rates of cell multiplication, DNA and protein synthesis, drop and an altered pattern of protein synthesis is detected by two-dimensional gel electrophoresis (Strickland and Madhavi, 1978; Linder et al., 1981). Linder et al. (1981) report that the relative rate of synthesis of some 26 polypeptides increases, while that of a further 27 polypeptides decreases. Specifically, it has been found that expression of the surface antigen SSEA-1 ceases (Solter et al., 1979), while the MHC antigen H-2 appears (Segal and Khoury, 1979)

If monolayers of F9 cells treated with retinoic acid are further exposed to compounds that elevate intracellular cyclic AMP levels (dibutyryl cyclic AMP, cholera toxin or the phosphodiesterase inhibitor, isobutylmethylxanthine; IBMX) a further differentiation event occurs and the cells become rounded and more refractile, and synthesise large quantities of basement membrane proteins (Kuff and Fewell, 1980; Strickland et al., 1980). Alternatively, if F9 cells are grown as floating aggregates in bacteriological petri dishes in the presence of retinoic acid, embryoid bodies, with an outer layer of visceral endoderm-like cells, are formed and AFP is secreted into the culture medium (Hogan et al., 1981).

By analogy with the differentiation of murine extra-embryonic endoderm lineages, it has been proposed that treatment of F9 cells with retinoic acid causes them to differentiate into primitive

endoderm and that subsequent manipulations (elevation of internal cyclic AMP or culture as aggregates) influence their differentiation to either parietal or visceral endoderm (Figure 1.4; Hogan et al., 1983).

1.3e Differentiation of F9 cells to parietal endoderm

The treatment of F9 monolayers with retinoic acid alone, usually 10^{-7}M, results in an increased expression of a number of proteins including type IV collagen, laminin and tissue plasminogen activator (TPA)(Strickland and Madhavi, 1978; Solter et al., 1979; Strickland et al., 1980). On this basis, the differentiated cells were originally identified as parietal endoderm (Strickland and Madhavi, 1978), although these markers do not distinguish between parietal endoderm and primitive endoderm cells (see Hogan et al., 1983). It was subsequently observed that treatment with 10^{-7}M retinoic acid and agents which increase intracellular cyclic AMP (e.g. 10^{-3}M dibutyryl cyclic AMP) produces a more extreme phenotype, which Strickland et al. (1980) have proposed is more closely analogous to parietal endoderm. Exogenous dibutyryl cyclic AMP alone does not invoke this response, but the full response can still be obtained if it is added after exposure to retinoic acid for 72 hours (Strickland et al., 1980).

The response to the addition of both inducing agents is apparent at two levels - alterations in the expression of certain genes and changes in the morphology of the cells.

The accumulation of laminin, type IV collagen and TPA is greatly increased when both retinoic acid and dibutyryl cyclic AMP are added to the culture medium of F9 cells (Strickland et al., 1980). Similarly, certain cell-surface proteins, designated p40, p45 and

Figure 1.4 The differentiation of F9 cells *in vitro*

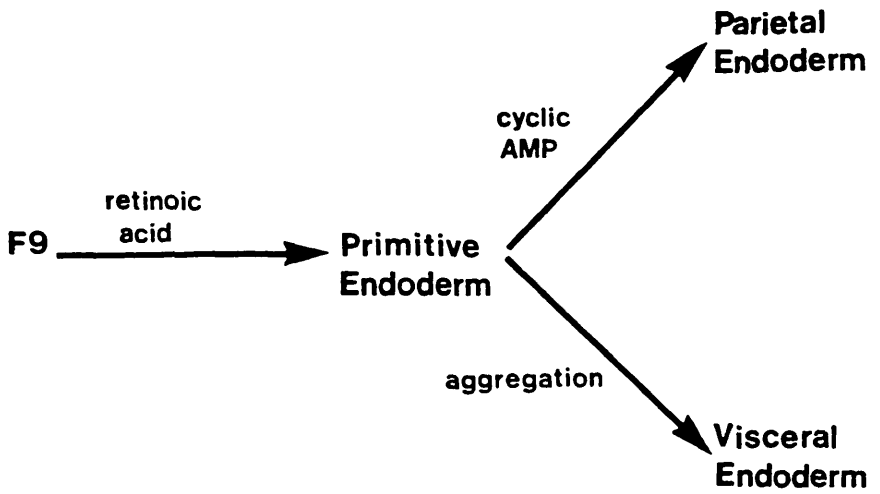
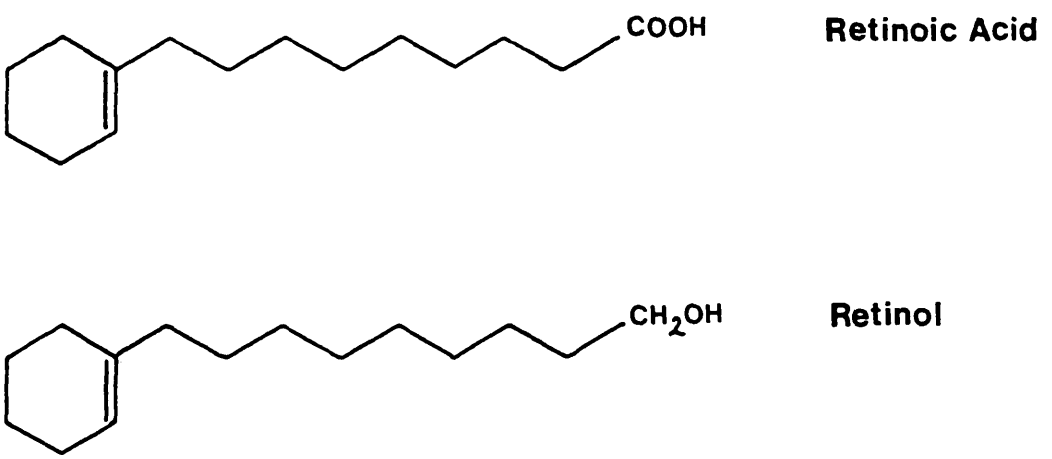


Figure 1.5 The structure of retinoic acid and retinol



p50, which show increased expression in response to retinoic acid alone, exhibit a much greater magnitude of accumulation when both agents are used (Joukoff et al., 1986). Undifferentiated F9 cells secrete fibronectin (Wolfe et al., 1979), but in accord with their proposed parietal endoderm phenotype, F9 cells treated with retinoic acid and dibutyryl cyclic AMP for 5 days synthesise little or no fibronectin (Cooper, A.R. et al., 1983). One difference between parietal endoderm cells and the differentiated F9 cells is that the former express high levels of the cytokeratin filaments TROMA 1 and LE61, whereas only a minority of the latter cells do so (cited in Hogan et al., 1983; Lane et al., 1983).

Secreted laminin, type IV collagen and other basement membrane constituents, including entactin, are incorporated into a matrix which is deposited beneath the differentiated cells. Thus, the differentiation of F9 cells has provided an in vitro system in which to study the regulation and biosynthesis of basement membrane components and their secretion and assembly into matrix. In undifferentiated F9 cells, laminin comprises only about 0.02% of the total translateable mRNA and protein populations, but increases 15-20 fold following 5 days' exposure to retinoic acid and cyclic AMP (Kurkinen et al., 1983c; Cooper, A.R. et al., 1983). Similar results have been obtained for the increase in type IV collagen mRNA (Kurkinen et al., 1982, 1983b). However, even following treatment with retinoic acid and dibutyryl cyclic AMP, both of these proteins are synthesised at rates much lower than those observed in parietal endoderm, in which they constitute 8% and about 7%, respectively, of the total ^{35}S-methionine-labelled proteins synthesised (Kurkinen et al., 1982, 1983c).

There is some controversy concerning the effect of retinoic acid

and dibutyryl cyclic AMP on the mitotic index of F9 cells. Strickland et al. (1980) report that doubling times of the differentiated cells are close to those of control cultures of undifferentiated cells. Increased doubling times have been reported in differentiated derivatives of the F9 sub-line OTF9-63 (Rosenstraus et al., 1982), while Campisi et al. (1984) report a decrease in the mitotic index of F9 cells following differentiation. Whether this is due to differences between the cell stocks held in these laboratories or due to differences in the culture conditions is not known. The F9 line used in this laboratory does not exhibit a significant modulation of its doubling time following induction (Cooper, A.R. et al., 1983), indicating that the cells do not cease dividing as a consequence of terminal differentiation . Hogan et al. (1983) have suggested that the reduced secretion and accumulation of matrix components in the in vitro system may be responsible for the increased mitotic index of our differentiated F9 cell line compared to the parietal endoderm in vivo.

Cells treated with both inducing agents show greater degree of morphological change compared with those treated with retinoic acid alone. The cells become rounded with many filopodia and often have numerous processes which overlap (Hogan et al., 1983). There is an expansion of the rough endoplasmic reticulum, which is presumably associated with the increased secretion of certain proteins, including the matrix components. These morphological characteristics do not revert upon removal of the inducing agents from the culture medium, indicating that the new phenotype is stable.

Kuff and Fewell (1980) report that F9 cells exposed to retinoic acid for several days and then to exogenous cyclic AMP have the morphology of bipolar neurons and express acetylcholinesterase but

not neurofilaments. These results have been extended by Liesi et al. (1983a) and Wartiovaara et al. (1984) who also observe properties of the neuronal phenotypes in differentiated, long-term cultures. F9 cells, seeded at low density and exposed to 10^{-7}M retinoic acid on day 2 and 10^{-3}M dibutyryl cyclic AMP on day 3, adopt a neuron-like morphology and express acetylcholinesterase by day 7 and neurofilaments by day 14. The authors do not, however, give details of what fraction of the cell population expresses a neuronal phenotype. One difference between these cultures and the differentiated cells described by other workers, is that they do not express detectable levels of type IV collagen, although laminin is synthesised. A more extreme neuronal phenotype is obtained if nerve growth factor is also added to the medium - the cells have more extensive processes and express tyrosine hydroxylase, a marker of adrenergic neurons. If this behaviour is not due to alterations in the F9 cell lines which are cultured in certain laboratories or due to low density culture in only 3% FBS, it is possible that differentiation in response to retinoic acid and cyclic AMP produces cells of a "mixed phenotype". This phenotype initially resembles parietal endoderm most closely but may subsequently become more neuronal. However, the differentiation of F9 cells to a neuronal phenotype in response to retinoic acid and cyclic AMP has not proved repeatable in this or other laboratories (B.Hogan pers. comm.) and the F9 cell line cultured in this laboratory expresses increasing levels of type IV collagen at least until 5.5 days after the addition of inducing agents (Kurkinen et al., 1982; Kurkinen et al., 1983b). Furthermore, the possibility that differentiation of F9 cells in response to retinoic acid and cyclic AMP produces a mixed neuronal/parietal endoderm phenotype does not preclude the use of

these cells to study various aspects of gene regulation, including the coordinate regulation of markers of parietal endoderm, the role of DNA methylation or the regulation of exogenous genes. It may, however, limit their usefulness as a model of parietal endoderm differentiation, although Strickland (1981) raises the possibility that the trophectoderm may direct the differentiation of parietal endoderm via mechanisms which raise intracellular levels of cyclic AMP.

1.3f Effects of retinoic acid and cyclic AMP on gene regulation

Retinoic acid (Figure 1.5) is a naturally-occuring oxidation product of vitamin A (retinol). It has been found to possess vitamin A-like activity in growth promotion, but cannot replace the requirement for retinol in vision or reproduction. However, in many *in vitro* systems retinoic acid is 100-1000 times more active than retinol (Sporn, 1984). In the differentiation of F9 cells, the active part of the retinoic acid molecule appears to be the carboxylic acid group on the C-15 atom. Modification of this group by reduction to the aldehyde or alcohol forms decreases the effectiveness of the molecule in promoting differentiation about 1,000-fold. In contrast, substitution of the 5,6-cyclohexenyl ring system with an aromatic ring does not significantly affect its biological activity (Strickland and Madhavi, 1978). Retinoic acid appears to be transported *in vivo* bound to albumin (Smith *et al.*, 1973) and it is not stored in appreciable quantities by any mammalian organ, but is metabolised rapidly and excreted (Lotan, 1980).

Wolbach and Howe (1925) first postulated a role for retinoids in proliferation and differentiation. They reported that vitamin A deficiency in rats causes epithelial cells to differentiate into

keratinising epithelium instead of mucociliary epithelium. The subsequent studies of Fell and Mellanby (1953) revealed that excess vitamin A causes the converse effect in organ cultures of chick epidermis. As described above, retinoic acid causes the differentiation of F9 EC cells *in vitro*; this is concomitant with the loss of their tumorigenicity *in vivo* (Strickland and Sawey, 1980). Retinoic acid also causes the loss of tumorigenicity in the promyelocytic leukaemia cell line HL-60 by causing the cells to differentiate into granulocytes; thus overcoming the proposed block to myeloid differentiation that causes tumorigenesis (Breitman *et al.*, 1981). There is also a body of evidence that retinoic acid can suppress the growth of certain tumours *in vivo*, by causing the stem cells to differentiate and form benign growths (Moon *et al.*, 1977; Bollag, 1979; see Sporn, 1984 for a review).

These data suggest that retinoic acid can influence gene expression in a variety of cells. This raises two questions: which genes are affected by retinoic acid; and how are they regulated? In F9 cells several retinoic acid-regulated proteins have been identified (see above). Two mechanisms have been proposed for this action. One possibility is that retinoic acid acts in a manner analogous to steroid hormones - binding to a cytoplasmic receptor and entering the nucleus where the receptor interacts directly with the chromatin. The second hypothesis is that retinoic acid affects gene expression via interaction with protein kinases. Retinoic acid is known to increase the intracellular concentration of cyclic AMP (Sporn, 1984), the further enhancement of this effect by exogenous cyclic AMP may account for the more extreme phenotype of cells induced by both agents. These hypotheses are discussed in more detail in Chapter 5.

Are retinoic acid and other retinoids involved in normal mammalian embryogenesis? In contrast to the anti-tumorigenic effects of retinoic acid described above, marked teratogenic effects of high doses of retinoids, including retinoic acid, have been reported in rat embryos in vivo (Sporn et al., 1981). It is unclear, in these experiments, whether the action of retinoic acid is direct or occurs indirectly through effects on maternal metabolism. The perturbations of normal embryonic development appear to be specific for cells of mesodermal origin, causing both vascular and skeletal abnormalities. Retinoic acid or retinol excess prevents the formation of the early embryonic vascular system in the yolk sacs of both rat and chick embryos (Morriss and Steele, 1977; Sporn, 1984), but similar effects are also caused by retinol deficiency in the 1-day old chick embryo (Sporn, 1984). Among the skeletal abnormalities caused by excess retinoic acid are severe cranofacial defects (Sporn et al., 1981) and limb defects that are typified by a reduction in the length of the long bones and fused, or missing, digits (Kochar, 1973). The latter observation has been extended in the developing chick limb, where it has been found that exogenous retinoic acid, applied to the anterior side of the limb bud produces mirror-image skeletal duplications along the anterior-posterior axis (Tickle et al., 1982; Summerbell, 1983). The degree of duplication is dependent upon the concentration of the retinoic acid applied. These effects are similar to those produced by grafting the zone of polarising activity (ZPA), which is believed to act as a scource of diffuseable morphogen on the posterior side of the limb bud (Tickle et al., 1975), to the same position. It is not known whether the morphogen produced by the ZPA is retinoic acid, but retinoic acid has recently been detected in the developing limb bud of the chick (G.Ikaly pers. comm.). Skeletal

positional-signalling effects have also been observed when exogenous retinoic acid is applied to regenerating or developing amphibian limbs (reviewed by Maden, 1985). The grafting of mesodermal tissue from retinoic acid-treated amphibian limb buds beneath untreated epidermis results in skeletal duplications, whereas the grafting of treated epidermis onto untreated mesoderm has no effect. This provides further evidence for the specificity of retinoic acid on mesodermal cells (Maden, 1983). Taken together with the effects of retinoids on epithelial cells, these results suggest a stringent requirement for retinoids in normal development with either deficiency or excess leading to abnormal development. However, the role of retinoic acid in early developmental processes, including those that give rise to the extra-embryonic lineages is unknown.

Cyclic AMP (3', 5'-cyclic adenylic acid) has been implicated as an intracellular mediator of hormone action and a regulator of gene expression (see Gancedo *et al.*, 1985 for review). The cytoplasmic level of cyclic AMP depends upon the balance of the activities of adenylate cyclase and phosphodiesterase. These can be manipulated *in vitro* by inhibitors of phosphodiesterase (e.g. IBMX) or by addition of cyclic AMP analogues to the medium; both of these techniques are used in the routine differentiation of F9 EC cells in this laboratory.

Cyclic AMP has been found to control functions as diverse as cell division, mitosis, transcription, translation, secretion and membrane permeability (Gancedo *et al.*, 1985). There appears to be only one high-affinity binding protein for cyclic AMP in mammalian cells - cyclic AMP-dependent protein kinase. The holoenzyme is composed of two regulatory and two catalytic sub-units; cyclic AMP binds to the regulatory sub-units and causes the dissociation of the catalytic

sub-units from the enzyme and activation of them. Cyclic AMP-dependent protein kinase is a highly specific enzyme, phosphorylating only few proteins at significant rates and only at certain of the available serine or threonine residues (Cohen, 1981). Since increases in intracellular cyclic AMP can have such a diverse range of effects, all of which are apparently mediated by cyclic AMP-dependent protein kinase , regulation of its action must occur at other levels. The specificity of cyclic AMP action may be imparted by the regulation of these target proteins. There is now considerable evidence that many of the substrate proteins are expressed in a tissue-specific manner e.g. troponin 1 in muscle (Cohen, 1981). Alternatively, the specificity may reside in the cyclic AMP-dependent protein kinase molecule since, although the catalytic sub-unit is constant, there is some evidence for the existence of two regulatory sub-units, RI and RII, that differ in their physichochemical properties and may be expressed in a tissue-specific manner (Bechtel et al., 1977). The regulatory sub-unit may perform a nuclear function, where it is transported under the influence of intracellular cyclic AMP concentration (Nesterova et al., 1981). These workers have further demonstrated that the expression of a particular protein, P15, by 3T3 cells is correlated with the transfer of the regulatory sub-unit to the nucleus, suggesting a mechanism by which cyclic AMP may influence transcription (Nesterova et al., 1982).

1.3g Differentiation of F9 cells to visceral endoderm

If F9 cells are cultured as floating aggregates in the presence of retinoic acid, a very different response is observed compared to that of monolayers in the presence of retinoic acid and cyclic AMP. A distinct outer epithelial layer can be seen by phase contrast

microscopy on the outside of the aggregates after four days. These aggregates have been called embryoid bodies by analogy with the ascitic embryoid bodies formed by certain EC cell lines *in vivo* (Hogan *et al.*, 1981). These F9 embryoid bodies may subsequently become cystic, developing a central, fluid-filled cavity.

A single exposure to retinoic acid for the first 24-48 hours is sufficient to invoke the full response, although differentiation of the outer layer is not manifest until day 4. The outer cells have both morphological and biochemical characteristics of visceral endoderm. They are polarised, with numerous apical microvilli, and have many vesicles, vacuoles and junctional complexes. In contrast, the inner cells of the embryoid body are unpolarised and, on the basis of their morphology, appear to be undifferentiated. Biochemical evidence that the outer cells are visceral endoderm is based on their increased synthesis of AFP (Hogan *et al.*, 1981; Grover *et al.*, 1983a). However, the levels of AFP mRNA expressed by the embryoid body cells are about 0.001% of the level detected in the intact visceral yolk sac (Hogan *et al.*, 1983). Synthesis of AFP is not confined to floating aggregates, since F9 monolayers, treated with retinoic acid and allowed to reach high density, also secrete AFP.

If aggregates are co-cultured with a parietal endoderm-like derivative of F9 cells, cells of the differentiated lineage form an epithelial layer around the aggregates and treatment with retinoic acid does not result in the formation of visceral endoderm-like cells (Rosenstraus *et al.*, 1983). This suggests that visceral endoderm-like cells do not form in the centre of the aggregate and migrate peripherally, and that an external position is important in the differentiation process. Decompaction of aggregates of PSMB EC cells with Fab fragments directed against cell surface components of F9 EC

cells also prevents the formation of an epithelial layer of visceral endoderm (Maillet and Buc-Caron, 1985). Taken together, these observations imply that intercellular interactions and cell position may be important in the expression of the visceral endoderm phenotype by F9 cells. This creates a striking analogy with the effects of other embryonic tissues on the phenotype of embryonic visceral endoderm (described in section 1.2d above).

The data of Grover et al. (1983a, 1983b) suggest that the formation of a basement membrane beneath the outer layer of cells and, in particular, laminin synthesis are important in creating an asymmetry which directs polarisation and subsequent differentiation. AFP synthesis cannot be detected until after the basement membrane has formed and the application of exogenous laminin, but not type IV collagen or fibronectin, prevents the differentiation of visceral endoderm-like cells. These results are discussed more fully, in the context of the regulation of gene expression, in Chapter 5.

1.4 Aims of these studies

As detailed above, the differentiation of parietal and visceral endoderm from a common bipotential stem cell population is one of the most well characterised developmental events during murine embryogenesis. It also has the additional advantage that certain aspects of the differentiation process can be modelled in vitro using F9 teratocarcinoma cells.

There are several features of the developmental programme governing the differentiation of these cell types which can be investigated using the techniques of molecular biology. One of these concerns the identification of genes that are subject to developmental or differential regulation during the determination and

differentiation event. When the research described in this thesis was undertaken a number of protein markers that are differentially expressed at high levels in each of the two differentiated cell types had been identified (laminin, type IV collagen and TPA for parietal endoderm, AFP and transferrin for visceral endoderm). Such tissue-specific markers are a necessary requirement for the study of the differentiation process, however, in its broadest sense, gene regulation can occur at several levels - the frequency and site of initiation of transcription, the processing, stability and translation of the transcript and the modification and turnover of the protein. Consequently, the identification of tissue-specific proteins, in itself, provides little information concerning the level at which developmental gene control acts. This problem can be overcome by using the techniques of molecular cloning which allow the additional characterisation of regulatory events which occur at the DNA and RNA level.

The initial aim of this research was to isolate and characterise developmentally-regulated transcripts preferentially expressed at high levels in the parietal endoderm of the mid-gestation mouse embryo. Three approaches were available to achieve this goal:

1. The cloning of known parietal endoderm-specific markers. This technique has been adopted by other members of this laboratory and has resulted in the molecular cloning of cDNAs for type IV collagen and laminin genes (Kurkinen _et al._, 1983b; Barlow _et al._, 1984, 1986).

2. The identification of parietal endoderm markers by screening genes already isolated by other groups, for those expressed at high levels in parietal endoderm. Using this approach the _c-fos_ gene was found to be abundantly transcribed in parietal endoderm cells (Chapter 3).

3. The isolation of cDNA clones of transcripts preferentially expressed in parietal endoderm by the differential screening of a cDNA library. This approach resulted in the identification of a 2.2Kb transcript expressed at high levels in the parietal endoderm (Chapters 4 and 5).

The studies performed subsequent to the identification of these genes as markers of parietal endoderm had two main objectives. First, the characterisation of the expression of both the transcripts and proteins encoded by these genes in murine extra-embryonic tissues and in F9 cells differentiated to an extra-embryonic endoderm phenotype. This will provide the necessary database of information for a detailed study of their developmental regulation and its control, both *in vivo* and *in vitro* (discussed in Chapter 6). Second, the detailed characterisation of the 2.2Kb RNA and the protein that it encodes with the ultimate goal of understanding the biological significance of its expression in parietal endoderm and other tissues.

Chapter 2

Materials and Methods

Materials

2.1 Chemicals

Those chemicals that were most commonly used in these studies were obtained from Fisons, Sigma, or BDH, and were of the highest purity available. Organic solvents were supplied by Fisons. Other specific reagents were obtained as follows:

Acrylamide	Bio-Rad "ultrapure" for nucleic acids Sigma for proteins
Agarose - high melting point	Sigma type II
- low melting point	Sigma or BRL
Amberlite MB-1	BDH
Amino acids (unlabelled)	Sigma
2-aminopurine	Cambrian Chemicals
Ampicillin	Beechams
Bis-acrylamide	Bio-Rad "ultrapure" for nucleic acids Sigma for proteins
Bromophenol blue	BDH
Caesium chloride (ultrapure)	Koch-Light Laboratories, Sigma
Coomassie brilliant blue	Sigma
Creatine kinase	Sigma
Creatine phosphate	Sigma
Dextran sulphate	Sigma
Dibutyryl cyclic AMP	Sigma
DMS	BDH
Dowex AG50	Bio-Rad
DTT	Sigma
EDTA	Fisons
EN^{3}HANCE	New England Nuclear
Ethidium bromide	Boehringer Mannheim
Formaldehyde	May and Baker
Formamide	Fisons
G-50	Sigma
Gelatin	Sigma
GF/C filter discs	Whatman

Glass powder	Eagle Ceramics Inc.
Glycerol	BDH, Fisons
Glycine	Fisons
Glyoxal (40% solution)	BDH
Guanidinium isothiocyanate	Fluka
HEPES	Sigma
Hydrazine	Eastman Kodak
Hydrogen peroxide	BDH
IBMX	Sigma, Aldrich Chemical Co.
IPTG	Sigma
Kanamycin	Sigma
2-mercaptoethanol	BDH
NP40	BDH
Nucleoside triphosphates	Boehringer Mannheim, BRL
Oligo(dT)-cellulose (type II)	Collaborative Research Inc.
Phenol (re-distilled)	BRL
Piperidine	BDH
Polyethylene glycol 6000	Koch-Light Laboratories
POPOP	Packard
PPO	Packard
Protein A agarose	Sigma
Protein A sepharose CL4B	Pharmacia
PVP	Sigma
Radioactive amino acids	Amersham International
Radioactive nucleotides	Amersham International, New England Nuclear
Radioactive phosphate	Amersham International
Repelcote	Hopkins and Williams
RNasin	BRL
Salmon sperm DNA	Sigma
Sarkosyl	Ciba-Geigy
SDS	Fisons
TEMED	BDH
Tetracycline	Sigma
Triton X-100	Sigma
tRNA (calf liver)	Sigma
tRNA (yeast)	Sigma
Trans-retinoic acid	Sigma
TCA	Fisons
Tris (Trizma base)	Sigma
Tunicamycin	Sigma
Urea	Schwarz Mann, Bio-Rad "ultra-pure"
Vanadyl ribonucleoside complex	BRL
X-GAL	Sigma
Xylene cyanol	Sigma

2.2 Enzymes

Alkaline phosphatase (Calf intestine)	Boehringer Mannheim
DNA polymerase 1 (Kornberg polymerase)	Boehringer Mannheim
DNase 1 (pancreatic)	Sigma
DNase 1 (RNase-free)	BRL
Klenow fragment	Amersham International
Lysozyme	Sigma
Pancreatin	BDH, Sigma
Proteinase K	Sigma
Retriction endonucleases	Boehringer Mannheim, BRL, New England Biolabs
Reverse transcriptase (AMV)	Anglian Biotechnology
RNase A	Sigma
RNase H	BRL
Sp6 polymerase	Promega Biotech
T4 DNA ligase	Boehringer Mannheim
T4 DNA polymerase	BRL
T4 polynucleotide kinase	Boehringer Mannheim
Terminal deoxynucleotidyl transferase (calf thymus)	Boehringer Mannheim, BRL

2.3 Miscellaneous

1,2,3 Kb DNA ladder	BRL
^{14}C-labelled protein markers	Amersham International
Dialysis tubing	Raven Instruments. Treated by boiling in 2mM EDTA (pH 8.0) for 10 minutes. Stored at $4^{o}C$ in dH_2O.
Dowex AG50	To equilibrate the Dowex beads they were washed in 100ml 1M NaCl, then in 100ml 1M HCl, twice in 100ml H_2O, and once in 100ml of 1M NaCl. The beads were stored at $4^{o}C$ in 1M NaCl, 0.01M EDTA, 0.1M Tris; the pH was adjusted to 7.5 with NaOH and 0.02% (w/v) sodium azide was added.
Glass powder	The powder was slurried in water, allowed to settle for 1 hour and the fines spun out of the supernatant. These were resuspended in an equal volume of concentrated nitric acid and boiled for 2 minutes in a fume hood. The glass was then spun out and washed in many changes of distilled water until the pH was neutral. The glass was stored in 1ml aliquots as a 50% (v/v) slurry in water.
G-50	30g of G-50 was added to 250ml TE (pH 8.0), autoclaved and stored at $-20^{o}C$.
M13 universal probe primer	BRL
Random DNA primer (6-mer)	P.L. Biochmicals

2.4 Buffers and solutions

All solutions were prepared using quartz-distilled, de-ionised water and were stored at room temperature unless otherwise stated.

Acrylamide (protein gels)	30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide; store foil-wrapped 4°C.
CAP buffer (10x)	100mM Tris.HCl (pH 9.5), 10mM spermidine, 1mM EDTA.
Cell lysis buffer	0.15M NaCl, 0.005M EDTA, 0.05M Tris-HCl (pH 8.0), 1% (v/v) NP-40.
Coomassie blue stain	30% (v/v) methanol, 10% (v/v) glacial acetic acid, 0.1% (w/v) Coomassie brilliant blue.
Denatured salmon sperm DNA	Salmon sperm DNA dissolved in water at 1mg/ml, sonicated to a length of 200-500bp, boiled for 20 minutes and water added to return concentration to 1mg/ml. Stored at -20°C.
Denhardt's solution (100x)	2% (w/v) BSA (Sigma, Fraction V), 2% (w/v) Ficoll, 2% (w/v) PVP. Stored at -20°C.
Dibutyryl cyclic AMP	Prepared as a 0.1M stock in DMEM, filter-sterilised and stored at 4°C.
DMS buffer	50mM sodium cacodylate (pH 8.0), 1mM EDTA. Stored at -20°C.
DMS stop buffer	1.5M sodium acetate (pH 7.0), 1M 2-mercaptoethanol. Stored at -20°C.
DMSO sample buffer	30% (w/v) DMSO, 1mM EDTA, 0.05% (w/v) xylene cyanol. Stored at -20°C.
Formamide (deionised)	Prepared by passage through Bio-Rad AG 501-X8 mixed-bed resin until pH is neutral. Recrystallised at 0°C and stored at -20°C.
Glyoxal (deionised)	40% glyoxal solution is stirred over changes of Bio-Rad AG501-X8

	mixed-bed resin until the pH is 5.5-6. The glyoxal is then stored at -20°C as 100µl aliquots.
Hydrazine stop buffer	0.3M sodium acetate, 0.1mM EDTA.
IBMX	Prepared as a 0.1mM stock in DMSO filter-sterilised and stored at 4°C.
IPTG	100mM (23.8mg/ml) in H_2O, stored at -20°C.
Kinase buffer (10x)	0.5M Tris.HCl (pH 7.5), 0.1M $MgCl_2$, 0.05M DTT. Stored at -20°C.
Klenow buffer (10x)	100mM Tris.HCl (pH 8.0), 50mM $MgCl_2$. Stored at -20°C.
Laemmli sample buffer (twice concentrated)	
a) Reducing	4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.125M Tris.HCl (pH 6.8), bromophenol blue.
b) Non-reducing	As above but omit 2-mercaptoethanol.
Ligase buffer (5x)	250mM Tris.HCl (pH 7.4), 50mM EDTA, 50mM DTT, 2.5mM ATP. Stored at -20°C.
NET buffer	0.4M NaCl, 0.005M EDTA, 0.05M Tris.HCl (pH 8.0), 1% (v/v) NP-40.
Nick translation buffer (10x)	0.5M Tris.HCl (pH 7.8), 100mM 2-mercaptoethanol, 50mM $MgCl_2$; stored at -20°C.
OLB	OLB is prepared from the following stock solutions: Solution A:1ml 1.25M Tris.HCl (pH 8.0), 0.125M $MgCl_2$ 18µl 2-mercaptoethanol 5µl 0.1M dATP in TE (pH 7) 5µl 0.1M dTTP in TE (pH 7) 5µl 0.1M dGTP in TE (pH 7) Solution B: 2M Hepes (pH 6.6 with NaOH) Solution C: random primer (6-mer) at 90 OD units/ml in TE. A, B and C are mixed in the ratio 1 : 2.5 : 1.5 to prepare OLB, which

	is stored at -20°C.
PBSA	0.17M NaCl, 3mM KCl, 1mM disodium hydrogen phosphate, 1.8mM potassium dihydrogen phosphate (pH 7.4).
Phenol:chloroform	Redistilled phenol, chloroform, isoamyl alcohol (50:50:1), equilibrated with TE (pH 8.0).
Pronase buffer	50mM Tris.HCl (pH 10), 0.15M NaCl, 0.1M EDTA.
Restriction enzyme buffers:	
Low salt buffer	10mM Tris.HCl (pH 7.5), 10mM $MgCl_2$, 1mM DTT.
Medium salt buffer	50mM NaCl, 10mM Tris.HCl (pH 7.5), 10mM $MgCl_2$, 1mM DTT.
High salt buffer	100mM NaCl, 50mM Tris.HCl (pH 7.5), 10mM $MgCl_2$, 1mM DTT.
Very high salt buffer	150mM NaCl, 50mM Tris.HCl (pH 7.5), 10mM $MgCl_2$, 1mM DTT.
RNA gel buffer (10x)	0.2M MOPS (pH 7.0 with acetic acid), 50mM sodium acetate, 10mM EDTA (pH 8.0).
RNA sample buffer	50% (v/v) glycerol, 1mM EDTA, 0.4% (w/v) bromophenol blue.
SDS-PAGE running buffer	1.44% (w/v) glycine, 0.1% (w/v) SDS, 0.3% (w/v) Tris, 0.045% (v/v) concentrated hydrochloric acid.
Sequencing sample buffer	80% (v/v) formamide, 1mM EDTA, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol.
SET buffer (20x)	3M NaCl, 20mM EDTA, 0.4M Tris.HCl (pH 7.8).
Sp6 transcription buffer (10x)	0.4M Tris.HCl (pH 7.5), 60mM $MgCl_2$, 20mM spermidine, 0.2M NaCl. Stored at -20°C.
SSC (20x)	3M NaCl, 0.3M sodium citrate.
STET	0.1M NaCl, 10mM Tris.HCl (pH 8.0),

	0.1mM EDTA, 0.5% (v/v) Triton X-100.
T4 polymerase buffer (10x)	330mM Tris.acetate (pH 7.9), 660mM potassium acetate, 100mM magnesium acetate, 5mM DTT. Stored at -20°C.
TAE buffer (1x)	40mM Tris.acetate (pH 8.0), 5mM sodium acetate, 1mM EDTA.
TBE (10x)	0.89M Tris , 0.89M boric acid, 20mM EDTA (pH 8.3).
TE	10mM Tris.HCl (appropriate pH), 1mM EDTA.
Terminal transferase buffer (5x)	1M potassium cacodylate (pH 6.9), 5mM cobalt chloride. Stored at +4°C, replenished monthly.
TES	10mM Tris.HCl (pH7.5), 1mM EDTA, 0.1% (w/v) SDS
TfbI	30mM potassium acetate, 100mM rubidium chloride, 10mM calcium chloride, 50mM manganese chloride, 15% (v/v) glycerol. pH adjusted to 5.8 with 0.2M acetic acid, filter sterilised and stored at -20°C.
TfbII	10mM MOPS, 75mM calcium chloride, 10mM rubidium chloride, 15% (v/v) glycerol. Adjust pH to 6.5 with KOH, filter sterilise and store at -20°C.
Toluene scintillant	0.068M PPO, 0.0005M POPOP, in toluene.
trans-retinoic acid	Prepared as a 0.01M stock in ethanol, stored foil-wrapped in the dark at 4°C. Fresh stock prepared weekly.
X-GAL	2% (w/v) in dimethylformamide, stored at -20°C.

2.5 Bacterial media and plates

All components of bacterial media were obtained from Difco.

2x TY agar	1.6% (w/v) bacto tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) bacto agar.
2x TY broth	As 2x TY medium but omit agar.
L-agar	1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) bacto agar.
L-broth	As L-agar but omit agar.
L-top agar	As L-agar but 0.7% (w/v) bacto agar.
Minimal agar	0.6% (w/v) disodium hydrogen phosphate, 0.3% (w/v) potassium dihydrogen phosphate, 0.1% (w/v) ammonium chloride, 0.05% (w/v) NaCl, 1mM magnesium sulphate, 0.1mM calcium chloride, 1mM thiamine HCl and 1.5% (w/v) minimal agar.
Psi-agar	2% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 0.5% (w/v) magnesium sulphate, 0.075% (w/v) KCl. pH adjusted to 7.6 with KOH and then add 1.4% (w/v) bacto agar.
Psi-broth	As psi-agar but omit agar.
SOB	2% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 10mM NaCl, 2.5mM KCl. Autoclaved and then filter-sterilised magnesium chloride and magnesium sulphate added, each to a final concentration of 10mM.

2.6 Cell culture media and solutions

DMEM	ICRF media supplies, NIMR media supplies
Foetal bovine serum	ICRF media supplies, NIMR media supplies
PBSA	ICRF media supplies, NIMR media supplies
Trypsin	ICRF media supplies; 0.8% (w/v) NaCl, 0.038% (w/v) KCl, 0.01% (w/v) disodium hydrogen orthophosphate, 0.01% (w/v) dextrose, 0.3% (v/v) Tris.HCl (pH7.7), 0.25% (w/v) trypsin, 10,000u sodium penicillin, 0.01% (w/v) streptomycin, phenol red; stored at -20^{o}C.
Versine	ICRF media supplies; 0.02% (w/v) EDTA in PBSA.

2.7 Dissection of murine tissues

For the staging of embryonic tissues, all mice were normally mated and the time of fertilisation taken to be 12pm on the night of mating. Mice were killed by cervical dislocation, and were dissected under aseptic conditions. Embryos were removed and dissected under sterile conditions. An excellent detailed account of the dissection of individual embryonic tissues may be found in Hogan et al. (1986).

The removal of trophectoderm cells from 13.5 day p.c. parietal yolk sacs was accomplished by manual dissection - these cells are dying at this stage and may be removed in sheets using fine forceps.

In certain experiments visceral yolk sacs were separated into mesodermal and endodermal components by the method of Levak-Svajger et al. (1969) as modified by Meehan et al. (1984). Yolk sacs were rinsed in calcium-free DMEM and incubated for 2 hours at 37°C in calcium-free DMEM containing 2.5% (w/v) pancreatin, 0.56% (w/v) trypsin, 0·5% (w/v) PVP and 20mM Hepes (pH 7.4). After incubation, the yolk sacs were transferred to DMEM containing 10% FBS and dissected using "watchmaker's" forceps. The mesoderm was distinguished from the endoderm by virtue of the blood vessels which it contains, these were easily recogniseable through the disecting microscope.

All mice were from the C3H/He breeding colonies at the Imperial Cancer Research Fund or National Institute for Medical Research

2.8 Cell culture and metabolic labelling

All cells and embryonic tissues were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.8a PYS-2 cells (Cooper et al., 1981).

PYS-2 is derived from the OTT6050 teratocarcinoma and is designated as a parietal endoderm cell line (Lehman et al., 1984). Cells were maintained in monolayer culture in DMEM containing 10% (v/v) FBS, and were sub-cultured by trypsinisation every 3 days.

For metabolic labelling, about 5×10^4 cells were seeded into a 35mm tissue culture dish 24 hours before use.

For the preparation of RNA cells were grown in 90mm tissue culture dishes and harvested before they had achieved confluence.

2.8b F9 cells.

I. Culture of undifferentiated cells.

F9 EC cells (see Chapter 1.3d) were maintained in DMEM containing 10% (v/v) FBS, in tissue culture dishes coated with 1% (w/v) gelatin. Cells were routinely sub-cultured every 2 days or before they had achieved confluence.

II. Differentiation to parietal endoderm (see Chapter 1.3e; Cooper et al., 1981).

For metabolic labelling approximately 2×10^4 cells were seeded onto gelatin-coated dishes in DMEM containing 10% (v/v) FBS, 0.05μm trans-retinoic acid, 1mM 1,6-dibutyryl-adenosine 3',5'-monophosphate (dibutyryl cyclic AMP) and 0.1mM IBMX. The inducing medium was changed every 48 hours.

For the preparation of nucleic acids, cells were treated as above except that they were grown in either 90mm or 240mm dishes and seeded at concentrations such that they would not have achieved confluence at the time of harvesting them.

III. Formation of embryoid bodies (see Chapter 1.3g; Hogan et al., 1981).

F9 cells were trypsinised briefly and gently pipetted so that they dispersed into clumps of about 20 cells. These clumps were seeded into bacteriological petri dishes in DMEM containing 10% (v/v) FBS and 0.05μm trans-retinoic acid. As the aggregates enlarged and formed embryoid bodies the cultures were split, to prevent overcrowding, using a wide-bore (agar) pipette.

2.8c F9Ac C19 cells.

The F9Ac C19 cell line (Solter et al., 1979; Howe and Solter, 1980; obtained from C.F.Graham) is an F9-derived line which synthesises large quantities of laminin and was originally identified as a parietal endoderm. However, other workers have suggested that these may be more closely analogous to primitive endoderm (see Hogan et al., 1983 for discussion). These cells were cultured in the same manner as F9 EC cells and, for RNA extraction, were seeded at a density of about 1×10^4 cells per cm^2 into 120mm dishes. RNA was extracted after culturing these cells for 15 hours. For metabolic labelling purposes 2×10^4 cells were seeded into 35mm culture dishes.

2.8d NIH-3T3 cells.

NIH-3T3 cells were provided by J.Bell and G.Foulkes and were

cultured in DMEM supplemented with 10% (v/v) FBS. Cells were routinely sub-cultured every 2 days.

For serum-stimulation experiments cells were grown to between 30 and 50% of confluence and then exposed to DMEM containing 0.5% (v/v) FBS for 36 hours in order to render them quiescent. The cells were then re-exposed to DMEM containing 10% (v/v) FBS to stimulate their re-entry into the cell cycle. The growth of cells during the course of the experiment was monitored by counting control cultures, grown in parallel, using a haemocytometer.

2.8e Culture of parietal endoderm and amnion cells.

These extra-embryonic tissues were dissected into DMEM containing 10% (v/v) FBS and 20mM HEPES buffer (pH 7.4). The tissues were washed and cultured in DMEM containing 10% (v/v) FBS. For ease of handling in labelling studies, amnions were pre-incubated in DMEM containing 300µg/ml hyaluronidase (Sigma type II) and washed thoroughly in DMEM to remove the copious extra-cellular glycosaminoglycans.

2.8f Metabolic labelling (Cooper et al., 1981; Mason et al., 1985).

I. ^{35}S-methionine.

For continuous-labelling experiments cells or tissues were cultured for 1 hour in 1ml of DMEM containing only 1µg/ml unlabelled methionine, together with 10% (v/v) FBS (rendered methionine-free by extensive dialysis against PBSA, ICRF media supplies, NIMR media supplies). Labelling was performed in 1ml methionine-free DMEM containing 10% (v/v) dialysed FBS and ^{35}S-methionine (usually 100µCi of specific activity 1000-1400Ci/mmol). For long labelling periods (>12 hours) the DMEM was supplemented with 1µg/ml methionine.

In pulse-chase experiments cells were washed twice in DMEM

containing 10% (v/v) FBS prior to incubation for the chase period in the same medium.

II. ^{32}P phosphate.

Membranes were incubated in phoshate-free DMEM for 15 minutes prior to the addition of 1mCi/ml carrier-free ^{32}P orthophosphate. Pulse chase analyses were carried out as above.

2.8g Inhibition of glycosylation.

To study the glycosylation of SPARC, the antibiotic tunicamycin was used at a concentration of 4µg/ml. Previous work has shown that this concentration of tunicamycin inhibits protein synthesis in PYS-2 cells by about 35% (Hogan et al., 1982b), but >2µg/ml are required to completely inhibit the glycosylation of colligin in these cells (Kurkinen et al., 1984). Cells were incubated for 1.5 hours in DMEM containing 5% (v/v) FBS and 4µg/ml tunicamycin, followed by 30 minutes in methionine-free DMEM supplemented with 5% (v/v) dialysed FBS. Labelling was performed in the same medium following the addition of 100µCi ^{35}S-methionine.

2.8h Trypsinisation.

To routinely sub-culture cells and to harvest cells in certain experiments, they were released from the substrate with trypsin-versine (1:4) pre-warmed to 37°C. Monolayers of cells were rinsed in trypsin-versine and detached in the same buffer for 5-10 minutes, depending on the cell line, at 37°C. The enzyme was subsequently inhibited by addition of DMEM containing 10% (v/v) FBS. Prior to re-seeding, cells were counted in a haemocytometer, followed by dilution as appropriate.

2.9 Immunology and protein biochemistry

2.9a Preparation of medium and cytoplasm.

Following metabolic labelling, the medium was removed and cell monolayers and membranes were washed briefly with two changes of PBSA. Washed cells and tissues were solubilised in 500µl of cell lysis buffer at 4°C for 3 minutes with intermittent vortexing. Cell lysates were centrifuged at 12,000*g* for 10 minutes at 4°C to remove insoluble material. These were either used immediately or glycerol was added to 50% (v/v) and the extract stored at -20°C; conditions which do not result in appreciable degradation of proteins (Cooper, 1983).

2.9b Measurement of incorporation of label into protein.

To measure total radioactivity incorporated in cell-free translations of RNA, aliquots were added to 1ml of 0.5M NaOH, 2.5% (v/v) hydrogen peroxide containing 0.5mg/ml unlabelled methionine and incubated at 37°C for 15 minutes to remove labelled methionine bound to its tRNA. Following incubation, 2ml of 25% (w/v) TCA were added and the precipitate collected on glass fibre discs (GF/C, Whatman), washed with 10% (w/v) TCA, twice with ethanol, dried and counted as described above.

2.9c Immunoprecipitation.

This technique is a modified version of that described by Kessler (1975)(Cooper *et al.*, 1981; Kurkinen *et al.*, 1982, 1983c).

100-500µl of culture medium or cell lysate were mixed with 1ml of NET buffer, containing 1-5µl of antiserum. After incubation for 30 minutes at room temperature, 30µl of a washed 50% (v/v) suspension of

protein A agarose or protein A sepharose in NET buffer were added and incubation continued for a further 1.5-15 hours at 4°C with gentle mixing on a rotator.

Immune complexes, bound to protein A sepharose or agarose, were collected by brief (15 second) centrifugation at 12,000g , washed twice with 1ml of NET buffer, and once with 1ml of 0.01M Tris-HCl (pH 6.8). Immunoprecipitated proteins were solubilised by the addition of 30µl of twice concentrated Laemmli sample buffer (reducing or non-reducing as appropriate)(Laemmli, 1970) and heating to 100°C for 3 minutes with occasional vortexing. Sepharose or agarose beads were then pelleted by centrifugation for 15 seconds at 12,000g , and the supernatant, containing immunoprecipitated proteins, was either loaded directly onto a gel or stored at -20°C.

In order to minimise the level of background radioactivity, due to non-specific purification of labelled proteins, the following points were considered important:

I. Thorough washing of the bound complexes, involving complete resuspension of the beads at each washing stage, in order to release "trapped" material.

II. The use of minimal volumes of protein A sepharose or agarose.

In addition, vortexing was avoided during resuspension of pellets, since it may shear complexes from the beads and reduce yields.

2.9d *In vitro* translation (Kurkinen *et al.*, 1982, 1983b).

Nuclease-treated rabbit reticulocyte lysates (Pelham and Jackson, 1976) were provided by J.R.Jenkins and R.Jackson. For *in vitro* protein synthesis, 25µl of reticulocyte lysate was incubated at 30°C for 3 hours in a final volume of 50µl containing 10-30µCi of ^{35}S-methionine, 50µM all amino acids except methionine, 200µg/ml calf

liver tRNA, 200μg/ml creatine kinase, 10mM creatine phosphate, 5mM 2-aminopurine, 100mM KCl, 0.5mM $MgCl_2$, 1000units/ml RNasin and 2-4μg total cellular RNA. Under these conditions, protein synthesis, as measured by the increase in TCA-insoluble, ^{35}S-methionine-labelled material, is linear up to about 4μg/ml poly(A)+ RNA which produces 60% of the protein synthesis observed at saturating levels of RNA. Translated proteins were either diluted with Laemmli sample buffer and analysed immediately by SDS-PAGE, or diluted into 1ml NET buffer for immunoprecipitation.

2.9e SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed essentially as described by Laemmli (1970). Resolving gels were either linear gradients of acrylamide concentration, or of uniform concentration. Gradients were formed by the progressive mixing of 11ml of each of two acrylamide gel mixes, using a gradient former attached to a peristaltic pump (see Table 2.1). Polymerisation of the gels was initiated by the addition of ammonium persulphate immediately before pouring. To produce a smooth interface the gel was overlaid with 1-2ml of distilled water. Uniform polymerisation was obtained after 20-30 minutes at room temperature.

The water overlay was removed, a spacer inserted and a "stacking" or "spacer" gel was poured over the resolving gel. The stacking gel polymerisation was complete within 10 minutes. Electrophoresis of protein samples was carried out at 25-30 volts overnight or at 120-150 volts for 3-5 hours in SDS-PAGE running buffer.

Radioactively labelled proteins were detected in SDS-polyacrylamide gels by fluorography (Bonner and Laskey, 1974) using the following protocol:

I. Fix for 1 hour or longer in 10% (v/v) glacial acetic acid,

Table 2.1 **SDS-PAGE gel mixtures**

	Gradient Gels		**Uniform Gels**			**Stacking Gel**
	6%	**12%**	**8%**	**10%**	**15%**	**3%**
Solution						
ml 3M Tris-HCl (pH 8.9)	1.55	1.55	3.1	3.1	3.1	-
ml 1M Tris-HCl (pH 6.8)	-	-	-	-	-	1.25
ml acrylamide (see solutions)	2.5	5	6.65	8.3	12.5	1.25
µl 10% (w/v) SDS	125	125	250	250	250	100
ml H_2O	2.1	4.54	14.8	13.2	6.6	7.5
ml glycerol	1.2	1.2	-	-	2.4	-
µl TEMED	6.25	6.25	12.5	12.5	12.5	10
µl 10% (w/v) APS	80	80	150	150	150	100
TOTAL VOLUME (ml)	12.5	12.5	25	25	25	10

30% (v/v) methanol.

II. Pour off fix and impregnate with 3 gel volumes of EN^{3}HANCE for 1 hour.

III. Precipitate with several changes of tap water over a one hour period.

Gels were dried onto Whatman 3MM filter paper, under vacuum, on a heated base (Bio-Rad gel dryer), and exposed to Kodak SB-5 or XAR-5 X-ray film in a light proof cassette at -70^{o}C. Films were developed and fixed using an Ilford automatic processor. To prevent cracking of the gel, 12 and 18% polyacrylamide gels were incubated for 30min in 1% (v/v) glycerol before drying.

2.9f Staining protein gels.

Gels were stained in Coomassie blue stain at room temperature, for at least two hours or until bands could be resolved. They were then destained in 30% (v/v) methanol, 10% (v/v) glacial acetic acid until optimum resolution was obtained.

2.9g Calibration of gels for molecular weight determination.

^{14}C-labelled protein markers were included in all gels to facilitate the determination of relative molecular weights. These were:

Myosin heavy chain	Mr 212,000
Phosphorylase B	Mr 100,000 and 92,000
Bovine serum albumin	Mr 69,000
Ovalbumin	Mr 46,000
Carbonic anhydrase	Mr 30,000
Lysozyme	Mr 14,300

The mobility of most proteins in SDS polyacrylamide gels is

proportional to the logarithm of their molecular weight (Weber and Osborn, 1969). Therefore the relative molecular weight of proteins between Mr 212,000 and 14,300 could be estimated by a semi-logarithmic plot of mobility versus molecular weight for the markers.

2.10 Molecular cloning and DNA techniques

2.10a Bacterial strains and storage.

The following strains of *E.coli* were used in these studies:

DH1 and DH5 (Hanahan, 1983 and unpublished) and HB101 (Boyer and Roulland-Dussiox, 1969) were used for routine cloning procedures and were grown on L-agar plates.

JM101 (Messing, 1979) was used in the propagation of M13 bacteriophage recombinants and was maintained on minimal media plates supplemented with 0.2% (w/v) glucose.

DIH101 (D.Ish-Horowicz, unpublished) is a derivative of HB101 containing an F plasmid which is maintained by selection with kanamycin. It was used as a host for pEMBL vectors and recombinants, and was grown on psi-agar plates containing 50µg/ml kanamycin.

Bacterial cultures were stored at -70°C following the addition of Hogness freezing medium (6.3% (w/v) dipotassium hydrogen phosphate, 0.45% (w/v) sodium acetate, 0.09% (w/v) magnesium sulphate, 0.9% (w/v) ammonium sulphate, 1.8% (w/v) potassium dihydrogen phosphate and 44% (v/v) glycerol) to 10% (v/v) and snap-freezing in liquid nitrogen.

Bacterial colonies grown on nitrocellulose or nylon membranes (e.g. libraries) were replicated onto fresh membranes and grown on L-agar plates containing 10% (v/v) glycerol and appropriate antibiotics until colonies were just visible. The filter was then removed from the plate and a second filter, pre-wetted on a separate, plate was pressed onto the colonies. The two filters were placed at -70°C.

2.10b Preparation of competent bacteria

I. Competent DH1, DH5 and HB101 (adapted from Hanahan, 1983 and M.Scott and V.Simanis unpublished).

Bacteria were freshly streaked onto an L-agar plate and incubated at 37°C until the colonies were about 2.5mm in diameter. 10 colonies were picked from the plate and dispersed in 1ml of SOB medium by gentle vortexing. This was inoculated into 100mls of SOB medium, prewarmed to 37°C, and the bacteria shaken at 275rpm in a 37°C incubator until the cell density was 4-7 x 10^7 cells/ml (usually 2-2.5 hours). Cell density was measured using a bacterial counter, since this had proved to be more reliable than measuring the OD_{550}. The cells were then chilled on ice for 5 minutes, and then pelleted at 6,000g at 4°C for 5 minutes. The bacteria were resuspended in 40mls of ice-cold TfbI either by gentle pipetting or gentle vortexing, and placed on ice for 5 minutes. The bacteria were then re-pelleted, as described previously, resuspended in 4mls of ice-cold TfbII and placed on ice for 15 minutes. Aliquots (usually 200µl) were then placed in 2.5ml wide-bottomed, screw-cap tubes (NUNC) on ice, snap-frozen in liquid nitrogen and stored at -70°C.

II. Competent DIH101 (D.Ish-Horowicz unpublished).

Bacteria were freshly streaked onto a psi-agar plate containing 50µg/ml kanamycin and grown overnight at 37°C. A single colony was then picked into 0.5ml of psi-broth containing 25µg/ml kanamycin and the culture was incubated at 37°C on a rotating wheel until it became cloudy. A further 2.5ml of pre-warmed psi-broth containing kanamycin was added to this and the culture grown in a shaking 37°C incubator until it became cloudy. Another 2.5ml of medium was then added and the culture grown until it had an OD_{550} of 0.15. The culture was then

inoculated into 100ml of pre-warmed (37°C) psi-broth containing 25µg/ml kanamycin and incubated, with vigorous shaking, until it had an OD_{550} of 0.22. The culture was chilled on ice for 5 minutes, and the bacteria were then pelleted at 1,500g at 4°C for 5 minutes. The supernatant was removed and the bacteria re-suspended in 40ml of TfbI. Following incubation on ice for 2 hours, the bacteria were re-pelleted and re-suspended in 4ml of TfbII, incubated on ice for 15 minutes, and were stored as previously described (section 2.10b I.).

III. Competent JM101.

1ml of an overnight culture of JM101 cells, grown in 2x TY broth, was inoculated into 200ml of 2x TY (pre-warmed to 37°C). The culture was grown, with shaking, at 37°C until the OD_{600} had reached 0.1. The bacteria were then cooled on ice, and pelleted at 4,000g for 5 minutes at 4°C. The pellet was gently resuspended in 100ml of fresh, ice-cold 0.1M calcium chloride and left on ice for 20 minutes. The bacteria were then pelleted again and gently resuspended in 5ml of 0.1M calcium chloride. The competent bacteria were used immediately, stored overnight at 4°C, or made 10% (v/v) with sterile glycerol and snap-frozen in aliquots for long term storage at -70°C.

2.10c Transformation of bacteria.

I. DH1 and HB101 (modified from M.Scott and V.Simanis, unpublished).

An aliquot of competent cells was incubated at room temperature until just thawed and then placed on ice for 10 minutes. DNA (up to 100ng/200µl competent cells and in a volume no larger than 20µl) was added and the bacteria were incubated on ice for 30 minutes. They were then heat-shocked for 2 minutes (3 minutes if stored in

Eppendorf tubes) at 37°C and returned to ice for 1 minute. 800µl of L-broth (room temperature) was then added and the cells incubated at 37°C for 1 hour with gentle shaking. The bacteria were then plated on L-agar plates containing the appropriate antibiotic and grown overnight.

II. Competent DIH101.

The competent DIH101 were thawed on ice and up to 5µl of a ligation mixture was added per 25µl of competent bacteria. The tube was placed on ice for 15 minutes and then heat-shocked at 42°C for 90 seconds before being returned to the ice bath for a further 2 minutes. 4 volumes of psi-broth were added to the bacteria and the culture was gently shaken at 37°C for 50-60 minutes. The bacteria were then plated on L-plates containing 50µg/ml kanamycin and an appropriate second antibiotic.

III. Competent JM101.

0.1ml aliquots of competent cells were mixed with 5-10µl of an M13 ligation mixture and incubated on ice for 15 minutes. The cells were then heat-shocked at 42°C for 2 minutes and replaced on ice for 3 minutes. The bacteria were added to 3ml of molten L-top agar containing 1.5mM IPTG and 0.025% (w/v) X-GAL, mixed and immediately poured onto a dry 2x TY plate. The plate was allowed to set before being incubated in an inverted position at 37°C overnight.

2.10d Preparation of plasmid and cosmid DNA

I. Miniprep (protocol supplied by D.Banville).

A single bacterial colony was picked and grown overnight in 2ml L-broth containing the appropriate antibiotic. 1.5ml of the overnight culture was centrifuged at 12,000g for 5 minutes and the supernatant discarded. The bacterial pellet was resuspended in 100µl of STET, followed by the addition of 10µl of freshly-prepared lysozyme (10mg/ml) in STET. The tube was placed in a boiling water bath for 45 seconds and then centrifuged for 10 minutes at 12,000g . The pelleted material was removed with a sterile toothpick and 100µl of isopropanol was added to the supernatant. Following incubation at -20°C for 10 minutes, the precipitated DNA was collected by centrifugation at 12,000g for 5 minutes. The supernatant was poured off and the pellet was carefully washed with 1ml of diethyl ether. The diethyl ether was poured off and any that remained was allowed to evapourate for 5 minutes at room temperature. The DNA was resuspended in 50µl of TE (pH 8.0) and stored at -20°C.

To remove RNA from the preparation, following digestion with a restriction endonuclease and prior to gel electrophoresis, 1µl of boiled RNase A (10mg/ml) was added to the digest. After incubation at room temperature for 10 minutes, the RNase was destroyed by the addition of sample buffer containing 1mg/ml proteinase K and incubation for a further 10 minutes. The sample was then loaded onto the gel.

II. Midiprep (Treisman, 1985).

A 100ml overnight culture of bacteria was centrifuged at 7,500g for 5 minutes, the supernatant was removed and the pellet was drained dry for 2 minutes. The bacteria were resuspended in 5ml of 50mM

glucose, 25mM Tris.HCl (pH 8.0), 10mM EDTA and then 10ml of fresh 0.2M NaOH, 1% SDS was added to the suspension. The solutions were mixed by swirling and placed on ice for 5 minutes. 5 ml of ice-cold 5M potassium acetate (pH 8.0) were added and the solutions were mixed well before being returned to the ice bath for a further 5 minutes. The lysate was centifuged at 9,500g for 5 minutes at 4°C and the supernatant poured through gauze into a fresh tube. 12ml of isopropanol was added to the supernatant and the solutions were mixed and centrifuged at 9,500g for 5 minutes. The supernatant was removed and the DNA was resuspended in 2ml of TE (pH 8.0). To precipitate most of the RNA from the preparation, 2ml of ice-cold 5M lithium chloride were added to the DNA and the tube was centrifuged at 15,000g for 5 minutes at 4°C. The supernatant was transferred to another tube and 8ml of ethanol were added to it. Following incubation at -70°C for 30 minutes, the precipitated nucleic acids were collected by centrifugation at 15,000g for 10 minutes. They were resuspended in 0.5ml TE (pH 8.0) containing 40µg/ml boiled RNase A and incubated at 37°C for 15 minutes to digest any remaining RNA molecules. DNA was then precipitated by the addition of 0.5ml of 13% (w/v) PEG 6000, 1.6M NaCl and incubation at 4°C for between 1 and 15 hours. The DNA was pelleted by centrifugation at 12,000g for 5 minutes and resuspended in 200µl of TE (pH 8.0). It was extracted twice with chloroform, then once with phenol, before being precipitated by the addition of sodium acetate to 0.3M and 2.2 volumes of ethanol. The precipitation was performed on dry ice for 15 minutes, the precipitate was collected at 12,000g for 15 minutes and resuspended in 100µl of TE (pH 8.0).

III. Maxiprep (D.Ish-Horowicz pers.comm.)

A 500ml bacterial culture was grown overnight in L-broth containing an appropriate antibiotic and harvested at 7,500g for 5 minutes. The supernatant was removed and the pellet was drained dry before being resuspended in 40ml of 50mM glucose, 25mM Tris.HCl (pH 8.0). The bacteria were lysed by the addition of 80ml of 0.2M NaOH, 1%(w/v) SDS and placed on ice for 5 minutes. 40ml of cold 5M potassium acetate (pH 4.8) were added and incubation on ice was continued for a further 5 minutes. Following centrifugation at 9,500g for 5 minutes at 4°C, the supernatant was collected and mixed with 0.6 volumes of isopropanol. The precipitate was collected by centrifugation at 9,500g for 5 minutes and resuspended in 6ml TE (pH 7) and the pH of the solution adjusted to pH 7.0 with 2M Tris base. The solution was then adjusted to 10.1ml with TE (pH 7) and 11.15g of caesium chloride dissolved in it, followed by the addition of 0.5ml of ethidium bromide (10mg/ml). The plasmid DNA was then banded at 100,000g for 18 hours. After extraction of the banded DNA from the tube, ethidium bromide was removed by passing the DNA solution through a Dowex AG50 column in a pasteur pipette. The column was washed with 1 volume of TE (pH 7.5) and the volume of the pooled eluent adjusted to 3 times the original volume of the banded DNA. 2 volumes of ethanol were added and the DNA precipitated at -20°C. The DNA was collected by centifugation at 15,000g for 15 minutes at 4°C and resuspended in TE. The DNA was then reprecipitated, washed with 70% (v/v) ethanol, dried and dissolved in TE (pH 8.0).

2.10e Preparation of single-stranded DNA.

I. M13 single-stranded DNA (modified from Messing, 1983)

100ml of 2x TY broth were inoculated with 1ml of an overnight culture of E.coli JM101. 1.5ml were dispensed into a sterile 10ml culture tube. This was inoculated with a single M13 plaque and shaken at 37°C for 5 hours. The culture was then transferred to a sterile Eppendorf tube and the bacteria were pelleted at 12,000g for 5 minutes. 1.2ml of the supernatant were transferred to a fresh tube, and the bacterial pellet was either discarded or used to prepare RF DNA by the miniprep procedure. The supernatant was re-centrifuged and 1ml transferred to a fresh tube. 200µl of 20% (w/v) PEG 6000, 2.5M NaCl were added and the tube was shaken and allowed to stand for 15 minutes. The viral particles were pelleted by centrifugation at 12,000g for 15 minutes and the supernatant was discarded. The viral pellet was resuspended in 100µl of TE (pH 8.0) and mixed by vortexing with 50µl of redistilled phenol (pre-saturated with TE, pH 8.0). The tube was allowed to stand for 15 minutes at ambient temperature, then vortexed again and centrifuged at 12,000g for 3 minutes. The aqueous phase was transferred to a fresh Eppendorf tube and the single-stranded DNA precipitated by the addition of sodium acetate to 0.3M and 250µl of ethanol. After standing the tube on dry ice for 15 minutes, the DNA was pelleted by centrifugation at 12,000g for 10 minutes. The pellet was washed with 1ml of cold (-20°C) ethanol and drained dry. The DNA was then re-dissolved in 50µl of TE (pH 8.0) and stored at -20°C.

II. pEMBL single-stranded DNA (A.Flavell, modified from Dente et al., 1983).

A single bacterial colony was picked from an L-agar plate containing 50µg/ml ampicillin and 50µg/ml kanamycin and inoculated into 5ml of L-broth containing 50µg/ml ampicillin and 20µg/ml kanamycin. The culture was grown overnight at 37°C. 1.4ml of 2x TY medium, supplemented with 0.2% (w/v) glucose and 50µg/ml ampicillin, were inoculated with 30µl of the overnight culture and incubated at 37°C for 2 hours with shaking. 6µl of f1 phage (10^{12} plaque-forming units per ml in TE, pH 8.0) were then added and incubation was continued for a further 5 hours. The culture was then transferred to an Eppendorf tube and centrifuged at 12,000g for 4 minutes. 1ml of the supernatant was added to 250µl of 20% (w/v) PEG 6000, 2.5M NaCl, shaken and allowed to stand at ambient temperature for 15 minutes. The viral particles were pelleted at 12,000g for 5 minutes, redissolved in 100µl of TE (pH 8.0) and extracted with 50µl of phenol. 95µl of the aqueous phase were added to 10µl of 3M sodium acetate and the DNA was precipitated following the addition of 2.5 volumes of ethanol. The DNA was collected by centrifugation at 12,000g for 3 minutes and the supernatant was removed. The pellet was washed with 1ml of ethanol, air dried and resuspended in 30µl of TE (pH 8.0).

2.1Of Preparation of high molecular weight genomic DNA.

I. Preparation from small amounts of tissue (Chapman et al., 1984).

Dissected tissues were washed in PBSA, spun down at 3,750g for 30 seconds and the PBSA was removed. 1ml of pronase buffer was added to the pelleted tissue and incubation carried out at 37°C for 30 minutes. DTT was then added to 1mM, SDS to 0.2% (w/v) and preboiled RNase A to

150µg/ml and the incubation was continued for a futher 90 minutes. SDS was then added to 0.8% (w/v) and proteinase K (pre-digested at 37°C for 15 minutes) was added to 150µg/ml. The mixture was then incubated at 37°C for 4 hours to overnight. The mixture was extracted twice with redistilled phenol, three times with chloroform:isoamyl alcohol (24:1) and, finally, once with ether. Traces of ether were boiled off for 15 minutes at 45°C. Sodium acetate was added to 0.3M and the DNA was precipitated with 2.5 volumes of ethanol, followed by incubation at -20°C overnight. The DNA was pelleted at 12,000g and the pellet washed twice in 70% (v/v) ethanol. The DNA was then lyophilised for 3-5 minutes, resuspended in 100µl of sterile distilled water and stored at 4°C.

II. Preparation from large tissues (D.Hanahan modified from Blin and Stafford, 1976)

About 0.7cm^3 of tissue was added to 700µl of 50mM Tris.HCl (pH 8.0), 100mM EDTA, 100mM NaCl, 1% (w/v) SDS and finely minced with a pair of scissors. 35µl of 10mg/ml proteinase K were added and the tube was shaken and incubated at 55°C overnight. Any undigested tissue was pelleted at 12,000g for 5 minutes. The supernatant was removed to a new tube with a wide-mouthed pipet and 25µl of 10mg/ml boiled RNase A were added to it. Following incubation at 37°C for 2 hours, the solution was extracted first with an equal volume of phenol and then with chloroform. The aqueous phase was transferred to a fresh tube and the DNA was precipitated by the addition of 2 volumes of isopropanol. The DNA was spooled onto a sealed capillary, dipped into 70% (v/v) ethanol and then into 100% ethanol, dried and re-dissolved in 500µl TE (pH 7.6).

2.10g Restriction enzyme analysis.

Restriction enzyme digestions were performed in low, medium or high salt buffers containing 100µg/ml BSA (Maniatis et al., 1982), with the exception of SmaI digestions, which were performed in 20mM KCl, 6mM $MgCl_2$, 6mM Tris.HCl (pH 8.0) and 100µg/ml BSA. Digests contained a final concentration of DNA no greater than 1µg/10µl and no more than 0.1 volumes of enzyme. Typical digests of cloned DNA were performed at 37°C (except TaqI; 70°C), using a 2-3 fold excess of enzyme. Digests of genomic DNA were incubated overnight with a 2-5 fold excess of enzyme. Digestion with more than one enzyme was performed simultaneously if the enzymes required the same buffer. If they required different buffers, the digest was extracted with phenol:chloroform and spun through a G-50 column equilibrated in the second buffer (see section 2.10n). The second enzyme and BSA were then added.

Digests were stopped by the addition of 0.1 volumes of 0.5M EDTA or by the addition of 0.1 volumes of a sample buffer for electrophoresis.

2.10h Gel electrophoresis of DNA.

I. Agarose gel electrophoresis (Maniatis et al., 1982).

Agarose was dissolved in 1x TBE by boiling, the solution was allowed to cool to about 50°C and ethidium bromide was added to 0.5µg/ml. Gels were formed in 20cm x 12cm plastic trays or in a BRL "minigel" apparatus, as required. Restriction enzyme digest products or other DNA samples were prepared containing 10% (v/v) sample buffer (50% (v/v) glycerol, 1x TBE, 0.1M EDTA, 0.2% (w/v) SDS, bromophenol blue and xylene cyanol). The samples were loaded and electrophoresis was performed in 1x TBE containing 0.5µg/ml ethidium bromide, either

at 1.5V/cm overnight or at up to 6V/cm until the desired separation had been achieved. the progress of electrophoresis was monitored by the migration of the bromophenol blue and xylene cyanol dyes. DNA in the gel was visualised by illumination with long wave ultra-violet light and photographed with a Polaroid camera. Estimation of DNA fragment sizes was obtained by comparison with restriction fragments of known size, e.g. BRL 1,2,3Kb ladder, HindIII or EcoRI fragments of bacteriophage lambda DNA or HinfI fragments of pAT153.

II. Polyacrylamide gel electrophoresis (Protocol supplied by D.Banville, slightly modified from Maniatis et al., 1975, 1982).

75ml gels were prepared containing 0.5x TBE, 0.065% (w/v) ammonium persulphate and usually 3.5% acrylamide (stock solution: 29% (w/v) acrylamide, 1% (w/v) bisacrylamide). The mixture was degassed under vacuum for several minutes, 25µl of TEMED were then added and the gel poured between two siliconised plates. The gel usually set within an hour at room temperature.

Samples, for example restriction digest fragments, were precipitated with ethanol, washed with 70% (v/v) ethanol, dried briefly and resuspended in 25µl of sample buffer (0.1x TBE, 5% (v/v) glycerol, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol). The samples were then loaded onto the gel and electrophoresis performed at 10V/cm in 0.5x TBE for about 2 hours. To visualise DNA, following electrophoresis, the gel was placed in about 200ml of electrophoresis buffer containing 1µg/ml ethidium bromide for 1 hour and then washed twice in tap water. The DNA was then visualised on a long wave ultra-violet transilluminator.

III. Denatured DNA in native polyacrylamide gels (Williams and Mason, 1985).

The KpnI-PstI DNA primer used to prepare the parietal endoderm cDNA library was strand separated on a native polyacrylamide gel. The purified fragment was ethanol precipitated and twice washed with 70% (v/v) ethanol. It was then resuspended in 10µl of DMSO sample buffer. A 40cm long, 1mm thick gel containing 8% (w/v) polyacrylamide, 0.24% (w/v) bisacrylamide and 0.5x TBE was prepared and pre-electrophoresed at 300V for 1 hour. The sample was then denatured at 90°C for 3 minutes, chilled briefly, and loaded onto the gel. Electrophoresis was performed at 300V until it was calculated, from the migration of the xylene cyanol dye, that the native fragment would have almost reached the bottom of the gel (single stranded DNA migrates with a lower mobility than double stranded DNA under these conditions). The gel was then exposed directly to X-ray film and the primer strand was excised from the gel and purified by electroelution.

IV. Denaturing urea/polyacrylamide gels.

Denaturing urea/polyacrylamide gels were generally used to separate the products of sequencing reactions. The products of chemical cleavage sequencing reactions were separated on 8% (w/v) ~ 20% (w/v) gels, while the products of chain termination reactions were analysed on 6% (w/v) gels. The 6% (w/v) gels were of two types; 40cm uniform gels and 60cm 0.5x-2.5x TBE buffer gradient gels (Biggin et al., 1983). The composition of the different gels is given in Table 2.2. Gels were prepared between glass plates which were separated by 0.3mm thick plasticard spacers and sealed with tape. One of the plates was siliconised with repelcote. Buffer gradient gels were prepared from 18ml of bottom gel mix and about 70ml of top gel mix. Buffer gradient

Table 2.2 **The compostition of urea/polyacrylamide denaturing gels**

	Uniform gels			**Gradient Gels**	
	6%	**8%**	**20%**	**Bottom gel**	**Top gel**
40% acrylamide	15ml	20ml	50ml	3.75ml	20ml
Urea	40g	40g	40g	11.5g	64.5g
10x TBE	5ml	5ml	5ml	6.25ml	7.5ml
H_2O	50ml	45ml	15ml	4.25ml	73.5ml
0.4% (w/v) bromophenol blue	-	-	-	0.5ml	-
10% APS	240µl	240µl	240µl	125µl	125µl
TEMED	120µl	120µl	120µl	25µl	150µl

gels were run at a constant power of 60W for about three hours, whereas uniform gels were run at between 1.7 and 2.5KV, the voltage being adjusted during the process to prevent overheating.

Following electrophoresis, the siliconised plate was removed and the gel was either covered with "cling film" and exposed to X-ray film or fixed. Gels were fixed by immersion in 10% (v/v) methanol, 10% (v/v) acetic acid for 20 minutes. Following fixation, the gel was drained, transferred to a sheet of 3MM paper and dried at 80°C under vacuum. The dried gel was then exposed to X-ray film.

2.10i Isolation and purification of restriction fragments.

I. Glass powder technique from LGT agarose (R.Krumlauf pers.comm.).

DNA fragments were separated in either HGT or LGT agarose gels prepared in 1x TAE buffer not TBE buffer. The gel was stained and a gel slice, containing the fragment of interest, was excised and transferred to an Eppendorf tube. The volume of the gel slice was estimated by weighing and 2 volumes of sodium iodide solution (90.8% (w/v) sodium iodide, 0.5% (w/v) sodium sulphite; stored foil-wrapped at 4°C) were added to it. The tube was incubated at 37°C until the gel had dissolved (about 15 minutes) and then 1µl of a 50% (v/v) slurry of glass powder was added for every 2µg of fragment. The mixture was vortexed and chilled on ice for 1 hour. The glass powder was pelleted (12,000g for 15 seconds) and the supernatant discarded. The powder was resuspended in half of the original gel volume of sodium iodide solution, the glass was spun down again, the supernatant was removed and this wash was repeated once more. The glass powder was then resuspended in half of the gel volume of ethanol wash solution (50% (v/v) ethanol, 0.1M NaCl, 10mM Tris.HCl

(pH 7.5), 1mM EDTA; stored at -20°C), the glass was repelleted and this wash was repeated once more. The last of the ethanol wash was removed from the powder with a capillary and, without allowing the glass to dry, a convenient volume (10-100µl) of elution buffer (TE pH 8.0) was added to the pellet. The DNA was eluted into this buffer at 37°C for 15 minutes, the glass was then spun down and the DNA solution transferred to a fresh tube.

II. Melting technique from LGT agarose (modified from Maniatis et al., 1982)

The desired DNA fragment was located within an LGT agarose gel and excised. The gel slice was transferred to an Eppendorf tube and melted at 65°C for 5 minutes. 1 gel volume of TE (pH 8.0) and an equal volume of phenol were added to the tube and votexed. The aqueous phase was removed to a fresh tube and the organic phase was extracted with 50µl of TE. The aqueous phases were pooled and extracted with an equal volume of phenol:chloroform, and then with chloroform alone. The aqueous phase was added to an equal volume of 5M ammonium acetate and the DNA precipitated by the addition of either 2.2 volumes of ethanol or 1 volume of isopropanol. Following incubation in dry ice for 20 minutes, the DNA fragment was recovered by centrifugation at 12,000g for 15 minutes. The fragment was re-dissolved in 100µl of 2.5M ammonium acetate and precipitated by the addition of 250µl ethanol followed by incubation in dry ice and centrifugation. The DNA pellet was washed in 70% (v/v) ethanol and resuspended in TE (pH 8.0) or ddH_2O.

III. Electroelution from polyacryamide or HGT agarose gels (adapted from Maniatis et al., 1982).

The fragment of interest was excised in a minimal volume of gel and placed inside a short length of dialysis tubing which was clipped at one end. A minimum volume of 0.5x TBE was added (usually 200-400µl, sufficient to cover the gel slice) and all air bubbles were removed. The tubing was clipped at the other end and electroelution was performed in 0.5x TBE at 150-200V for 2 hours. The current was reversed for 45 seconds and the buffer was removed from the bag to an Eppendorf tube. The inside of the bag and the gel slice were thoroughly rinsed with 200µl of 0.5x TBE, which was then pooled with the buffer that was originally recovered from the bag. The fragment was precipitated by the addition of 0.1 volumes of 3M sodium acetate and 2.2 volumes of ethanol, followed by cooling on dry ice for 15 minutes. The fragment was pelleted at 12,000g for 15 minutes, washed with 70% (v/v) ethanol, and resuspended in TE (pH 8.0).

2.10j Southern blotting and hybridisation (modified from Southern, 1975)

DNA was transferred to nitrocellulose (Sartorius or Schleicher and Schull) or to nylon (Gene Screen Plus, NEN) membranes as follows. Agarose gels were soaked in 0.5M NaOH, 1.5M NaCl for 1 hour to denature the DNA, and then neutralised by soaking in 1M Tris.HCl pH 8.0, 1.5M NaCl for 1 hour. Gels containing restriction digests of genomic DNA were treated with 0.25M HCl for 30 minutes prior to denaturation to partially depurinate the DNA and facilitate efficient transfer (Wahl et al., 1979). The neutralised gel was placed on a pre-soaked sheet of Whatman 3MM paper that was in contact with a reservoir of 20x SSC, and the membrane, pre-wetted with 10x SSC,

placed on top of the gel. Two sheets of 3MM paper, soaked in 10x SSC were placed over the membrane, followed by a weighted stack of tissues to act as a wick. Transfer of genomic DNA was allowed to proceed overnight. Multiple filters could be obtained from single gels containing fragments of cloned DNA by allowing transfer to proceed for 30 minutes, and replacing the first membrane with a fresh one. Transfer to the second filter was allowed to occur for 1-1.5 hours before this was replaced by a third filter. After transfer, the membranes were washed in 6x SSC (not nylon membranes), air dried and baked at 80^{o}C for 2 hours. They were then ready for hybridisation with labelled probes as described below.

Two hybridisation procedures were routinely used for Southern blots, dot blots (section 2.10) and Northern blots (section 2.11d). A high strigency protocol was used to detect homologous hybrids, while a low stringency procedure was used to detect non-identical, related hybrids or in inter-species hybridisation studies.

I. Hybridisation at high stringency (slightly modified from Wahl et al., 1979).

The baked filter was wet in 6x SSC and then pre-hybridised for 12-24 hours in 20-50ml of 50% (v/v) formamide, 5x SSC, 5x Denhardt's solution, 50mM sodium phosphate buffer (pH 7.0), 250μg/ml denatured salmon sperm DNA and 0.1% (w/v) SDS at 42^{o}C. The pre-hybridisation solution was then removed and the filter was hybridised to a ^{32}P-labelled probe in 50% (v/v) formamide, 5x SSC, 1x Denhardt's solution, 20mM sodium phosphate buffer (pH 7.0), 100μg/ml denatured salmon sperm DNA, 0.1% (w/v) SDS and 10% (w/v) dextran sulphate at 42^{o}C. Probes were labelled to a specific activity of about 1-3 x 10^{8}cpm/μg using the nick translation or oligonucleotide priming methods (sections 2.10n) and were used at a concentration of about

10ng/ml of hybridisation solution. Hybridisation was performed for between 12 and 48 hours depending upon the complexity of the probe or the target DNAs. Filters were then washed twice in 2x SSC, 0.1% (w/v) SDS at room temperature and then at 68°C for 30 minutes. They were then washed twice in 0.1x SSC, 0.1% (w/v) SDS at 68°C for 15 minutes. The filters were then exposed to pre-flashed X-ray film (Kodak XAR-5) with an intensifying screen.

II. Hybridisation at low stringency (McGinnis et al., 1984).

Prehybridisation and hybridisation were performed, for the same periods of time as described above, in 43% (v/v) formamide, 5x SSC, 5x Denhardt's solution, 50mM sodium phosphate buffer (pH 7.0), 0.1% (w/v) SDS and 250µg/ml yeast tRNA at 37°C and at a probe concentration of 10ng/ml. Following hybridisation, the filters were washed 6 times in 2x SSC, 0.1% (w/v) SDS for 5 minutes at room temperature and then twice in the same buffer at 50°C for 15 minutes. Filters were then exposed to X-ray film as described above.

To remove a probe from a filter, so that it could be hybridised with a different probe, the filter was washed in 20mM NaOH for 20 minutes. The filter was then washed in several changes of 1M Tris.HCl (pH 8.0), 1.5M NaCl and then in 6x SSC before proceding to a prehybridisation step.

2.10k DNA dot blots.

DNA (usually 0.01-1µg) in 0.3M NaOH was boiled for 10 minutes and chilled on ice. An equal volume of 4M ammonium acetate was added and the DNA was applied, over 5 minutes, to nitrocellulose (pre-wetted with 2x SSC) using a dot blot or slot blot manifold (Scleicher and Schull). The wells were then washed with 500µl 1M ammonium acetate.

The filter was washed in 5x SSC, allowed to air dry and then baked for 2 hours at 80°C. Prehybridisation, hybridisation and washing were performed as described in section 2.10j. Removal of a previous probe to allow the filter to be re-used was also performed as described in section 2.10j.

2.10l Subcloning and ligations.

Vector DNA was prepared by restriction enzyme digestion and phosphatase treatment (sections 2.10g and 2.10n) was purified and isolated from an agarose gel (sections 2.10h and 2.10i). The isolated vector DNA was resuspended in TE (pH 8.0) at a concentration of 20ng/μl.

Ligations generally contained about 20-40ng of vector and between 1 and 3 molar equivalents of insert DNA. Vector and insert DNA were ligated in a volume of 10-20μl containing 1x ligase buffer, 100μg/ml BSA and either 1-10u of T4 DNA ligase for "blunt end" reactions and 0.1-1u for "sticky end" ligations. The reactions were incubated overnight at 15°C and the products were then used to transform competent bacteria (sections 2.10b and 2.10c).

2.10m Sequencing DNA.

Two different sequencing procedures were utilised in these studies; the chemical cleavage method of Maxam and Gilbert (1977, 1980) and the dideoxy chain termination procedure of Sanger et al. (1977).

I. Chemical cleavage.

In the chemical cleavage method of sequencing DNA, single- or double-stranded DNA is uniquely end-labelled and partially cleaved at

each of the four bases in four separate reactions. The cleavage products are then separated on a denaturing polyacrylamide gel and visualised by autoradiography.

To obtain sequence from a particular DNA fragment it was uniquely end-labelled close to the region of interest. This was accomplished by cleaving the DNA with a restriction enzyme, labelling the DNA at the cutting site, and then cleaving with a second restriction endonuclease. In general, 1-5μg of cloned DNA was digested with the appropriate first restriction endonuclease. The reaction mixture was then extracted with phenol, ethanol-precipitated and the DNA strand of interest was labelled by a method appropriate to the nature of the end generated by the restriction enzyme (i.e. protruding, blunt or recessed). Several labelling procedures were used in these studies; these are summarised in Table 2.3 and described in detail in section 2.10n. The labelled DNA was then spun through a G-50 column equilibrated in TE (pH 8.0), extracted with phenol and precipitated with ethanol. The DNA was then cleaved with a second restriction endonuclease and the products were separated on an agarose gel. The fragment of interest was located in the gel either by staining with ethidium bromide or by autoradiography, and was purified by the glass powder technique (section 2.10i). The DNA was ethanol-precipitated twice from 2.5M ammonium acetate, washed with 70% (w/v) ethanol, lyophilised briefly, and resuspended in 32μl of ddH_2O.

The chemical cleavage procedures which are central to this technique involve three consecutive steps - the modification of a particular base or bases, followed by the removal of that base from its sugar and then strand scission at that sugar. The reactions used in these studies were as follows:

Table 2.3 **Enzyme and radioactive nucleotide combinations which were used to label DNA for sequencing by chemical cleavage**

End to be labelled	Enzyme	Radioactive nucleotide	
5' extended or blunt	T4 polynucleotide kinase[1]	γ^{32}P-dATP	>4,000Ci/mM
5' recessed	T4 polynucleotide kinase[1]	γ^{32}P-dATP	>4,000Ci/mM
3' extended or blunt	Terminal transferase	α^{32}P-ddATP	800Ci/mM
	T4 DNA polymerase	α^{32}P-dNTP	800Ci/mM[2]
3' recessed	Klenow fragment	α^{32}P-dNTP	800Ci/mM[2]

1. Labelling conditions were different for the two classes of ends.
2. The precise radioactive nucleotide used was dependent on the sequence of the site to be labelled.

G reaction.

5µl of the solution of labelled DNA were added to 200µl of DMS buffer and the mixture chilled to 0°C. 1µl of DMS was then added and the mixture was vortexed briefly and placed on ice for 45 seconds. The reaction was then terminated by the addition of 50µl of DMS stop buffer and placed on ice.

G+A reaction.

10µl of the labelled fragment was cooled to 0°C and 25µl of 88% (v/v) formic acid at 4°C were added to it. The reaction was allowed to proceed for 10 minutes at room temperature before it was terminated by the addition of 200µl of hydrazine stop buffer and placed on ice.

C+T reaction.

10µl of the labelled fragment were added to 10µl of ddH_2O and the mixture cooled to 0°C. 30µl of hydrazine were then added and the mixture was incubated at room temperature for 10 minutes. The reaction was stopped by the addition of 200µl of hydrazine stop buffer and placed on ice.

T reaction.

5µl of labelled DNA were added to 15µl of 5M NaCl and the solution was cooled to 0°C. 30µl of hydrazine were then added and the reaction was performed at room temperature for 10 minutes before being terminated by the addition of 200µl of hydrazine stop buffer. It was then placed on ice.

Following the base-specific reactions the products from all four reactions were cleaved using the same procedure. 1µl of tRNA (5µg/ul)

was added, followed by 750µl of ethanol to precipitate the DNA and tRNA carrier. The precipitation was chilled for 15 minutes on dry ice and then centrifuged at 12,000g for 5 minutes. The nucleic acid pellet was resuspended in 250µl of 0.3M sodium acetate and re-precipitated with ethanol. The pellet was then washed in 95% (v/v) ethanol and lyophilised briefly. It was resuspended in 100µl of 1M piperidine and incubated at 90°C for 30 minutes. The piperidine was then removed in the lyophiliser. The DNA pellet was resuspended in 10µl of ddH_2O, which was then frozen on dry ice and then removed in the lyophiliser. This was repeated twice more and the DNA was then resuspended in 10-30µl of sequencing sample buffer.

The products of the cleavage reactions were analysed on 40cm denaturing urea/polyacrylamide gels (section 2.10h). By analysing the products of one set of reactions on a 20% gel and performing four separate runs on an 8% gel it was possible to obtain the sequence of the 260 nucleotides adjacent to the site of labelling (see Table 2.4).

Table 2.4 **Sequence derived from 40cm uniform denaturing gels**

Gel	Xylene cyanol migration	Nucleotides resolved
20%	12cm	2 - 56
8%	20cm	19 - 105
8%	40cm	74 - 184
8%	60cm	135 - 214
8%	80cm	175 - 260

Following electrophoresis, the gels were fixed, dried onto Whatman 3MM paper and exposed to X-ray film.

II. Chain termination.

The dideoxy chain termination method of sequencing DNA is based on the inability of DNA polymerase 1 to further extend DNA molecules after a dideoxynucleotide has been incorporated. This is a consequence of the lack of the 3' hydroxyl group on the dideoxy nucleotide which is present in a deoxynucleotide and participates in the formation of a phosphate ester linkage with the alpha-phosphate of an incoming deoxynucleoside triphosphate. Sequencing by the chain termination method involves the annealing of the DNA of interest, which is usually single-stranded, to an oligonucleotide primer. A DNA strand, complementary to the template, is extended from the primer in the presence of all four deoxynucleotides and one dideoxy-nucleotide using the Klenow fragment of E.coli DNA polymerase 1. For example, in the case of the reaction containing dideoxy ATP (ddATP) the enzyme will either incorporate a dATP molecule and chain extension will continue, or it will incorporate a ddATP molecule and extension will terminate. By adjusting the ratio of the concentrations of ddATP and dATP a family of extension products of different lengths, but with common 5' ends, can be generated which are complementary to a considerable length of the template DNA. By performing four separate reactions, each with a different dideoxynucleotide, four different sets of fragments are generated with an extension product terminated at every position on the template. Since the extension products only differ in the position of their 3' ends, fractionation of the four reactions on denaturing polyacrylamide gels generates a ladder of fragments, each differing from the next by a single nucleotide. One of the deoxynucleotides included in the extension reactions is radio-labelled and, hence, the fragment ladder can be visualised by autoradiography. The sequence of

the extended DNA strand is read directly from the autoradiograph.

Single-stranded template DNA was produced using either the M13 or pEMBL vectors (Messing and Vieira, 1982; Dente *et al.*, 1983). In these studies, three methods were used to generate DNA fragments of the *Sparc* cDNAs for sequencing by the chain termination method. First, certain restriction fragments were gel-purified (section 2.10i) and cloned directly into the multiple cloning sites in all four pEMBL vectors (pEMBL 8+, 8-, 9+ and 9-). The use of all four vectors allowed the orientation and strand of the subcloned fragment that was encapsidated as single-stranded DNA to be varied, such that sequence could be obtained from both ends of the fragment and from either DNA strand. The subcloning of these fragments into the pEMBL vectors was performed as described in section 2.10l and the ligation products transformed into *E.coli* DIH101 (section 2.10c) which were then plated and grown on L-agar plates containing 50µg/ml ampicillin. Other restriction fragments were subcloned into the M13mp8 and mp9 vectors in a similar manner and the ligation products transformed into *E.coli* JM101.

The second method of fragment preparation involved the sonication of large cDNA inserts to produce a random set of smaller fragments for sequencing (Bankier and Barrell, 1983). 30µg of an insert were purified on a 3.5% polyacrylamide gel (sections 2.10h and 2.10i). The gel-purified cDNA insert was extracted once with phenol, then with phenol:chloroform and finally with chloroform:isoamylalcohol (24:1). The DNA was then ethanol precipitated and resuspended in 100µl of 1x Klenow buffer containing all four deoxynucleotides at a concentration of 0.25mM. 10u of the Klenow fragment of DNA polymerase 1 were added and the reaction incubated at 37°C for 30 minutes. The reaction was extracted with phenol and spun through a G-50 column equilibrated in

TE (pH 8.0). The DNA was ethanol-precipitated and resuspended in TE (pH 8.0) at a concentration of 1μg/μl. To prevent the preferential cloning of fragments generated from the ends of the cDNA, it was self-ligated to produce closed-circle molecules. The ligation reaction was performed overnight at 15°C in a total volume of 200μl of 1x ligase buffer containing 15μg of DNA, 0.1mg/ml BSA and 20u of T4 DNA ligase. Following incubation, the ligation mixture was extracted with phenol:chloroform and precipitated with ethanol. The DNA was resupended in 500μl of TE (pH 8.0) containing 0.2M NaCl and sonicated on ice for 3 minutes in 5 second bursts, allowing 10 seconds between bursts for cooling. The sonicated DNA was ethanol-precipitated, washed with 95% (v/v) ethanol and resuspended in 28μl TE (pH 8.0). The ends of the DNA fragments were then repaired with T4 DNA polymerase; T4 polymerase buffer was added to 1x, all four deoxynucleotides to 0.1mM and 20u T4 polymerase. The reaction was incubated at 15°C for 3-4 hours, extracted with phenol:chloroform, ethanol-precipitated and resuspended in 50μl TE (pH 8.0). The DNA was fractionated in a 1.5% LGT agarose gel and fragments between 100 and 400bp were purified from the gel. The fragments were sub-cloned into M13mp8 at the SmaI site as described in section 2.101 and the ligation products transformed into JM101.

The third method of generating restriction fragments for sub-cloning and sequencing involved the use of the enzymes AluI and Sau3A (Messing, 1983). 3μg of purified cDNA insert was digested with each of the enzymes in separate reactions. Digestion was monitored on a 1.5% (w/v) agarose gel. The products of the AluI digest were sub-cloned into the SmaI site of M13mp8 and the Sau3A fragments were introduced into the BamHI site of M13mp8 according to the procedure described in section 2.101 and the products were transformed into

JM101.

Single-stranded DNA was isolated from the recombinants according to the procedures described in section 2.10e. All sequencing reactions were performed on M13 or pEMBL templates using a universal M13 oligonucleotide primer. The primer was annealled to the template as follows. A 10µl reaction was set up containing 0.5µg template DNA, 0.25pmole of primer and 1.5x Klenow buffer. The mixture was incubated at 55-60°C for 1-2 hours and then allowed to cool to room temperature. 15µCi of ^{35}S-dATP (>600Ci/mM) and 1u of Klenow polymerase were then added to the annealling mix . Initially, the sequencing reactions were performed in 1.5ml Eppendorf tubes, but it was subsequently found to be more convenient to perform the reactions in the wells of microtitre plates. Each sequencing reaction contained the following, in order of addition, 2.5µl of the annealling mix and 2µl of the relevant nucleotide mix (Table 2.5). The solutions were spotted onto the sides of the tubes or wells, and the tubes or plates were spun briefly to start the reaction. The reaction was allowed to proceed for 20 minutes at room temperature, after which 2µl of chase mix (0.25mM of each deoxynucleotide) were added, and the reaction allowed to proceed for a further 20 minutes. The reaction was terminated by the addition of 4µl of sequencing sample buffer. The mixtures were then heated at 90°C for 2 minutes and the reaction products were analysed on 6% denaturing polyacrylamide gels (section 2.10h). Two types of gel were routinely employed either 60cm 0.5-2.5x TBE buffer gradient gels or 40cm uniform 6% gels. Following electrophoresis gels were fixed, dried onto 3MM paper and exposed to X-ray film as described in section I. Exposures were generally for 1-3 days. To identify M13 clones containing the same DNA insert in a common orientation, "T-tracking" was performed involving only the T

Table 2.5 **Composition of nucleotide mixes for sequencing by chain termination**

	Reaction			
	A	**C**	**G**	**T**
Component				
0.5mM dCTP	20µl	1µl	20µl	20µl
0.5mM dGTP	20µl	20µl	1µl	20µl
0.5mM dTTP	20µl	20µl	20µl	1µl
0.1mM ddATP	80µl	-	-	-
0.1mM ddCTP	-	80µl	-	-
0.3mM ddGTP	-	-	80µl	-
0.5mM ddTTP	-	-	-	80µl
TE (pH 8.0)	20µl	20µl	20µl	20µl

nucleotide mix, and the products were analysed as above.

2.10n Radioactive labelling of nucleic acids.

I. Nick translation (adapted from Rigby et al., 1977)

Nick translations were performed in 1x nick-translation buffer containing 2μM of each unlabelled deoxynucleotide, 1mg/ml BSA, 8pM of each alpha-labelled deoxynucleotide (3000Ci/mM; 10mCi/ml), and usually 0.25μg of DNA, in a final volume of 25μl. DNase 1 (1μl, containing 100pg DNase 1/μg DNA) was added to the mixture and the reaction was left at ambient temperature for 1 minute. Kornberg polymerase (E.coli polymerase 1, 1μl; 10u/μl) was then added and the reaction was incubated at 14°C for 2hours. The reaction was stopped by the addition of 25μl TES.

Unincorporated labelled nucleotides were removed in a spun G-50 column (Maniatis et al., 1982). A 0.7ml column of G-50 was prepared in a 1ml disposeable syringe, equilibrated in TES, and pre-spun at 1,500g for exactly 3 minutes. The nick translation reaction mixture was loaded onto the column and spun into an Eppendorf tube at 1,500g for exactly 3 minutes. The collected labelled DNA was then denatured by boiling for 3 minutes and added to the hybridisation buffer.

II. Labelling restriction fragments with a random primer (Feinberg and Vogelstein, 1984a, 1984b).

Restriction fragments were separated on a 1-2% (w/v) LGT agarose gel in TAE buffer. The gel was stained for 20-30 minutes in 1 litre of H_2O containing 0.2μg/ml ethidium bromide. The desired fragment was excised cleanly with a razor blade and weighed in an Eppendorf tube.

3ml of H_2O per gram of gel was added to the tube and incubation at 100°C performed for 7 minutes. The fragment was then stored at -20°C until it was required.

For labelling, the fragment was reboiled for 3 minutes and then cooled for 10-60 minutes in a 37°C water bath. The following reaction mixture was set up:

10µl of OLB

2µl BSA (10mg/ml)

5µl ^{32}P-dCTP (2,000-4,000 Ci/mM)

DNA in agarose up to 32µl

H_2O to a final volume of 50µl

2u of Kornberg polymerase (E.coli DNA polymerase 1)

Incubation was carried out at room temperature for 2.5 hours for 30-50ng of template, 5 hours for 10-30ng of template or overnight for <10ng of template. The reaction was terminated by the addition of 200µl of 20mM NaCl, 20mM Tris.HCl (pH 7.5), 2mM EDTA, 0.25% (w/v) SDS, 1µM dCTP. The unincorporated labelled nucleotide was then removed using a G-50 spun column as described above.

III. Ribonucleotide probes.

Ribonucleotide probes were prepared from the Sp6 series of vectors (Melton et al., 1984). The reaction components were assembled in the following order: 11µl ^{32}P-dGTP (400-3,000Ci/mM; 10mCi/ml), 2µl 10x Sp6 transcription buffer, 1µl DTT (200mM), 1µl RNasin (25u/µl), 2µl 5mM CTP, TTP and ATP, 1µl (10-20u) Sp6 polymerase and finally 2µl (1-2µg) linearised DNA. The first three components were pre-warmed to 37°C prior to addition. The reaction was incubated at 37°C for 60-90 minutes and the template was then degraded by the addition of 5µl RNase-free DNase 1 (200µg/ml containing 1,000u/ml RNasin), 10µl yeast

tRNA (10mg/ml) and 10µl ddH_2O, followed by incubation at 37°C for 15 minutes. The reaction was terminated by the addition of 5µl of stop mix (50mM EDTA, 0.1% (w/v) SDS, 100mM Tris.HCl (pH7.5) and phenol red).

The probe was separated from the unincorporated labelled nucleotide by fractionation on a 1.5ml Bio-Rad agarose A1.5M (100-200 mesh) column equilibrated and run in 10mM Tris.HCl (pH 7.4), 1mM EDTA, 0.1% (w/v) SDS. The phenol red dye co-migrated with the free nucleotide and was used to monitor the progress of the separation. The excluded peak of radioactivity, containing the labelled RNA, was pooled and added directly to the hybridisation buffer.

IV. Labelling with Klenow polymerase (Maniatis et al., 1982).

Labelling of recessed 3' ends with the Klenow fragment of E.coli polymerase 1 was performed either in 1x Klenow buffer or by addition of the enzyme and nucleotides directly to a restriction enzyme digest reaction. Typical reactions contained 1-2µg digested DNA, 0.1mM each unlabelled deoxynucleotide, 1-5µl of an appropriate alpha-labelled deoxynucleotide (400-3,000Ci/mM; 10mCi/ml) and 1-10u Klenow polymerase. Reactions were incubated at 37°C for 15 minutes and were terminated by the addition of an equal volume of TES. Unincorporated nucleotides were removed by the spun column procedure.

V. Labelling 5' ends with T4 polynucleotide kinase (J.Williams and D.Banville).

The 5' terminal phosphate was removed from the products of restriction enzyme digests of DNA with calf alkaline phosphatase. The same procedure was used for the phosphatase treatment of both vectors and for DNA fragments to be kinase-labelled. DNA was ethanol

precipitated and then resuspended in 15µl of 1x CAP buffer per µg of DNA. To this was added 1µl/µg DNA of 40u/ml calf intestinal alkaline phosphatase and the reaction incubated for 30 minutes at 37°C for protruding 5' ends. For blunt ended fragments or those with recessed 5' ends incubation was performed for 15 minutes at 37°C and then for 15 minutes at 56°C, a second aliquot of enzyme was then added and the procedure was repeated. In all cases the phosphatase was then inactivated by incubation at 70°C for 1 hour. Vector DNA was then further prepared as described in section 2.101.

Phosphatase-treated DNA that was to be kinased was denatured at 100°C for 3 minutes and cooled briefly on ice. The following components were added to the denatured DNA: 50-100µCi γ^{32}P-dATP (>4,000Ci/mM; 10mCi/ml), 0.1 volumes of 10x kinase buffer and 5-10u T4 polynucleotide kinase and the reaction was incubated at 37°C for 1 hour. Unincorporated labelled nucleotide was removed in a spun G-50 column. If further restriction digestion was required, e.g. for sequencing by the method of Maxam and Gilbert, a phenol extraction was performed prior to the spun column.

VI. Labelling with T4 polymerase (modified from O'Farrell, 1981).

T4 polymerase was used to label protruding 3' ends due to its strong 3'-5' exonuclease activity. Typical reactions were performed on 1-3µg digested DNA in 20µl 1x T4 polymerase buffer containing 100µg/ml BSA, 3-5µl of an appropriate alpha-labelled deoxynucleotide (400-3,000Ci/mM; 10mCi/ml), 0.1mM each of the three unlabelled deoxynucleotides and 2.5-10u T4 polymerase. The reaction was incubated at 37°C for 30 minutes and terminated by the addition of and equal volume of TES. Unincorporated nucleotides were then removed on a spun G-50 column.

VII. Labelling with terminal transferase.

Restriction fragments (0.1-1.5µg) were ethanol-precipitated and resuspended in 15µl of 1x terminal transferase buffer containing 50µCi of α^{32}P-ddATP (3000Ci/mM; 10mCi/ml). Twenty units of terminal transferase were added and the reaction was incubated at 37°C for 1 hour. Following incubation, the reaction was extracted with phenol and unincorporated labelled nucleotide removed by a spun G-50 column.

VIII. Labelling M13 DNA with the universal probe primer.

The following components were assembled in a final volume of 10µl: 2ng of M13 universal probe primer, 50ng of M13 template single-stranded DNA and 1.5x Klenow buffer. This mixture was heated to 90°C for 3 minutes and cooled slowly to room temperature to allow annealling of the primer to the template DNA. To start the synthesis and labelling of the second DNA strand, 1µl 0.1M DTT, 1µl 0.5mM unlabelled nucleoside triphosphates, 1µl of ^{32}P-dATP (400Ci/mM; 10mCi/ml) and 1µl of Klenow fragment (1u/µl) were added. The reaction was incubated for 1 hour at 25°C and then stopped by the addition of 1µl of 0.5M EDTA (pH 8.0). The probe was used immediately without denaturation.

2.10o Preparation of a parietal endoderm cDNA library (modified from Gubler and Hoffman, 1983).

The cDNA library which I constructed to isolate sequences at the 5' end of the 2.2Kb transcript was primed both with oligo dT_{14}, and also a single stranded primer which was generated from an existing cDNA in the following manner. The primer was obtained from a KpnI-PstI fragment of pPE.220, a fraction (5%) of which had been kinase-

labelled at the KpnI site to act as a tracer. Following denaturation, the primer was separated from the unlabelled complementary strand on an 8% native strand separating gel (section 2.10h). Autoradiography revealed that the two strands were separated by a distance of about 2cm by this technique as determined from an adjacent lane containing fragments labelled on both strands, and the primer was purified from the gel by electroelution. The purified primer was then kinase-labelled to high specific activity.

The first cDNA strand was synthesised on 2.5µg of parietal endoderm poly(A)+ RNA which had been incubated at room temperature for 10 minutes in 10mM methylmercuric hydroxide, to remove secondary structures in the RNA, followed by the addition of 2-mercaptoethanol to a final concentration of 40mM. The first strand synthesis was performed in a final volume of 50µl containing 50mM Tris.HCl (pH 8.3), 50mM KCl, 10mM $MgCl_2$, 100µg/ml oligo dT_{14}, 2ng KpnI-PstI primer, 10mM DTT, 1mM each dATP, dTTP and dGTP, 0.5mM dCTP, 30µCi α^{32}P-dCTP (3,000Ci/mM; 10mCi/ml) to monitor the yield of cDNA, 20u RNasin and 50u reverse transcriptase. The reaction was incubated at 42°C for 1 hour and terminated by the addition of 2.5µl 0.5M EDTA. The incorporation of labelled nucleotide into TCA-insoluble material indicated that 1µg of cDNA had been synthesised.

The products of the first strand synthesis were spun through a G-50 column which had been pre-equilibrated in 1x second strand buffer (20mM Tris.HCl (pH 7.5), 5mM $MgCl_2$, 10mM ammonium sulphate and 0.1M KCl). The collected volume was measured and adjusted to 95µl containing 1x second strand buffer, 50µg/ml BSA, 40µM each dATP, dTTP, dCTP, and dTTP and, again, a further 30µCi of dCTP was included to monitor the yield. The reaction was initiated by the addition of 30u E.coli polymerase 1 and 3u RNaseH and incubated for 1 hour at

12°C and then at 22°C for a further hour. Following incubation, the reaction was stopped by the addition of 4µl 0.5M EDTA, phenol extracted, precipitated twice from 2.5M ammonium acetate with ethanol and resuspended in TE (pH 8.0). The calculated yield of double stranded cDNA was about 0.8µg.

A homopolymer tail of G residues was added to 200ng of the double stranded cDNA in a total reaction volume of 100µl containing 1x terminal transferase buffer, 0.1mM DTT, 100µg/ml BSA, 40µM dGTP and 30u terminal deoxynucleotidyl transferase. The reaction was incubated for 1 hour at 37°C and was heat-inactivated at 65°C for 10 minutes and stored at -20°C.

The vector (pUC8) was prepared as follows. 20µg of pUC8 were digested overnight with 200u PstI. The linearised vector was purified free from any uncut vector DNA on a 0.8% (w/v) agarose gel, followed by electroelution into a dialysis bag. The vector was further purified by phenol extraction and two sequential ethanol precipitations. 5µg of the vector was then C-tailed in 50µl of 1x terminal transferase buffer, containing 5mg/ml BSA, 0.9mM dCTP and 50u terminal deoxynucleotidyl transferase for 50 minutes at 22°C. The enzyme was then inactivated at 65°C for 5 minutes and the tailed vector was stored at -20°C.

Annealing of the vector and cDNA was performed in 0.1M NaCl. The annealing reactions were placed in a water bath at 60°C for 15 minutes, the heater was then turned off and the annealing mix was allowed to cool slowly to 40°C. The annealing was chilled on ice and then used to transform competent (10^8cfu/µg) E.coli DH5 (section 10c). The transformants were plated on agar plates containing 50µg/ml ampicillin. Pilot reactions revealed that the optimum annealing ratio was 1ng cDNA:30ng vector. 15ng of cDNA was annealed to vector at this

ratio in 150µl of 0.1M NaCl and the products were used to transform 2.5mls of competent DH5 bacteria. The library was plated onto Pall-Biodyne nylon membranes on 24 x 24 cm agar plates containing ampicillin and was grown for 15 hours until the colonies were about 1mm in diameter. A library of about 4 x 10^3 recombinants was generated and was replicated and screened as described below.

2.10p Hybridisation to DNA from bacterial colonies.

The genomic and cDNA libraries which were used in these studies are described in Table 2.6. These were replicated and screened essentially as described by Hanahan and Meselson (1980). Libraries were replicated onto either nitrocellulose or nylon membranes and grown on agar plates containing the appropriate antibiotic. For long term storage the colonies were grown on plates that were supplemented with 10% (v/v) sterile glycerol and stored at -70°C as a "sandwich" with a fresh filter which had been pre-wet on a similar plate.

For screening, colonies were grown to a diameter of no more than 1mm and were then lysed and the liberated DNA was fixed as follows (Maniatis et al., 1982). The filters were transferred, colony side up onto 3MM paper pre-soaked in 10% (w/v) SDS for 3 minutes, then to 3MM soaked in 0.5M NaOH, 1.5M NaCl for 5 minutes and finally to 3MM soaked in 1.5M NaCl, 0.5M Tris.HCl (pH 8.0) for 5 minutes. The filters were allowed to dry at room temperature for 1 hour and then baked at 80°C for 2 hours.

Prior to pre-hybridisation, bacterial debris was removed from the filters by washing them in 50mM NaOH for 20 minutes, followed by neutralistion in several changes of 0.5M Tris.HCl (pH 8.0), 1.5M NaCl. The filters were prehybridised in 20-50ml of 5x SSC, 5x Denhardt's solution, 0.1% (w/v) SDS and 100µg/ml denatured salmon

Table 2.6 **Libraries that were screened in these studies**

Library	Nature	Vector	Antibiotic	Reference.
Differentiated F9 cells	cDNA	pAT153	Tetracycline	Kurkinen et al. 1983a
Parietal endoderm	cDNA	pUC8	Ampicillin	Kurkinen et al. 1983c
Parietal endoderm	cDNA	pUC8	Ampicillin	This thesis
129/SV mouse liver	genomic	pcos2EMBL	Kanamycin	Poutska et al. 1984

sperm DNA for between 4 and 16 hours at 65°C. Hybridisation was performed in 10-15mls of pre-hybridisation buffer for 16 hours at 65°C.

2.10q Hybridisation to M13 plaques (Mason and Williams, 1985a)

For hybridisation to M13 plaques, the plates containing the phage plaques were cooled at 4°C prior to replication since this helps to prevent the softer top agar being withdrawn on the membrane. Plaques were replicated onto nitrocellulose membranes as described above. Filters were baked immediately, without any lysis or denaturation steps. Prehybridisation, hybridisation and washing were performed as described in section 2.10p.

2.10r Quantitation of nucleic acids by spectrophotometry.

To measure the concentration of nucleic acids, the optical density of a dilution of the preparation in ddH_2O was measured at 260 and 280nm. An OD_{260} of 1 corresponds to approximately 50µg/ml for double-stranded DNA and 40µg/ml for single-stranded DNA and RNA. The ratio of the two readings (260/280) provides an estimation of the purity of the preparations; the ratio for pure DNA is about 1.8, while pure RNA is about 2.0.

2.10s Precipitation of nucleic acids.

DNA or RNA were precipitated from solutions containing 0.3M sodium acetate, or 0.3M NaCl, or 2.5M ammonium acetate with either 2.2 volumes of ethanol or 1 volume of isopropanol. The nucleic acids were allowed to precipitate on dry ice for 15 minutes, or at -70°C for 1-2 hours, or at -20°C overnight and then collected at 12,000g for 15 minutes. The nucleic acid pellet was washed with 70% (v/v) ethanol

and re-dissolved in ddH_2O or an appropriate buffer.

2.10q Autoradiography.

Filters or gels wrapped in cling-film were exposed to pre-flashed X-ray film, in cassttes or wrapped in several layers of black plastic, at -70°C. All films were processed in an Ilford automatic processor.

Autoradiograms were scanned, prior to the saturation of the film by the signal, using a Zeineh soft laser densitometer.

2.11 RNA techniques

2.11a Preparation of RNA from tissues and cells.

I. Small-scale preparation from tissues (adapted from Maniatis et al., 1982).

Dissected tissues were washed briefly in PBSA and then homogenised in 3.5mls of 4M guanidinium isothiocyanate, 5mM sodium citrate, 0.1M 2-mercaptoethanol, 0.5% (w/v) Sarkosyl using a glass homogeniser. At this stage the homogenate could be snap-frozen in liquid nitrogen and stored at -20°C. The RNA was spun through a 1ml cushion of 5.7M caesium chloride in 0.1M EDTA (pH 7.5) in a polyallomer tube at 35,000rpm for 15 hours at 20°C. The supernatant was carefully removed and the walls of the tube dried thoroughly. The RNA pellet was then re-dissolved in 100µl of 10mM Tris.HCl (pH 7.4), 5mM EDTA, 1% (w/v) SDS and extracted once with an equal volume of chloroform:butanol (4:1 (v/v)). The aqueous phase was removed to a sterile Eppendorf tube and the organic phase was re-extracted with 100µl of 10mM Tris.HCl (pH 7.4), 5mM EDTA, 1% (w/v) SDS. The two aqueous phases were combined and re-extracted with another 100µl of chloroform:butanol. The aqueous phase was removed and 20µl of 3M sodium acetate (pH 5.2) added to it. Following the addition of 2.2 volumes of ethanol to precipitate the RNA, the tube was placed on dry ice for 15 minutes and then spun at 10,000rpm for 15 minutes at 4°C. The supernatant was discarded and the RNA pellet rinsed with 70% (v/v) ethanol at 4°C. The ethanol was discarded and the pellet dried in a lyophiliser for about 90 seconds. The pellet was then redissolved in 20-100µl of autoclaved, distilled water and stored at -70°C.

II. Large-scale preparation from tissues (adapted from Auffrey and Rougeon, 1979).

Tissues were homogenised in 3M lithium chloride, 6M urea (filter-sterilised; 10-20ml per gram of tissue) using an Ultra-Turex homogeniser for 1 minute at maximum speed. The homogenate was allowed to stand at 4°C for 12-15 hours and was then spun at 15,000g at 4°C for 20 minutes. The supernatant was discarded and the pellet redissolved in 0.5%(%)SDS, 20mM Tris.HCl (pH 7.4), 1mM EDTA, 0.5%(%)2-mercaptoethanol (10mls per gram of original tissue). This was then extracted with 5ml re-distilled phenol, mixing the two phases with the homogeniser for 3 minutes at maximum speed. 5mls of chloroform:isoamyl alcohol were then added to the homogenate and mixing continued for a further 30 seconds. The phases were separated by centrifugation at 1,500g for 5 minutes. The organic phase was discarded, leaving behind any interface material, and the process was repeated until no more interface material was left. Ammonium acetate was then added to the aqueous phase to a final concentration of 0.3M and the RNA was precipitated overnight at -20°C following the addition of 2 volumes of ethanol. The precipitate was collected at 15,000g for 20 minutes at 4°C, washed with ice-cold 70% (v/v) ethanol and lyophilised briefly. The pellet was then resuspended in sterile, distilled water and stored at -70°C.

III. Large-scale preparation from cultured cells.

Cell cultures, generally containing about 10^9 cells were rinsed twice in ice-cold PBSA. 3M lithium chloride, 6M urea was added to them (4ml per 90mm dish) and the cells placed on ice for 5 minutes. The cells were scraped off using a "rubber policeman" and homogenised using an Ultra-Turex homogeniser. The remainder of the protocol was

as described above (section II.).

2.11b Selection of poly(A)+ RNA (Maniatis et al., 1982).

A column of oligo(dT)-cellulose (0.2ml per mg of starting total RNA), equlibrated in sterile loading buffer (1x loading buffer: 20mM Tris.HCl (pH 7.6), 0.5M NaCl, 1mM EDTA, 0.1% (w/v) SDS) was prepared in a 1ml disposeable syringe plugged with sterile polyallomer wool. The column was then washed with 3 column-volumes each of sterile H_2O, then 0.1M NaOH, 5mM EDTA and finally sterile H_2O again (the pH of the final effluent was <8). The column was then washed with 5 volumes of sterile loading buffer.

RNA, in sterile H_2O, was heated to 65°C for 5 minutes. An equal volume of 2x loading buffer was added to it and the sample cooled to room temperature. It was then loaded on the column and the flow-through collected, heated to 65°C again, cooled and reapplied to the column. This process was repeated once more. The column was then washed with 5-10 column-volumes of loading buffer, followed by 4 volumes of loading buffer containing 0.1M NaCl. Poly(A)+ RNA was then eluted in 2-3 column volumes of sterile 10mM Tris.HCl (pH 7.4), 1mM EDTA, 0.05% (w/v) SDS. The selected RNA was then precipitated for 15 minutes on dry ice following the addition of sodium acetate (pH 5.2) to a final concentration of 0.3M and 2.2 volumes of ethanol. The RNA was pelleted at 12,000g and the pellet rinsed with 70% (v/v) ethanol, lyophilised briefly, resuspended in sterile distilled water and stored at -20°C.

2.11c Quantitation of RNA - spectrophotometry and ^{3}H-poly(U) assay.

RNA was initially quantitated by determination of its optical density (OD) at 260nm. An OD of 1 corresponds to about 40µg/ml for RNA.

For comparative analyses RNA concentrations were standardised by ^{3}H-poly(U) assay (protocol supplied by J.Williams, adapted from Bishop et al., 1974b). This technique measures the hybridisation of labelled polyuridine to the poly(A), mainly poly(A) tails, present in an RNA sample. Hybridisation is linear only over a certain range of concentrations of poly(A). The linear range was determined, in each assay, using a set of poly(A) standards (0, 2, 4, 8, 12 and 15ng of poly(A), each standard point being measured in triplicate). Several different amounts of RNA (usually 2, 4 and 6µg of total RNA) were assayed for each sample in order to obtain measurements which fell centrally in the linear region of the standard curve. These readings were used to determine the relative concentrations of poly(A) between different samples.

Each RNA sample was added to 2ml of 2x SSC containing 5µl of ^{3}H-poly(U) (480 cpm/ng; 50ng/µl) in a 5ml siliconised glass test tube and vortexed briefly. To eliminate errors, each RNA dilution was assayed in triplicate. The samples were placed at 45^{o}C for 10 minutes and then put on ice for 10 minutes. 20µl of boiled pancreatic RNase A was then added and the samples incubated on ice for a further 15 minutes. 100µl of 1mg/ml BSA was added as a carrier and the poly(A)-poly(U) hybrids were precipitated by the addition of 220µl of 100% (w/v) TCA, followed by vortexing and incubation on ice for 5 minutes. This last step was performed in batches of no more than 10 samples since poly(U) has been found to break down in TCA (J.Williams and A.Tsang pers. comm.). The precipitated nucleic acids were collected

on GF/C filters, washed twice with 15mls 10% (w/v) TCA and then with 10mls ethanol. The filters were then dried and counted in toluene scintillant. The amount of poly(A) in each sample was calculated from the standard curve.

2.11d Gel electrophoresis of RNA, Northern blotting and hybridistion

The gel (usually 0.75% or 1% (w/v)) was prepared by melting HGT agarose in distilled water and cooling to 55^{o}C before adding RNA gel buffer to 1x and formaldehyde to 2.2M final concentrations. RNA samples were prepared at a final RNA concentration of up to 1mg/ml in 1x RNA gel buffer, 50% (v/v) deionised formamide, 2.2M formaldehyde. The samples were incubated at 55^{o}C for 15 minutes, RNA sample buffer was added to a final concentration of 1x and then the samples were loaded onto the gel. When required, kinase-labelled DNA markers (about 5,000 cpm for an overnight exposure) were boiled for 5 minutes and loaded directly onto the gel. Electrophoresis was performed at 120-150V for 1-2 hours in 1x RNA gel buffer.

Following electrophoresis the gel was washed for 5 minutes in double-distilled water. After washing in distilled water, the RNA was transferred to nitrocellulose or nylon membranes as described in section 2.10j and hybridisation was also performed as described in that section. To allow a filter to be re-used the previous probe was removed by gently boiling the filter in ddH_2O for 10 minutes. The filter was then soaked in 2x SSC and then pre-hybridisation and hybridisation were performed in the usual manner.

To stain 28S and 18S rRNA for markers, an extra lane of about 10µg total RNA was electrophoresed. This lane was cut away from the rest of the gel and stained in 1x RNA gel buffer containing 1µg/ml ethidium bromide. The stained gel was then photographed on an ultra-

violet transilluminator.

2.11e RNA dot (slot) blots

The slot blot apparatus (Schleicher and Schull) was soaked for 2 hours in 1M sodium acetate containing 200µg/ml denatured salmon sperm DNA. RNA samples (up to a final concentration 0.5µg/ml) were prepared in 8.3mM sodium phosphate buffer (pH 7), 1.2M deionised glyoxal in a final volume of 26.5µl and incubated at 50°C for 1 hour. Following incubation, 200µl 20x SSC were added to the samples and they were applied to nitrocellulose using the slot blot manifold. Application was performed over a period of 5 minutes and the wells were then washed with 500µl of 20x SSC. The filter was removed from the apparatus, allowed to air dry and then baked at 80°C for 2 hours. Prehybridisation, hybridisation and washing were then performed as described in section 2.10j and stripping a probe from the filter to facilitate the re-use of the filter was performed as described above (section 2.11d).

2.11f Hybrid-selection.

Hybrid-selection was performed as described by Maniatis et al. (1982). 30µg of clones pPE.220 and pF9.25 were digested with EcoRI and PstI respectively. The digests were phenol extracted, ethanol precipitated and resuspended in 60µl ddH_2O. The DNA was heated to 100°C for 10 minutes, chilled briefly and 60µl of 1M NaOH was added to it. The mixture was incubated at room temperature for 20 minutes and then neutralised with 60µl of 1M NaCl, 0.3M sodium citrate, 0.5M Tris.HCl (pH 8.0) and 1M HCl. It was then applied, in 5µl aliquots to $3mm^2$ nitrocellulose filters. The filters were allowed to dry in air

for 1 hour and were twice washed with 6x SSC. The filters were allowed to dry before baking them at 80°C for 2 hours. Following baking, they were washed twice in sterile ddH_2O at 100°C.

The hybridisation solution (100µl of 65% (v/v) deionised formamide, 20mM PIPES (pH 6.4), 0.2% (w/v) SDS, 0.4M NaCl and 100µg/ml calf liver tRNA), containing either 17µg poly(A)+ RNA from monolayers of F9 cells differentiated to parietal endoderm for 5 days or 20µg poly(A)+ RNA from PYS-2 cells, was pre-warmed to 70°C for 10 minutes and then added to the filters. Hybridisation was performed at 50°C for 3 hours, after which the filters were washed 10 times in 1ml of 10mM Tris.HCl (pH 7.6), 0.15M NaCl, 1mM EDTA and 0.5% (w/v) SDS at 65°C and twice in the same buffer without SDS. The filters were transferred to a siliconised tube and 500µl ddH_2O containing 30µg calf liver tRNA was added to them. The tube was placed in a boiling water bath for 1 minute and then the solution was snap frozen on dry ice. The samples were thawed and the filters were removed. Following phenol extraction, ethanol precipitation and washing in 70% (v/v) ethanol, the RNA was resuspended in 5µl ddH_2O for *in vitro* translation (section 2.9d).

2.11g Primer extension.

Primer extension was performed according to the procedure of Williams and Mason (1985). The primer was derived from a HpaII-DdeI fragment of clone pIM.22 (see Figure 4.4) and was strand separated on a urea/polyacrylamide gel (see Figure 4.7). The single stranded primer was purified by electroelution from a gel fragment and kinase-labelled to a specific activity of 5×10^6dpm/pM. Hybridisations contained about 1µg of poly(A)+ RNA, 17.5pg (2.5fmoles) of primer, 0.4M NaCl and 10mM PIPES (pH 6.4) in a final volume of 10µl. The

hybridisations were drawn into glass capillaries which were sealed and incubated, submerged in a waterbath, overnight. The optimum annealing temperature for this primer-template combination was found to be 70°C. After hybridisation the mixture was expelled into 90µl of reaction buffer to give a final volume of 100µl which contained 50mM Tris.HCl (pH 8.2), 10mM DTT, 6mM $MgCl_2$, 25µg/ml actinomycin D, 0.5mM all four deoxynucleotides and 10u reverse transcriptase. The extension reaction was performed at 42°C for 1 hour and the products were precipitated with ethanol. The pellet was washed with 95% (v/v) ethanol and analysed on a urea/polyacrylamide gel.

2.11h Transcription from nuclei *in vitro*.

The RNA probes for nuclear "run off" assays were prepared by a modification of the methods of Greenberg and Ziff (1984), Turcotte *et al.* (1985) and Gilroy *et al.* (1985). Nuclei were extracted from about 4×10^8 cells with 25ml of lysis buffer (10mM Hepes pH 7.9, 10mM NaCl, 3mM $MgCl_2$ and 0.05% NP-40), centrifuged at 15,000g through a cushion of lysis buffer containing 30% sucrose, washed and repelleted through a cushion of 30% sucrose in lysis buffer. Nuclei were resuspended in 50mM Hepes pH 7.9, 40% (v/v) glycerol, 5mM $MgCl_2$, 0.1mM EDTA at 5×10^7 nuclei per ml and stored at -70°C.

For labelling, 100µl of nuclei were diluted with an equal volume of 2x transcription buffer (50mM Hepes pH 7.9, 120mM KCl, 20mM DTT, 30mM 2-mercaptoethanol, 50mM magnesium acetate, 2mM $MnCl_2$, 1mM EDTA, 8mM phosphoenolpyruvate, 6µg/ml pyruvate kinase, 2mM fructose-1,6-diphosphate, 1% Tween 80, 2mM thymidine diphosphate, 1mM ATP, 1mM CTP, 1mM UTP, 10µM GTP, 1000u/ml RNasin, 0.4mg/ml heparin and 100µCi α ^{32}P-GTP (3,000Ci/mM)). Incubation was at room temperature for 1 hour. Vanadyl ribonucleoside complexes were then added to 2mM,

together with 80µg of yeast tRNA and 6µg of RNase-free DNase, and the reaction was incubated for a further 30 minutes at ambient temperature. Following the addition of Tris.HCl (pH 7.5) to 10mM, EDTA to 5mM, SDS to 1% (w/v) and 40µg of proteinase K, the mixture was incubated at 37°C for 1 hour before extracting twice with phenol:chloroform and once with chloroform. The labelled RNA was ethanol-precipitated twice and resuspended in TE (pH 7.5). Routinely, 2-3 x 10^7cpm were incorporated into TCA-insoluble material from 5 x 10^6 nuclei.

A slot blot containing 10µg of each target DNA was prepared as described in section 2.10k. The filter was baked for 2 hours and then prehybridised at 42°C overnight in 4x SSC, 50mM sodium phosphate (pH 7.0), 1x Denhardt's solution, 0.2% (w/v) SDS, 250µg/ml yeast tRNA and 50% (v/v) formamide. Hybridisation was carried out for 72 hours at 42°C in the same buffer, with a final probe concentration of 1 x 10^7cpm/ml. The filter was then washed in 2x SSC, 0.1% (w/v) SDS at room temperature and then at 68°C. It was then washed in 2x SSC containing 20µg/ml boiled RNase A for 1 hour at room temperature. Finally, the filter was washed in 2x SSC, 0.1% (w/v) SDS for 1 hour at 68°C and exposed to pre-flashed X-ray film with an intensifying screen.

CHAPTER 3

The expression of c-fos mRNA and protein in murine extra-embryonic tissues

Introduction

Many genes were being cloned and investigated when this work commenced, but one group, the cellular proto-oncogenes, stood out as being especially worthy of study in a developmental context. Not only were these genes well characterised, but their pleiotropic effects in neoplasia suggested that they might be involved in the regulation of growth and differentiation during development. Indeed, such a role had been postulated for these cellular transforming genes well in advance of their actual discovery (Comings, 1973).

3.1 Cellular and viral oncogenes

The acutely oncogenic retroviruses induce a wide range of malignancies, often with a latency of only a few weeks (reviewed in Teich *et al.*, 1982). Within their genomes they contain sequences (*v-onc*) which have been acquired from the vertebrate genome (see Bishop and Varmus, 1982 for review). These *v-onc* sequences are required for neoplastic transformation, but their integration into the viral genome often inactivates genes which are essential for viral replication and propagation. Therefore, most acutely oncogenic retroviruses are propagated in association with a closely-related helper retrovirus which provides the functions lost as a result of *v-onc* insertion (Bishop and Varmus, 1982). To date, nearly twenty

different retroviral oncogenes with cellular proto-oncogene homologues have been identified, and perhaps thirty more cellular proto-oncogenes have been detected by transfection, or as a result of amplification, translocation or mutagenesis (Bishop, 1985a). The v-onc genes and their cellular homologues show considerable conservation at both the nucleic acid and protein levels (Bishop and Varmus, 1982). The cellular counterparts of two v-onc genes have been shown to have transforming ability in vitro when linked to a retroviral long terminal repeat (LTR) which contains promoter and enhancer sequences (Blair et al., 1981; DeFeo et al., 1981). Moreover, transformation in vivo, as a consequence of the activation of a cellular proto-oncogene in situ, by upstream insertion of a retroviral LTR has also been demonstrated (Hayward et al., 1981; Payne et al., 1982).

3.2 Cellular proto-oncogenes in growth and development

The c-onc homologues of the retroviral v-onc sequences show a remarkable degree of conservation throughout the vertebrate subphylum and some, for example c-ras, may be conserved throughout the Metazoa (Varmus, 1984). Indeed, two ras genes have been identified in the yeast Saccharomyces cerevisiae (DeFeo-Jones et al., 1983; Papageorge et al., 1984). Although activity of both is required spore viabilty (Tatchell et al., 1984), dysfunction of either gene can be complemented by the human c-ras^{Ha} gene (Kataoka et al., 1985); suggesting that the biochemical function of the ras gene has been conserved during evolution.

In view of their dramatic effects on cellular growth and differentiation during neoplasia, it has been widely suggested that the cellular oncogenes may play key roles in these processes in the

normal cell (Graf and Beug, 1978; Bishop, 1978; 1981; 1983). This is supported by the findings that the c-sis gene encodes the B chain of platelet-derived growth factor (PDGF; Waterfield et al., 1983; Doolittle et al., 1983), v-erb-A has homology to oestrogen receptor cDNA clones (Green et al., 1986), v-erb-B is homologous to the epidermal growth factor (EGF) receptor (Downward et al., 1984), and c-fms is related to the receptor for the mononuclear phagocyte growth factor, CSF-1 (Sherr et al., 1985). Consequently, the c-onc genes might prove to be expressed in a tissue- and stage-specific manner during development. Indeed, a temporal correlation between c-onc expression and differentiation has been demonstrated for c-src in the retina (Sorge et al., 1984) and for c-myc and c-myb during haematopoiesis in vitro (Westin et al., 1982a; 1982b).

When the research detailed in this chapter was undertaken, the expression of transcripts from four cellular proto-oncogenes (c-fos, c-abl, c-mos and c-ras^{Ha}) had been studied in the developing mouse and its associated extra-embryonic tissues (Muller et al., 1982). Only one of these c-onc genes, c-fos, showed a dramatic variation in its level of expression between tissues; high levels of c-fos mRNA were detected in the placenta (days 10 and 18 p.c.), whereas much lower levels were found in the whole embryo (days 10 and 13 p.c.) and the combined amnion and visceral yolk sac (days 10 and 12 p.c.). The high levels of c-fos expression in the placenta offered a plausible explanation for the observation that the entire 9 day p.c. mouse conceptus expressed 10-fold more c-fos RNA than the 10 day p.c. embryo which had been dissected-free of extra-embryonic tissues.

The c-fos proto-oncogene is the cellular homologue of the transforming gene of the FBJ-murine sarcoma virus (FBJ-MSV; Curran et al., 1982). Fos has been detected in two different mouse osteosarcoma

virus isolates: FBJ-MSV, isolated from a spontaneous tumour in a CF-1 mouse (Finkel et al., 1966) and FBR-MSV, derived from a radiation-induced osteosarcoma (Finkel et al., 1968). In addition, a modified cellular fos gene has been isolated from a spontaneous mouse osteosarcoma (N.Teich pers. comm.). While FBJ-MSV and FBR-MSV will transform a variety of cell lines in vitro (Curran and Teich, 1982a; Curran and Verma, 1984), they specifically give rise to osteosarcomas in vivo (Finkel et al., 1966; 1975; Finkel and Biskis, 1968; Ward and Young, 1976). However, some viral isolates are restricted in the range of inbred mouse strains in which they are sarcomagenic. These restricted isolates induce tumours in Fv-$1^{n/n}$ mice (e.g. C3H/He) but not in Fv-$1^{b/b}$ mice (e.g. BALB/c)(Curran and Teich, 1982a). The osteosarcomas contain a variety of different transformed cell types including fibroblasts, giant cells and osteocytes. One, or all, of these cell types may be the target cell for the virus, or they may be differentiated derivatives of a transformed pluripotential stem cell.

Before these experiments were initiated, little was known about the c-fos protein. Antisera, derived by injecting allogeneic (Wistar x Fischer) F1 rats with 208F rat fibroblasts which had been non-productively transformed with FBJ-MSV (RS2 cells), had identified the v-fos protein product as an Mr 55,000 protein (p55; Curran and Teich, 1982b). In addition to $p55^{v\text{-}fos}$, the tumour-bearing rat sera (TBRS-2 and -3) immunoprecipitated an Mr 39,000 protein not encoded by FBJ-MSV sequences but only detected in FBJ-MSV-transformed cells (Curran and Teich, 1982a, 1982b).

This chapter describes experiments designed to extend the data of Muller et al. (1982), concerning the developmental expression of c-fos, to the separated extra-embryonic tissues including the parietal endoderm. These experiments were carried out with a v-fos DNA probe

and with a rabbit antiserum raised specifically against a C-terminal c-fos peptide.

Results

3.3 Expression of c-fos mRNA in murine extra-embryonic tissues

Extra-embryonic tissues were dissected from 13.5 day p.c. mouse embryos and the expression of c-fos mRNA within them was determined by Northern analysis using a v-fos probe (Figure 3.1A, lanes 2-6). RNA was extracted from the tissues and initially quantitated by spectrophotometry. To facilitate comparison of c-fos expression between tissues the poly(A) content of each of the RNA samples was estimated by ^{3}H-poly(U) assay. Equal amounts of polyadenylated RNA were separated by gel electrophoresis, transferred to nitrocellulose and hybridised with a v-fos probe. The v-fos probe used in these and subsequent experiments was a BglII-SalI fragment obtained from a genomic clone containing integrated proviral DNA (Curran et al., 1982). This probe contained 987bp of v-fos sequence which were derived from the second, third and fourth exons of c-fos. In detail, the most 5' 841bp of the probe (with respect to the c-fos transcript) contained only 8 dispersed, single base substitutions when compared to the c-fos gene. The next 104bp of c-fos were deleted in the v-fos probe (Van Beveren et al., 1983) and the remaining 146bp of the probe contained only two further single base changes. Due to the 90% homology between the probe and c-fos sequences, the blots were washed at a slightly reduced stringency (1xSET, 0·1% (w/v) SDS at 65^{o}C). After washing and exposure to X-ray film, the autoradiograms were quantitatively evaluated by densitometry scanning.

At 13.5 days p.c. the highest levels of the 2.2Kb c-fos transcript

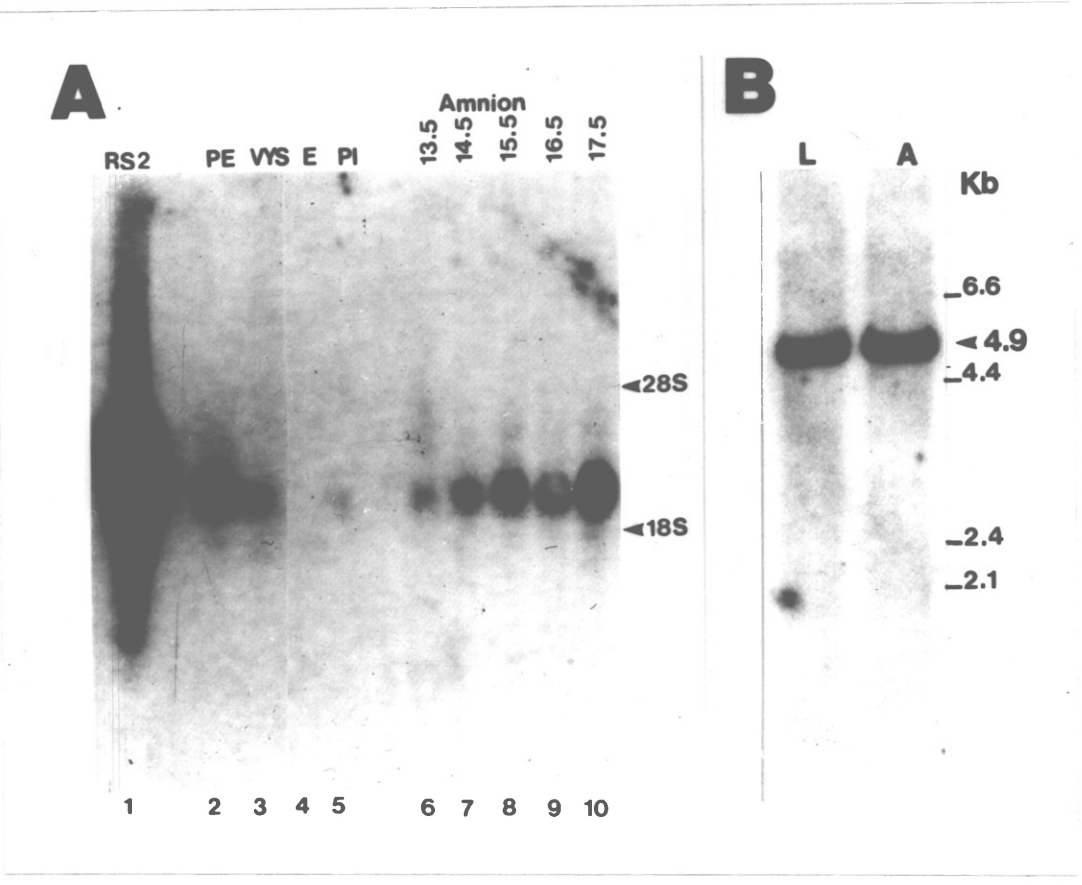


Figure 3.1 **Expression of c-fos transcripts in extra-embryonic tissues of the 13.5 day p.c. mouse embryo and during amnion development.**

A. Equal amounts of total cellular RNA, standardised by ^{3}H-poly(U) assay against 10µg of 13.5 day p.c. placenta RNA, were analysed on a 0.75% (w/v) formaldehyde/agarose gel, transferred to nitrocellulose, and hybridised with a nick-translated v-fos probe. Lane 1, RS2 rat cells (208F rat fibroblasts transformed with v-fos); lanes 2-5,tissues from 13.5 day embryos: lane 2, parietal endoderm; lane 3, visceral yolk sac; lane 4, embryo alone; lane 5, placenta. Lanes 6-10, amnion dissected from embryos between 13.5 and 17.5 days of development. The autoradiogram was exposed for seven days.

B. Southern blot analysis of 10µg of genomic DNA from adult liver (L) and 17.5 day amnion (A). DNA was digested with EcoRI, run on a 1% (w/v) agarose gel, transferred to nitrocelluose and probed with the same v-fos probe. Exposure to X-ray film was for three days.

were detected in the parietal endoderm which had been dissected-free of trophoblast. The visceral yolk sac expressed more c-fos than either the amnion or placenta at 13.5 days p.c. and no transcripts were detected in the embryo. RNA from these tissues was also analysed with v-fms (Donner et al., 1982) and c-myc (Vennstrom et al., 1981) probes (data not shown). Transcripts from the c-fms proto-oncogene were only detected in the placenta; these data conflicts with those of Muller et al. (1983b) which report similar levels of c-fms mRNA in the combined visceral yolk sac and amnion to those found in the placenta. No c-myc transcripts were detected in the extra-embryonic tissues. However, due to the very small amounts of tissue available - one parietal yolk sac contains about 10^5 parietal endoderm cells and provides approximately 1μg of total cellular RNA - low levels of expression of c-fos, c-myc and c-fms would not be detected.

The temporal expression of c-fos was studied, in the same manner, in the amnion, visceral yolk sac, and placenta during late gestation (days 13.5 to 17.5 p.c.). A dramatic increase in the level of c-fos transcripts was detected in the amnion during this period (Figure 3.1A, lanes 6-10). Densitometry-scanning indicated that the highest steady-state levels of c-fos mRNA in the amnion (day 17.5 p.c.) were slightly less than half those of the 3.2Kb v-fos transcript detected in 208F rat fibroblasts transformed with v-fos (Figure 3.2C). Southern blot analysis of genomic DNA extracted from amnions at 17.5 days p.c. and adult mouse liver, which expresses c-fos mRNA at comparatively low levels (Muller et al., 1982), suggested that this dramatic increase in expression was not accompanied by any gross changes in the c-fos gene structure, for example amplification (Figure 3.1B). Little variation in the levels of c-fos transcripts was detected, over the same period, in the placenta or the visceral

yolk sac (Figure 3.2), although the slight increase in the placenta was reproducible in separate experiments (data not shown).

3.4 C-fos mRNA levels in differentiating F9 cells

Since c-fos transcripts were found to be abundant in parietal endoderm, the levels of c-fos were measured throughout the course of differentiation of F9 teratocarcinoma cells to a parietal endoderm phenotype. Preliminary experiments, performed on total cellular RNA in the manner described in Chapter 3.3 above, failed to detect c-fos transcripts in either undifferentiated F9 EC cells or in F9 cells which had been differentiated to a parietal endoderm phenotype. Consequently, poly(A)+ RNA was prepared and used in this study. No increase in c-fos mRNA was detected in the differentiated F9 cells when compared to stem cells using Northern blot or slot blot techniques (Figure 3.3A and 3.3C). However, a reproducible transient increase in c-fos transcripts was detected between 15 and 90 minutes after addition of retinoic acid and dibutyryl cyclic AMP (Figure 3.3A, slots 2 - 5). This response was maximal at 30 minutes post-induction with levels about three-fold greater than those detected in undifferentiated cells (Figure 3.3A, slot 4). Northern analysis confirmed that the low levels of hybridisation seen in the slot-blot analysis were due to 2.2Kb c-fos transcripts and not caused by non-specific background signals (Figure 3.3C). Control experiments, performed under identical conditions, demonstrated that over the same period there was a fifteen- to twenty-fold increase in transcripts of type IV collagen (Kurkinen et al., 1983b), laminin B1 and B2 chains (Barlow et al., in press) and Sparc (Figure 3.3A and Chapter 5).

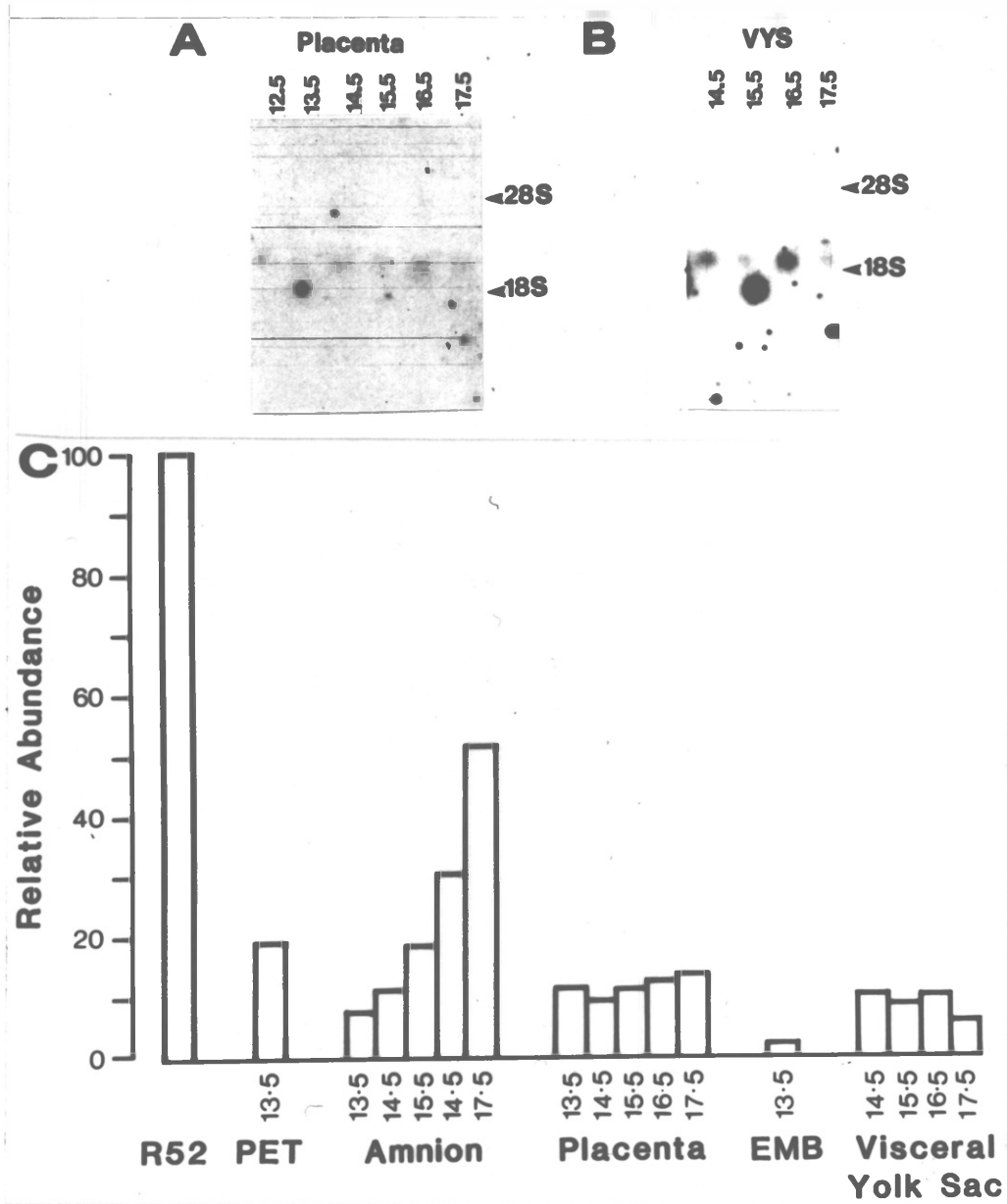


Figure 3.2 **Expression of c-fos transcripts in the developing placenta and visceral yolk sac.**

A and B. Equal amounts of poly(U)-assayed total RNA were standardised and analysed on Northern blots as described for Figure 3.1. Both filters were exposed to X-ray film for 5 days. A: placental RNA between 12.5 and 17.5 days p.c. B: visceral yolk sac RNA between 14.5 and 17.5 days p.c.

C. A histogram showing the relative levels of c-fos in extra-embryonic tissues was obtained by scanning autoradiograms, from two separate experiments in each case, with a soft laser densitometer. Levels of v-fos in RS2-transformed rat fibroblasts were given an arbitrary value of 100.

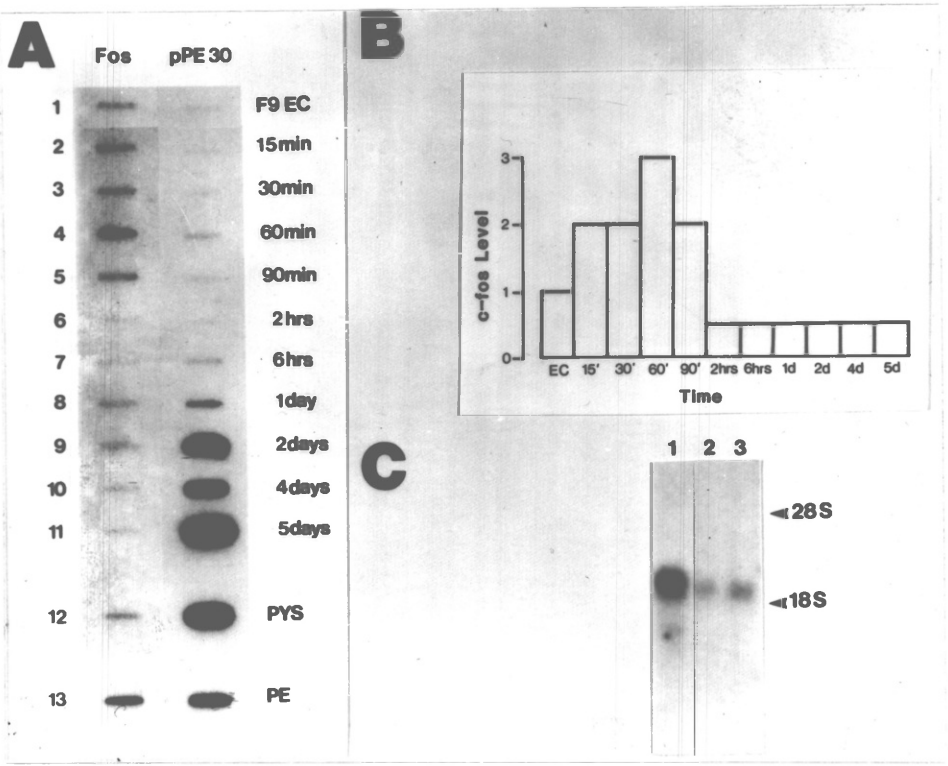


Figure 3.3 **c-fos transcripts in differentiating F9 cells.**

A. Poly(A)+ RNA was isolated from F9 cells at various times after the addition of retinoic acid (RA) and dibutyryl cyclic AMP (dBcAMP) and analysed on a slot blot using a v-fos probe (left row). The filter was reprobed with a cDNA from the Sparc gene (pPE30; right row; see Chapter 5.1a). Slot 1, 1µg poly(A)+ RNA from F9 EC cells. Slots 2-11, 1µg of poly(A)+ RNA isolated from differentiating F9 cells at the times shown on the figure (t=0 is the time of first addition of inducers). Slot 12, 1µg poly(A)+ RNA from PYS-2 cells. Slot 13, 10µg total cellular RNA from 13.5 days p.c. parietal endoderm. Exposure times were 7 days for the c-fos lane and 15 hours for the pPE30 lane.

B. Histogram plot of c-fos expression obtained by densitometry-scanning the slot-blot in A; the level in EC cells was given an arbitrary value of 1.

C. Northern blot analysis of c-fos transcripts in 10µg total RNA from 13.5 day p.c. parietal endoderm cells (lane 1), 4µg poly(A)+ RNA from F9 cells 5 days after adding RA and dBcAMP (lane 2) and 4µg poly(A)+ RNA from PYS-2 cells (lane 3) and using the same probe as in A.

3.5 Expression of c-fos protein in parietal endoderm and amnion

Studies using TBRS-3, performed in collaboration with Deborah Allen (I.C.R.F.), failed to immunoprecipitate c-fos from either cell extracts or nuclear pellets from parietal endoderm and amnion cells labelled overnight with ^{35}S-methionine, although high levels of p55$^{v\text{-}fos}$ were detected in labelled protein extracts from RS2 cells in control experiments (data not shown). Nuclear localisation of p55$^{v\text{-}fos}$ had been detected in RS2 cells by indirect immunofluorescence techniques (T.Curran, D.Allen and N.Teich pers. comm.). Using the same antiserum, indirect immunofluorescence studies on 13.5 day p.c. parietal endoderm cells attached to Reichert's membranes were inconclusive - nuclear antigen was detected but cytoplasmic filaments and Reichert's membrane were also stained (data not shown). Control experiments on RS2 cells detected nuclear antigen, although there was also some cytoplasmic staining.

On the basis of these results, I could not determine whether TBRS-3 did not recognise c-fos protein in immunoprecipitations or by staining fixed cells or whether c-fos protein is not synthesised by cultured amnions or parietal endoderm cells.

Antibodies have been raised in rabbits against a number of proteins, including SV40 large-T and small-t antigens, polyoma middle-t antigen, interferon and c-myc, by injecting short peptides coupled to a carrier. These peptides corresponded to amino acid sequences predicted by the relevant DNA sequence (Walter et al., 1980, 1981; Shimizu et al., 1981; Harvey et al., 1982; Walter and Doolittle, 1982; Rabbitts et al.,1985). Computer analysis of the predicted c-fos amino acid sequence (Van Beveren et al., 1983) by the methods of Chou and Fassman (1978) and Hopp and Woods (1981) suggested that the carboxy-terminal region of c-fos would be hydro-

philic and, if located on the surface of the molecule, immunogenic. In collaboration with Dr Alan Smith and members of his laboratory (National Institute for Medical Research), the peptide:

Tyr.Asp.Ser.Leu.Ser.Ser.Pro.Thr.Leu.Leu.Ala.Leu

corresponding to the eleven C-terminal amino acids predicted for the mouse c-fos protein and N-terminal tyrosine, for coupling to a carrier and for titration, was synthesised. This was bound to bovine serum albumin and injected into a rabbit to raise an antiserum. The affinity purified antiserum was active for immunoprecipitating I^{125}-labelled peptide at dilutions down to 1 in 1000 (A.Taylor pers. comm.; Mason, I.J. et al.,1985).

In common with the tumour-bearing rat serum (TBRS-3), the anti-peptide serum did not immunoprecipitate c-fos protein from the 10,000-g supernatant of extracts from either 13.5 day p.c. parietal endoderm or amnion cells which had been labelled overnight with ^{35}S-methionine (data not shown). However, both the anti-peptide serum and TBRS-3 immunoprecipitated proteins of Mr 46,000 and Mr 39,000 from extracts of parietal endoderm cells that had been methionine-labelled for 15 minutes (Figure 3.4A, lanes 2 and 3). The immunoprecipitation of these two proteins was specifically blocked if the antipeptide serum was pre-incubated with the peptide it was raised against (Figure 3.4A, lane 4), and neither protein was recognised by normal rat serum (Figure 3.4A lane 1). However, the Mr 39,000 protein was not immunoprecipiated by antipeptide serum if the cell extract was denatured by boiling in SDS prior to incubation with the antiserum (B.Hogan, unpublished results). It was concluded, therefore, that the Mr 46,000 protein was the c-fos protein and that the Mr 39,000 protein was non-covalently associated with it. Similar results were obtained from short ^{35}S-methionine labellings of amnions (Figure 3.4C

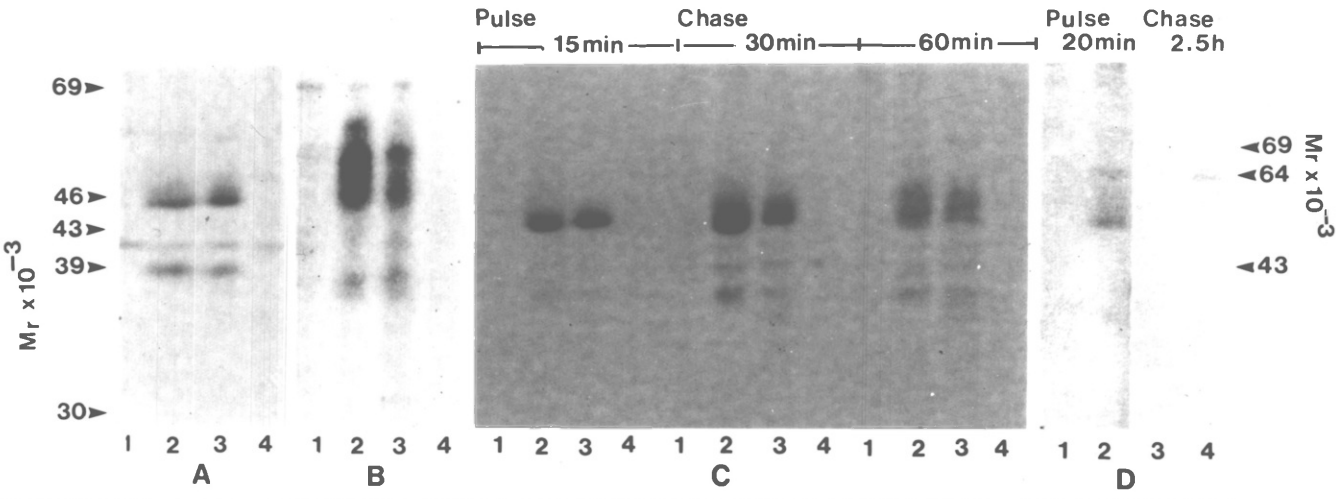


Figure 3.4 **Expression of c-fos protein in parietal endoderm and amnion.**

A. Parietal endoderm cells attached to 30 Reichert's membranes were labelled for 15 minutes with ^{35}S-methionine and extracted (Chapter 2.8f). Equal aliquots of the 10,000-g supernatant were immunoprecipitated with normal rat serum (1), TBRS-3 (2), c-fos anti-peptide serum (3) and anti-peptide serum pretreated with peptide (4).

B. Parietal endoderm cells attached to 28 Reichert's membranes were labelled for 2 hours with ^{32}P before extraction and immunoprecipitation (as in A).

C. Thirty amnions from 17.5-day embryos were labelled for 15 minutes with ^{35}S-methionine. Ten were then extracted and immunoprecipitated (as in A). The remainder were transferred to medium containing excess cold methionine and incubated for 30 and 60 minutes before extraction and immunoprecipitation (as in A).

D. Reichert's membranes were labelled for 20 minutes with ^{35}S-methionine; half were then extracted and immunoprecipitated with anti-fos peptide serum pre-treated with peptide (1) or anti-fos peptide serum alone (2). The remainder were chased for 2.5 hours (as in C) and then extracted and immunoprecipitated with pre-treated anti-fos peptide serum (3) and anti-fos peptide serum alone (4).

All samples were analysed by SDS-PAGE on 8% gels.

and B.Hogan unpublished results). These data suggested that de novo synthesis of c-fos protein ceased in prolonged cultures of these tissues and that c-fos protein that was synthesised early in the culture period was degraded. This ceasation of de novo synthesis in cultured parietal endoderm cells is not a property common to all proteins, since when parietal endoderm cells are cultured overnight, under identical conditions to those used above, label is continuously incorporated into basement membrane components (Cooper, 1983).

In Figure 3.4A, diffuse, higher molecular-weight bands can be seen above the Mr 46,000 component after only 15 minutes' labelling. This suggested that the newly synthesised c-fos protein undergoes rapid modification. This modification was investigated in both parietal endoderm cells and amnion cells by pulse chase experiments using both the antipeptide serum and TBRS-3. In the amnions, after 15 minutes' labelling followed by chases of 30 and 60 minutes, there was a decrease in the amount of labelled Mr 46,000 protein and a concomitant increase in the amount of diffuse, higher molecular-weight components (Figure 3.4C lanes 2 and 3). Control experiments in which the antipeptide serum was pre-incubated with free peptide failed to immunoprecipitate these components (Figure 3.4C lane 4). Similar results were obtained from pulse chase experiments using parietal endoderm (Figure 3.4D); after 2.5 hours the c-fos protein was chased to a band of Mr 64,000 (Figure 3.4D; amnion data not shown). The apparent half-life of the c-fos protein, as estimated from the disappearance of labelled protein in the pulse chase experiments, was about 60 minutes. No change in the molecular weight of the Mr 39,000 component was observed.

Curran and Teich (1982b) have shown that $p55^{v\text{-}fos}$ is phosphorylated on serine residues in extracts of FBJ-MSV-transformed NRK cells

(RKS3) and FBJ-MSV-transformed rat fibroblasts (RS1). Experiments in which parietal endoderm or amnion cells were labelled with ^{32}P-orthophosphate demonstrated that at least some of the post-translational modification of c-fos in these cells was due to phosphorylation. After labelling for 2 hours, radioactivity was found specifically in c-fos components of Mr 50,000-64,000 and the Mr 39,000 protein was also phosphorylated. When phophatase inhibitors (NaF, sodium pyrophosphate and orthovanadate) were included in the lysis buffer identical results were obtained (Mason et al., 1985), demonstrating that the smear of c-fos protein was not due to de-phosphorylation during extraction and immunoprecipitation

Discussion

3.6 C-fos mRNA is expressed at high levels in parietal endoderm.

In these studies, the level of c-fos transcripts was measured in the 13.5 days p.c. mouse embryo and its associated extra-embryonic tissues by Northern blot hybridisation. This technique was used in preference to dot blot hybridisation since multiple, independently-regulated, mature RNAs have been found to be transcribed from certain c-onc genes (Venstrom and Bishop, 1982; Muller et al, 1982) - the latter technique would not detect this phenomenon. These hybridisation techniques measure the steady-state level of a transcript within a tissue at the time of extraction of the RNA. The level detected reflects the balance between its rate of transcription and degradation; both of which may vary between different transcripts within the same cell (see, for example, Harpold et al., 1979; McKnight and Palmiter, 1979; Greenberg and Ziff, 1984).

There is no entirely satisfactory means of quantitating RNA to

facilitate comparison of the abundance of a particular transcript between tissues. The commonly used methods of quantitation are: spectrophotometry, ^{3}H-poly(U) assay, and hybridisation of a transcript, the level of which is assumed to be invariant (actin is commonly used). These techniques all require possibly erroneous assumptions to be made: the ratio of mRNA to total RNA is invariant between tissues (spectrophotometry), the polyadenylated RNA content per cell is a constant value (poly(U) assay), or the ratio of the level of a particular transcript to total mRNA remains constant in different tissues. Therefore, some caution should be exercised when interpreting any RNA hybridisation data which describes the relative levels of a transcript between tissues. In these experiments the ^{3}H-poly(U) assay was used, because I had found it to be the most sensitive quantitation technique.

In the initial experiments of this study, a comparison of embryonic and extra-embryonic expression of c-fos was made at 13.5 days p.c., and not later, since the parietal yolk sac breaks down during the following 24-48 hours. At 13.5 days p.c. the highest levels were detected in the parietal endoderm which had been dissected-free of trophoblast (Figure 1) and about 5-fold less c-fos mRNA was detected in the visceral yolk sac (endoderm plus mesoderm). Levels of c-fos-hybridising transcripts in the separated 18 days p.c. visceral endoderm and mesoderm are approximately equal (Muller et al., 1983b; Adamson et al., 1983) but whether this is also the case in 13.5 days p.c. tissues is not known. Lower relative levels of c-fos were detected in the placenta than have been reported by other workers (Adamson et al., 1983). This may be a consequence of the different methods used to quantitate the RNA samples prior to analysis. In my experiments the samples were standardised by ^{3}H-

poly(U) assay to contain equal amounts of poly(A) (mostly poly(A) tails), whereas in the experiments of Muller et al. (1982; 1983a; 1983b) and Adamson et al. (1983), RNA was quantitated by spectrophotometry for total RNA content. The placenta, unlike the parietal and visceral endoderm, contains a mixture of cell types, some or all of which may synthesise c-fos. Adamson et al. (1983) have demonstrated that the level of c-fos transcripts are about 15-fold greater in the outer, transferrin receptor- and alkaline phosphatase-negative portion of the placenta, than in the inner part, which expresses both of these markers. The outer portion of the placenta consists largely of foetal-derived trophectoderm (cytotrophoblast and giant cells) with some maternal decidua, whereas the inner portion contains cells from the parietal and visceral yolk sacs, endothelial cells, trophoblast cells and fenestrated cells lining maternal blood sinuses. In view of the high levels of c-fos in the outer placenta, which is mostly trophoblastic, I compared the levels of c-fos mRNA in the whole parietal yolk sac with those in the parietal yolk sac manually dissected-free of trophoblast. Lower levels were detected in the intact parietal yolk sac, than were detected if it was dissected-free of trophoblast, suggesting that c-fos transcripts are more abundant in the parietal endoderm than in the trophoblast (data not shown).

Co-expression of the c-myc and c-sis proto-oncogenes in the same cells of the first trimester human cytotrophoblast (Goustin et al., 1985) has prompted speculation that they are both involved in autocrine regulation of the "pseudomalignant" invasive growth properties of this tissue. C-fms is expressed in the murine placenta during late gestation (Adamson et al., 1983; Muller et al., 1983b). Although the co-expression of c-fms and c-fos in individual placental

cells has yet to be investigated, they may be ultimately be found to be part of a common cellular process within this organ.

3.7 Studies on the c-fos protein.

The amino acid compositions of the v-fos and c-fos proteins have been predicted from their nucleic acid sequences (Van Beveren et al., 1983, 1984; Van Straaten et al., 1984). The FBJ-MSV v-fos sequences have a coding capacity of 381 amino acids which would produce a protein with a predicted molecular weight of 41,601. The v-fos protein has been detected using tumour-bearing rat sera (TBRS) and migrates with an Mr of 55,000 on SDS-PAGE (Curran and Teich, 1982b). The mouse c-fos sequences encode a 380 amino acid protein which differs in its C-terminal 48 amino acids from the v-fos protein, although there are only five differences in the preceding 332 amino acids (Van Beveren et al., 1983).

3.7a. C-fos protein expression in parietal endoderm and amnion.

Initial immunoprecipitation experiments, performed with TBRS-3 on parietal endoderm and amnions which were labelled overnight, failed to detect the c-fos protein (data not shown; Van Beveren et al., 1983). Indirect immunofluorescence studies on parietal endoderm and amnion cells, also using TBRS-3, were inconclusive, although this may have been due to loss of the c-fos protein from the nucleus under our fixation conditions as detected by Adamson et al. (1985). Curran et al. (1984) have reported difficulty in detecting v-fos protein in the FBJ-MSV-transformed cell line RS2. However, we were able to immunoprecipitate and immunostain v-fos in this cell line (D.Allen unpublished data). This initially suggested that our failure to detect c-fos using TBRS-3 was due to antigenic differences between

the viral and cellular proteins.

In order to study the c-fos protein, an antipeptide serum was raised against the eleven C-terminal amino acids of the predicted mouse and human c-fos proteins. The high titre antiserum which was obtained is in contrast to the poor immunogenicity of an identical peptide subsequently reported by others (Curran et al., 1985a) and attributed to poor peptide solubility. Both the anti-fos peptide serum and TBRS-3 immunoprecipitated an Mr 46,000 protein from amnion and parietal endoderm cells labelled for 15 minutes. After similar short labellings, Curran et al. (1984, 1985a), using both TBRS-3 and an anti-fos peptide serum directed against a central region common to both c-fos and v-fos, have immunoprecipitated c-fos protein of Mr 54,000-55,000 from 208F fibroblasts transformed by c-fos linked to a viral LTR. Calculation of the size of the c-fos protein from the markers provided by Curran et al. (1984, 1985a) would appear to give c-fos an apparent Mr nearer 48,000 and not, as published, 54,000. However, the difference in the size of the c-fos protein, which I and Curran et al. detect, might also have been due to differences in the SDS-PAGE systems or to the protein being modified differently in the various cell types studied (Curran et al. have used transformed cells whereas I studied embryonic material). Assuming that the c-fos protein detected after short labelling is unmodified, the difference between the predicted (Mr 40,000) and observed (Mr 46,000) molecular weight is probably due to the high proline content (34 of 380 residues) or due to charge distribution. Similar discrepancies between the observed and predicted sizes of the unmodified proteins have been reported for v-fos (Mr 41,601 and 55,000 respectively; Curran and Teich, 1982b) and c-myc (Mr 48,812 and 64,000; Watt et al., 1985) - these are also rich in proline residues.

Indirect immunoflourescence studies of v-fos using TBRS-3, in a CHO cell line in which v-fos is amplified, and of c-fos in dissociated amnion cells, using TBRS-3 and an anti-fos peptide serum directed against the central domain of c-fos, has detected nuclear antigen which is excluded from the nucleoli (Curran et al., 1984, 1985). Similarly, Adamson et al. (1985), using an N-terminal anti-peptide serum, report nuclear localisation of c-fos in mouse extra-embryonic tissues. These tissues include parietal endoderm, and the intensity of the fluorescence and immunostaining correlates with the transcript levels that have previously been reported for most tissues. The authors demonstrate the specificity of this staining by its abolition when the antiserum is pre-treated with free peptide. However, the nuclei of the 19 days p.c. inner and outer placenta have an equal intensity of fluorescence and all cell types within each layer appear to express c-fos, whereas RNA hybridization studies have detected 2-3 fold more c-fos transcripts in the outer placenta at this stage (Adamson et al., 1983). The apparent discrepancy between these results may be due to translational regulation or due to the lower sensitivity of the immunofluorescence technique. Adamson et al. (1985) also report low levels of c-fos antigen in the nuclei of cells in which hybridisation studies had previously detected very few or no transcripts e.g foetal tissues, suggesting that c-fos expression, at varying levels, may be common to all cells. In contrast to the anti-peptide serum used by Adamson and her co-workers, our anti-fos peptide serum only weakly detected nuclear c-fos when used in immunofluorscence analyses (B.Hogan and E.Adamson unpublished data). Immunoprecipitation following sub-cellular fractionation has subsequently confirmed the nuclear localisation of c-fos and v-fos proteins (Curran et al., 1984).

3.7b. C-fos protein is associated with a Mr 39,000 protein.

An understanding of the interactions of v-onc and c-onc proteins with components of normal and transformed cells will be essential in elucidating their functions. Many of the c-onc genes have protein kinase activity (Sefton, 1985) and a number of their possible protein substrates and some phospholipid substrates have been identified (Radke and Martin, 1979; Cooper, J.A. et al., 1983; Cooper and Hunter, 1984; Sugimoto et al., 1984). Proteins of Mr 90,000 and 50,000, which bind $pp60^{src}$, have been identified (Brugge et al., 1981) and, in addition, src may form a complex with polyoma middle T antigen (Courtneidge and Smith, 1983).

A fos-associated Mr 39,000 protein (p39) was originally detected as an FBJ-MSV transformation-specific antigen (Curran and Teich, 1982a), co-precipitating with v-fos in TBRS immunoprecipitates. It was not among the products of in vitro translation of FBJ-MSV RNA (Curran and Teich, 1982b). p39 was therefore thought to be a cellular protein that was antigenically related to v-fos and induced by FBJ-MSV transformation or, alternatively, an unrelated protein which was non-covalently associated with v-fos.

In my studies, the C-terminal anti-fos peptide serum immunoprecipitated an Mr 39,000 protein, together with c-fos, from parietal endoderm and amnion cells. The immunoprecipitation of this protein and c-fos was specifically blocked by preincubation with free peptide, however, if the extract was denatured, prior to immunoprecipitation, c-fos was still recognised but immunoprecipitation of the Mr 39,000 protein was abrogated. Similar results have been obtained with TBRS-3 (Chapter 3.5; Curran et al., 1984) and with an anti-peptide sera directed against the middle region and amino terminus of the c-fos protein (Curran et al., 1985; Muller et al.,

1986a). Using an N-terminal antipeptide serum, Adamson *et al.* (1985) have immunoprecipitated p39 in association with *v-fos* but do not detect an Mr 39,000 protein on Western blots of amnion extracts. Identical results are reported by Muller *et al.* (1986a). Tryptic peptide mapping of the *v-fos* and *c-fos* proteins and their associated p39 proteins has indicated that each p39 protein is not closely related to the *fos* protein with which it co-precipitates (Curran and Teich, 1982b; Kruijer *et al.*,1984). However, while it is likely that the p39 associated with *c-fos* is identical to that associated with *v-fos*, this has yet to be confirmed by peptide mapping. Sub-cellular localisation studies (Curran *et al.*, 1985a) have revealed that *c-fos* is only associated with p39 in the nucleus. Taken together, these data suggest that a protein of Mr 39,000 (p39) is non-covalently associated with *c-fos* in the nucleus *in vivo* and that the regions of *c-fos* which have been used to generate antipeptide sera are exposed when the two are complexed. Curran *et al.* (1984), using the same labelling procedure as myself and evaluating incorporation by densitometry scanning, report variable ratios of p39 to *c-fos* in *c-fos*-transformed fibroblasts. In contrast, I always found that less ^{35}S-methionine was incorporated into p39 than into *c-fos* in extra-embryonic tissues.

Currently, the function of p39 and its possible expression in the absence of *c-fos* are unknown, but it provides the first potential substrate or cofactor for the *fos* gene product.

3.7c. C-fos protein is phosphorylated and rapidly turned over.

The c-fos protein is modified to forms with a higher apparent molecular weight in extra-embryonic tissues, differentiating macrophage cell lines, transformed fibroblasts and growth factor-stimulated fibroblasts (Figure 3.4; Curran et al., 1984; Muller et al., 1984b, 1986a; Kruijer et al., 1984). Inhibition of immuno-precipitation from amnion cells with peptide (Figure 3.4C), and tryptic peptide analysis of protein from serum-stimulated fibroblasts (Kruijer et al., 1984) has confirmed that these are all forms of c-fos. The range of apparent molecular weights detected is from 46KD to 62KD (Chapter 3.5) and from 55KD to 72KD (Curran et al., 1984; Kruijer et al., 1984). The difference in these data may reflect differences in the SDS-PAGE systems used or specific differences in the modification of c-fos between the cell types studied. The possibility that modification of the carboxy-terminus prevented the detection of the lowest mobility forms in my experiments was discounted because identical results were obtained when TBRS-3 was used (TBRS-3 is directed against the v-fos protein which has a different C-terminus to c-fos). A molecular weight shift in the fos-associated protein, p39, from 38KD to 39KD has been reported in c-fos-transformed fibroblasts (Curran et al., 1984) but was not detected in parietal endoderm or amnion cells in my studies. Whether this difference is central to fos-dependent transformation or reflects differences in the cell types or analytical methods used is unclear.

The modification of c-fos is accompanied by a decrease in immuno-precipitable protein during pulse chase experiments (Figure 3.4C and D; Curran et al., 1984; Muller et al., 1984b, 1986a). Again, the range of different antigenic sites in c-fos recognised by the

antisera which have been used in these studies precludes this being the result of a loss of antigenicity during modification. Therefore, c-fos protein is rapidly turned over concomitant with its modification, indeed, the modification of c-fos may be part of the degradative process. The half life of the protein has not been accurately determined in any of the cells studied, but estimations from the decrease in labelled material during pulse chase analyses suggest a half-life of between 30 minutes and 2 hours (Chapter 3.5; Curran et al., 1984; Muller et al., 1984b). This rapid turnover has also been reported for another nuclear oncogene, c-myc, which has a half-life of between 30 and 90 minutes (Rabbitts et al., 1985; Watt et al., 1985). These data suggest that levels of both c-fos and c-myc are stringently regulated and that, at least in certain extra-embryonic tissues, c-fos may be continuously required in a dynamic process. However, c-fos has a short half-life in transformed fibroblasts (Curran et al., 1984); indicating that elevated levels of c-fos, due to perturbation of its degradation pathway, are not involved in transformation in vitro.

There are a number of known modifications of nuclear proteins, including phosphorylation, acetylation, poly(ADP)-ribosylation and ubiquitination (McGhee and Felsenfeld, 1980). Both v-fos (Curran and Teich, 1982b), c-fos (Figure 3.4B; Curran et al., 1984) and p39 (Figure 3.4B) are phosphorylated. This may account, at least in part, for the decrease in mobility of c-fos since phosphorylation has been shown to have this effect on certain proteins e.g. cyclic AMP-dependent protein kinase and glycogen synthase (Zoller et al., 1979; Picton et al., 1982). However, the possibility of other modifications, for example glycosylation, has not been investigated. Curran and Teich (1982b) have demonstrated that v-fos is

phosphorylated on one, or more, serine residues. If this also proves to be the case for c-fos, the proto-oncogenes c-mil/raf and c-mos, for which a serine kinase activity has been postulated (Cooper and Hunter, 1984; Moelling et al., 1984), may be involved in its processing and regulation. Two other nuclear oncogenes are also phosphorylated - c-myc (Persson et al., 1986) and SV40 large T antigen (Van Roy et al., 1983). It has been suggested (Bishop, 1985b) that this modification may affect the ability of the protein to bind DNA, however, the phosphorylated forms of c-myc (pp64 and pp67) appear to be short-lived precursors of a more stable form (pp65) which is not phosphorylated (Persson et al., 1986). It remains to be determined whether the different forms of c-fos and c-myc are all biologically active, whether they perform separate functions, and whether rapid modification is involved in their turnover.

3.7d. C-fos protein synthesis ceases when parietal yolk sacs and amnions are cultured overnight.

Using both TBRS-3 and anti-fos peptide serum, I consistently failed to immunoprecipitate c-fos from extracts of parietal endoderm cells cultured on Reichert's membranes and amnions, that had been labelled overnight, although the cells continued to incorporate label into other proteins. Similar results have been obtained when dissociated amnion cells are cultured overnight and levels of both c-fos protein and RNA subsequently measured (T.Curran pers. comm.). However, c-fos was immunoprecipitated after short (15 minutes to 2 hours) labelling periods (Figure 3.4). These results suggest that c-fos synthesis is terminated in prolonged culture of parietal endoderm and amnion cells.

Indirect immunofluorescence studies on dissociated amnion cells in

culture have shown that, while strong nuclear fluorescence is observed 2 to 4 hours after placing the cells in culture, c-fos levels have clearly decreased after 7 hours and the protein is barely detectable by 12 hours (Muller et al., 1986a). This indicates that continued c-fos expression might require a factor, or factors, which are either absent or rapidly depleted in the culture medium (Dulbecco's modified Eagle's medium supplemented with 10% FBS). A variety of growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), tumour growth factor, 10% mouse plasma, fibroblast-conditioned medium, insulin and transferrin, fail to maintain c-fos levels when added to the culture medium (Muller et al., 1986a). Colony stimulating factor (CSF-1) and interleukin-3 (IL-3) also do not prevent the decrease in c-fos protein (Muller et al., 1986a), although they have been shown to regulate c-fos expression in macrophages and mast cells respectively (Muller et al., 1985b; Conscience et al., 1986). In contrast, one, or more, unidentified factors of Mr <3,500, present in medium conditioned by both 18-20 days p.c. placentae and embryos but not visceral yolk sacs, maintain c-fos expression in vitro in a concentration-dependent fashion (Muller et al., 1986a). Furthermore, placental-conditioned medium can re-induce c-fos protein expression in cultured amnion cells, although it remains to be seen whether placental-conditioned medium can also restore c-fos expression in parietal endoderm cells.

The factor(s) responsible for the regulation of c-fos has not yet been identified and its role in c-fos regulation in vivo remains to be determined. In view of the endocrine nature of the placenta, it is possible that the regulatory factor is a hormone. C-fos has been found to be hormonally regulated in rat thyroid follicular cells by

thyrotropin (TSH), which stimulates proliferation of the thyroid cells (Tramontano et al., 1986). The placenta is the scource of several peptide hormones that are homologues of hormones synthesized in other tissues (Krieger, 1982; Boime et al., 1982). Indeed, during late gestation in rodents, the removal of the pituitary, which secretes somatotropin (growth hormone), does not block the progression of pregnancy if a functional placenta is retained (Newton and Beck, 1939; Gardner and Allen, 1942) and chromosomal parental origin effects on foetal growth thought to act through the placenta have also been described (Cattanach and Kirk, 1985). The rodent placenta secretes at least two forms of placental lactogen (Robertson and Friesen, 1981; Robertson et al., 1982; Colosi et al., 1982; Soares et al., 1983), proliferin (Linzer et al., 1985) and a proliferin-related protein (PRP; Linzer and Nathans, 1985); all of which have homology to somatotropin (growth hormone) and may be considered as candidates for the effector molecule regulating c-fos expression in amnion cells. The same placental factors, circulating in the foetal blood, may account for the ability of embryo-conditioned medium to restore c-fos expression in cultured amnions.

3.8 C-fos mRNA levels increase in the amnion during late gestation.

The large increase in c-fos transcripts detected in the combined visceral yolk sac and amnion during late gestation (Adamson et al., 1983; Muller et al., 1983b) appears to be confined to the amnion (Figures 3.1A and 3.2B). In situ hybridisation to c-fos transcripts in the 17 days p.c. mouse amnion, indicates that mRNA levels are approximately equal in the mesodermal and ectodermal layers at this time (Deschamps et al., 1985). The slight increase in c-fos mRNA in the whole placenta complements data of Adamson et al. (1983) which

show increasing levels of c-fos transcripts in the inner placenta and constant levels in the histochemically distinct outer portion during this period. Comparison of c-fos protein levels in parietal endoderm cells by immunoflourescence reveals higher levels of nuclear antigen at 13.5 days p.c. than at 8.5 days p.c.; suggesting that a modulation in c-fos synthesis or degradation occurs during the intervening period (Adamson et al., 1985). Thus, the relative levels of c-fos expression increase in three extra-embryonic tissue during gestation, although, in the case of the amnion or inner placenta, it is not known whether the increase in RNA is reflected at the protein level.

Since the placental factor(s) regulates amniotic c-fos protein expression in vitro in a concentration-dependent manner (Muller et al., 1986a), it is possible that increasing concentrations of the same factor(s) causes the increase in c-fos mRNA in the amnion in vivo as gestation procedes. The increase in c-fos protein in the parietal endoderm occurs when c-fos levels in the amnion are relatively low (Figure 3.1A). Therefore, regulation of c-fos in the amnion may be accomplished by a separate process, or by an inability of the amnion cells to respond to the regulatory molecule(s) at earlier stages of development. As discussed above, hormones secreted by the placenta may regulate this temporal modulation in c-fos expression. Interestingly, the increase in c-fos protein in parietal endoderm coincides with maximum placental expression of murine placental lactogen, murine proliferin mRNA, and murine PRP mRNA, while the increase in the amnion during late gestation is accompanied by a second increase in the level of murine placental lactogen (Kelly et al., 1973; Soares et al., 1983; Linzer et al., 1985; Linzer and Nathans, 1985). Whether the mouse secretes more than one placental lactogen, as demonstrated for the rat, or only produces a

single molecule remains the subject of controversy (see Colosi et al., 1982; Robertson et al., 1982). It may also prove significant that an increase in c-fms levels in the placenta accompanies the amniotic increase in c-fos (Adamson et al., 1983).

The highest levels of c-fos transcripts in extra-embryonic tissues occur in the amnion of the term mouse, and human, embryos and are similar to those detected in fibroblasts transformed by c-fos or FBJ-MSV in vitro or in FBJ-MSV-induced osteosarcoma in vivo (Figures 3.1A and 3.2C; Adamson et al 1983; Muller et al., 1983c; Miller et al., 1984). However amnion cells, are not transformed and, furthermore, do not give rise to tumours when transplanted into nude mice (Adamson et al., 1985). The reason for this may lie in the regulation of the translation of c-fos. C-fos will transform NIH-3T3 cells in vitro if the gene is linked to a viral LTR and a 67bp region of the gene, located between the stop codon and the poly(A) addition site, is deleted (Miller et al., 1984; Meijlink et al., 1985). Similar results can be obtained by deletion of part of the C-terminal coding sequences (Miller et al., 1984); this correlates with the position of the frame-shift mutation in v-fos (Van Beveren et al., 1983) and a deletion or insertion in a spontaneous osteosarcoma (N.Teich pers. comm.). The presence of the viral LTR alone produces levels of c-fos mRNA similar to those detected in the transformed state but is insufficient for transformation in itself (Miller et al., 1984), but the deletion in the 3' non-coding sequences coincides with greatly increased levels of c-fos protein. This has prompted speculation that the translation of the c-fos transcript is negatively regulated by the interaction of its 3' non-coding sequences with the c-fos protein (Miller et al., 1984). Although, Curran et al. (1985) report only low levels of c-fos in the immunoprecipitations of cytoplasmic extracts

from transformed cells.

While some reports (e.g. Verma *et al.*, 1984) claim that *c-fos* protein levels are similar in amnions and transformed cells, the actual Western blot data presented by Muller *et al.* (1986a) reveal much higher levels of protein in $5x10^4$ *c-fos*-transformed NIH-3T3 cells than in $3x10^6$ amnion cells. Therefore, amnion cells may not be transformed because *c-fos* protein levels are relatively low due to regulation at the level of translation or protein stability. In contrast, levels of *c-fos* protein in serum-stimulated NIH 3T3 cells are transiently greater than those detected in *c-fos*-transformed cells (Muller *et al.*, 1986a), suggesting that either continuous expression at high levels or other, as yet unidentified, factors are required for transformation of cultured fibroblasts by *c-fos*.

3.9 *High levels of c-fos expression are not required in differentiating F9 cells for expression of the parietal endoderm phenotype.*

The F9 embryonal carcinoma stem cell provides a model system with which both cellular growth and differentiation processes can be investigated *in vitro*. This cell line can be differentiated into two cell types, visceral and parietal endoderm, mimicking a developmental event which occurs *in vivo*. The presence of *c-fos* protein and high levels of *c-fos* mRNA were detected in the parietal endoderm and led me to speculate that *c-fos* might be involved in the establishment and maintenance of the parietal endoderm phenotype. This hypothesis was investigated using the F9 culture system.

Surprisingly, slightly lower levels of *c-fos* mRNA were detected in F9 cells differentiated to parietal endoderm than in undifferentiated cells and levels in the EC cells were much lower than in embryonic

parietal endoderm. In addition, only low levels were detected in the parietal endoderm cell line PYS-2. A study of c-fos mRNA abundance during the course of differentiation of F9 cells to parietal endoderm revealed a small, transient increase in transcripts between 15 and 90 minutes after adding inducers. Early, transient increases in c-fos mRNA are often a consequence of challenging cultured cells with differentiation- or proliferation-promoting agents (see Chapter 3.10 and Table 3.1). Dibutyryl cyclic AMP stimulates transient high levels of c-fos expression during the differentiation of PC12 cells to a sympathetic neuron phenotype (Deschamps et al., 1985) - perhaps the transient modulation of c-fos during the differentiation of F9 cells is a consequence of elevated levels of cyclic AMP. While it is possible that this minor modulation in transcript levels plays a role in the establishment of the parietal endoderm phenotype in vitro, continuous high levels are not apparently required either for differentiation or maintenance of the phenotype. It is conceivable that the expression of high levels of c-fos in F9 cells differentiated to parietal endoderm may require factors which are absent from the culture medium, as is the case for amnion cells (Muller et al., 1986a). However, formation of embryoid bodies, with an external layer of visceral endoderm-like cells, from F9 cells is accompanied by a six-fold induction of c-fos mRNA over three days in two F9 lines. In addition, a third F9 EC line, re-isolated from a tumour in a 129/J mouse, constitutively expresses high levels of c-fos in culture but maintains the EC phenotype (E.Adamson pers. comm.).

Transient increases in c-fos in vitro are often accompanied, or followed, by increases in c-myc transcripts (see Chapter 3.10). However, differentiation of F9 cells to parietal endoderm causes a

twenty-fold decrease in steady-state levels of c-myc mRNA (Campisi et al., 1984) between 8 and 16 hr after adding inducers (Dean et al., 1986). Although this occurs after the modulation in c-fos which I detected, it should be noted that Dean et al. use five-fold more retinoic acid and dibutyryl cyclic AMP than I did and, consequently, the kinetics may not be comparable between the two sets of data. The decrease in c-myc transcript levels is the result of post-transcriptional regulation and occurs both in the absence of dibutyryl cyclic AMP and if establishment of the parietal endoderm phenotype is blocked with sodium butyrate (Dean et al., 1986). Several other genes, including Sparc (Figure 3.3 and Chapter 5.1), also show a decrease in steady state RNA levels during the first 24 hours after addition of inducers. Therefore, it is possible that an early event in the action of retinoic acid on F9 cells is the activation of an RNase and that the early increase in c-fos after adding inducers may be involved in this process.

Expression of exogenous c-fos genes transfected into undifferentiated F9 cells can lead to a dramatic change in their morphology and growth characteristics (Muller et al., 1984c). However, the results are confusing since although expression of exogenous c-fos mRNA and protein in the F9 cells is an absolute requirement for this change, it is, in itself, insufficient to cause the expression of the new phenotype. Some clones of apparently undifferentiated cells express high levels of c-fos and, on continued culture, give rise to cells of the altered phenotype (Ruther et al., 1985). This has led the authors to speculate that differentiation results from spontaneous events in culture which act in concert with the c-fos gene product. The presence of the polyoma origin and early region apparently enhances the frequency of differentiation, presumably by

increasing the frequency of the "spontaneous event".

The differentiated derivative does not have a morphology consistent with any known F9 derivative or other identified cell type. It appears as colonies of enlarged, flattened cells growing in an epitheloid fashion for a limited number of cell divisions. The differentiated cells express TROMA-1, TROMA-3, and type IV collagen, in common with parietal endoderm, but do not express laminin. TROMA-1 is also expressed by visceral endoderm, but these altered cells do not express AFP. The issue is further clouded because the levels of these biochemical markers varies between cells of an individual differentiated clone and between clones of cells in the same dish. Furthermore, the differentiated cells can still respond to retinoic acid and cyclic AMP, resulting in a change in morphology towards that associated with parietal endoderm and a more uniform expression of TROMA-1 (Ruther et al., 1985). The continued ability of the morphologically altered cells to respond to inducing agents implies that they are not terminally differentiated. Since an F9 EC cell line exists which constitutively expresses high levels of c-fos (E.Adamson pers. comm.), the differentiation of cells in response to transfected c-fos may be an artefact arising from the transfection process or the method of selection of transfected cells.

Are the differentiated cells derived from the undifferentiated population of F9 cells? Muller et al. (1984c) cotransfected c-fos with pSV2-neo and Ruther et al. (1985) transfected c-fos constructs with the SV40-driven neomycin gene linked in tandem. In both sets of experiments transfected cells were selected on the basis of G-418 (geneticin) resistance. These SV-neo constructs utilise the SV40 early region enhancer and promoter sequences to direct the expression of the neomycin gene which confers resistance to G-418, however, the

SV40 early region promoter is relatively inactive in undifferentiated F9 cells (Herbomel et al., 1983; Sleigh and Lockett, 1985; Gorman et al., 1985). Therefore, since the SV40 early promoter is considerably more active in differentiated F9 cells (Sleigh and Lockett, 1985; Gorman et al., 1985) and undifferentiated cells may have a lower inherent capacity to take up DNA (Sleigh and Lockett, 1985), use of the SV-neo constructs may select for an altered sub-population of F9 cells. The maximum frequency of G-418 resistant colonies reported by Muller et al. (1984c) was 300 per 10^6 (approximately 1 in 3,000) and our F9 cell cultures contain one cell showing a differentiated (parietal endoderm) phenotype per 400 scored (Cooper, 1983). This may also help to explain the observation that constructs containing the origin of replication, promoter, enhancer and amino terminal 40% of the large T antigen from polyoma virus induce most morphologically altered colonies (Ruther et al., 1984). Polyoma virus expression in EC stem cells is also blocked at the level of transcription and the enhancer becomes more active in differentiated cells (Dandolo et al., 1983; Linney and Donerly, 1983). Alternatively, it is also possible that the F9 EC cells used in these experiments can transcribe efficiently from these viral promoters.

If the c-fos gene and neomycin constructs are being transfected into, and expressed in, the F9 stem cell population, an alternative problem in the interpretation of these data arises from evidence that F9 EC cells may contain a trans-acting repressor molecule (or molecules) which reduces transcription from viral enhancers (Gorman et al., 1985). Transfection of SV40 and polyoma early regions may dilute this factor and, consequently, activate cellular genes previously repressed by the same molecule. This hypothesis predicts that the transfer of viral genes is an integral part of the process

by which c-fos induces the change in F9 cellular morphology. Moreover, the transfer of other proto-oncogenes e.g. c-ras, and even viral genes themselves, can induce differentiation of F9 cells (U.Ruther unpublished data, cited in Muller, 1986c). Therefore, the significance of the data of Muller et al. (1984c) and Ruther et al. (1985) is, as yet, unclear, although it provides evidence that c-fos expression is, in itself, insufficient to differentiate F9 cells to parietal or visceral endoderm.

The apparent discrepency between levels of c-fos RNA in parietal endoderm and F9 cells differentiated to the parietal endoderm phenotype may not reflect a major difference between the in vitro system and the developmental event which it is thought to model. My results suggest that parietal endoderm cells rapidly stop synthesising c-fos when placed in culture, therefore, factors required for c-fos expression in these cells and differentiated F9 cells may be missing from the culture medium.

3.10 Towards the function of the c-fos gene.

It has been postulated that the cellular oncogenes are involved in either growth or differentiation processes during the normal development of the organism. The induction of c-fos expression by external signals (Table 3.1) has resulted in speculation that it may regulate both proliferation and differentiation .

3.10a. C-fos and growth control.

Many of the cellular oncogenes are believed to function either as growth factors or in the transduction of growth signals. This has prompted investigators to suggest that the nuclear proto-oncogenes (c-fos, c-myb and c-myc) serve as molecular switches which direct the

b) C-fos and differentiation.

Cell type / Tissue	Agent	Derivative	Fos expression[1]	Reference
PC12 pheochromocytoma	NGF	Sympathetic neurons	Large transient increase	Kruijer et al., 1985
	dBcAMP	" "	" " "	" " " "
	Potassium	" "	" " "	" " " "
	DEX	Chromaffin cells	No change	" " " "
	EGF	No effect	Large transient increase	" " " "
U-937 monocyte	TPA	Macrophages	Large increase	Mitchell et al., 1985
HL-60 monomyelocyte	TPA	Macrophages	Large increase	Muller et al., 1984a
	Vitamin D3	Monocytes	No change	Muller et al., 1985b
	RA	Granulocytes	" "	" " " "
	DMSO	"	" "	Mitchell et al., 1985
WEHI-3B monomyelocyte	G-CSF + ActD	Monocytes	No change	Gonda and Metcalf, 1984
F9 embryonal carcinoma	RA + dBcAMP	Parietal endoderm	Small transient increase	This thesis
	RA + agg.	Embryoid bodies	3-fold induction	E.Adamson pers. comm.

1. C-fos expression is scored at the protein level in some data and as the steady-state RNA level in others.
2. The modulation in c-fos was detected after 8 hours; earlier time points were not taken.

Abbreviations.

PDGF - platelet-derived growth factor; FGF - fibroblast growth factor; EGF - epidermal growth factor; TPA - 12-O-tetradecanoylphorbol-13-acetate; CSF-1 - colony-stimulating factor 1; IL-3 - interleukin 3; dBcAMP - dibutyryl cyclic AMP; UV - ultra violet; NGF - nerve growth factor; DEX - dexamethasone; RA - retinoic acid; DMSO - dimethylsulphoxide; G-CSF - granulocyte-colony stimulating factor; ActD - actinomycin D; agg. - aggregation.

Table 3.1 Induction of c-fos.

a) C-fos and proliferation.

Cell type / Tissue	Agent	Effect	Fos expression[1]	Reference
Quiescent BALB/c 3T3 fibroblasts	Serum	Proliferation	Large transient increase	Greenberg and Ziff, 1984
	PDGF	"	" " "	" " " "
	FGF	"	" " "	" " " "
	EGF	"	No change in fos transcription	" " " "
	TPA	"	Large transient increase	" " " "
Quiescent NIH 3T3 fibroblasts	Serum	Proliferation	Large transient increase	Muller et al., 1984b
	PDGF	"	" " "	Kruijer et al., 1984
	FGF	"	" " "	Muller et al., 1984b
	EGF	"	Small transient increase	" " " "
	Wounding	"	Large transient increase	Muller et al., 1986b
Quiescent mouse embryo fibroblasts	Serum	Proliferation	Large transient increase	Muller et al., 1984b
Quiescent 208F rat fibroblasts	Serum	Proliferation	Large transient increase	Curran et al., 1985b
Quiescent primary macrophages	CSF-1	Proliferation	Large transient increase	Muller et al., 1985
	Serum	"	" " "	" " " "
Quiescent PB-3C mast cells	IL-3	Proliferation	Large increase	Conscience et al., 1986
Liver	Partial hepatectomy	Regeneration	Induced	Cited in Verma, 1986
FRTL5 rat thyroid follicular cells	Thyrotropin	Proliferation	Large transient increase	Tramontano et al., 1986
	dBcAMP	"	" " "	" " " "
A431	EGF	Growth arrest	Large transient increase	Bravo et al., 1985a
A431 clone 16	"	No effect	" " "	" " " "
A431 clone 15	"	Increased growth	" " "	" " " "
Human skin fibroblasts	UV light	Growth arrest	Small increase[2]	Angel et al., 1985
	TPA	" "	" "	" " " "

transition from the resting to the growing state. C-fos levels remain low and unchanged during the normal cell cycle in synchronously growing fibroblast populations in vitro (Bravo et al., 1986), but serum-stimulation of quiescent fibroblasts (BALB/C 3T3 cells, NIH 3T3 cells, 208F rat fibroblasts, or mouse embryo fibroblasts) is accompanied by a transient induction and accumulation of high levels of c-fos mRNA and protein (Greenberg and Ziff, 1984; Muller et al., 1984b; Curran et al., 1985b). An increase in the rate of c-fos transcription can be detected within 5 minutes of serum addition (Greenberg and Ziff, 1984) and does not require de novo protein synthesis (Greenberg et al., 1986). This suggests that stimulation of c-fos expression may be directly involved in the transduction of the growth signal, rather than a secondary response to changes the in cellular growth state.

Growth factors can act either as "competence factors" or as "progression factors" (Stiles et al., 1979). Quiescent fibroblasts exposed to a competence factor (e.g. PDGF) become "competent" for growth but require subsequent treatment with a progression factor (e.g. platelet-poor plasma, PPP) to proceed through G1 and into S phase. Treatment with PPP alone has no effect on cellular growth states. The competence factors PDGF and FGF (fibroblast growth factor) both induce transient high levels of c-fos mRNA and protein, whereas stimulation with PPP alone only weakly induces c-fos expression (Kruijer et al., 1984; Muller et al., 1984b; Bravo et al., 1985b). EGF is not a competence factor and has a weaker mitogenic effect on NIH 3T3 cells than PDGF or FGF. EGF only induces a small increase in c-fos mRNA in NIH 3T3 cells and does not stimulate c-fos transcription in BALB/C 3T3 cells (Greenberg and Ziff, 1984; Muller et al., 1984b). This suggests that c-fos functions in the transition

to the competent state . Competence can be induced in fibroblasts following release from growth arrest in S phase (Scher et al., 1979) and this is accompanied by accumulation of c-fos protein (Bravo et al., 1986). However, Bravo et al. (1986b) also find that c-fos is inducible in the G1 and G2 phases. Confluent monolayers of fibroblasts can also be made competent by wounding in the presence of PPP (Stiles et al., 1979); this procedure causes a rapid, transient induction of c-fos protein in the cells surrounding the wound (Muller et al., 1986b). Although continuous expression of high levels of c-fos is not required to maintain the competent state (Bravo et al., 1985b), these data strongly suggest that modulation of c-fos expression may be involved in the induction of competence in vitro.

There is, however, a body of data which is difficult to reconcile with the evidence presented above:

1. An increase in c-fos transcript levels accompanies growth arrest of human skin fibroblasts in S or G2 phases (Angel et al., 1985).

2. Fibroblasts transformed by c-fos remain dependent upon the presence of PDGF for growth (Bravo and Muller unpublished data cited in Muller et al., 1986c). While this implies that these cells are serum-dependent and therefore they are not fully transformed, it also suggests that if c-fos induces competence it must act in concert with other factors. A possible candidate for this role is c-myc. Growth factor induction of c-fos is commonly followed by a transient increase in c-myc transcripts (Greenberg and Ziff, 1984; Muller et al., 1984b; Curran et al., 1985b). Microinjection of c-myc protein is sufficient to render quiescent fibroblasts competent for growth (Kaczamarek et al., 1985), however, expression of c-myc genes transfected into fibroblasts allows them to enter S phase, when

cultured in PPP, but they are not completely growth factor-independent (Armelin et al., 1984). Furthermore, induction of c-myc via the activation of protein kinase C is insufficient to elicit the full mitogenic response of PDGF (Coughlin et al., 1985). It is therefore possible that c-myc and c-fos must be co-expressed to induce the full competence state in fibroblasts.

3. Treatment of A431 human epidermoid carcinoma cells with EGF results in abrupt, and complete, arrest of proliferation (MacLeod et al., 1986). This is accompanied by rapid, concentration-dependent induction of c-fos and c-myc (Bravo et al., 1985a). A similar response is detected in an A431 clone which does not respond to EGF, and in another that increases proliferation when challenged by EGF (Bravo et al., 1985a). Coupling of cyanogen bromide to EGF cleaves the molecule, rendering it non-mitogenic, but still able to bind to its receptor with 10% of the efficiency of native EGF (Shechter et al., 1979) and to induce c-fos expression (Bravo et al., 1985a).

4. The high levels of c-fos detected during mouse development are apparently unrelated to proliferation. First, if the high levels of c-fos mRNA detected in extra-embryonic tissues reflect their high rate of growth during development, why are only low levels detected in the embryo proper, which is also growing very rapidly? Second, amnion cells stop synthesising c-fos when placed in culture, yet they retain their proliferative capacity (Muller et al., 1986a). Third, although no detailed study of the proliferative capacity of parietal endoderm cells has been published, the available data suggest that parietal endoderm cells have a low mitotic index and that most parietal endoderm cells arise by division from a primitive endoderm population or from visceral endoderm. This suggests that c-fos expression within the parietal endoderm is not related to

proliferation.

3.10b. C-fos and differentiation.

As discussed previously, the transfection of c-fos into F9 teratocarcinoma stem cells, together with other unidentified processes, can induce a dramatic change in cellular phenotype. The relevance of this to actual developmental events remains unclear, but evidence is accumulating that suggests that c-fos plays a part in other differentiation pathways.

Both murine bone marrow and primary cultures of macrophages express relatively high levels of c-fos (Muller et al., 1984a). A rapid increase in c-fos expression, accompanies the differentiation of HL-60 monomyelocytic cells and U-937 monocytic cells into macrophages in vitro but levels do not change when HL-60 cells differentiate into monocytes or granulocytes (Table 3.1). Following a 30-fold transient increase in c-fos transcripts during macrophage differentiation; levels of c-fos mRNA remain about 10-fold greater than those detected in untreated HL-60 cells although protein rapidly diminishes (Muller et al., 1984a; Verma et al., 1985) In contrast, a large decrease in c-myc is detected during macrophage differentiation.

c-fos is also regulated by the growth state of cultured haematopoeitic cells. Lack of colony stimulating factor 1 or serum-starvation causes primary cultures of bone marrow macrophages to become quiescent. The re-addition of either agent causes the cells to proliferate, with a concomitant increase in c-fos and c-myc (Muller et al., 1985b). The expression of c-fos and c-myc in growth-stimulated macrophages differs from that of cultured fibroblasts in that c-myc expression precedes c-fos by several hours and that high

levels of c-fos are detected in asynchronous cultures of proliferating macrophages. Similarly, interleukin-3 stimulation of PB-3C mast cell proliferation results in the induction of c-fos and c-myc (Conscience et al., 1986).

A second system in which c-fos is differentially regulated in vitro during differentiation is the differentiation of PC12 pheochromocytoma cells to either sympathetic neurons or chromaffin cells (Table 3.1). Differentiation to sympathetic neurons is accompanied by a transient increase in c-fos, whereas no increase accompanies differentiation to chromaffin cells.

3.10c. Conclusions and perspectives.

At the present time it is difficult to assimilate the wealth of data on c-fos expression which has already accumulated into a coherent hypothesis for its function. This could be due, at least in part, to problems in extrapolating from data obtained largely from cell culture systems. Most cultured cells were initially derived from tumours and, therefore, may have a very different biochemistry and physiology from the cell type and cellular processes which they are thought to model in vivo. Another problem is that c-fos is regulated at both the transcriptional and translational levels - much of the preceding data was obtained purely from studies on c-fos transcription and may therefore be incomplete.

C-fos protein accumulates rapidly in the nucleus where it presumably performs its cellular function. With the possible exception of cultured macrophages, its expression is regulated or maintained in vitro by a wide variety of extracellular signals. C-fos may function in the coupling of these signals to transcriptional processes. Certain of the factors which regulate its expression (e.g.

PDGF and 12-O-tetradecanoylphorbol-13-acetate) also activate protein kinases. Thus, kinase activation may provide the route by which c-fos can be induced without protein synthesis in many different systems.

A single, common function for c-fos, which could account for its stimulation in both proliferative and differentiation processes (e.g a response to stress or the destruction of existing nuclear transcripts prior to a change of state), is difficult to reconcile with it not being universally induced in these events. It is also possible that the c-fos protein is multifunctional with its role being determined by the cell type within which it is expressed either by modification or by interaction with other factors (e.g. p39 or c-myc).

CHAPTER 4

The cloning and immunological characterisation of SPARC - a novel marker of parietal endoderm

Introduction

The differential screening of a cDNA library, using probes from two different RNA populations, can select for sequences which have different abundances in the tissues from which probes were prepared. This approach has been successfully applied to the isolation of developmentally regulated mRNAs from both Dictyostelium discoideum (Williams and Lloyd, 1979) and Xenopus laevis (Dworkin and Dawid, 1980). In these studies the same technique was utilised to isolate genes which are preferentially expressed in the parietal endoderm. The use of parietal endoderm and visceral yolk sac cDNA probes in the differential screening of a cDNA library facilitated the isolation of cDNAs from transcripts that are differentially expressed in these tissues. These cloned markers can subsequently be used in investigations into gene regulation during the binary decision which gives rise to either parietal endoderm or visceral endoderm during murine development. For this purpose, the markers would ideally be expressed at only low levels in the primitive endoderm, however, the small cell number and inaccessibility of this tissue precluded its use in the isolation and characterisation of the cDNAs. This problem was partially overcome by the use of the F9 EC cell line to model the

differentiation of parietal endoderm *in vitro*. The cDNAs were isolated from a library prepared from poly (A)+ RNA from F9 cells differentiated to a parietal endoderm phenotype, ensuring that they were expressed in the model system. Their expression in both differentiated and undifferentiated F9 cells was subsequently examined on Northern blots.

This chapter describes the isolation of cDNA clones containing overlapping sequence spanning the length of a 2.2Kb mRNA that was originally identified by the differential screening approach. This transcript is expressed at higher levels in parietal endoderm than in the visceral yolk sac and, unlike the *c-fos* gene (Chapter 3), its expression is greatly increased when F9 cells differentiate to a parietal endoderm phenotype. The primary sequence of the transcript is described and the conceptual translation of this sequence has been used to identify and characterise the protein which it encodes.

Results

4.1 *Isolation of overlapping cDNA clones differentially expressed in the parietal endoderm and visceral yolk sac.*

4.1a. Differential screening of a cDNA library from differentiated F9 teratocarcinoma cells.

In order to isolate cDNA clones from transcripts which are differentially expressed in the parietal and visceral endoderm, and also expressed in F9 cells differentiated to a parietal endoderm phenotype, a cDNA library prepared using poly(A)+ RNA from differentiated F9 cells was screened with parietal endoderm and visceral yolk sac cDNA probes (Kurkinen *et al.*, 1983a; M.Kurkinen

unpublished data). The visceral yolk sac cDNA probe was largely derived from visceral endoderm transcripts since the mesoderm only contributes about 20% of the total RNA of the visceral yolk sac (Andrews et al., 1982b). The essential details of this library (see also Figure 4.1) were that it was prepared from poly(A)+ RNA from monolayers of F9 cells that had been exposed to retinoic acid and dibutyryl cyclic AMP for 5 days. This RNA was size-selected to enrich for transcripts encoding the high molecular weight basement membrane components laminin, type IV collagen and entactin, as determined by cell-free translation followed by immunoprecipitation and SDS-PAGE. cDNA copies of the enriched fractions were inserted into the PstI site of the vector pAT153 (Twigg and Sherrat, 1980) using homopolymer tails and transformants were selected by growth on agar containing tetracycline. A library of 1200 E.coli HB101 transformants was generated and picked into individual microtitre wells for storage and screening. The library was replicated onto nitrocellulose filters and grown on agar plates containing the antibiotic tetracyclin. The colonies were grown for 10 hours, lysed in situ and the liberated DNA was denatured and bound to the filters. The library was differentially screened with ^{32}P-labelled cDNA probes prepared from 100ug of total RNA from parietal endoderm and visceral yolk sac (Williams and Lloyd, 1979; Kurkinen et al., 1983a) . A total of 54 colonies containing cDNA clones which apparently hybridised preferentially to the parietal endoderm probe were isolated from this primary screening and were designated pF9.1 to pF9.54 (Figure 4.2A and M.Kurkinen unpublished data). Plasmid DNA was prepared from all of these colonies and the uncut DNA subjected to electrophoresis on 0.7% (w/v) agarose gels, transferred to nitrocellulose, and hybridised, in a secondary screening, with the same two cDNA probes

Figure 4.1 Construction details of cDNA libraries used to isolate clones.

A Differentiated F9 library (Kurkinen et al., 1983a)

20mg total cellular RNA from differentiated F9 cells

Size select RNA to be larger than 28S on a Biogel A-50M column

Translate in vitro and pool fractions containing transcripts for basement membrane proteins

Prepare poly(A)+ RNA from pooled fractions

Synthesise first cDNA strand on 20µg poly(A)+ RNA with reverse transcriptase

Cleave hairpins with nuclease S1

Synthesise second strand with reverse transcriptase

Add C-tail with terminal transferase

Anneal into pAT153 which has been G-tailed at the Pst1 site

Transform into E.coli HB101

B Parietal endoderm library (Kurkinen et al., 1983b)

25µg poly(A)+ RNA from parietal yolk sacs

Synthesise both cDNA strands with reverse transcriptase using oligo dT14 as a primer

Fill in with klenow fragment

Ligate to Sal1 linkers

Cleave hairpins with nuclease S1

Ligate to EcoR1 linkers

Digest to completion with Sal1 and EcoR1

Size-select cDNA to be larger than 500bp on a sepharose 4B column

Ligate into pUC8 at the EcoR1 and Sal1 sites

Transform into E.coli DH1

C Parietal endoderm library (This thesis)

2.5µg poly(A)+ RNA from parietal yolk sacs dissected free of trophoblast

Synthesise first cDNA strand with oligo dT14 and a single-stranded primer derived from an existing clone

Synthesise second strand with DNA polymerase 1 in the presence of RNase H

Add G-tail with terminal transferase

Anneal into pUC8 which has been C-tailed at the Pst1 site

Transform into E.coli DH5

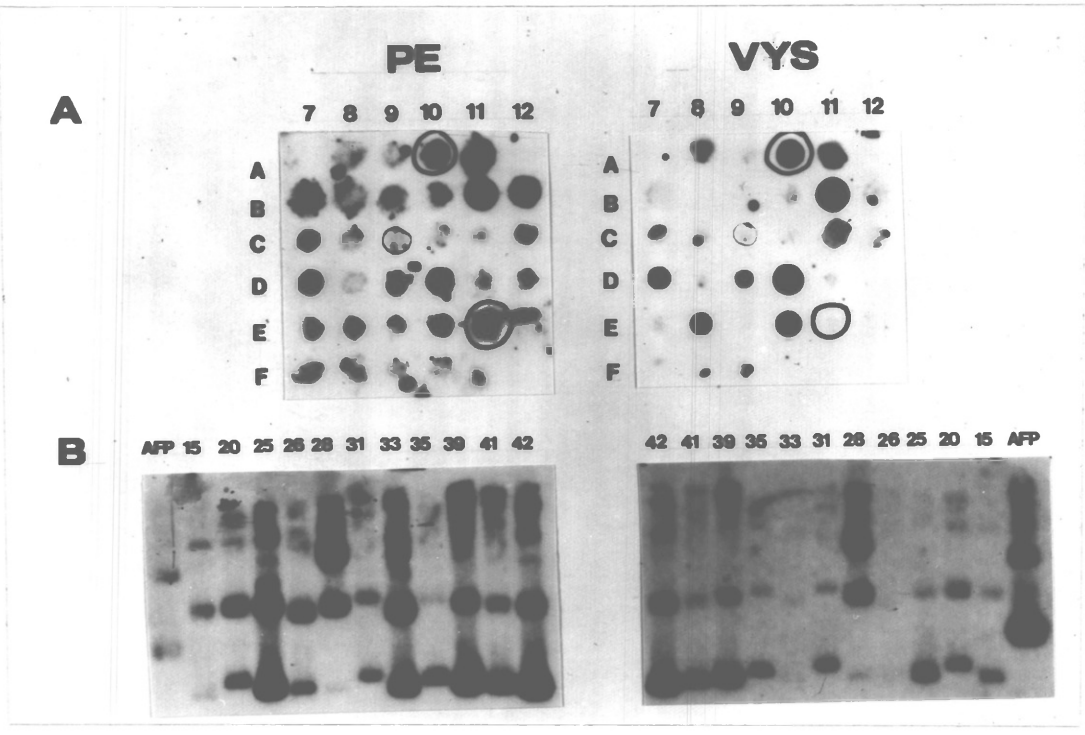


Figure 4.2 **Identification of parietal endoderm-specific cDNAs**

A. Duplicate copies of the differentiated F9 cDNA library were grown on nitrocellulose, lysed in situ, fixed and baked. These were differentially hybridised to cDNA probes prepared from parietal endoderm and visceral yolk sac RNA at a probe concentration of about 10^6 cpm/ml. The filters were washed at high stringency and exposed to X-ray film for 3 days. The left hand panel shows part of microtitre plate 2 probed with parietal endoderm (PE) cDNA and the right panel shows a duplicate filter probed with visceral yolk sac (VYS) cDNA. The colony in position E11 contained clone pF9.33.

B. Secondary screening of a selection of the 54 parietal endoderm "positive" pF9 series of clones. Uncut plasmid DNA was electrophoresed on a 0.7% (w/v) agarose gel and transferred to nitrocellulose. Duplicate filters from the same gel were probed with PE or VYS cDNA probes under the same conditions as in A. The filters were exposed to X-ray film for 4 days. The AFP clone (pAFP1) was included as a positive control for the VYS probe and to check the contamination of the parietal endoderm probe by visceral endoderm RNA. The figures were provided by Dr M.Kurkinen.

(Figure 4.2B and M.Kurkinen unpublished data). On the basis of the results of the secondary screening, the recombinants were divided into 3 groups:

Group 1. Clones which hybridised strongly to parietal endoderm cDNA and only very weakly to visceral yolk sac cDNA (e.g. clones pF9.26 and pF9.33 in Figure 4.2B).

Group 2. Clones which hybridised to transcripts of approximately equal abundance in both tissues (e.g. clone pF9.42 in Figure 4.2B).

Group 3. Clones which hybridised to transcripts which were only slightly less abundant in visceral yolk sac RNA than in parietal endoderm RNA (e.g. clone pF9.31 in Figure 4.2B).

There are a number of possible explanations for the difference between the hybridisation results from the primary and secondary screening. Probably the most important single factor was that the visceral yolk sac cDNA probe used in the initial screening had a lower specific activity than the parietal endoderm probe (M.Kurkinen pers. comm.). However, false positives are commonly detected in the differential screening of DNA liberated from lysed bacterial colonies and may arise from non-specific binding of the probe to certain colonies (J.Williams pers. comm.). The non-specific hybridisation to bacterial debris was eliminated by secondary screening of purified plasmid DNA on Southern blots. In addition, the heterogeneous nature of the probes may also contribute to this effect, causing higher non-specific "background" signals than would be obtained if a homogeneous probe was used.

The rest of this chapter concerns my studies on a number of clones selected from Group 1.

4.1b. Hybridisation of the Group 1 clones to RNA on Northern blots.

The nature of the transcripts from which the group 1 clones were derived was investigated by Northern blot analysis. Hybridisation of the nick-translated clones to total parietal endoderm and visceral yolk sac RNA on Northern blots yielded 4 clones which hybridised to 2.2Kb transcripts (pF9.25, pF9.33, pF9.52 and pF9.54; Figure 4.3A). The size of the transcripts were determined by their migration relative to 18S (2,060 nucleotides; Wang and Gudas, 1983) and 28S (5,303 nucleotides; Wang and Gudas, 1983) rRNA standards. Information, obtained by densitometry scanning of these autoradiograms, suggested that the 2.2Kb transcripts were approximately five-fold more abundant in the parietal endoderm than in the visceral yolk sac. Furthermore, the levels of the transcripts were significantly elevated in both F9 cells differentiated to a parietal endoderm phenotype and in the parietal endoderm cell line, PYS-2, compared to undifferentiated F9 cells (Figure 4.3B and data not shown). One of these clones, pF9.25, also hybridised to a 0.7Kb transcript of approximately equal abundance in both parietal endoderm and visceral yolk sac RNA (Figure 4.3A).

To determine whether all four clones hybridised to the same 2.2Kb transcript, DNA from each clone was digested with the restriction endonuclease PstI to separate the cDNA insert from vector sequences. The digested DNA was separated on an agarose gel, transferred to nitrocellulose, and probed with the nick-translated cDNA insert from plasmid pF9.33. This cDNA insert hybridised strongly to the cDNA sequences of the other three clones, but not to vector sequences on the same filter (Figure 4.3C). All four cDNAs must therefore have been derived from the same, or closely related, 2.2Kb transcripts. The sequence of the four cDNA inserts was determined by the chemical

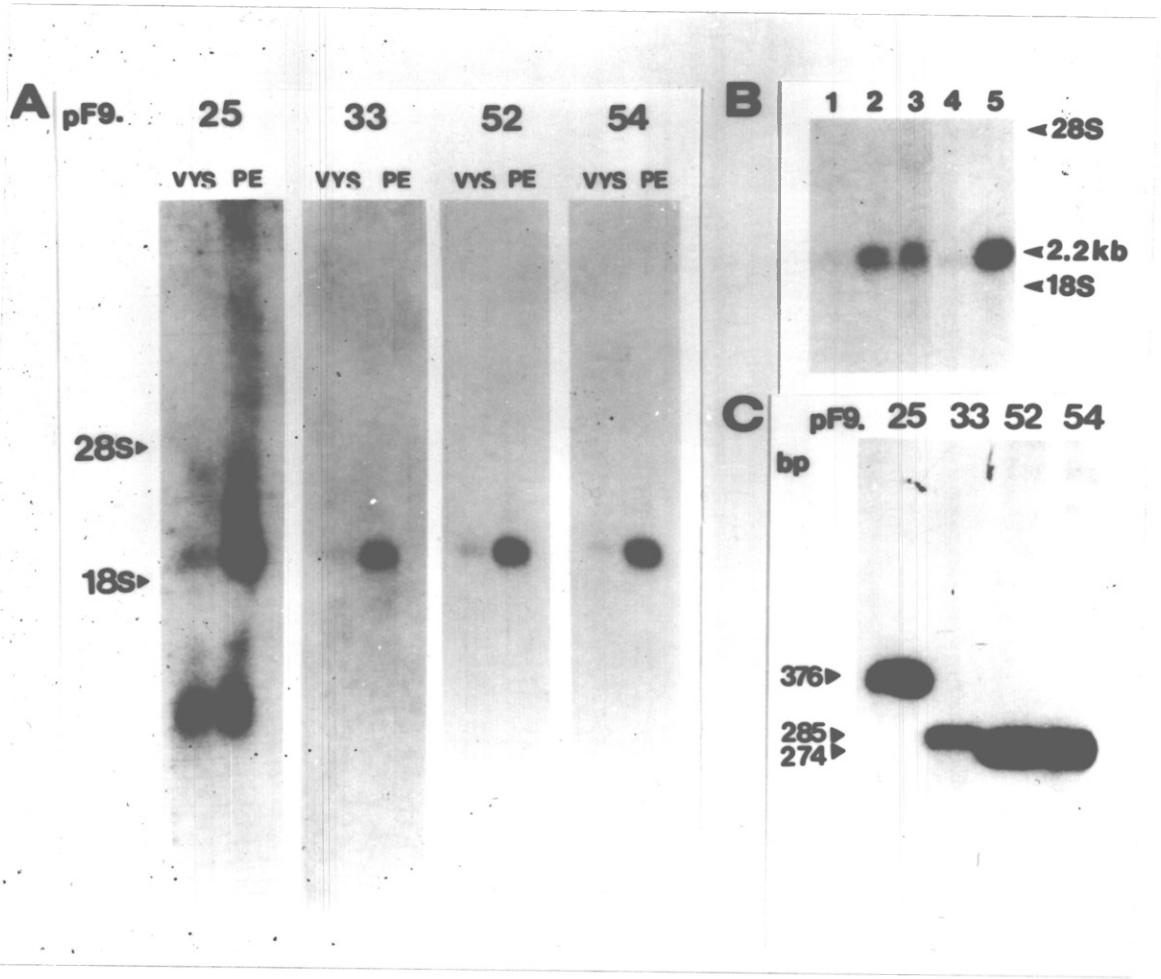

A
pF9.
25
33
52
54
VYS PE
VYS PE
VYS PE
VYS PE
28S
18S
B
1 2 3 4 5
28S
2.2kb
18S
C
pF9. 25 33 52 54
bp
376
285
274

Figure 4.3 **Characterisation of four parietal endoderm-specific cDNAs**

A. Hybridisation of differentially expressed clones to parietal endoderm and visceral yolk sac RNA. 10µg of total cellular RNA from parietal endoderm dissected-free of trophoblast (PE), and visceral yolk sac (VYS) RNA was standardised by ^{3}H-poly(U) assay and electrophoresed on a 0.75% (w/v) formaldehyde/agarose gel. The RNA was transferred to nitrocellulose by the Northern blot technique, and separate strips were hybridised with the nick-translated cDNAs pF9.25, pF9.33, pF9.52 and pF9.54. The filters were washed at high stringency and exposed to X-ray film overnight. Hybridisation was evaluated by densitometry scanning. The positions of the 28S and 18S ribosomal RNAs are marked on the figure and were obtained by staining the gel with ethidium bromide prior to transfer of the RNA.

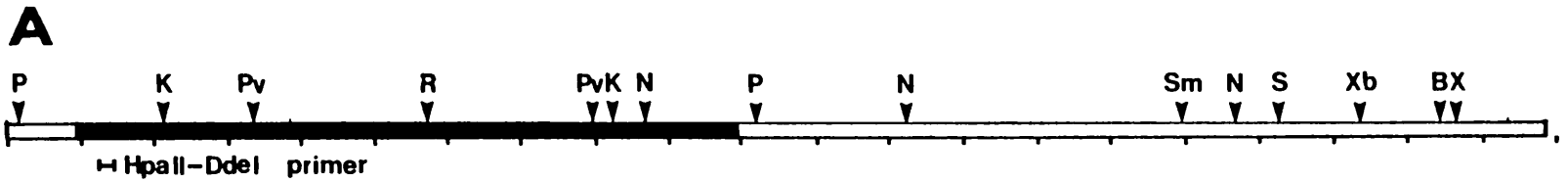
B. Differential expression of a 2.2Kb mRNA, hybridising to pF9.33, in F9 teratocarcinoma cells. Clone pF9.33 was hybridised to 10ug of total cellular RNA from undifferentiated F9 cells (lane 1); F9 cells treated with retinoic acid and dibutyryl cyclic AMP for 5.5 days (lane 2); PYS-2 cells (lane 3). In a separate experiment, it was hybridised to 10µg of total RNA from visceral yolk sac (lane 4) and parietal endoderm (lane 5). Technical details were as described in A.

C. Cross-hybridisation of the cDNA inserts from the pF9 clones. The cDNA clones, pF9.25, pF9.33, pF9.52 and pF9.54, were digested with PstI and the products analysed on a 1.5% (w/v) agarose gel. Following transfer to nitrocellulose by the Southern blot procedure, these were hybridised with the nick-translated insert from clone pF9.33. The blots were washed at high stringency and exposed to film for 4 hours. The sizes of the hybridising fragments were estimated from the position of DNA markers (HinfI fragments of pAT153).

cleavage procedure of Maxam and Gilbert (1980) and analysis of this data confirmed that all four clones contained overlapping regions of homologous sequence. The nucleotide sequence was identical within these regions - indicating that the cDNAs were all derived from transcripts of the same gene (see Figure 4.4 for their relative alignment). Clones pF9.52 and pF9.54 spanned an identical sequence of 240 nucleotides, suggesting that they were derived from the same transformant which may have been picked twice into the microtitre wells. Clone pF9.33 had a run of 22 adenosine residues beginning 13 nucleotides downstream of a canonical hexanucleotide polyadenylation signal (AATAAA; Proudfoot and Brownlee, 1976). This indicated that all four clones had been derived from the 3' end of the 2.2Kb RNA and the presence of the poly(A) tail and poly(A) addition signal suggestedthat it might be an mRNA transcript.The sequence ofclone pF9.25 began at the poly(A) tail and was identical to sequence obtained from the other three clones for 178 nucleotides 5' to the poly(A) tail (see Figure 4.4). However, the 122 nucleotides which preceded this region of complete identity had no homology to sequence obtained from clones pF9.33, pF9.52 and pF9.54, which indicated that this 122bp region of pF9.25 hybridised to the 0.7Kb RNA detected on Northern blots.

The small sizes, extreme 3' location, and conceptual translation of these clones suggested that they contained little, if any, coding sequences; therefore, to facilitate the further characterisation of this gene longer cDNAs were sought.

Figure 4.4



B

100 bp

clone	nucleotides
pF9.33	1870–2070
pF9.52	1813–2053
pF9.54	1813–2053
pF9.25	1892–2070
pPE.30,7,27	570–2079
pPE.20	809–2079
pPE.220,144	570–1162
pIM.3	1–1162
pIM.22	5–1162
pIM.27	8–1162
pIM.39	196–733
pIM.40	370–1162

Figure 4.4 **Restriction mapping and alignment of cDNA clones from the 2.2Kb transcript.**

A. Restriction map generated by analysis of the cDNA clones encoding the 2.2Kb transcript. The long open reading frame obtained from the translation of the sequence data obtained from cDNAs (Figure 4.6) is filled in. The position of the HpaII-DdeI primer used in the primer extension studies is indicated below the restriction map.

Restriction endonulease sites are: B: BlgII; K: KpnI; N: NcoI; P: PstI; Pv: PvuII; R: EcoRI; S: SacI; Sm: SmaI; X: XmaI; Xb: XbaI.

B. Relative alignment of cDNA clones derived from the 2.2Kb transcript as determined from restriction mapping and sequence analysis. The broken line indicates the region of pF9.25 that is not present in the other cDNAs and both A and B are drawn to the same scale to facilitate direct comparisson.

4.1c. Screening parietal endoderm libraries to isolate longer cDNAs from the 2.2Kb transcript.

To isolate longer clones, the cDNA insert from pF9.33 was used to screen a parietal endoderm cDNA library provided by M.Kurkinen (Figure 4.1B; Kurkinen et al., 1983b). Thirty clones, which hybridised to pF9.33, were obtained from screening 22,000 recombinants, these were designated pPE.1 to pPE.30, and restriction mapping and comparisson with the pF9 series of clones indicated that all of these cDNAs extended from the region of the poly(A) tail. The longest clones - pPE.30, pPE.7, and pPE.27 - had insert sizes of approximately 1500bp (Figure 4.4B). On Northern blots of parietal endoderm RNA, the cDNA insert from pPE.30 only hybridised to a 2.2Kb RNA and did not hybridise to the 0.7Kb transcript (see also Figure 4.5).

In order to obtain clones extending closer to the cap site of the 2.2Kb RNA, an EcoRI-PstI fragment spanning the most 5' region of pPE.30 (Figure 4.4A) was used to re-screen the same library. This screening produced 365 hybridising colonies. Ninety-six of these were picked, re-screened, and plasmid DNA was prepared from them using the "miniprep" procedure. Restriction mapping revealed that all 96 clones contained an identical insert of about 590 nucleotides (see for example pPE.220, pPE.144 and pPE.596 in Figure 4.4B) which extended to the same position as the EcoRI site at the 5' end of the pPE.30 cDNA. The presence of an EcoRI site at exactly the same position in so many cDNAs indicated that this enzyme site was already present in the cDNA sequence, and was not the result of linker addition. In the construction of this library, SalI linkers were ligated to the 3' end of the cDNA and EcoRI linkers were added to the 5' end. The cDNA was then digested with SalI and EcoRI prior to ligation into the pUC8

vector at its EcoRI and SalI sites. The presence of an internal EcoRI site in the cDNA would result in the loss of the sequences 5' to the internal site from the library - these fragments would have EcoRI sites at both ends and, consequently, would not be cloned efficiently. Such a cDNA fragment could be cloned, as a low-frequency event, in association with a separate EcoRI-SalI fragment, but a probe which spanned the EcoRI site in the cDNA would have provided the only means of identifying such a recombinant. Since all 96 clones had cDNA inserts of the same size, they must have been primed from the same position with oligo dT_{14}. Subsequent sequence analysis of clones pPE.30, pPE.7, and pPE.27 (Chapter 4.2 and Figure 4.6) revealed a string of 16 adenosine residues within the 2.2Kb transcript 592 nucleotides 3' to the EcoRI site in the cDNAs and demonstrated that clones pPE.220, pPE.144 and pPE.596 had been internally primed from this sequence.

Clone pPE.30 contained 1509 nucleotides preceeding the poly(A) tail. Thus, after 100 residues, the estimated average size of a poly(A) tail (Brawerman, 1981) were deducted from the estimated transcript size (2.2Kb), it was predicted that about 600 nucleotides of the transcript remained to be cloned. This was in good agreement with the sizes of the transcripts of both AFP (Miura et al., 1979) and c-fos (Van Beveren et al., 1983), both of which comigrated with the 2.2Kb transcript on Northern blots.

In order to isolate the missing sequences, a second parietal endoderm library was constructed. The essential details of the preparation of this library are as follows. In addition to excess oligo dT_{14}, which would prime from the internal run of adenosines as well as the poly(A) tail, a single stranded primer, generated from sequences close to the EcoRI site of pPE.30, was used to prime the

first cDNA strand. The primer was prepared from a KpnI-PstI restriction fragment of pPE.30 (Figure 4.4). The primer strand complementary to the 2.2Kb transcript was separated from the "message" strand. This was achieved, following denaturation, by electrophoresis on an 8% non-denaturing polyacrylamide gel and the single-stranded primer was purified from the gel by electroelution. The cDNA library was constructed using a modification of the method of Gubler and Hoffman (1983). This protocol eliminates the nuclease S1-mediated cleavage of the hairpin loop in the double stranded cDNA which often results in the loss of 5' terminal sequences (Land et al., 1981). Pilot reactions were used to optimise the first and second strand cDNA synthesis conditions to produce products of average length 1 to 1.5Kb. The library was prepared from 2.5µg of parietal endoderm poly(A)+ RNA with a calculated excess of the two primers. This produced approximately 0.8µg of double stranded cDNA as estimated from the incorporation of a radioactive tracer. One tenth of the cDNA was analysed on a 1% (w/v) agarose gel together with a track of labelled "1Kb ladder" DNA markers (BRL). The gel was dried down onto Whatman 3MM paper and exposed to X-ray film. The autoradiogram revealed that the average size of the double stranded cDNA was about 1.5Kb, with the largest molecules detected being >5Kb. This suggested that clones extending to the RNA cap site of the 2.2Kb transcript could be expected. Approximately 200ng of the cDNA was G-tailed in the presence of cobalt ions to allow the terminal transferase to utilise recessed, as well as extended, 3' ends. Pilot reactions were performed to determine the optimum ratio for annealing cDNA to C-tailed pUC8 vector - for this preparation of cDNA and vector, the optimum annealing conditions, in terms of transformants/µg cDNA, were found to occur at the ratio of 1ng cDNA

to 30ng vector. To prepare the library 15ng of cDNA was annealed to vector and transformed into competent (10^8 cfu/μg) E.coli DH5. The annealing titrations indicated that this would produce a library of approximately 10^4 recombinants. The library was plated and grown on nylon membranes laid on agar plates which contained ampicillin select for transformants. However, only $4x10^3$ transformants were obtained; the cause of this 10-fold reduction was subsequently traced to the particular batch of nylon filters on which the library was grown.

In order to eliminate recombinants which were derived from any contaminating double stranded primer, the library was screened with the EcoRI-KpnI fragment of pPE.30 located 5' to the KpnI-PstI primer fragment (see Figure 4.4). Forty positive signals were obtained (designated pIM.1 to pIM.40) and restriction mapping revealed that 8 of these recombinants contained sequences 5' to the EcoRI site in pPE.30. Five of these clones terminated at approximately the same position; 550bp upstream of the EcoRI site (pIM.3, pIM.22, pIM.27 in Figure 4.4B and pIM.20, pIM.38 data not shown). This suggested that either the 5' end of the transcript had been reached or a region of strong RNA secondary structure had prevented further extension of the cDNA. Sequencing subsequently revealed that six of the eight clones had been primed from the position of the internal run of adenosine residues (nucleotides 1147-1162 Figure 4.6) and one, pIM.38, had been primed from the restriction fragment primer; indicating that both strategies had generated clones from the 2.2Kb transcript.

4.1d. Isolation of cDNA clones from the 0.7Kb transcript.

In order to further determine the relationship of the 0.7Kb transcript to the 2.2Kb transcript, both of the parietal endoderm cDNA libraries were differentially screened with the cDNA inserts from pF9.25 and pF9.33. No signals, which were exclusive to the pF9.25 insert, were obtained from the library prepared with linkers. This was probably due to the size-selection of the cDNA in the construction of the library since 6 signals which were specific to pF9.25 were obtained from the G-C tailed parietal endoderm library (Figure 4.5A). DNA was isolated from these colonies (designated pTF.47, .51, .56, .60, .71 and .75) and the relationship of their cDNA inserts was determined by restriction mapping (Figure 4.5B). Analysis of this data revealed that pTF.75 contained all of the sequences present in the other clones. When pTF.75 was used to probe parietal endoderm RNA on a Northern blot, it only hybridised to the 0.7Kb transcript. Therefore, it was concluded that the 0.7Kb and 2.2Kb transcripts were unrelated and that sequences derived from both RNAs were present in pF9.25 as the result of an artefact of the cloning procedure during the construction of the F9 cDNA library

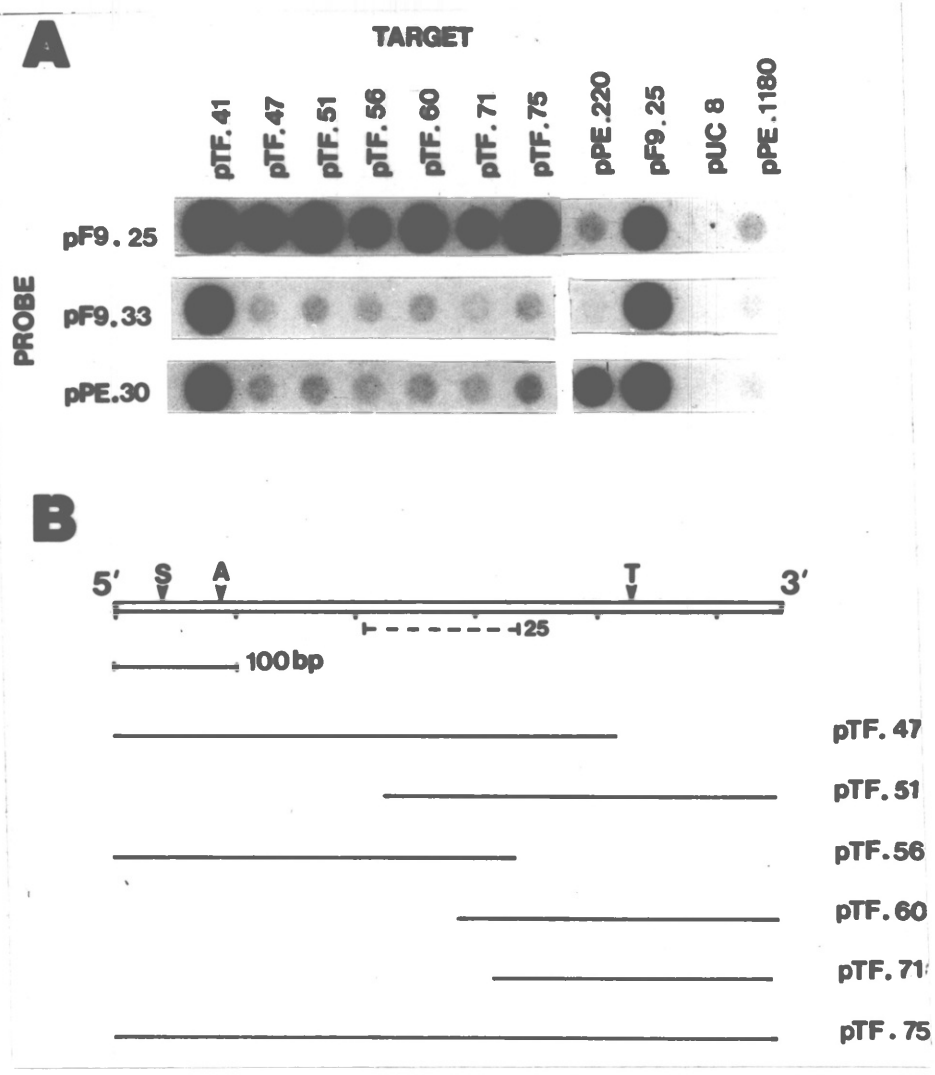


Figure 4.5 **Identification and alignment of cDNAs from the 0.7Kb transcript.**

A. Secondary screening of the clones derived from the 0.7Kb transcript. 1µg of DNA from each of the clones isolated from the primary screening of the library was hybridised with the ^{32}P-labelled cDNA inserts from clones pF9.25, pF9.33 and pPE.30. The clones pPE.220, pF9.25, pUC8 (vector) and pPE.1180 (type IV collagen; Kurkinen et al., 1983b) were included as positive and negative controls. Hybridisation and washing were performed at high stringency and the blots were exposed overnight.

B. Alignment and preliminary restriction map of the cDNAs derived from the 0.7Kb transcript. The position of the homologous sequences present in pF9.25 is indicated by the broken line. Restriction enzyme cutting sites are: S: SacI; A: AvaII; T: TaqI.

4.2 Sequencing of cDNA clones and primary sequence of the predicted protein

All of the clones derived from the 2.2Kb transcript that are illustrated in Figure 4.4, were sequenced. This eliminated any artefacts which may have arisen during the cloning procedures since sequence from each region of the transcript was obtained from at least three different cDNAs. The DNA sequence was always determined from both strands of each clone; this procedure confirms that the sequence data is correct and is particularly important because it removes errors which arise from band compressions on the autoradiograms of sequencing gels and from base modifications e.g. methylation. In my experience, the former is most often encountered when using the chain termination sequencing procedure of Sanger et al. (1977) and the latter only arises when the chemical cleavage procedure (Maxam and Gilbert, 1977, 1980) is used. Both of these sequencing techniques were employed in this study.

4.2a. Sequencing the "pF9" and "pIM" series of clones.

The sequence of the "pF9." and the "pIM." series of clones was obtained by the chemical cleavage method. This technique is dependent on the availability of convenient restriction enzyme sites, both for labelling the DNA strand to be sequenced and the subsequent purification of the labelled fragment. DNA was cleaved with a restriction enzyme which cut at a site adjacent to the region of the clone from which sequence was to be obtained. The terminal nucleotide of the strand to be sequenced was labelled with ^{32}P. Following inactivation of the labelling enzyme and the removal of unincorporated label, the DNA was cleaved with a second restriction enzyme. The fragment to be sequenced was separated from other

labelled fragments on a low melting-point agarose gel and purified by the glass powder technique. The uniquely-labelled DNA was partially cleaved at each of the four bases in four separate reactions using the chemical cleavage procedure of Maxam and Gilbert (1980) and the products of the sequencing reactions were separated in adjacent lanes on 35cm denaturing (urea/acrylamide) gels at high voltage. The gels were fixed, dried onto Whatman 3MM paper and exposed to X-ray film. Sequence could be obtained from up to 260 nucleotides adjacent to the labelled site using a single set of cleavage reactions if the reaction products were analysed on one 20% gel, and three staggered loadings on an 8% gel. The duration of the exposures which were required to obtain clear sequence data varied from a few hours, for sequence very close to the labelled nucleotide, to 7 days for those which were most distant.

4.2b. Sequencing the "pPE" series of clones.

The nucleotide sequences of the "pPE" series of clones was derived using the chain termination procedure. The cDNA inserts of clones pPE.30, pPE.7 and pPE.27 were subcloned directly into the vectors pEMBL 8+, 8-, 9+ and 9- (Dente et al., 1983). These vectors contain the β-lactamase gene, the multiple cloning site from either pUC8 or pUC9 located in the β-galactosidase gene and a region of phage f1 DNA which contains the f1 origin of replication. The latter element confers upon the pEMBL vectors the property of being encapsidated as single stranded DNA when the bacteria in which they are maintained (DIH101; D.Ish-Horowicz unpublished) are superinfected with phage f1. pEMBL DNA is incorporated into the secreted virion capsids as efficiently as phage f1 DNA and either plasmid strand is specifically encapsidated, dependent upon the orientation of the f1 origin of

replication within the vector. In the pEMBL+ vectors the antisense strand of the β-galactosidase gene is found in the virions and in the pEMBL- vectors the sense strand is incorporated. Bacteria transformed with pEMBL DNA are resistant to ampicillin and recombinants can be scored when the bacteria are plated on agar containing the chromogenic substrate X-Gal.

The cDNA inserts were purified from the three clones as EcoRI-HindIII fragments. These were subcloned into the EcoRI and HindIII sites of all four pEMBL vector, so that sequence data could be obtained from both ends of the cDNAs and in both strands. Recombinants were picked and viral particles, containing single-stranded template were isolated following the superinfection of log phase cultures of these bacteria with f1. The pEMBL and f1 DNA was isolated from the virions which accumulated in the medium over the following 5-7 hours. The template DNA was sequenced by the chain-termination procedure of Sanger et al. (1977), as modified for use with ^{35}S-labelled nucleotides. Chain extension was initiated from the M13 universal sequencing primer (Messing, 1983) which hybridises specifically to the pEMBL template, thus making it unnecessary to purify the template free of f1 DNA. The products of the sequencing reactions were analysed on 60cm denaturing, buffer-gradient gels (Biggin et al., 1983). The gels were fixed, dried down, and exposed to X-ray film for 2-4 days. Using the combination of ^{35}S label and gradient gels, it was possible to obtain the sequence of up to 350 nucleotides adjacent to the sequencing primer from a single set of reactions in one loading.

A similar "forced cloning" approach, using the vectors M13mp8 and M13mp9 (Messing and Vieira, 1982), was used to completely sequence the smaller clones, pPE.220, pPE.144 and pPE.596. The cDNA insert of

each of these clones was isolated as an EcoRI-HindIII fragment. The purified insert was then cleaved at the PstI site (see Figure 4.4) and the two fragments produced were subcloned directionally into both M13 vectors. Single-stranded template DNA for the sequencing reactions was prepared from recombinant (colourless) plaques and was sequenced as described above.

To obtain the sequence of the remaining regions of clones pPE.30, pPE.7 and pPE.27 a "shotgun" approach utilising the chain termination sequencing technique (Bankier and Barrell, 1983) was employed. 30µg of the cDNA insert from clone pPE.30 was purified and "end-polished" with the Klenow fragment of DNA polymerase I. It was then self-ligated to form "closed-circles" and the ligated DNA was fragmented by sonication. Pilot sonications, initially on vector DNA and then on the ligated insert of pPE.30, had demonstrated that sonication on ice for 3 minutes would generate fragments of 200-400bp which is the optimal size for sequencing. Sonication was performed in 5 second bursts for a total of 3 minutes, allowing 10 seconds between bursts for cooling. The DNA was then blunt-ended with T4 polymerase. The fragments which had been generated were separated on a 2% native low melting-point agarose gel and fragments between 100 and 400bp were extracted, purified, and ligated into M13mp8 at the SmaI site. The ligation products were transfected into competent ($5x10^6$ cfu/µg) E.coli JM101 (Messing et al., 1981). However, after plating out the bacteria and allowing them to grow overnight, only 30 recombinant (colourless) plaques were obtained. Single-stranded DNA was prepared from these recombinants and sequenced as described above. The sequence which was obtained was assembled using a programme on the ICRF DEC20 computer.

As expected, due to the small number of clones which were

generated, certain areas of sequence were only obtained in one strand and there were some discontinuities in the sequence which prevented the assembly of a continuous sequence. The problem which caused so few shotgun clones to be isolated was traced to the sonication procedure, but it was not identified (C.Pears and J.Williams pers. comm.). Therefore, I adopted an alternative shotgun procedure (Messing, 1983) to complete the sequencing of pPE.30 and to sequence pPE.27 and pPE.7. The cDNA inserts from these clones were digested with the enzymes AluI and Sau3A in separate reactions. These enzymes both have four base pair recognition sites and, therefore, are statistically predicted to cut once in every 256bp. Two different enzymes were used to produce fragments from each clone, in order to generate overlapping sequence and thus facilitate assembly of the data. The products of the AluI digests were subcloned into M13mp8 at the SmaI site and the Sau3A fragments into the same vector at the BamHI site. The ligation products were transfected into JM101 and after overnight growth, each set of fragments had generated >200 recombinants. Initially, 24 recombinants were picked from each plate and single stranded DNA was prepared from them. T-tracking was used to identify which of the recombinants had the same insert in an identical orientation and thus prevent repeated sequencing of the same fragment of DNA. Sequencing was performed as previously described and added to that obtained from the shotgun clones.

Regions for which sequence data had only been obtained from one strand were eliminated by the use of the single-stranded templates from these regions as probes to identify their complements. The M13 vector sequences, in the clone to be used as a probe, were labelled using the M13 universal probe primer (BRL) and the Klenow fragment of DNA polymerase 1. The probe primer hybridises upstream of the cDNA

Figure 4.6 ctd.

Sequences complementary to the primer fragment used in primer extension experiments (section 4.2d) are underlined. The single potential N-linked glycosylation site is indicated by an arrow. The C-terminal amino acid sequence which was used to generate an anti-peptide serum is boxed.

sequences in the template and the complementary DNA strand is synthesised away from the insert. The labelling was performed under conditions in which the concentration of labelled nucleotide limited the reaction and left the cDNA sequence in the template single stranded and available for hybridisation to its complementary strand without denaturation. These probes were used to screen nitrocellulose plaque lifts of the phage containing AluI and Sau3A fragments . In all instances, phage plaques containing single stranded DNA complementary to the probe sequences were identified and their inserts sequenced.

4.2c. The sequence of the 2.2Kb transcript.

The assembled sequence, derived from all of the clones, is shown in Figure 4.6. Sequence obtained from each of the parietal endoderm cDNAs was always identical to that generated from homologous regions of other clones. Searches of nucleotide sequences in the EMBL and NIH databases with this sequence, even under greatly reduced stringency, failed to reveal homology to any other sequence. A comparison of the sequence obtained from the F9-derived cDNAs (pF9. series) with that of the parietal endoderm-derived clones also revealed exact sequence homology. However, although the AATAAA polyadenylation signal was in an identical position in both sets of clones, the poly(A) tail was added after nucleotide 2070 in the F9 cDNAs and after nucleotide 2079 in the parietal endoderm clones . An interesing feature in the sequence of the 2.2Kb transcript is the run of 16 adenosine bases (nucleotides 1147-1162) in an A-rich region spanning 39 residues (80% adenosine). Another feature of the sequence is a tetranucleotide (GCCT) which is repeated seven times consecutively between nucleotides 41 and 68.

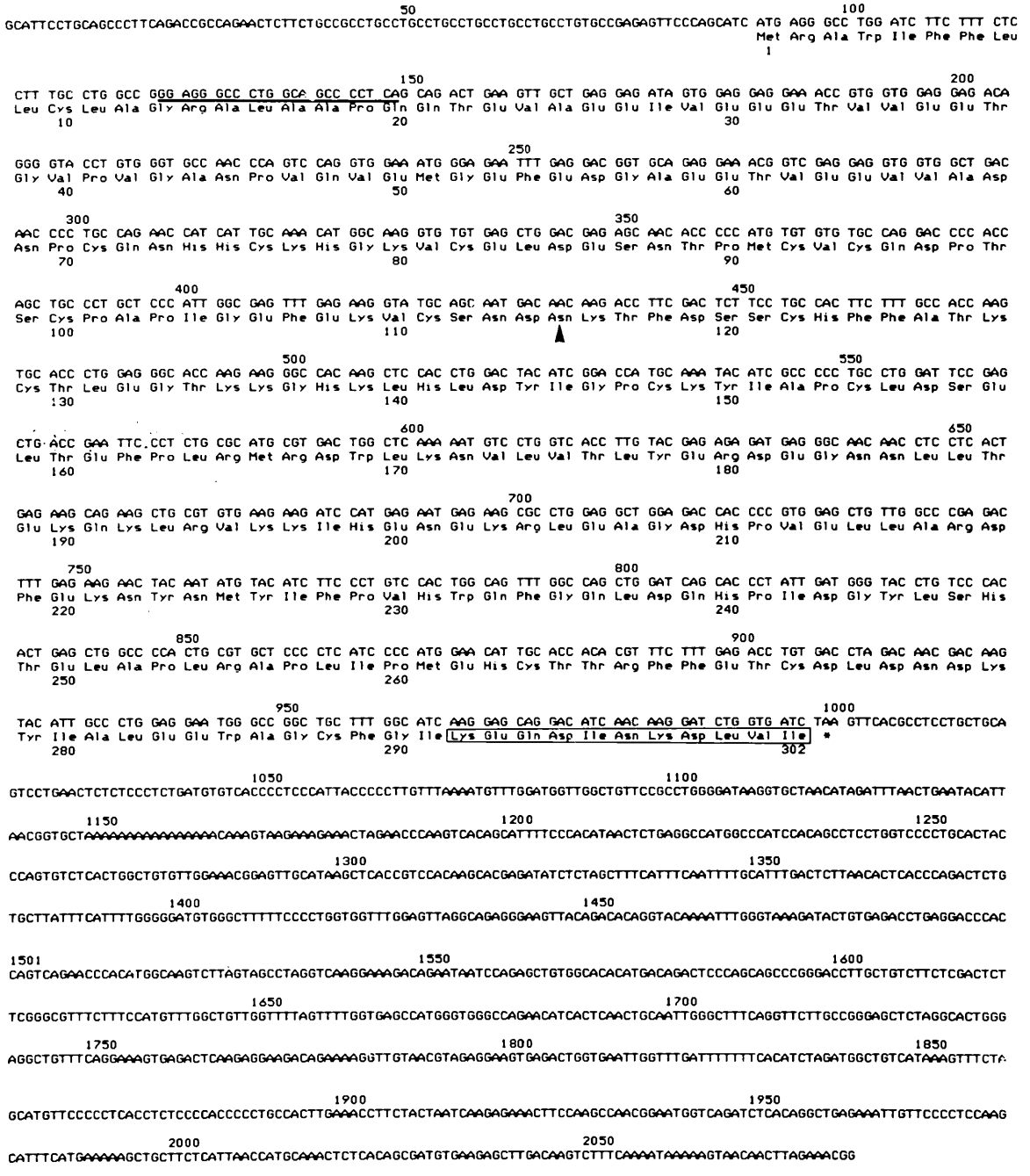


Figure 4.6 **Nucleotide sequence and predicted amino acid sequence from the differentially expressed 2.2Kb RNA.**

Sequence was obtained from the overlapping cDNAs from the parietal endoderm libraries illustrated in Figure 4.4.

4.2d. Primer extension to determine whether the entire 2.2Kb transcript had been cloned.

The assembled sequence of the 2.2Kb transcript obtained from the cDNAs contained 2079 nucleotides (Figure 4.6) and the addition of 100 nucleotides, the estimated average length of a poly(A) tail, to this figure suggested that most, if not all, of the 2.2Kb RNA was represented in the cDNAs which had already been isolated and sequenced. Further support for this hypothesis was derived from the observation that the five clones which extended closest to the cap site terminated within 8 nucleotides of each other. This suggested that either the clones extended to the 5' end of the transcript or that a strong region of RNA secondary structure had caused the reverse transcriptase enzyme to cease synthesis of the first cDNA strand. The latter hypothesis was considered unlikely since, prior to preparation of the cDNA, the RNA had been pre-treated with methyl-mercuric hydroxide to remove secondary structures.

To determine the position of the 5' end of a transcript a combination of primer extension and S1 mapping procedures should be used since the artefacts observed with the two methods are quite different (Williams and Mason, 1985). However, cosmids containing the genomic sequences from which the 2.2Kb transcript is derived (Chapter 5.4) were not sufficiently well-characterised to allow the generation of a probe for S1 analysis. Therefore, only primer extension was possible.

A primer was generated from a HpaII-DdeI fragment of clone pIM.22 (nucleotides 127-148; see Figure 4.4A). A search of the nucleic acid databases revealed that this primer sequence had no homology to sequences in the databases, even when parameters which would detect 60% homology over 10 nucleotides were used. The 22 nucleotide primer

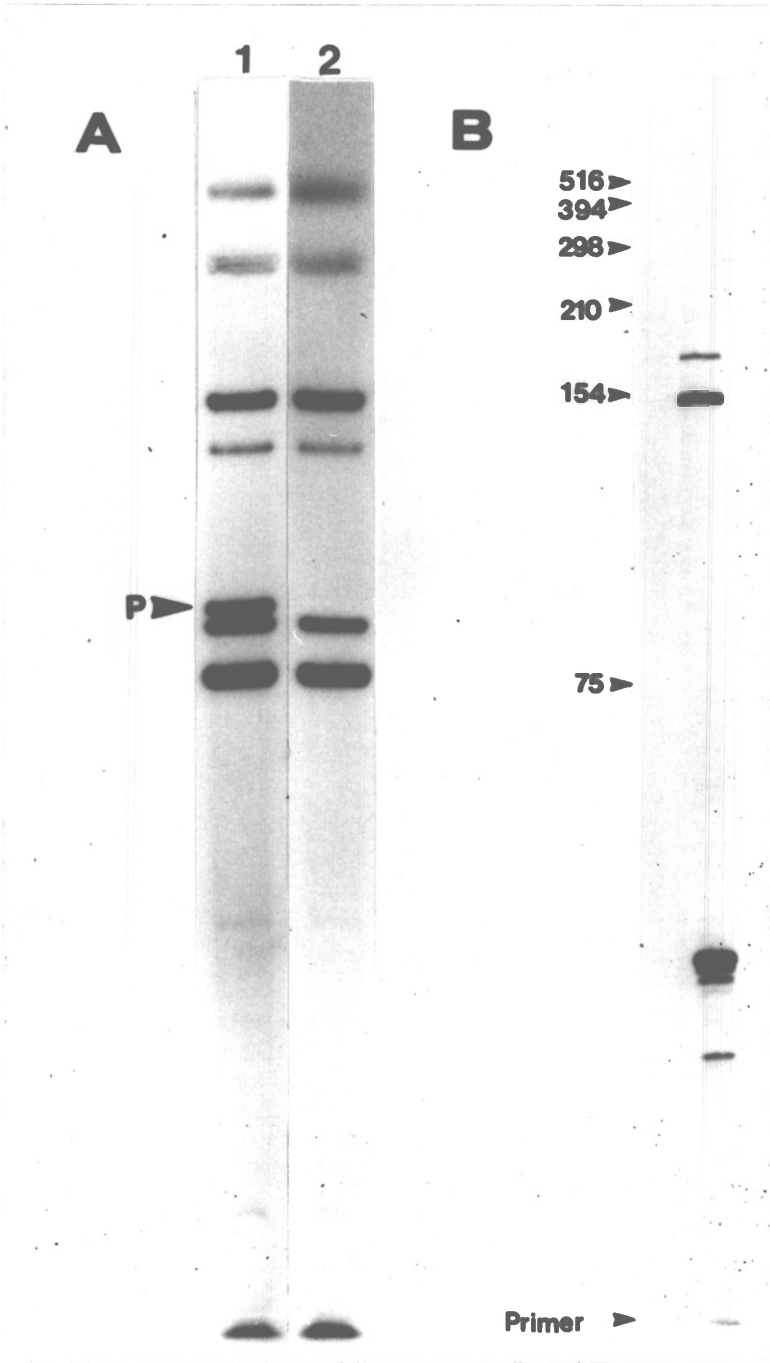

1
2
A
B
516
394
298
210
154
P
75
Primer

Figure 4.7 **Primer extension to locate the position of the 5' end of the 2.2Kb transcript.**

A. Preparation of a single-stranded primer complementary to the 2.2Kb RNA. The BamHI-KpnI fragment containing the most 5'cDNA sequences was initially purified from clone pIM.22. This was digested with HpaII and DdeI and a small amount of the digest products were labelled with T4 polynucleotide kinase and γ^{32}P-dATP as a tracer. The pooled digest products were separated on a 20% preparative urea/acrylamide gel and exposed to X-ray film (lane 1). The 22 nucleotide primer (P) had been separated from its complementary strand. The primer fragment was excised from the gel, accurate excision was monitored by autoradiography (lane 2), and the primer was purified from the gel by electroelution. The primer was then kinase-labelled to high specific activity.

B. Primer extension on parietal endoderm RNA. 17.5pg of primer were hybridised to 1µg of parietal endoderm poly(A)+ RNA at 70°C overnight in a sealed glass capillary. The hybridisation products were expelled into extension buffer and extended from the primer with reverse transcriptase. The extension products were denatured and analysed on an 8% urea/acrylamide gel together with denatured, ^{32}P-labelled fragments of pBR322 as markers. The gel was fixed, dried down and exposed overnight. The molecular weight markers, shown in base pairs in the figure, are denatured HinfI fragments of pAT153.

was ^{32}P-labelled with T4 polynucleotide kinase and separated from its 21 nucleotide complementary strand on a 20% denaturing urea/polyacrylamide gel (Figure 4.7A). The single stranded primer was excised from the gel, electroeluted into a dialysis bag and further purified. Primer extension, using the protocol of Williams and Mason (1985), was performed on 1μg of poly(A)+ RNA from parietal endoderm, differentiated F9 cells, and the parietal endoderm cell line F9AcC19, with 17.5pg of primer. When the products were analysed on an 8% denaturing gel an identical pattern of extension products was produced (Figure 4.7B and data not shown) from each RNA sample. Two abundant transcripts (44 and 148 nucleotides long) and a minor transcript (178 nucleotides long) were detected. Much longer exposures (14 days) failed to detect any additional transcripts larger than the 178 nucleotide product, although many more termination products were detected in the region of the 44 nucleotide product, indicating that secondary structures (e.g. stem-loop structures) within this region of the transcript may have caused premature termination of the extension reaction. Reactions performed over a range of hybridisation temperatures (45^{o}C-80^{o}C) revealed that the proportion of label incorporated into each product was constant until 75^{o}C, when all of the products disappeared simultaneously. This indicated that the extension products were primed from identical RNA sequences. To confirm this, a scaled-up primer extension was performed on 40μg of poly(A)+ RNA from differentiated F9 cells. The 44 and 148 nucleotide products were isolated from the denaturing gel and sequenced by the chemical cleavage method. The sequences of both of these transcripts were exact complements of the predicted sequence of the 2.2Kb RNA. In addition, the 148 nucleotide product terminated at the same nucleotide as the cDNA clone which extended closest to

the RNA cap site (pIM.3). There was not enough label incorporated into the 178 nucleotide product (<400 cpm) to allow its complete sequence to be determined, however, a single cleavage reaction, the "C+T" reaction, was performed on it and data from this revealed that it was transcribed from the 2.2Kb RNA and that its length was not due to "snap-back" copying of the cDNA strand. This latter result was to be expected since actinomycin D had been included in the extension reactions to prevent "snap-back" artefacts.

The interpretation of these results which I currently favour is that the termination after about 44 nucleotides is due to effects of secondary structure and that the products of 148 and 178 nucleotides represent a major and a minor start site for transcription (see discussion). Many genes transcribed by RNA polymerase II have multiple start sites for transcription which yield mRNA sequences differing in the length of their 5' non-coding region (Williams and Mason, 1985). However, confirmation of this hypothesis awaits data from S1 mapping experiments and until then the isolation of full-length cDNAs cannot be confirmed.

4.2e. Translation of the predicted sequence of the 2.2Kb RNA.

Conceptual translation of the sequence of the 2.2Kb transcript gave a unique long open reading frame between the first methionine codon (AUG; nucleotide 90) and the stop codon TAA (nucleotide 996)(Figure 4.6). Analysis of the deduced sequence of 302 amino acids revealed four distinct protein domains:

Domain I, residues 1-17, presents a classical signal sequence (von Heijne, 1985), strongly suggesting that the protein is secreted. A probable cleavage site is predicted within residues 18-20.

Domain II, residues 23-68, is very rich in acidic residues (17/46),

particularly glutamic acid (15/46). When analysed by the method of Chou and Fassman (1978), this domain is strongly predicted to form alpha-helix.

Domain III, the central region of the predicted protein (residues 69-170), contains 11 cysteine residues and the unique potential N-linked glycosylation site, Asn-X-Thr (Pless and Lennarz, 1977; Struck et al., 1978), at residue 115.

Domain IV, which encompasses residues 171-302, contains a large proportion of charged residues. Approximately equal numbers of acidic (Glu and Asp; 21/132) and basic (Arg, Lys and His; 22/132) amino acids are present and these charged residues are particularly abundant between positions 189 and 210 (14/22). This domain also contains three cysteine residues near the C-terminus.

Taken together, the sequence data and the abundance of the 2.2Kb transcript on Northern blots predicted that parietal endoderm and differentiated F9 cells secrete large amounts of an acidic protein, rich in disulphide bonds, which may also be glycosylated. The estimated molecular weight of the unmodified protein, including the signal sequence, is 34,411 and the predicted signal sequence has a molecular weight of 1,950.

4.2f. Sequence of the 0.7Kb transcript.

Restriction mapping of the cDNA clones from the 0.7Kb mRNA, with 18 different restriction endonucleases, revealed few internal restriction enzyme sites (see Figure 4.5B). However, using the chemical cleavage procedure, preliminary sequence data were obtained from the most 5' 322 nucleotides and the most 3' 109 nucleotides of clone pTF.75. This revealed that the 122bp sequence in pF9.25 which hybridises to the 0.7Kb transcript was located towards the centre of

clone pTF.75 (Figure 4.5B). The 3' end of clone pTF.75 contained sequence derived from a poly(A) tail together with a hexanucleotide (AATAAA) polyadenylation signal 18 nucleotides 5' to the polyadenylation site, and the 322 nucleotides at the 5' end of clone pTF.75 contained two open reading frames which extended from the 5' end of the clone to the end of the region which has been sequenced. It should be stressed that these data are very preliminary and require the complete sequence to be obtained and possibly the isolation of longer cDNAs before they can be fully evaluated. However, a search of the databases, with the sequence which had been obtained to date, revealed no homology to other sequences. In addition, no complementarity which would allow the 0.7Kb and 2.2Kb transcripts to hybridise and which might account for the origin of clone pF9.25, was found when sequence from pTF.75 and pF9.25 was compared with that derived from the 3' end of the 2.2Kb transcript.

4.3 Identification of the protein encoded by the 2.2Kb transcript by hybrid-selection and in vitro translation.

Hybrid-selection followed by *in vitro* translation of the selected transcript is a procedure which has often been used to identify the polypeptide encoded by a cloned DNA fragment. In this procedure DNA is bound to an inert support (e.g nitrocellulose) and hybridised, under conditions of DNA excess, to an RNA population containing the transcript of interest. The mRNA which hybridises to the filter-bound sequences is then recovered, translated in a cell-free protein translation system, and the translation products are analysed by SDS-PAGE.

Initial hybrid-selection experiments were performed using the clones pF9.33, pPE.30 and pPE.220 following the procedure described

by Maniatis et al. (1982). The denatured cDNA clones were immobilised on nitrocellulose and used to select the 2.2Kb RNA from 100µg of total RNA from parietal endoderm cells. In vitro translation of the selected RNA was carried out in the nuclease-treated rabbit reticulocyte lysate in the presence of ^{35}S-methionine and the products were analysed by SDS-PAGE on 10-20% gradient gels. The gels were fixed, treated with ^{3}H-Enhance and exposed to X-ray film. Prolonged exposure failed to reveal any proteins other than those which were present in control translations in which no exogenous RNA was added to the lysate. Similar results were obtained when the experiments were repeated using an alternative hybrid-selection procedure (Mason and Williams, 1985a). When the unselected RNA was recovered it could be translated to give high molecular weight polypeptides (>100KD; data not shown), demonstrating that the RNA had not been degraded during the hybridisation. In contrast, using the same protocol, cDNA clones had been identified which selected the mRNA encoding a type IV collagen chain (Kurkinen et al.,1983b) and two laminin B chains (Barlow et al., 1984). Therefore, to investigate the possibility that the in vitro translation product of the 2.2Kb transcript co-migrated with an endogenous ^{35}S-methionine-labelled protein of the reticulocyte lysate, the translation products from parietal endoderm RNA selected with pF9.33 were analysed by J.Garrels (Cold Spring Harbour Laboratory) on 20 x 25cm two-dimensional gels (Garrels, 1979)(Figure 4.8). As a negative control, the vector sequences (pAT153) in clone pF9.33 were also used to select transcripts from the same RNA population. Fluorograms of these gels revealed two closely-migrating acidic proteins which were specifically selected by clone pF9.33. Furthermore, these migrated with approximately the same Mr as two proteins present in the

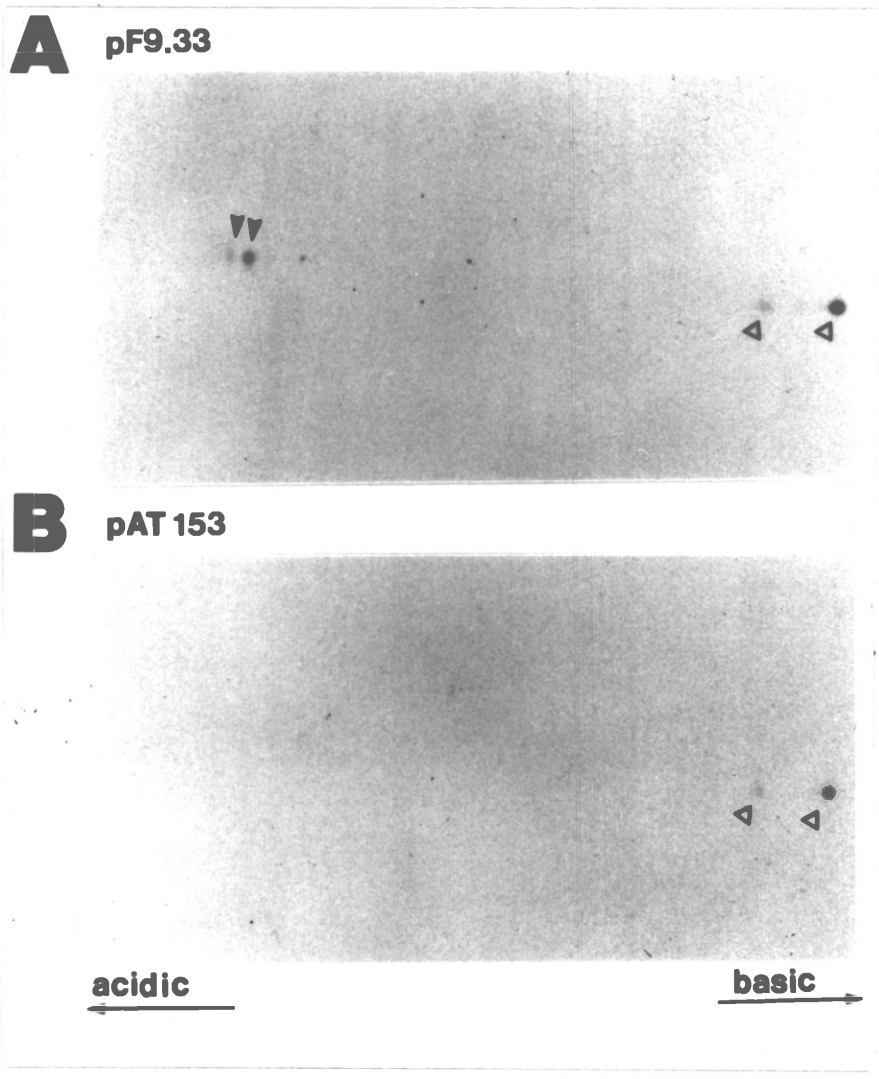


Figure 4.8 **Hybrid-selection and in vitro translation of the 2.2Kb RNA**

RNA was selected from 100µg total parietal endoderm RNA at 50°C for 3 hours using clone pF9.33 (A) and, as a control, pAT153 (B). The selected RNA was isolated and translated in vitro in the nuclease-treated rabbit reticulocyte lysate. The products were analysed by two-dimensional gel electrophoresis by Dr J.I.Garrels. The samples were electrophoresed on pH 4 to 8 isoelectric focusing gels and subsequently on 7.5% slab gels. The fluorograms were exposed for 35hours. The translation products of RNA specifically selected by clone pF9.33 are indicated by the solid arrowheads, whereas non-specific proteins that are endogenous to the lysate are indicated by open arrowheads.

translation products of both the pF9.33 and pAT153 hybrid-selections (Figure 4.8) and these latter proteins were also present in translations in which no exogenous RNA was added. Unfortunately, the pI and Mr values of the four proteins could not be determined due to the lack of known protein "standards" on the gels. However, this data provided the first evidence that the 2.2Kb transcript might encode an acidic protein.

While these data initially suggested that two closely-related transcripts, encoding different proteins, were selected by pF9.33, subsequent studies have demonstrated that this was due to incomplete reduction of the protein. If the sample buffer is freshly-prepared just before reduction of the protein and its analysis by SDS-PAGE, only a single translation product is detected (I.Mason, A.Taylor and B.L.M.Hogan data not shown). This is a common property of proteins with extensive intra-molecular disulphide bonding (J.Garrels pers. comm.) and, subsequently, the native protein was also found to exhibit this behaviour (A.Taylor pers. comm.)

To facilitate the characterisation of the protein product of the 2.2Kb transcript, a peptide corresponding to the hydrophilic C-terminal sequence of the predicted protein:

Lys.Glu.Glu.Asp.Ile.Asn.Lys.Asp.Leu.Val.Ile

was synthesised, coupled to keyhole limpet haemocyanin as a carrier molecule and injected into rabbits to obtain antibodies (A.Taylor, this laboratory). The antiserum produced was active against substrate-bound peptide at dilution of 1:4000 and in immuno-precipitation assays at 1:1000 (A.Taylor unpublished data; Mason *et al.*, 1986a)

This antiserum was used to immunoprecipitate the *in vitro* translation products from 4µg of total cellular RNA from F9 cells

differentiated to the parietal endoderm phenotype. The immunoprecipitated proteins were analysed by SDS-PAGE on a 6-12% gradient gel under reducing conditions. A protein of Mr 47,500 was detected and its immunoprecipitation was specifically blocked when the antiserum was pre-incubated with the C-terminal peptide (Figure 4.9 lanes 3 and 4). Some other proteins were also recognised by the antiserum but they were still detected when blocked antipeptide serum was used.

To confirm that this protein was the product of the 2.2Kb transcript, poly(A)+ RNA from differentiated F9 cells was hybrid-selected with clone pPE.220 following the procedure of Maniatis et al. (1982). When the translation products of the selected RNA were immunoprecipitated and analysed by SDS-PAGE on a 6-12% gradient gel, a single protein of Mr 47,500 was detected (Figure 4.9 lane 5). This protein exactly comigrated with a labelled protein which was detected when no exogenous RNA was added to the lysate (Figure 4.9 lane 2), but which was not immunoprecipitated by the antipeptide serum. Subsequent immunoprecipitations of RNA translated in vitro, followed by analysis by SDS-PAGE, demonstrated that transcripts encoding the Mr 47,500 protein were abundant in RNA from parietal endoderm and differentiated F9 cells and much less abundant in RNA from visceral endoderm and undifferentiated F9 cells (data not shown).

The observed molecular weight of the protein on SDS-PAGE (47,500) was much greater than predicted (34,411) from the primary amino acid sequence. It is known that certain protein modifications, including phosphorylation, can occur in the reticulocyte lysate (H.Pelham pers. comm.). To investigate the possibility of phosphorylation increasing the apparent Mr of the protein, RNA from differentiated F9 cells was translated in the reticulocyte lysate supplemented with 500μCi of

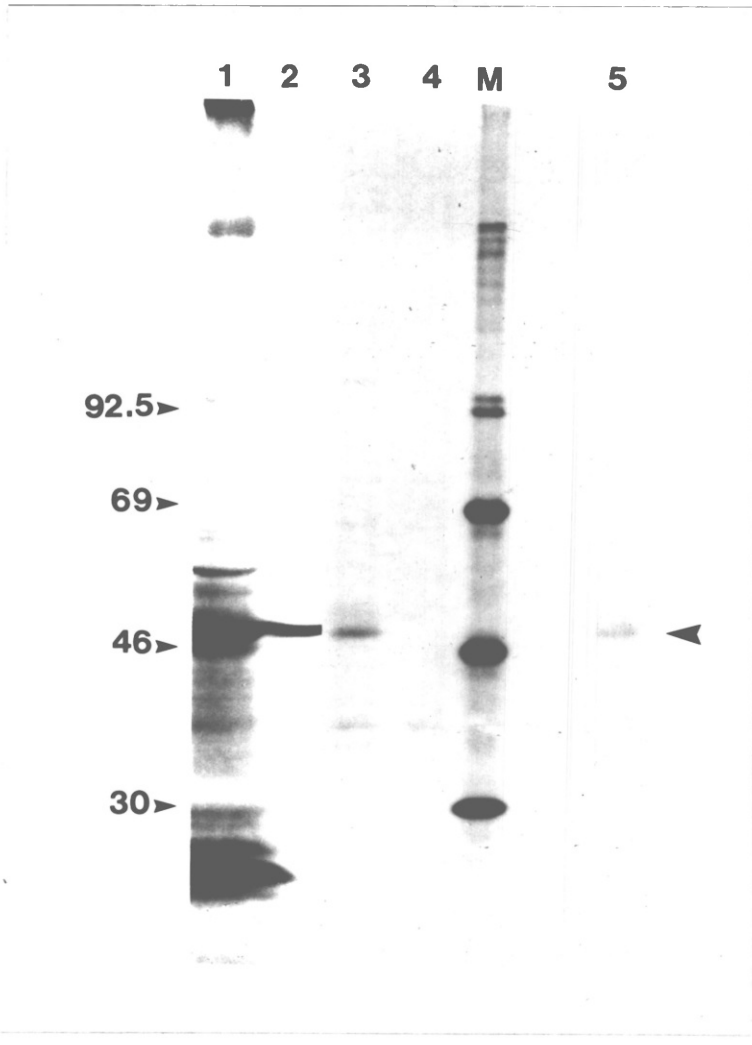


Figure 4.9 **C-terminal antipeptide serum immunoprecipitates the protein encoded by the 2.2Kb RNA**

RNA from differentiated F9 cells was translated in the nuclease-treated reticulocyte lysate and the products analysed on a 6-12% gel under reducing conditions. Lane 1: total proteins translated from 0.2µg of the RNA recovered after removal of the RNA hybridising to pPE.220; lane 2: control, lysate with no added RNA; lane 3: protein immunoprecipitated with antipeptide serum from lysate primed with 4µg total RNA; lane 4: as lane 3 but the antiserum was preincubated with free peptide; lane 5: 17µg of poly(A)+ RNA was selected with pPE.220. Bound RNA was eluted, translated in vitro and immunoprecipitated with the antipeptide serum. 0.2µg of the unbound RNA was translated and analysed in lane 1. The large arrowhead indicates the specifically-selected and immunoprecipitated Mr 47,500 translation product.

γ^{32}P-ATP. Proteins were immunoprecipitated with the antipeptide serum and the products analysed by SDS-PAGE. The Mr 47,500 protein was not labelled under these conditions, although, when the total translation products were analysed other phosphorylated proteins were detected.

4.4 The 0.7Kb transcript encodes an Mr 10,000 protein

To determine whether the 0.7Kb transcript encoded a protein, the clone pTF.75 was also used in hybrid-selection and in vitro translation experiments. When the products were analysed on 6-12% gels no specific translation products were detected. However, analysis on an 18% gel detected a protein of Mr 10,000, which was not present in the translation products of RNA selected by vector sequences (pUC8; Figure 4.10). The proteins, which are present in both tracks, were also detected in translations in the absence of exogenous RNA. The low molecular weight smear, seen in Figure 4.10, is possibly due to label being retarded by the haemoglobin protein in the lysate. However, again these data concerning pTF.75 are very preliminary and the protein encoded by the 0.7Kb transcript requires further characterisation.

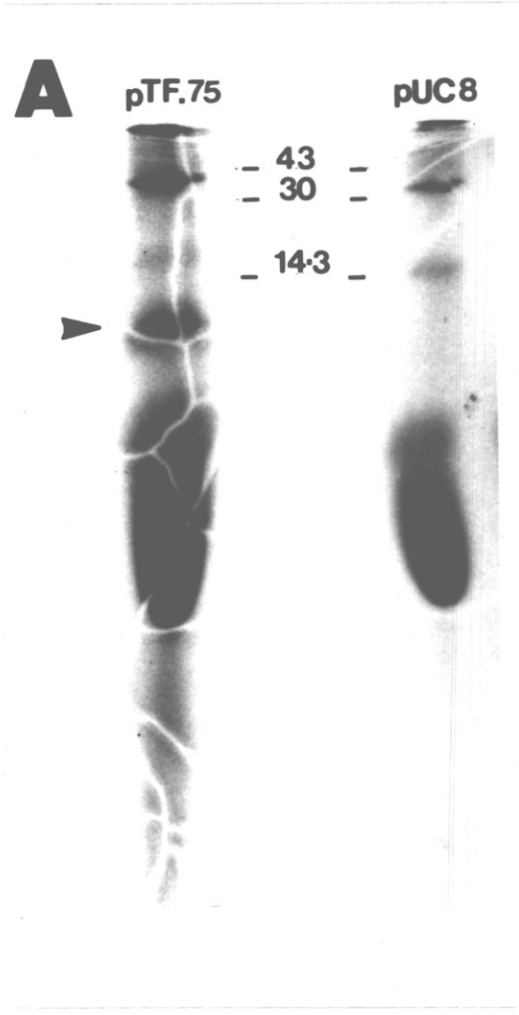


Figure 4.10 **Hybrid-selection and in vitro translation of the 0.7Kb RNA**

Clone pTF.75 was used to select the 0.7Kb transcript from 20µg of poly(A)+ RNA from PYS-2 cells. As a control, the vector sequences (pUC8) present in clone pTF.75 were used to select from the same RNA. The RNA selected by pUC8 and pTF.75 was isolated and translated in the nuclease-treated reticulocyte lysate. In each case, half of the translation products were analysed on an 18% slab gel.

4.5 Peptide antibodies recognise a Mr 43,000 secreted glycoprotein

The antipeptide serum was used to precipitate proteins from the culture medium of 13.5 day parietal endoderm cells, attached to Reichert's membranes, which had been labelled overnight with ^{35}S-methionine. The antipeptide serum immunoprecipitated a Mr 43,000 secreted protein from the culture medium (Figure 4.11A lane 1) and the precipitation of this protein was specifically blocked when the antiserum was preincubated with peptide (Figure 4.11A lane 2). This protein apparently contained about 25% of the ^{35}S-methionine incorporated into proteins that accumulated in the medium of parietal endoderm cells over 14 hours (estimated by scanning a shorter exposure of Figure 4.11A lane 3). Additionally, it was the only protein precipitated from lysates of the parietal endoderm cells (Figure 4.11A lane 4) and was also found to be a major secreted product of both PYS-2 cells (Figure 4.11A lanes 5 and 6) and F9Ac C19 cells (Figure 4.11C) Moreover, monolayers of F9 cells secreted 20-40 fold more of this protein after 5 days' exposure to retinoic acid and dibutyryl cAMP than did undifferentiated cells (Figure 4.11B). Analysis of labelled protein immunoprecipitated from the medium of F9Ac C19 cells under non-reducing conditions (Figure 4.11C lane 5 and compare with lane 3) revealed a dramatic increase in the mobility of the protein - these data are consistent with a high content of intramolecular disulphide bonds.

The covalent linkage of oligosaccharide side chains to protein can be accomplished through asparagine residues (N-linked), through serine and threonine (O-linked) or, as in collagen, through hydroxylysine residues. The antibiotic tunicamycin inhibits N-linked glycosylation by preventing the formation of the dolichol phosphate-linked oligosaccharide precursor (Takatsuki et al., 1971; Tkacz and

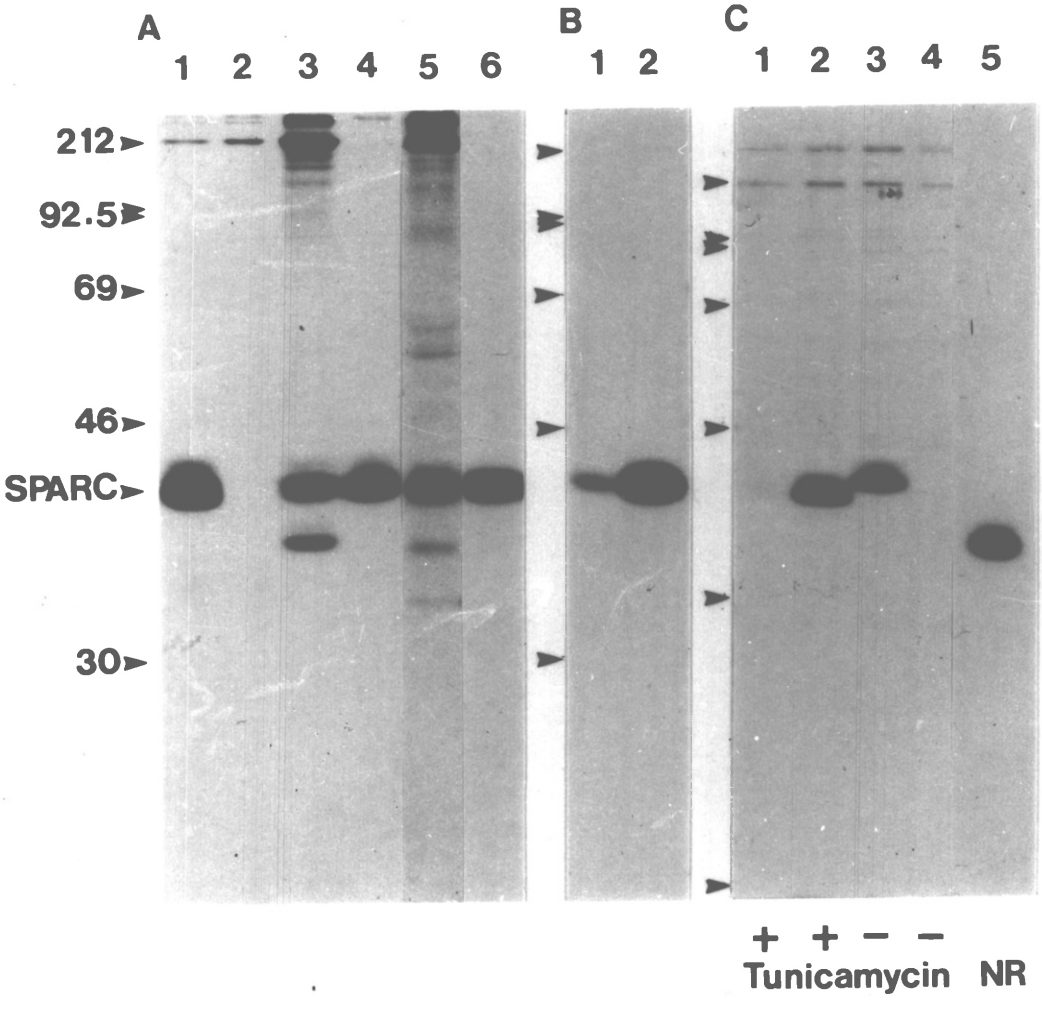

A
1 2 3 4 5 6
B
1 2
C
1 2 3 4 5
212
92.5
69
46
SPARC
30
+ + − −
Tunicamycin
NR

Figure 4.11 **Antipeptide sera recognise an Mr 43,000 glycoprotein**

A. Specific immunoprecipitation of Mr 43,000 by the antipeptide serum. Approximately $4x10^6$ parietal endoderm cells attached to 40 Reichert's membranes were labelled for 14.5hr with 50μCi ^{35}S-methionine and $1x10^6$ PYS-2 cells were labelled for 16hr. Lane 1: protein immunoprecipitated from 1/8 of the culture medium of parietal endoderm cells using 3μl of antipeptide serum; lane 2: as lane 1 but antiserum was preincubated with free peptide; lane 3: 1/200 of total culture medium from parietal endoderm cells; lane 4: protein immunoprecipitated from 1/4 of detergent lysate of parietal endoderm cells; lane 5: 1/200 total overnight culture medium of PYS cells; lane 6: protein immunoprecipitated from 1/8 culture medium using 3ul of antipeptide serum. All samples were analysed on a 10% gel and the positions of ^{14}C-labelled markers are indicated by arrowheads.

B. Synthesis of Mr 43,000 by F9 teratocarcinoma cells. Undifferentiated F9 cells and cells differentiated for 5 days in the presence of retinoic acid and cyclic AMP were labelled with 50μCi ^{35}S-methionine for 10hr. Aliquots of the culture medium, containing equal amounts of TCA precipitable radioactivity. The samples were immunoprecipitated and analysed as in A. The autoradiogram was evaluated by densitometry scanning.

C. Effects of tunicamycin. F9AcC19 cells were preincubated for 2hr in medium with or without 4μg/ml tunicamycin before labelling for 4.5hr with 100μCi ^{35}S-methionine. Mr 43,000 was immunoprecipitated from equal aliquots of the cell lysate using 5μl of antipeptide serum and analysed on a 10% gel under reducing conditions. Lanes 1 and 2: incubated with tunicamycin; lanes 3 and 4 control cells. Lanes 1 and 4: immunoprecipitated with antiserum preincubated with peptide. Lane 5: SPARC protein analysed under non-reducing conditions.

Lampen, 1975; Hubbard and Ivatt, 1981). F9AcC19 and PYS-2 cells were treated with tunicamycin prior to labelling with ^{35}S-methionine. The cells were lysed and the lysates immunoprecipitated with antipeptide serum. A comparison of the protein precipitated from tunicamycin-treated cells with that from extracts of untreated cells revealed a reduction in the Mr of the protein of about 1,300 (Figure 4.11C lanes 2 and 3). This data is consistent with the addition of an N-linked oligosaccharide to the unique potential glycosylation site at residue 115.

Taken together, the data obtained from hybrid-selection experiments and studies on the mature protein confirm the predictions made from the analysis of the primary amino acid sequence. The 2.2Kb transcript encodes a Secreted Protein which is Acidic and Rich in Cysteine residues, which we have refered to as **SPARC** (Mason et al., 1986a).

Discussion

4.6 Molecular cloning of SPARC

The data presented above have identified SPARC as an abundant secreted product of parietal endoderm and parietal endoderm cell lines. The mRNA encoding this protein, measured as a fraction of the total polyadenylated RNA content, is expressed at lower levels in visceral endoderm (see Chapter 5.2) and intact visceral yolk sac than in parietal endoderm. Furthermore, the levels of SPARC mRNA and secreted protein increase dramatically when F9 cells are differentiated to the parietal endoderm phenotype. Thus, SPARC provides a potential marker for future gene-regulation studies concerning the differentiation of parietal endoderm.

4.6a. Theoretical considerations concerning the isolation and sequence of Sparc cDNAs.

The first Sparc cDNAs were identified by the differential screening of a cDNA library of 1200 recombinants prepared from poly(A)+ RNA from differentiated F9 cells. Solution hybridisation data indicate that the average eukaryotic cell contains 10,000 to 30,000 different mRNA sequences (Bishop et al., 1974a; Galau et al., 1976). Analysis of the complexity of mRNA populations generally assumes the existence of three abundance classes - high, medium and low - and the proportion of the mRNA in each class has been estimated for an SV40-transformed human fibroblast cell line (MGFSV451-1; Williams et al., 1977). If these data are introduced into the formula of Clarke and Carbon (1976), it is predicted that it is necessary to generate a library of 7,200 recombinants in order to have a 99% probability that all of the medium and high abundance polyadenylated transcripts are represented at least once (Williams, 1981). Since only 1200 clones were obtained, it is probable that certain medium abundance transcripts were absent from the library, severely limiting the number and nature of the parietal endoderm-specific genes which could be isolated.

A further consideration, which affects the properties of the clones which could be isolated, arises from the cDNA probes and the nature of the screening technique. Studies have suggested that the lowest abundance of a particular sequence in the probe, that will produce a hybridisation signal above background, is 0.05-0.1% (Williams and Lloyd, 1979; Dworkin and Dawid, 1980). Similarly, to demonstrate that a sequence is at a higher level in one RNA population than in another, there must be at least a five-fold difference between the two populations (Williams, 1981). Therefore,

the methods used to isolate clones would only have identified medium to high abundance mRNAs in the differentiated F9 population, which also constitute a minimum of 0.25-0.5% of the parietal endoderm polyadenylated RNAs and 0.05-0.1%, or less, of the visceral yolk sac population. The relative abundance of SPARC transcripts in these tissues, predicted from the above theoretical considerations, was confirmed by Northern blot analysis (Figure 4.3 and Chapter 5).

A similar approach has been used by L.Gudas and her colleagues to isolate differentially regulated cDNAs from F9 cells differentiated to a parietal endoderm phenotype. These workers prepared two cDNA libraries, containing 900 and 1200 recombinants respectively, with poly(A)+ RNA from F9 cells 72 hours after induction of differentiation with retinoic acid and dibutyryl cAMP (Wang and Gudas, 1983; Wang *et al.*, 1985). The libraries were screened with cDNA prepared from RNA extracted from undifferentiated F9 cells and from differentiated cells 72 hours after induction. Twelve differentially expressed cDNAs were isolated which were most abundant in the differentiated F9 cDNA population. These were placed in 7 groups based on the results of Northern analysis and hybrid-selection experiments (Wang *et al.*, 1985). Although cDNAs encoding part of a laminin B chain and a type IV collagen chain were isolated in this manner (Wang and Gudas, 1983), the results of Northern blot hybridisation and hybrid-selection followed by *in vitro* translation experiments with the other cDNA clones suggests that SPARC-encoding cDNAs were not isolated (Wang *et al.*, 1985). One possible explanation for this is that SPARC mRNA is insufficiently abundant in the F9 cells after only 3 days of differentiation; resulting in its absense from the small libraries which were screened or in it being below the minimum abundance in the probe population which would allow its

detection. Studies presented in the following chapter indicate that SPARC mRNA in F9 cells 72hr post-induction are about 6-fold less than in the mRNA population of 13.5 day p.c. parietal endoderm cells. Alternatively, there may be differences in SPARC expression between the F9 cell lines used in the two laboratories.

4.6b pF9.25 contains cDNA derived from two unrelated transcripts.

One anomalous clone, pF9.25, was identified from the screening of the differentiated F9 cell cDNA library. This clone also hybridised to a 0.7Kb transcript in addition to the SPARC transcript. The subsequent cloning and characterisation of cDNAs specific to the 0.7Kb transcript demonstrated that it was unrelated to the SPARC mRNA. pF9.25 could not have arisen by the insertion of two cDNAs into the same vector, since the library was prepared by homopolymer tailing. The explanation for the origin of this clone which I favour is that, during the synthesis of the first cDNA strand, the reverse transcriptase molecule "jumped" from the SPARC transcript to the 0.7Kb RNA. The lack of sequence homology in the region of the 0.7Kb-SPARC fusion would suggest that this was not due to hybridisation of the two transcripts. Preliminary data from hybrid-selection and *in vitro* translation experiments suggest that this 0.7Kb transcript is a mRNA encoding a protein of Mr 10,000.

4.6c. The structure of the SPARC transcript.

Two parietal endoderm cDNA libraries had to be screened in order to isolate cDNA clones spanning most of the SPARC mRNA, due to the presence of an EcoRI site in the cDNA. When the first of these libraries was screened a total of 395 SPARC cDNA clones was isolated. However, only 30 of these originated from the poly(A) tail, while the remaining 365 were primed from a run of sixteen A residues present in the 3' non-coding sequences of the SPARC transcript. A possible explanation for the increased abundance of internally-primed cDNAs is that the extension of cDNA from the poly(A) tail was blocked at nucleotide 1162 by hybridisation of the oligo dT_{14} primer to the string of adenosine residues. If the hairpin required for the synthesis of the second cDNA strand could not be formed in this region, such cDNAs would be lost from the library, resulting in a disproportionate number of clones primed from the internal site.

A second interesting feature of the nucleotide sequence of the SPARC transcript is the tetranucleotide motif (GCCU) which is repeated seven times consecutively at the 5' end of the transcript (nucleotides 41-68). No other sequence present in the databases contains a repeated sequence homologous to this. It is tempting to speculate that this sequence plays a regulatory role in either the transcription or translation of SPARC. Sequences which regulate transcription have been identified in the body of both the immunoglobulin heavy chain gene, located in the intron between the joining and first constant exons (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Neuberger, 1983), and 3' to the mRNA cap site in the alpha- and beta-globin genes (Charnay *et al.*, 1984). There is also evidence for the regulation of the translation and accumulation of the *c-fos* transcript by sequences within its 3' non-coding region

(Miller et al., 1984; Treisman, 1985).

S1 mapping analyses using genomic clones are required to confirm the isolation of full-length cDNA clones. However, in the absence of these data, my interpretation of the primer extension studies is that there are two transcription initiation sites for the SPARC mRNA, corresponding to the termination sites of the 148 and 178 nucleotide extension products, and that the clone pIM.3 extends to the cap site of the major transcript. This is based on the following observations. First, several cDNA clones terminate in the same region as the 148 nucleotide extension product. However, no cDNA clones terminate at the position of the equally abundant 44 nucleotide primer extension transcript. This is probably due to the pre-treatment of the RNA with methylmercuric hydroxide prior to cDNA synthesis to remove secondary structures from within the RNA. In contrast, the RNA which was used in the primer extension reactions was not pre-treated in this manner. These data suggest that the termination after about 44 nucleotides in the primer extension products was due to secondary structure in the RNA and, by inference, that the termination after 148 nucleotides was not due to similar structural effects. Second, sequence analysis indicates that the 178 nucleotide transcript is not the result of a "snap back" artefact (the self-copying of the cDNA strand). Third, although use of poly(A)+ RNA and a single stranded primer can detect the 5' end of transcripts located several hundred nucleotides away from the primer (Williams and Mason, 1985), prolonged exposure failed to reveal any transcripts greater than 178 nucleotides. Finally, the total length of cloned sequence is sufficient to account for the size of the SPARC transcript detected on Northern blots.

Densitometry-scanning of the autoradiogram shown in Figure 4.7B, indicates that the major transcription start site is used

approximately 12 times more frequently than the minor site in parietal endoderm cells.

When the sequence of the parietal endoderm cDNA clones was compared with sequence of SPARC transcripts in F9 cells obtained from both cDNAs and primer extension, the two were found to be identical except with respect to the site of addition of the poly(A) tails. The AAUAAA polyadenylation signal is located at exactly the same position (nucleotides 2054-2059) in both the F9-derived cDNAs and in the parietal endoderm clones. However, in the clones pF9.25 and pF9.33 the poly(A) tail is added after nucleotide 2070, whereas in the parietal endoderm clones it occurs after nucleotide 2079. The poly(A) tail is often added immediately after the dinucleotide sequences CA or TA (Birnstiel et al., 1985), although this is not the case for SPARC transcripts in either F9 or parietal endoderm cells.

In recent years, considerable progress has been made towards an understanding of the processes involved in the termination of transcription and the polyadenylation of eukaryotic messenger RNAs. Papovavirus, adenovirus and several cellular genes have been shown to be transcribed beyond the 3' ends of their mature transcripts (Ford and Hsu, 1978; Nevins and Darnell, 1978; Fraser et al., 1979; Nevins et al., 1980; Hofer and Darnell, 1981; Weintraub et al., 1981; Hofer et al., 1982; Amara et al., 1984; Frayne et al., 1984; Mather et al., 1984). In the case of the murine major B globin gene, this primary transcript is terminated at a precise location (Salditt-Georgieff and Darnell, 1983), and these termination sequences also function efficiently, but in an orientation-dependent fashion, when inserted downstream of the adenovirus E1A gene (Falck-Pedersen et al., 1985).

These data suggest that the formation of the 3' end of a mature polyadenylated RNA involves two processes:

I. The endonucleolytic cleavage of the precursor at the polyadenylation site.

II. The addition of the poly(A) tail, presumably by a poly(A) polymerase enzyme.

The sequence AAUAAA is found 11-30 nucleotides upstream of most polyadenylation sites (Proudfoot and Brownlee, 1976), including the SPARC polyadenylation sites. Fully-functional, naturally occuring variants of this consensus sequence have been identified in the transcripts of certain genes. These include the genes encoding hepatitus B virus surface antigen (UAUAAA; Simonsen and Levinson, 1983), chicken lysozyme (AUUAAA; Jung et al., 1980) and mouse pancreatic alpha-amylase (AUUAAA; Hagenbuchle et al., 1980). Other functional variants include AAUUAA, AAUACA, AAUAAU, AAUAAC, and CAUAAA (Wickens and Stefenson, 1984; Mason and Williams, 1985b). While these data suggest that the only absolute requirement, within the hexanucleotide, is the uridine at position 3, it is noticeable that only a single base change from the consensus occurs in any particular sequence and that the majority of transcripts (including SPARC) contain the consensus sequence.

There is a large body of evidence which suggests that this hexanucleotide sequence plays an essential role in the cleavage/polyadenylation process and that certain base substitutions within the element impair or abolish its function. For example, the removal of this sequence from SV40 late mRNAs prevents them being polyadenylated (Fitzgerald and Shenk, 1983). In one case of alpha-thalassaemia, the human α-2 globin gene has the hexanucleotide modified to AAUAAG and mature transcripts of the gene extend beyond the normal 3' end (Higgs et al., 1983) and a similar modification causes a human beta-thalassaemia (Orkin et al., 1985). Montell et al.

(1983) have demonstrated that transcripts of the adenovirus early region 1A, in which the concensus is changed to AAGAAA by oligonucleotide mutagenesis, are cleaved very inefficiently, however, all of the cleaved transcripts are polyadenylated. This demonstrates that the hexanucleotide sequence is required for efficient cleavage, but the role of this sequence in directing the addition of the poly(A) tail to the cleaved transcript is uncertain. Polyadenylation may be directed by other sequences, RNA secondary structure or by the endonuclease itself. The transcripts of the histone genes are not polyadenylated in most vertebrate tissues and do not contain the AAUAAA consensus sequence, but the primary transcripts of two histone genes are also cleaved from a larger precursor at the 3' end (Hentschel et al., 1980; Hentschel and Birnstiel, 1981; Krieg and Melton, 1984). This would appear to suggest that there are two different endonucleolytic processes in vertebrates, only one of which is associated with polyadenylation. However, a fraction of the histone mRNA population in Xenopus oocytes is polyadenylated (Levinson and Marcu, 1976), so the 3' processing of histone transcripts and those containing the AAUAAA hexanucleotide may share certain common elements.

SPARC transcripts are apparently polyadenylated on different nucleotides in F9 and parietal endoderm cells, but the same AAUAAA hexanucleotide is used in both cases. There are a number of possible explanations for this phenomenon. First, since only two cDNAs from F9 cells and four from parietal endoderm cells were sequenced in this region, it is possible that sampling variation has led to the failure to detect polyadenylation at both sites in each cell type. In this respect, it is known that transcripts of bovine prolactin and mouse ribosomal L30 protein are polyadenylated at several distinct sites,

within an 11-14nt region downstream of a single AAUAAA sequence (Sasavage et al., 1982; Wiedemann and Perry, 1984). In the case of SPARC transcripts this possibility can be investigated using S1 nuclease protection techniques.

Second, it is possible that the variation in SPARC polyadenylation is due to tissue-specific effects. In the Xenopus B1 globin transcript, two polyadenylation sites are used but with different frequencies (Mason and Williams, 1985b). Developmental or tissue-specific variation in the site of polyadenylation of transcripts from a single gene have been demonstrated for transcripts of the immunoglobulin μ chain, vimentin and calcitonin genes (Rogers et al., 1980; Capetanaki et al., 1983; Rosenfeld et al., 1983). However, in all the above examples, different AATAAA sequences direct the polyadenylation and, in the case of the immunoglobulin and vimentin transcripts, differential RNA splicing, rather than direct regulation of 3' cleavage and polyadenylation, may be responsible for this. Moreover,the regulation of polyadenylation of these genes involves the utilisation of different AAUAAA sequences, in contrast, the same sequence is used in SPARC transcripts in both F9 and parietal endoderm cells.

Finally, it is possible that a second element within the immature SPARC transcript is responsible for the variation in the site of polyadenylation which I detected. Direct evidence for a second element which directs cleavage and polyadenylation comes from studies in which the deletion of sequences 3' to the polyadenylation sites of hepatitus B surface antigen, adenovirus E2A antigen and bovine growth hormone, reduce the efficiency and accuracy of polyadenylation (Simonsen and Levinson, 1983; Gil and Proudfoot, 1984; McDevitt et al., 1984; Woychick et al., 1984). A similar element, which is

required for correct processing, has been detected downstream of the cleavage site of the histone H2A gene (Birchmeier et al., 1983). The element is located within 86 nucleotides of the cleavage site in all three of the polyadenylated transcripts. In the case of the bovine growth hormone gene, this element may act through the formation of secondary stem-loop structures with the AAUAAA and the exposure of cleavage sites on the loops (Woychick et al., 1984). Two sequences which are present in many polyadenylated transcripts may form part of the cleavage/polyadenylation signal. Berget (1984) identified a loosely-defined concensus sequence, CAPyUG, which may be located upstream or downstream of the cleavage site and sequences fitting the concensus PyGTGTTPyPy are often located downstream of the cleavage site (McLaughlan et al., 1985). It should be noted that no sequences showing close homology to the former sequence are located within the SPARC transcript in close proximity to the poly(A) addition site. In contrast, the histone genes also have a conserved downstream sequence; this decanucleotide is well-conserved between transcripts from different histone genes and between different vertebrate species (Birnstiel et al., 1985).

The most recent advances in our knowledge of the cleavage/polyadenylation process have been obtained using exogenous RNA substrates, generated from Sp6 vectors, in nuclear extracts from HeLa cells (Moore and Sharp, 1984, 1985; Hart et al., 1985). These experiments have confirmed that sequences 3' to the polyadenylation site are required for the accurate cleavage of adenovirus L2 and SV40 early region transcripts. In the case of the SV40 template, processing is enhanced by the presence of the RNA cap. The recovery of a 3' RNA fragment following cleavage, has demonstrated that cleavage is an endonucleolytic cut rather than a "chew-back" process.

The use of an ATP analogue to inhibit polyadenylation does not inhibit cleavage, demonstrating that the two processes can be uncoupled. A body of evidence suggests that small nuclear ribonucleoproteins (snRNPs) may be involved in the cleavage/polyadenylation process as well as in RNA splicing. Berget (1984) demonstrated that the AAUAAA and CAPyUG sequences could base-pair with the U7 small nuclear RNA (snRNA). The same snRNA contains sequences complementary to the conseved sequences flanking the 3' end of the histone mRNA (Birnstiel et al., 1985). Antisera which precipitate the U1, U2, U5, U4/U6 and U7 snRNPs, as well as those believed to be specific for the U1 snRNP, inhibit the in vitro cleavage/polyadenylation of L3 RNA (Moore and Sharp, 1984, 1985). Furthermore, anti-U RNA sera precipitate RNA fragments containing the AAUAAA hexanucleotide (Hashimoto and Steitz, 1986). These obsevations can be incorporated into a third hypothesis to account for the variation in the polyadenylation of SPARC: sequence differences downstream of the polyadenylation site which affect RNA secondary structures and/or the binding of snRNPs define different cleavage sites in transcripts in F9 cells and C3H/He murine parietal endoderm cells. This hypothesis can be partly tested by the direct comparisson of sequences of Sparc genomic DNA from F9 cells and C3H/He mice, and further investigated using Sp6-derived transcripts in nuclear extracts.

4.7 Characterisation of the SPARC protein

The conceptual translation of the sequence provided by the overlapping cDNA clones produced a single long open reading frame between the first methionine codon (nucleotide 90) and a stop codon (nucleotide 996). It should be noted that the open reading frame

continues 5' to the first methionine residue and is uninterrupted to the end of the nucleotide sequence derived from both the longest cDNA clone and the 148 nucleotide primer extension product. However, in the absence of evidence to the contrary and in view of the properties of the protein which are discussed below, I believe that this methionine residue represents the first amino acid of the protein encoded by this transcript.

Several predictions about the nature of the protein were made from analysis of the primary amino acid sequence. The protein was expected to be secreted, acidic, possibly glycosylated and to contain many intramolecular disulphide bonds.

To test these hypotheses and to characterise the protein further, an antiserum directed against the C-terminal of the predicted protein was raised in rabbits. The use of this antiserum in hybrid-selection and *in vitro* translation experiments confirmed that the open reading frame encoded a protein. The apparent Mr of the reticulocyte lysate translation product encoded by the SPARC mRNA (47,500) was much greater than that predicted from the conceptual translation (34,411). There are a number of possible explanations for this apparent discrepency. The aberrant migration may be due to the effects of the highly charged nature of certain regions of the protein reducing the mobility of the protein, for example the glutamic acid-rich N-terminal region may have this effect. In addition, the reduced mobility could also be due to other modifications of the protein in the lysate, although I found no evidence that the *in vitro* translation product was phosphorylated.

Analysis of the translation products of hybrid-selection of SPARC mRNA on two dimensional gels revealed two closely-migrating proteins with slightly different pI values, this was also observed when the

immunoprecipitated products of *in vitro* translations and when the mature, secreted protein were studied on 2-D gels (B.Hogan and J.I.Garrels pers. comm.). This was subsequently found to be the result of incomplete reduction of SPARC. Alternative splicing, resulting in the translation of two different proteins which are transcribed from the same gene, has been reported for a number of proteins, including fibronectin (Schwazenbauer *et al.*, 1983) and alpha-crystallin (King and Piatgorsky, 1983), however, sequence of the SPARC coding region has been derived from several cDNAs and offers no evidence for differential splicing of the transcript. Therefore, it is probable that the *Sparc* gene encodes only a single protein.

4.7a. SPARC is secreted.

Secretory and membrane-bound proteins are cleaved from precursors which contain a signal sequence of between 13 and 30 amino acids, usually between 18 and 20 residues. This sequence directs the nascent polypeptide from the ribosome through the membrane of the rough endoplasmic reticulum and is rapidly removed in the lumen (Blobel and Dobberstein, 1975). All signal sequences have a similar gross structure, consisting of a positively charged N-terminal region, a central hydrophobic region and a more polar C-terminal region which defines the cleavage site. The fine-structure of these three domains has been analysed, at the amino acid level for a large number of eukaryotic secreted proteins (von Heijne, 1985). A comparison of the N-terminal sequences of the SPARC protein with the data presented by von Heijne strongly suggested that it was a secreted protein (Figure 4.12). The predicted signal sequence has a very short N-region consisting of a single charged residue (Arg), a feature it shares

Figure 4.12 The signal sequence of Sparc.

Sparc sequence:

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Residue	Met	Arg	Ala	Trp	Ile	Phe	Phe	Leu	Leu	Cys	Leu	Ala	Gly	Arg	Ala	Leu	Ala	Ala	Pro	Gln

Signal region

Most frequent residues in the H/C region (von Heijne, 1985)	Residue					Leu	Leu	Leu	Leu Ala	Leu	Ala Gly	Gly			Ala	Leu	Ala	Ala		
	Position					-10	-9	-8	-7	-6	-5	-4			-3	-2	-1	+1		

with human influenza haemagluttinin (von Heijne, 1985). A positive charge immediatly after the first methionine residue is a common feature of signal sequences. The N-region is followed by an H-region of 10 hydrophobic residues and then a polar C-region of five amino acids. Most eukaryotic signal sequences have a C-region of 5-6 residues. Although there is great variation in the length and amino acid composition of eukaryotic signal sequences the most frequent residue at any given position in the H/C region has been determined (von Heijne, 1985). If a gap is inserted at the position of the arginine residue (position 14) in the SPARC sequence it contains the most frequent amino acid at almost every position (Figure 4.12). The presence of a cysteine residue in the H-region is unusal, but has been previously reported in the case of rat T and K prekininogen signal sequences (Furoto-Kato *et al.*, 1985).

The prediction that the protein would be secreted was confirmed by immunoprecipitation of labelled proteins from the medium of a number of cell types, including parietal endoderm. A protein of Mr 43,000 was specifically immunoprecipitated from the culture medium by the antipeptide serum. The figure of 43,000 for the relative molecular weight of SPARC should be considered an approximation since the most closely migrating standard, ovalbumin, has a reported molecular weight range of 43,000-46,000. The difference in Mr between the mature protein and the nascent polypeptide detected in analysis of *in vitro* translations is due, at least in part, to the presence of the signal sequence in the SPARC protein from the latter protein scource. The SPARC protein was found to be a major secreted product of the parietal endoderm - SPARC constitutes about 25% of the ^{35}S-methionine-labelled protein accumulating in the culture medium of parietal endoderm.

4.7b. SPARC is acidic and contains intramolecular disulphide bonds.

The acidic nature of SPARC predicted from the primary sequence and analysis of the translation products of hybrid-selection on 2-D gels, was confirmed for both the cytoplasmic and secreted protein when analysed in the same way (Mason et al., 1986a); both forms of the protein were found to have a pI of 4.3. When the mature SPARC is analysed under non-reducing conditions the mobility of the protein increases, a behaviour which is consistent with a high content of intramolecular disulphide bonds. This data also suggests that SPARC is not covalently associated with another protein in the medium through disulphide bonds.

4.7c. SPARC is glycosylated.

The primary sequence analysis of the SPARC protein revealed a single potential site for N-linked glycosylation - the asparagine residue (position 115) in the sequence Asn-Lys-Thr. However, in certain proteins, fewer than half of these sites are glycosylated, therefore, other determinants such as accessibility of the site, may also be important (Marshall, 1974; Beeley, 1977). Since the potential glycosylation site lay within the cysteine-rich domain, it was possible that it would not be available for glycosylation. However, the use of tunicamycin to inhibit N-linked glycosylation of SPARC in cultures of F9AcC19 and PYS-2 cells, resulted in an increase in the mobility of the ^{35}S-labelled SPARC protein. During these experiments unglycosylated SPARC continued to accumulate in the culture medium These data suggested that the mature SPARC protein is glycosylated at residue 115 in these cell lines and that inhibition of glycosylation does not prevent secretion.

The glycosylation of SPARC has been studied in more detail using ^{3}H-mannose labelling and the enzyme endo-B-N-acetylglucosaminidase H (ENDO-H). The ^{3}H-mannose labelling of secreted SPARC protein in cultures of PYS-2 cells confirmed that it is glycosylated (Mason et al., 1986a; A.Taylor unpublished data). Treatment with tunicamycin abolished ^{3}H-mannose labelling of the protein in these experiments, indicating that the mature SPARC protein has no O-linked oligosaccharides. ENDO-H cleaves off newly-attached oligosaccharide precursors, cutting between the N-acetylglucosamine residues, in asparagine-linked, large, high-mannose oligosaccharide side chains (Tarentino and Maley, 1974; Robbins et al., 1977). ENDO-H digestion of SPARC, labelled for 15min with ^{35}S-methionine resulted in a 1,250 reduction in its Mr (A.Taylor cited in Mason et al., 1986a). This provided further evidence that a large, N-linked oligosaccharide molecule is added to SPARC. The transfer of the oligosaccharide precursor from a lipid carrier (dolichol phosphate; Li et al., 1978; Liu et al., 1979; Hubbard and Robbins, 1980) to asparagine residues on secreted proteins probably occurs in the lumen of the rough endoplasmic reticulum (Czichi and Lennarz, 1977; Hubbard and Robbins, 1979). This process, which is inhibited by tunicamycin, may be co-translational (Kiely et al., 1976) or it may take place after release of the nascent polypeptide from the ribosome (Schubert, 1970). The oligosaccharide is sensitive to ENDO-H, while it is within the rough and smooth endoplasmic reticulum, at the time of the removal of three glucose residues (Grinna and Robbins, 1979). The next stage of processing, the hydrolysis of four mannose residues, probably occurs in the Golgi body, where the alpha-1,2-mannosidases required for this event are located (Tulsiani et al., 1982). At this stage the oligosaccharide is still sensitve to ENDO-H. However, the subsequent

processing steps which take place in the Golgi are insensitive to ENDO-H.

Pulse-chase analyses of ^{35}S-labelled cytoplasmic and secreted SPARC proteins from PYS-2 cells have revealed that the protein is not continuously modified in the manner described for c-fos in the previous chapter (Mason et al., 1986a). These studies also suggest that the signal sequence is cleaved and the oligosaccharide added to SPARC within 15min of the initiation of synthesis of the nascent polypeptide, since only one labelled form of SPARC was detected in cell lysates after this, the earliest time point. Furthermore, SPARC did not accumulate within the cell, suggesting that all of the synthesised protein is secreted. After a chase period of 120min, labelled SPARC was almost undetectable in the cell lysates and accumulation in the culture medium had plateaued. This suggests that secretion of SPARC by PYS-2 cells is via a constitutive rather than a regulated pathway (Kelly, 1985). Studies using $^{35}SO_4$ and $^{32}PO_4$ have failed to detect further modification of SPARC by sulphation or phosphorylation (B.Hogan and A.Taylor cited in Mason et al., 1986).

4.8 Towards the identity of SPARC.

4.8a. SPARC is not a basement membrane component.

A major function of parietal endoderm cells is the secretion of a very thick (5μm by mid-gestation) basement membrane - Reichert's membrane. Metabolic labelling and in vitro translation studies have demonstrated that at least 15% of the total protein synthesis of parietal endoderm cells is devoted to known basement membrane components; type IV collagen (7%) and laminin plus entactin (8%)(Hogan and Cooper, 1982; Kurkinen et al., 1982, 1983c). The

abundance of the SPARC protein secreted by parietal endoderm cells suggested that it might be a novel basement membrane component. Indirect immunofluorescence studies with the antipeptide serum have failed to stain either the matrix secreted by PYS-2 cells or Reichert's membranes from 8.5 day p.c. mouse embryos, under conditions which gave positive staining with an antiserum directed against laminin and entactin (B.Hogan cited in Mason et al., 1986a). Using a combination of Western blotting and immunoprecipitation techniques, the relative incorporation of ^{35}S-methionine labelled SPARC and laminin into Reichert's membranes by cultured parietal endoderm cells from 12.5 day p.c. mouse embryos has been compared with the accumulation of these proteins in the medium. The data reveal that the levels of SPARC accumulating in the medium were 60-fold greater than those incorporated into matrix, whereas approximately 4 times more laminin is incorporated into Reichert's membranes than is present in the medium (Mason et al., 1986a). The most likely explanation of these data is that SPARC is not a major integral basement membrane component. The small amounts of SPARC detected within Reicherts membrane may be due to non-specific entrapment of the protein during basement membrane synthesis, possibly following cell death and lysis.

4.8b. SPARC is structurally and antigenically related to bovine endothelial cell 43K glycoprotein.

No significant homology was detected between SPARC and protein or nucleotide sequences in the NIH, EMBL, Dayhoff or Doolittle databases. Additionally, the SPARC cDNA sequence showed no homology to that of tissue-type plasminogen activator (sequence supplied by F.Grosveld), an abundant secreted product of parietal endoderm cells

which is also not a matrix component. However, the SPARC protein shares many common features with a 43K protein (Sage et al., 1984), which was originally recovered as 1.1% of the total secreted protein from medium conditioned by bovine aortic endothelial cells (summarised in Table 4.1 and Figure 4.13). Sage et al. (1984) have detected this 43K protein in the conditioned medium of a parietal endoderm cell line (HR9). Furthermore, low stringency hybridisation of the SPARC cDNA pPE.220 to poly(A)+ RNA from bovine retinal endothelial cells detects a 2.2Kb transcript (Table 5.2). The two proteins from parietal endoderm and bovine aortic endothelial cells have been shown to have similar chromatographic properties, glycosylation properties, and tryptic cleavage patterns, and are not components of the extracellular matrix (Sage et al., 1984; Mason et al., 1986a). Additional evidence for their homology was provided by the observation that the anti-SPARC peptide serum cross-reacted with the purified bovine protein on Western blots (Mason et al., 1986a). This suggested that the two proteins might have homology at their carboxy-termini. To investigate the possible sequence homology between the two proteins further, the N-terminal sequence of the purified bovine 43K protein (provided by H.Sage) was compared with the predicted sequence of the SPARC protein obtained from the cDNA clones. The best alignment of the two sequences is shown in Figure 4.13. The four N-terminal amino acids of the bovine protein are identical to those at the predicted cleavage site of murine SPARC and the remaining sequence has close homology. Analysis of the overall amino acid composition of SPARC and that provided of the bovine protein (Sage et al., 1984) also reveals an overall similarity between the two proteins. We therefore concluded that SPARC and the bovine 43K protein are closely related, if not homologous.

Table 4.1 **Common properties of SPARC and bovine 43K protein**

Attribute	**SPARC**	**43K**	**Reference**
Apparent Mr when reduced	43K	43K	1,2,3.
Increased mobility when non-reduced	+	+	1,2,3.
Secretion by parietal endoderm cell lines	+	+	1,2,3.
Expressed by bovine endothelial cells	+	+	2,3.
Expressed by human foreskin fibroblasts	+	+	2,3.
Expressed by HT1080 cells	-	-	2,3.
Sulphated	-	-	1,2.
Phosphorylated	-	-	1,2.
Glycosylated	+	+	1,2,3.
Resistant to proteases	+	+	1,2.
Trypsin cleavage pattern	similar		1,2.
Incorporation into matrix	-	-	1,2.
Heparin-binding	-	-	1,2
Gelatin-binding	-	-	1,2

References:

1. Mason et al., 1986a.

2. Sage, 1984.

3. I.Mason this thesis.

Figure 4.13 Comparison of N-terminal sequences of bovine 43K with murine Sparc.

Sparc	Ala	Pro	Gln	Gln	Thr	Glu	Val	Ala	Glu	Glu	Ile	Val	Glu
43K	Ala	Pro	Gln	Gln	——	Glu	Ala	Leu	Asp	Glu	Cys	Val	
								Pro			X		Val

No function has yet been determined for the bovine 43K protein, although studies with purified 43K protein have apparently ruled out identity to a wide range of serum proteins, proteases and protease inhibitors. These include serine proteases, collagenase, plasminogen activator and plasminogen activator inhibitors (Sage et al., 1984, 1986). However, it should be noted that during its purification, the 43K protein was denatured in 6M urea. While denatured proteins,e.g. trypsin and chymotrypsin inhibitors, may readopt their native configuration and function (Creighton, 1975), the possibility that purified bovine 43K is functionally inactive cannot be ruled out. The bovine protein is capable of forming a complex with two serum proteins in vitro - bovine serum albumin (Sage et al., 1984) and, probably more specifically,a novel Mr 70,000 protein (Sage, 1986). Attempts to show an albumin-binding activity in ^{35}S-labelled SPARC by treating medium conditioned by PYS-2 cells with BSA-sepharose have been unsuccessful (Mason et al., 1986a). However, all secreted SPARC may already be complexed with albumin or the Mr 70,000 protein. Data from the staining of gels, on which immunoprecipitates from PYS-2 cells had been electrophoresed, in order to identify any proteins co-precipitated with SPARC, suggested that it is co-precipitated with an unlabelled, Mr 68,000 protein (A.Taylor, I.Mason and B.Hogan unpublished observations). However, the precipitation of this protein was not blocked when the antiserum was preincubated with free peptide prior to the immunoprecipitation. This suggests that the Mr 68,000 protein is not complexed with SPARC. A second difference between bovine 43K and SPARC has been detected by pulse-chase analysis of the secreted protein. The bovine protein is converted, in the medium of cultured aortic endothelial cells, from an Mr 38,000 form to a final form of Mr 43,000 (Sage et al., 1986) over a period of 6 to 21 hours,

whereas SPARC secreted by all the murine cells and cell lines has only been detected as a 43K form. Pulse-chase analysis of SPARC synthesised by PYS-2 cells only detected a 43K form in the medium and in cell lysates (Mason et al., 1986a). However, these data require studies to be performed on the same cell type using antisera to both proteins before they can be fully evaluated.

The identity and possible function of the SPARC protein are further discussed in the final chapter, following the presentation of data concerning its tissue-specific expression and regulation.

CHAPTER 5

Developmental and transformation-sensitive expression of the Sparc gene

Introduction

The previous chapter describes the molecular cloning and immunological characterisation of the *Sparc* gene. SPARC was isolated on the basis of its differential expression in parietal endoderm versus visceral yolk sac, and its induction during the differentiation of F9 embryonal carcinoma cells *in vitro*. Hence, the *Sparc* gene provides a molecular marker with which developmental gene regulation can be studied, both *in vitro* and *in vivo*, using the approach known as "surrogate" or "reverse" genetics.

The principle of reverse genetics can be broken down into four separate elements. First, a marker is isolated which can be used to study gene regulation in the tissue of interest. Second, modulation of the expression of this gene in the experimental system is examined. Third, a cloned fragment of genomic DNA which encodes the marker gene is introduced into the system and the accuracy of its expression is verified by comparison with the endogenous gene. A means by which the exogenous and host genes can be distinguished is required - this is accomplished by a modification of the former,for example by the insertion of a DNA linker. Fourth, modified forms of the gene are introduced in order to characterise those sequences within the gene which are essential for the correct regulation of the gene. Subsequently, these control elements can be used to identify *trans*-acting regulatory molecules.

This chapter describes the expression of the *Sparc* gene both *in vivo* and *in vitro*. These studies will provide the basis for the future investigation of its regulation in cell cultures, by DNA-mediated transfection, and *in vivo* using transgenic mice. These data also supply further information concerning the function of the SPARC protein. Finally, I describe the isolation of genomic clones containing the *Sparc* gene unit.

5.1 Expression of SPARC in differentiating F9 embryonal carcinoma cells

Metabolic labelling, followed by immunoprecipitation, and Northern blot analyses demonstrated that the levels of secreted SPARC protein and cellular SPARC mRNA increase when F9 cells differentiate in response to retinoic acid and dibutyryl cyclic AMP (Chapters 4.1b and 4.5). The following series of experiments was performed in order to provide more detailed information about induction of SPARC expression during the differentiation of F9 cells.

a. Time course of SPARC induction during the differentiation of F9 EC cells to parietal endoderm.

To study the expression of SPARC mRNA during the differentiation of monolayer cultures of F9 EC cells in response to retinoic acid, dibutyryl cyclic AMP and isobutylmethylxanthine, duplicate 24cm x 24cm dishes of cells were incubated with inducers for various periods of time as described in Chapter 2.8b. Fresh medium containing inducing agents was added after 48 and 96 hours. Total cellular RNA was prepared from the cells, and this was standardised by ^{3}H-poly(U) assay. Equal amounts of polyadenylated RNA (approximately 10μg of total RNA), on a slot blot, were hybridised with pPE.220 which had

been labelled by the oligonucleotide-priming procedure. The accumulation of SPARC transcripts during the differentiation of F9 EC cells to parietal endoderm is shown in Figure 5.1A and expressed graphically in Figure 5.1. F9 EC cells were found to contain low levels of SPARC transcripts. As previously described, after differentiation for 5.5 days, levels of SPARC mRNA had increased more than 10-fold as estimated by densitometry-scanning of the autoradiogram. The graphic representation revealed four distinct phases in the accumulation of SPARC transcripts during the course of differentiation. During the first 24 hours levels of SPARC mRNA decreased by about 50%. This was followed by a rapid accumulation of transcripts between 1 and 2 days post-induction. The rate of accumulation of SPARC transcripts decreased between the second and third day of exposure to inducing agents, but subsequently increased again during the following two days. An identical pattern of expression was observed when the experiment was repeated using the Northern blot technique (data not shown). After 5.5 days of differentiation to the parietal endoderm phenotype, SPARC mRNA did not accumulate to the levels detected in parietal endoderm *in vivo*, although the differentiated F9 cells did express more SPARC than was detected in the parietal endoderm cell line, PYS-2 (Figure 5.1). When the blot was reprobed with pTF.75, levels of the 0.7Kb transcript were found to remain constant throughout the differentiation process (data not shown).

The level of SPARC transcripts also increased in F9 aggregates cultured in retinoic acid for 8 days to form embryoid bodies containing an outer layer of visceral endoderm-like cells (Figure 5.1). However, the amount of SPARC mRNA detected in the RNA extracted from embryoid bodies was only equivalent to that detected between days 2

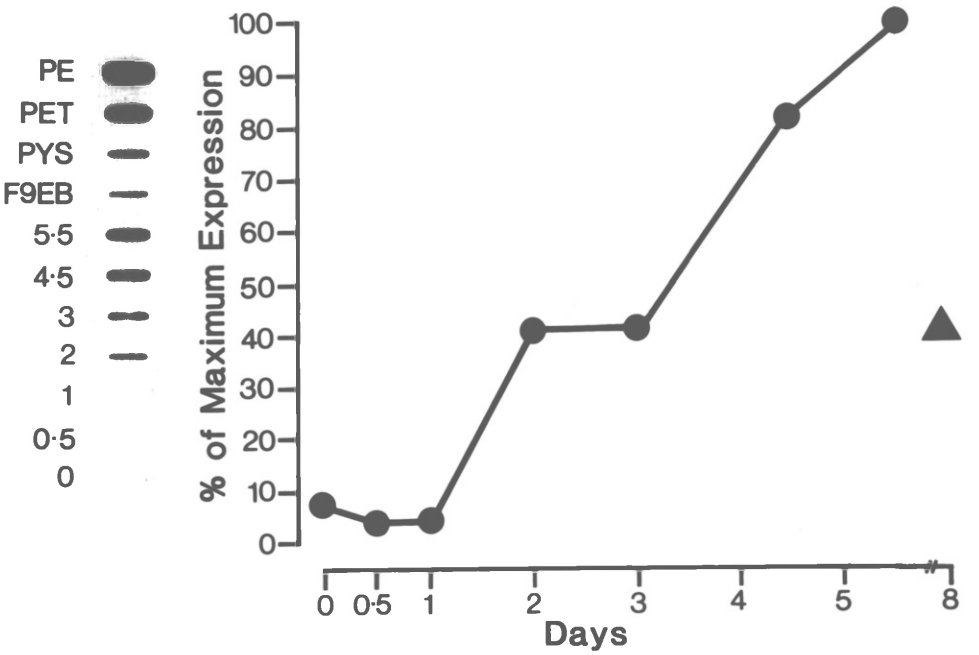


Figure 5.1 **SPARC RNA levels in F9 cells differentiating into parietal endoderm in response to retinoic acid and dibutyryl cyclic AMP.**

Slot blot analysis of total cellular RNA (about 10µg) normalised by ^{3}H-poly(U) assay. Denatured RNA was transferred to a nylon membrane using a slot blot manifold and hybridised with ^{32}P-labelled pPE.220. The blot was subsequently washed under stringent conditions and exposed to pre-flashed X-ray film for three days. Slots are: 0: undifferentiated F9 cells; 0.5 - 5.5: RNA extracted from F9 cells treated with retinoic acid and cyclic AMP for between 0.5 and 5.5 days; F9EB: F9 embryoid bodies after 8 days' exposure to retinoic acid; PYS: the parietal endoderm cell line PYS-2; PET: parietal yolk sac (endoderm plus trophoblast); PE: parietal endoderm (manually dissected free from the trophoblast).

The autoradiogram was quantitatively evaluated by densitometry scanning and the results expressed graphically as a percentage of SPARC mRNA levels obtained after 5.5 days of differentiation (circles). The relative level of SPARC RNA in embryoid bodies after 8 days' exposure to retinoic acid is also shown (triangle).

and 3 during differentiation into parietal endoderm.

b. Increase in the rate of transcription of the *Sparc* gene during the differentiation of F9 EC cells.

The experiments described above demonstrated that the steady-state level of SPARC mRNA increases during the differentiation of F9 EC cells to parietal endoderm. This could be the result of either an increase in the transcription rate of the *Sparc* gene or an increase in the stability of the SPARC transcript.

Nuclear "run off" assays are widely accepted as a method of assessing the rate of gene transcription independent of post-transcriptional mechanisms which regulate transcript abundance (McKnight and Palmiter, 1979; Groudine *et al.*, 1981; Greenberg and Ziff, 1984; Greenberg *et al.*, 1985). Nuclei are isolated and nascent transcripts are elongated *in vitro* in the presence of one or more radioactive ribonucleotides. It is assumed that re-initiation of transcription does not occur or is negligable, so that the amount of label incorporated into a particular transcript is proportional to the number of RNA polymerase molecules transcribing the gene when the nuclei were harvested. Consequently, this technique can be used to determine the rate of transcription of a particular gene in different tissues. However, Turcotte *et al.* (1985) have demonstrated that re-initiation can occur during the incubation process and that this can be inhibited by the inclusion of 0.1mg/ml heparin in the *in vitro* reaction mixture. Heparin has been shown to inactivate free RNA polymerases and to inhibit initiation but not elongation (Dynan and Burgess, 1979; Schiaffonati *et al.*, 1982). Subsequently, the transcription probe is hybridised to cloned DNA which has been denatured and immobilised on nitrocellulose.

In vitro nuclear transcription probes were generated, in the presence of heparin (see Chapter 2.11h for details of procedure) from 5 x 10^6 nuclei which had been isolated from undifferentiated F9 cells or from F9 cells treated for 5 days with retinoic acid, dibutyryl cyclic AMP and isobutylmethylxanthine. During the reaction, 2-3 x 10^7cpm were incorporated into trichloroacetic acid-insoluble material. The probe was hybridised for 72 hours (as described in Chapter 2.11h), at a probe concentration of 1 x 10^7cpm/ml to plasmid DNA (10μg) which had been denatured and bound to nitrocellulose, . The filter was subsequently washed at high stringency and in the presence of RNaseA to degrade any unhybridised probe. The relative transcription rates of the genes studied is shown in Figure 5.2.

The relative rate of transcription of the *Sparc* gene was found to increase following differentiation to parietal endoderm. However, the magnitude of the stimulation could not be determined since the level of SPARC transcription in EC cells was below the detection limit of the assay. An elevation in transcription was also detected for the laminin B1 and B2 genes. Surprisingly, there was no apparent increase in the transcription of pPE.992 - a cDNA clone which hybridises to a >8Kb RNA that is more abundant in differentiated F9 cells than in F9 EC cells (M.Kurkinen and D.Barlow unpublished data). Detection of of transcripts of cytochrome oxidase subunit II, a gene which is encoded by the mitochondrial genome, indicated that mitochondria were also present in the nuclear preparation.

Northern analysis has demonstrated that β_2-microglobulin transcripts are expressed at higher levels in differentiated derivatives of F9 cells than in EC cells (Croce *et al.*, 1981; Morello *et al.*, 1982; Rosenthal *et al.*, 1984) and *c-ras*^{Ha} transcript levels show only slight variation between F9 EC cells and differentiated F9 cells (Campisi *et al.*, 1984). However, transcription of both of these

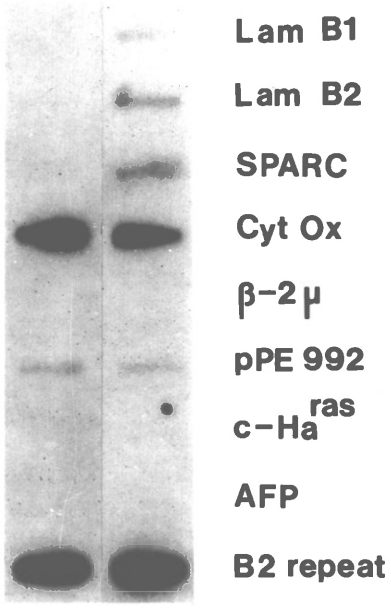


Figure 5.2 **Nuclear transcription analysis of gene expression in F9 cells before and after differentiation to parietal endoderm**

10μg of denatured, immobilised plasmid DNA was hybridised with 10^7cpm/ml of ^{32}P-labelled RNA from nuclei isolated from cells before (left panel) and after (right panel) 5 days' exposure to retinoic acid and dibutyryl cyclic AMP. The blot was washed as described in Chapter 2.11h and exposed to pre-flashed X-ray film for 7 days. Slots are: Lam B1: laminin B1 chain (pPE.49; Barlow et al., 1984); Lam B2: laminin B2 chain (pPE.9; Barlow et al., 1984); SPARC (pPE.220); Cyt.Ox.: mitochondrial cytochrome oxidase subunit II (pAG.88; Scott et al., 1983); murine B2 repeat (E10; supplied by D.Murphy; P.Brickell, D.Latchman, D.Murphy and P.W.J.Rigby, unpublished data); β-2μ: β_2-microglobulin (Croce et al., 1981); pPE.992: a parietal endoderm-specific cDNA (D.P.Barlow, unpublished data); c-ras^{Ha} (supplied by N.Teich) and AFP (Latchman et al., 1984).

genes could not be detected in this experiment. This was possibly due to the sensitivity of the assay. As expected, AFP, which was included as a negative control, failed to produce a hybridisation signal.

The results of this experiment may have been influenced by changes in the relative amounts of RNA which are transcribed by different polymerases in the two probes. For instance, the apparent increase in the transcription rate of the Sparc gene, which has been described above, could be due to an overall increase in the proportion of RNA which is transcribed by RNA polymerase II in the differentiated cells relative to the EC cells, without any change in the rate of transcription of the Sparc gene. RNA polymerase II transcription generates mRNA and is specifically inhibited by low concentrations (1μg/ml) of alpha-amanitin (Kedinger et al., 1970). Alpha-amanitin was used to estimate the relative levels of RNA polymerase II transcription in nuclear transcription probes from differentiated and undifferentiated F9 cells. The effect of the inclusion of alpha-amanitin on the incorporation of α^{32}P-GTP into trichloroacetic acid-insoluble material is shown in Table 5.1. These results suggest that the relative level of RNA transcribed by polymerase II decreases by about 30% during the differentiation of F9 cells to parietal endoderm. This is in accord with the overall decrease in protein synthesis reported by Linder et al. (1981). Therefore, I concluded that the increase in the level of SPARC RNA in the probe derived from the nuclei of differentiated F9 cells was not due to an overall increase in the levels of transcription due to polymerase II relative to polymerases I (rRNA) and III (tRNA and B2 repetetive elements), but was the result of increased transcription of the Sparc gene in the differentiated cells.

Table 5.1 **RNA polymerase II transcription in isolated nuclei from differentiated and undifferentiated F9 cells.**

Nuclei	Alpha-amanitin	Incorporation (cpm)	Reduction in incorporation by alpha-amanitin
F9 EC	-	2.49×10^6	
F9 EC	+	9.43×10^5	63%
F9 (PE)	-	3.8×10^5	
F9 (PE)	+	2.33×10^5	39%

In vitro nuclear transcription probes were generated from 5×10^6 nuclei from undifferentiated (F9 EC) and differentiated (F9 PE) monolayer cultures of F9 cells in the presence (+) or absence (-) of alpha-amanitin (1µg/ml). The incorporation of labelled nucleotide into elongated RNA chains was estimated by trichloroacetic acid precipitation.

c. SPARC mRNA is induced during the formation of F9 embryoid bodies.

Increased expression of SPARC mRNA was detected in F9 embryoid bodies following 8 days' exposure to retinoic acid (Figure 5.1). Studies by Adamson and her co-workers have suggested that the formation of a mature embryoid body, with an outer layer of visceral endoderm-like cells, occurs over a period of several days (Grover et al., 1983a, 1983b; Grover and Adamson, 1985; Grover and Adamson, 1986). Morphological and biochemical evidence has sub-divided this process into separate stages. The first 3-4 days are characterised by rapid proliferation which produces an aggregate of similar cells. Increased synthesis of the basement membrane component, laminin, occurs on the second day. Initially, matrix (laminin, type IV collagen and fibronectin) is deposited uniformly between the cells. However, by day 4 it is apparent that the outer cells are synthesizing large amounts of laminin which are deposited beneath the outer layer in a basement membrane. It is at this time that the outer cells first express markers of the differentiated phenotype (AFP, ENDO-A, ENDO-B). Therefore, it was important to determine whether increased SPARC expression occured before, during or after the time that endoderm markers are detected for the first time.

RNA was prepared from embryoid bodies following exposure to retinoic acid for various lengths of time and assayed for SPARC expression on Northern blots (Figure 5.3). Levels of SPARC mRNA were found to be reduced in aggregates cultured for 24 hours in the presence of retinoic acid, when compared to levels in monolayers of F9 EC cells. SPARC transcripts accumulated rapidly during the following 5 days, but then plateaued at a level about three-fold lower than in F9 cells treated with retinoic acid and dibutyryl cyclic AMP. Levels of the 0.7Kb transcript detected with clone pTF.75

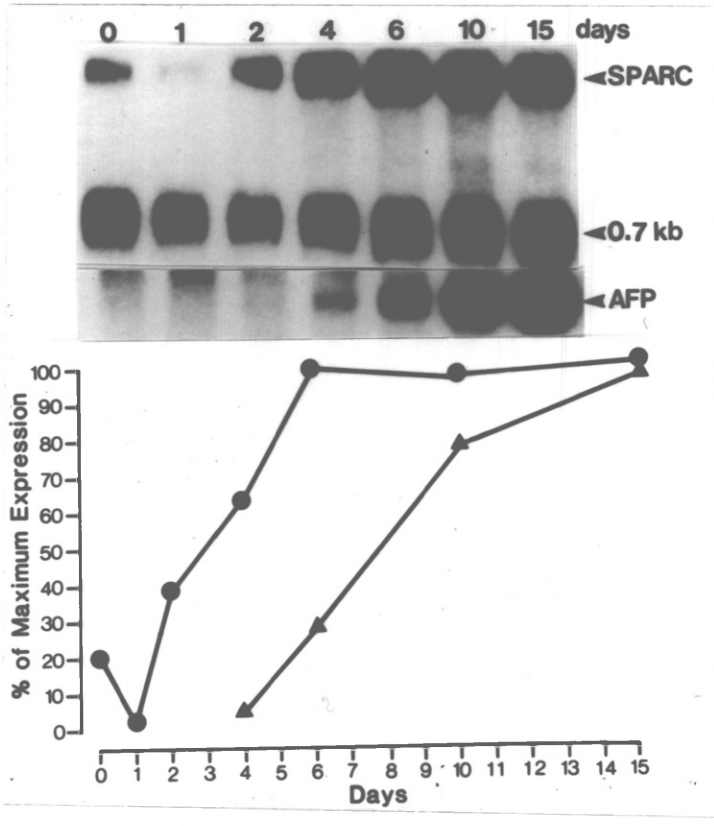


Figure 5.3 **SPARC mRNA levels in F9 cell aggregates differentiating into embryoid bodies in response to retinoic acid**

A. 3.5µg of poly(A)+ RNA extracted from F9 EC cell monolayers (0) and aggregates at various times after exposure to retinoic acid (1-15 days) was separated on a 1% (w/v) formaldehyde/agarose gel, transferred to nitrocellulose and hybridised with the ^{32}P-labelled cDNA inserts from clones pPE.220 (SPARC) and pTF.75 (0.7Kb transcript). Following hybdidisation, the blot was washed at high stringency and exposed to pre-flashed X-ray film for three days.

B. Hybridised probe DNA was removed from the blot used in A as described in Chapter 2.11d. The blot was reprobed with ^{32}P-labelled AFP antisense RNA produced from a construct in the vector pSp65 (HincII.440; Krumlauf et al., 1985) using the procedures described in Chapter 2.10n. The blot was exposed to X-ray film for two days.

C. The autoradiograms in A and B were scanned and the results expressed graphically as a percentage of the maximum levels of SPARC (circles) and AFP (triangles) detected.

exhibited little variation (<25% as estimated by denstiometry scanning) during the course of differentiation to parietal endoderm (data not shown) and embryoid body formation (Figure 5.3); these sequences served as an internal control.

To monitor the differentiation of outer, visceral endoderm-like cells the Northern blot was reprobed with an SP6-derived RNA probe, complementary to AFP transcripts. Use of this type of probe increased the sensitivity of the assay and facilitated the earliest detection of AFP transcripts. Even following long exposure (10 days) to X-ray film, AFP transcripts could not be detected earlier than day 4. Thereafter, the level of AFP transcripts continued to increase to day 15 (Figure 5.3) as reported by Young and Tilghman (1984). Hence, the accumulation of SPARC transcripts during the formation and maturation of embryoid bodies precedes the appearance of the visceral endoderm marker, AFP.

5.2 Studies of the expression of SPARC in embryonic and adult tissues and cultured cell lines

a. SPARC expression in murine extra-embryonic tissues.

The expression of SPARC mRNA was studied by Northern blot analysis in the foetus and extra-embryonic tissues of the 13.5 day p.c. mouse embryo (Figures 5.4A and 5.5). Highest levels of SPARC mRNA were detected in the parietal endoderm. Lower levels of SPARC mRNA were detected in the other extra-embryonic tissues - visceral yolk sac, placenta and amnion. Western blotting has detected SPARC protein in amniotic fluid and isolated amnions secrete SPARC into the culture medium (Mason et al., 1986b). Very little SPARC mRNA was detected in RNA extracted from the whole embryo. As estimated by densitometry scanning of the autoradiogram, the level of SPARC mRNA in the

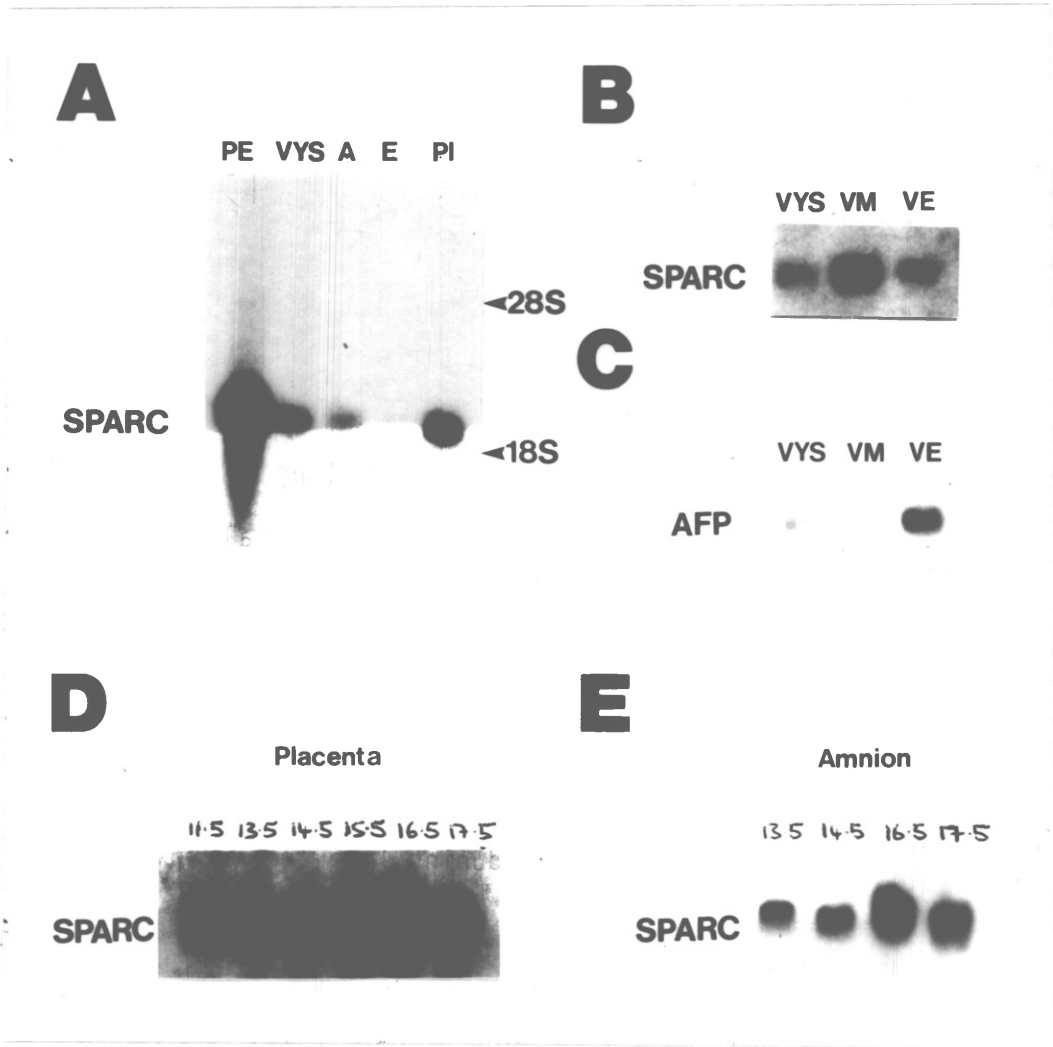

A
PE VYS A E PI
SPARC
28S
18S
B
VYS VM VE
SPARC
C
VYS VM VE
AFP
D
Placenta
11·5 13·5 14·5 15·5 16·5 17·5
SPARC
E
Amnion
13·5 14·5 16·5 17·5
SPARC

Figure 5.4 **SPARC expression in murine extra-embryonic tissues**

Approximately 10µg of total cellular RNA, standardised against 10µg of total RNA from 13.5 day p.c. mouse placenta by ^{3}H-poly(U) assay, was analysed on Northern blots, as described in Figure 5.3, using the cDNA insert from pPE.220 as a probe.

A. SPARC expression in tissues from the 13.5 day p.c. conceptus.

PE: parietal endoderm dissected-free of trophoblast; VYS: visceral yolk sac; A: amnion; E: whole embryo; Pl: placenta. The autoradiogram was over-exposed to detect the low levels of SPARC mRNA in the embryo. The position of the 28S and 18S rRNA markers was derived by ethidium bromide staining of an extra lane of placental RNA.

B. SPARC expression in the visceral yolk sac.

VYS: 5µg total RNA from visceral yolk sac; VM: 10µg of total RNA from visceral mesoderm; VE: 10µg total RNA from visceral endoderm. The autoradiogram was exposed for 2 days.

C. AFP expression in the visceral yolk sac.

AS B, but the filter was reprobed with AFP and exposed to X-ray film for 8 hours.

D. SPARC expression in the placenta during late gestation.

RNA was extracted from whole placentae between 11.5 and 17.5 days p.c. and probed as in A. The autoradiogram was exposed for 2 days.

E. SPARC expression in the amnion during late gestation.

RNA was extracted from amnions between 13.5 and 17.5 days p.c. and analysed as in A. The autoradiogram was exposed for 2 days.

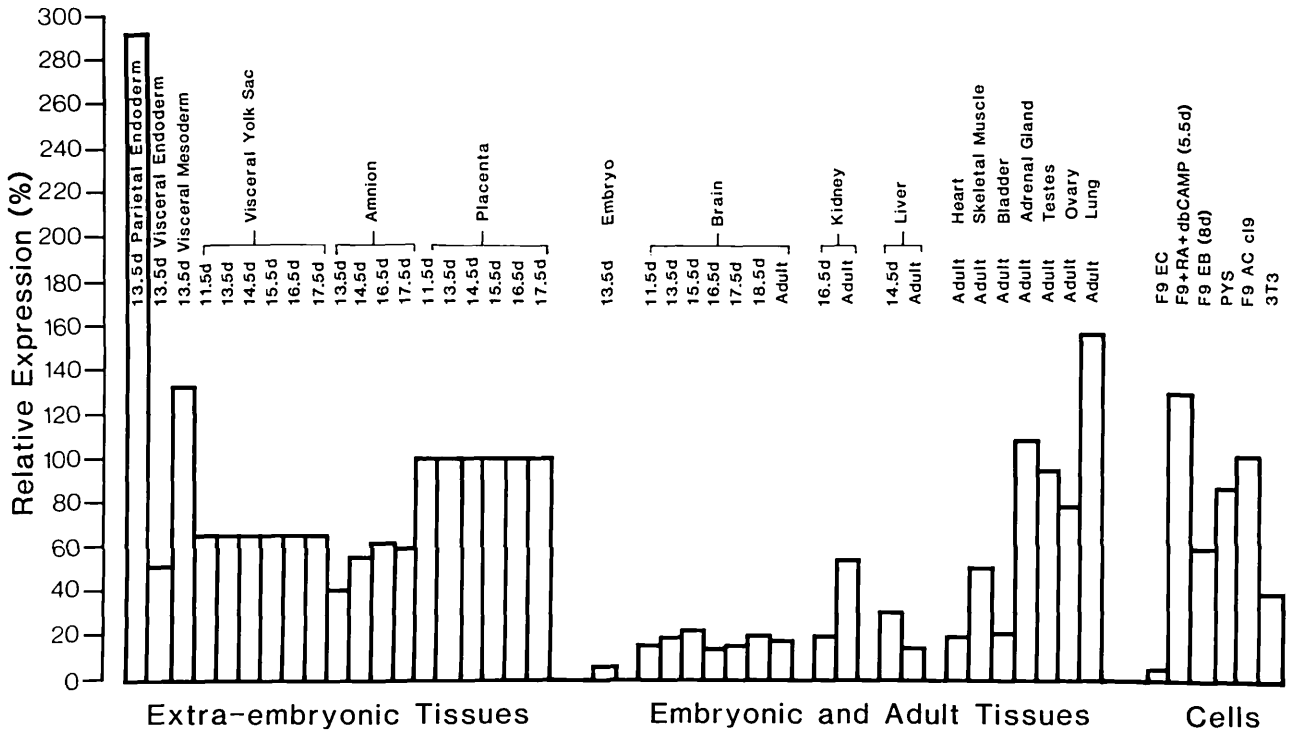


Figure 5.5 **SPARC mRNA levels in adult and embryonic tissues and cell lines**

Equal amounts of total or poly(A)+ RNA, standardised by ^{3}H-poly(U) assay, from a variety of tissues were analysed on Northern blots as described in Figure 5.3 using the cDNA insert from pPE.220 as the probe. Each experiment included a sample of either total or poly(A)+ RNA, as appropriate, from the same preparation of 13.5d mouse placental RNA. This provided an internal standard to allow comparison of the results between different experiments. The relative level of 2.2Kb SPARC mRNA was determined by densitometer scanning of the autoradiograms, and the optical density values were expressed graphically as a percentage of the level in placenta (placenta was given the arbitrary value of 100%).

parietal endoderm is nearly fifty-fold greater than in the intact foetus.

To study SPARC expression in the separated visceral endoderm and visceral mesoderm, visceral yolk sacs were treated with pancreatin and trypsin. The tissues were then separated manually and were distinguished by the blood vessels which are present only in the mesodermal layer. Northern analysis of RNA extracted from the separated tissues (Figure 5.4B), demonstrated that the level of SPARC expression in the mesodermal tissue (blood islands, blood vessels and connective tissue) was about 3-fold greater than in the visceral endoderm. Reprobing the same blot with AFP indicated that the mesoderm was not significantly contaminated with endoderm (Figure 5.4B). The level of SPARC expression in the intact visceral yolk sac is consistent with the data of Andrews *et al.* (1982b) which demonstrate that the mesoderm contributes only about 20% of the total RNA of this organ. These results also indicated that the expression of SPARC mRNA is six- to seven-fold greater in the parietal endoderm than in the visceral endoderm.

Increased expression of *c-fos* mRNA in the amnion during late gestation is described in Chapter 3. When SPARC expression in the placenta, visceral yolk sac and amnion was investigated during the same period, no change in SPARC mRNA levels were detected in the placenta (Figures 5.4C and 5.5) or visceral yolk sac (Figure 5.5) and only a small modulation was observed in the amnion (Figures 5.4D and 5.5). High levels of 2.2Kb transcripts which hybridised with *Sparc* cDNA probes under stringent conditions were also detected in human term placenta (data not shown). This result indicates that *Sparc*-related sequences are not only present in man but are transcribed in at least one human extra-embryonic tissue.

b. SPARC expression in adult mouse tissues.

SPARC mRNA expression was examined in a range of adult mouse tissues by Northern blot hybridisation (Figures 5.5 and 5.6A). Highest levels of SPARC transcripts were detected in the lung, testis, ovary and adrenal gland. SPARC expression has been detected in several fibroblast cell lines *in vitro* (Table 5.2) and approximately 50% of the lung tissue comprises a fibroblast capsule (H.Sage pers. comm.). The adrenal gland, testis and ovary are all endocrine glands which secrete steroid hormones. The production of steroid hormones (aldosterone and cortisol) in the adrenal gland is restricted to the cortex and recent *in situ* hybridisation studies have detected high levels of SPARC mRNA in all three zones of the adrenal cortex but not in the medulla (P.Holland pers. comm.). These data suggest that SPARC expression in these three organs might be related to the synthesis and secretion of steroid hormones.

Secretion of SPARC protein has been detected in primary cultures of ovarian granulosa cells (B.Hogan pers. comm.; Table 5.2). These cells are believed to be responsible for the rupture of the ovarian follicle which results in the release of the oocyte. It is believed that tissue plasminogen activator (TPA), secreted by the granulosa cells in response to increased levels of follicle-stimulating hormone (FSH) and luteinising hormone (LH), is responsible for the reduction in the tensile strength of the follicle which ultimately leads to its rupture (Strickland and Beers, 1976; Beers and Strickland, 1978; Liu *et al.*, 1981; Wang and Leung, 1983; Canipari and Strickland, 1985; Ny *et al.*, 1985). In common with SPARC, TPA expression is elevated when F9 monolayers are treated with retinoic acid and dibutyryl cyclic AMP (see Chapter 1.3e). It was therefore of interest to investigate whether SPARC expression in the ovary was regulated by FSH or LH. The

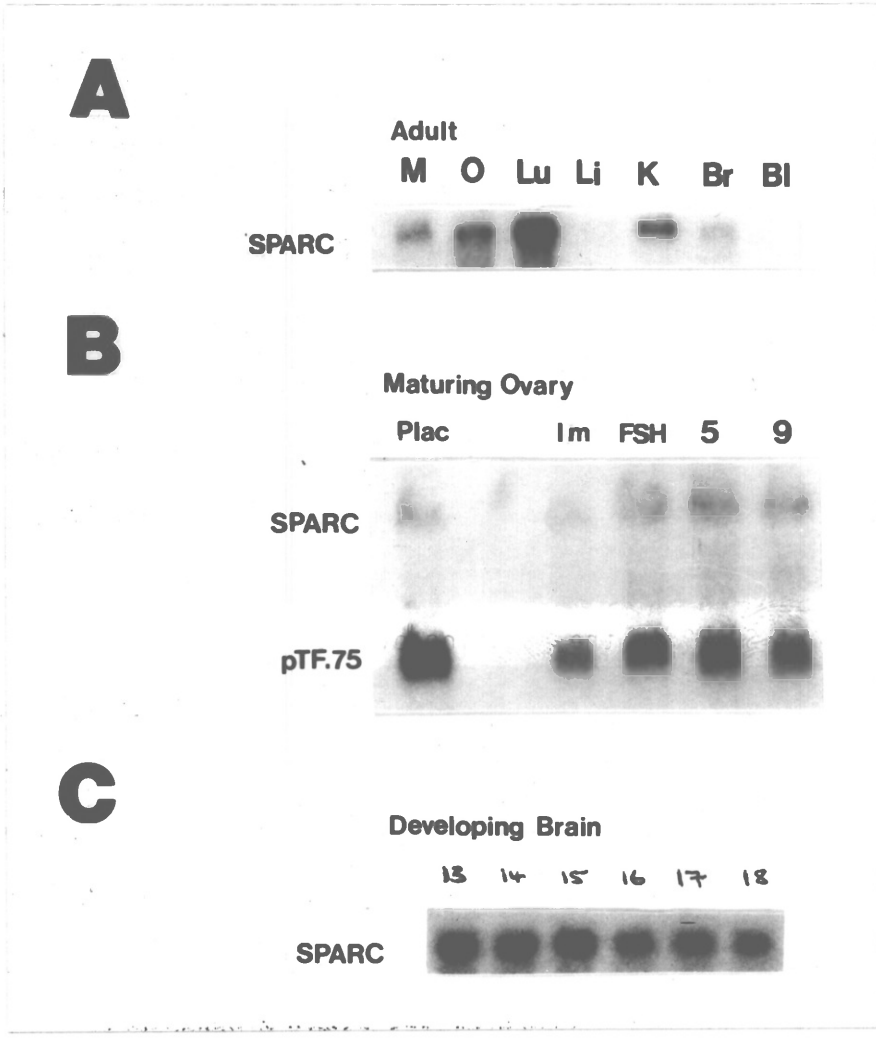

A
Adult
M O Lu Li K Br Bl
SPARC
B
Maturing Ovary
Plac Im FSH 5 9
SPARC
pTF.75
C
Developing Brain
13 14 15 16 17 18
SPARC

Figure 5.6 **SPARC mRNA expression in adult tissues and developing brain.**

The expression of SPARC transcripts was investigated in a range of tissues by Northern blot analysis as described in Figures 5.3 and 5.4. In all cases the RNA loadings were standardised by ^{3}H-poly(U) assay against 10µg of total cellular RNA from 13.5 day p.c. mouse placenta.

A. Expression of SPARC in adult mouse organs.

RNA was prepared from the pooled tissues of 4 C3H/He adult females. Li: Liver M: skeletal muscle; O: ovary; Lu: lung; K: kidney; Br: brain; Bl: bladder. The autoradiogram was exposed overnight.

B. Expression of SPARC in the maturing ovary.

RNA was extracted from the ovaries of pre-pubertal mice (Im), from the ovaries of immature mice 48 hours after administration of follicle-stimulating hormone (FSH) and from ovaries 5 and 9 hours after injection of human chorionic gonadotrophin (5 and 9). Also included was a lane of 13.5 day p.c. placental RNA (Plac) as an internal standard. The autoradiogram was exposed overnight.

C. Expression of SPARC in the developing brain.

RNA was prepared from the brains of embryonic mice between 13.5 and 18.5 days p.c. The autoradiogram was exposed for 2 days.

Table 5.2 **Expression of SPARC mRNA and protein in cultured cells**

Cell line	SPARC expression	
	mRNA	Protein
1. EC cells and their derivatives		
F9 EC	+	+
PCC4 (EC)[1]	+	ND
P19 (EC)[2]	+	ND
F9 differentiated to parietal endoderm	+++++	+++++
F9 embryoid bodies (8 days)	+++	ND
PYS-2 (parietal endoderm-like)	++++	+++++
F9ACc19 (parietal endoderm-like)	+++++	+++++
F9ACc19 vesicles	++++	ND
2. Primary cells		
Human foreskin fibroblasts[3]	++	ND
Pig chondrocytes[4]	+++++	ND
Bovine retinal endothelial cells[5]	+	ND
Human keratinocytes[6]	+	ND
Type I astrocytes (rat optic nerve)[7]	ND	+++
3. Fibroblast cell lines		
NIH-3T3	++	++
RS2[8]	++	ND
NR6[9]	++	++
Balb/c 3T3	++	ND
4. Transformed cell lines		
RAG (mouse adenocarcinoma)[10]	-	ND
2102Ep (human testicular carcinoma)[11]	-	ND
208F (*v-fos*-transformed RS2)[12]	+	ND
ANN-1 (*v-abl*-transformed NIH-3T3)[13]	+	+
pv-src (*v-src*-transformed NIH-3T3)[14]	+	+
NR6-Ab14 (*v-abl*-transformed NR6)[15]	ND	+
NIH-Ab14 (*v-abl*-transformed NIH-3T3)[15]	ND	+
SV3T3.C138 (SV40-transformed Balb/c 3T3)[16]	-	ND
HT1080 (fibrosarcoma)[17]	-	ND

Levels of SPARC protein and RNA expression are indicated relative to those detected in PYS-2 cells on a scale of - (no detectable expression) to +++++ (equivalent or greater than PYS-2 cells.

1. Jacob *et al.*, 1983.
2. Jones-Villeneuve *et al.*, 1982.
3. RNA a gift from C.Schneider.
4. RNA provided by B.Hogan.
5. RNA provided by B.Hogan.

....continued overleaf

Table 5.2 continued....

6. Cells provided by F.Watt.
7. Cells provided by J.Cohen.
8. RNA provided by D.Allen. Curran and Teich, 1982a.
9. Cells provided by J.Bell. Pruss and Herschman, 1977.
10. RNA provided by F.Benham. Klebe et al., 1982.
11. RNA provided by F.Benham. Andrews, 1983.
12. RNA provided by D.Allen. Curran and Teich, 1982a.
13. Cells provided by J.Bell. Mathey-Prevot and Baltimore, 1985.
14. Cells provided by J.Bell. Scher and Siegler, 1975.
15. Cells provided by A.Gerhardt.
16. RNA provided by D.Murphy. Scott et al., 1983.
17. RNA provided by F. Grosveld. Rasheed et al., 1974

ovaries of pre-pubertal (4 week-old) female mice can be prematurely and synchronously matured by intraperitoneal injection of FSH and LH. In my experiments maturation was induced by injection of 5iu of FSH, followed 48 hours later by luteinising hormone in the form of human chorionic gonadotrophin (5iu) as detailed in Hogan et al. (1986). Ovulation occurs between 8 and 12 hours after injection of human chorionic gonadotrophin. This treatment results in super-ovulation with the release of as many as 20 oocytes per ovary. Therefore the number of hormone-stimulated granulosa cells is greater than in a normal ovulation of 5 to 8 oocytes per ovary. RNA was extracted from the ovaries of two hormonally-induced female mice at the following times: 48 hours after injection of FSH, 5 hours and 9 hours after injection of LH and from untreated pre-pubertal females. Northern blot analysis, followed by densitometry scanning revealed less than a 50% increase in the levels of SPARC, and the 0.7Kb RNA which hybridises with pTF.75, following the administration of hormones (Figure 5.6B). These data indicate that levels of SPARC expression are not significantly affected by FSH and LH in the ovary as a whole. However, this experiment cannot rule out increases or decreases in SPARC expression that are restricted to a sub-set of ovarian cells e.g. follicular granulosa cells.

I detected lower levels of SPARC transcripts in a variety of other adult organs including heart, skeletal muscle, kidney, liver and brain (Figures 5.5 and 5.6A). Although only low levels of SPARC expression were detected in the brain, high levels of SPARC expression were detected in astrocytes derived from the neonatal rat cortex (Table 5.2). The numbers of astroglial cells in the brain increases during late gestation (M.Raff pers. comm.), but analysis of SPARC mRNA expression in the embryonic brain revealed a slight

decrease in SPARC levels during this period of development (Figure 5.6C). Lower levels of SPARC were detected in embryonic (16.5 days p.c.) than in adult kidney and slightly higher levels detected in embryonic (14.5 days p.c) liver than in the adult organ (Figure 5.5). In a study of cultured primary cells, high levels of SPARC expression were detected in pig chondrocytes, whereas lower levels were detected in bovine retinal endothelial cells and human keratinocytes (Table 5.2).

These data provide general information concerning the expression of SPARC in organs of the adult mouse. The full significance of these observations concerning SPARC expression *in vivo* and *in vitro* must await further studies on the function of the protein and *in situ* hybridisation of cDNA probes to tissue sections.

c. SPARC expression in growing and quiescent fibroblasts.

Data presented in Table 5.2 demonstrates that SPARC is expressed in a number of actively-growing, cultured fibroblast cell lines. As discussed in Chapter 3.10, *c-fos* expression is transiently increased when quiescent fibroblasts are stimulated to enter the growth cycle, for example by the addition of serum. We have detected high levels of both *c-fos* and SPARC expression in the parietal endoderm, and this raised the possibilty that SPARC might be modulated during serum stimulation in a similar manner to *c-fos*. Moreover, a report by Edwards *et al.* (1985) indicated that a 2.1 Kb mRNA, which encodes an Mr 48,000 polypeptide, increases in abundance between 6 and 18 hours after serum-induction of fibroblasts. The possibility that this sequence had homology with *Sparc* was investigated.

Sub-confluent NIH-3T3 fibroblasts were rendered quiescent by exposure to low serum for 30 hours, as described by Treisman (1985),

and then stimulated with fresh medium containing 10% foetal bovine serum. Determination of cell density, using a haemocytometer, prior to serum deprivation and at 24 and 30 hours post-starvation confirmed that the cells had been rendered quiescent. Total cellular RNA was isolated from quiescent cells and at 30 minutes, 6 hours and 18 hours after re-exposure to high levels of serum and was subjected to Northern blot analysis (Figure 5.7). In agreement with previous reports (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Muller *et al.*, 1984b), a large increase in *c-fos* expression was detected 30 minutes post-stimulation. When the same filter was probed with SPARC (pPE.220) and pTF.75, little change in the expression of either of these transcripts could be detected, although there was perhaps slightly more SPARC mRNA in the quiescent fibroblasts.

These data suggest that SPARC and the gene identified by Edwards *et al.* are not homologous and that levels of SPARC expression do not change significantly during the transition from the quiescent to the growing state.

5.3 SPARC expression is decreased in transformed fibroblast cell lines

R.G.Martin (1981) defines transformation as the "acquisition of growth characteristics not exhibited by the parental cells". As a result of oncogenic transformation, cells acquire growth properties which are characteristic of malignant tumour cells (Tooze, 1981)(in my studies the term "transformation" implies oncogenic transformation). Many of these novel growth properties are observed following treatment of cultured cells with oncogenic agents. Thus, *in vitro* transformation has been regarded as analogous to spontaneous tumorigenesis *in vivo*.

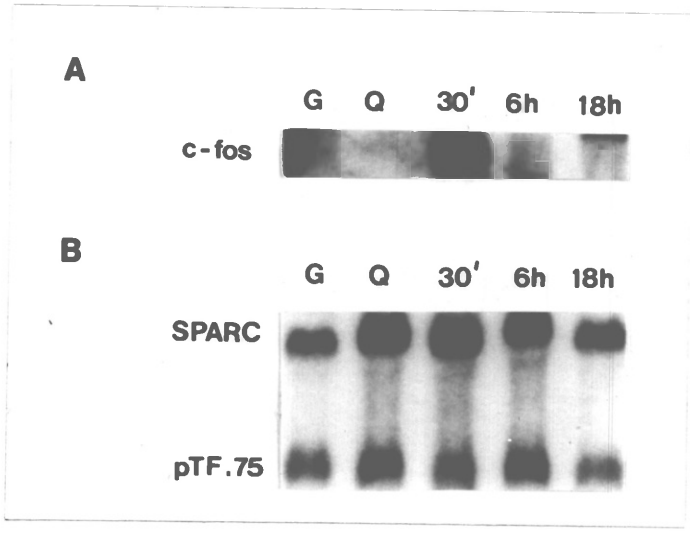


Figure 5.7 **SPARC expression in quiescent and serum-stimulated fibroblasts.**

Equal amounts of total cellular RNA (20μg) from growing, quiescent and serum-stimulated NIH-3T3 fibroblasts was hybridised, on a Northern blot as described in Figure 5.3, with nick-translated c-fos (A) and SPARC (pPE.220) and pTF.75 (B) probes. Lanes are: G; growing NIH-3T3 cells (40-60% of confluence); Q; quiescent NIH-3T3 cells and serum-stimulated cells 30 minutes, 6 hours and 18 hours after the addition of medium containing 10% foetal bovine serum.

Growth properties which are often exhibited by transformed cells include:

I. The ability to grow in low serum.

II. Loss of density-dependent inhibition of growth.

III. Anchorage-independent growth.

IV. Tumour formation in syngeneic or immuno-suppressed animals.

Martin (1981) has suggested that these growth properties constitute a hierarchy, with criterion IV, the ability to produce tumours, as the most stringent criterion and growth in low serum the least stringent. All cells which can give rise to tumours invariably possess the other three growth properties. Different transformed clones, obtained from the same parental population by the same manipulation, can exhibit variation (from "full" to "minimal") in the transformation properties which they possess (Rigby et al., 1980; Martin, 1981). Moreover, certain primary cells in culture possess some of these properties; primary cultures of both chondrocytes (Horwitz and Dorfman, 1970) and aortic endothelial cells (Laug et al., 1980) can be grown in soft agar. This strengthens the position that cells can only be defined as transformed with respect to the parent population and that possession of growth properties characteristic of transformed cells is, in itself, not proof that a cell has been transformed.

The differentiation of F9 EC cells in response to retinoic acid and dibutyryl cyclic AMP is concommitant with the loss of their ability to induce tumours in syngeneic animals (see Chapter 1.3f). A ten- to twenty-fold increase in SPARC transcripts occurs during this differentiation event, so it was therefore of interest to investigate SPARC expression in transformed cells, and, where possible, to compare this with expression in their parental cell population.

Studies of the process of transformation *in vitro* have greatly contributed to our knowledge of the less accessible process *in vivo*. However, the fibroblast cell lines which are commonly used to study transformation *in vitro* (e.g. Balb/c 3T3 or NIH-3T3) are aneuploid and have infinite growth potential in culture - properties which are common in tumour cells but not exhibited by primary fibroblast cultures. Thus these cells are transformed with respect to primary fibroblast cultures but aquire a more extreme phenotype in response to treatment with tumorigenic agents. However, SPARC expression in primary cultures of human foreskin fibroblasts is very similar to that detected in NIH-3T3 cells (Table 5.2).

The expression of SPARC mRNA or protein was examined in a variety of transformed fibroblast lines and, where possible, was compared with levels of SPARC expression in the parent population. In initial experiments, total cellular RNA was prepared from ANN-1 cells (NIH-3T3 cells transformed with Abelson murine leukaemia virus; Scher and Siegler, 1975), pv-src cells (NIH-3T3 cells transformed with *v-src* ; Mathey-Prevot and Baltimore, 1985) and from untransformed NIH-3T3 cells. Expression of SPARC mRNA was evaluated by Northern blot analysis, followed by densitometry scanning as described previously (Figure 5.8A). Approximately eight-fold less SPARC mRNA was detected in the transformed cells than in the control RNA from NIH-3T3 cells. This was also reflected in the levels of secreted SPARC protein as determined by metabolic labelling (B.Hogan and A.Taylor pers. comm.). However, the NIH-3T3 cell line studied in this analysis was not the population from which ANN-1 or pv-src had been derived. Furthermore, the two transformed cells lines had been established for some time, which may have resulted in changes in SPARC expression that are unrelated to the transformation process or expression of the

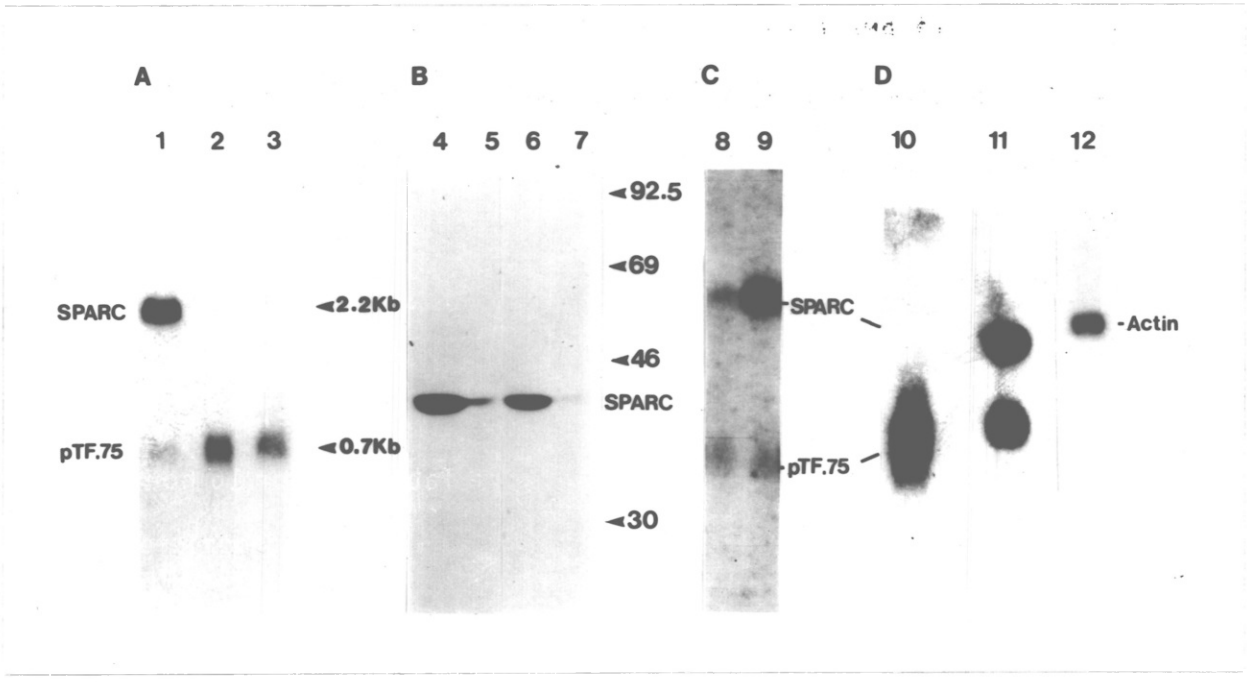

A
B
C
D
1 2 3
4 5 6 7
8 9
10
11
12
SPARC
pTF.75
2.2Kb
0.7Kb
92.5
69
46
30
SPARC
SPARC
pTF.75
Actin

Figure 5.8 **Expression of SPARC protein and RNA in fibroblast lines transformed *in vitro*.**

SPARC expression was examined by Northern blot analysis as described in Figure 5.3 (A,C and D) or by immunoprecipitation followed by SDS-PAGE and fluorography (B).

A. SPARC mRNA expression in ANN-1 and pv-src cells.

20μg of total RNA from pv-src and ANN-1 cells was hybridised with pPE.220 and pTF.75. Lane 1: NIH-3T3 cells; lane 2: ANN-1 cells; lane 3: pv-src cells. The blot was exposed to pre-flashed X-ray film for 2 days.

B. SPARC protein expression in NR6 and NIH-3T3 cells recently-transformed by *v-abl*.

"Normal" (NR6 and NIH-3T3) fibroblasts and their transformed derivatives (NR6-Ab14 and NIH-Ab14) were labelled for 16 hours with 86μCi/ml ^{35}S-methionine and SPARC recovered by immunoprecipitation from aliquots of medium containing equal amounts of trichloroacetic acid-precipitable material. Samples were analysed on a 10% SDS gel under reducing conditions. Lane 4: NIH-3T3; lane 5: NIH-Ab14; lane 6: NR6; lane 7: NR6-Ab14.

C. SPARC mRNA expression in RS2 cells and 208F fibroblasts.

10μg of total RNA from RS2 cells (lane 8) and 208F fibroblasts (lane 9) was analysed as in A. The autoradiogram was exposed for 2 days.

D. SPARC mRNA expression in SV3T3 C138.

5μg of poly(A)+ RNA from SV3T3 C138 (lane 10) and 1μg of poly(A)+ RNA from 13.5 day p.c. mouse placenta was analysed as in A. The blot was exposed to X-ray film for 5 days. Lane 12: 1μg of the same preparation of poly(A)+ RNA from SV3T3 C138 was reprobed with actin, to demonstrate that the RNA preparation was not degraded.

transformed phenotype. Therefore, SPARC expression was examined in two recently-transformed cell lines, NR6-Ab14 and NIH-Ab14, together with their parental populations. These cell lines, which had only been passaged 28 times, were established by transformation of NR6 and NIH-3T3 fibroblasts, respectively, with v-abl (A.Gerhardt and G.Foulkes unpublished data). Cells were labelled overnight with ^{35}S-methionine and secreted SPARC protein was recovered by immunoprecipitation of aliquots of the culture medium containing equal amounts of TCA-precipitable radioactivity. Analysis of the precipitated protein by SDS-PAGE and quantitative fluorography revealed that both of the transformed cell lines exhibited a large decrease in secreted SPARC protein when compared to the parental cell lines (Figure 5.8B)

Both the v-abl and the v-src proteins have cytoplasmic tyrosine kinase activity; it was therefore possible that the decrease in SPARC expression was a response to increased tyrosine phosphorylation, rather than a consequence of transformation. Therefore SPARC expression was compared between 208F rat fibroblasts and RS2 cells (208F fibroblasts non-productively transformed by v-fos; see Chapter 3.2). The v-fos gene product is nuclear and no kinase activity has been documented for it. Levels of SPARC mRNA were found to be 5-10 fold less in RS2 cells than in their parental cell line (Figure 5.8C). In addition, no SPARC mRNA could be detected in 5µg of poly(A)+ RNA from an SV40-transformed line established from Balb/c 3T3 cells (SV3T3 C138; Rigby et al., 1980; Scott et al., 1983)(Figure 5.8D), the adenocarcinoma cell line, RAG (Table 5.1), a human testicular carcinoma, 2102Ep (Table 5.1) or in a human fibrosarcoma line, HT1080 (Table 5.1). Subsequent re-probing of these RNA preparations with an actin probe demonstrated that they were not

degraded.

These data suggest that decreased SPARC expression may be a general feature of the transformation of fibroblasts *in vitro* and a property of at least some tumour cells *in vivo*. It is also interesting to note that the levels of SPARC detected in fibroblasts transformed with *v-abl*, *v-src* and *v-fos* are similar to those found in F9 EC cells since the latter cells are also transformed.

5.4 The isolation of genomic clones of the Sparc gene

Sparc genomic clones were required to allow the structure and organisation of the gene unit to be determined and to facilitate subsequent investigations to identify those sequences which are involved in its regulation *in vivo* and *in vitro*. The genomic library which was used to isolate these clones was supplied by H.Lehrach and A.M.Frischauf (Poustka *et al.*, 1984). This library was generated from partial Sau3A digestion of high molecular weight liver DNA from 129/Sv inbred mice. Fragments, of average lengths 50-100 Kb, were cloned into the vector pcos 2EMBL at the BamHI site, which allowed for the selection of recombinants on the basis of resistance to kanamycin and sensitivity to tetracycline.The library was transformed into *E.coli* BHB3175 and the average insert size was reported to be 45Kb.

The library had been amplified once in Dr Lehrach's laboratory and was amplified for a second time in our own. Following amplification, I determined that the library had a titre of 6×10^6 colony-forming units per microlitre by serial dilution. Calculations suggested that 3×10^5 recombinants would have to be screened to have a 99% probability of isolating a cosmid containing *Sparc* sequences, assuming that such recombinants had not been lost or reduced in

abundance during amplification. Approximately 6 x 10^6 colonies were plated onto nylon membranes on four 24cm x 24cm L-agar plates containing kanamycin (30μg/ml). This density of plating was such that individual colonies could not be distinguished following overnight incubation at 37°C. Duplicate copies were made of each filter and the colonies grown, lysed and the DNA fixed to the nylon membrane *in situ*. The library was screened using the cDNA insert from clone pPE.220 as a probe. From this screening, 38 duplicate positive signals were obtained, eight of these were selected, and the areas on the master plates containing these colonies were diluted into L-broth containing kanamycin. These recombinants were purified through two further rounds of screening, at which point single, well-isolated colonies could be picked. Three different recombinants were selected for subsequent studies - p30.G1, p30.G3 and p30.G5. These were grown in large-scale liquid culture and the DNA isolated for further characterisation. Several *c-fos* cosmids were also isolated from this library (I.Mason and D.Murphy cited in Treisman, 1985).

A particular problem of cloning large fragments of DNA is the rearrangement of the sequences in the bacteria - deletions of large sections of DNA are a particular problem. To determine whether any of the *Sparc* genomic isolates had been rearranged, DNA from all three cosmids was digested with BamH1 and electrophoresed on a 0.7% (w/v) agarose gel. The separated fragments were transferred to nitrocellulose and hybridised sequentially with the cDNA inserts from clones pF9.33 and pPE.220 and also with a cDNA fragment containing the sequences upstream of the internal EcoRI site in the *Sparc* cDNA clones. After washing the filters at high stringency and exposure to X-ray film, it was found that clones p30.G1 and p30.G3 produced an identical pattern of fragments which hybridised with the probes (see

Figure 5.9 for p30.G3). These results indicated that most, if not all, of the Sparc cDNA sequences were located in three BamHI fragments of approximate sizes 6Kb, 4.5Kb and 3Kb. However, in clone p30.G5, the 6Kb fragment was absent and a novel 12Kb fragment, which hybridised to the 5' cDNA sequences, was identified. These data suggested that no large rearrangements in the region encoding the Sparc gene product were present in clones p30.G1 and p30.G3, but that a rearrangement had occured in p30.G5. Clone p30.G3 was subsequently selected for detailed characterisation.

The insert size of p30.G3 was estimated to be in excess of 40Kb. This was too large to be easily restriction-mapped by conventional methods. I therefore used the Sparc cDNA clones to identify restriction fragments containing Sparc sequences, which could subsequently be sub-cloned to facilitate detailed restriction mapping of the gene. Southern blots of p30.G3 DNA, digested with a range of restriction endonucleases, either singley or in combination, were probed with fragments of the Sparc cDNA clones (Figure 5.9A). This allowed a preliminary restriction map of the genomic clone to be generated (Figure 5.9C).

There are obvious problems with this approach:

1. Genomic fragments which contain only short stretches of sequence present in the cDNA probe may not be detected or only produce a weak hybridisation signal which may not be distinguishable from partial digestion products.
2. The presence of intron sequences in the genomic DNA can lead to errors in the interpretation of data.

Assuming that such artefacts were not present in these data, my results indicated that the Sparc gene unit might span about 20Kb. Restriction fragments of p30.G3 have subsequently been subcloned and

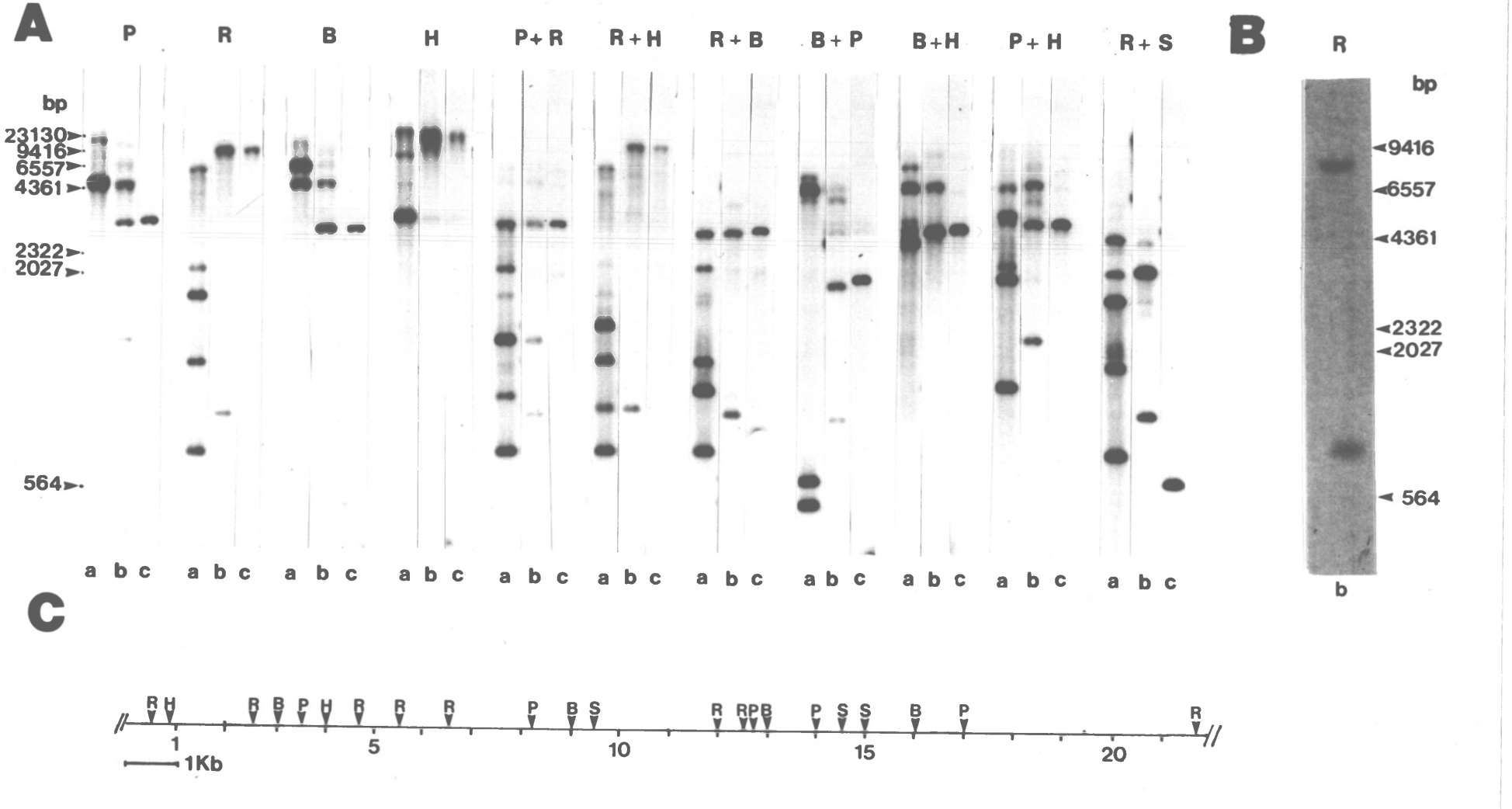

A
P
R
B
H
P+R
R+H
R+B
B+P
B+H
P+H
R+S
bp
23130
9416
6557
4361
2322
2027
564
a b c
B
R
bp
9416
6557
4361
2322
2027
564
b
C
1
5
10
15
20
1Kb

Figure 5.9 **Analysis of the SPARC gene unit.**

A. Restriction mapping of the cosmid clones using cDNA probes.

1µg of p30.G3 was digested with a variety of restiction endonucleases, either singly or in combinations. The digested DNA was separated on a 1% (w/v) agarose gel and transferred to a nylon membrane. The filter was hybridised to an EcoR1 fragment of pIM.3 (see Figure 4.4; lanes a). The blot was washed at high stringency and exposed to pre-flashed X-ray film overnight. The same filter was then probed with the cDNA inserts from pPE.220 (lanes b) and pPE.33 (lanes c). Abreviations: B; BamH I; H: Hind III; P: Pst I; R: EcoRI; S: Sma I.

B. Analysis of the SPARC gene in genomic DNA.

10µg of genomic DNA from C3H/He mouse liver were digested with EcoRI, separated on a 0.7% (w/v) agarose gel and transferred to nitrocellulose. The DNA was hybridised with nick-translated pPE.220, washed at high stringency and exposed to X-ray film for 2 days.

C. Restriction map of the SPARC gene unit.

Restriction map of the region of genomic DNA containig the Sparc gene based on data obtained from A and subsequent analysis by J.McVey (pers. comm.).

are being utilised in a detailed examination of the structure of the Sparc gene (J.McVey pers. comm.)

Comparison of results obtained from the Southern blot analysis of p30.G3 with similar analyses of genomic DNA, isolated from the livers of C3H/He mice and digested with the same enzymes, revealed an identical pattern of hybridisation with all of the cDNA probe fragments (compare Figures 5.9A and 5.9B, and data not shown). These data confirmed that p30.G3 did not contain any large sequence rearrangements and also that Sparc was probably a single-copy gene. Studies on the chromosomal location of Sparc, using both in situ hybridisation and recombinant inbred strains, have recently confirmed that there is a single copy of the Sparc gene per haploid genome and that this is located in the central region of mouse chromosome 11 (Mason et al., 1986b). There are very few polymorphic markers for mouse chromosome 11, consequently, these studies will provide a firm basis for the future linkage of cloned genes on this chromosome. Results from Dr Uta Franke's laboratory show that the human Sparc gene is on chromosome 5.

To determine whether Sparc had homology with other sequences in the mouse genome, probes spanning the entire length of the Sparc transcript were hybridised to digested genomic DNA on Southern blots under conditions of low stringency. The conditions used should allow the detection of sequences with greater than 70% homology to Sparc (McGinnis et al., 1984). This experiment failed to detect sequences other than those which had been detected at high stringency and which were present in p30.G3, suggesting that Sparc is not a member of a multigene family. Experiments performed under similar conditions of low stringency detected Sparc related sequences in the human genome, as had been predicted from the results of Northern blot analysis of

human placental and fibroblast RNA, but failed to detect homologous sequences in the Drosophila melanogaster or Aplysia genomes.

Discussion

5.5 Gene expression during the differentiation of F9 cells to parietal endoderm

Sparc is one of several genes which exhibit a considerable increase in expression during the differentiation of F9 EC cells to parietal endoderm in response to retinoic acid and dibutyryl cAMP. Other cloned genes which have similar patterns of expression include laminin B chains (Wang and Gudas, 1983; Barlow et al., 1984; 1986), type IV collagen chains (Kurkinen et al., 1983b; Wang and Gudas, 1983; Marotti et al., 1985) and several unidentified sequences (Wang et al., 1985; M.Kurkinen and D.Barlow pers. comm.). Following treatment with inducing agents for 5.5 days, the level of SPARC mRNA increases between 10 and 20 fold and metabolic labelling studies have revealed that 20 to 40 times more SPARC protein is secreted. Kurkinen et al. (1982, 1983c) have reported that the elevation in the levels of type IV collagen and laminin mRNAs, as assayed by in vitro translation and immunoprecipitation, are of a similar magnitude. In common with SPARC, the increase in laminin-encoding transcripts is also reflected in the accumulation of secreted laminin (Cooper, A.R. et al., 1983).

It has been proposed that retinoic acid functions through a simliar mechanism to steroid hormones (Lotan, 1980). Cells which are responsive to steroid hormones contain specific protein receptors in the cytoplasm which form a complex with the hormone molecule. Recent evidence suggests that the hormone-receptor complex is either formed

or transferred to the nucleus where it interacts with chromatin and influences gene expression. Inhibition of retinoic acid metabolism does not prevent the differentiation of F9 cells, suggesting that, like the steroids, retinoic acid acts directly (Williams and Napoli, 1985a, 1985b). Cytoplasmic proteins which bind to retinoic acid (CRABP - cellular retinoic acid binding protein) and its metabolite retinol (CRBP - cellular retinol binding protein) have been identified in many normal and transformed cells (Lotan, 1980), including F9 cells (Mertz et al., 1985). Where studied, these have all been found to be of similar sizes (Mr 16,000 or 2S). Furthermore, bovine CRABP and rat CRBP have homology at the amino acid level with each other and other cellular retinoid-binding proteins (Sundeli et al., 1985). The levels of CRABP and CRBP increase during the first 96 hours of F9 cell differentiation in response to exogenous retinoic acid and dibutyryl cyclic AMP (Mertz et al., 1985; Eriksson et al., 1986) and this increase is most rapid during the first 24 hours. Jetten and Jetten (1979) have reported nuclear localisation of a retinoic acid-CRABP complex in the EC line PCC4-aza1R following exposure to 10^{-6}M ^{3}H-retinoic acid for 2 hours and nuclear localisation of a retinoic acid-CRABP complex has also been reported in other cell types in vitro (Lotan, 1980; Boyd et al., 1984). In addition, two mutant EC cell lines which fail to differentiate in response to retinoic acid have little or no CRABP activity (Schindler et al., 1981; Sherman et al., 1981). Taken together, these data suggest that retinoic acid may act in a manner analogous to steroid hormones. The progesterone and glucocorticoid receptors bind to specific sequences in the genes which they regulate (Mulvihill et al., 1982; Dean et al., 1983; Pavyar et al., 1983; Karin et al., 1984; Renkawitz et al., 1984; von der Ahe et al., 1985). The DNA

sequences specifically recognised by the glucocorticoid receptor *in vitro* have been shown to render a heterologous promoter (Herpes simplex virus thymidine kinase promoter) glucocorticoid-responsive *in vivo* (Chandler *et al.*, 1983). However, no data concerning the ability of CRABP or CRBP to bind to DNA, either in the presence or absence of retinoids is, as yet, available.

It has been demonstrated that the steroid hormone oestrogen stimulates the accumulation of ovalbumin transcripts in the hen oviduct and vitellogenin RNA in the liver of *Xenopus laevis*. For both genes, regulation has been demonstrated at the levels of transcription and mRNA stability (Palmiter and Carey, 1974; McKnight and Palmiter, 1979; Shapiro and Brock, 1985). The increase in the levels of SPARC mRNA and protein, following the differentiation of F9 cells to parietal endoderm, has been found to be due, at least in part, to an increase in the transcription rate of the *Sparc* gene. I also found, in accord with the results of Wang *et al.* (1985), that transcription of the laminin B1 mRNA also increases. However, the B1 clone, that I used, hybrid-selects mRNAs encoding both laminin B1a and B1b chains under stringent hybridisation conditions (Barlow *et al.*, 1984), so the signal detected will reflect the cumulative transcription levels of both sequences. I have also detected an increase in the transcription of laminin B2 mRNA. In addition, increases in the transcription rates of a type IV collagen chain and the intracisternal A-particle (IAP), following the treatment of F9 monolayers with retinoic acid and dibutyryl cyclic AMP, have been reported (Wang *et al.*, 1985; Howe and Overton, 1986). The relative rates of transcription of the different genes cannot be compared by this procedure because the hybridisation signal will be affected by factors which are unrelated to the rate of transcription. These

include the length of the primary and processed nuclear transcripts, variation in the specific activity of individual probe transcripts due to their base composition and the length of target DNA available for hybridisation.

Wang et al. (1985) have demonstrated that exposure to retinoic acid alone increases the transcription rate of the laminin B and type IV collagen genes and that this rate of transcription is further increased in the presence of dibutyryl cyclic AMP. Positive transcriptional regulation by dibutyryl cyclic AMP in other systems has been demonstrated for the lactate dehydrogenase, phosphoenol-pyruvate carboxykinase, tyrosine aminotransferase and prolactin genes (Chapkiewicz et al., 1982; Murdoch et al., 1982; Jungman et al., 1983). However, the effects of cyclic AMP are not detected until 16 hours after induction in F9 cells (Morita et al., 1985). It is known that retinoic acid increases intracellular protein kinase activity and cyclic AMP concentration (Evian et al., 1981; Plet et al., 1982). It is possible, therefore, that the effect of exogenous cyclic AMP is to enhance cellular processes initiated by retinoic acid and that this results in increased transcription of certain genes already induced by retinoic acid.

Steroid hormones are also known to influence message stability. We found that levels of nascent nuclear transcripts hybridising to pPE.992 were not elevated in differentiated F9 cells, despite an increse in the steady-state levels of this transcript during F9 differentiation. One explanation for these results is that there is post-transcriptional modulation of transcripts hybridising to pPE.992. Preliminary studies on the influence of α-amanitin on the levels of laminin B1 transcripts have suggested that the presence of dibutyryl cyclic AMP increases the stability of this mRNA (D.Barlow

pers. comm.). Levine et al. (1984a) have isolated sequences which exhibit a reduction in expression following the differentiation of F9 cells. It has been demonstrated that retinoic acid negatively regulates the transcription of these genes, but that dibutyryl cyclic AMP appears to act post-transcriptionally to further reduce transcript levels, providing further evidence of a post-transcriptional role. However, there are alternative explanations for the observations concerning the regulation of pPE.992. For instance, Bentley and Groudine (1986) have recently suggested that the c-myc gene is regulated, in differentiated HL60 cells, by the attenuation of transcript elongation. The design of my experiments and those of the other workers cited above would not have identified transcriptional control of this type, and it is formally possible that this could account for my observations on the transcription of pPE.992. Bentley and Groudine also report transcription on both strands of the c-myc gene and this, too, cannot be ruled out in my experiments.

SPARC mRNA and protein can be detected in undifferentiated F9 cells (Figure 5.1 and Figure 4.3). F9 EC cells have a low spontaneous rate of differentiation (Sherman and Miller, 1978), but this is apparently insufficient to account for the detected levels of SPARC expression. F9 EC cells express approximately twenty-fold less SPARC than differentiated cells. However, the spontaneous rate of differentiation of our EC cultures was estimated, by the detection of cytoplasmic laminin and entactin using indirect immunofluorescence, to be 1 cell in 400 (Cooper, A.R. et al., 1983). Since 98% of the EC cells differentiate to parietal endoderm in response to 4 days' exposure to retinoic acid, dibutyryl cyclic AMP and isobutylmethyl-xanthine (Cooper, A.R et al., 1983), a much larger increase in SPARC

expression would be expected if it is only expressed in differentiated cells present in the EC cell cultures. Therefore, it is probable that SPARC, in common with laminin (Cooper, A.R. et al., 1983; Wang and Gudas, 1983; Wang et al., 1985; Barlow et al., 1986), type IV collagen (Wang and Gudas, 1983; Wang et al., 1985) and entactin (Cooper et al., 1983), is expressed in F9 stem cells. Marotti et al. (1985) detect little or no type IV collagen mRNA in undifferentiated F9 cells, but disparities in the expression of these genes detected between laboratories may be due to variations in the F9 cultures. It can be concluded from these studies that the increased expression of the Sparc, laminin B1 and laminin B2 and possibly the type IV collagen genes is due to an increase in transcription from a lower, "basal" level in the EC stem cells, rather than due to the "switching on" of completely repressed or inactive genes. This type of regulation is compatible with either the activation by retinoic acid and dibutyryl cyclic AMP of positive regulatory elements within these genes or the removal of negative regulatory influences. In this context, it is interesting that the SV-40, Moloney murine leukaemia virus, and polyoma genomes are transcribed more efficiently in differentiated F9 cells than in undifferentiated stem cells. There is a body of evidence which suggests that this is due, at least in part, to negative regulation of transcription in the undifferentiated cells (Dandolo et al., 1983; Gorman et al., 1985). The regulatory activity in EC cells, which is lost on differentiation, closely resembles that of the adenovirus E1A gene (Jones, 1986). Both activities repress the enhancers of polyoma and SV40 (Borelli et al., 1984) and fail to block transcription of the polyoma virus mutant ECF9-1 which replicates efficiently in EC cells (Hen et al., 1986). These data suggest that the repressor activity in

undifferentiated EC cells and E1A block transcription in a similar fashion. However, whether the loss of this activity from EC cells is a cause or a consequence of differentiation has still to be ascertained. As speculation, it is possible that the stimulation of Sparc, laminin and type IV collagen transcription during differentiation is accomplished via the same processes which release the block on viral transcription.

Retinoic acid and dibutyryl cyclic AMP may act through separate or identical cis-acting sequences to stimulate SPARC expression, and these may ultimately be identified as enhancer elements. It has been proposed that the steroid hormone receptors bind to and activate enhancer elements which stimulate transcription (Parker, 1983). The rat liver glucocorticoid receptor recognises specific nucleotide sequences in close proximity to the mouse mammary tumour virus which are required for hormonal induction in gene transfer experiments (Chandler et al., 1983; Pavyar et al., 1983; Schneidereit et al., 1983; Haynes et al., 1983). Similar sequences have been found in the human metallothionein gene II_A (Karin et al., 1984) and in the chicken lysozyme gene (Renkawitz, 1984), which are also regulated by glucocorticoids. By analogy, it is possible that homologous retinoic acid- and cyclic AMP-responsive regulatory elements are located within the Sparc, laminin and type IV collagen genes. These might be identified by a combination of "footprinting" and sequencing techniques.

Figure 5.1 shows the accumulation of SPARC transcripts during the differentiation of F9 cells to parietal endoderm. A decrease in the level of SPARC mRNA was detected during the first 24 hours of differentiation. Type IV collagen has been reported to undergo a similar transient in expression six hours after adding inducers (Wang

and Gudas, 1983). A twenty-fold decrease in c-myc mRNA levels during this period has also been described (Campisi et al., 1984). This has been attributed to post-transcriptional regulation (Dean et al., 1986). These workers have postulated that the regulation of c-myc is related to growth arrest and not to processes involved in the expression of differentiation-specific markers. It may also be related to the 80% loss of anchorage-independent growth which occurs, in response to retinoic acid, during this period (Rodrigues et al., 1985). Levine et al. (1984a) have identified several other sequences which exhibit a reduction in expression during this period and have suggested that these effects are independent of dibutyryl cyclic AMP. However, certain transcripts, including laminin (Wang and Gudas, 1983; Wang et al., 1985) and type IV collagen (Wang and Gudas, 1983; Marotti et al., 1985; Wang et al., 1985) and a homeobox-containing transcript (Colberg-Poley et al., 1985) exhibit increased levels after the first 24 hours of differentiation. This suggests that the decrease in SPARC transcript levels is not part of a global reduction in RNA accumulation during the earliest part of the differentiation process.

After the first 24 hour period, the accumulation of SPARC mRNA is biphasic. A sharp increase in the rate of accumulation of SPARC transcripts occurs between 24 and 48 hours post-stimulation; this has been documented for several other sequences (Wang and Gudas, 1983; Kurkinen et al., 1983b; Wang and Gudas, 1985; Barlow et al., 1986). Between 48 and 72 hours the level of SPARC expression does not increase significantly. Since the culture medium and inducing agents were replenished after 48 hours it was possible that this influenced the expression of SPARC mRNA. However, when the same RNA time-course was probed with laminin, no plateau was observed during this period

(D.Barlow pers. comm.). Furthermore, no change in the rate of SPARC accumulation was detected following the replenishment of medium on day 4. Marotti et al. (1985) have reported a similar decrease in the rate of accumulation of type IV collagen transcripts, but this occured between 24 and 48 hours after induction and was not detected by Kurkinen et al. (1983b) or Wang and Gudas (1983). Again these quantitative and qualitative variations may be due to differences between the F9 cells held by the different groups or due to disparity in the culture materials.

Between 3 and 5.5 days post-induction SPARC, laminin B1 and B2 and type IV collagen transcripts continue to accumulate. However, levels of the homeobox-containing transcript decrease (Colberg-Poley et al., 1985). It is during this period that the first increases in secreted entactin protein (Cooper, A.R et al., 1983) and cellular IAP transcripts (Howe and Overton, 1986) are detected.

The studies described above have revealed complex patterns of gene expression during the differentiation of F9 monolayers in response to retinoic acid and dibutyryl cyclic AMP and there is evidence that this is accomplished by post-transcriptional, as well as transcriptional, regulation. As envisaged when my studies were initiated, these processes can now be investigated in DNA-mediated transfection analyses utilising cloned markers, including Sparc.

5.6 Expression of SPARC during the formation of embryoid bodies

If F9 EC cells are cultured in bacterial petri dishes, they do not attach to the plastic but form aggregates in suspension and if these aggregates are exposed to retinoic acid, they differentiate into embryoid bodies which consist of an inner core of EC cells surrounded by an outer layer of visceral endoderm-like cells (Hogan et al.,

1981). My initial observation, that SPARC mRNA levels are elevated in embryoid bodies compared to undifferentiated F9 cells, was expanded in a study of the timing of SPARC induction. During the first 24 hours of culture, SPARC mRNA levels decrease (Figure 5.3). A similar decrease has been reported for laminin B1 transcripts, but not laminin B2 mRNA (Grover et al., 1986). Thereafter, SPARC transcripts increase five-fold in aggregates cutured for 6 days and subsequently remain largely unaltered. In accord with the results of Young and Tilghman (1984), the first AFP transcripts are not detected until day 4, but continued to accumulate thereafter. The basement membrane components, chondroitin sulphate proteoglycan, α1- and α2-type IV collagen, laminin B1, and laminin B2, all show very different patterns of RNA accumulation during the differentiation process (Grover et al., 1986), none of which resemble that of Sparc. However, major histocompatibility complex H2 and actin transcript levels do increase during the first five days and then plateau (Young and Tilghman, 1984). Consequently, it appears that SPARC expression is not coordinately regulated with those of basement membrane components, but may share certain common features with H2 and actin.

F9 embryoid bodies consist of two major cell populations; an outer layer of visceral endoderm-like cells, as determined by morphological and biochemical criteria, and an inner core of apparently undifferentiated F9 EC cells. It is not known whether increased SPARC expression is induced only in the outer cells, or if the inner cells also respond to retinoic acid and express higher levels of SPARC. This possibility can be resolved by in situ hybridisation techniques. If only the outer visceral endoderm layer expresses high levels of SPARC, the amount of SPARC which we detect in embryoid bodies will be reduced by RNA derived from the inner, undifferentiated cells. Thus,

the highest levels of SPARC expression in the outer cells may be similar to that detected in F9 monolayers treated with retinoic acid and cyclic AMP.

It has been proposed that the early increase in the synthesis of matrix proteins during the formation of embryoid bodies is involved in the deposition of a basement membrane between the outer layer of cells and the inner core (Grover et al., 1983a, 1983b; Grover and Adamson, 1985). These authors suggest that the presence of a basement membrane allows the outer cells to express a polarised morphology and, subsequently, to differentiate into visceral endoderm. This hypothesis is supported by the expression of basement membrane components which increases during the first four days and, in the case of chondroitin sulphate proteoglycan, α1-type IV collagen and laminin B1, subsequently decrease (Grover et al., 1986). In accord with this hypothesis the addition of laminin or anti-laminin serum to the culture medium prevents the differentiation of the outer epithelium, presumably by interfering with the ability of the cells to assume a polarised morphology. It is possible that SPARC, which increases during the period of basement membrane deposition, is also involved in the differentiation of visceral endoderm and may subsequently be required to maintain the visceral endoderm phenotype.

5.7 Early events may be the same during the differentiation of F9 cells to parietal endoderm and the formation of embryoid bodies

Experimental manipulation of teratocarcinoma stem cells in vitro is believed to mimic certain murine developmental events. More specifically, the F9 embryonal carcinoma cell line has been used as a model system with which to study the developmental events which lead to the formation of parietal and visceral endoderm. Immunological and

nucleic acid hybridisation techniques have now identified many markers which exhibit differential expression within this system (including laminin, type IV collagen, entactin, AFP, IAP, transferrin and SPARC). The data provided by these markers, together with studies of cell morphology, can be used to evaluate how faithfully the F9 system duplicates the developmental events which occur *in vivo*.

Parietal endoderm and visceral endoderm arise from a common stem cell population - the primitive endoderm. It has been suggested that F9 EC cells represent a pre-primitive endoderm cell type, possibly inner cell mass cells. However, Adamson and Hogan (1984) report that F9 cells express low levels of certain differentiation markers, EGF receptor and transferrin, which are not expressed in certain other EC cell lines, e.g. PC13 and PCC4. This observation prompted the authors to suggest that F9 EC cells may already be partially differentiated along the endoderm pathway. However, undifferentiated F9 cells still retain certain EC cell characteristics e.g. expression of SSEA-1 antigen (Solter and Knowles, 1978).

Strickland (1981) has proposed that addition of retinoic acid to monolayers of F9 cells converts them to primitive endoderm-like cells. This effect has been demonstrated to be irreversible (Strickland and Madhavi, 1978; Solter *et al.*, 1979; Linder *et al.*, 1981). The subsequent differentiation of these cells is determined by external influences which are dependent on the culture conditions. This process is summarised in Figure 1.4, which suggests that a common pathway exists during the early stages of F9 differentiation to parietal or visceral endoderm and that these events mimic the derivation of primitive endoderm in the mouse embryo. If this model is correct, it would be expected that, during this period, the regulation of gene activity, as manifested by the expression of

biochemical markers, is the same for both the parietal endoderm and visceral endoderm pathways. Furthermore, during primitive endoderm formation, F9 cells should readily switch to the other pathway by altering the culture conditions.

My studies on the expression of SPARC mRNA during the differentiation of F9 cells along either pathway demonstrates that a decrease in expression during the first 24 hour period, followed by a rapid increase during the following 24 hours is common to both processes. Retinoic acid may affect all of the cells in the F9 aggregates at this time. Grover and Adamson (1986) have studied the the early events in the differentiation of F9 cells, in suspension, to either parietal endoderm or embryoid bodies. Using metabolic labelling, they report that, during the initial 24 hours, the levels of secreted fibronectin and type IV collagen, and cytoplasmic laminin A and B chains are the same in both pathways. Furthermore, the abundance of these proteins is unaffected by increasing concentrations of retinoic acid and dibutyryl cyclic AMP until after this initial phase.

If the two manipulations produce the same initial events, we must ask for how long the differentiation events follow a common course? My data concerning SPARC expression would suggest a figure of 24-48 hours, while the data of Grover and Adamson would suggest that gene regulation is the same for both pathways only during in the first 24 hours. In contrast, Marotti et al. (1985) have reported that levels of type IV collagen in F9 monolayers treated with 10^{-7}M retinoic acid alone increase until day 3, whereas Strickland and Madhavi (1978) report that the response to retinoic acid requires 2 days. However, equivalent levels of type IV collagen are achieved after 24 hours' exposure to the same concentration of retinoic acid in the presence

of dibutyryl cyclic AMP. Thus, if a common pathway, involving the formation of a primitive endoderm intermediate, exists when F9 cells are differentiated to parietal endoderm or visceral endoderm, the derivation of the primitive endoderm cell type may be accelerated in the presence of dibutyryl cyclic AMP and possibly by culture as aggregates.

The data presented above are based on the appearance and accumulation of markers of differentiation, but there may be a lag between the irreversible commitment to the formation of primitive endoderm and the appearance of these markers. Levine et al. (1984b) have reported that 2mM sodium butyrate inhibits the effects of retinoic acid, but only if added during the first 8 hours; suggesting that certain retinoic acid-induced events are complete within this period.

It was predicted that, in addition to exhibiting common patterns of gene expression, F9 cells at early stages of differentiation should be readily converted to the alternate pathway. Grover and Adamson have demonstrated that F9 aggregates, cultured in retinoic acid and cyclic AMP for 24 hours and then exposed to retinoic acid alone for 8 days, morphologically resemble cystic embryoid bodies and secrete increased levels of AFP. The ability of aggregates to convert from the parietal endoderm to the visceral endoderm pathway persists for 3 days, but thereafter the cells are irreversibly differentiated. However, Morita et al. (1985) have found that the effects of dibutyryl cyclic AMP on laminin and type IV collagen secretion in monolayer cultures are reversible during at least the first 5 days of culture. It has been shown for laminin that this is due to a decrease in transcript levels. These effects take 48 hours to occur and there is a concomitant reversion of cell morphology. These observations are

consistent with a reversion to a primitive endoderm cell type, although it has yet to be accertained whether or not these cells can subsequently differentiate into visceral endoderm. The addition of dibutyryl cyclic AMP to the medium of F9 embryoid bodies causes both the inner and outer cells to differentiate into a parietal endoderm phenotype (Grover and Adamson, 1986). This ability to "trans-differentiate" persists even in 9 day-old embryoid bodies and complements experiments of Hogan and Tilly (1981) on the ability of visceral endoderm to differentiate into parietal endoderm *in vitro* (see Chapter 1.2c)

Thus, although these two lines of evidence suggest that common processes occur early in the differentiation of F9 cells to both parietal and visceral endoderm and involve the formation of a primitive endoderm-like intermediate, the data also imply that the "differentiated" cells still retain some plasticity.

5.8 *Transformation-sensitive regulation of the Sparc gene*

Changes in the expression of cellular genes are a general feature of oncogenic transformation. These alterations presumably determine many of the novel biochemical, behavioural and growth properties of the transformed cell. Williams *et al.* (1977) studied changes in gene expression between an SV40-transformed fibroblast cell line and its parental cell using mRNA/cDNA hybridisation in solution. They reported that approximately 3% of the transcripts present in the transformed cell were absent in the parental cell, while about 5% of the parental sequences were absent in the transformed line. However, more subtle changes would not have been detected, since these experiments were performed under conditions of mRNA excess. Studies on SV40- and polyoma-transformed BHK21 hamster cells suggest that

quantitative changes occur in the levels of many proteins as a result of transformation by these agents and most of these changes involved decreases in the relative abundance of the protein in the transformed cell (Bravo and Celis, 1980).

In my studies, SPARC expression was found to be reduced in transformed fibroblast lines relative to the parental cells. Moreover, SPARC could not be detected in cell lines established from an adenocarcinoma, a testicular carcinoma or a fibrosarcoma. In the previous chapter the homology between SPARC and a secreted bovine protein, 43K, is described. Sage and her co-workers have failed to detect 43K in a range of transformed cells including murine haemangiopericytoma, human Wilm's tumour and human astrocytoma (Sage et al., 1984). The latter is particularly interesting since high levels of SPARC expression have been detected in neo-natal rat astrocytes (Table 5.2). In common with SPARC, 43K was not detected in HT1080 cells (Sage et al., 1984). These data suggest that reduced levels of SPARC expression may be a general feature of transformed cells, however, many more transformed cell lines need to be examined before we can be certain of this.

The experiments which I have performed cannot ascertain whether or not SPARC is involved in the transformation process. It is interesting, however, that SPARC expression increases in parallel with matrix protein expression during the differentiation of F9 cells and loss of the ability to form tumours in vivo. Decreased expression of matrix proteins is a common feature of oncogenic transformation of fibroblasts in vitro (Hynes, 1973; Adams et al., 1977; Krieg et al., 1980; Fagan et al., 1981; Liau et al., 1985; Schmidt et al., 1985; Trueb et al., 1985). However, recent work by Setoyama et al. (1985) suggests that the reduced expression of matrix proteins is unrelated

to the maintenance of the transformed phenotype. These workers describe mutant lines of v-mos-transformed fibroblasts with elevated levels of those matrix proteins (type I collagen and fibronectin) which were repressed following transformation and the revertant lines still retain their tumorigenic capacity. This demonstrates that not all of the transformation-sensitive proteins play a role in the expression of the transformed phenotype.

While the role of SPARC in the transformation process remains uncertain, it will be interesting to determine the nature of its regulation during the in vitro transformation of fibroblasts. If the Sparc gene proves to be regulated at the transcriptional level, it might provide a means of identifying trans-acting regulatory molecules which are induced by the transforming agent or as a secondary effect of transformation.

5.9 Towards the function of the Sparc gene

The data presented in this chapter describe the expression of transcripts from the single-copy Sparc gene both in vivo and in vitro. The primary aim of these studies was to provide the necessary background information for future studies directed towards the identification of the regulatory sequences which control its expression in vivo and in vitro. However, these data may also provide information concerning the function of the Sparc gene product.

Sparc expression was studied in very few homogeneous cell populations in vivo, but the highest levels of expression were detected in an extra-embryonic cell population - the parietal endoderm. The parietal endoderm is a homogeneous population of cells which are specialised for the synthesis of a thick basement membrane, known as Reichert's membrane. Consequently, many of the major

secreted proteins of these cells are basement membrane components (Chapter 1.2c). SPARC constitutes about 25% of the ^{35}S-methionine-labelled protein that accumulates in the medium of parietal endoderm cells cultured *in vitro* (Figure 4.11), but is not a major integral component of Reichert's membrane (Mason *et al.*, 1986a)

The parietal endoderm cells are part of a bi-layered extra-embryonic membrane - the parietal yolk sac. The parietal yolk sac is a dynamic tissue and undergoes extensive remodelling throughout post-implantation development. It expands into the uterine stroma without creating blood clots which would impede the filtration of fluid through Reichert's membrane. During the expansion of the parietal yolk sac, Reichert's membrane first thickens, then becomes thinner and eventually breaks down before the end of pregnancy (Clark *et al.*, 1975; Dickson, 1979). The increase in parietal endoderm cell number which accompanies the expansion of the yolk sac appears to occur either by continual recruitment from a primitive endoderm population or by transdifferentiation of visceral endoderm rather than by proliferation of existing parietal endoderm cells (Chapter 1.2c). These data are consistent with my results which suggest that SPARC expression is not involved in cell proliferation - SPARC expression does not change significantly when NIH-3T3 fibroblasts are rendered quiescent and it is only expressed at low levels in the foetus as a whole. Likewise, levels of secreted bovine 43K in confluent and sub-confluent cultures of aortic endothelial cells are unaffected by culture in the presence or absence of serum (Sage *et al.*, 1984).

In view of the close contact of the parietal yolk sac with the maternal blood sinuses (Figure 1.3), it is possible that SPARC may be involved in the transport of maternally-derived metabolites or ions into embryonic tissues. This might account for its high levels of

expression in extra-embryonic tissues relative to the foetus proper.

Alternatively, the secretion of SPARC may be related to the migratory properties of the parietal endoderm cells or to the assembly and re-modelling of Reichert's membrane. In this context, it has already been demonstrated that parietal endoderm, like many other migratory tissues and tissues undergoing remodelling, secretes large amounts of the protease, tissue plasminogen activator (Strickland et al., 1976, 1980; Marotti et al., 1982; Mullins and Rohrlich, 1983). But the high levels of SPARC mRNA expression detected in primary cultures of pig chondrocytes would seem to preclude a role in cell migration, since these cells are not motile. Moreover, TPA expression is often elevated in transformed cells (Mullins and Rohrlich, 1983) whereas SPARC expression, in common with that of matrix components, appears to be reduced. Increased expression of bovine 43K is detected when endothelial cells are subjected to "culture shock" conditions which cause them to re-synthesise an extracellular matrix beneath their basal surface (Sage, 1986; Sage et al., 1986). In view of this possible association of SPARC with matrix metabolism, it is of interest that astrocytes secrete both SPARC and laminin (Liesi et al., 1983b) in vitro and that increased laminin synthesis by astrocytes, in vivo, occurs transiently following brain injury. Liesi (1984) has proposed that this increase in laminin synthesis may be involved in axonal regeneration; a process which is accompanied by the increased synthesis of acidic proteins of Mr 43,000-49,000 (Skene, 1984). However, it is difficult to reconcile an exclusive role for SPARC in the secretion and assembly of extracellular matrix with the detection of relatively high levels of SPARC mRNA in the testis, ovary and adrenal cortex. Perhaps SPARC plays a more generalised role in the secretion process? If so, high levels of

SPARC expression might be expected in tissues involved in the digestive process e.g. salivary glands, pancreas and small intestine.

The data which I have accumulated offer only very preliminary evidence concerning the function of SPARC. Its expression in nearly all of the cells and organs examined suggests that it may perform a housekeeping function, for instance in the synthesis of extracellular matrix - a property common to most cells - but that elevated levels of SPARC play a role in the specialised functions of certain cell types over and above extracellular matrix synthesis. Obviously, these data can only be fully evaluated following further experiments directed towards the understanding of the function of SPARC and identifying those cells in which it is expressed.

CHAPTER 6

Post-script - murine SPARC is homologous to bovine osteonectin

Following the completion of Chapters 4 and 5 of this thesis the amino-terminal sequence of bovine osteonectin was published (Young, et al., 1986). The sequence was derived from both adult and foetal protein and also from a cDNA clone and shows extensive homology to both bovine 43K and SPARC (Figure 6.1). Subsequent communication with the authors has revealed that the entire amino acid sequences of bovine osteonectin and SPARC have more than 90% homology (Figure 6.2), and that the cDNA sequences are highly homologous (70%-90%) in their coding regions, 5' untranslated sequences and close to the poly(A) addition site (Figure 6.3). Moreover, the genomic organisation of bovine osteonectin is very similar to that of Sparc, consisting of a 3' exon of about 1100bp and six uniformly small exons of about 150bp (Findlay et al., 1986; J.McVey pers. comm.). In common with the interpretation of my primer extension studies, bovine osteonectin has been found to have two transcription initiation sites (M.Bolander and J.D. Termine pers. comm.) These results, taken together with the inability to detect Sparc-related genes on Southern blots of mouse genomic DNA using probes from sequences >70% homologous to osteonectin under conditions of low stringency, strongly suggest that Sparc is the murine osteonectin gene.

Osteonectin was originally isolated from 4M guanidinium.HCl, 0.05M EDTA extracts of foetal bone and migrated with an Mr of 32,000 (close to the predicted Mr of the SPARC protein) on Sepharose CL-4B equilibrated and run in extraction buffer (Termine et al., 1981a).

Figure 6.1 Amino-Terminal Homology Between Bovine Osteonectin, Bovine 43K and murine SPARC.

Bovine cDNA-derived osteonectin[1]	Ala Pro Gln Gln Glu Ala Leu Pro Asp Glu Thr Glu Val Val Glu Glu Thr Val Ala
Foetal bovine osteonectin[1]	Ala Pro Gln x Glu Ala Leu Pro Asp Glu x Glu Val Val Glu Glu x Val Ala
Adult bovine osteonectin[1]	x Pro Glx x Glu Ala Leu Pro Asp Glx Glu Glx Val Glu Glu Glu Leu Val Ala
Bovine 43K[2]	Ala Pro Gln Gln Glu Ala Leu Pro Asp Glu Cys x Val Val
SPARC[2]	Ala Pro Gln Gln Glu Val Ala - - Glu Glu Ile Val Glu Glu Glu Thr Val Val
	Thr

The amino-terminal sequences of bovine osteonectin and SPARC were derived from translation of cDNA sequence, and amino-terminal sequence of bovine 43K, foetal and adult osteonectins was obtained by sequencing purified protein.

References: 1. Young et al., 1986.
2. Mason et al., 1986a; This Thesis.

Figure 6.2 Dot-Matrix Comparison of the Predicted Protein Sequences of SPARC and Osteonectin.

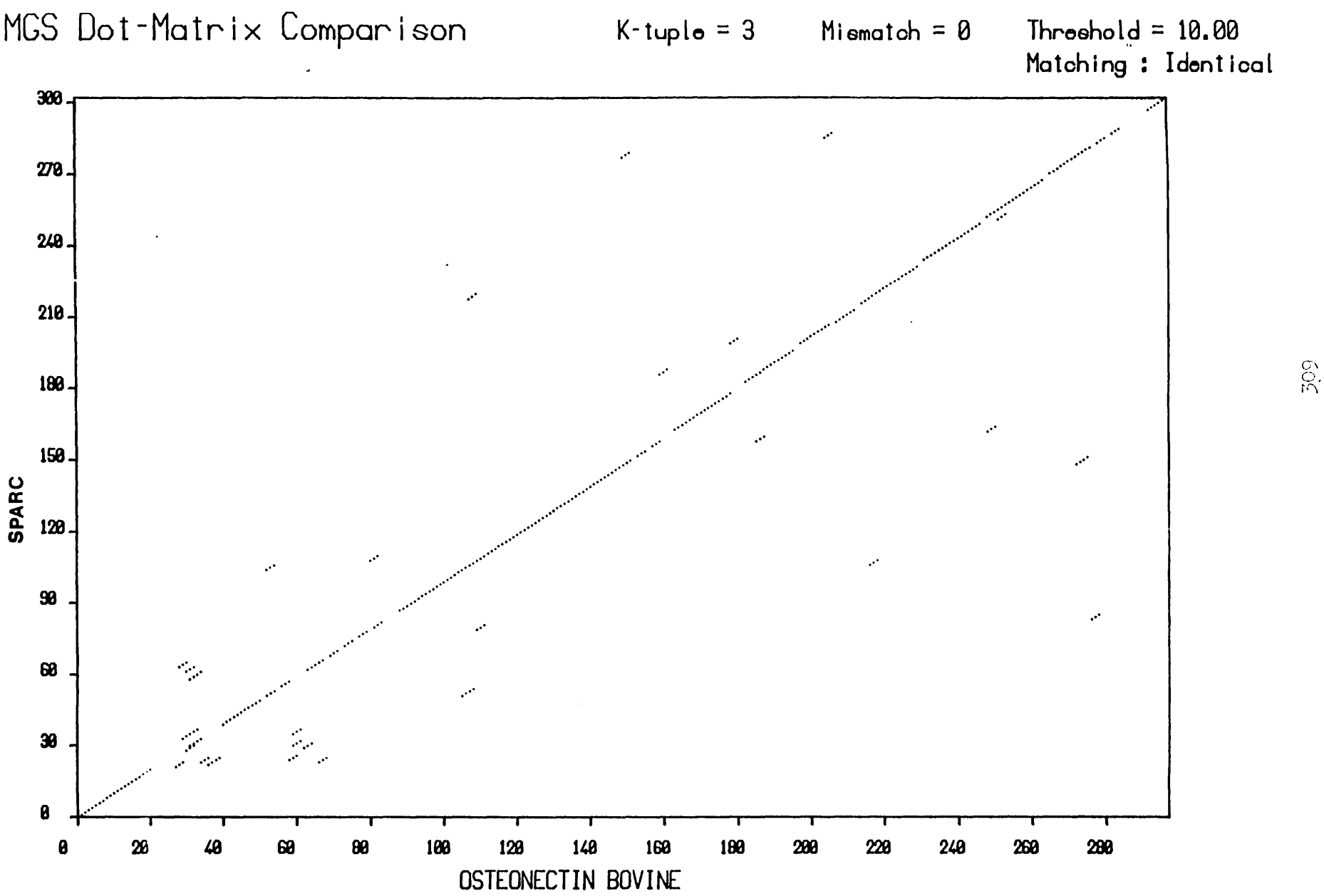
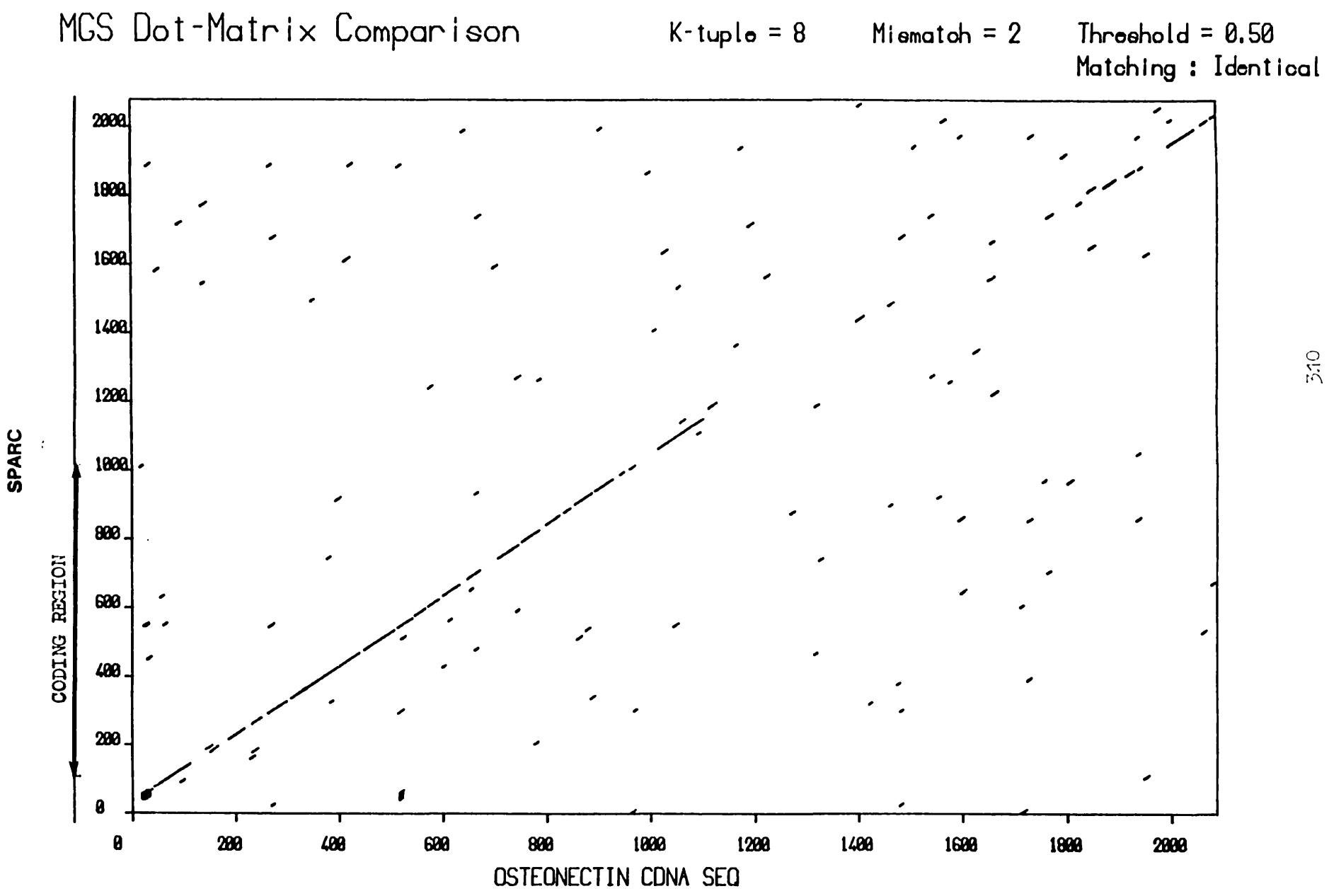


Figure 6.3 Dot-Matrix Comparison of SPARC and Osteonectin cDNA sequences



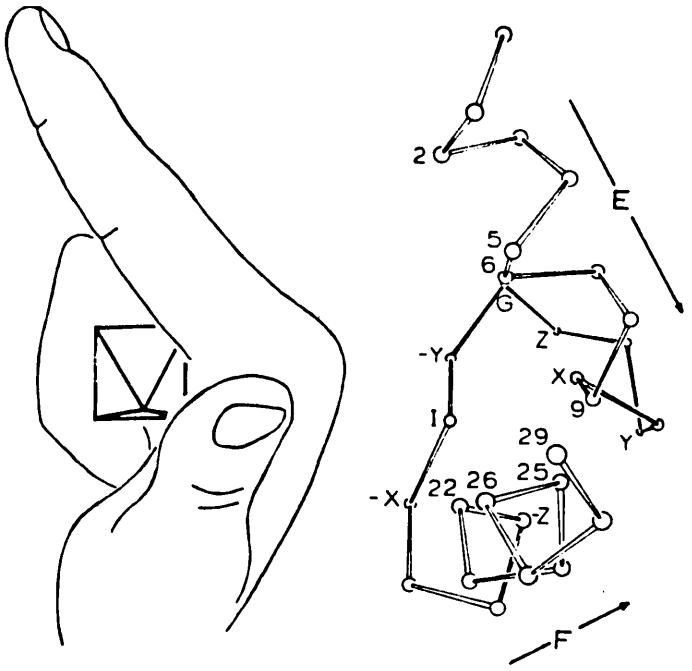
In common with SPARC, immunoprecipitated osteonectin analysed by SDS-PAGE under reducing conditions migrates with an Mr of about 43,000 (Termine et al., 1981a; Whitson et al., 1984; Romberg et al., 1985). Moreover, the cell-free translation product of osteonectin mRNA migrates with an Mr 6,000-9,000 greater than the secreted form (Otsuka et al., 1984; Kuwata et al., 1985; Young et al., 1986).

On the basis of indirect immunofluorescence and ELISA assays of cell extracts, osteonectin was originally believed to be bone-specific (Termine et al., 1981b). The purified protein binds strongly to calcium, hydroxyapatite and type I collagen, and promotes the deposition of calcium phosphate material onto type I collagen in vitro (Termine et al., 1981b; Romberg et al., 1985). It was from these properties that the name osteonectin was derived ("osteo": bone; "nectare": to bind or link). In foetal calf bone one osteonectin molecule is present for every 4-5 triple-stranded collagen molecules (Termine et al., 1981a). It is of particular interest that osteonectin is one of three proteins depleted in the bones of Holstein cattle with the congenital disease, bovine osteogenesis imperfecta; a condition which is characterised by extensive demineralisation of the bones (Termine et al., 1984).

In view of the calcium-binding properties of osteonectin, the predicted amino acid sequence of SPARC was analysed for potential calcium-binding domains. A classical EF-hand was identified close to the carboxyl terminus of SPARC, between residues 265 and 292. The molecular configuration of an EF-hand is illustrated in Figure 6.4A and comprises two alpha helices (the index finger and thumb in the diagram) separated by a calcium-binding loop. EF-hands do not exhibit great amino acid homology, but all have considerable structural homology (reviewed in Kretsinger, 1982). To maintain the structure of

Figure 6.4 The Structure of the SPARC EF-Hand.

A.



B.

α-helix → EF-HAND (X Y Z -Y -X -Z) ← α-helix

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Concensus EF-Hand	x	h	x	x	h	h	x	x	C h	D A	x	D	x	D E	G	x	I	h D	x	x	D E	h	x	x	h	h	x	x	h
SPARC EF-Hand	C 265	T	T	R	F	F	E	T	C	D	L	D	N	D	K	Y	I	A	L	E	E	W	A	G	C	F	G	I	K 298

A. Structure of the EF-Hand (from Kretsinger, 1979).

B. Comparison of the SPARC EF-Hand with the EF-Hand concensus sequence from Kretsinger (1980). The notation used is the standard 1 letter amino acid code, h is any hydrophobic residue and x is any residue.

the alpha-helices residues 2,5,6,9 and 22, 25,26, 29 are hydrophobic (h). The calcium-binding domain contains 6 calcium-binding ligands which are assigned to the vertices of an octahedron and designated X, Y, Z, -Y, -X and -Z (Figure 6.4A and B). The residue at -Y is always isoleucine (I) since the oxygen atom involved in the calcium binding is provided by the main chain peptide. In contrast, the other 5 positions can be occupied by the following amino acids which all have oxygen-containing side-chains: aspartic acid, asparagine, glutamic acid, serine or threonine. Glycine is frequently present at position 15, although in the case of SPARC (Figure 6.4B) and scallop myosin (Kretsinger, 1982) this position is occupied by lysine.

All of the proteins with EF hands that have been identified to date are cytoplasmic and are functionally-regulated through calcium-dependent configurational changes (e.g. calmodulin, troponin C, S-100, parvalbumin and myosin light chains). In contrast, SPARC is the first extra-cellular protein in which an EF-hand has been identified and, since extracellular calcium levels are about 1mM, it is likely that the calcium binding loop of the secreted protein will always have a bound calcium. This suggests that SPARC either has a structural requirement for calcium or that it may be involved in the transport of this ion. With respect to the former hypothesis, it is interesting that the SPARC EF-hand contains two cysteine residues (positions 9 and 25) which have the potential to bind and further stabilise the EF-loop. Cysteine residues have been found at position 9 in parvalbumin molecules of certain species of fish, but no instance of a cysteine at position 25 has previously been documented.

Studies on the expression of osteonectin mRNA have several similarities with my studies on the Sparc gene. In common with SPARC, osteonectin is expressed at relatively high levels in chondrocytes

and at low levels in bovine brain and liver. However, highest levels of osteonectin are detected in bone cells where it constitutes about 0.1% of the poly(A)+ RNA (Young et al., 1986). Two osteonectin transcripts are detected in chick embryo fibroblasts, although Southern blot hybridisation is consistent with only a single copy of the gene per haploid genome. A further similarity with my studies is that both of these transcripts are undetectable following the transformation of chick embryo fibroblasts with Rous sarcoma virus.

CHAPTER 7

Summary and Concluding Remarks

This thesis describes research designed to identify and characterise transcripts which are preferentially expressed at high levels in the parietal endoderm of the developing mouse embryo. Two mRNAs which have this property were identified - the transcripts of the c-fos and Sparc genes. Subsequent studies were conducted to gain further insight into the expression of these genes and the nature of the proteins which they encode. These data will provide the basis for future research in two main areas: the regulation of their expression and the biological roles of the proteins which they encode. The following is a brief summary of the conclusions which have been derived from my studies.

a) Expression of the c-fos proto-oncogene in murine extra-embryonic tissues

The expression of the c-fos gene in murine extra-embryonic tissues was studied on Northern blots. At 13.5 days p.c. c-fos transcript levels were found to be most abundant in the parietal endoderm. During the differentiation of F9 cells to a parietal endoderm phenotype, c-fos exhibits only a small modulation, between 30 and 90 minutes of the addition of inducing agents, before returning to basal levels that are considerably less than those detected in parietal endoderm. Northern blot analysis of c-fos RNA levels in the other extra-embryonic tissues during mid- to late-gestation revealed that the increase in c-fos detected in the combined visceral yolk sac and

amnion which had been reported by other workers was confined to the amnion. In this tissue, c-fos mRNA increases about 10-fold in its abundance between 13.5 and 17.5 days p.c.

The expression of c-fos protein was investigated in the parietal endoderm and amnion using an anti-serum directed against a peptide corresponding to the C-terminal amino acids of c-fos. The synthesis of the c-fos protein ceases in prolonged cultures of both of these tissues, but metabolic labelling for short periods detected Mr 46,000 c-fos protein in extracts from both of these tissues. The protein was rapidly modified to forms with higher Mr values (46,000-64,000). Labelling studies with radioactive orthophosphate revealed that the protein was phosphorylated. In addition, a phosphoprotein of Mr 39,000 was found to be associated with c-fos in both of these tissues.

b) Characterisation of the SPARC transcript and protein

SPARC was identified by the differential screening of a cDNA library prepared with RNA from F9 cells differentiated to a parietal endoderm phenotype. The screening was performed with cDNA prepared from parietal endoderm and visceral yolk sac RNA, and SPARC was identified as a 2.2Kb transcript which was preferentially expressed in the former tissue. Longer SPARC cDNAs were isolated by screening two parietal endoderm libraries and these were sequenced. The results of primer extension analysis of the 5' end of the SPARC mRNA suggested that cDNA clones had been isolated from the entire length of the transcript. In addition, SPARC genomic clones were also isolated, comparative analysis of these clones and genomic DNA on Southern blots suggests that only a single copy of the Sparc gene is present per haploid genome in the mouse.

Conceptual translation of the assembled sequence indicated that SPARC transcripts contained a single long open reading frame. Analysis of the amino acid sequence of the predicted protein suggested that the protein would be secreted, acidic, have many intramolecular disulphide bonds and might be glycosylated. An antiserum raised against the C-terminal sequences of the predicted protein was used to identify SPARC and test these predictions. Hybrid-selection, followed by *in vitro* translation and immunoprecipitation provided direct and unambiguous proof that the 2.2Kb transcript encoded a protein which migrated with an Mr of 48,000 on SDS-PAGE under reducing conditions and analysis on two-dimensional gels suggested that the protein was acidic. Metabolic labelling studies using parietal endoderm cells and parietal endoderm cell lines revealed that SPARC was secreted and studies with the antibiotic tunicamycin proved that it was glycosylated. The Mr of the secreted protein on SDS-PAGE under reducing conditions was 43,000, but this was decreased to 39,000 under non-reducing conditions, indicating that SPARC contains intramolecular disulphide bonds.

Considerable homology has been detected between SPARC and two bovine proteins, 43K and osteonectin. Structural and immunological homology has been detected between SPARC and 43K, while the predicted amino acid sequences of SPARC and bovine osteonectin are more than 90% homologous. Low stringency hybridisation studies have failed to detect SPARC-related sequences in the mouse genome and I therefore concluded that SPARC is the murine homologue of bovine osteonectin.

c) Studies on the expression of the Sparc gene

Northern analysis revealed that SPARC mRNA was expressed at high levels in the parietal endoderm of the 13.5 day p.c. mouse embryo. Quantitative evaluation of these data revealed that expression of SPARC in this tissue was about 6-fold greater than in the visceral endoderm and about 50-fold greater than in the intact embryo. Studies performed on mid- to late-gestation extra-embryonic tissues revealed that, unlike c-fos, SPARC expression remains largely unchanged during this period. Levels of SPARC transcripts also increase in F9 aggregates cultured in retinoic acid, although this induction preceeds the formation of a visceral endoderm-like epithelial layer and the expression of AFP.

When these studies were extended to adult tissues, high levels of SPARC transcripts were detected in the lung, adrenal gland, testis and ovary. When SPARC expression was analysed in the maturing ovary, no change in transcript levels were detected during the maturation process leading up to ovulation.

Studies on the expression of SPARC during the serum-stimulation of quiescent mouse fibroblasts revealed little variation in SPARC transcript levels with the growth state of the cells. In contrast, the levels of both SPARC mRNA and secreted protein are decreased in transformed fibroblasts compared to their parental cell lines.

d) Future prospects

My studies have provided information concerning the expression of both c-fos and SPARC transcripts in murine extra-embryonic tissues and studies on the Sparc gene were extended to the adult. Since the adult tissues, as well as certain of the extra-embryonic tissues,

contain several mophologically and functionally distinct cell types, more precise data concerning the expression of SPARC and c-fos will require the use of in situ hybridisation or indirect immunofluorescence and immunostaining techniques. In this respect, it will be of particular interest to determine whether c-fos is expressed in the same cells as the other oncogenes which are known to be transcribed in the placenta (c-src, c-myc and c-fms). To date, in situ hybridisation has already revealed that the high levels of SPARC expression which I detected in the adult adrenal gland are confined to the steroid-producing cortex, and studies are underway to determine whch cells express SPARC in other endocrine organs including the testis, ovary and placenta (P.Holland and S.Harper pers. comm.). The same techniques will allow SPARC and c-fos expression to be investigated at earlier stages of murine development. In particular, it is important to examine expression in the primitive endoderm. The results of the latter experiment will determine whether these genes are developmentally regulated during the differentiation of parietal endoderm. My hybridisation studies have already revealed that SPARC is expressed in the visceral endoderm, although at levels about 6-fold less than in visceral endoderm. Thus, although there is differential regulation of the gene between the two tissues, Sparc is not a particularly useful marker with which to study the developmental choice between the two cell types.

Studies on the regulation of SPARC expression in vivo, using the F9 system, currently appear to have the greatest potential. I have already demonstrated that both SPARC mRNA and secreted protein levels increase during the differentiation of monolayers of F9 cells to a parietal endoderm phenotype, due to an increase in the transcription

rate of the *Sparc* gene. However, further experiments are required to determine the temporal nature of this increase. In particular, it will be of interest to determine whether the initial decrease in steady-state levels of SPARC mRNA is due to a decrease in the rate of transcription, or, as in the case of *c-myc* (Dean *et al.*, 1986), to a decrease in the stability of the transcript. Additionally, experiments using cycloheximide may be used to investigate the effects of inhibition of *de novo* protein synthesis on the early stages of SPARC induction and on the accumulation of the SPARC transcript. Information is also required concerning such questions as the ability of retinoic acid alone to induce the increase in SPARC expression, whether cyclic AMP affects the rate of transcription and stability, and how long the cells need to be exposed to retinoic acid for before the effects of cyclic AMP are manifest.

In contrast to *Sparc*, *c-fos* mRNA undergoes only a small modulation in expression early in the differentiation of F9 cells to parietal endoderm, before returning to basal levels that are considerably less than those detected in the parietal endoderm proper. Since cultured parietal endoderm and amnion cells cease expression of *c-fos* when placed in culture, and, in the case of the amnion cells expression can be restored by placental conditioned medium, the possibility that *c-fos* may be induced in differentiated F9 cells and cultured parietal endoderm cells by the same factors can be investigated.

The availability of *Sparc* genomic clones will allow the characterisation of those elements within the body of the gene that regulate its expression *in vitro* in the F9 system and, subsequently, *in vivo*. Elegant studies of this type have already been performed by Tilghman and her co-workers using the AFP gene (Scott and Tilghman, 1983; Young and Tilghman, 1984; Krumlauf *et al.*, 1985). Mutagenesis

of the Sparc gene in vitro, followed by DNA-mediated transfection may be used to determine those cis-acting sequences within the gene unit that are required for the increase in transcription during the differentiation of F9 cells to parietal endoderm. It will be of particular interest to study the effect of deleting the tetranucleotide repeat in the 5' non-coding sequences of the SPARC transcript. Subsequently, the question of whether these cis-acting sequences also regulate the increase in SPARC expression in F9 embryoid bodies and whether they are present in other genes which also undergo an increase in transcription during the differentaition of F9 cells to parietal endoderm e.g. laminin and type IV collagen can be adressed. Ultimately, it will be important to establish whether the cis-acting sequences that regulate Sparc expression in F9 cells are also required for its correct regulation in the parietal endoderm in vivo through the use of transgenic mice.

At another level, the role of DNA methylation in the regulation of SPARC expression, both in vivo and in vitro can be investigated. There is already evidence which suggests that a general reduction in cytosine methylation occurs during the differentiation of F9 cells (Morello et al., 1983; Tanaka et al., 1983; Young and Tilghman, 1984; Razin et al., 1984), although this finding is complicated by the observation that growth conditions (culture in vivo or in vitro) can affect the the degree of methylation of discrete genes (Erickson et al., 1984). More data are required concerning the methylation of individual genes during the differentiation of F9 cells since the data currently available are sparse and somewhat confusing: both AFP and albumin are demethylated during the differentiation of visceral endoderm-like cells in F9 embryoid bodies, whereas an expressed H-2K gene is hypermethylated in differentiated derivatives of F9 cells

(e.g. F9Ac C19; Tanaka *et al.*, 1983). Surprisingly, although treatment of differentiated cells with 5-azacytidine reduces H-2K expression it fails to induce expression of AFP or albumin in F9 EC cells. In this context, studies on the expression of SPARC in differentiating F9 cells may provide additional data which can subsequently be compared with methylation patterns *in vivo* in the parietal and visceral endoderm.

Future studies on the SPARC and *c-fos* proteins will undoubtedly focus on their biological functions. More information is required concerning the sub-nuclear localisation of *c-fos*, its interactions with chromatin and the nature of the *c-fos*-associated protein, p39. The recent data concerning the calcium-binding potential of the SPARC protein require further investigation and, in view of the homology with osteonectin, studies of SPARC expression in developing bones are required. The availability of cDNA clones will allow large quantities of both proteins to be synthesised in prokaryotic systems, and the purified protein can be used in both physical and biological studies. Alternatively, the availability of these clones allows the function of the two proteins to be evaluated through the use of constructs expressing antisense RNA to inhibit the translation of the endogenous gene (see Weintraub *et al.*, 1985 for a review).

The *Sparc* gene has been mapped to the region of sub-band B1 on mouse chromosome 11 (Mason *et al.*, 1986b). The linkage map of this chromosome reveals that a number of murine developmental lesions map to this region, including *vestigial tail*, *vibrator*, and *trembler*. In addition, parental origin effects on foetal growth which may act through the placenta have been located in this region (Cattanach and Kirk, 1985). While *Sparc* may not be involved in these defects, it provides the first polymorphic marker within this region which can be

used to map these defects more precisely and possibly to clone the genes involved. In this respect Sparc has also been used to map two haematopoietic growth factors on mouse chromosome 11 to a 150Kb fragment (D.Barlow pers. comm.). Interestingly, these growth factors are, like Sparc, located on human chromosome 5.

APPENDIX

Abbreviations

AFP	Alphafoetoprotein
bp	Base pairs
BSA	Bovine serum albumin
cfu	Colony-forming units
dBcAMP	Dibutyryl cyclic AMP
ddH_2O	Double-distilled water
DMS	Dimethyl sulphate
DMSO	Dimethylsulphoxide
DTT	Dithiothrietol
EC	Embryonal carcinoma
EDTA	Disodium ethylene diamine tetraacetate.$2H_2O$
EGF	Epidermal growth factor
FBJ-MSV	Finkel, Biskis and Jinkins murine sarcoma virus
FBR-MSV	Finkel, Biskis and Reilly murine sarcoma virus
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
HGT	High gelling temperature
HPRT	Hypoxanthine phosphoribosyl transferase
IAP	Intracisternal A-particle
IBMX	Isobutylmethylxanthine
IPTG	Isopropyl-2-D-thio-galactopyranoside
Kb	Kilobases
LGT	Low gelling temperature
LTR	Long terminal repeat
MOPS	Morpholinopropanesulphonic acid
NP-40	Nonidet P40
OD	Optical density
PBSA	Phosphate-buffered saline A
p.c.	Post-coitum
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
pers. comm.	Personal communication
PIPES	1,4-piperazinediethanesulphonic acid
POPOP	1,4-bis-(2-(4-methyl-5-phenyloxazolyl))-benzene
PPO	2,5 diphenyloxazole
PPP	Platelet-poor plasma
PVP	Polyvinylpyrrolidone
RA	Retinoic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl (lauryl) sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis

TBRS	Tumour-bearing rat serum
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylene diamine
TPA	Tissue plasminogen activator
tRNA	Transfer RNA
u	Units
X-GAL	5-bromo-4-chloro-3-indolyl-2-galactoside

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