

In vitro analysis of chick limb development

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ABSTRACT

The limb bud develops as a mesenchymal outgrowth beneath an inductive epithelium known as the apical ectodermal ridge (AER). Cell differentiation occurs in a proximal-distal direction, whilst proliferation and thus generation of new tissues occurs at the distal tip of the bud in a region known as the progress zone.

This thesis describes experiments using the high-density micromass culture system to investigate some contemporary issues in limb development.

Ectoderm grafted onto micromass cultures inhibits the formation of cartilage. This effect was investigated and found not to be mediated by components of the extracellular matrix. AERs grafted onto micromass cultures result in the formation of a mesenchymal outgrowth. This effect was shown to involve mitogenic stimulation and secretion of an extensive extracellular matrix. The ability of cells from different proximo-distal locations cultured for different periods of time to respond to the effect of a grafted AER was investigated. The influence of different culture media was also analysed. The results suggest that the ability to respond to factors produced by the AER is lost in cells that have progressed too far along the pathway of chondrogenic differentiation.

Peptide growth factors have been identified throughout embryogenesis and have marked effects upon differentiation and proliferation of cells *in vitro*. The effects of several growth factors known to be present during limb development, upon the differentiation and proliferation of limb bud cells in micromass culture has been examined. bFGF stimulated proliferation and cartilage differentiation whilst inhibiting muscle differentiation. TGF- β stimulated cartilage differentiation and blocked the effects of bFGF on proliferation and muscle differentiation.

Homeobox-containing genes have been shown to encode positional information during *Drosophila* embryogenesis and are increasingly implicated in vertebrate development. Expression of the mouse homeobox genes *Hox-7.1* and *Hox-8.1* in the limb bud is described. Transcripts were retained *in vitro* in cultured tips and in micromass cultures prepared from mouse limb bud cells. In addition the distribution of the chick homologue of the *Hox-7.1* gene in normal limb development and in micromass culture was also investigated. *In vitro* transcripts were retained in proximal cell cultures but not in distal cell cultures. However transcription of *Hox-7.1* was induced in distal cultures beneath a grafted AER.

Finally the distribution of the retinoic acid receptor beta during normal limb development has been investigated. A high level of RAR- β transcripts were continually expressed in proximal regions, thus suggesting a role for this receptor in the development of the shoulder. In reciprocal proximal distal grafting experiments RAR- β behaved in a position-dependent manner, with transcripts consistently downregulated at the distal tip of the bud. Expression of RAR- β in micromass culture was consistent with the behaviour of RAR- β *in vivo*.

The results obtained in micromass culture appear to reflect *in vivo* development, and thus support the use of this culture system as an *in vitro* model for aspects of early limb development.

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GENERAL INTRODUCTION

The limb develops as a mesenchymal outgrowth from the flank, capped by an inductive epithelium, which is responsible for maintaining cells at the distal tip of the bud in a proliferative, undifferentiated state. Cellular condensation precedes overt differentiation of both cartilage and muscle and begins in the proximal-most region of the bud. Differentiated tissues are thus laid down in a proximodistal direction. The anteroposterior axis of the limb is established by a polarising region present in the posterior part of the bud.

The first morphological signs of limb development occur at stage 17 (Hamburger and Hamilton, 1951) when the flank mesoderm induces a thickening in the overlying ectoderm. Proliferation of the mesenchyme causes outgrowth of the bud from the flank. The ectoderm at the distal tip of the bud is transformed from a normal columnar to a higher pseudostratified epithelium known as the apical ectodermal ridge (AER). The importance of the AER in maintaining limb outgrowth was first demonstrated by Saunders (1948), who showed that surgical removal of the AER resulted in limb truncations. This is substantiated in embryos homozygous for the mutation *limbless* (Prahlad *et al.* 1979). In this mutation a small mesenchymal outgrowth in the region of the developing limbs forms at early stages, subsequently however the presumptive limb bud cells degenerate and no limb structures are formed. The finding that normal limb development can be induced in these embryos by replacing the ectodermal hull with one from a normal embryo shows that this mutation is caused by failure to form an AER (Carrington and Fallon, 1988).

Saunders (1948) also showed that the structures which eventually developed following removal of the AER depended on the stage of development. Thus at early stages only the most proximal structures

developed, whilst at later stages more distal elements formed in addition to the proximal elements. These results confirmed the findings of his carbon mapping experiments and provided the first unequivocal evidence that the pattern of the limb is laid down temporally in a proximo-distal sequence (also shown by Summerbell, 1974). Other experiments (Summerbell and Lewis, 1975) showed that when distal tips from older wings were grafted onto proximal stumps from younger wings limbs consisting of a humerus attached to digits developed, with the normally intervening radius and ulna absent. Similarly young tips grafted onto old stumps resulted in the formation of wings containing two humerus elements followed by a radius and ulna and digits. Thus little if any regulation occurred at the junction of the grafts, instead each part developed autonomously. These experiments ruled out the existence of long range signals controlling the specification of proximo-distal pattern. Instead it appeared that proximo-distal positional identity was already programmed into the limb tissue.

Combining these sets of observations Wolpert and co-workers came up with a "progress zone" model of limb development, where the progress zone is defined as the region of distal mesenchyme maintained in a labile state with respect to positional identity by the influence of the overlying AER: this being the only region of the limb where positional identity is labile. This model dictates that positional information along the proximo-distal axis of the limb is designated by the amount of time a cell has spent in the progress zone; thus with increasing time, which is most likely measured in terms of the number of cell divisions, the positional identity specified within the progress zone becomes more distal (Summerbell *et al.* 1973; Wolpert *et al.* 1975).

The development of cartilage in the chick limb bud begins with the condensation of mesenchyme cells in the proximal region at stage

23. Limb bud cells prior to condensation are separated by large intercellular spaces, although intercellular contact is achieved by means of long filopodia. Condensation results in an increased cell density and seems to be brought about by migration rather than an increase in the rate of mitosis, although Gould *et al.* (1972) claimed that the increase in cell density was a result of the failure of these cells to move apart after division. This is accompanied by the cells assuming a rounded morphology with large surface area contacts between apposing cells. Later the precartilage cells begin to separate from one another due to the secretion of cartilage-specific matrix (Thorogood and Hinchcliffe 1975). Cell-cell contact is apparently maintained throughout and is therefore presumably significant in the process of *in vivo* chondrogenesis.

In vitro, the micromass culture technique is often employed for the study of chondrogenesis. First devised by Ahrens *et al.* (1977), this involves culturing cells at high-density which both facilitates extensive cell-cell contact and also ensures that all cells are surrounded by extracellular matrix, apart from the layer in contact with the culture dish. In these respects it would appear that this culture technique mimics the condensation phase limb bud. Cottrill *et al.* (1987b) demonstrated that in mixed cell cultures prechondrogenic and non-chondrogenic cells sort out, presumably on the basis of differential adhesion. This culture phenomenon reflects normal development in that prechondrogenic and non-chondrogenic cells become separated from each other during the condensation of the prechondrogenic cells. Coelho and Kosher (1991) have recently shown, by the scrape-loading/dye transfer technique, that in cultures prepared from distal tip mesenchyme, little gap junctional activity is found after 3 hours of culture, but from 24 hours onwards extensive communication is observed. In cultures prepared from whole limbs of

stage 23-24 embryos, gap junction activity seems to be restricted to the nodules developing as cartilage and is absent from intervening non-chondrogenic areas. A detailed analysis of gap junctional communication within the developing limb has not yet been performed. However contacts resembling gap junctions have been detected by electron microscopy in the precartilaginous region (Gould *et al.* 1972) thus further suggesting that this system provides a good model for the investigation of the early differentiative events that occur *in vivo*.

The aim of the work described in this thesis has been to explore some unresolved issues in limb development using micromass culture. In turn the results presented suggest that this system accurately reflects certain aspects of *in vivo* development, and may therefore prove useful in the future as a system in which to assay actions of growth factors and genes implicated in development.

CHAPTER 1

EPITHELIAL-MESENCHYMAL INTERACTIONS *IN VITRO*

SECTION 1.1 - ANTI-CHONDROGENIC EFFECT OF NON-RIDGE ECTODERM

INTRODUCTION

Ectoderm-induced inhibition of chondrogenesis

The first evidence showing that the ectoderm inhibits the differentiation of cartilage in adjacent mesenchyme came from the work of Kosher *et al.* (1979). They cultured 400-500 μ m distal tips of stage 25 chick wing buds over a period of 5 days either intact, with the AER surgically removed but retaining the dorso-ventral ectoderm, or with all ectoderm removed by brief trypsinisation. Those explants without ectoderm differentiated into a mass of cartilage, whereas in explants surrounded by dorso-ventral ectoderm only the cells in the central, core region of the explant formed cartilage. Directly beneath the ectoderm, tissue resembling dermal and subcutaneous connective tissue developed. The total DNA content of all explants was similar, thus suggesting that large scale cell death of a pre-dermal connective tissue cell type had not occurred in explants lacking ectoderm; it was therefore concluded that the dorso-ventral ectoderm had influenced the cells directly beneath itself preventing them from differentiating into cartilage as is apparently their preferred fate in this culture situation. Instead these cells are diverted into forming dermal connective tissue. These results were consistent with the finding that in micromass culture cells from all regions of the limb bud were able to differentiate into cartilage (Ahrens *et al.* 1977; Solursh *et al.*

1981a), although *in vivo* cartilage differentiation is restricted to cells of the central core of the bud.

Solursh *et al.* (1981b) investigated this phenomenon further using the experimental approach of Globus and Vethamany-Globus (1976), whereby limb ectoderm was grafted onto micromass cultures. Ectoderm from various sites within the embryo was grafted onto micromass cultures of stage 23-4 whole chick limbs. Such cultures normally give rise an extensive pattern of cartilage nodules when grown in serum-containing medium. However when ectoderm from the limb (stage 26), flank (stage 24), maxillary process (stage 24) or even the retinal pigmented epithelium was grafted onto these cultures, cartilage differentiation was inhibited beneath and within a zone of approximately 200 μ m around the grafted ectoderm. After several days of culture the cells in these areas were differentiating as loose connective tissue. This inhibitory effect still occurred when ectoderms were separated from the cultures by nucleopore filters of pore size 0.6 μ m and 0.1 μ m. Since cell processes do not cross 0.1 μ m pore filters, it was concluded that the inhibitory effect of the ectoderm on chondrogenesis is not dependent on cell contact. The inhibitory effect was not however transmitted across millipore filters of standard thickness (150 μ m); thus the ectodermally produced factor which inhibits chondrogenesis would appear to be incapable of diffusing more than 150 μ m from the ectoderm. Ectoderm grafted for 24 hours and then removed left no irreversible antichondrogenic effect, since normal cartilage differentiation was seen in these cases. Ectoderm grafted onto 4 day cultures did not effect the already differentiated cartilage, although the cells were closer together than in control areas and there appeared to be an increased collagen content in the extracellular matrix.

Using the same experimental system, Gregg *et al.* (1989)

investigated the time course of the anti-chondrogenic effect, employing *in situ* hybridisation to detect collagen type II transcripts. A decrease in collagen type II transcripts was detected in the cells beneath the ectoderm as little as 3 hours later. When ectoderms were grafted onto 3 day-old micromass cultures, a less well-defined inhibition of type II collagen transcripts was detected 4 days later, although directly beneath the ectoderm the level of transcripts was reduced. Ectoderm grafting had no effect upon the levels of type I collagen transcripts within these cultures. These findings imply that the ectoderm exerts a rapid inhibitory effect on either the transcription of collagen type II or the stability of collagen type II transcripts. The decreased inhibitory effect of ectoderm grafted onto 3 day old micromass cultures suggests that the ectoderm acts upon the early labile limb bud cells in part by inhibiting cartilage differentiation.

In vivo support for the presence of an ectodermal inhibitor of cartilage differentiation was demonstrated by Hurle and Ganan (1986) and Hurle *et al.* (1989). They removed the marginal or dorsal ectoderm from the interdigital space of stage 27-31 chick leg buds. At stages 28 and 29 over 90% of experimental limbs exhibited a Peanut agglutinin (PNA)-staining cell aggregate within the interdigital space, of which approximately 50% went on to develop into Alcian blue staining ectopic cartilage nodules. Thus removal of ectoderm allowed cartilage to form in locations that would normally exhibit programmed cell death.

Solursh *et al.* (1981a) suggested that the inhibition of cartilage differentiation seen in micromass culture was consistent with *in vivo* development in which a non-chondrogenic sleeve of approximately 150-160 μ m extends beneath the surface ectoderm surrounding a chondrogenic core. It was therefore proposed that the ectoderm plays a major role in determining the pattern of the developing limb, by restricting

cartilage differentiation to the core of the bud. However, the *in vivo* findings of Martin and Lewis (1986) have since cast doubt on this initial interpretation. These authors used UV irradiation to destroy large areas of the dorsal ectoderm of stage 17-19 chick wings. The ectoderm disappeared within 24 hours and did not regenerate. Dorsal soft tissues were also severely depleted. Irradiated wings either remained approximately normal in shape or curved dorsally. Despite extensive denudation of ectoderm, those limbs which retained a normal shape always developed a complete and normally proportioned cartilage pattern, thus suggesting that non-ridge ectoderm does not exert any direct control over the development of the limb cartilage pattern. The limbs which curved dorsally exhibited pattern deformities, suggesting that the topology of cartilage differentiation depends upon the shape of the limb bud. It therefore seems more likely that the function of the anti-chondrogenic effect is to ensure that a loose connective tissue dermis develops beneath the ectoderm.

Extracellular matrix components as regulators of chondrogenesis

The exact sequence of intracellular events leading to the synthesis of cartilage-specific gene products in the developing limb bud remains unknown, however one possibility is that the process is initiated by interactions between mesenchymal cells and extracellular matrix (ECM) molecules. ECM molecules bind to specific cell surface receptors known as integrins. Integrins consist of heterodimers of transmembranous proteins, which bind polymerised actin on the interior of the cell membrane. Binding of ECM molecules to the mesenchymal cells would therefore result in changes in the actin cytoskeleton, which could cause changes in the organisation of other intracellular cytoskeletal elements, thus leading to a change in cell shape. Such

changes might then lead to the synthesis of cartilage-specific matrix molecules, either by affecting transcription at the level of the nucleus or translation of mRNAs in the cytoplasm. In support of this hypothesis are the findings that ECM components have been shown to affect both the proliferation and differentiation of a large number of cells in culture; the effect of each component determined by the cell type and the culture conditions (reviewed by Watt, 1986). In the developing limb bud, the role of ECM molecules as initiators of cartilage differentiation is suggested by the fact that fibronectin, collagen type I and a chondroitin sulphate proteoglycan (PG-M) all show increased expression in the core region of stage 22-23 limb buds before the onset of cell condensation and overt cartilage differentiation (Dessau *et al.* 1980; Kimata *et al.* 1986).

In vitro evidence also suggests that the extracellular matrix could mediate the ectoderm-induced antichondrogenic effect. Solursh *et al.* (1982) devised a system for growing limb bud mesenchyme cells in collagen type I gels which permitted chondrogenic differentiation. If ectoderm was placed on top of these gels the number of Alcian blue staining cartilage foci or collagen type II-positive cells was drastically reduced, not only in the region of the ectoderm but throughout the gel, thus indicating that the factor responsible for the inhibition was diffusible. Cells grown on collagen gels preconditioned with limb ectoderm for 4 days were also prevented from differentiating into cartilage. However the ectoderm was not able to prevent collagen type II accumulation by the same cells grown in an agarose gel (Solursh *et al.* 1984). Thus it was proposed that the ectoderm acts by modifying the underlying collagenous extracellular matrix, which in turn effects the mesenchyme cells by preventing their differentiation into cartilage.

Fibronectin is an extracellular matrix protein which appears

early in development and plays a significant role in early migratory movements of cells such as gastrulation and neural crest migration (reviewed by Thiery *et al.* 1989). Cell surface fibronectin receptors (reviewed by Ruoslahti and Pierschbacher, 1987) have been identified in many species including chick. In migratory cells these receptors are distributed diffusely throughout the membrane, whilst stationary cells develop aggregates of receptors which are bound to fibronectin externally, and talin and vinculin internally to which actin filaments can attach and thus form aligned stress fibres (Thiery *et al.* 1989). Swalla and Solursh (1984) demonstrated that exogenous fibronectin added to stage 23-24 proximal mesenchyme cultures resulted in decreased chondrogenesis, whereas distal cultures were unaffected. Zanetti *et al.* (1990) developed an ectoderm-conditioned medium which inhibited cartilage differentiation of limb bud mesenchyme cells grown on collagen gels by 60-100%. This inhibition was mimicked by both the addition of chick cellular and human plasma fibronectin. However, Western blot analysis of this medium and experiments utilising peptides which mimic the cell-binding domain of the fibronectin molecule showed that the inhibition was not due to fibronectin. The inhibitory effect was blocked by cytochalasin D, an agent which induces cell rounding, and also by JG22, an antibody which recognises β integrin chains.

The findings that fibronectin inhibits chondrogenesis *in vitro* are contradictory to reports that fibronectin is present in the precartilaginous condensation and early cartilage blastema (Dessau *et al.* 1980; Tomasek *et al.* 1982). These findings suggest that fibronectin plays a role in the initial condensation and possibly the onset of cartilage differentiation *in vivo*. The response of cultured cells to fibronectin therefore may not correspond to the response of cells to fibronectin *in vivo*. Thus, since fibronectin is relatively absent from peripheral mesenchyme beneath non-ridge ectoderm, it seemed reasonable

that there might be a correlation between inhibition of cartilage differentiation and the absence of fibronectin.

Heparan sulphate has also been identified in the prechondrogenic limb bud of the chick (Kimata *et al.* 1986) and in confluent cultures derived from chick limb buds (Pennypacker *et al.* 1978). In fact during the condensation phase of chick limb bud development, the prechondrogenic mesenchyme produces heparan sulphate in greater quantities than other GAGs (Vasan, 1986); although immunofluorescent localisation of heparan sulphate has only revealed low level labelling throughout the mesenchyme of stage 23 limb buds, compared to intense labelling of the basement membrane (Shinomura *et al.* 1990). Heparan sulphate proteoglycans are composed of glycosaminoglycan (GAG) chains covalently bound to a protein core which contains a plasma membrane domain (Fransson, 1985). These cell surface proteoglycans function as receptors for various extracellular matrix components (Koda *et al.* 1985) and participate in cellular processes such as adhesion (Cole and Glaser, 1986) and division (Ratner *et al.* 1985); this role is supported by the findings that the ECM molecules collagen, fibronectin and laminin all contain specific GAG-binding domains (Sakashita *et al.* 1980, Hayman *et al.* 1982, Laterra *et al.* 1983). In addition, heparan sulphate present in both the extracellular matrix and associated with the cell surface binds bFGF amongst other growth factors. Cell surface heparan sulphate acts as a low affinity binding site for bFGF and is essential in initiating the cellular response to bFGF (Klagsbrun and Baird, 1991).

Kosher and Lash (1975) found that the chondrogenic effect of the notochord on somites was considerably impaired if notochords were exposed to enzyme treatments designed to remove cell surface-associated proteoglycans. Similarly San Antonio *et al.* (1987) reported that addition of heparan sulphate and the structurally related GAGs heparin,

dermatan sulphate and dextran sulphate led to a specific stimulation of chondrogenesis. Furthermore, addition of heparin and heparan sulphate prevented the inhibition of cartilage differentiation which occurred when limb mesenchyme cells immobilised in collagen gels were grown in ectoderm-conditioned medium or in the presence of exogenous fibronectin (Zanetti *et al.* 1990). However Hadhazy *et al.* (1983) reported that high doses of heparin and other polyanions inhibited chondrogenesis of limb bud mesenchyme *in vitro*.

Hyaluronate is also present in large quantities in the intercellular spaces of the early, pre-condensation limb bud (Singley and Solursh, 1981). As the precartilage and premuscle masses condense, cells within them begin to express cell-surface hyaluronate binding sites (Knudson and Toole, 1987). In other systems hyaluronate binding sites are involved in the endocytosis of hyaluronate prior to its degradation (McGuire *et al.* 1987; Fraser and Laurent, 1989) and also in cell aggregation (Underhill, 1982). Toole *et al.* (1989) have proposed that the acquisition of hyaluronate binding sites is a fundamental step enabling precartilage and premuscle cells to condense in the developing limb. They have also interpreted the ectoderm inhibitory effect in relation to hyaluronate. Co-cultures of ectoderm and mesoderm cells isolated from stage 21-24 limb buds were found to produce twice as much hyaluronate as the sum of that produced by each cell type alone.

Ectoderm-conditioned medium also stimulated hyaluronate synthesis by mesodermal cells (Knudson and Toole, 1988). Antibodies against TGF- β inhibited this effect, and TGF- β mimicked the stimulatory effect of the ectoderm. Thus they concluded that the ectoderm produces a factor related to TGF- β that causes the subjacent mesenchyme to maintain a high rate of hyaluronate synthesis relative to the central condensing mesenchyme, thereby preventing cartilage differentiation and ensuring the development of loose dermal connective tissue beneath the ectoderm.

As has been previously stated, binding of ECM molecules to cell surface integrin receptors results in a rearrangement of the actin cytoskeleton, leading to a change in cell shape. Expression of the differentiated cartilage phenotype has been shown to be responsive to changes in cell shape; a rounded configuration favours cartilage differentiation, whereas treatments which cause cell flattening result in loss of the cartilage phenotype. For example, Archer *et al.* (1982) demonstrated that chick mesenchyme cells maintained in a rounded configuration by culture on a semi-adhesive substrate of poly(HEMA), as apposed to flattened on tissue culture plastic, produced greater amounts of sulphur-containing cartilage matrix. Zanetti and Solursh (1984) showed that chondrogenesis could be stimulated by treating subconfluent limb mesenchyme cultures with cytochalasin D, a microfilament disrupting agent which causes cells to assume a spherical configuration. Cytochalasin D also reversed the inhibition of chondrogenesis caused by growing proximal limb cells in hydrated collagen gels in the presence of exogenous fibronectin and successfully alleviated the inhibition of chondrogenesis brought about by growing mesenchyme cells in collagen gels preconditioned with ectoderm (Zanetti and Solursh, 1986). However, Brown and Benya (1988) have shown that the dihydrocytochalasin (DHCB) induced reexpression of the chondrocyte phenotype involves changes in the organisation of the actin cytoskeleton without inducing a change in cell shape. Chondrocytes treated with retinoic acid (RA) stopped synthesising cartilage-specific products and became flattened. These changes were associated with a rearrangement of the actin cytoskeleton, in that the unorientated stress fibre pattern characteristic of chondrocytes was replaced by a more ordered array of fibres concentrated at the cell periphery. DHCB

treatment resulted in disruption of the RA-induced pattern and resumption of a less orientated pattern within 48 hours. This preceded the resumption of proteoglycan and collagen type II synthesis, which occurred after 4 and 6 days respectively. However this reexpression of the cartilage phenotype was not accompanied by a resumption of a rounded cell shape. In addition, the modification of the actin cytoskeleton by DHCB was not sufficient to cause resumption of proteoglycan and collagen type II synthesis in the absence of serum.

The finding that cell shape changes are not necessary for modification of the cartilage phenotype is also supported by the results of Horton and Hassell (1986), who cultured chondrocytes in methylcellulose, in which cells exhibit a spherical configuration. Retinoic acid modulation of the cells resulted in loss of cartilage specific protein synthesis i.e. chondrogenic phenotype, without a change in cell shape. If these cells were subsequently retrieved and plated on plastic they flattened, thus showing that the change in cell shape is secondary to initial molecular or biochemical effects. Gregg *et al.* (1989) showed that grafting ectoderm onto 24 hour old micromass cultures resulted in a rapid down regulation of type II collagen transcripts in the cells beneath the ectoderm after only 3 hours, whereas these same cells did not flatten (as judged by EM histology) until after 2 days. Therefore, at least in these cases, it appears that cell flattening is a secondary consequence of the direct inhibition of chondrogenesis and not the primary cause, as has been previously claimed.

It thus seems that the organisation of the actin cytoskeleton plays an important role in establishing expression of the chondrocyte phenotype, and in some cases this is associated with a change in cell shape, although this is apparently not an essential requirement of this process. Since the binding of ECM components to a cell via

integrin receptors causes a rearrangement of the actin cytoskeleton, this suggests that cell-ECM interactions could play an important role in the process of chondrogenesis.

Role of cyclic AMP in chondrogenesis

Other work suggests that the interaction between ECM molecules and mesenchyme cells is responsible for the initial process of condensation during limb bud chondrogenesis, but the intracellular events leading to expression of the cartilage phenotype involve elevation of cyclic AMP (cAMP) levels, possibly in response to endogenous prostaglandin synthesis rather than control mediated by changes in the organisation of the cytoskeleton.

This hypothesis is supported by several lines of evidence. Firstly, agents that elevate cAMP levels in both organ culture and cell culture also promote chondrogenesis (Ahrens *et al.* 1977; Solursh *et al.* 1981a). This effect has been confirmed at the nuclear level by Kosher *et al.* (1986), who demonstrated that addition of dibutyryl cAMP resulted in an increase in the levels of cartilage proteoglycan core protein and collagen type II mRNA in micromass cultures derived from stage 25 distal tip cells. Evidence that this may correspond to the mechanism of cartilage differentiation employed by limb bud cells comes from the work of Biddulph *et al.* (1984), who showed that a transient increase in endogenous cAMP levels in micromass cultures of chick limb bud cells corresponds to the time judged to represent the onset of chondrogenesis. Prostaglandin E₂ (PGE₂) appears to be a good candidate molecule for stimulating this increase in cAMP levels. Exogenous PGE₂ stimulates chondrogenesis in organ culture and micromass culture of chick limb bud cells; in each system its effects are dramatically potentiated by addition of the phosphodiesterase inhibitor theophylline

(Kosher and Walker, 1983; Gay and Kosher, 1984). Exogenous PGE₂ also results in an increase in cAMP levels in micromass cultures (Ballard and Biddulph, 1983). Cultures prepared from distal mesenchyme cells are most responsive to PGE₂ during the initial 48 hours of culture during the onset of chondrogenesis, when they respond by an increase in adenylate cyclase activity and cAMP levels, thus suggesting that these cells may possess PGE₂ receptors coupled to adenylate cyclase. The reduced response to PGE₂ observed after 3 days in culture is not caused by a decrease in intracellular cAMP reserves or by an inhibitory action of cartilage-specific matrix molecules on PGE₂ receptors (Capehart *et al.* 1990); therefore it would appear that either the affinity of the PGE₂ receptor or the efficiency with which it is coupled to adenylate cyclase must change as cells express the differentiated cartilage phenotype. The finding that cultured cells are maximally responsive to exogenous PGE₂ during the initial 48 hours of culture and appear to possess adenylate cyclase-coupled PGE₂ receptors might therefore be related to the dramatic increase in endogenous adenylate cyclase activity and intracellular cAMP which also occurs within the first 48 hours of culture (Biddulph *et al.* 1988). This suggestion is supported by the finding that PGE₂ is the major prostaglandin species synthesised by limb bud cells in micromass culture (Gay and Kosher, 1985). The peak of PGE₂ corresponds to the timing of the onset of chondrogenesis, and this correlates with the finding that maximal PG synthesis is observed in precondensation and condensation stage limb buds, after which time PG levels progressively decrease (Biddulph *et al.* 1984).

The mechanism whereby cAMP leads to the expression of cartilage specific genes remains unknown, however one apparent action of cAMP is to cause phosphorylation of the non-histone protein PCP35.5_b, which is expressed in precartilage cells but is absent from differentiated cartilage. PCP35.5_b is also absent from cells of the myogenic lineage,

thus suggesting that it may be specific to cells of the chondrogenic lineage. This phosphorylation appears to be mediated by a cAMP-dependent protein kinase, occurs within the first 5 hours of culture and is greatly enhanced upon addition of dbcAMP. It has been suggested that phosphorylation of PCP35.5_b may result in conformational changes in the arrangement of DNA, thus resulting in exposure of cartilage-specific genes and hence allowing new patterns of gene expression (Leonard and Newman, 1987).

Experimental approach

This investigation attempted to discern whether or not molecules of the extracellular matrix were involved in the ectoderm inhibitory effect in micromass culture. Initially immunofluorescent localisation was carried out to determine which ECM components, if any, were absent from sub-ectoderm mesenchyme but present in the remaining chondrogenic mesenchyme. Five components were found to exhibit this distribution but of these only fibronectin and heparan sulphate were present at the time of ectoderm grafting. The amounts of these matrix components only increased in areas of the culture not associated with the grafted ectoderm, thus it seemed plausible that the absence of these components might be responsible for preventing the cells from becoming cartilage. Heparan sulphate, heparin and fibronectin were therefore added separately and in combination to cultures for 24 hours prior to ectoderm grafting to see if the inhibitory effect of the ectoderm could be prevented. Hyaluronate was also added to cultures in order to test the hypothesis of Toole and co-workers, who claimed that the ectoderm stimulates underlying mesenchyme to produce hyaluronate, which in turn prevents cell condensation and thus inhibits cartilage differentiation. The role of fibronectin was further investigated using synthetic

peptides (kindly donated by Dr.M.Humphries) based on the GRGDS peptide which mimics the cell-binding domain of the fibronectin molecule and thus disrupts cell-fibronectin interactions. TGF- β , a stimulator of cartilage differentiation, was also added to cultures to see if the ectoderm anti-chondrogenic effect could be blocked. Finally an attempt was made to produce a conditioned medium from which it might be possible to determine the nature of the inhibitory factor.

METHODS

Micromass culture

Stage 22-23 (Hamburger and Hamilton, 1951) chick embryos were removed from the egg into cold phosphate-buffered saline (PBS). The amnion was torn open and the limb buds removed with curved forceps. Limb buds were then transferred to a Petri dish containing Hanks buffered salt solution (HBSS) with 10% foetal calf serum (FCS). 300-350 μ m distal tips were cut using a tungsten needle and were then incubated in 2% trypsin in HBSS on ice. After 30 minutes the trypsin was removed and replaced by either HBSS + FCS or culture medium containing 10% serum in order prevent further tryptic activity upon the tissue. With the Petri dish on an ice block, the epithelium was removed from each tip using tungsten needles and discarded. The remaining mesenchyme tips were then transferred to a microcentrifuge tube and the cells dissociated by gentle pipetting up and down with a Gilson pipette. The cells were spun for 4 minutes at 6500 rpm into a pellet. The supernatant was carefully removed with a flame-drawn Pasteur pipette, and the pellet resuspended in 1ml of 1:2 mixture of HBSS + FCS and culture medium (culture medium only in later experiments). 10 μ l of cell suspension was then placed in a haemocytometer and the number of cells determined. The number of micromass cultures that could be made was calculated and the cell suspension spun into a pellet as before. After the supernatant had been discarded, the pelleted cells were resuspended at a density of 2×10^7 /ml in a 1:2 mixture of HBSS + FCS and culture medium in the early experiments and in culture medium only in later experiments. The volume added was calculated as 10 μ l for every micromass. 10 μ l droplets of cell suspension containing 2×10^5 cells were then plated onto sterile 35mm plastic culture dishes (Sterilin) and placed in a humidified, 5% CO₂:95% air, 37°C incubator for 60-90

minutes. Each dish was then flooded with culture medium which was replaced in full or in part every 24 hours.

For the characterisation experiments cultures were also prepared from proximal limb tissue. In these cases the proximal-most 500 μ m of stage 22-23 limb buds was dissected and used to make micromass cultures as described above.

The composition of F12 culture medium was:

90% Ham's F12 nutrient mixture

10% foetal calf serum (FCS)

1% antibiotic/antimycotic

2mM glutamine

200 μ g/ml ascorbate

(All tissue culture medium ingredients were supplied by Gibco)

Ectoderm grafting

Stage 24 limb buds were dissected in HBSS + FCS. Distal tips were removed and the bud bisected along the longitudinal axis. The resulting limb pieces consisted of dorsal and ventral ectoderm meeting at either the anterior or posterior edge. At the distal extremity of this edge a small amount of AER often remained, which was removed. These pieces were incubated in 2% trypsin for 30-45 mins. After washing in cold medium each piece was transferred to a culture dish containing 1 day old micromass cultures that had previously been cooled for 10 minutes at 4°C. The ectoderm was removed using tungsten needles, positioned above the culture and gently pushed down. The medium was then withdrawn and the cultures returned to the incubator. Every 1.5-2 hours a few drops of medium were placed onto the culture to prevent dehydration. After 3-4 hours when the ectoderm had attached, the cultures were flooded with fresh medium.

Wholemount staining of micromass cultures

Cartilage differentiation

Cultures were fixed overnight in 1/2 strength Karnovsky's (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1M phosphate buffer - see Karnovsky, 1965). After several rinses in PBS, cultures were stained for 3-4 hours with 1% Alcian blue (BDH Chemicals Ltd.), pH 1.0. Excess stain was removed by washing with PBS. Cultures were then dehydrated in consecutive 5 minute washes in 50%, 70%, 90% and 100% alcohol respectively before being covered in glycerol. Providing cultures are kept out of direct sunlight this appears to be an effective method of storage.

Immunofluorescent localisation of matrix molecules

Cultures were fixed in cold 70% alcohol and kept overnight at 4°C. Cultures were then gently rehydrated with alcohol:PBS mixtures. The PBS used for immunofluorescent washes consisted of 1.42g Na₂HPO₄, 0.49g NaH₂PO₄.2H₂O and 9.00g NaCl dissolved in 1 litre of distilled water. When the cultures were in PBS, a circle was marked around them with a diamond pencil. The PBS was then removed and the cultures incubated for 15 minutes in medium containing 10% serum to reduce non-specific binding. The dishes were then carefully dried with tissue paper except for the marked area containing the culture. 20µl of the primary antibody were then placed onto the culture, the lid was replaced to prevent evaporation and the culture left for 1 hour. The culture dish was then washed 3 times with PBS, dried as before and the second antibody applied for 45 minutes in the dark. Some of the primary antibodies were used at full strength. The others and the FITC-conjugated second antibodies were prediluted in PBS containing 1% Tween

20 (permeabilising agent), 0.1% BSA and 0.1% sodium azide (preservative). The dish was then washed with PBS as before. A coverslip was then mounted with a DABCO-based mountant which preserves fluorescence. The mountant was made by dissolving 0.6g Tris in 66ml distilled water, the pH adjusted with HCl to 8.5. 33ml glycerol was then added followed by 15g polyvinyl alcohol. The mixture was warmed and stirred on a magnetic hotplate before 2.5g 1,4-Diazabicyclo[2.2.2.]octane (DABCO) was added. Mountant in use was kept at 4°C, whilst excess was stored at -20°C.

Wholmount stained cultures were observed and photographed under phase contrast and ultraviolet illumination on an Zeiss Axiovert 405M inverted microscope.

In initial experiments cultures were pretreated with 0.25U/ml chondroitinase/ 0.45U/ml hyaluronidase (both from Sigma) for 30 minutes prior to incubation in primary antibody, to prevent masking by other extracellular components. However the staining patterns were identical to those obtained without this pretreatment and therefore this was discontinued with the exception of the 3B3 (Chondroitin sulphate) antibody which required chondroitinase pretreatment to ensure that all chondroitin sulphate side-chains were recognised.

Hyaluronate localisation

Hyaluronate was detected using a biotinylated hyaluronan binding region link protein complex (gift of Dr. M. Bayliss, Kennedy Institute, London and Prof. C. Archer, Cardiff) as described by Ripellino *et al.* (1985, 1988).

Cultures were washed several times in PBS before fixation overnight at 4°C in 3% formaldehyde, 0.5% cetylpyridinium chloride, 30mM NaCl in 0.1M phosphate buffer, pH 7.4. Cultures were then briefly washed in PBS before incubation with blocking serum (10% serum for 15

minutes) and then 2 hour incubation in 50 μ g/ml biotinylated binding region probe. Cultures were then washed 3 times for 5 mins in PBS before incubation in avidin-5nm gold conjugated IgG (Biocell) diluted 1:50 in PBS for 1 hour. Following several 5 min washes in PBS the gold was enhanced with silver (using silver solutions from Sigma) in subdued light for 5-10 mins. Non-biotinylated hyaluronan binding region link protein was used as a control.

Table of antibodies

The following antibodies were used in these experiments:

antibody	designation	source	ref.
<hr/>			
keratan sulphate	MZ15	Dr. F. Watt	Zanetti <i>et al.</i> (1985)
fibronectin	B3-D6	Developmental studies Hybridoma Bank	Gardener and Fambrough (1983)
heparan sulphate	33-2	"	Bayne <i>et al.</i> (1984)
laminin	31	"	Bayne <i>et al.</i> (1984)
procollagen I	M-38	"	
collagen type I		Prof. C. Archer	- affinity-purified
collagen type II			polyclonals
chondroitin sulphate	3B3		Caterson <i>et al.</i> (1990)

Addition of matrix components

Cultures were plated and grown in F12 medium containing 100 μ g/ml of fibronectin, heparan sulphate or heparin (all from Sigma). The above molecules were all dissolved in F12 medium to establish stock solutions, which were then further diluted before addition to cultures.

In addition some cultures were grown in 100 μ g/ml fibronectin and 100 μ g/ml heparan sulphate. After 24 hours ectoderms were grafted onto cultures as previously described. Cultures were then fixed at 72 hours and stained with Alcian blue.

In a separate set of experiments cultures were plated and grown in F12 medium containing different concentrations of hyaluronate (Sigma). Ectoderms were not grafted onto these cultures, however the effect on cartilage differentiation was determined.

Addition of GRGDS and related peptides

Micromass cultures of 350 μ m distal tips were plated and grown in F12 medium containing 10% serum for the first 24 hours of culture. At 24 hours the medium was replaced with defined medium (DM - described on pp.133) containing 500 μ g/ml one of the following peptides: GRGDS, GRGES, GRGD, SDGR, SDGRG, DGR or GRGGDS. The medium was replaced daily. Cultures were then fixed at 3 or 4 days in cold 70% alcohol prior to wholemount staining, as described previously, with antibodies to fibronectin, collagen type II or keratan sulphate.

Addition of TGF- β

Distal cell cultures were fed F12 medium containing 10ng/ml TGF- β 1 (R&D Systems) for either the entire culture period or from 24 hours of culture onwards. Ectoderms were grafted onto the cultures after 24 hours as previously described. The medium was replaced daily. 72 hour old cultures were then fixed and stained with Alcian blue and the extent of cartilage differentiation determined.

Ectoderm-conditioned medium

Several months were spent trying to prepare a conditioned medium which would consistently inhibit cartilage differentiation. Initially

attempts were made to produce a single cell suspension of ectoderm. Ectoderms were removed from fore- and hindlimbs and then incubated in different concentrations of trypsin and EDTA for about 30 minutes. Unfortunately clean and complete separation of ectoderm cells was never achieved. Intact sheets of ectoderm were then grown in culture and medium collected. Ectoderms were removed from fore- and hindlimbs and grown in 600 μ l of DM for 3-4 days. The ectoderms formed rounded, vacuolated structures. The conditioned medium was harvested and fed undiluted or diluted 1:1 with DM to freshly plated distal limb micromass cultures.

RESULTS

Characterisation of inhibitory effect

Figure 1.1.01 A,B shows a distal cell culture and a proximal cell culture respectively, fixed after 72 hours and stained with Alcian blue. Ectoderm was grafted onto each culture after 24 hours. Alcian blue staining is absent under the ectoderm and in a zone of approximately 150 μ m surrounding the edge of the ectoderm. The same inhibition is seen in both distal and proximal cultures grown in both serum-containing and serum-free medium.

In order to ascertain whether or not the non-chondrogenic zone surrounding the ectoderm had initially been in direct cell contact with the ectoderm, cultures were photographed immediately after ectoderm grafting at 24 hours of culture, and then again after 72 hours of culture following fixation and staining with Alcian blue. The initial size of the ectoderm was then superimposed onto the Alcian blue stained cultures (Fig. 1.1.01 C,D). In 20 out of 20 cases, performed on several different occasions, the non-chondrogenic zone extended further than the initial size of the grafted ectoderm, i.e. into areas that had never been in direct cell contact with the ectoderm.

Immunolocalisation of extracellular matrix components

The results of the immunolocalisation of various extracellular matrix components in 72 hour cultures are summarised in Table 1.1.1. 5 of the 8 components localised were found throughout the cartilaginous region of the cultures but were absent or present in very much reduced amounts in the area beneath and surrounding the grafted ectoderm; these components were fibronectin, heparan sulphate, keratan sulphate, collagen type II and chondroitin sulphate. Fibronectin was extremely

abundant in the cartilaginous region of the culture. In the zone surrounding the ectoderm it was virtually absent, and beneath the ectoderm it was present in vastly reduced amounts, as can be clearly seen in wholemounts (Fig. 1.1.03 A,B) and cryosections (Fig. 1.1.02). Heparan sulphate was less abundant than fibronectin, although it exhibited the same pattern of localisation. It was found at high levels in the cartilage-forming part of the culture but absent from beneath the ectoderm and the surrounding zone (Fig. 1.1.03 C,D). Keratan sulphate was very abundant in the cartilaginous part of the culture but was completely absent from the area of ectodermal influence (Fig. 1.1.04 B). Collagen type II was again abundant in the cartilaginous region of the culture and absent from the area associated with the ectoderm, although individual bundles of fibres were seen crossing the zone surrounding the ectoderm (Fig. 1.1.04 A). Chondroitin sulphate showed a similar pattern of distribution to keratan sulphate, being present at high levels in the cartilaginous part of the culture but completely absent from the area of ectodermal influence (Fig. 1.1.05 C). The other 3 ECM components assayed were laminin, type I collagen and hyaluronate. Laminin was present throughout the culture at low levels but was higher under the ectoderm (Fig. 1.1.05 D). Collagen type I stained the edges of the ectoderm and also individual cells which resemble myoblasts morphologically (Fig. 1.1.05 A,B). Hyaluronate was not detected either under the ectoderm or in the surrounding culture (Fig. 1.05 E).

Three of the five components absent from the area of ectodermal influence but present in the chondrogenic part of the cultures were then visualised at different time points. Fibronectin was present at low levels in 24 hour old cultures. Ectoderms were grafted at 24 hours of culture. 24 hours later, at 48 hours, the amount of fibronectin had increased slightly both under the ectoderm and in the rest of the

culture. By 72 and 96 hours the amount of fibronectin had greatly increased in the cartilaginous parts of the culture but not in the area of ectodermal influence (Fig. 1.1.06). Heparan sulphate was also present at a low level at 24 hours. With increasing time the quantity of heparan increased especially in the area immediately outside of the area of ectodermal influence, however the level of heparan beneath the ectoderm did not increase with time (Fig. 1.1.07). Keratan sulphate was not present in the culture at 24 hours, however at 48 and 72 hours it was present in large quantities throughout the culture apart from the area of ectodermal influence, where it was absent (Fig. 1.1.08).

Addition of matrix components

Cultures were plated and maintained in medium containing either 100 μ g/ml fibronectin, heparan sulphate, heparin or a combination of 100 μ g/ml fibronectin with 100 μ g/ml heparan sulphate. The results are summarised in Table 1.1.2. In all cases ectoderm added at 24 hours still inhibited cartilage formation to the same extent as in control cultures. It was also noted that none of these treatments resulted in a significant increase in the amount of cartilage matrix produced.

In a separate set of experiments, cultures were plated and maintained in medium containing different concentrations of hyaluronate, although ectoderms were not added. Again the amount of cartilage produced did not differ from control levels (Table 1.1.2).

Addition of GRGDS and related peptides

The results of peptide additions are summarised in Table 1.1.3. The addition of three peptides, namely GRGES, GRGD and GRGGDS, resulted in significant cell detachment and therefore no cartilage was formed in these cases. Addition of the other peptides, including the GRGDS sequence, did not effect cell attachment or the amount of cartilage

differentiation. Micrographs showing the immunofluorescent localisation of fibronectin and keratan sulphate are shown for each peptide in Figures 1.1.09 and 1.1.10.

Addition of TGF- β

The inhibitory effect of the ectoderm on cartilage differentiation was also seen in cultures which had been treated with 10ng/ml TGF- β 1 for either the whole incubation period or from the time that the ectoderm was present, that is the last 48 hours of culture (Table 1.1.4). This occurred despite the fact that the amount of cartilage differentiation seen in TGF- β treated cultures was markedly enhanced.

Ectoderm-conditioned medium

Attempts to make a conditioned medium which consistently inhibited cartilage differentiation were unsuccessful. Firstly it proved impossible to obtain a single cell suspension of ectoderm cells which could have been plated out into a large dish. Dissected ectoderms were then incubated in medium for 3-4 days, the medium was removed and fed to freshly prepared micromass cultures. However cartilage differentiation was never substantially reduced in these cultures.

Table 1.1.1 - Immunolocalisation of matrix components

matrix component	area of ectodermal influence	chondrogenic region	no. cases
<hr/>			
fibronectin	-	+	9
heparan sulphate	-	+	8
keratan sulphate	-	+	4
collagen type II	-	+	4
chondroitin sulphate	-	+	3
laminin	+	+	4
collagen type I	-	- ^a	4
hyaluronate	-	-	5

a: single cells stained which resemble myoblasts morphologically

Table 1.1.2 - Addition of matrix components

matrix component	dose (μ g/ml)	ectoderm inhibition	cartilage differentiation	no. cases
<hr/>				
heparan sulphate	100	+/-	+/-	6
heparin	10	+/-	+/-	8
	100	+/-	+/-	12
fibronectin	100	+/-	+/-	8
hyaluronate	1000	n	+/-	12
	100	n	+/-	12
	10	n	+/-	8
	1	n	+/-	6

key: + increase

+/- no change

- decrease

n: not determined

Table 1.1.3 - Addition of peptides

peptide sequence	cell detachment	keratan sulphate	collagen II	fibronectin
<hr/>				
control	-	+	+	+
GRGDS	-	+	+	+
GRGES	+	-	-	+
GRGD	+	-	-	+
GRGGDS	+	-	-	+
DGR	-	+	+	+
SDGR	-	+	+	+
SDGRG	-	+	+	+

key: + positive

- negative

Table 1.1.4 - Addition of TGF- β

time present	ectoderm	cartilage	no.cases
	inhibition	differentiation	
<hr/>			
0-72 h	+/-	+	7
24-72 h	+/-	+	5

key: + increase

+/- no change

- decrease

Figure 1.1.01

3 day old micromass cultures with ectoderms grafted after 24 hours. (A) distal cell culture, (B) proximal cell culture. e = grafted ectoderm, c = Alcian blue stained cartilage, i = area of ectodermal influence in which cartilage differentiation is inhibited. Scale bar = 200 μ m.

A grafted ectoderm exerted the same inhibitory effect on cartilage differentiation in both distal and proximal cell cultures. The area surrounding the ectoderm, in which cartilage differentiation was also inhibited was approximately 150 μ m.

(C) and (D) illustrate the results of an experiment to determine whether cells in the area of ectodermal influence were initially in contact with the ectoderm. (C) Phase contrast view of a grafted ectoderm taken shortly after grafting at 24 hours of culture. (D) The same culture after 72 hours of culture stained with Alcian blue. The initial area occupied by the ectoderm is shown by the dotted line. Scale bar = 200 μ m.

The ectoderm anti-chondrogenic effect was seen in cells which have never been in direct cell contact with the grafted ectoderm.

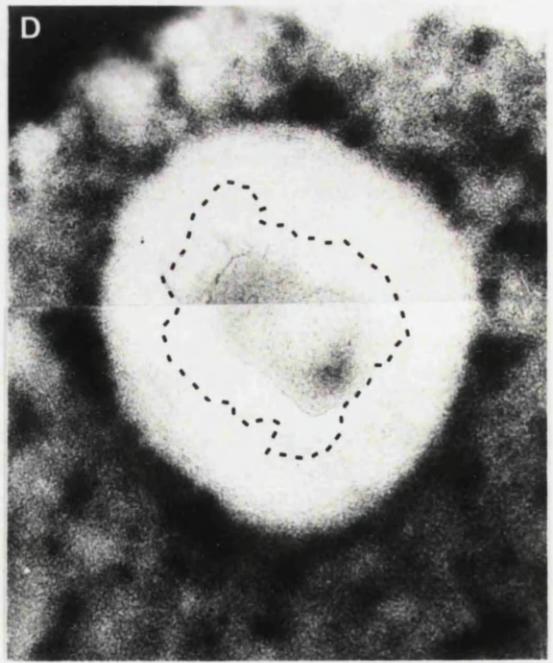
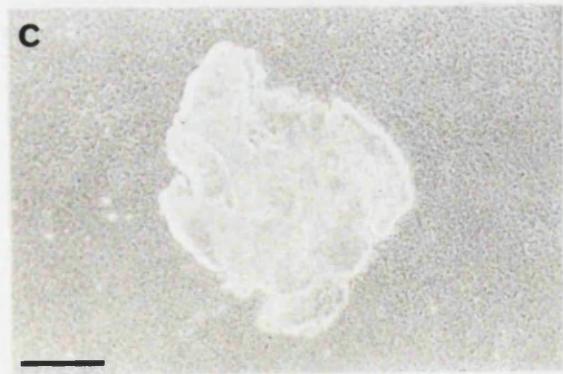
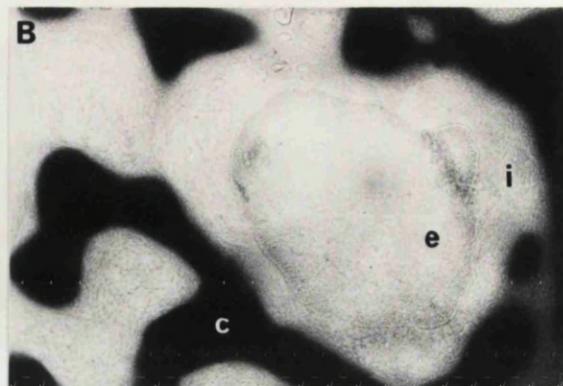
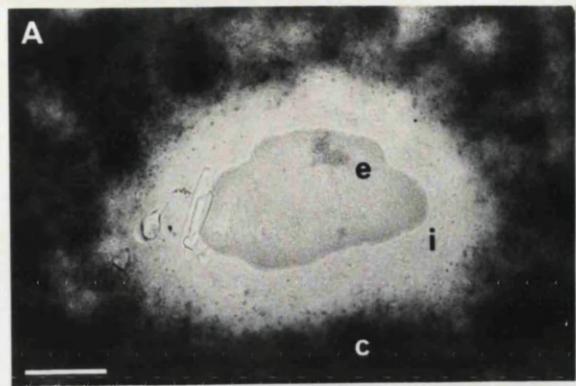


Figure 1.1.02

Phase contrast (A,C) and corresponding immunofluorescent (B,D) micrographs of 7 μ m cryosections through 72 hour distal cell micromass cultures containing ectoderm grafts and stained with antibody to fibronectin. The extent of the ectoderm is indicated with arrowheads; e = ectoderm.

Scale bar = 200 μ m.

Fibronectin staining was significantly reduced beneath the grafted ectoderm.

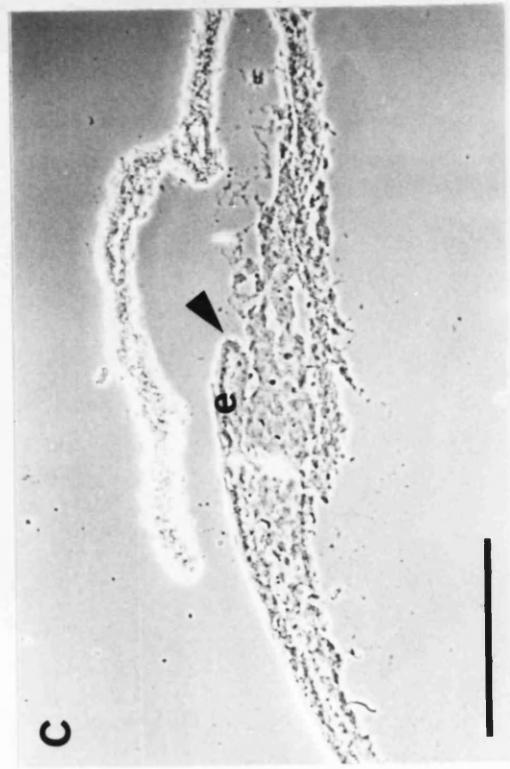
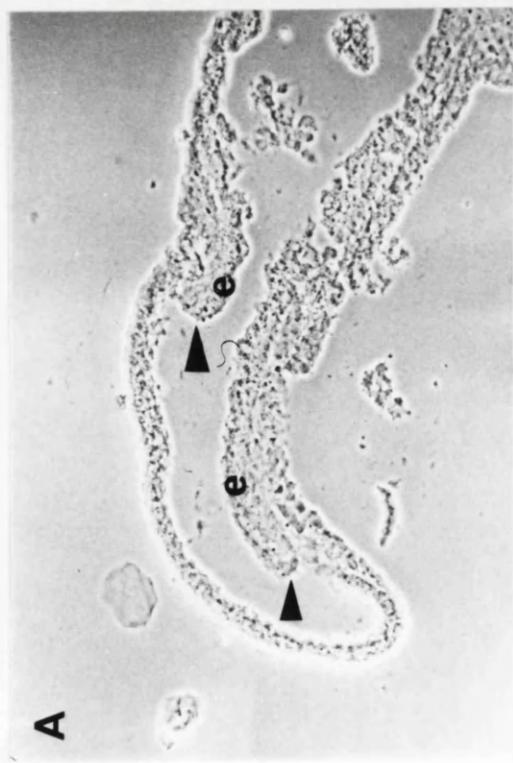


Figure 1.1.03

Wholemount immunolocalisation preparations showing 72 hour cultures with ectoderm grafts. Phase contrast (A) and corresponding immunofluorescent (B) micrograph of an ectoderm graft stained with antibody to fibronectin. Phase contrast (C) and corresponding immunofluorescent (D) micrograph of an ectoderm graft stained with antibody to heparan sulphate.

Scale bar = 200 μ m.

Fibronectin and heparan sulphate staining were seen in the cartilage forming areas of the cultures, but were substantially reduced in the area beneath the grafted ectoderm and also in the surrounding area of ectodermal influence.

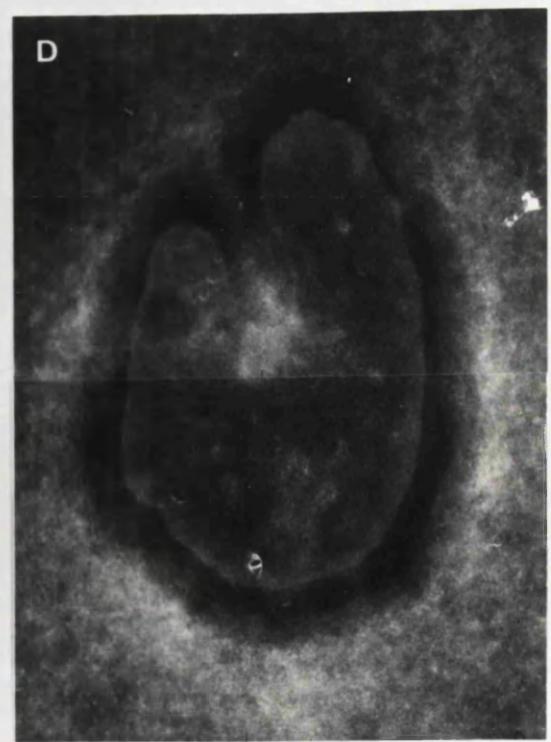
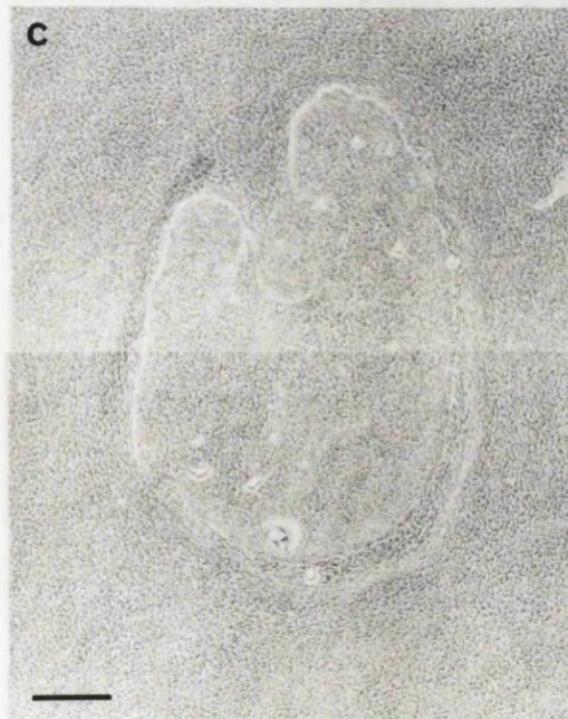
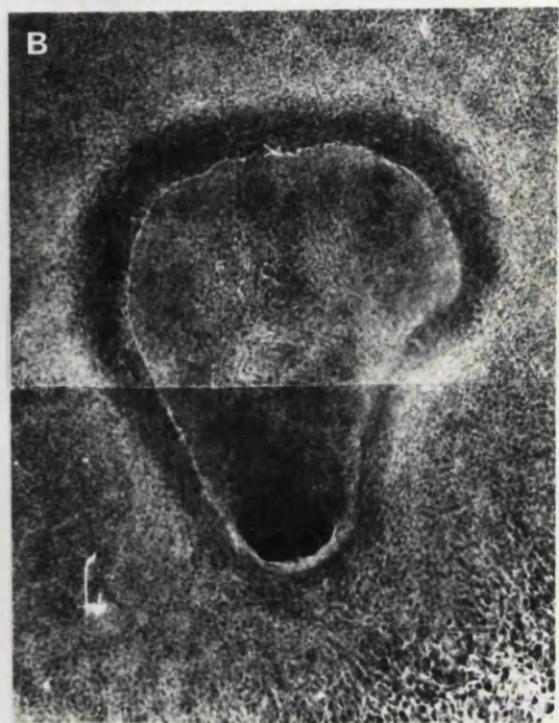


Figure 1.1.04

Immunofluorescent micrographs of 72 hour cultures stained with antibody to (A) collagen type II and (B) keratan sulphate. The outer limits of the grafted ectoderm are indicated with arrowheads.

Scale bar = 200 μ m.

Collagen type II and keratan sulphate were present in large quantities in the cartilage forming areas of the culture. Keratan sulphate was completely absent from the area beneath the grafted ectoderm and the surrounding area of ectodermal influence. Collagen type II was virtually absent from these regions with the exception of a small number of fibres which were seen both beneath the ectoderm and also crossing the area of ectodermal influence.

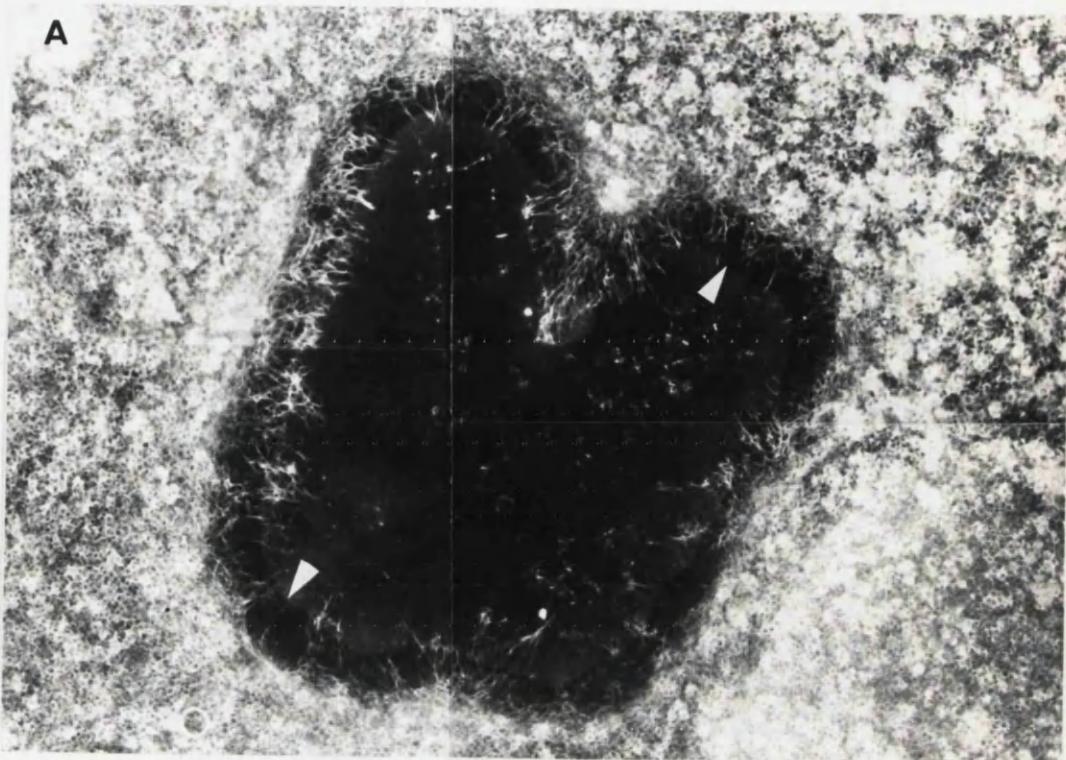
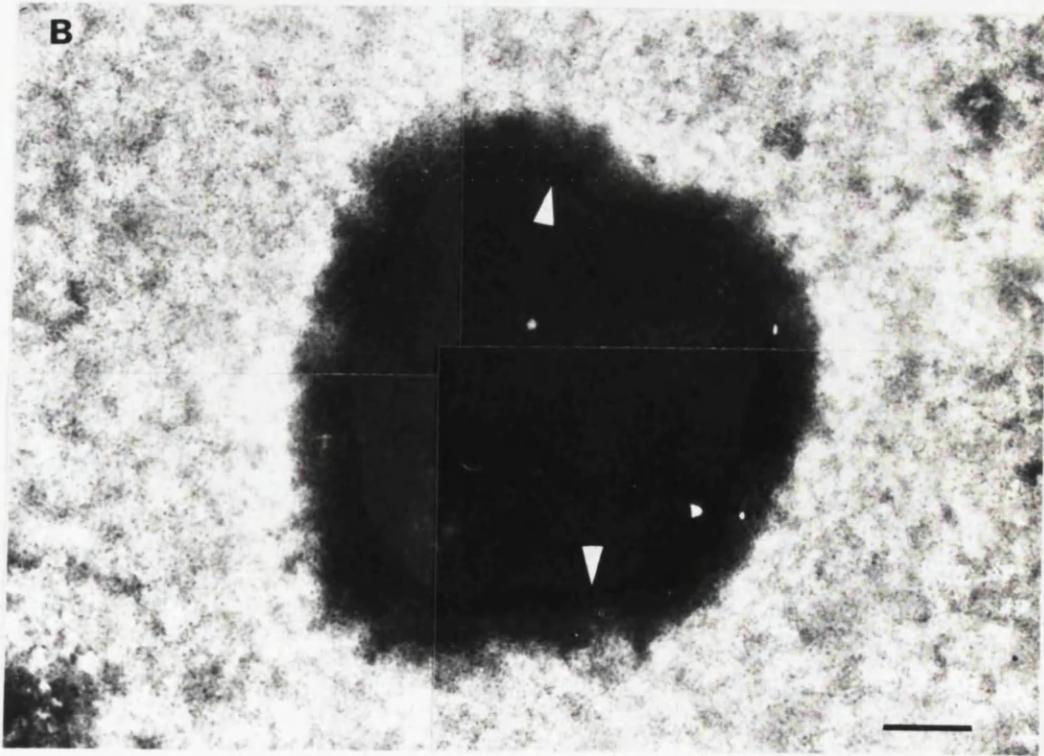
A**B**

Figure 1.1.05

Immunofluorescent micrographs of 72 hour cultures stained with antibodies against collagen type I (A,B), chondroitin sulphate (C), laminin (D) and unbound hyaluronate (E).

Scale bars = 200 μ m.

Collagen type I stained the edge of the ectoderm and also a population of cells within the cultures (arrowhead) which morphologically resemble myoblasts. Chondroitin sulphate staining was seen in the cartilage forming region of the culture but was absent from the area beneath the ectoderm and the surrounding area of ectodermal influence. Laminin staining was seen at the edge of the grafted ectoderm, but in no other locations within the cultures. Unbound hyaluronate was not detectable anywhere within these cultures.

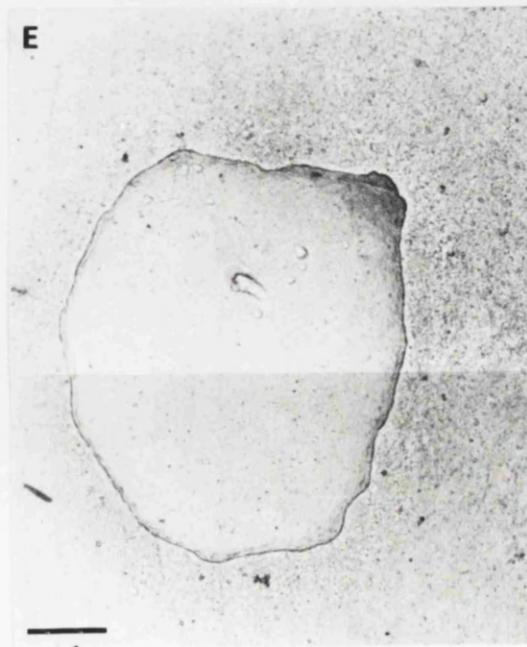
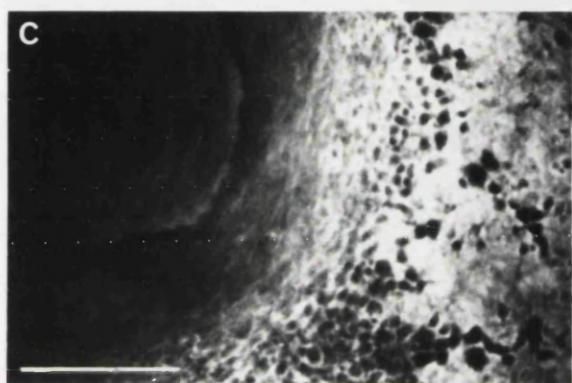
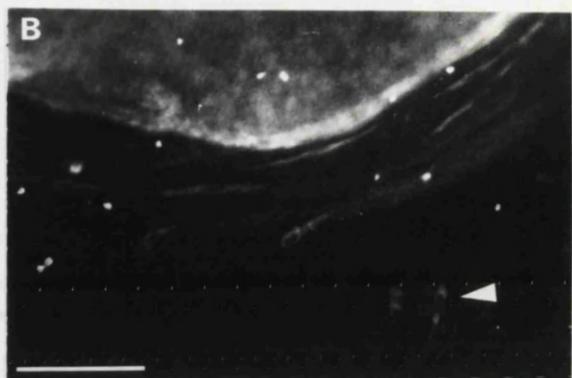
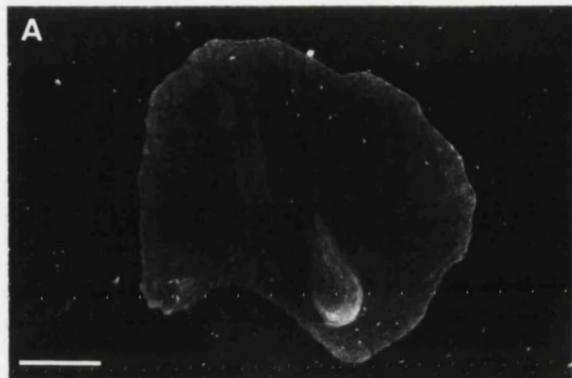


Figure 1.1.06

Phase contrast (A,C,E,G) and corresponding immunofluorescent (B,D,F,H) micrographs of cultures fixed and stained with antibody to fibronectin after 1 day (A,B), 2 days (C,D), 3 days (E,F) and 4 days (G,H). Cultures fixed after 2 days of culture or more had ectoderms grafted onto them at 24 hours of culture.

Scale bars = 200 μ m.

Fibronectin staining was present after 1 day of culture at low levels. With increasing time the amount of fibronectin staining increased in the cartilage forming area of the culture but remained relatively constant at a low level beneath the ectoderm and in the surrounding area of ectodermal influence.

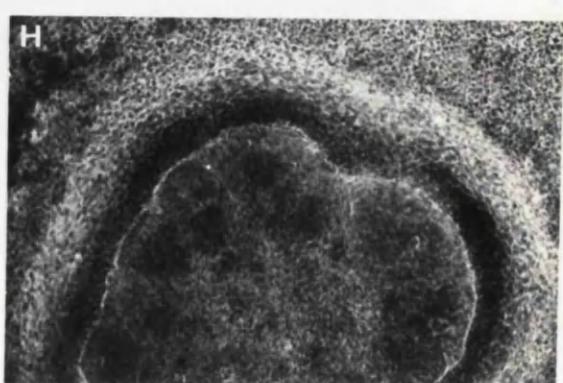
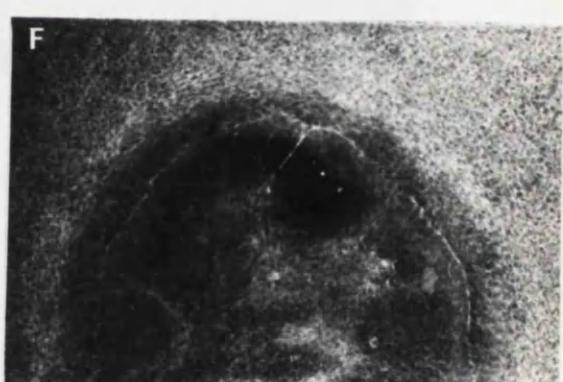
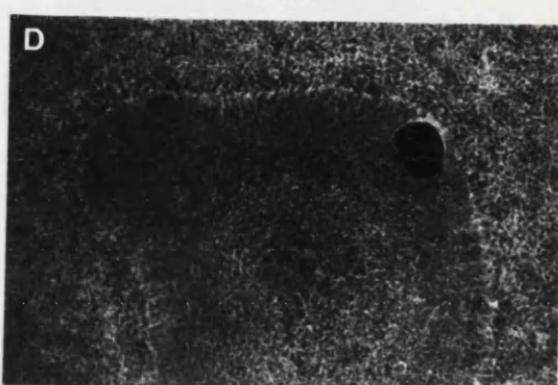
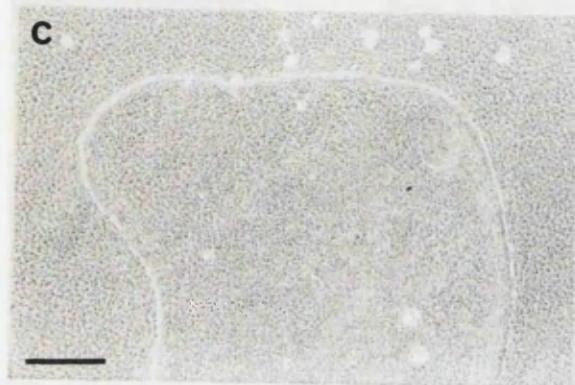
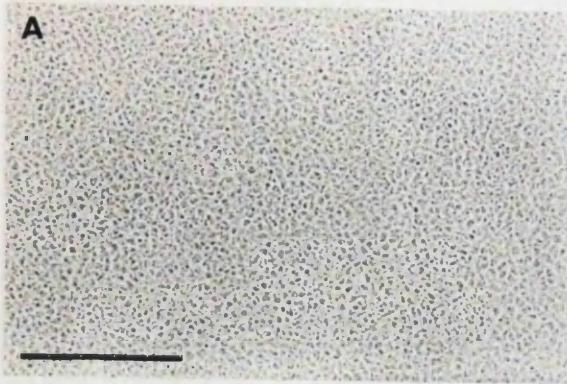


Figure 1.1.07

Phase contrast (A,C,E,G) and corresponding immunofluorescent (B,D,F,H) micrographs of cultures fixed and stained with antibody to heparan sulphate after 1 day (A,B), 2 days (C,D), 3 days (E,F) and 4 days (G,H) of culture. Cultures fixed after 2 days of culture or more had ectoderms grafted onto them at 24 hours of culture.

Scale bar = 200 μ m.

Heparan sulphate staining was seen in 1 day cultures. After 2 and 3 days of culture the level of heparan sulphate has increased in the cartilage forming region of the cultures but remained very low beneath the ectoderm and in the surrounding area of ectodermal influence. After 4 days of culture the area of ectodermal influence still exhibited very little heparan sulphate staining. Staining in the cartilage forming region on the culture has increased yet further, but in addition staining of the ectoderm was also observed in several cases.

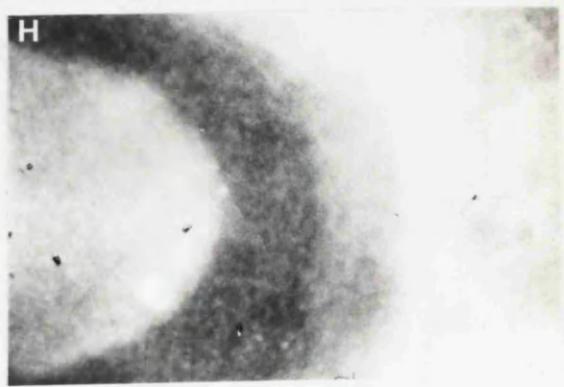
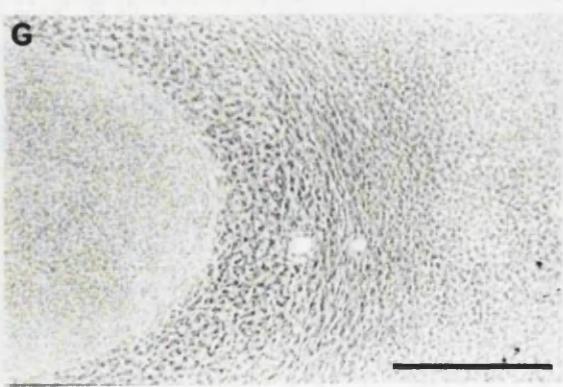
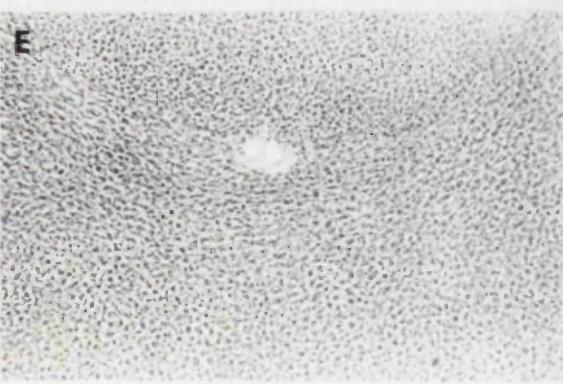
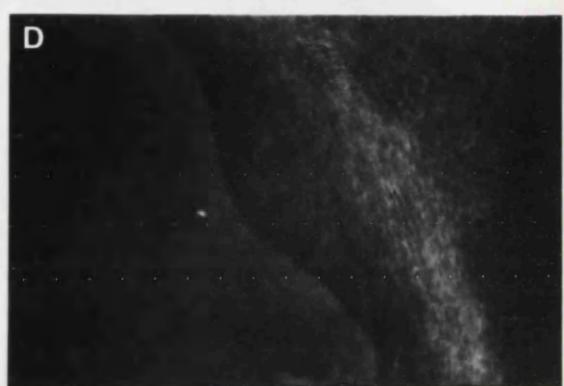
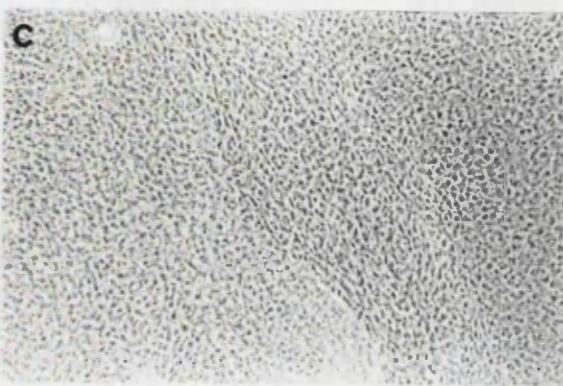
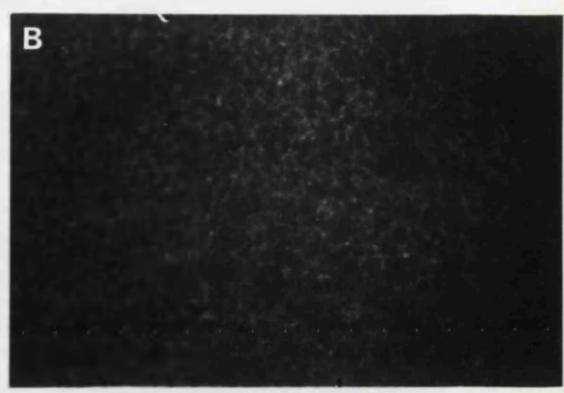
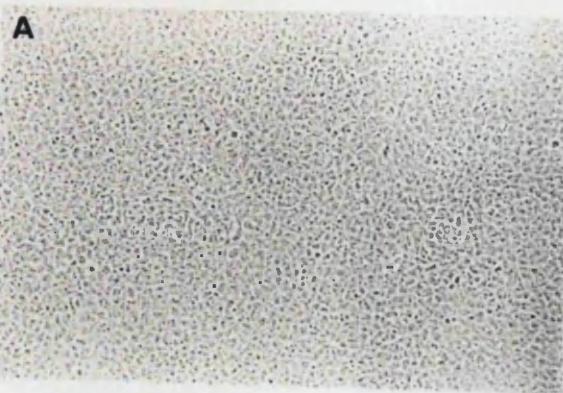


Figure 1.1.08

Phase contrast (A,C,E,G) and corresponding immunofluorescent (B,D,F,H) micrographs of cultures fixed and stained with antibody to keratan sulphate after 1 day (A,B), 2 days (C,D), 3 days (E,F) and 4 days (G,H) of culture. Cultures fixed after 2 days of culture or more had ectoderms grafted onto them at 24 hours of culture.

Scale bars = 200 μ m.

Keratan sulphate staining was not seen in 1 day cultures. With increasing incubation period, the amount of keratan sulphate staining increased substantially in the cartilage forming areas of the culture, but staining was not seen beneath the ectoderm or in the surrounding area of ectodermal influence.

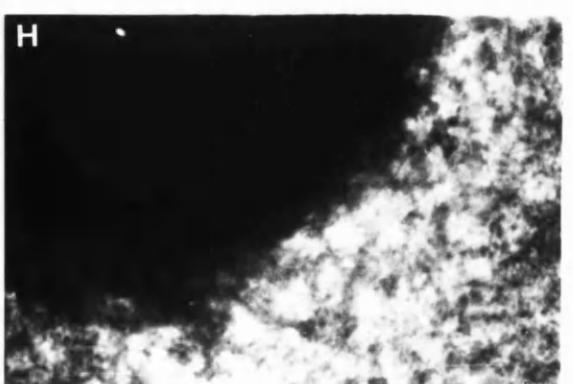
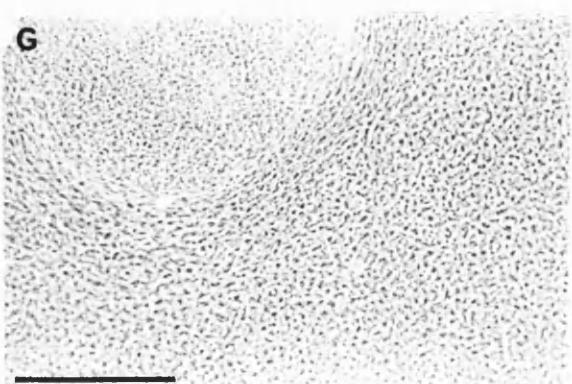
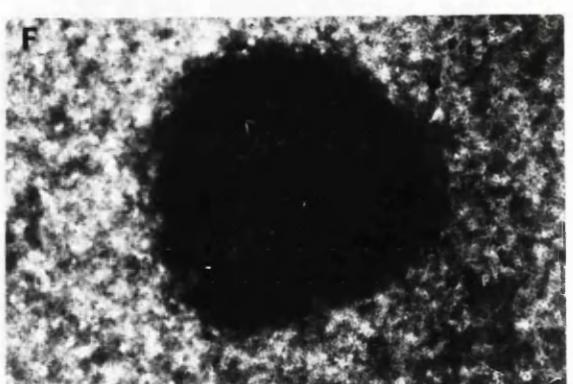
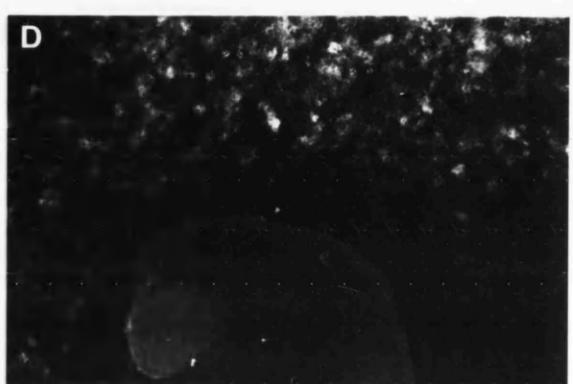
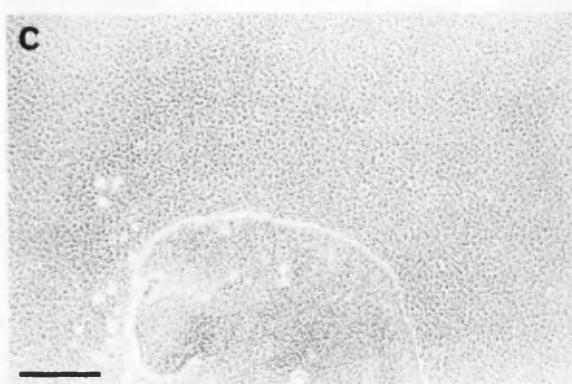
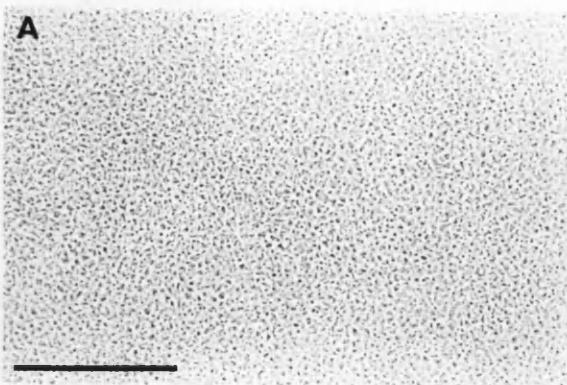


Figure 1.1.09

Phase contrast (A,D,G,J) and immunofluorescent (B,C,E,F,H,I,K,L) micrographs of 3 day distal cell micromass cultures grown in F12 for the first 24 hours of culture and then transferred to DM (A,B,C) or DM containing 0.5mg/ml of the following peptides: GRGDS (D,E,F), GRGES (G,H,I) or GRGD (J,K,L). Phase contrast and the corresponding immunofluorescent micrographs of cultures stained with antibody to fibronectin are shown (A,B,D,E,G,H,J,K) in addition to immunofluorescent micrographs of cultures stained with antibody to keratan sulphate (C,F,I,L).

Scale bar = 200 μ m.

Cultures grown in DM alone or in the presence of GRGDS maintained a healthy morphology and differentiated into cartilage, whereas a large number of the cells grown in the presence of the peptides GRGES and GRGD detached from the cultures. However the cells attached to the tissue culture plastic remained and elaborated a fibronectin-rich matrix, however the remaining cell density was insufficient to allow cartilage formation.

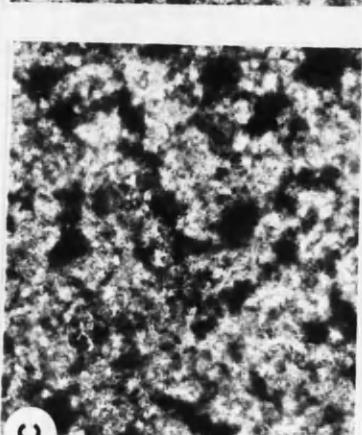
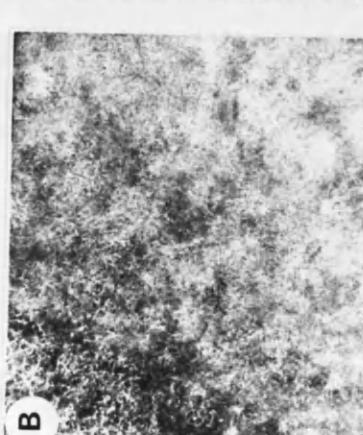
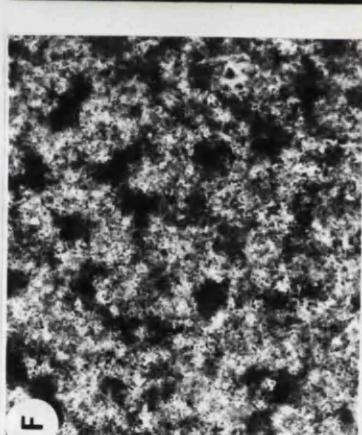
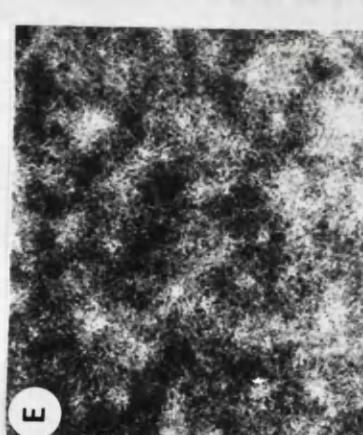
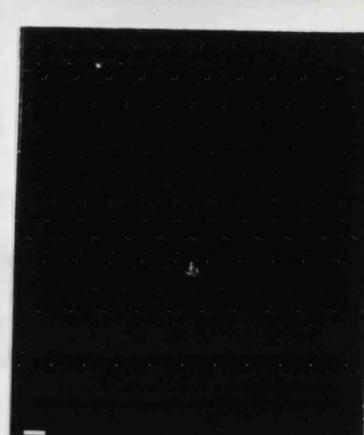
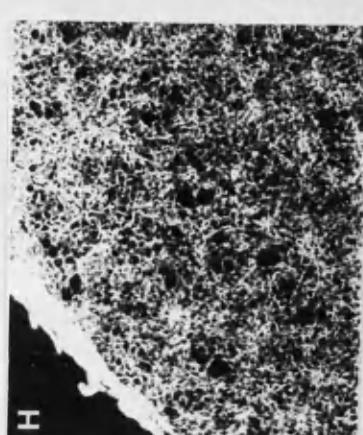
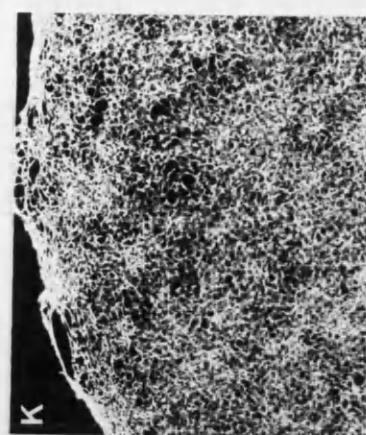
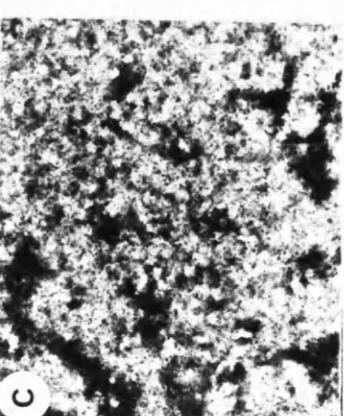
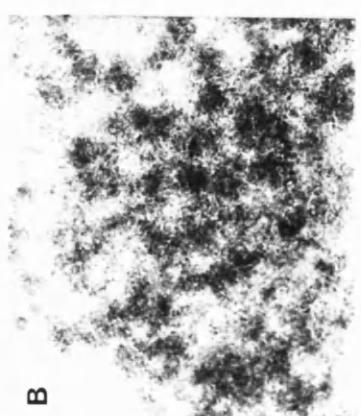
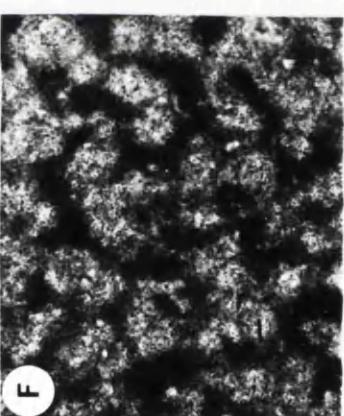
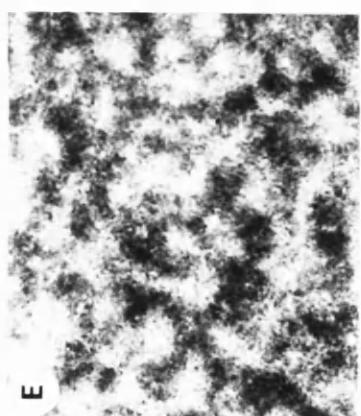
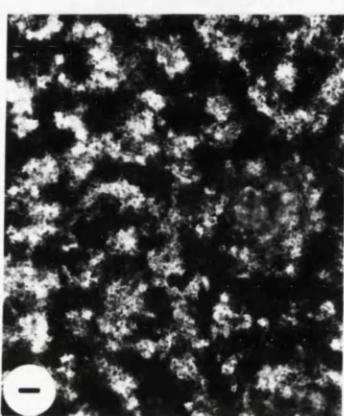
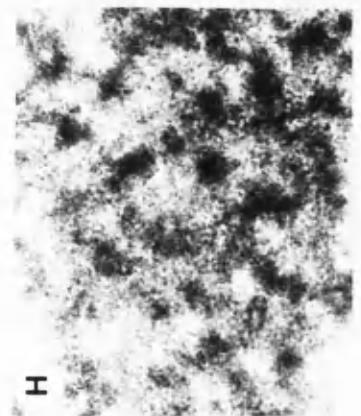
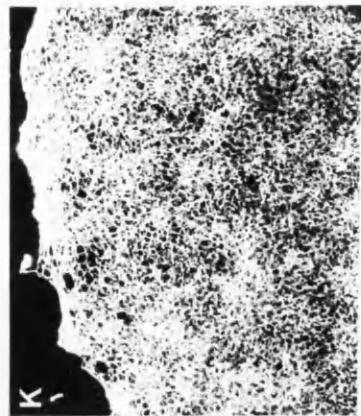
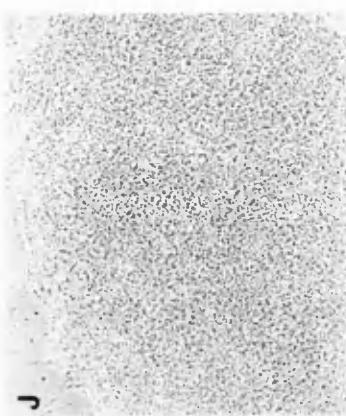


Figure 1.1.10

Phase contrast (A,D,G,J) and immunofluorescent (B,C,E,F,H,I,K,L) micrographs of 3 day distal cell micromass cultures grown in F12 for the initial 24 hours of culture and then maintained in DM containing 0.5mg/ml of the following peptides: SDGRG (A,B,C), SDGR (D,E,F), DGR (G,H,I) or GRGGDS (J,K,L). Phase contrast and corresponding immunofluorescent micrographs of cultures stained with antibody to fibronectin (A,B,D,E,G,H,J,K) in addition to immunofluorescent micrographs of cultures stained with antibody to keratan sulphate (C,F,I,L).

Scale bar = 200 μ m.

Cultures grown in the presence of SDGRG, SDGR or DGR all exhibited a healthy morphology and differentiated into cartilage. A large proportion of cells grown in the presence of the GRGGDS peptide detached from the culture. The remaining cells elaborated a fibronectin-rich matrix, but an insufficient density of cells remained for cartilage formation to occur.



DISCUSSION

Characterisation

Ectoderm grafted onto micromass cultures of limb bud cells results in a potent inhibition of cartilage differentiation, irrespective of the initial proximo-distal location of the mesenchyme cells or the composition of the culture medium. Comparison of the initial size of the grafted ectoderm with the final size of the area of cartilage inhibition shows that the antichondrogenic effect extends to cells that have never been in direct cell contact with the ectoderm. This confirms previous reports that the inhibitory influence acts over a limited distance (Solursh *et al.* 1981b).

Immunolocalisation of ECM components

It seems likely that ECM molecules may have a causative role in the initiation of limb bud chondrogenesis, especially since the spatially restricted distribution of several matrix components coincides with the area of mesenchyme which will undergo condensation prior to cartilage differentiation (Dessau *et al.* 1980; Kimata *et al.* 1988). The immunolocalisation of matrix components reported here, revealed the presence of a relatively deficient extracellular matrix in the area of ectodermal influence, at least compared to the cartilage forming area of the cultures. This is consistent with the *in vivo* situation where ultrastructural studies have shown that the peripheral mesenchyme beneath the dorso-ventral ectoderm is also relatively deficient in matrix components, containing occasional fibrillar material resembling immature collagen fibrils and also hyaluronate (Thorogood and Hinchcliffe, 1975; Singley and Solursh, 1981).

7 out of the 8 matrix components localised were found to be absent from the zone of ectodermal influence. The presence of laminin

underneath the ectoderm presumably indicates the formation of a basement membrane, however collagen type I and heparan sulphate were not abundant beneath the ectoderm whereas these two components are also associated with the basement membrane beneath the ectoderm *in vivo*. Collagen type I was also absent from the cartilaginous region of the culture, whereas *in vivo* reports suggest that it is associated with the precartilaginous condensation (Dessau *et al.* 1980). Type II collagen becomes detectable in large quantities as cells of the proximal condensation differentiate into cartilage, thus it is not surprising that the cartilage forming areas of these cultures stain intensely for collagen type II, whereas the region of ectodermal influence does not synthesise this component. The presence of chondroitin sulphate in the cartilage forming areas of the cultures and absence from the area of ectodermal influence is also consistent with *in vivo* reports. There are two major chondroitin sulphate proteoglycans synthesised in early limb buds. PG-M is present in the distal mesenchyme and precartilaginous condensation, whereas PG-H is cartilage-specific and does not appear before overt cartilage differentiation; the antibody used in this study would probably not discriminate between these two proteoglycans.

Interestingly hyaluronate could not be detected in the cultures in the area beneath the ectoderm. This suggests that either this model system is not reflecting the true *in vivo* situation, or that any hyaluronate present is masked from detection by optimal binding of proteoglycans. However, since chondroitin sulphate and keratan sulphate were absent from beneath the ectoderm, this suggests that there are few, if any, proteoglycans in this region. This therefore suggests that large amounts of hyaluronate may not be produced by the mesenchyme subjacent to the ectoderm contrary to the report of Singley and Solursh (1981). It should be noted however that hyaluronate was identified ultrastructurally in their paper. Immunological identification of

hyaluronate in the limb bud has not yet been reported. Keratan sulphate was only detected in the cartilage-forming regions of the culture as expected since keratan sulphate proteoglycans are synthesised as a component of differentiated cartilage matrix, although the antibody used in this study recognises cartilage-specific and non-cartilage associated keratan sulphate. In view of this fact it is not surprising that keratan sulphate is absent from 24 hour cultures and increases with increased incubation period in areas that are differentiating as cartilage. Fibronectin and heparan sulphate were also absent from the area of ectodermal influence, although they were present in the cartilage-forming area of the cultures. In the developing limb bud, fibronectin is abundant in all regions except beneath the dorso-ventral ectoderm, however immunolocalisation of heparan sulphate in similar stage limb buds has shown an even distribution throughout the mesenchyme with increased abundance in the basement membrane beneath the ectoderm. It is therefore not surprising that fibronectin and heparan sulphate are present in 24 hour old cultures, albeit in small quantities. With increasing time their levels increase in areas destined to form cartilage but not in the zone of ectodermal influence. In several cases heparan sulphate staining of the ectoderm but not of the surrounding area of ectodermal influence in 96 hour cultures was seen; this probably represents staining of the basement membrane. Heparan sulphate is apparently less abundant than fibronectin, although the antibodies may not be of the same efficacy. It thus seems that the ectoderm prevents the synthesis of fibronectin and heparan sulphate in the adjacent mesenchyme. In order to determine if the absence of these molecules prevents this mesenchyme from differentiating into cartilage, fibronectin and heparan sulphate were added to cultures to see if the ectoderm-induced antichondrogenic effect could be prevented.

The finding that the addition of heparin, heparan sulphate and fibronectin did not prevent the inhibitory effect of the ectoderm suggests that their absence from beneath the ectoderm is not the cause of the inhibition of cartilage but is more probably a secondary consequence of this effect. Thus the cells are diverted from a chondrogenic fate and hence their secretion of fibronectin and heparan sulphate ceases. The addition of these factors did not increase the amount of cartilage matrix produced either, suggesting that these factors are not involved in the stimulation of matrix synthesis, although these results do not rule out the possibility that these components are necessary for the initial onset of cartilage-specific gene expression. The addition of heparan sulphate and heparin did not prevent the ectoderm-induced inhibition of chondrogenesis. Previous reports are somewhat confusing, for example San Antonio *et al.* (1987) claim that addition of 10 μ g/ml heparan sulphate or 1 μ g/ml heparin significantly stimulated chondrogenesis in micromass cultures of whole limb and distal limb mesenchyme, however at higher doses heparin (up to 200 μ g/ml) increased nodule number but decreased sulphate incorporation. Doses higher than 10 μ g/ml heparan sulphate were not tested. In my experiments doses of 100 μ g/ml heparan sulphate or heparin did not significantly effect cartilage differentiation as judged by Alcian blue staining. Thus my experimental findings that the addition of heparan sulphate or heparin did not prevent the ectodermal inhibition of chondrogenesis together with the fact that heparan sulphate levels were very low in cultures until cartilage differentiation had begun suggests that the heparan sulphate proteoglycan does not play a significant role in the process of chondrogenesis. This contradicts the conclusions drawn by Vasan (1986) and San Antonio *et al.* (1987) who postulated that

heparan sulphate or a heparan sulphate-like proteoglycan plays a regulatory role in chondrogenesis by promoting the initial expression of the cartilage phenotype. It seems more probable that heparan sulphate may influence cartilage growth after differentiation has begun.

The finding that the addition of hyaluronate did not effect the amount of cartilage differentiation, together with the fact that hyaluronate could not be detected in any region of these cultures suggests that this molecule does not play a role in the ectoderm-induced inhibition of chondrogenesis or indeed the differentiation of cartilage.

The addition of fibronectin neither prevented the antichondrogenic effect of the ectoderm or affected the amount of cartilage matrix production. This last point confirms the previous report of Swalla and Solursh (1984), who showed that exogenous fibronectin did not effect chondrogenesis of distal cells in micromass culture, although it inhibited chondrogenesis in cultures derived from whole limb buds. The role of the fibronectin-cell interaction was further investigated using a panel of peptide sequences. Two cell binding sites have currently been identified on the fibronectin molecule. Of these the GRGDS site appears to be the most commonly used, since fibronectin function has been successfully inhibited in a large number of experimental systems by addition of the GRGDS peptide (reviewed by Yamada, 1989), including *in vivo* and *in vitro* studies involving chick embryo cells (Boucaut *et al.* 1984; Horwitz *et al.* 1985; Chen *et al.* 1986). In most systems addition of 100 μ g/ml GRGDS effectively impairs fibronectin function by 50-100%. Thus it can be concluded that in this experimental system, interaction between cells and the GRGDS binding site of fibronectin does not play a significant role in either cell attachment or in cartilage differentiation.

However three peptides tested did interfere with cell attachment. The common sequence between the three is GRG, a sequence which is repeated in the heparin-binding and collagen-binding domains of the fibronectin molecule (Kornblihtt *et al.* 1985). Frenz *et al.* (1989) showed that the peptide GRGD was equally competent as GRG in blocking translocation of heparin-coated beads in micromass cultures of distal and whole limb mesenchyme. However they did not report a significant loss of cells from the GRGD-treated cultures, although the peptide was added at lower concentrations of 10-100 μ g/ml. Presumably these peptides compete with cell surface receptor(s) involved in attachment of the cells to the matrix rather than the culture dish, since a layer of cells remains on the tissue culture plastic and appears quite healthy after 24 hours of culture in the presence of these peptides. The GRGES peptide has so far only been shown to interfere with the attachment of melanoma cells to fibronectin; in all other cell types tested it has proved ineffective. It therefore remains possible that a further cell binding domain exists on the fibronectin molecule, as yet undefined, but which is blocked by the GRGES peptide and is utilised by melanoma cells and also a population of chick limb mesenchymal cells, at least in this culture system. Assuming that these three peptides interfere with the same cell-matrix interaction, the affected site must have a highly specific conformation, since addition of the peptide GRGDS, which also contains a GRG sequence, had no effect.

Addition of the peptide growth factor TGF- β also failed to prevent the inhibitory effect of the ectoderm, despite the fact that TGF- β stimulates cartilage differentiation. TGF- β may either act directly to stimulate the transcription of cartilage-specific gene expression (Kulyk *et al.* 1989) or might act indirectly via stimulation of matrix secretion. TGF- β is a potent stimulator of matrix accumulation in a large number of different cell types. It acts both by

increasing the synthesis of matrix components such as fibronectin and collagen type I and also by inhibiting the release of matrix degrading enzymes, such as proteases and collagenase (reviewed by Rizzino, 1988). TGF- β also up-regulates expression of integrins in some cell types (Heino *et al.* 1989).

It has been suggested that the ECM molecule tenascin (formerly known as myotendinous antigen) might be involved in the process of chondrogenesis *in vivo*. Tenascin is prominent in the perichondrium of actively growing foetal cartilages and in the condensing mesenchyme surrounding the budding epithelial rudiments of the mammary gland, hair follicle and molar tooth (Chiquet-Ehrismann *et al.* 1986). In high-density cultures of 4 day old chick embryo wing buds, tenascin was detected after 2 days, finally becoming associated with cartilaginous nodules. Exogenous tenascin also reversed the inhibition of chondrogenesis caused by growing limb bud cells on fibronectin coated dishes; an effect which may have been mediated by a change in cell shape, since cells grown on tenascin coated dishes or treated with tenascin were more rounded and more likely to detach (Mackie *et al.* 1987). However, in the developing limb bud tenascin cannot be detected immunologically before stage 25, when it is present in the proximal tissue surrounding the developing cartilage element (Shinomura *et al.* 1990). Thus it appears that tenascin is not involved in the early stages of cartilage formation, at least not in the developing limb bud.

The results presented here indicate that the ectoderm-induced inhibition of cartilage differentiation is not mediated by depriving subjacent mesenchyme of the ECM molecules fibronectin, heparan sulphate or hyaluronate. It seems more likely that the absence of these components from the mesenchyme is a secondary consequence of the inhibition of cartilage differentiation set up by a different mechanism. This is likely to be a diffusible factor which acts directly

at the level of the nucleus to prevent the transcription of cartilage-specific genes. However, these findings do not rule out the hypothesis that interactions between mesenchymal cells and ECM molecules are essential for the initial condensation of mesenchyme. ECM components present in the region corresponding to the precartilaginous condensation include fibronectin, collagen type I and the chondroitin-sulphate proteoglycan PG-M. Whereas chondrogenesis can be achieved by growing cells on or in collagen type I gels, *in vitro* evidence suggests that fibronectin inhibits chondrogenesis; however PG-M has been shown to bind to fibronectin, collagen type I and hyaluronate. Thus interaction of matrix components *in vivo* may result in exposure of certain domains and masking of others. In turn prechondrogenic and non-chondrogenic cells may express integrins of different specificities, and these might change throughout the process of condensation and onset of chondrogenesis.

The sequence of intracellular events leading to the expression of cartilage-specific genes is also unknown, although it might be dependent on elevation of intracellular cAMP. The findings that condensation stage limb buds synthesise prostaglandins, and that these prostaglandins cause elevation of cAMP in mesenchymal cells *in vitro* lend firm support to this hypothesis. In view of this it would be interesting to know if the mesenchyme beneath the ectoderm differed from prechondrogenic mesenchyme either in its ability to synthesise PGE₂ or in its lack of adenylate cyclase-coupled PGE₂ receptors. However there are many other points at which a cell can exert control over the cAMP system therefore even differences in these parameters could not be considered conclusive. It would also be interesting to know if the cells beneath the ectoderm possessed the PCP35.5 protein. If it were present and phosphorylated in response to exogenous cAMP then presumably it is not involved in the process of chondrogenesis.

In the long run the only way to resolve the antichondrogenic phenomenon is to isolate and identify the ectoderm produced factor which elicits this effect. Probably the best way of doing this is to establish an ectodermal cell line from mouse embryos, since chick cells are more difficult to transfect. Large numbers of cells could then be grown, the conditioned medium harvested and the factor purified. It would appear that the system described here would act as a good assay to determine if the cloned cells did produce the antichondrogenic factor and eventually on which to test the purified factor.

INTRODUCTION

Mitogenic effect of the AER

During the early stages (stage 17-20) of limb bud outgrowth, the rate of cell proliferation remains relatively constant throughout all regions of the bud. Between stages 21-23 the rate of proliferation decreases, however the rate of decline is much greater in the proximal central region, which corresponds to the area undergoing condensation and destined to form cartilage. Thus by stage 23 the rate of proliferation in the subridge region is substantially higher than the rate in the proximal region (Hornbruch and Wolpert, 1970; Janners and Searls, 1970). These findings can be interpreted in two ways. Either the AER exerts a constant mitogenic activity, but the mesenchymal response changes with time or alternatively, the mitogenic activity of the AER may decrease progressively with time. The experiments of Rubin and Saunders (1972) tend to suggest that the first interpretation is more likely, in that an older AER grafted onto younger mesenchyme still promoted the rate of outgrowth characteristic of that mesenchyme. Similarly, an AER from a younger limb grafted onto older mesenchyme did not result in excessive proliferation and growth. Thus the mitogenic effect of the AER appears to be constant throughout development; the fact that the rate of proliferation decreases slowly in the subridge mesenchyme must therefore be due to a change in the response of these cells with time, such as an increase in the length of the cell cycle.

These results also suggest that outgrowth during embryogenesis is not achieved by an increase in the rate of cell proliferation in the outgrowing tissue, but is the result of a steeper decline in the rate of proliferation in adjacent tissues. This hypothesis is supported by

evidence concerning outgrowth of the limb bud from the flank. At stage 16 the rate of proliferation in the prospective limb tissue is identical to the rate in the adjacent flank tissue, however during stage 17 the rate of proliferation decreases drastically in the flank whilst that in the limb remains relatively constant (Searls and Janners, 1971). Development of the medial and lateral nasal processes is apparently achieved by the same mechanism (Minkoff and Kuntz, 1977).

The mitogenic factors produced by the AER remain unknown, although transcripts of FGF-4 (formerly K-FGF) have recently been identified in the posterior region of the AER during mouse limb development (Niswander and Martin, submitted). Since this member of the FGF family is secreted by cells, it provides a good candidate molecule for ensuring both continued proliferation of distal mesenchyme and posteriorly directed outgrowth of the limb bud. Bone morphogenetic protein 2A (BMP-2A), a member of the TGF- β superfamily which most closely resembles the *Drosophila* protein *decapentaplegic* (*dpp*), has also been localised to the AER, although its effects on the undifferentiated mesenchyme cells are not yet known.

Progress zone

The evidence for an anatomically distinct region beneath the AER corresponding to the progress zone is limited, in terms of differential ECM composition since no ECM component is present exclusively in the distal region of the limb bud. The distal mesenchyme does however contain both fibronectin and collagen type I from stages 19 onwards (Dessau *et al.* 1980). Collagen type I appears to be evenly distributed within the precartilaginous limb bud, although an increase in staining is observed at the ectodermal-mesodermal border. In contrast fibronectin is most abundant in the distal region, although it is also

found in the precartilage condensation (Tomasek *et al.* 1982). The chondroitin sulphate proteoglycan PG-M, which is structurally distinct from the chondroitin sulphate proteoglycan synthesised by differentiated cartilage (PG-H), is also abundant in the distal mesenchyme from stage 19 onwards. Although in common with fibronectin, PG-M is also present in the precartilaginous condensation at stages 23-24 (Shinomura *et al.* 1990). It has been reported that precondensation stage limb buds produce heparan sulphate in greater quantities than other GAGs (Vasan, 1986), although immunofluorescent localisation in stage 23 limb buds showed that whilst the basement membrane labels intensely, a much lower level of heparan sulphate is found distributed evenly throughout the mesenchyme. Although the heparan sulphate proteoglycan syndecan has been localised in mesenchyme beneath inductive epithelia in other locations such as the frontonasal process and the developing tooth bud (Vainio *et al.* 1989).

The existence of an anatomically distinct progress zone has received more conclusive support from the discovery and localisation of homeobox genes (see Chapter 3). Of particular interest are the genes *Hox-7.1* which is expressed in the distal mesenchyme as well as in distinct anterior and posterior domains, and the *Hox-4.8* gene, which is restricted to the most distal and posterior limb mesenchyme. In addition, transcripts of the putative growth factor *Wnt-5a* become localised to the AER and distal mesenchyme in 10 day mouse limb buds and *Hox-8.1* transcripts have been localised in the AER and a narrow rim of underlying mesenchyme.

Previous in vitro experimentation

The AER-mesenchyme interaction has been studied *in vitro* by several investigators. Globus and Vethamany-Globus (1976) cultured

mesenchyme from stage 19-20 limb buds as monolayers (6×10^6 cells/ml) in MEM containing 10% FCS and 5% 9-day chick embryo extract. After 24 hours AERs from stage 19 limb buds were isolated and grafted onto these cultures. 24 hours later the AER had caused a localised outgrowth of cells beneath it, thus showing that the mesenchyme cells were both able to maintain the ridge after 1 day in culture and to respond to the mitogenic factors produced by the AER. Solursh *et al.* (1981b) confirmed these findings by grafting AER containing ectoderm onto 1 day micromass cultures of stage 23-24 whole limbs grown in F12 or MEM containing 10% FCS and 0.5% high-molecular weight fraction of chick embryo extract. Further investigations of this system (Reiter and Solursh, 1982) revealed that the mesenchyme beneath the grafted AER exhibited a higher mitotic index than other regions of the culture. The AER induced outgrowth first became apparent about 24 hours after grafting and continued to grow for the duration of the incubation period.

Maintenance of the functional properties of AERs cultured in the absence of mesenchyme has also been investigated. Feinberg and Saunders (1979) claimed that the AER retained its functional properties after being cultured for up to 12 hours; however they did not state the composition of the medium in which the AER was maintained and Boutin and Fallon (1984) found that the addition of 5 μ g/ml insulin maintained the AER for at least 24 hours in culture according to histological examination for necrotic cells, whereas without the addition of insulin AERs degenerated quickly exhibiting approximately 50% cell death after 12 hours in culture.

Experimental aims

The experiments described in this chapter have been based on the method of Solursh *et al.* (1981b), in which freshly prepared AER has been grafted onto micromass cultures. The purpose of these experiments

has been to further investigate the interaction between the AER and mesenchyme in the micromass culture system in an attempt to evaluate this system's accuracy as a model of *in vivo* development. The ECM composition of the sub-ridge mesenchyme *in vitro* was first determined using immunofluorescent localisation to see if this corresponded to previous *in vivo* reports. Secondly, a comparison of the ability of cultures, made from distal or proximal cells and grown in culture for different periods of time, to maintain and respond to a grafted AER was made. Finally, the ability of cultures to maintain and respond to a grafted AER in different culture media was investigated.

Frontonasal mass cultures were prepared from the central tissue between the developing nasal slits of stage 24 embryos (see Wedden *et al.* 1987). Flank cultures were prepared from the lateral flank mesenchyme between the fore- and hind limb buds of stage 22-24 embryos.

METHODS

Micromass cultures

Micromass cultures were prepared from either 350 μ m distal limb bud tips or from the most proximal 500 μ m of stage 22-23 limb buds. Cultures were prepared as described previously (pp.29) and were plated and maintained in F12 medium. Cultures in the final section were plated and grown in four different culture media, the composition of which are shown below:

ingredient	F12	F12A	F12B	DM + S
Ham's F12 nutrient mixture	90%	45%	90%	45%
Dulbecco's modified Eagle's medium (DMEM)	-	45%	-	45%
Foetal calf serum (FCS)	10%	10%	10%	10%
2mM glutamine	+	+	+	+
200 μ g/ml ascorbate	+	+	+	+
5 μ g/ml insulin	-	-	+	+
10 μ g/ml transferrin	-	-	+	+
20nM hydrocortisone	-	-	+	+
1% antibiotic/antimycotic	1%	1%	1%	1%

AER grafting

AERs were prepared from stage 22 limb buds. 350 μ m distal tips were cut with tungsten needles and incubated in 2% trypsin on ice for 30 minutes. After this time the trypsin was replaced with F12 medium. Several tips were then transferred to the dish containing micromass cultures which had previously been cooled at 4°C for 5-10 minutes.

Working on an ice block, the ectodermal hull containing the AER was removed and placed on top of a culture with care taken to ensure that the mesenchymal surface was in contact with the culture. AERs were gently pushed down on to the culture and the medium removed. Dishes were returned to the incubator for 1.5-2 hours, after which they were observed under the dissecting microscope and gently wetted with 50-100 μ m of culture medium. This process was repeated usually one further time; on the third occasion the cultures were flooded with medium.

Immunofluorescent localisation

i) *wholemount*

Cultures were fixed at various intervals in cold 70% alcohol and stored at 4°C. Antibody staining was as previously described (see pp.31).

ii) *wax sections*

The procedure used for immunolocalisation in wax-embedded sections was derived from that of Sainte-Marie (1962). Cultures were removed from the culture dish with a rubber policeman and fixed in 99% cold 96% alcohol with 1% glacial acetic acid overnight at 4°C. Cultures were then processed as follows:

100% alcohol	- 20 mins	}
100% alcohol	- 20 mins	} at 4°C
xylene	- 20 mins	}
50% xylene: 50% wax	- 20 mins	
wax	- 30 mins	
wax	- 30 mins	

7 μ m sections were then cut on a Spencer 800 wax microtome,

mounted on gelatin-coated slides and baked on at 60°C overnight. Slides were stored in dessicated boxes at 4°C until staining. The sections were demarcated using a diamond pencil, dewaxed in histoclear for 10 minutes and then rehydrated through a series of alcohols into PBS. The area surrounding the sections was dried with tissue and 20-30µl of primary antibody applied to each section. Slides were then placed in humidified boxes for 1 hour. Unbound antibody was removed by three washes in PBS. Slides were then redried and the sections incubated in 20-30µl of FITC-conjugated second antibody, again in a humidified box in the dark. After a final three PBS washes sections were coverslipped using DABCO mountant and examined under UV illumination on a Zeiss microscope.

(The antibodies used in this study have been previously described on pp.33)

Autoradiography

Cultures were incubated in medium containing either 10µCi/ml (24 hour AER grafts) or 25µCi/ml (72 hour AER grafts) ³H-thymidine for 6 hours before fixation in 1/2 Karnovsky's fixative overnight at 4°C. Cultures were then processed according to the following schedule:

0.1M Phosphate buffer - 5 mins

50% alcohol - 20 mins

0.1% Alcian green in

70% alcohol - 10 mins

70% alcohol - 20 mins

90% alcohol - 20 mins

100% alcohol - 10 mins

- 10 mins

- 10 mins

Propylene oxide - 15 mins

- 15 mins

50% propylene oxide/

50% araldite - 1 hour

Araldite - overnight

Fresh araldite - 4 hours

Araldite was made by mixing 10g epoxy resin (Araldite CY212), 10g hardener (DDSA) and 0.8g accelerator (BDMA) on a magnetic hotplate at 40°C. When streaks were no longer seen in the mixture 0.4ml plasticiser (dibutyl phthalate) was added. All chemicals were supplied by Agar Aids.

Processed cultures were then orientated in plastic moulds under the dissecting microscope before polymerisation overnight at 60°C.

1 μ m araldite sections were cut on a Cambridge Huxley ultra-microtome using glass knives made on a LKB Knifemaker. Boats made of adhesive foil tape were attached to the front of the knives and the edges sealed with dental wax. The boats were filled with distilled water onto which cut sections float. Sections were mounted on 0.5% gelatin coated slides and dried on a hotplate. At regular intervals several sections were mounted on a test slide, were stained with 1% Toluidine Blue, 1% sodium tetraborate (BDH Chemicals Ltd.) in distilled water and observed under the microscope. This was to ensure that only sections through the AER grafts were mounted on the slides for autoradiography.

The emulsion (Ilford L.4 Nuclear Research emulsion in gel form) was melted in a glass measuring cylinder placed in a 43°C water bath. When molten, 15ml of emulsion were poured into a glass dipping jar which already contained 15ml of 2% glycerol in distilled water. The mixture was slowly stirred, with care taken to avoid introducing air bubbles. Clean slides were dipped every few minutes until an even layer

of emulsion was achieved. The experimental slides were then dipped.

After wiping the emulsion from the back of the slide, the slides were placed on cooled metal plates. Slides were then left to dry overnight before being packaged into light-proof boxes. Slides were stored at 4°C for 2-3 weeks before developing.

Slides were developed in Kodak D19 developer at room temperature for about 5 minutes. After rinsing in water the slides were fixed in Kodafix and washed in running tap water. Sections were stained with Toluidine Blue for 30 seconds on a hotplate. When dry, coverslips were mounted with Clearmount.

Sections were initially observed and photographed under brightfield illumination, however it was often difficult to distinguish between labelled cells (black) and unlabelled cells (purple) in black and white photographs. Furthermore none of the cells beneath the AER appeared to be labelled. Upon further examination it was clear that these cells were indeed labelled, but at a lower intensity than labelled cells in other parts of the culture. Therefore the only way to show this labelling was to photograph the sections under darkfield illumination.

Cell proliferation

The rate of cell proliferation after 24 hours of culture was determined by BrdU uptake as described on pp.136.

RESULTS

Immunolocalisation of ECM components in sub-ridge mesenchyme

Apical ectodermal ridges (AERs) were grafted onto distal cell micromass cultures at 24 hours of culture and allowed to develop for a further 48 hours. In all cases examined, a mesenchymal outgrowth was seen beneath the AER. This either took the form of one large outgrowth or 2-3 smaller outgrowths. The results of ECM localisation in sub-ridge mesenchyme are summarised in Table 1.2.1. The mesenchymal outgrowths stained intensely for fibronectin especially in the regions directly adjacent to the AER. Closer examination showed that this staining was indeed confined to the mesenchyme and not seen in the AER (Fig. 1.2.01 A-D). In contrast, the ectoderm of the AER stained strongly for keratan sulphate, whereas non-ridge ectoderm did not exhibit staining. This was confirmed by immunostaining of paraffin sections through an AER-induced outgrowth (Fig. 1.2.01 D-H). Sub-ridge outgrowths also stained strongly for heparan sulphate, chondroitin sulphate, procollagen I and collagen type I (Fig. 1.2.02 A-D). Collagen type II staining was not detected (Fig. 1.2.02 E). Laminin staining was seen in the whole ectoderm, although staining in the AER appeared to be more intense (Fig. 1.2.02 F). The ridge ectoderm also stained with the hyaluronate binding region probe, however the same staining was seen with the control probe (Fig. 1.2.02 G,H).

Comparison of the response of different aged distal cell and proximal cell cultures to a grafted AER

AER grafts examined after 24 hours

Fibronectin and keratan sulphate were used as markers for the mesenchyme and AER respectively. 24 hours after AER grafting, a band of fibronectin staining was seen beneath the grafted AER in most cultures

examined (Fig. 1.2.03 A-D), although the staining beneath an AER grafted onto a 1 day distal cell culture appeared the most extensive and intense (Fig. 1.2.03 A,B). The specificity of fibronectin staining for the mesenchyme was confirmed by growing ridges on plastic for 24 hours. No staining for fibronectin was seen underlying the AER (Fig. 1.2.03 E,F); however AERs grown on plastic tissue culture dishes for 24 hours maintained their characteristic appearance and exhibited strong staining for keratan sulphate (Fig. 1.2.03 G,H).

The results of tritiated-thymidine incorporation are summarised in Table 1.2.4a. The percentage of cells in S-phase beneath the grafted AER is consistently higher than in other parts of the culture in both distal and proximal cultures by approximately 7%. Distal cultures also contained twice as many cells in S-phase as proximal cultures (Fig. 1.2.04).

24 hours after AER grafting was deemed too short a period to compare the responses of distal and proximal cell cultures of different ages for several reasons. Firstly, the size of induced mesenchymal outgrowths was quite small, thus making comparisons between different cultures difficult. Secondly, ridges grown on plastic for the same period of time retain a normal morphology and stain for keratan sulphate, thus keratan sulphate staining cannot be used as a marker for ridge maintenance at this time point and finally, the difference in the rate of proliferation beneath the AER as compared to other regions of the culture is relatively small at this stage.

AER grafts examined after 72 hours

The results of grafting apical ridges onto micromass cultures of either distal or proximal cells are summarised in Tables 1.2.2a and 1.2.2b respectively. Only one day old distal cultures were able to respond to the grafted AER by producing a substantial outgrowth beneath

the AER. These outgrowths exhibited intense staining for fibronectin and keratan sulphate (Fig. 1.2.05 A-D). 2 and 3 day old distal micromass cultures did not produce these outgrowths apart from in one experimental run. Accordingly, the incidence of an increased production of fibronectin beneath the AER only occurred in a few cases, in the majority of cases no increase in fibronectin staining was seen (Fig. 1.2.05 E,F). Although a large number of AERs grafted onto 2 and 3 day distal cell cultures did show staining for keratan sulphate (Fig. 1.2.05 G,H).

The majority of 1, 2 and 3 day old proximal micromass cultures all responded to a grafted AER, however there was only a very small outgrowth of the culture beneath this which stained strongly for fibronectin (Fig. 1.2.06 A,B,E,F). The limited response of proximal cell cultures was supported by the finding that only a fragment of AER could be detected morphologically and by keratan sulphate staining 3 days after grafting (Fig. 1.2.06 C,D,G,H). The response was similar in proximal cultures irrespective of the length of time they had been cultured before receiving the AER graft.

The tritiated thymidine incorporation data show that cells beneath the AER exhibit a rate of proliferation approximately twice that of cells in other areas of the cultures in both the distal and proximal cultures (Table 1.2.4b). The proportion of cells in S-phase beneath the AER grafted onto distal cultures (28.6%) is also much greater than the corresponding proportion in proximal cultures (17.5%) (Fig. 1.2.07). It is also interesting to note that the proportion of cells in S-phase has decreased in all regions of the cultures as compared to the corresponding data at 24 hours (compare Tables 1.2.4a and 1.2.4b).

The results of grafting AERs onto cultures made from flank tissue or from the frontonasal mass (FNM) are summarised in Table 1.2.3. FNM

cultures exhibited an increase in fibronectin staining beneath a grafted AER (Fig. 1.2.08 A,B), although a morphologically identifiable outgrowth was detected in only 3 out of 13 cases. AER tissue was also identifiable by keratan sulphate staining in 3 out of 5 cases (Fig. 1.2.08 C,D). In contrast, flank cultures never exhibited an increase in fibronectin staining (Fig. 1.2.08 E,F) or formed an outgrowth in response to a grafted AER. In 3 out of 5 cases, keratan sulphate staining was seen, but in these cases the whole ectoderm stained rather than just the areas that corresponded to AER tissue (Fig. 1.2.08 G,H).

Analysis of the effect of media composition on the ability of AER grafts to cause outgrowths from the culture

The results of the ability of a grafted AER to cause an outgrowth from cultures grown in different media are summarised in Table 1.2.5. AERs grafted onto cultures grown in F12 resulted in large outgrowths which stained intensely for fibronectin and keratan sulphate (Fig. 1.2.09 A,B). Cultures grown in DM + S occasionally exhibited an increase in fibronectin staining in the region of the culture beneath the AER, however in none of the cases examined was the AER capable of inducing a significant outgrowth from the culture (Fig. 1.2.09 G). The AER did however still stain with keratan sulphate (Fig. 1.2.09 H) and could be distinguished morphologically. In order to determine which elements of the DM + S culture medium were responsible for preventing the cultured cells from responding to the effect of a grafted AER, AERs were grafted onto cultures grown in F12 containing 50% DMEM (F12A) and F12 supplemented with insulin, hydrocortisone and transferrin (F12B). Cultures grown in F12A responded in an identical manner to those grown in F12, that is the AER induced a substantial outgrowth from the culture which stained intensely for fibronectin (Fig. 1.2.09 C). The

overlying AER also stained strongly for keratan sulphate (Fig. 1.2.09 D). Cultures grown in F12B exhibited intermediate behaviour between that of cultures grown in F12 and those grown in DM + S, in that fibronectin-staining outgrowths were seen, but these were reduced in comparison to the sizes of outgrowths seen in cultures grown in F12 or F12A (Fig. 1.2.09 E). Keratan sulphate staining of the AER was however equally strong to that seen in cultures grown in F12 and F12A (Fig. 1.2.09 F).

In a further set of experiments AERs were plated directly onto tissue culture dishes and their behaviour observed over a period of several days. After 3-4 days in culture, they were fixed and stained with keratan sulphate. AERs grown in DM + S retained the best morphology and exhibited the strongest and most extensive staining with keratan sulphate. In contrast, only a small fragment of the AERs grown in the other three media retained a ridge-like morphology, and consequently keratan sulphate staining was greatly reduced. Thus the differential survival of the AER in the different media did not reflect the differential effect with respect to outgrowth formation seen in the different media. The different responses seen must therefore reflect the ability of the mesenchyme to react to the signals produced by the AER. Since the AER results in an increased rate of proliferation, it seemed possible that a difference in the rate of cell proliferation in the different media might explain the difference in response. The results of BrdU incorporation into 24 hour cultures maintained in the different media are summarised in Table 1.2.6. Cultures grown in DM + S exhibited the highest rate of proliferation, whilst F12 and F12A cultures exhibited the lowest level, however the difference between the rates of proliferation in the different media was not significant. It did appear however that cells grown in DM + S had progressed further along the pathway of chondrogenic differentiation. This was supported

by two findings. Firstly, the digestion with trypsin and EDTA took considerably longer for cultures grown in DM + S, with cultures grown in F12B also taking longer than F12 and F12A cultures. Secondly, cultures grown in the different media were fixed and stained with Alcian blue after 1, 2 and 3 days of culture. At all time points DM + S cultures exhibited a greater amount of staining than the other cultures.

Table 1.2.1 - Immunolocalisation of ECM components in sub-AER

mesenchyme

ECM component	sub-AER mesenchyme	AER
<hr/>		
Fibronectin	+	-
Keratan sulphate	-	+
Heparan sulphate	+	-
Chondroitin sulphate	+	-
Procollagen I	+	-
Collagen type I	+	-
Collagen type II	-	-
Laminin	-	+ ^a
Hyaluronate	- ^b	- ^b

a: the whole ectoderm stained, although staining in the AER was more intense

b: staining was obtained both with the binding region probe and the control probe

Table 1.2.2a - AER grafts onto distal cultures

age of culture time of AER grafting	Fibronectin	Keratan sulphate	Outgrowth
1 day	7 (7)	6 (6)	13 (13)
2 day	2 (6)	3* (5)	2 (11)
3 day	1 (5)	5 (6)	1 (11)

* whole ectoderm stained in 2 cases

Table 1.2.2b - AER grafts onto proximal cultures

age of culture at time of AER grafting	Fibronectin	Keratan sulphate	Outgrowth
1 day	7 (9)	7* (7)	12 (16)
2 day	7 (7)	6 (6)	13 (13)
3 day	6 (7)	3 (4)	9 (11)

* whole ectoderm stained

Table 1.2.3 - AER grafts onto cultures of flank or frontonasal mass

tissue used to make culture	Fibronectin	Keratan sulphate	Outgrowth
Frontonasal mass	6 (8)	3 (5)	3 (13)
Flank	0 (9)	3* (5)	0 (14)

* whole ectoderm stained including
possible fragments of AER in 2 cases

key: number in brackets = number of cases

Table 1.2.4a - Tritiated thymidine incorporation into 24 hour cultures

culture	% labelled cells in non-ridge culture	% labelled cells under AER
distal	31.8	39.1
proximal	16.1	23.0

Table 1.2.4b - Tritiated thymidine incorporation into 72 hour cultures

culture	% labelled cells in non-ridge culture	% labelled cells under AER
distal	13.5	28.6
proximal	8.7	17.5

Table 1.2.5 - Effect of different media on response of distal cell cultures to a grafted AER

culture medium	fibronectin staining	keratan sulphate staining	outgrowth
F12	8 (8)	4 (4)	10 (10)
F12A	8 (8)	2 (2)	10 (10)
F12B	8 (8)	2 (2)	10 ^a (10)
DM + S	4 (9)	4 (4)	0 (13)

a: partial outgrowth, i.e. substantially smaller than those obtained in F12 or F12A

Table 1.2.6 - Effect of different media on cell proliferation

culture medium	% BrdU incorporation at 24 hours
F12	12.0
F12A	12.5
F12B	13.4
DM + S	15.1

Figure 1.2.01

Phase contrast (A,C,E,G) and corresponding immunofluorescent (B,D,F,H) micrographs of 3 day old distal cell micromass cultures onto which an AER had been grafted at 24 hours of culture. Cultures were stained with antibody to fibronectin (A-D) or keratan sulphate (E-H).

(A,B) The mesenchymal cell outgrowths (m) exhibited intense staining for fibronectin, particularly in the regions directly beneath the AER.

Scale bar = 200 μ m.

At higher magnification (C,D) it was clear that this staining was confined to the mesenchyme and was absent from the AER; a = AER.

Scale bar = 100 μ m.

In contrast, the AER itself stained strongly for keratan sulphate (E,F), whilst the surrounding non-ridge ectoderm exhibited no staining; e = non-ridge ectoderm. Scale bar = 200 μ m.

Sections through such an outgrowth embedded in paraffin wax (G,H) confirmed that this staining was confined to the AER and was absent from the mesenchyme. a = AER, m = mesenchymal outgrowth.

Scale bar = 50 μ m.

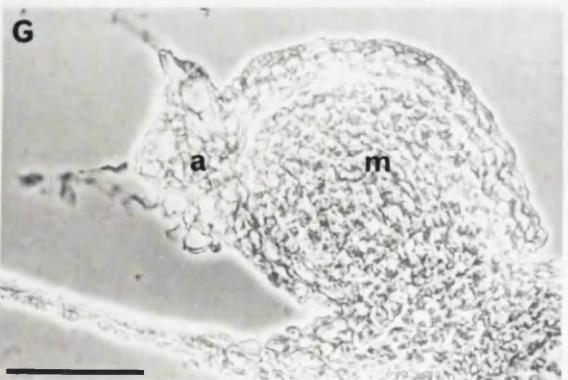
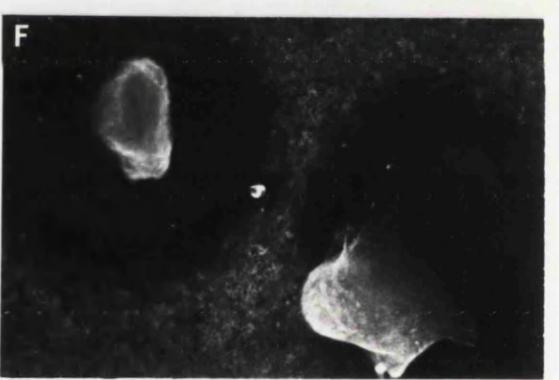
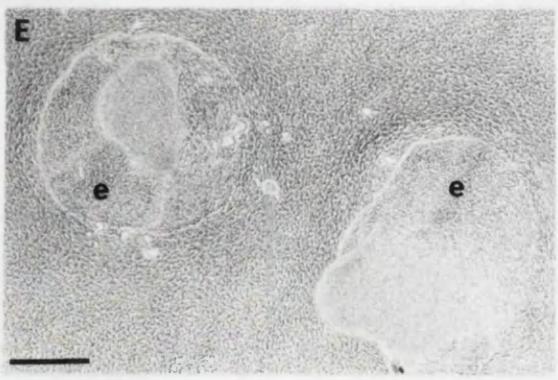
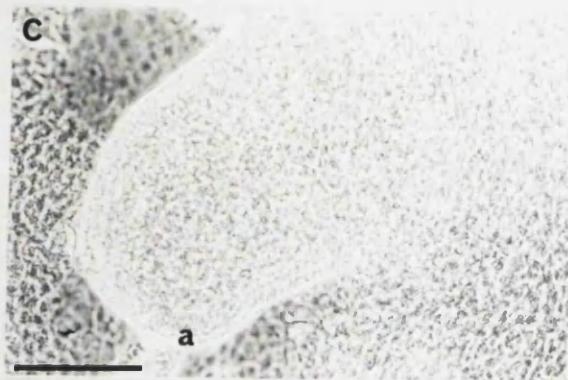
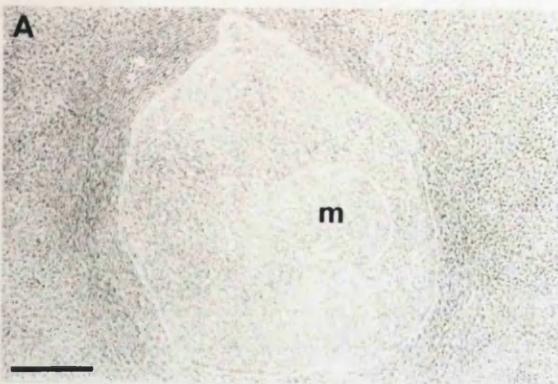


Figure 1.2.02

Immunolocalisation of extracellular matrix components in 3 day distal cell micromass cultures onto which AERs had been grafted at 24 hours of culture. Immunofluorescent micrographs showing staining with antibody to heparan sulphate (A), chondroitin sulphate (B), procollagen I (C), collagen type I (D), collagen type II (E) and laminin (F). (G) and (H) show the localisation of hyaluronate binding region probe and the control probe respectively. AER-induced outgrowths are indicated by arrowheads in (D) and (E).

Scale bar (A,B,C,F,G,H) = 200 μ m, scale bar (D,E) = 100 μ m.

The mesenchyme outgrowths stained strongly for heparan sulphate, chondroitin sulphate, procollagen I and collagen type I. Staining was not seen with antibody to collagen type II. Laminin stained the whole ectoderm, although staining of the AER did appear to be more intense. The AER, but not the surrounding non-ridge ectoderm, also stained with both the hyaluronate binding region probe and the non-biotinylated control probe.

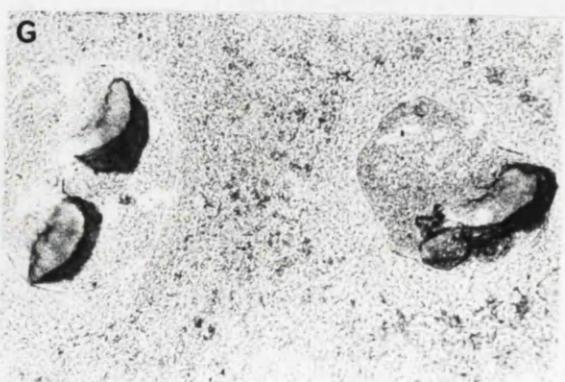
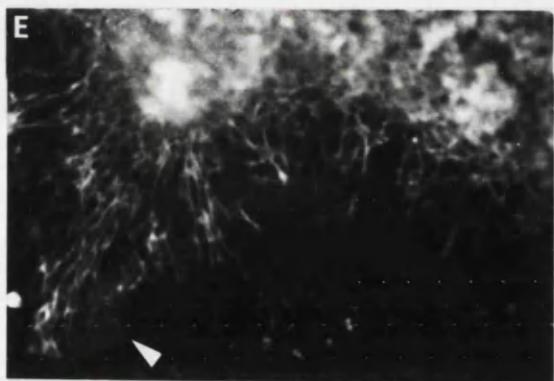
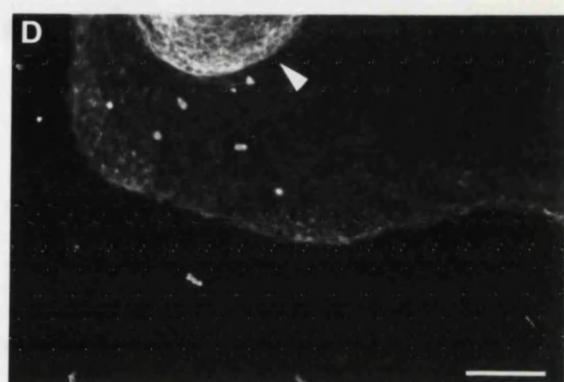
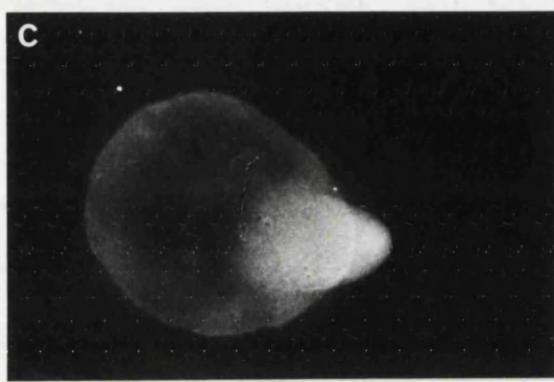
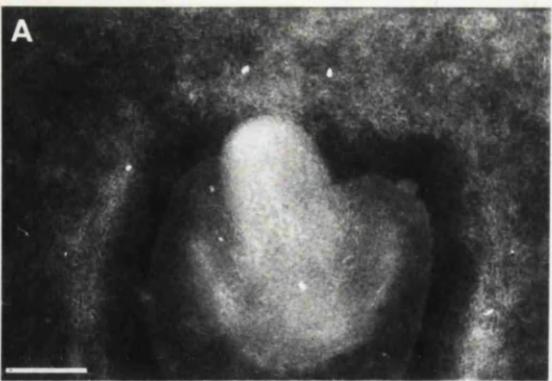


Figure 1.2.03

Phase contrast (A,C,E,G) and corresponding immunofluorescent (B,D,F,H) micrographs. (A,B) 48 hour old distal culture, onto which an AER was grafted 24 hours previously, stained with antibody to fibronectin. (C,D) 4 day old distal cell micromass culture onto which an AER had been grafted 24 hours previously stained with antibody to fibronectin. (E-H) AERs plated onto tissue culture plastic and maintained in F12 medium for 24 hours, before fixing and staining with fibronectin (E,F) and keratan sulphate (G,H) antibodies. a = AER.

Scale bar = 200 μ m.

Fibronectin staining was seen under AERs grafted onto both 1 day and 3 day old distal micromass cultures, however the staining observed in the 1 day culture was more intense than that in the 3 day culture. In addition the mesenchyme beneath the grafted AER on the 1 day culture appeared to have grown out from the culture somewhat whereas this was not seen in the 3 day culture.

AERs grown on tissue culture plastic did not exhibit fibronectin staining, unlike many of the surrounding non-ridge ectoderm cells. However intense keratan sulphate staining was confined to the AER cells.

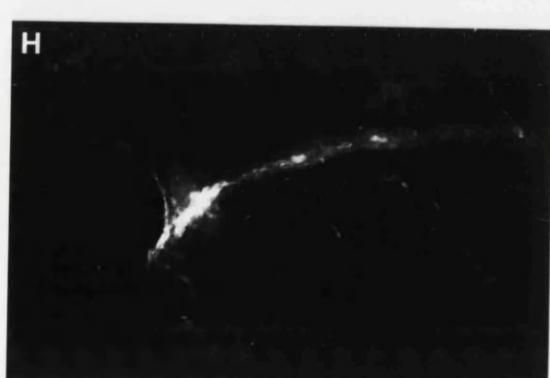
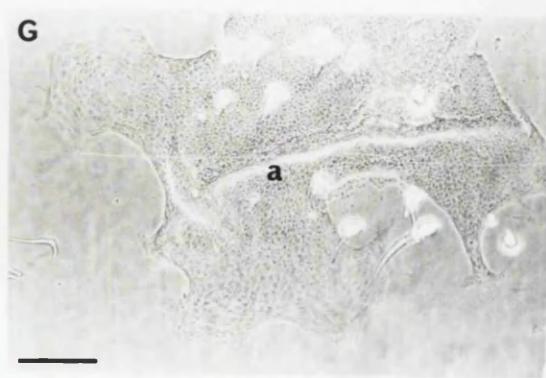
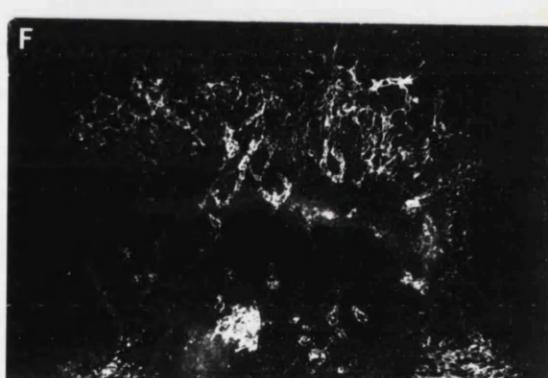
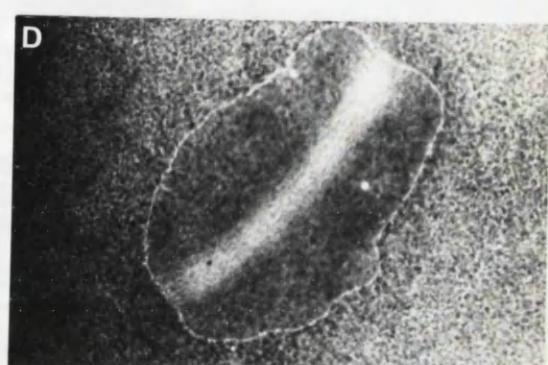
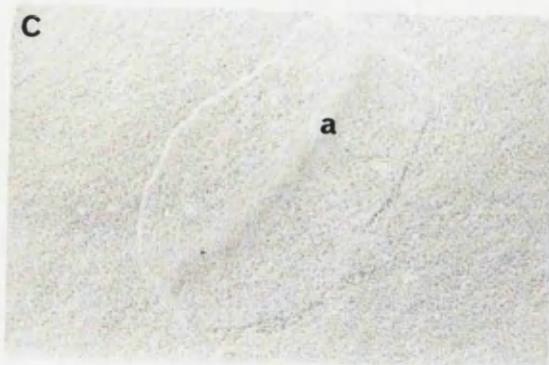
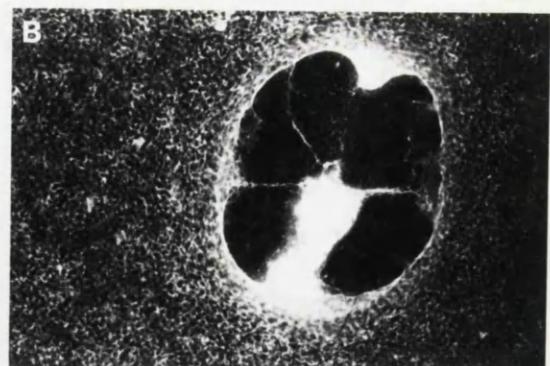


Figure 1.2.04

Light-field (A,C,E,G) and corresponding dark-field (B,D,F,H) micrographs showing cells labelled with tritiated thymidine. (A,B) illustrate the area beneath the grafted AER, and (C,D) illustrate a region of the same culture not including the grafted AER of a 2 day distal culture, the AER having been grafted onto the culture after 24 hours. (E,F) represent the region beneath the grafted AER of a 2 day proximal cell culture onto which an AER had been grafted after 24 hours. (G,H) represent a region of the same culture not including the grafted AER. The inner extent of the ectoderm is indicated by arrowheads; a = AER.

Scale bar = 50 μ m.

The proportion of labelled cells beneath the AER in both distal and proximal cultures was approximately 7% greater than the proportion in other parts of the cultures. In addition the proportion of labelled cells in distal cultures was almost twice that in proximal cultures. Cells beneath the AER in both cultures were less strongly labelled than cells in other regions of the cultures presumably due to the reduced amount of tritiated thymidine able to cross the AER. These cells were however readily seen if the sections were photographed under dark-field illumination.

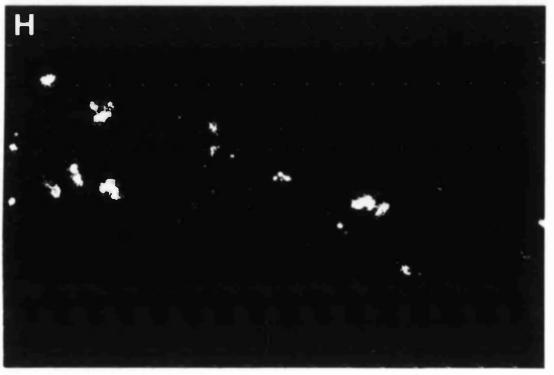
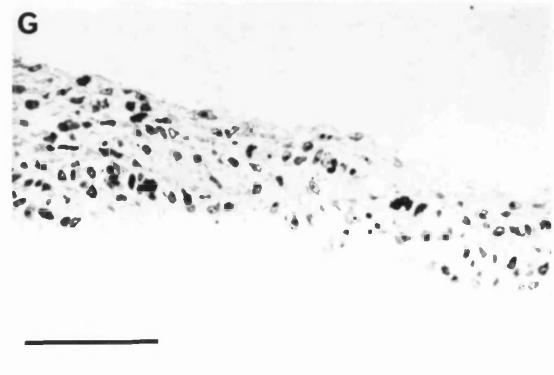
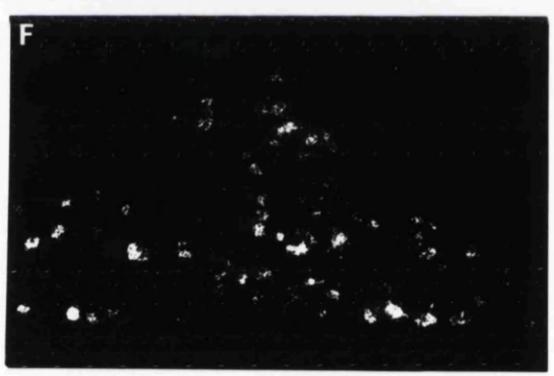
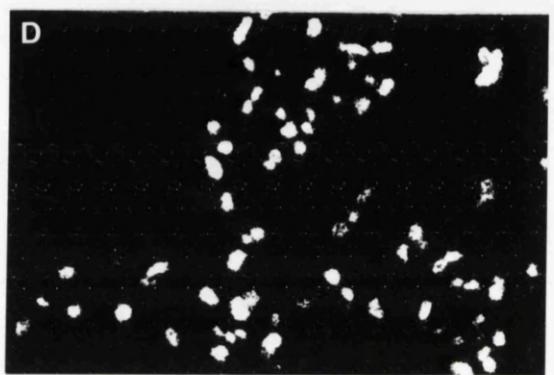
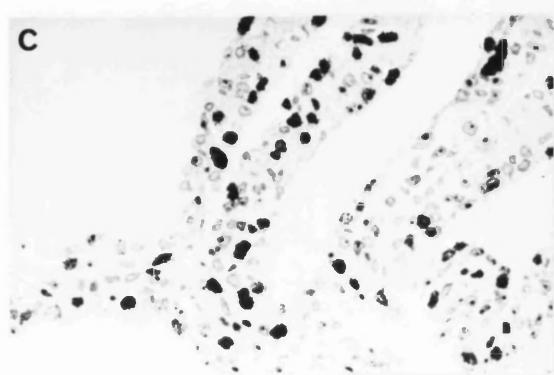
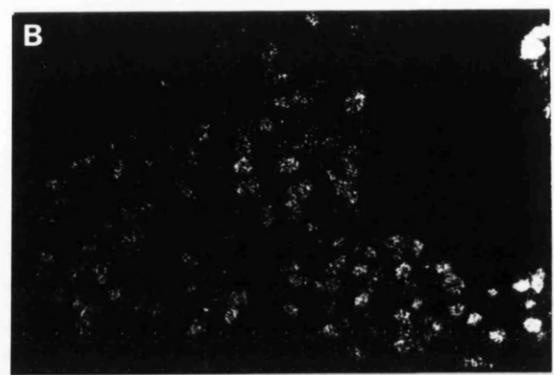
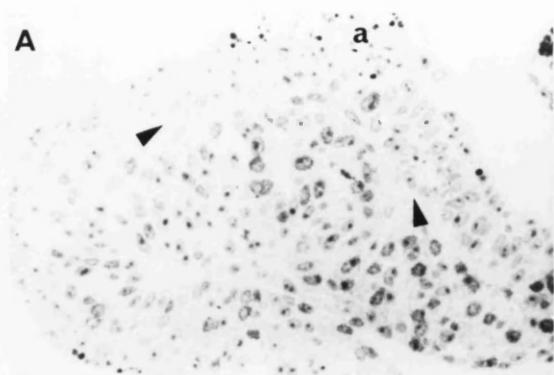


Figure 1.2.05

Phase contrast (A,C,E,G) and corresponding immunofluorescent (B,D,F,H) micrographs of a 4 day distal cell culture onto which an AER had been grafted after 24 hours of culture (A-D), and a 6 day distal cell culture onto which an AER had been grafted after 72 hours of culture (E-H). (A,B,E,F) show cultures stained with antibody to fibronectin and (C,D,G,H) show cultures stained with antibody to keratan sulphate.

Scale bars = 200 μ m.

Distal cultures that received an AER after 24 hours of culture responded by producing a large mesenchymal outgrowth, which stained intensely for fibronectin especially in the region just beneath the grafted AER. The grafted AER in turn stained intensely for keratan sulphate. Distal cultures grown for 72 hours before receiving an AER graft did not produce outgrowths and an increase in fibronectin staining beneath the grafted AER was not seen, even though staining with keratan sulphate revealed that the AER appeared to be maintained in these grafts.

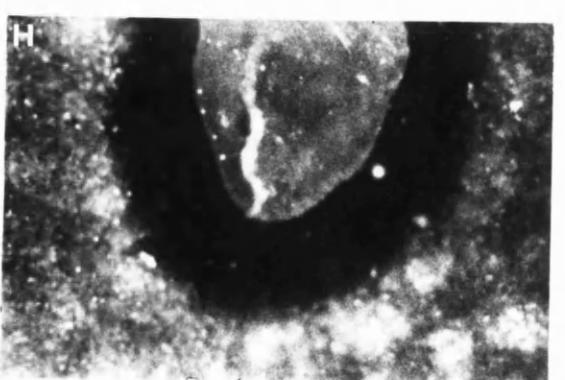
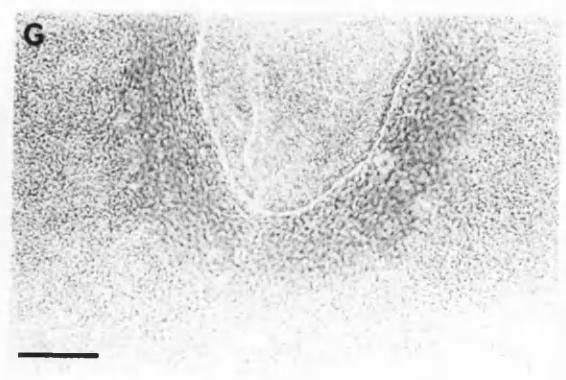
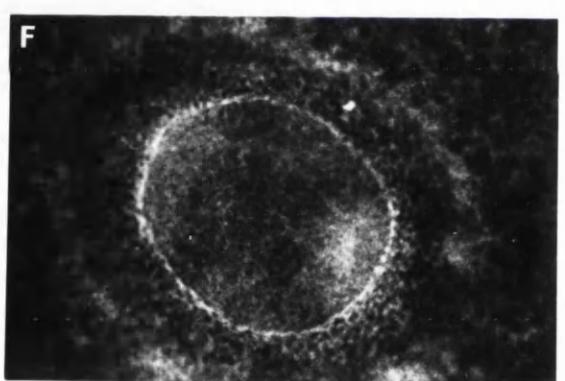
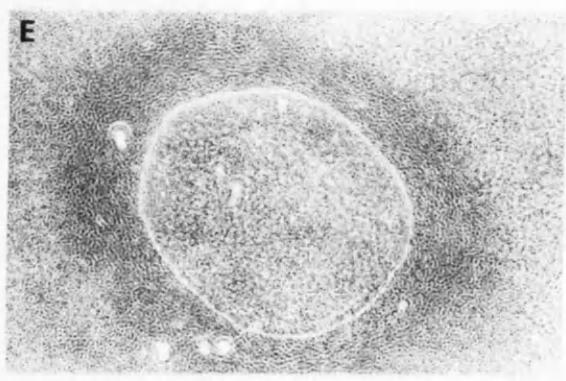
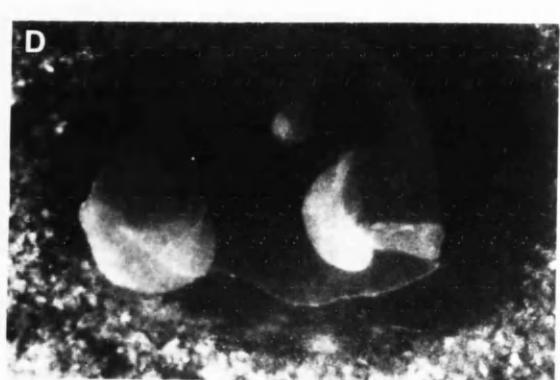
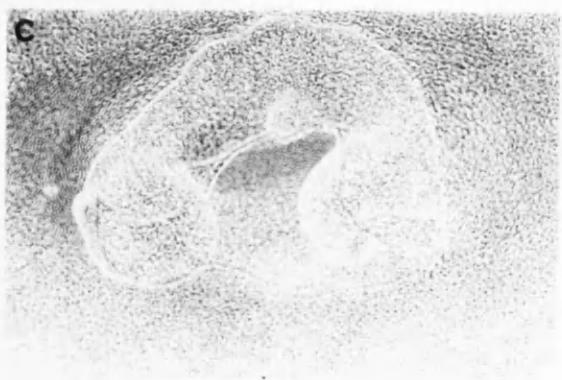
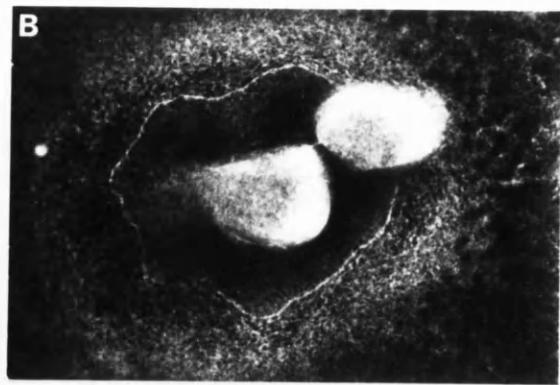
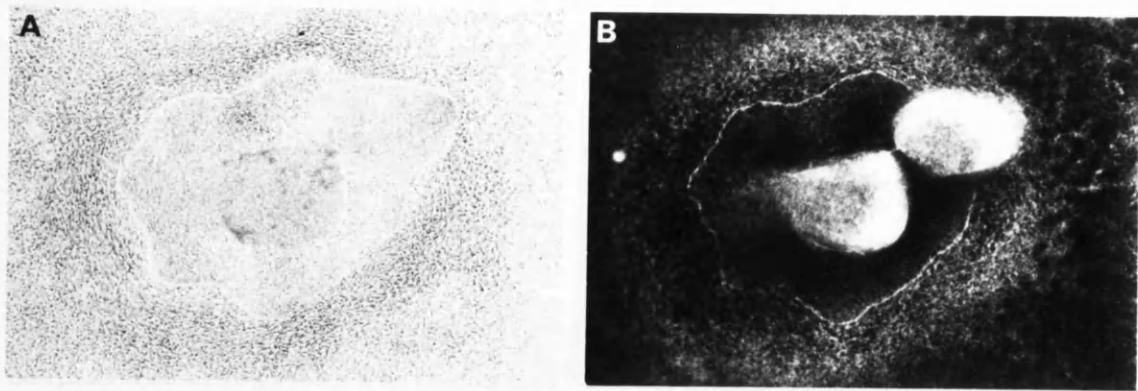


Figure 1.2.06

Phase contrast (A,C,E,G) and corresponding immunoofluorescent (B,D,F,H) micrographs of a 4 day old proximal cell micromass culture onto which an AER was grafted after 24 hours (A-D) and a 7 day old proximal cell culture onto which an AER was grafted after 72 hours (E-H). (A,B,E,F) show cultures stained with antibody to fibronectin, and (C,D,G,H) show cultures stained with antibody to keratan sulphate. Arrowheads on the phase contrast views indicate fragments of retained AER.

Scale bar = 200 μ m.

Proximal cells grown for 1 day, 2 days and 3 days in culture all responded to a grafted AER in a similar fashion. A small fibronectin-staining outgrowth was seen from the cultures. Staining with keratan sulphate revealed that this corresponded to the maintenance of only a small fragment of AER in these cultures.

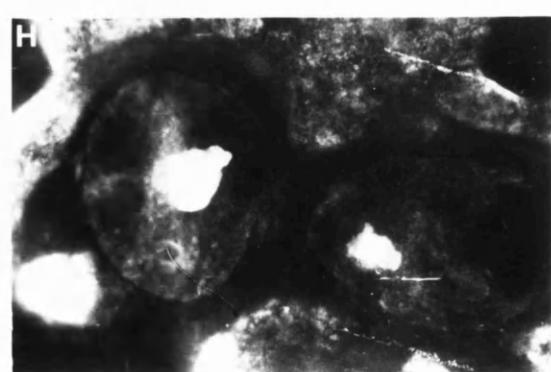
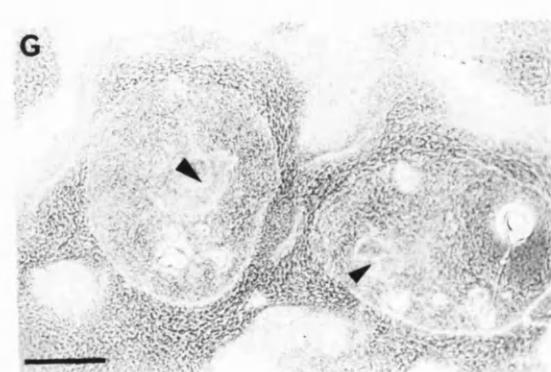
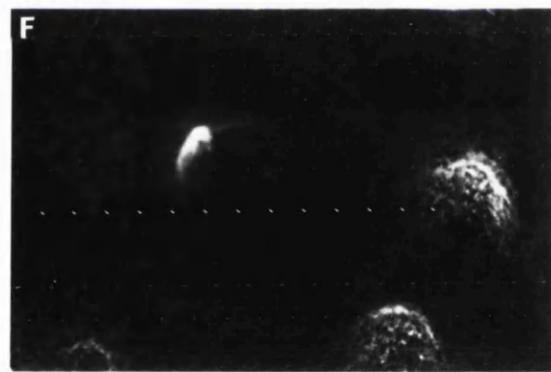
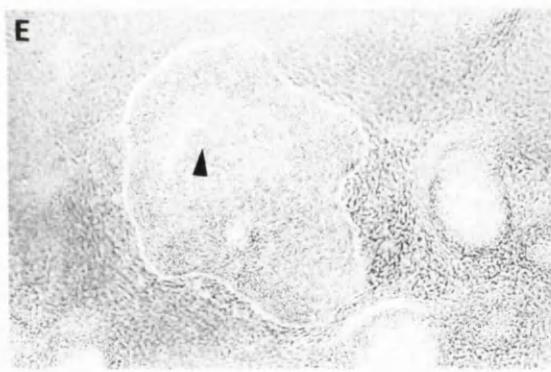
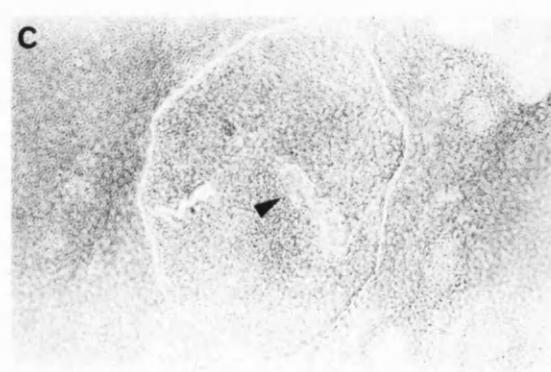
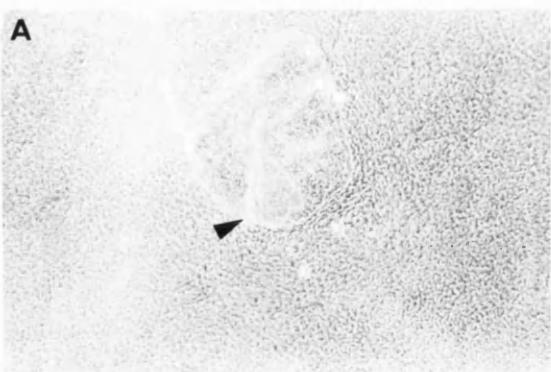


Figure 1.2.07

Light-field (A,C,E) and corresponding dark-field (B,D,F) micrographs showing tritiated thymidine labelling of a 4 day old distal cell culture (A-D) and a 4 day old proximal cell culture (E,F), both of which received AER grafts after 24 hours. (A,B) show the AER and underlying mesenchymal cell outgrowth of the distal cell culture. (C,D) show a region of the same culture not associated with the AER. (E,F) show the AER and underlying mesenchyme of the proximal cell culture; a = AER.

Scale bar = 50 μ m.

In both distal and proximal cell cultures the proportion of labelled cells beneath the AER was twice that in other regions of the cultures, although the proportion of labelled cells in distal cultures was substantially higher than in the equivalent regions of proximal cultures.

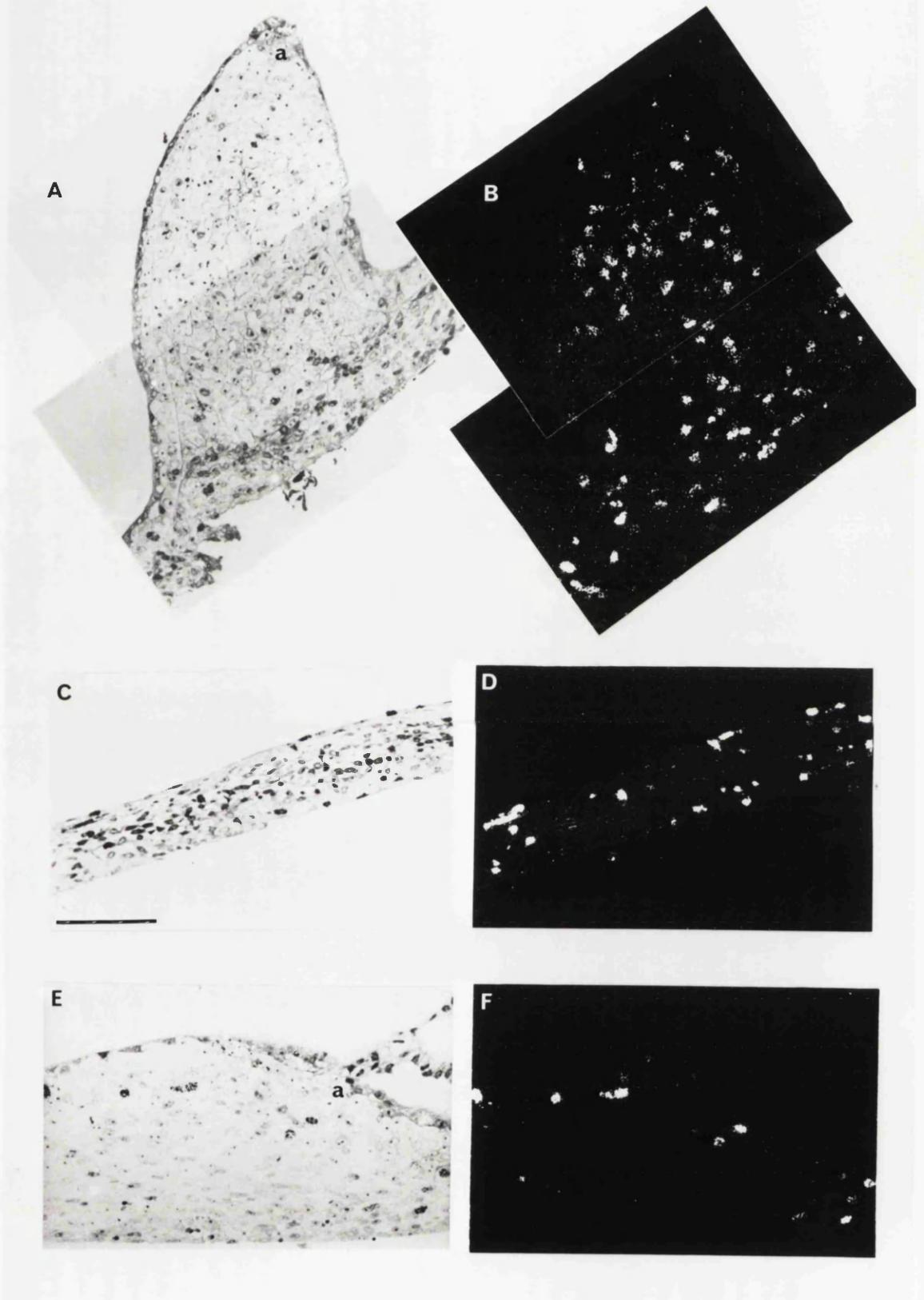


Figure 1.2.08

Phase contrast (A,C,E,G) and corresponding immunofluorescent (B,D,F,H) micrographs of 4 day old frontonasal mass (A-D) and flank (E-H) cultures onto which AERs had been grafted after 24 hours. (A,B,E,F) show cultures stained with antibody to fibronectin, and (C,D,G,H) show cultures stained with antibody to keratan sulphate.

Scale bar = 200 μ m.

Frontonasal mass cultures responded to a grafted AER with an increase in fibronectin staining, which in a few cases was associated with a small outgrowth from the culture. The AER was maintained in these cultures and exhibited keratan sulphate staining. In contrast, an increase in fibronectin staining or outgrowth from the culture was never seen in flank cell cultures. A morphologically recognisable AER was only seen in 2 out of 5 cases, although in 3 out of 5 cases the whole ectoderm stained for keratan sulphate.

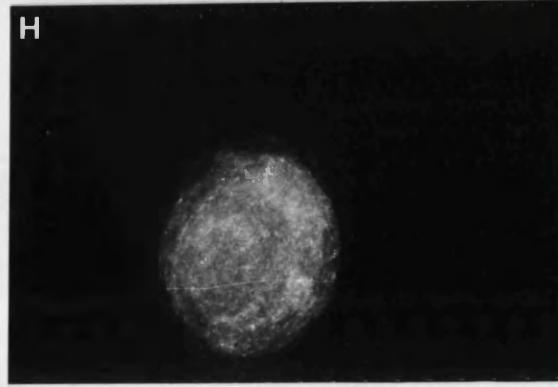
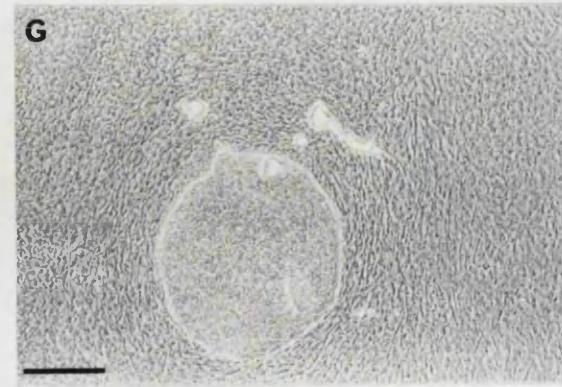
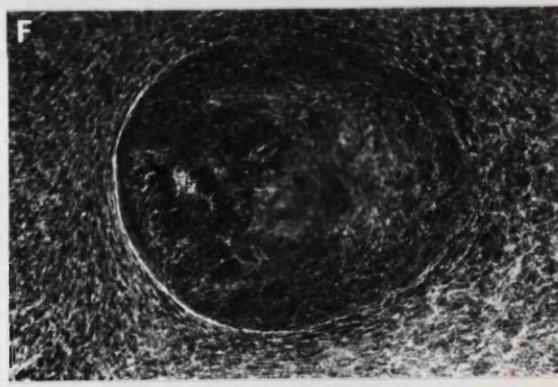
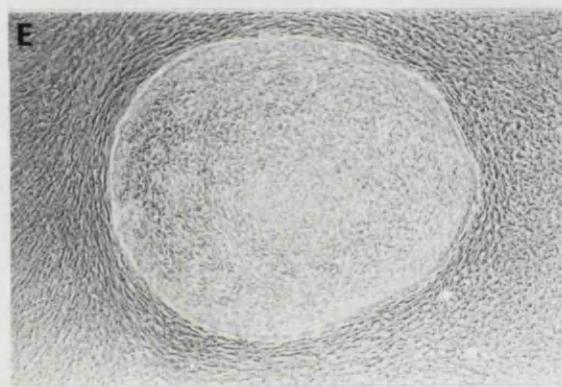
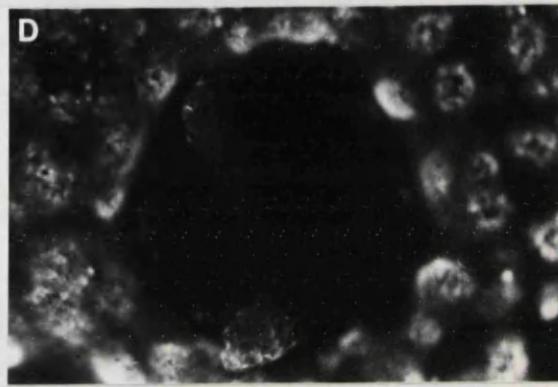
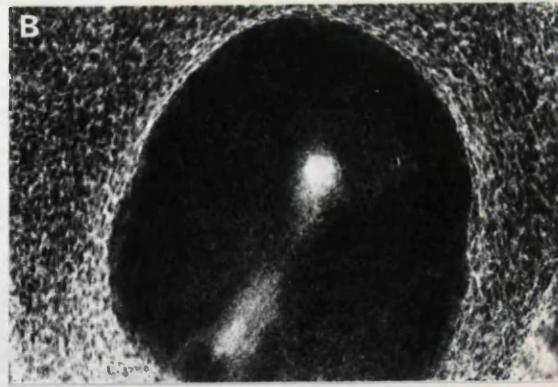
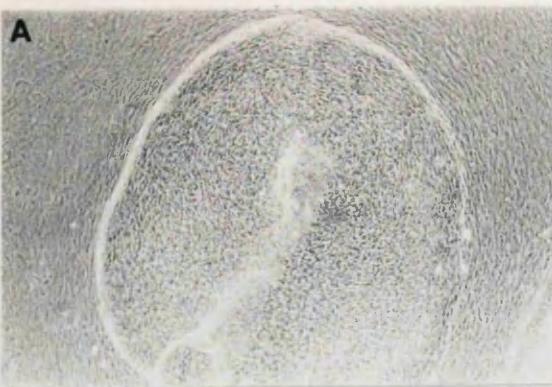
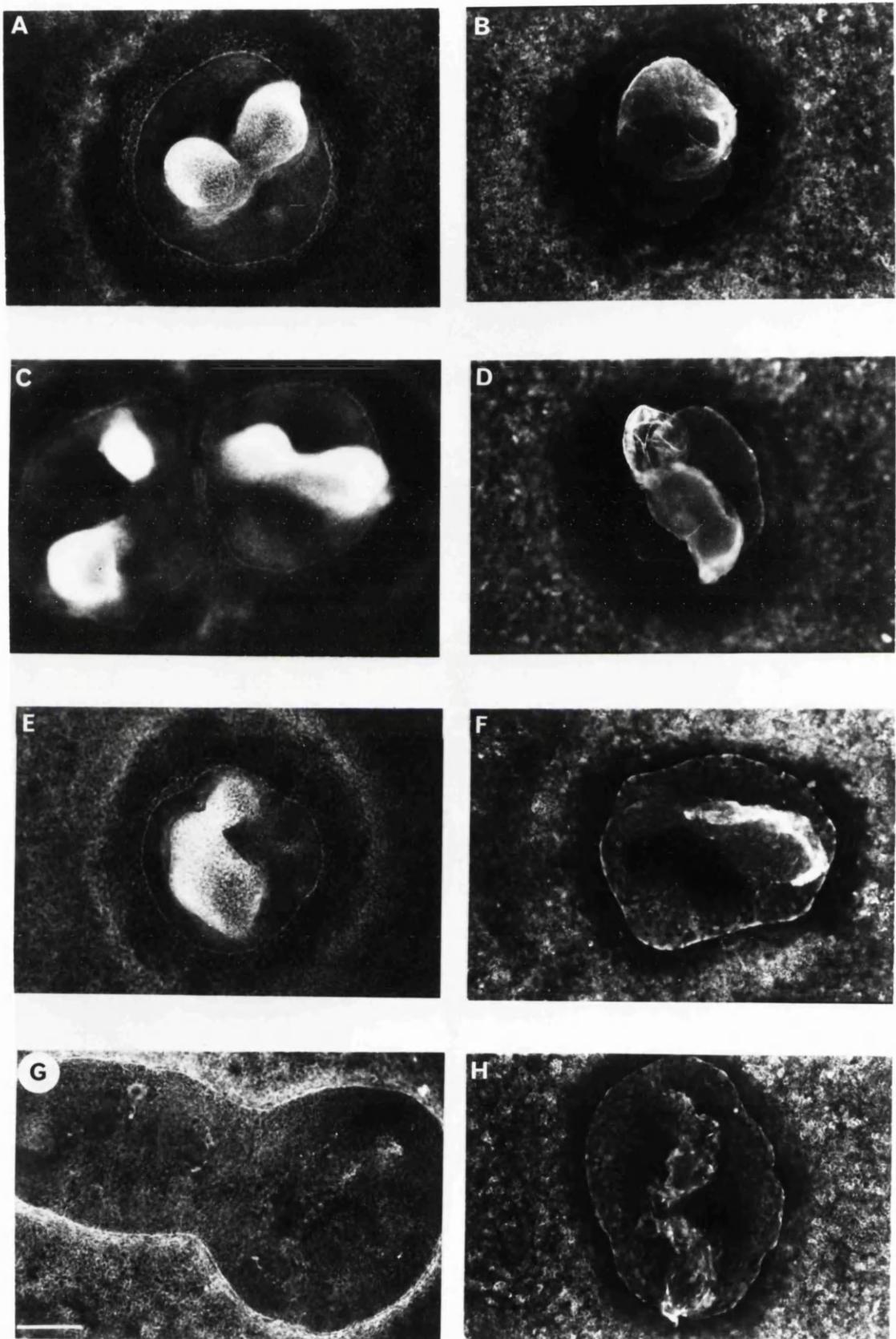


Figure 1.2.09

Immunofluorescent micrographs of 4 day old distal cultures onto which AERs had been grafted after 24 hours of culture stained with antibodies to fibronectin (A,C,E,G) and keratan sulphate (B,D,F,H). Cultures were grown in F12 (A,B), F12A (C,D), F12B (E,F) or DM + S (G,H).

Scale bar = 200 μ m.

Cultures grown in F12 and F12A responded to a grafted AER by producing large mesenchymal outgrowths which stain strongly for fibronectin. Cultures grown in F12B also produced outgrowths which stained for fibronectin, however these were often smaller than those seen in the F12 or F12A. The grafted AERs stained strongly for keratan sulphate in all three media. In contrast cultures grown in DM + S never responded to a grafted AER with a fibronectin-staining outgrowth, although the AER stained with keratan sulphate.



Immunolocalisation of ECM components

Fibronectin has been identified in developing limb buds, where it is most abundant in the distal mesenchyme beneath the AER. The finding that mesenchyme outgrowths induced by a grafted AER stain intensely for fibronectin thus agrees with the *in vivo* situation. It should be noted that fibronectin is also expressed in other regions of the cultures, but the staining is characteristically stronger beneath a grafted AER, and thus it served as a marker for the mesenchymal response to a grafted AER in this study.

Keratan sulphate proteoglycans are synthesised during cartilage differentiation, therefore the strong staining of cartilage nodules in these cultures with keratan sulphate antibody was expected. However keratan sulphate also appears to be a characteristic feature of AER ectoderm, since it is not detectable in non-ridge ectoderm. This is supported by previous studies which found that the AER exhibited strong staining with the lectin Peanut agglutinin which recognises sugar residues common to keratan sulphate (Auld and Tickle - unpublished observations; Croucher and Tickle, 1989). It therefore served as a marker of AER maintenance in this system, although in about 30% of cases the whole ectoderm stained for keratan sulphate. In these cases it was possible to identify the staining which corresponded to fragments of AER, however this does show that keratan sulphate is probably not the marker of choice in this system.

The identification of large amounts of heparan sulphate proteoglycan in the mesenchyme beneath a grafted AER, but not beneath non-ridge ectoderm suggests that this is a specific feature of the early proliferating mesenchyme of the progress zone. This is supported by the localisation of the heparan sulphate proteoglycan syndecan to

mesenchyme beneath inductive epithelia in the developing frontonasal mass and tooth bud (Vainio *et al.* 1989). Although in developing limb buds it has been reported that heparan sulphate is most abundant in the basement membrane beneath the ectoderm, with only a very low level present throughout the mesenchyme, including the distal mesenchyme beneath the AER. Chondroitin sulphate was also found to be very abundant in the mesenchyme beneath a grafted AER, and this supports *in vivo* findings that the chondroitin sulphate proteoglycan PG-M is most abundant in the distal mesenchyme of the precondensation stage limb bud. Procollagen and collagen type I were also produced in abundant quantities in sub-ridge mesenchyme. These findings are again consistent with *in vivo* studies, although it is interesting that other regions of the cultures did not exhibit collagen type I staining, whereas it has been reported that type I collagen is ubiquitously distributed in early developing limb buds. Laminin presumably stains the basement membrane of the grafted ectoderm, although it appears to be more abundant in the basement membrane of the AER. Staining of the AER was also seen with the hyaluronate binding region probe; however the control probe showed an identical staining pattern, therefore staining was not identifying unbound hyaluronate. Since the control probe was non-biotinylated, this suggests that the staining seen was non-specific, caused by binding of the avidin-gold conjugated antibody to the AER ectoderm. Although this again illustrates that the AER ectoderm is different from the remaining non-ridge ectoderm. Collagen type II was absent from mesenchyme beneath a grafted AER as expected, since this ECM component is undetectable in developing limb buds until stage 24, when it is synthesised by cells in the proximal limb condensation which are differentiating into cartilage (Dessau *et al.* 1980). The findings that keratan sulphate and collagen type II were absent from the sub-AER mesenchyme serves as an additional control demonstrating that the strong staining seen with some of the

other antibodies is not the result of artifactual non-specific binding.

These results indicate that the matrix elaborated by the mesenchymal cells beneath an AER is considerably richer than that produced by mesenchyme cells beneath non-ridge ectoderm (see section 1.1). It has been suggested that increased synthesis and accumulation of matrix beneath the AER might facilitate outgrowth by providing a matrix suitable for the invasion of proliferating cells (Tomasek *et al.* 1982). However, a more attractive hypothesis is that the accumulation of proteoglycans is necessary to trap growth factors produced by the overlying AER, thereby restricting their actions to cells of the progress zone. This hypothesis is supported by the discovery that proteoglycans act as specific binding sites for a variety of growth factors. Heparan sulphate, both in the extracellular matrix and associated with the cell surface, binds bFGF. In fact cell surface heparan sulphate acts as a low affinity receptor for bFGF and appears to be essential in order for cells to respond to this growth factor (Klagsbrun and Baird, 1991). In addition chondroitin sulphate side chains of the proteoglycan serglycin bind the growth factor platelet factor 4 (Périn *et al.* 1988) and TGF- β binds to the proteoglycan betaglycan via the core protein. Another matrix molecule which binds TGF- β is decorin. TGF- β stimulates the synthesis of decorin, which therefore acts as a reservoir of TGF- β , in turn restricting its activity to the area of its synthesis (Ruoslahti and Yamaguchi, 1991). Direct evidence showing that the distal mesenchyme *in vivo* acts in this manner comes from the fact that the putative growth factor *Wnt-5a* is restricted to the distal mesenchyme (Gavin *et al.* 1990). This is a member of the *Wnt* family of factors which are known to act over very short distances due to their high affinity for ECM components.

Mitogenic effect of the AER

The results of the thymidine incorporation show that the rate of proliferation in mesenchyme beneath the AER after 24 hours is only 7% higher than the rate seen in other parts of the cultures, in both distal and proximal cell cultures. This finding supports the earlier reports that the rate of cell proliferation in the distal mesenchyme does not differ from that in other regions of the early precondensation stage limb bud. As limb bud development proceeds, the rate of proliferation decreases in all areas, but the decline is most dramatic in the condensing precartilage and premuscle masses. Thus at later stages of development the distal mesenchyme exhibits a higher rate of proliferation than the other areas of the limb, although the rate at the distal tip has also decreased. The results presented here reflect this *in vivo* situation exactly. 72 hours after AER grafting the rate of proliferation has dropped throughout the culture as cartilage differentiation has become more extensive; but now the rate of proliferation in the mesenchyme beneath the AER is more than 50% greater than that in other parts of the culture, although it has decreased by 10% over the last 48 hours. The work of Rubin and Saunders (1972) suggested that the AER exerts a constant mitogenic effect, since ectodermal hulls grafted onto mesenchyme of a different developmental stage caused normal completion of development. If the mitogenic effect of the AER decreased with time then young ectodermal hulls grafted onto older mesenchyme would be expected to cause excessive proliferation and similarly older hulls grafted onto younger mesenchyme would result in a decrease in proliferation. Thus limbs with duplications and truncations would be expected respectively. However the rate of proliferation in the distal mesenchyme does decrease during development. This suggests that the rate of proliferation is limited by the intrinsic nature of

the limb mesenchyme cells, in that as the cells become more distal in positional character they appear to exhibit a longer cycling time and thus lower rate of proliferation, even though the mitogens secreted by the AER may be exactly the same.

Comparison of the ability of different cultures to respond to a grafted AER

Only one day old micromass cultures of distal limb bud mesenchyme were able to respond to a grafted AER by producing a substantial cellular outgrowth which stained intensely for fibronectin. In addition, the AER covering this outgrowth stained strongly for keratan sulphate. Interestingly, 2 and 3 day old distal cell cultures were unable to respond in a similar manner except in one atypical experimental run. This suggests that by 2 days of culture distal cells have progressed too far along the pathway of cartilage differentiation and have thus lost the ability to respond to certain factors produced by the AER which cause mesenchymal cell outgrowth. However it should be noted that cells beneath the grafted AER are diverted from forming cartilage in both 2 and 3 day cultures. These results suggest that the antichondrogenic effect of the ectoderm and the mitogenic effect of the AER are two separate, unrelated effects. The antichondrogenic effect appears to be very strong since it is capable of causing inhibition of the cartilage phenotype in cells which are some distance down the pathway of chondrogenic differentiation. However, these results suggest that the mitogenic effect of the AER is a very different effect which requires mesenchyme cells able to respond to the signals produced by the AER, and in turn capable of producing the factors necessary to maintain the AER.

Cottrill *et al.* (1990) grafted micromass cultures made from distal cells of quail limb buds into chick limbs. Cells grown in

culture for 1, 2 and 3 days and then grafted beneath the AER dedifferentiated, proliferated and contributed to a wide range of connective tissue within the limb; however cells cultured for 5 days only gave rise to cartilage or perichondrium. One day old cultures grafted to a proximal position also gave rise to a variety of connective tissues but cells cultured for 2 days or more only gave rise to cartilage and perichondrium. The conclusions of this work were that the cartilage phenotype is labile and can be reversed. The results presented here support this view in that cartilage differentiation was inhibited under grafted AER and the surrounding non-ridge ectoderm in all cultures, including those maintained for 3 days. However the results presented here extend the work of Cottrill *et al.* since they show that distal cells cultured for more than one day lose the ability to return to the undifferentiative, proliferative state characteristic of progress zone cells, which is determined in this assay as the ability to produce a mesenchymal outgrowth beneath a grafted AER.

The finding that proximal cultures after 1,2 and 3 days of culture were all capable of responding to a grafted ridge is puzzling. However it is interesting to note that in all cases only a tiny fragment of morphologically recognisable AER remains and this is supported by a tiny outgrowth which stains intensely for fibronectin. Since proximal cultures contain a mixture of cell types, it is possible that only one of these cell types is capable of responding to and supporting a grafted AER. Thus only in the areas where a grafted AER is on top of a sufficient number of these cells is it capable of surviving and thus in turn inducing an increase in the rate of proliferation of these underlying cells, thereby causing an outgrowth from the culture. Proximal cultures contain at least three cell types. Firstly myoblasts which have migrated into the limb from the somites, secondly cells which are destined to differentiate into cartilage and finally, cells

which are destined to differentiate into fibroblasts of the loose connective tissues of the limb. In order to investigate whether precartilage or prefibroblast-type cells were mediating the only visible AER:culture interactions in these cultures, AERs were grafted onto cultures prepared from lateral flank, which form a sheet of fibroblast-type cells in culture, and from the frontonasal mass, which forms virtually a complete sheet of cartilage in culture. The results of these experiments suggest that the precartilage cells are likely to be the cells which respond to the AER and in turn maintain small fragments of it in these cultures. However a similar mechanism of development is employed by the FNM and limb bud *in vivo*, since both exhibit mesenchymal proliferation and outgrowth beneath an inductive epithelium. Thus some other property common to limb bud cells and cells of the frontonasal mass could be responsible for enabling them to respond to a grafted AER and the fact that most FNM cells are also destined to form cartilage could therefore be irrelevant.

The fact that the composition of the culture medium also modulates the response of mesenchyme cells to a grafted AER allowed further investigation of the mechanism of this interaction. The culture medium which prevented mesenchymal outgrowth in response to a grafted AER (DM + S) also resulted in the highest rate of proliferation and the best maintenance of the AER. This ruled out the possibility that mesenchymal outgrowth might be dependent on the initial rate of proliferation of the culture. Also it showed that maintenance of the AER was not sufficient to cause mesenchymal outgrowth. The other feature of cultures grown in DM + S is that they elaborated a greater quantity of matrix, as determined by Alcian blue staining and the increased incubation in trypsin/EDTA needed to dissociate these cultures. Thus it appears that cells cultured in DM + S had advanced further along the pathway of chondrogenic differentiation than their

counterparts cultured in less rich media. These conclusions agree with the earlier finding that distal cell micromasses cultured for 2 or 3 days lost the ability to respond to a grafted AER in terms of mesenchymal cell outgrowth. There must be therefore a point at which distal mesenchyme cells grown in micromass lose the ability to respond to a grafted AER, at least in terms of forming a large cellular outgrowth. This point is reached somewhere between 24 and 48 hours of culture for cells grown in F12 medium, and by 24 hours in cells grown in DM + S. The reason for this loss of competence is presumably caused by the loss of ability to respond to mitogens secreted by the AER. For example these cells may no longer synthesise receptors for various growth factors. Alternatively, conformational changes in the arrangement of DNA may result in these cells losing the ability to express genes characteristic of the proliferating, undifferentiated progress zone cells.

CHAPTER 2

EFFECT OF GROWTH FACTORS ON THE PROLIFERATION AND DIFFERENTIATION OF LIMB BUD MESENCHYME CELLS

INTRODUCTION

TGF- β

TGF- β belongs to a complex family of growth factors, which exhibit homology across a large evolutionary spectrum, and include the *Drosophila* growth factor *decapentaplegic*, which is involved in dorso-ventral patterning and gastrulation and the *Vg-1* gene product, which is localised to the vegetal hemisphere in amphibian oocytes and is implicated in mesoderm induction. *TGF- β* growth factors are biologically active as 25 kDa disulphide-linked dimers of which 5 isoforms have currently been identified (see Jakowlew *et al.* 1991). *TGF- β* is strongly implicated in both endochondral and intramembranous ossification, since transcripts for *TGF- β 1* and *TGF- β 2* and *TGF- β* peptide have been detected in cells at all stages of the osteocyte lineage (Lehnert and Akhurst, 1988; Pelton *et al.* 1989; Heine *et al.* 1987). In addition, *TGF- β* expression is restricted to the mesenchymal components of several tissues including the gastro-intestinal tract and respiratory system. The finding that *TGF- β* transcripts and peptide are also spatially and temporally restricted to the forming endocardial cushions as well as the mesenchyme of developing hair follicles and tooth buds, suggests that *TGF- β* may also play a significant role in mediating epithelial-mesenchymal interactions. It is becoming apparent from numerous *in vitro* studies that *TGF- β* may have a unique role in controlling the

function of many other substances of critical significance in embryogenesis, such as other peptide growth factors, growth factor receptors and structural and adhesion molecules of the extracellular matrix (reviewed by Rizzino, 1988).

Although TGF- β does not appear to play a role in the early events of limb development, other members of the TGF- β superfamily are strongly implicated. Bone morphogenetic protein-2A (BMP-2A) has been identified in the thickened ventral epithelium of 9.5 day mouse embryo limb buds; becoming localised within the differentiated AER by 10.5 days (Lyons *et al.* 1990). BMP-4 is also present at these stages of early development. At 10.5 days it is present in the AER and throughout the mesenchyme showing a graded distribution with the greatest expression in the anterior and distal part of the limb bud (Jones *et al.* 1991).

TGF- β 2 mRNA was first detected in the perichondrium surrounding 13.5 day mouse limb cartilage elements, although staining in other parts of these cartilages was not seen. By 15.5 days transcripts were much more abundant, corresponding to the onset of active periosteal ossification (Pelton *et al.* 1989). Lehnert and Akhurst (1988) failed to detect TGF- β 1 RNA in limb cartilage under conditions of high-stringency hybridisation, although at lower stringency hybridisation was seen, perhaps suggesting that at low stringency another isoform of TGF- β was detected since they are all closely related at the nucleotide level. Similarly TGF- β peptide was first detected in limb buds in the periosteum at the onset of ossification (Heine *et al.* 1987). In contrast, Leonard *et al.* (1991) detected endogenous TGF- β like activity in acetic acid extracts of freshly isolated mesenchyme from the distal tips of 5 day chick embryos and in 1 day old micromass cultures prepared from this tissue, as determined by the inhibition of proliferation of Mv1Lu mink lung epithelial cells. They also claimed

that a polyclonal antibody directed against a TGF- β 1-like peptide detected immunoreactivity in micromass cultures prepared from stage 22/23 whole limb buds 18 hours after plating. However it seems likely that the endogenous TGF- β -like activity they detected could have been due to the presence of other members of the TGF- β superfamily, such as BMP-4. Hayamizu *et al.* (1991) implanted agarose beads soaked in solutions of TGF- β 1 into chick limb buds at different stages of development. Reasonable survival levels were only obtained with beads soaked in 0.02 μ g/ml TGF- β 1 or less. The general effect was a reduction in various limb skeletal elements which were consistent with the stage of operation and the position of the bead. There appeared to be a specific time in which this effect took place, for example beads implanted at stage 19 and left in place for 24 hours had no effect, whereas those implanted at stage 19 and left for 48 hours or those implanted into the humeral region at stage 22-24, resulted in reduction of the humerus. A small incidence of ectopic cartilage formation was seen when beads were implanted proximally in stage 25-26 limbs. This work, together with *in vitro* studies, suggests that the effect of exogenous TGF- β depends, at least in part, on the state of differentiation of the cell in question.

Lyons *et al.* (1989) have proposed that the expression of different members of the TGF- β superfamily is required at distinct stages of differentiation during the process of chondrogenesis. BMP-2A is the first member of the TGF- β family expressed in the sequence of chondrogenesis. It is localised to the condensing, precartilaginous mesenchyme but is undetectable in the resting, proliferating and differentiating chondrocytes (Lyons *et al.* 1989). TGF- β 2 is first detected at a later stage in the chondroblasts of the perichondrium (Pelton *et al.* 1989), whilst Vgr-1 is expressed at the final stage of chondroblast differentiation in the hypertrophic cells (Lyons *et al.*

1989). Thus the initial expression of BMP-2A and possibly others may promote the condensation and differentiation of mesenchyme into actively proliferating chondroblasts and chondrocytes, which results in the expression of a different set of TGF- β -like gene products, including TGF- β 2, in the more differentiated cells. The newly induced growth factors then may regulate the proliferation and differentiation of the cells in which they are produced in an autocrine manner and may act on additional cell types in the chondrocyte differentiation pathway in a paracrine manner to ensure coordinated progression through the lineage. Finally the expression of Vgr-1 in hypertrophic cartilage may promote the terminal differentiation of these cells, and affect the proliferation of chondrocytes from which the hypertrophic cells arise. Experimental support for this model includes the ability of BMP-2A to induce ectopic bone formation *in vivo* in a process involving the migration, proliferation and condensation of mesenchymal cells (Wozney *et al.* 1988). Additionally, *in vitro* TGF- β 1 and TGF- β 2 can induce foetal rat muscle mesenchymal cells embedded in agarose gels to undergo chondrogenic differentiation and synthesise cartilage-specific macromolecules such as type II collagen (Seyedin *et al.* 1986, 1987). TGF- β also stimulates GAG synthesis by cultured growth-plate chondrocytes (Hiraki *et al.* 1988) and TGF- β 1 and TGF- β 2 have been shown to stimulate the accumulation of type II collagen and cartilage-specific core proteoglycan mRNA in micromass cultures of both whole limb and distal tip mesenchyme (Kulyk *et al.* 1989). Even a transient exposure for 2 hours at the beginning of the culture period is sufficient to stimulate increased cartilage differentiation compared to control cultures. TGF- β was also able to stimulate cartilage differentiation in subconfluent cultures of limb mesenchyme, which under normal conditions do not differentiate into cartilage.

The effect of TGF- β on myogenesis has been investigated *in vitro*.

Massagué *et al.* (1986) showed that TGF- β inhibited the fusion of myoblasts from rat skeletal muscle and chick embryos. Addition of 0.3nM TGF- β inhibited differentiation as determined by the absence of muscle specific proteins α -actin, myosin light and heavy chains and troponin; however if TGF- β was removed the cells differentiated immediately. TGF- β was ineffective at inhibiting differentiation once the myoblasts had become committed to myogenesis, however both myoblasts and myotubes had equal numbers of receptors to TGF- β and both responded to TGF- β by increased synthesis of fibronectin and collagen. It was postulated that TGF- β may exert its inhibitory influence by increased production of fibronectin since myoblasts treated with exogenous fibronectin assumed a similar morphology to TGF- β treated cells and also failed to differentiate. Florini *et al.* (1986) showed that TGF- β inhibited differentiation of L6 rat myoblasts with maximal inhibition at 0.5ng/ml, despite the presence of 30 μ M insulin in the culture medium. TGF- β was shown to have no mitogenic effect upon these myoblasts, thus its inhibitory effect was not caused by forcing cells to reenter the cell cycle rather than undergo fusion. TGF- β also inhibited the differentiation of mouse C2 (skeletal muscle type) and BC2H1 (smooth muscle type) myoblasts at 5ng/ml in a differentiation medium containing 10 μ g/ml insulin and 5 μ g/ml transferrin, and TGF- β exerted post commitment inhibition of BC3H1 cell differentiation as seen by the block of creatine kinase induction and loss of cell surface acetylcholine receptors (Olson *et al.* 1986). This inhibitory effect on muscle cell differentiation has been confirmed by the finding that TGF- β inhibits the transcription of the muscle regulatory gene MyoD1 in cultured 23A2 and C2C12 myoblasts (Vaidya *et al.* 1989). These *in vitro* results support the findings that TGF- β peptide and TGF- β 1 and TGF- β 2 mRNAs are undetectable in the myotome portion of somites and the subsequent somatic skeletal muscle (Heine *et al.* 1987; Lehnert and

Akhurst, 1988; Pelton *et al.* 1989).

TGF- β is a potent inhibitor of proliferation in most cells *in vitro*, however both inhibitory and stimulatory effects have been reported for mesenchyme-derived cells. In these cases proliferation is stimulated at low doses of TGF- β and is mediated by the stimulation of autocrine PDGF-AA secretion; however at higher doses TGF- β causes a decrease in PDGF receptor α and thus proliferation ceases (Battegay *et al.* 1990). In addition to such direct effects upon transcription, TGF- β also mediates many of its actions via stimulation of extracellular matrix production. TGF- β has been shown to stimulate both the synthesis of fibronectin and collagen and their incorporation into the extracellular matrix in a variety of different cell types (Ignatz and Massagué, 1986). Rosen *et al.* (1988) found that addition of 0.4nM TGF- β to cultured chondroblasts decreased production of type II collagen and cartilage proteoglycan. These effects were mimicked by addition of exogenous fibronectin, and blocked by addition of the microfilament disrupting agent dihydrocytochalasin B (DHCB). In addition TGF- β modulates the actions of other growth factors by inhibiting the induction of matrix degrading enzymes such as collagenase and enhancing the production of the specific metalloproteinase inhibitor TIMP (Edwards *et al.* 1987). Finally TGF- β is also capable of influencing the way cells interact with the extracellular matrix. TGF- β 1 and TGF- β 2 both stimulate expression of integrin receptors by enhancing transcription of these genes and also by increasing the speed of posttranslational processing (Ignatz and Massagué, 1987).

bFGF

Seven members of the FGF family have now been identified. At the amino acid level there is approximately 50% homology between each protein, however the C- and N-termini regions show greater divergence.

Four genes encoding distinct high-affinity FGF receptors have also been identified, each gene encoding multiple proteins derived by alternative mRNA splicing. These proteins most likely differ in their ability to bind various members of the FGF family. bFGF also binds to heparan sulphate proteoglycan (HSPG) associated with both the cell surface, which acts in effect as a low affinity binding site (Moscatelli, 1987), and the extracellular matrix. Cell surface HSPG is thought to deliver the bFGF to high-affinity receptors, which internalise the protein and thus initiate the cellular response. It has been shown in several systems that in the absence of cell surface HSPG, bFGF does not bind to the high-affinity receptors. This effect can be reversed by the addition of exogenous heparin (Bernard *et al.* 1991). Thus it appears that cell surface heparan sulphate modulates bFGF activity by facilitating binding to high affinity receptors and that the low affinity sites are therefore directly involved in bFGF signalling (Klagsbrun and Baird, 1991). The importance of members of the FGF family in normal development has been shown by the work of Martin and colleagues who have identified a temporally and spatially restricted sequence of expression of three members of the FGF family during mouse gastrulation (Niswander and Martin - submitted).

Two studies have identified FGF activity in developing chick limb buds. Seed *et al.* (1988) found that the level of FGF in the chick limb bud is higher than in the rest of the body until stage 27 (5 days), it then undergoes a transient decrease between 6 and 7 days, after which it increases again but remains lower than the level in the rest of the body. In contrast, Munaim *et al.* (1988) identified bFGF in limb bud extracts and found the highest levels at the earliest stages of limb development (stage 18), which corresponds to the time when proliferation is the major cellular activity in the limb whereas by stage 22-24, when condensation and differentiation are beginning, the

level of bFGF had decreased by about 60%. However using an antibody raised against human bFGF, Joseph-Silverstein *et al.* (1989) could only detect bFGF within striated muscle precursor cells of the myotome as they migrated into the limb bud and at later stages within the differentiating myotubes developing alongside the cartilaginous elements. In 12 day embryos staining of skeletal muscle remained but was much less intense than in the developing muscle at earlier stages of development. Aono and Ide (1988) discovered that there is a gradient of responsiveness to a factor produced by the posterior fragments of stage 22-23 limb buds, in that the proliferation of anterior cells is most enhanced by the factor whilst posterior cells are unaffected. This action was mimicked by the addition of 100ng/ml FGF; whereas addition of 10 μ g/ml insulin or 10ng/ml EGF stimulated proliferation of all regions of the limb equally. This correlates with the finding that polarising region grafts to the anterior margin of the limb resulted in marked growth in the anterior region (Cooke and Summerbell, 1980) whilst similar grafts to the posterior margin did not stimulate growth (Javois and Iten, 1981). Thus they concluded that the posterior zone of polarising activity (ZPA) produces an FGF-like factor. In support of this hypothesis is the recent finding that FGF-4 (formerly known as K-FGF) has been localised to the posterior portion of the AER in developing mouse limbs (Niswander and Martin - submitted). Since this is a secreted form of FGF it is likely that it acts on the posterior mesenchyme cells in addition to any autocrine role it may also possess. Wanaka *et al.* (1991) recently reported the distribution of FGF receptor mRNA in the developing rat embryo. Receptor levels were highest in the distal region of the limb. At later stages of development the receptor was found in the compact mesenchyme beneath the epidermis and in the perichondrium, but was absent from the centres of ossification. Thus these findings suggest that bFGF plays a significant role in limb

development, being involved in the stimulation of cell proliferation in the early stages of bud formation and at later stages in the distal progress zone, ensuring continued distal outgrowth in a posterior direction.

In vitro, FGF effects the proliferation and differentiation of a variety of different cell types. FGF tends to stimulate proliferation and differentiation of cells of the chondrogenic lineage, for example growth plate and articular chondrocytes show increased DNA synthesis and GAG synthesis following addition of FGF at a dose of 0.4 ng/ml (Inoue *et al.* 1989; Hiraki *et al.* 1988). In contrast FGF stimulates proliferation but inhibits differentiation of cultured myoblasts. Clegg *et al.* (1987) showed that the differentiation of the mouse myoblast cell line MM14 was inhibited by bFGF, with maximal inhibition seen at 0.1 ng/ml. This was however reversible, since upon removal of bFGF cells withdrew from the cell cycle and expressed muscle-specific proteins within 6-7 hours, followed by fusion after 12-14 hours. Lathrop *et al.* (1985) showed that the inhibition of BC3H1 cell differentiation caused by addition of bFGF involved restricting cells at a point 4-6 hours within the G₁ phase of the cell cycle and was therefore independent of reentry into the cell cycle. Investigating the same system, Spizz *et al.* (1986) showed that the bFGF-induced downregulation of the differentiated muscle marker, creatine kinase mRNA, was blocked by cyclohexamide, an inhibitor of protein synthesis; thus demonstrating that this downregulation required the synthesis of additional gene products. More recently the inhibitory effect of bFGF on muscle cell differentiation has been confirmed by the findings that bFGF inhibits transcription of the muscle regulatory genes MyoD1 in 23A2 and C2C12 myoblasts (Vaidya *et al.* 1989) and myogenin in BC3H1 cells (Brunetti and Goldfine, 1990).

Insulin-like growth factors are proteins with a similar structure to insulin. They circulate in plasma bound to carrier proteins, possess receptors which are structurally distinct from the insulin receptor and stimulate cell replication. Their function is to stimulate cell proliferation and thus they are important in long-term growth (reviewed by Clemmons, 1989). IGF-I and IGF-II exert their growth promoting effects through the IGF-I receptor. The IGF-II receptor is equivalent to the mannose-6-phosphate receptor and appears to be involved in lysosomal degradation of protein. One of the IGF binding proteins that has been isolated is found in the amniotic fluid. This contains an RGD sequence and therefore presumably binds to the cell surface, thus potentiating the effect of IGF-I by facilitating contact with its target cells. Beck *et al.* (1987) found that IGF-II transcripts were predominant over IGF-I transcripts in developing rat embryos. IGF-II is strongly expressed in the liver and yolk sac and in mesodermally derived structures in the process of differentiation. Smith *et al.* (1987) identified type -I and -II IGF receptors in membrane preparations from 9-12 day mouse embryos, with IGF-I and -II receptors substantially more abundant than the insulin receptor. IGF-I specific binding proteins were also detected in 9-12 day embryo homogenates and immunoreactive IGF-I was detected in 10 and 14 day embryo extracts.

IGF-II transcripts have been detected in the developing limb buds of 12 day old rat embryos, where they appear to be most abundant in the distal and peripheral mesenchyme (Stylianopoulou *et al.* 1988). Using an anti-human IGF-I antibody which recognised both chick IGF-I and IGF-II, Ralphs *et al.* (1990) found that IGF peptide was restricted to the distal and peripheral regions of developing limb buds and was absent from the precartilaginous condensing mesenchyme. IGF peptide was

observed both inside cells and in the extracellular matrix, including some cells of the ectoderm.

IGF-II transcripts have been reported in cells of the myocyte lineage throughout their development from myoblasts to multinucleated skeletal muscle fibres (Beck *et al.* 1987; Stylianopoulou *et al.* 1988). The IGF synthesised by developing muscle cells seems to act in an autocrine manner, since immunological detection revealed the accumulation of IGF peptide in the myotome and myocardium (Ralphs *et al.* 1990).

Cells of the chondrogenic lineage express IGF-II transcripts from the precartilaginous condensation stage through to the mature chondrocyte stage, although transcripts are not detected in hypertrophying cartilage cells (Beck *et al.* 1987; Stylianopoulou *et al.* 1988). In mature cartilage and bone IGF-II transcripts are only detected in the perichondrium and periosteum. Interestingly, the observed distribution of IGF peptide is complementary, in that IGF peptide is absent from condensing and differentiated cartilage until the appearance of hypertrophying cells. Similarly IGF peptide is not detected in the perichondrium or periosteum of mature bone, but is found in the subjacent chondrocytes and osteocytes (Ralphs *et al.* 1990). Thus immature cells of the chondrogenic lineage synthesise IGFs which accumulate in adjacent tissues and therefore presumably act in a paracrine manner on other cell types.

IGFs appear to be important mitogens for a wide variety of cells, derived from all three embryonic germ layers. IGF-I and IGF-II cannot however recruit quiescent cells into the cell cycle, although once cells are brought into the cell cycle by competence factors such as FGF or PDGF, IGFs act as potent mitogens and function synergistically with these factors stimulating a rapid increase in cell proliferation. In addition to stimulation of growth, IGFs also stimulate differentiation

in some cell types, for example IGF-I stimulates proteoglycan synthesis in cultured proliferative and resting rat growth plate chondrocytes (Makower *et al.* 1988). Insulin acting through the IGF-I receptor has also previously been shown to stimulate the proliferation and differentiation of rat L6 myoblasts (Ewton and Florini, 1981), and the differentiation of primary chick myoblasts (Schmid *et al.* 1983). The finding that IGFs stabilise specific messenger RNAs in 3T3 cells suggests that IGFs may also play a role in the maintenance of certain differentiated phenotypes (Zumstein and Stiles, 1987).

Activin A

The mesoderm-inducing factor (MIF) present in the conditioned medium of the *Xenopus* cell line XTC has been purified and identified as the *Xenopus* homologue of mammalian activin A (Smith *et al.* 1990; van den Eijden-Van Raaij *et al.* 1990). Activin A is responsible for the release of follicle-stimulating hormone from the anterior pituitary and for the differentiation of erythroid haematopoietic progenitor cells in the adult; however in *Xenopus* embryos this factor is responsible for inducing mesoderm formation from the animal hemisphere cells at the blastula stage. Activin A is a member of the TGF- β superfamily, however the homology between activin and TGF- β is fairly low; this is supported by the finding that activin was 300 times less effective than TGF- β 2 in the TGF- β specific assay involving inhibition of CCL-64 mink lung epithelial cell proliferation (Smith *et al.* 1990).

Pasteurella multocida toxin

Pasteurella multocida toxin (PMT) is the most potent and effective mitogen known for Swiss 3T3 cells, with a half-maximal stimulation of DNA synthesis at 1-2pM. PMT also stimulated DNA synthesis in passaged human and mouse embryo fibroblasts more

effectively than a range of established growth factors. The mitogenic activity is mediated by a 150kDa protein, which is heat-labile and requires translocation into cells via endosomes. The intracellular mechanism of action is unknown, however it appears to involve stimulation of inositol phosphates (Rozengurt *et al.* 1990).

Experimental aims

Growth factors have been identified in embryos and are implicated in a wide range of developmental processes therefore two factors, namely TGF- β and bFGF, which are both known to be present in chick embryos were chosen and assayed for their effects upon cartilage and muscle cell differentiation and cell proliferation.

The defined medium employed in these experiments contains a high level of insulin, which is known to cross react with the IGF-I receptor. IGFs are also present in the developing limb and have been shown to interact with FGF and TGF- β in other systems, thus the above experiments were repeated in the absence of insulin in order to determine the contribution played by insulin in the differentiation of limb bud cells in these cultures.

In addition the effect of activin and PMT were assayed. Finally the effects of the putative morphogen retinoic acid (RA) were assayed as a prelude to the *Hox-7.1* and RAR- β work detailed in later chapters. Retinoids have been shown to inhibit chondrogenesis in high-density cultures of chick (Bee and Jeffries, 1987; Wedden *et al.* 1987) and mouse mesenchyme (Lewis *et al.* 1978, Pennypacker *et al.* 1978, Zimmerman and Tsambaos 1985), and to induce dedifferentiation of cartilage (Solursh and Meier, 1973; Horton and Hassell, 1986).

Defined medium

In order to accurately assay the effects of exogenously added growth factors or other putative morphogens, it is necessary to eliminate serum from the culture medium. The defined medium used in these experiments was devised by B.Gregg and M.Noble as one which supports the growth and differentiation of chick embryo limb cells in high-density culture; it does not differ significantly from the defined medium described by Kujawa *et al.* (1989), which was found to be optimal for the growth and chondrogenic differentiation of stage 24 limb mesenchyme cells. A characteristic feature of this medium is the high level of insulin it contains. Insulin or insulin-like growth factors (IGFs) have been shown to be essential components of a variety of defined media and are known to increase DNA and protein synthesis (Kujawa *et al.* 1989).

The medium also includes ascorbate, which has been shown to be an essential cofactor in collagen synthesis and is also important in the secretion of glycosaminoglycans by cultured chondrocytes (Prockop *et al.* 1979; Hajek and Solursh, 1977), for example Paulsen and Solursh (1988) showed that addition of 50 µg/ml ascorbate increased matrix production twofold over that in defined medium alone without a significant increase in DNA content. Since ascorbate is also ubiquitously distributed throughout chick embryos at an approximate concentration of 140-200µg/ml over the first 5 days of incubation (Chinoy *et al.* 1974; Rinaldini, 1960), and has a half-life of only one hour at physiological pH (Bissel *et al.* 1980) its incorporation into defined medium at 200 µg/ml seemed justified. However Archer *et al.* (1990) showed that inclusion of ascorbate in F12 medium resulted in a decrease in the number of differentiated muscle cells. This was especially pronounced after 3 or 4 days in culture, however the effect

was less marked after 48 hours, which is the time at which muscle differentiation was determined in these experiments.

The addition of transferrin enhances myogenic differentiation in defined medium without affecting chondrogenesis (Paulsen and Solursh, 1988). Kujawa *et al.* (1989) reported that transferrin alone has little effect, but when added together with insulin it improves cellular morphology without affecting DNA or protein synthesis.

Bovine serum albumen (BSA) increased cell replication rates and resulted in the production of more numerous and larger cartilage nodules (Kujawa *et al.* 1989). Kaplowitz *et al.* (1982) have also reported the need for serum protein at low levels (0.2%) in order to minimise cell detachment. BSA is also required in these experiments as a carrier for the exogenously added growth factors, especially bFGF.

METHODS

Micromass culture

For the experiments described in this chapter, micromass cultures were prepared from 350 μ m tips of distal limb mesenchyme obtained from stage 22-23 chick embryos. Cultures were prepared as described previously (pp.29). In the sections describing the assaying of TGF- β 1, TGF- β 2, bFGF, activin and PMT, pelleted cells were resuspended and plated in a 1:2 mixture of HBSS + 10% FCS and defined medium (DM). For the assay of retinoic acid (RA) cells were resuspended and plated in either DM alone or DM containing 10% serum. TGF- β 1 and bFGF were obtained from R&D systems and diluted in accordance with manufacturers instructions. TGF- β 2 and activin were kindly provided by Dr. J. Smith (NIMR, Mill Hill, London). PMT was a gift from Prof. Rozengurt (ICRF, Lincoln's Inn). Factors were diluted to final concentrations in DM. 200 μ l of medium containing growth factors was fed to 2 cultures in a 16mm diameter well of a 4-well multiwell plate (Nunc) and replaced daily. Due to the nature of the culture dishes, only one culture was grown per well when the result needed to be photographed; however the result in all assays was consistent regardless of whether 1 or 2 cultures were grown per well.

The composition of the defined medium used in these experiments was as follows:

50% Hams F12 nutrient mixture (Gibco)

50% Dulbecco's modified Eagles medium (DMEM) (Gibco)

2mM glutamine (Gibco)

5 μ g/ml insulin (Sigma)

10 μ g/ml transferrin (Sigma)

20nM hydrocortisone (Sigma)

200 μ g/ml ascorbate (Sigma)

1% antibiotic/antimycotic (Gibco)

0.29% bovine serum albumin (Miles scientific).

Alcian blue extraction

Using a method based on that of Hassell and Horigan (1982), 3 day old cultures were stained with 1% Alcian blue in 3% acetic acid at pH1 (3% acetic acid + 1% HCl) for 2 hours. Cultures were then rinsed in 3% acetic acid at pH1 for 5 minutes, 3% acetic acid at pH2.5 for 1 minute and distilled water for 3 minutes. The bound Alcian blue was then extracted into 600 μ l of 6M guanidine hydrochloride overnight in an orbital incubator, since mechanical agitation was necessary for complete extraction of the most heavily stained cultures. The absorbance of each culture well was then measured at 600nm on a Unicam spectrophotometer.

Immunolabelling of muscle cells

Muscle cells were detected with a monoclonal antibody, F39, which recognises fast skeletal muscle myosin heavy chain (Ralphs *et al.* 1989). This antibody was kindly supplied by Dr. G. Dhoot (Royal Veterinary College, London).

Cultures were stained as wholemounts. They were fixed in cold 70% alcohol and gradually rehydrated in PBS. After incubating for 15 minutes in medium containing 10% FCS, to reduce non-specific binding, cultures were incubated in 150 μ l of diluted F39 for 1 hour. F39 was diluted 1:40 in PBS (containing 1% Tween 20, 0.1% BSA and 0.1% sodium azide). Cultures were then washed for 5 minutes in PBS three times and incubated in 150 μ l of 5nm gold-conjugated IgG (Biocell) for 45 minutes. Following two further washes for 5 minutes in PBS and two in distilled water, the labelled cells were enhanced with silver; cultures were incubated in 150 μ l of a 1:1 mixture of silver enhancer solution A and

silver enhancer solution B (Sigma) in the dark. Cultures were observed every few minutes until the labelled cells were clearly visible. The enhancing solution was then removed, the cultures washed and mounted in glycerol. On a few occasions cultures were incubated with FITC-conjugated second antibody and observed under UV illumination. Cells were counted and photographed on a Zeiss Axiovert 405 microscope.

Cell proliferation

The percentage of cells in S-phase, i.e. that is duplicating their DNA, was determined by BrdU (5-bromodeoxyuridine) uptake with immunofluorescent localisation and by tritiated-thymidine uptake with autoradiography in one initial experiment.

Cell smears

Two cultures per well were grown in 16mm multiwell dishes. For immunofluorescence, cultures were incubated in 25 μ M BrdU for 3 hours. For autoradiography, cultures were incubated in 10 μ Ci/ml tritiated-thymidine for 3 hours. In both cases cultures were then removed from the dish by gentle agitation in 0.1mg/ml trypsin, 0.001M EDTA in HBSS for 2-10 minutes. Cultures which had produced a large amount of cartilaginous matrix were pretreated by incubation in 0.1mg/ml collagenase (Sigma) at 37°C for 20-30 minutes. (In later experiments collagenase was routinely added to the trypsin/EDTA, since it was found that this dramatically reduced background staining). 500 μ l of serum-containing medium was then added to each well to prevent further tryptic activity on the cells and the entire contents of each well transferred to a microcentrifuge tube. The cultures were then dissociated mechanically using a Gilson pipette, and the cells pelleted by spinning for 4 minutes at 6500 rpm in a microcentrifuge. The supernatant was removed with a flame-drawn Pasteur pipette and the

pellet resuspended in 40 μ l of medium. 20 μ l aliquots were then spread over a small area of a 0.5% gelatin-coated slide. Smears were allowed to air dry. Slides for immunofluorescence were then fixed and permeabilised in 70% alcohol for 10 minutes prior to storage at -20°C for up to 2 weeks. Slides for autoradiography were kept at 4°C before dipping in emulsion.

Staining

Slides were defrosted and the smear marked out with a diamond pencil. Smears were then wetted in PBS for a few minutes before being hydrolysed in 1.5N HCl at 50°C for 10-15 minutes. Smears were then washed twice in PBS for 5 minutes. The area around the smear was then blotted dry, the slide laid horizontally in a humidified box and 50 μ l of anti-BrdU, a monoclonal antibody raised against BrdU (Becton-Dickinson, California), diluted 1:10 in PBS was added to each smear. Smears were incubated for 1 hour at 37°C. Following three 5 minute washes in PBS the area around the smear was blotted dry as before and 50 μ l of fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG (Dakopatts, Denmark), diluted 1:50 in PBS, was added to the smear. After an incubation of 45 minutes in the dark, the smears were washed three times in PBS and coverslips were mounted with a DABCO-based mountant (see pp.32).

Smears were observed immediately after staining. 1000 cells were counted under phase contrast illumination; the number of these cells that exhibited fluorescence under UV illumination was also counted and thus the percentage of cells in S-phase determined.

Cell smears prepared for autoradiography were dipped in emulsion and processed as described previously (pp.81). When developed they were counterstained with toluidine blue and observed under bright-field illumination. As described above for BrdU labelled cells, the

proportion of thymidine-labelled cells in a population of 1000 randomly chosen cells enabled the percentage of cells in S-phase to be determined.

RESULTS

Assay of TGF- β 1, TGF- β 2 and bFGF

Cartilage differentiation

The results of cartilage differentiation as measured by Alcian blue extraction are shown in Figure 2.01, with some corresponding Alcian blue stained wholemounts shown in Figure 2.02. TGF- β 1, TGF- β 2 and bFGF all increased cartilage differentiation in a dose dependent manner with approximately equal effect, the results being statistically significant ($P<0.001$) for 10ng/ml doses of each factor, with lower significance levels at lower doses i.e. $P<0.02$ at 1ng/ml, $P<0.1$ at 0.1ng/ml. TGF- β 1 and bFGF together acted in an additive manner increasing measurable cartilage differentiation still further, this being significant compared to the amount of cartilage differentiation seen in cultures treated with the corresponding doses of TGF- β ($P<0.01$) and bFGF ($P<0.001$) alone.

Muscle differentiation

The results of muscle differentiation in response to growth factor addition are shown in Figure 2.03 - 2.06. As can be seen from Figure 2.03, the addition of either TGF- β 1 or TGF- β 2 did not significantly reduce myogenesis ($P>0.02$, $P>0.05$ for the number of muscle cells that differentiated in 10ng/ml doses of TGF- β 1 and TGF- β 2 respectively when compared to the number that differentiated in DM). However, bFGF suppressed myogenic differentiation in a dose-dependent manner, for example, a dose of 1ng/ml bFGF reduced the number of differentiated muscle cells by 49%, whereas a dose of 10ng/ml reduced the number by 80%; both results are statistically significant ($P<0.001$ compared to DM). Figure 2.04 shows the effect of adding TGF- β and bFGF together. As can be seen the inhibitory effect of bFGF on muscle cell

differentiation was blocked by the addition of TGF- β 1, which increased the number of differentiating cells to that seen in control cultures. This result is also statistically significant ($P<0.001$ for the number of muscle cell in bFGF compared to that in bFGF + TGF- β 1). Not only is the number of muscle cells restored, but also their characteristic bipolar, branched morphology; whereas in bFGF the muscle cells that differentiated were rounded and smaller than those in control cultures. This can be clearly seen in immunostained cultures of myogenic cells (Fig. 2.06).

Some previous literature had shown that FGF does not inhibit but delays myogenic differentiation; therefore in order to investigate whether bFGF is acting in this manner in this system, the number of differentiated muscle cells was also determined at 72 and 96 hours of culture. The results shown in Figure 2.07 indicate that FGF inhibited muscle differentiation, rather than merely delaying the onset of differentiation. In fact the number of muscle cells began to decrease in both control and bFGF treated cultures with increasing length of incubation.

Cell proliferation

The number of cells in S-phase at different times and with different growth factors is shown in Figure 2.08. The rate of proliferation was greatest after 24h of culture and decreased substantially thereafter as differentiation proceeded. At 24h, the proportion of cells grown in bFGF that were in S-phase was significantly higher than that in control cultures ($P<0.02$). TGF- β decreased the proportion of labelled cells by 5.4% compared to controls but this is not statistically significant. When TGF- β and bFGF were added together, TGF- β blocked the stimulation of proliferation seen in bFGF treated cultures resulting in a similar proportion of labelled

cells to that seen in control cultures; this is statistically significant compared to the proportion of labelled cells seen with bFGF alone ($P<0.01$). The proportion of labelled cells at 48 and 72 hours of culture did not differ significantly from control cultures with any growth factor treatment.

Effect of insulin

In the absence of insulin a large proportion of the cells detached from the culture dish unless FGF was also present. Therefore it was impossible to repeat many of the above experiments. However it was possible to investigate the behaviour of cultures grown in bFGF and bFGF with TGF- β without insulin.

Figure 2.09 shows the result of the Alcian blue extraction and Figure 2.10 shows Alcian blue stained wholermounts of cultures grown in the presence and absence of insulin. It can be clearly seen that considerably less cartilage matrix was produced in cultures grown in the absence of insulin. These results are statistically significant between cultures grown in bFGF alone and bFGF in the presence of insulin ($P<0.001$), and between cultures grown in TGF- β and bFGF in the absence and presence on insulin ($P<0.001$).

Figure 2.09 and Figure 2.11 show the effect of insulin on muscle cell differentiation. The suprising result is that in the absence of insulin bFGF did not inhibit muscle cell differentiation. The number of muscle cells that differentiated in FGF with insulin is significantly lower than the number that differentiated in FGF without insulin ($P<0.001$). As previously shown the number of muscle cells in FGF with insulin is also significantly lower than the number which develop in TGF- β , FGF and insulin ($P<0.001$). However in the absence of insulin, TGF- β did not increase the number of muscle cells that differentiated in bFGF-containing medium.

Assay of activin and PMT

Figure 2.12 shows the results of the assay of activin and PMT. The experiments were only repeated twice due to a limited supply of each factor, so standard deviations and levels of significance have not been calculated. PMT had no effect on cartilage or muscle differentiation but increased proliferation slightly. Activin had little effect on cartilage differentiation or muscle differentiation at the lower dose of 16ng/ml, but at 160ng/ml cartilage differentiation was greatly increased and muscle cell differentiation inhibited by over 50% (Fig. 2.13). At both doses cell proliferation was reduced, the proportion of labelled cells decreased by approximately 5% with a dose of 16ng/ml and by 8% at 160ng/ml.

Assay of retinoic acid

Cartilage differentiation

The effect of retinoic acid on cartilage differentiation is shown in Figures 2.14 - 2.16. In proximal cell cultures RA inhibited chondrogenesis in a dose dependent manner. A dose of 0.1 μ g/ml reduced the amount of cartilage matrix production slightly, although this result is not statistically significant; whereas at doses of 1 μ g/ml and 10 μ g/ml chondrogenic differentiation was completely inhibited, these results are statistically significant ($P<0.01$). In distal cultures a dose of 0.1 μ g/ml RA slightly increased the amount of cartilage produced, however, as with the proximal cultures, a dose of 1 μ g/ml or 10 μ g/ml RA inhibited cartilage differentiation completely, this result also being statistically significant ($P<0.001$). RA had identical effects on cultures grown in the presence or absence of 10% serum.

In proximal cell cultures RA reduced the rate of proliferation in a dose dependent manner with cultures grown in serum exhibiting a slightly higher proliferative rate than those grown in the absence of serum (Figure 2.17). At 1 μ g/ml the decrease in the proportion of labelled cells was significant compared to the proportion in control cultures ($P<0.001$). In distal cell cultures a dose of 0.1 μ g/ml RA stimulated proliferation slightly. At 1 μ g/ml the rate of proliferation was similar to that in control cultures, whilst 10 μ g/ml RA resulted in a substantially reduced rate of cell proliferation ($P<0.05$ compared to the proportion of labelled cells in control cultures).

Figure 2.01

Histogram to show the amount of cartilage matrix production, as measured by Alcian blue extraction, from pairs of micromass cultures grown in defined medium with or without growth factors at the indicated concentrations for 3 days. Data are the means of 3-5 separate experiments. Bars indicate standard deviations.

TGF- β 1, TGF- β 2 and bFGF all stimulated increased cartilage matrix synthesis in a dose-dependent manner. TGF- β and bFGF added together enhanced matrix synthesis yet further in an apparently additive manner.

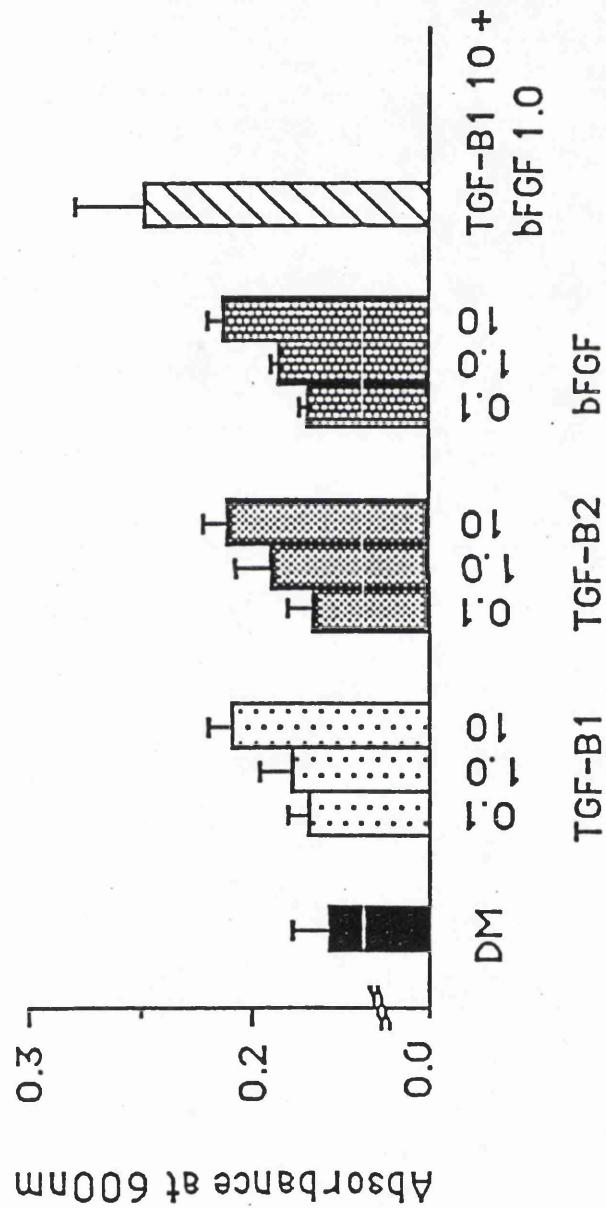


Figure 2.02

Alcian blue stained wholemounts of 3 day micromass cultures grown in (A) DM only, (B) 0.1 ng/ml TGF- β 2, (C) 1 ng/ml TGF- β 2, (D) 10 ng/ml TGF- β 2, (E) 1 ng/ml bFGF, (F) 10 ng/ml bFGF, (G) 10 ng/ml TGF- β 1 and (H) 10ng/ml TGF- β 1 and 10 ng/ml bFGF.

Scale bar = 1 mm.

Addition of TGF- β increased Alcian blue staining cartilage matrix in a dose-dependent manner. At a dose of 10ng/ml TGF- β 1 or TGF- β 2 cultures formed a homogenous sheet of cartilage, similar to that produced by cultures grown in the presence of 10% serum. Addition of bFGF also resulted in a dose-dependent increase in cartilage matrix synthesis, however a more nodular pattern of cartilage differentiation was obtained. Addition of TGF- β and bFGF together resulted in even greater cartilage matrix production, with the nodular pattern characteristic of bFGF treatment persisting.

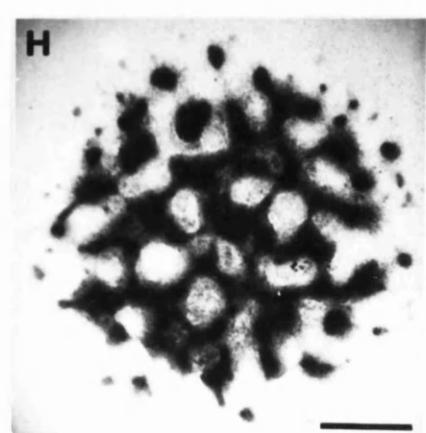
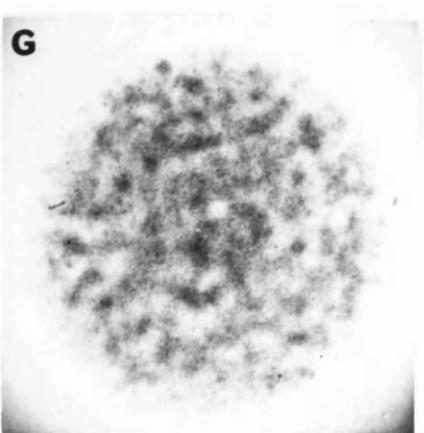
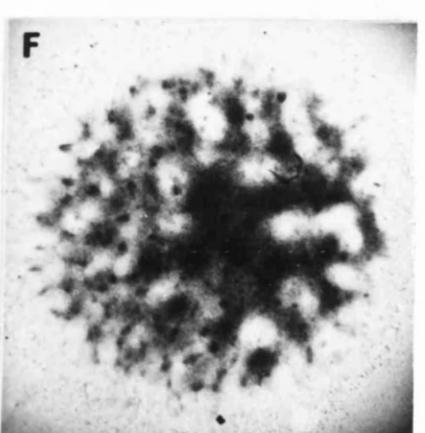
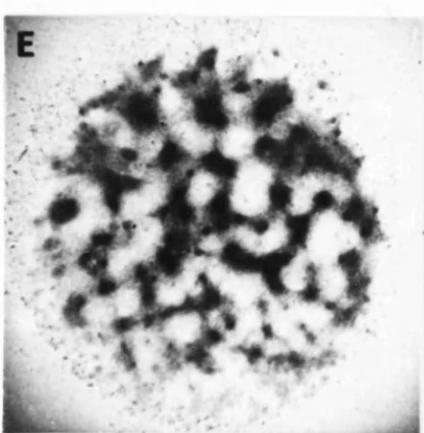
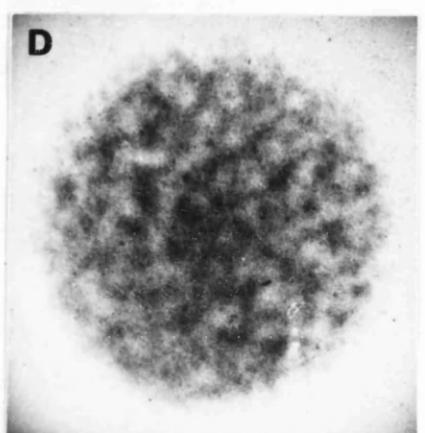
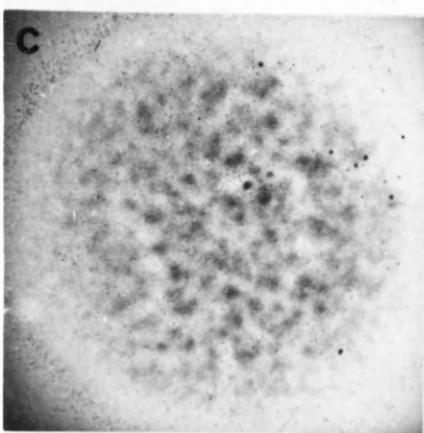
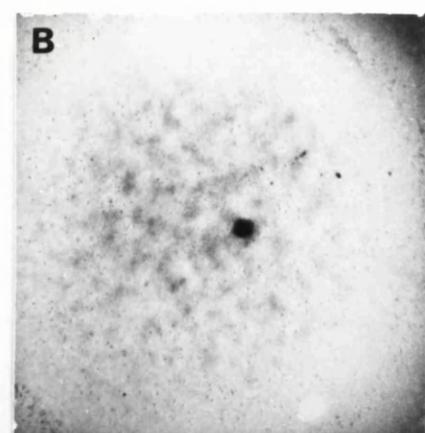
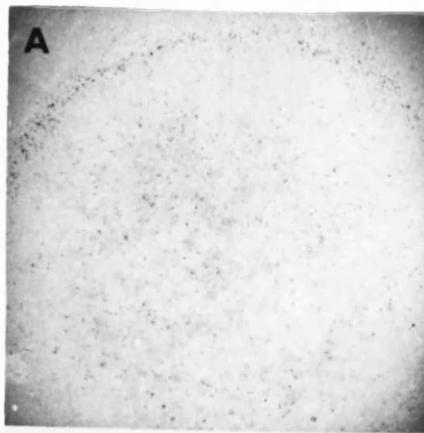


Figure 2.03

Histogram to show the number of differentiated muscle cells present in 2 day micromass cultures grown in DM alone or with the addition of growth factors. Data are the means of 4-7 cultures carried out on 2-4 separate occasions. Bars indicate standard deviations.

The number of muscle cells that differentiated was not significantly decreased by doses of TGF- β 1 or TGF- β 2 that significantly increased cartilage matrix synthesis, however addition of bFGF resulted in a dose-dependent inhibition of muscle cell differentiation, with approximately 50% inhibition caused by a dose of 1ng/ml.

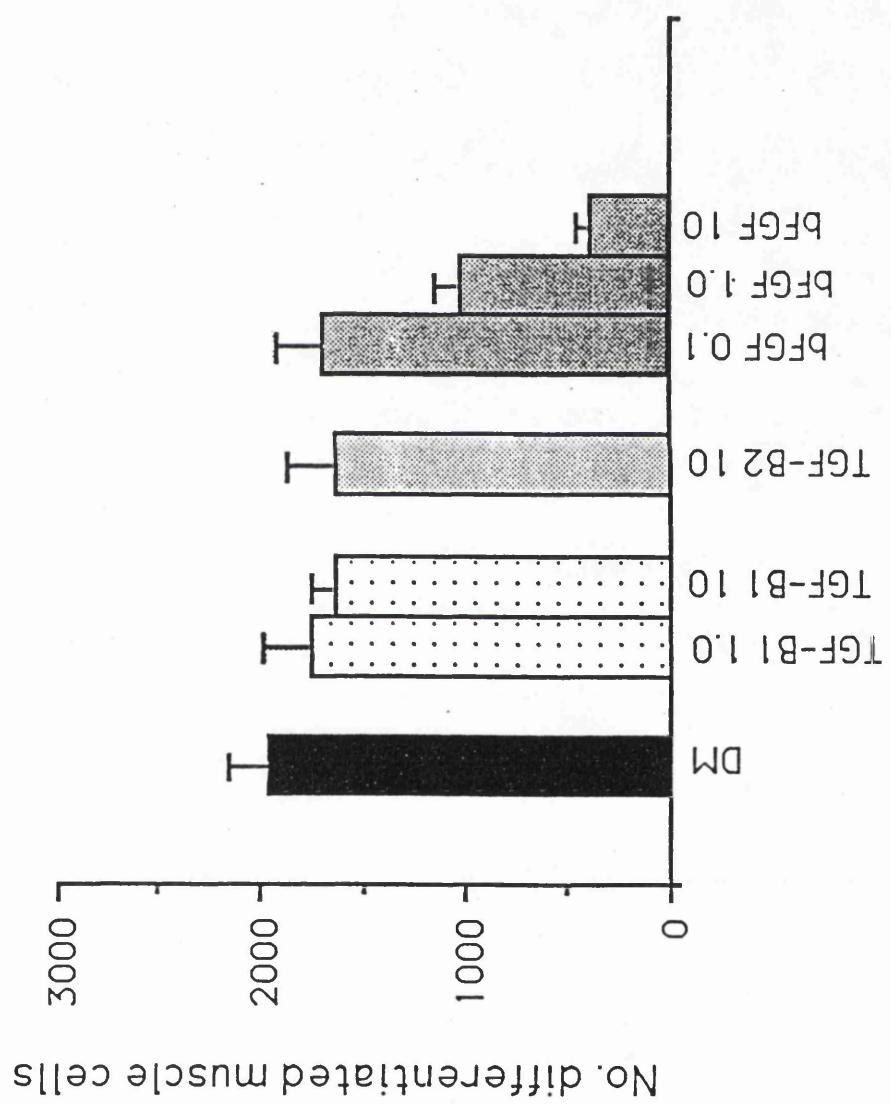


Figure 2.04

Histogram to show the number of differentiated muscle cells in 2 day old micromass cultures grown in DM, DM + 10 ng/ml bFGF and DM + 10 ng/ml bFGF + 10 ng/ml TGF- β 1. Data are the means for 4-6 cultures carried out on 2-3 separate occasions. Bars indicate standard deviations. The inhibition of muscle cell differentiation caused by bFGF was prevented by addition of TGF- β along with bFGF.

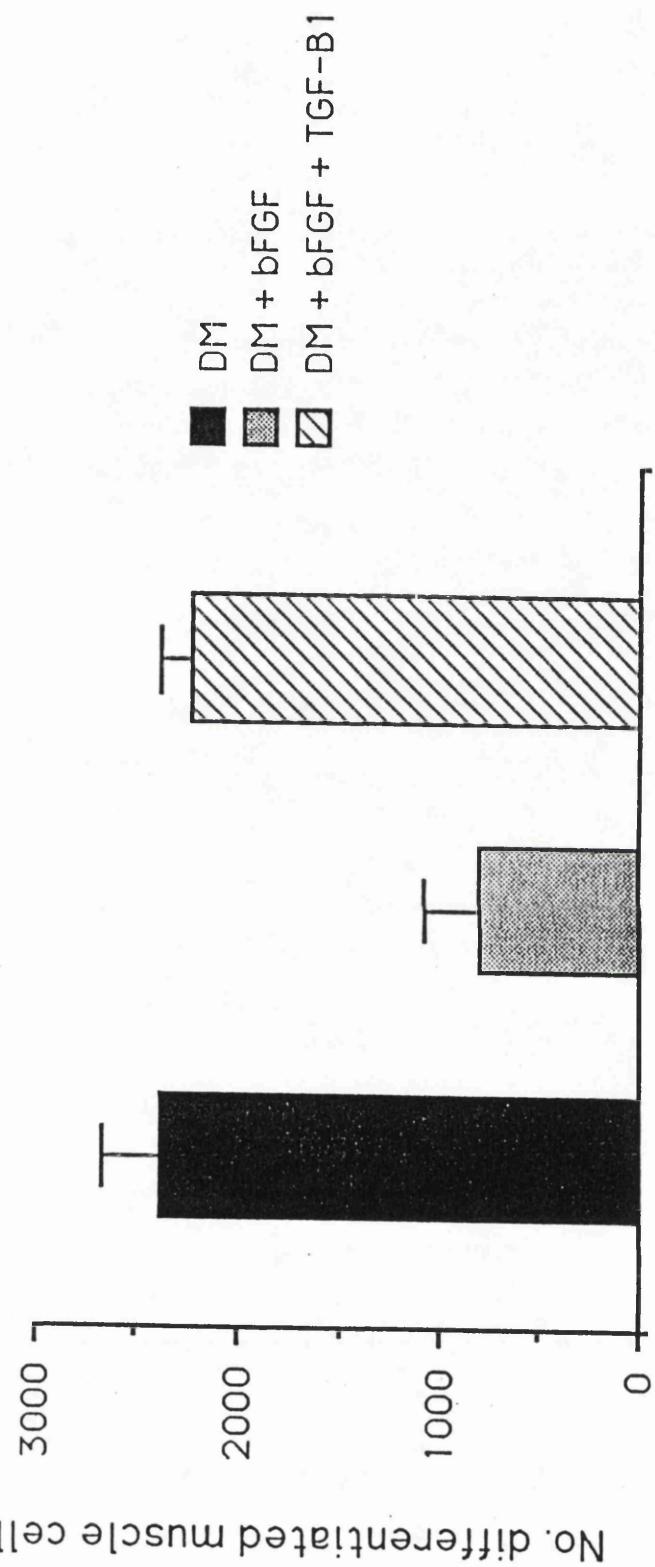


Figure 2.05a

Wholmount 2 day cultures stained with myosin heavy chain antibody and silver enhanced. Differentiated muscle cells are seen as single cells scattered throughout the cultures. Cultures were grown in (A) DM, (B) DM + 0.1ng/ml TGF- β 1, (C) 1ng/ml TGF- β 1 and (D) 10ng/ml TGF- β 1.

Scale bar = 500 μ m.

The number of differentiated muscle cells was only slightly decreased by addition of TGF- β .

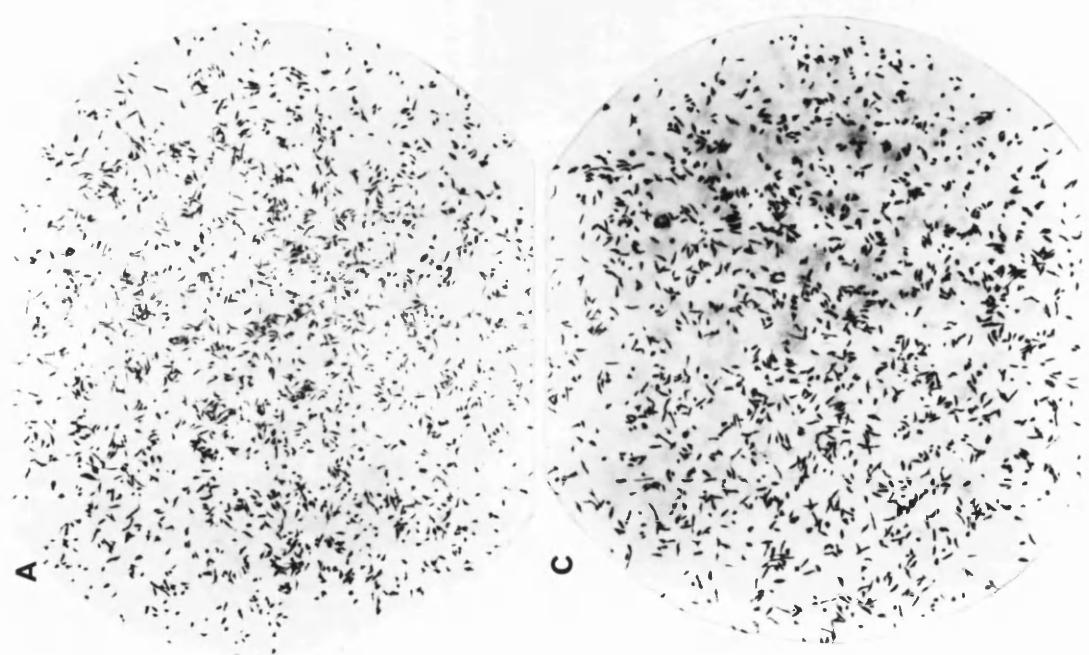
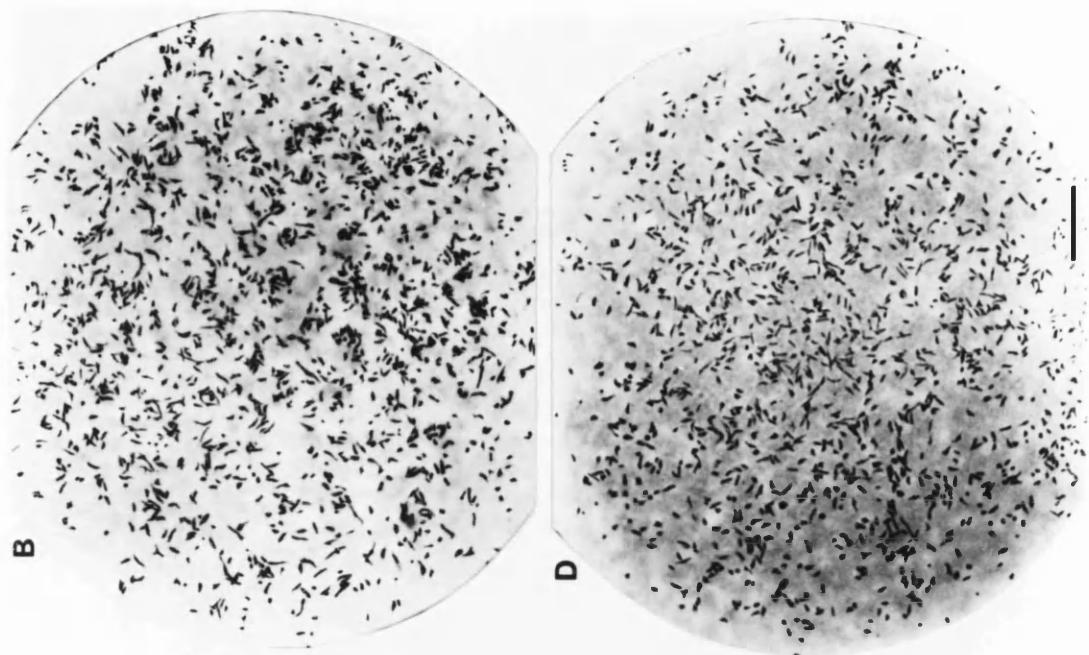


Figure 2.05b

Wholemount 2 day micromass cultures stained with myosin heavy chain antibody and enhanced with silver. Cultures show the dose dependent inhibitory effect of bFGF addition on myogenic differentiation and how this is blocked by the addition of TGF- β 1. Cultures were grown in DM with the addition of 0.1 ng/ml bFGF (A), 1ng/ml bFGF (B), 10ng/ml bFGF (C) or 10ng/ml bFGF + 10ng/ml TGF- β 1 (D). Scale bar = 500 μ m.

The number of differentiated muscle cells decreased with increasing dose of bFGF. At a dose of 10ng/ml the number of differentiated muscle cells was decreased by 77%, those cells which expressed myosin heavy chain were rounded rather than exhibiting the typical bipolar morphology of muscle cells in control cultures. Addition of TGF- β blocked the inhibitory effect of bFGF, restoring both the number and the morphology of differentiated muscle cells.

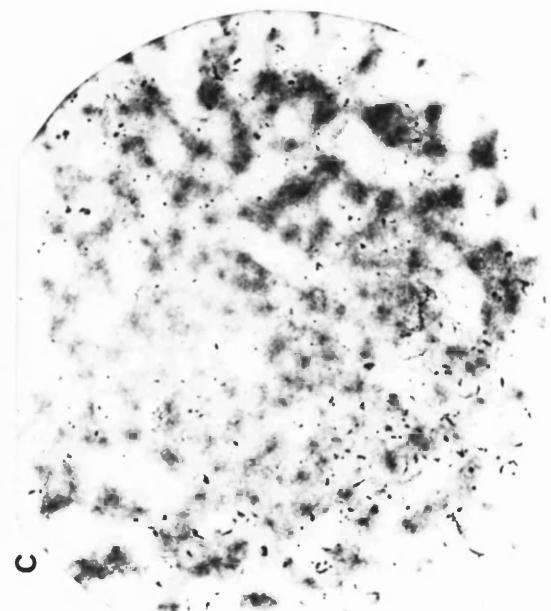
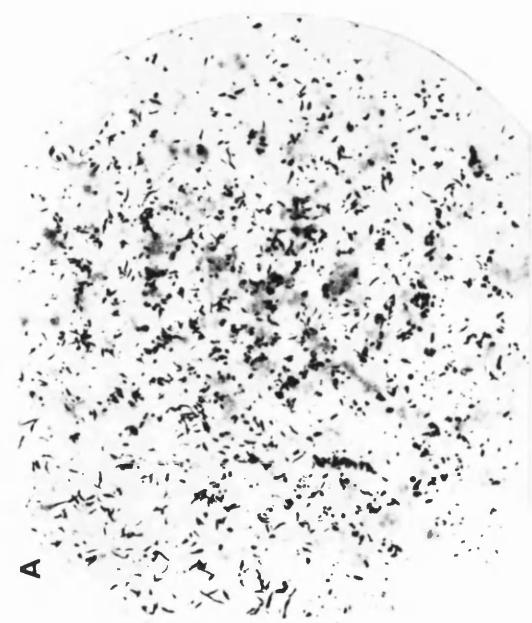
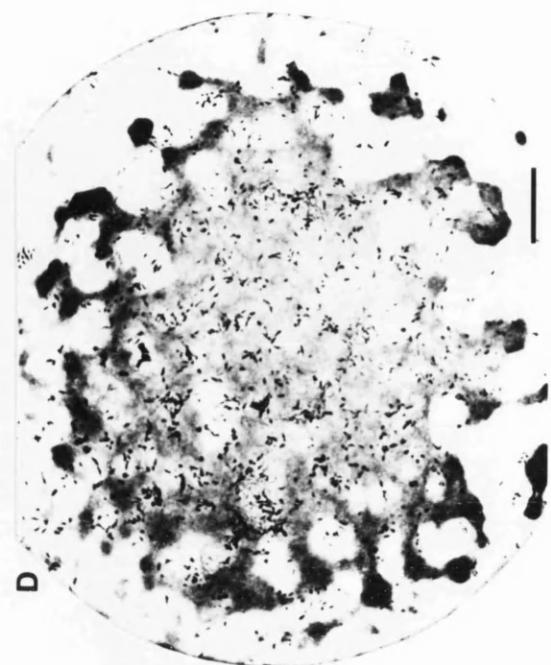
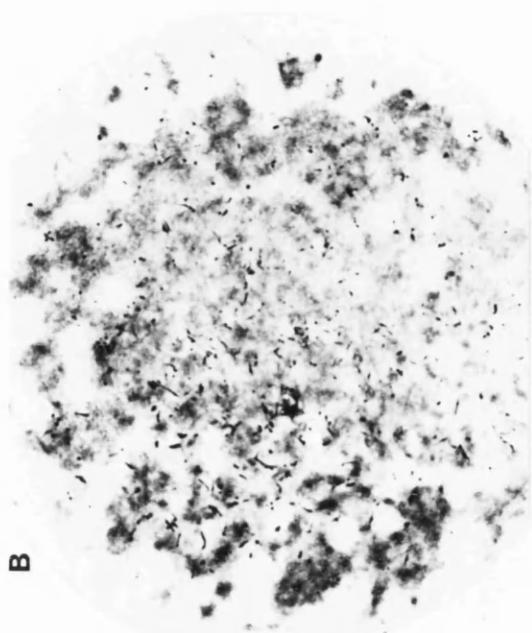


Figure 2.06

Phase contrast (A,C,E) and corresponding immunofluorescence (B,D,F) micrographs of 2 day micromass cultures stained with myosin heavy chain antibody and incubated in FITC-conjugated IgG. Cultures were grown in DM (A,B), DM + 10 ng/ml bFGF (C,D) or DM + 10 ng/ml bFGF + 10 ng/ml TGF- β 1 (E,F).

Scale bar = 200 μ m.

Note that the number of differentiated muscle cells in bFGF is reduced, and that they exhibit a rounded morphology with no cell processes unlike the typical bipolar muscle cells found in control cultures (B). Addition of TGF- β along with bFGF blocked this effect of bFGF (F). Note also how the differentiated muscle cells tend to aggregate in the internodular areas of the culture. c = cartilage nodule.

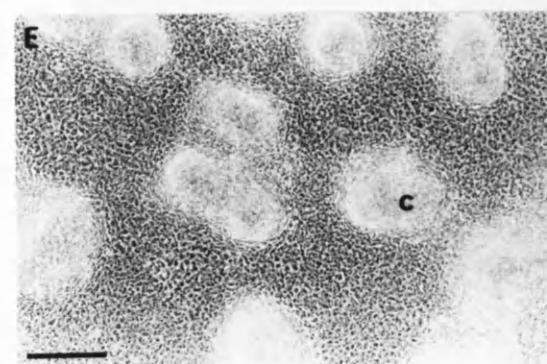
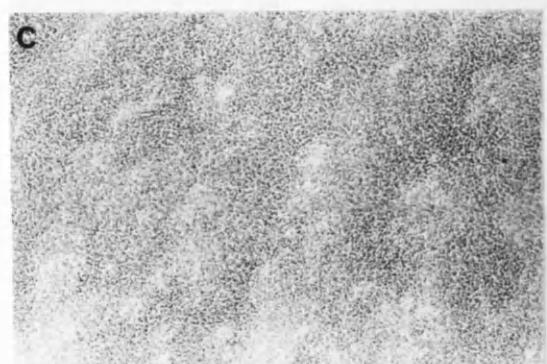
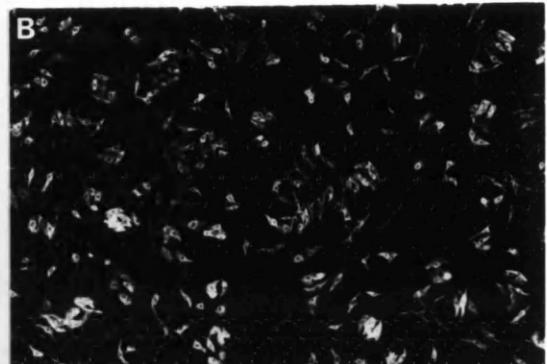
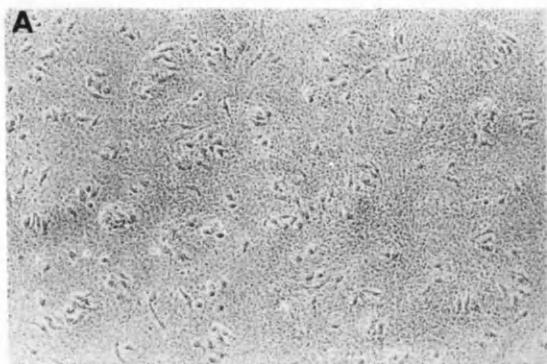


Figure 2.07

Histogram showing the number of muscle cells present after different intervals in culture grown in either DM or in DM + 10 ng/ml bFGF. Data are the means of 4-6 cultures performed on 2-3 separate occasions. Bars indicate standard deviations.

The number of muscle cells had decreased in both control cultures and in cultures grown in 10ng/ml bFGF by 96 hours of culture, thus showing that bFGF inhibited muscle cell differentiation in this system rather than merely delaying the onset of differentiation.

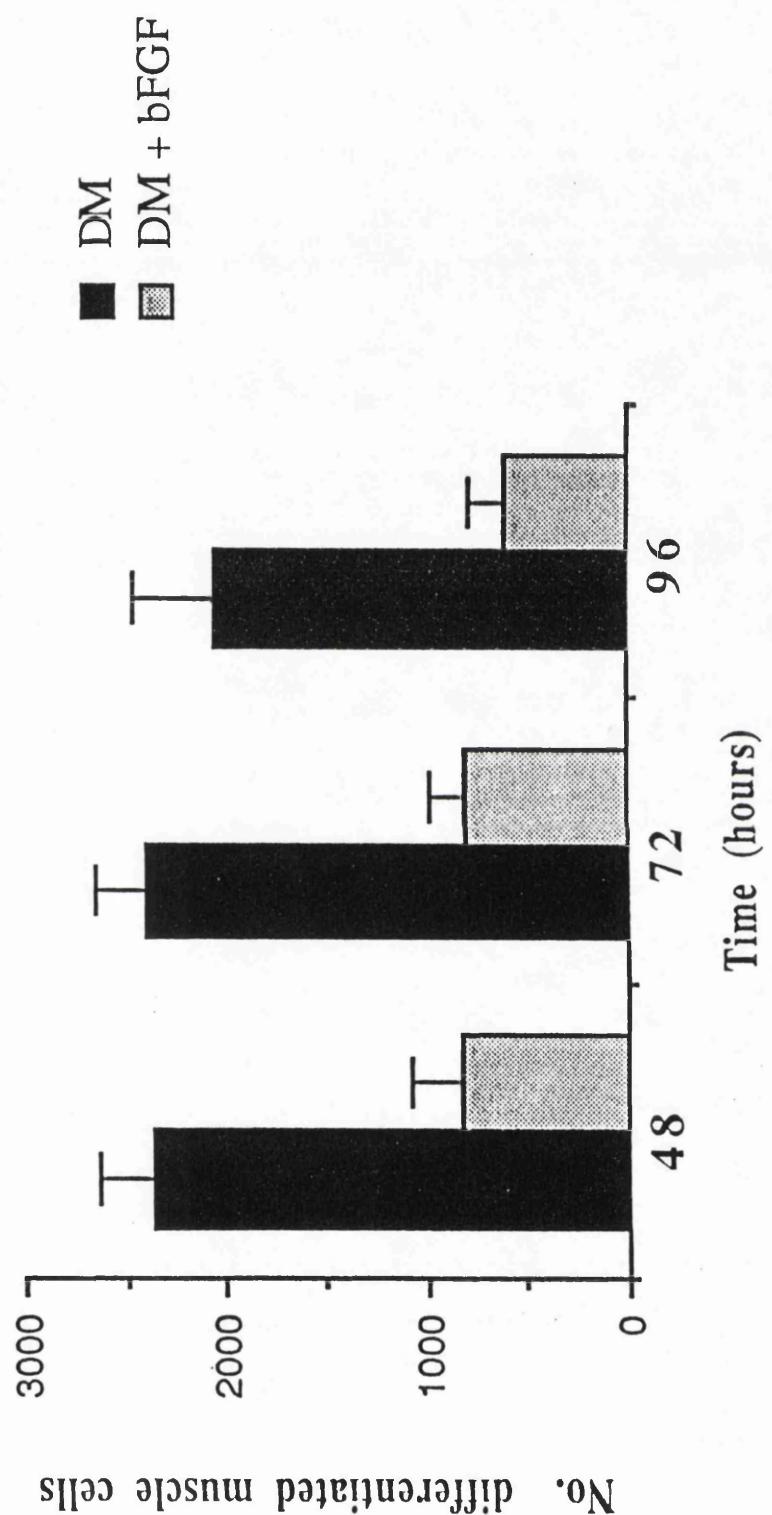


Figure 2.08

Histogram showing the percentage of cells in S-phase as determined by either tritiated thymidine or BrdU incorporation over a 3 hour period. Cultures were grown in either DM, DM+ 10 ng/ml bFGF, DM + 10 ng/ml TGF- β 1 or DM + 10 ng/ml bFGF + 10 ng/ml TGF- β 1, and incorporation measured at 24 hour intervals. Bars indicate standard deviation.

The rate of proliferation was highest at 24 hours of culture in all cultures, afterwhich it declined rapidly as cells differentiated. The rate of proliferation was substantially enhanced by addition of bFGF at 24 hours. In contrast TGF- β decreased the rate slightly, and addition of TGF- β along with bFGF blocked the stimulatory effect of bFGF, reducing proliferation to the level seen in control cultures.

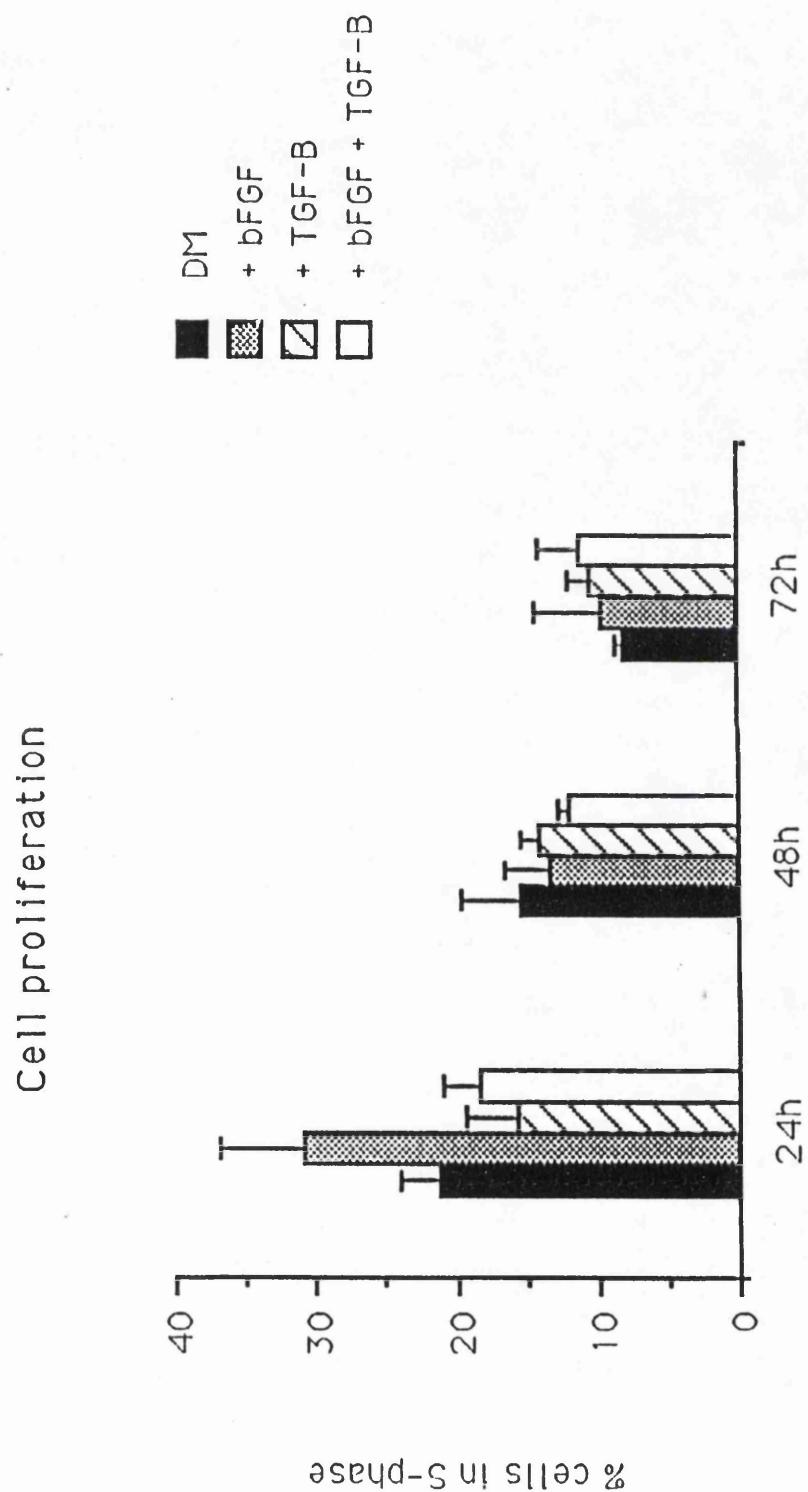


Figure 2.09

Graphs showing the effect of removing insulin from the medium.

Cartilage differentiation

Histogram showing the amount of Alcian blue extracted from pairs of 3 day micromass cultures grown in DM + 10 ng/ml bFGF or DM + 10 ng/ml bFGF + 10 ng/ml TGF- β 1 with or without insulin (5 μ g/ml). Bars indicate standard deviations.

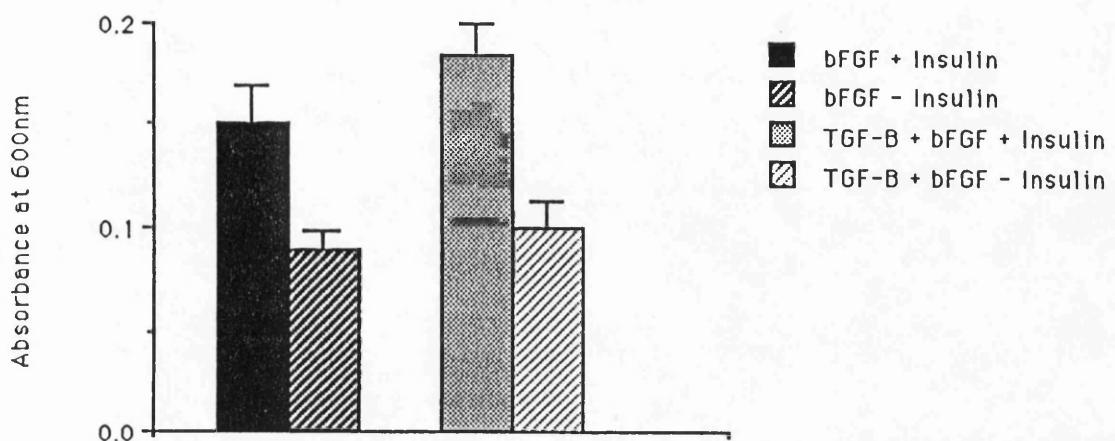
In the absence of insulin the amount of cartilage matrix produced was significantly reduced in both cultures grown in bFGF alone and those grown in bFGF and TGF- β .

Muscle differentiation

Histogram showing the number of muscle cells in 2 day cultures grown in DM + 10 ng/ml bFGF or DM + 10 ng/ml bFGF + 10 ng/ml TGF- β 1 with or without insulin.

In the absence of insulin the number of muscle cells which differentiated in the presence of bFGF doubled, thus the inhibitory effect of bFGF on muscle cell differentiation was lost. Although in cultures grown in TGF- β and bFGF the number of muscle cells in the absence of insulin was slightly decreased.

Effect of insulin - cartilage differentiation



Effect of insulin - muscle differentiation

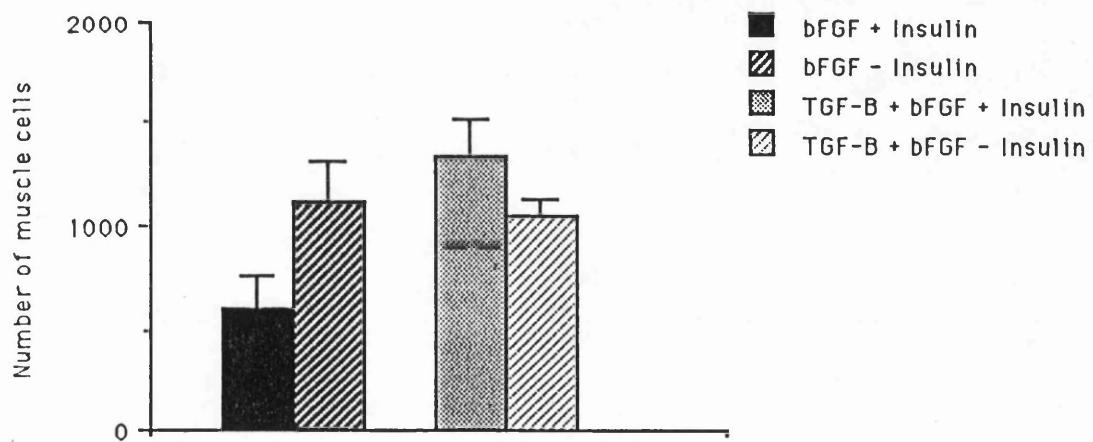


Figure 2.10

Alcian blue stained wholemounts of 3 day micromass cultures grown in the presence (A,C,E,G) or absence (B,D,F,H) of 5 μ g/ml insulin. (A,B) DM, (C,D) DM + 10 ng/ml TGF- β 1, (E,F) DM + 10 ng/ml bFGF, (G,H) DM + 10 ng/ml TGF- β 1 + 10 ng/ml bFGF.

Scale bar = 1 mm.

In the absence of insulin cartilage matrix production was substantially reduced in all cultures. In cultures grown in DM or with TGF- β no cartilage differentiation was detectable. In cultures grown in bFGF or bFGF with TGF- β cartilage differentiation was detectable, but was significantly reduced in comparison to cultures grown in the presence of insulin.

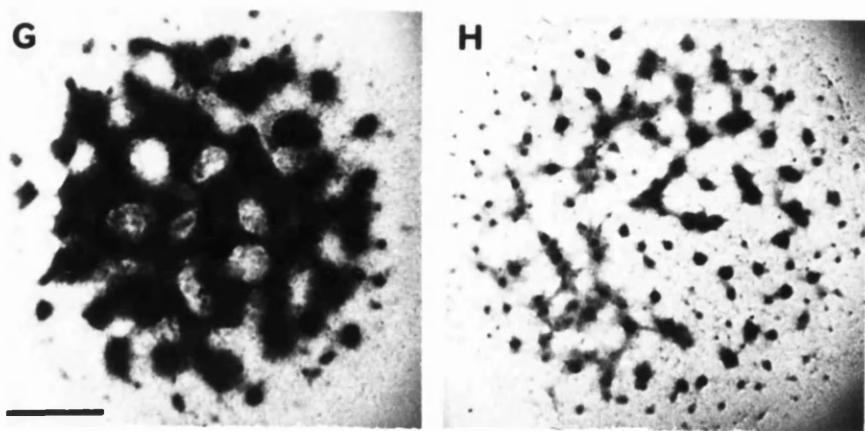
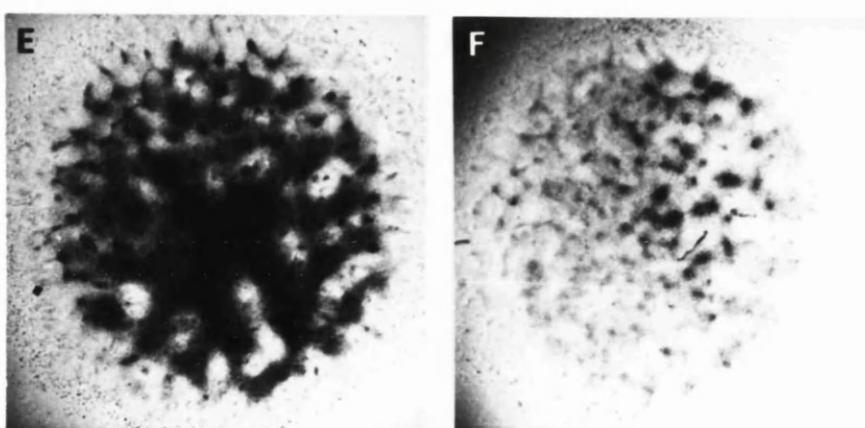
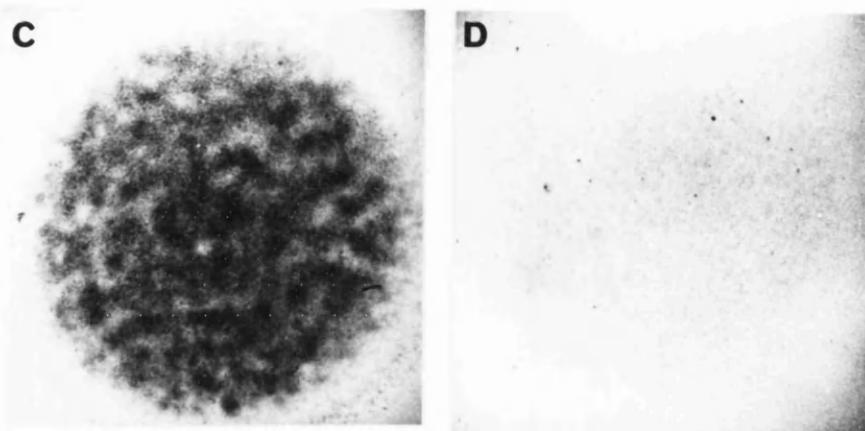
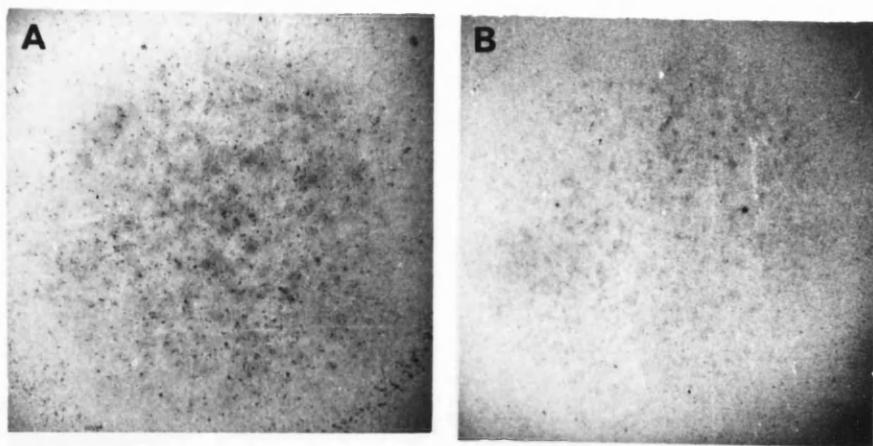


Figure 2.11

Phase contrast (A,C,E,G) and corresponding immunofluorescence (B,D,F,H) micrographs of 2 day cultures stained with myosin heavy chain antibody and FITC-conjugated IgG. (A,B,E,F) were grown in the presence of insulin, whilst (C,D,G,H) were grown in its absence. (A-D) were supplemented with 10 ng/ml bFGF, (E-H) were supplemented with 10 ng/ml bFGF + 10 ng/ml TGF- β 1.

Scale bar = 200 μ m.

The inhibition of muscle differentiation caused by bFGF in the presence of insulin was not seen when insulin was removed from the medium. In addition the morphology of the muscle cells became more typical, with a larger proportion exhibiting cell processes. In bFGF and TGF- β containing medium, the presence of insulin resulted in the differentiation of slightly more muscle cells, but the difference was not significant.

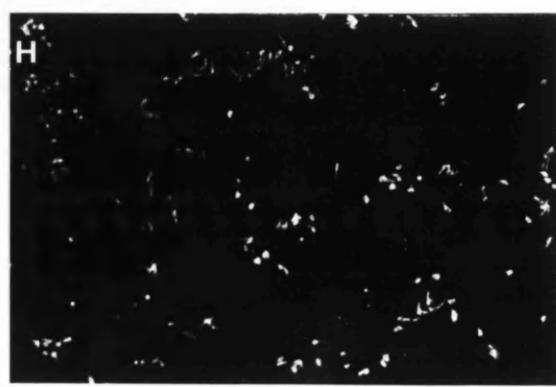
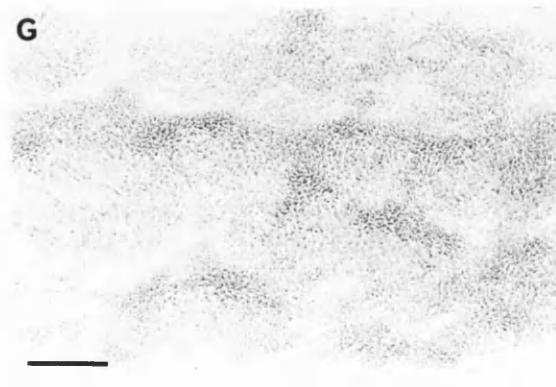
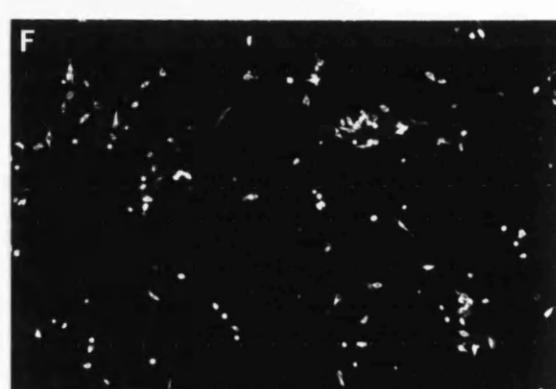
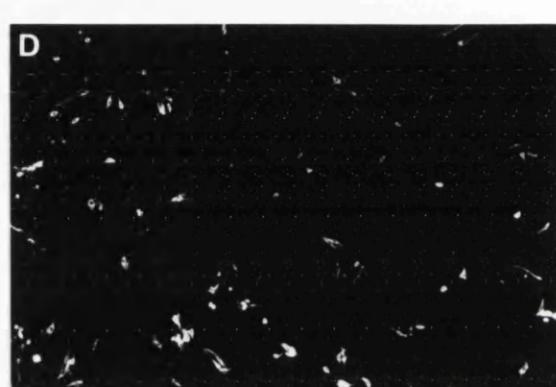
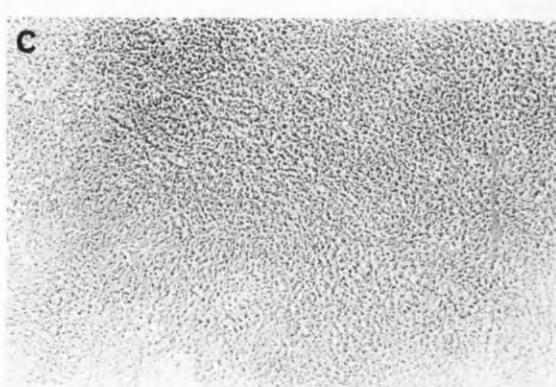
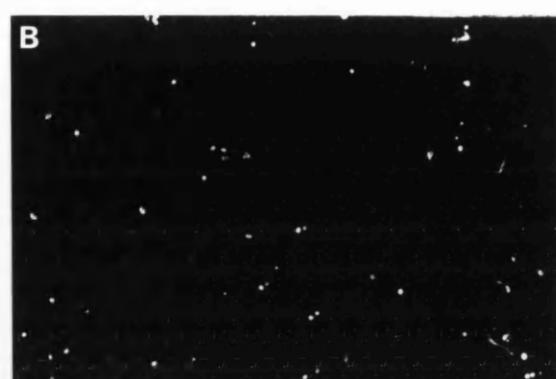


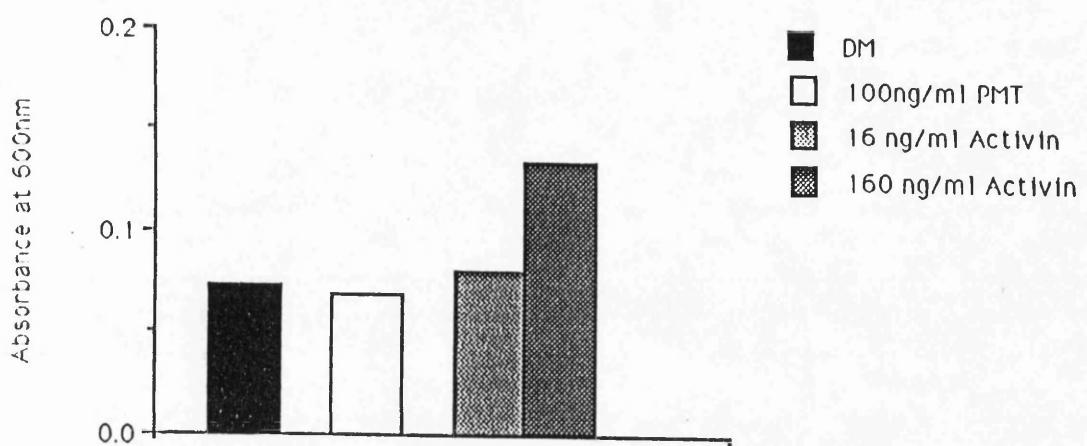
Figure 2.12

Histograms showing the effects of PMT and activin addition upon cartilage differentiation, muscle differentiation and cell proliferation. Each experiment was repeated twice so standard deviations are not given.

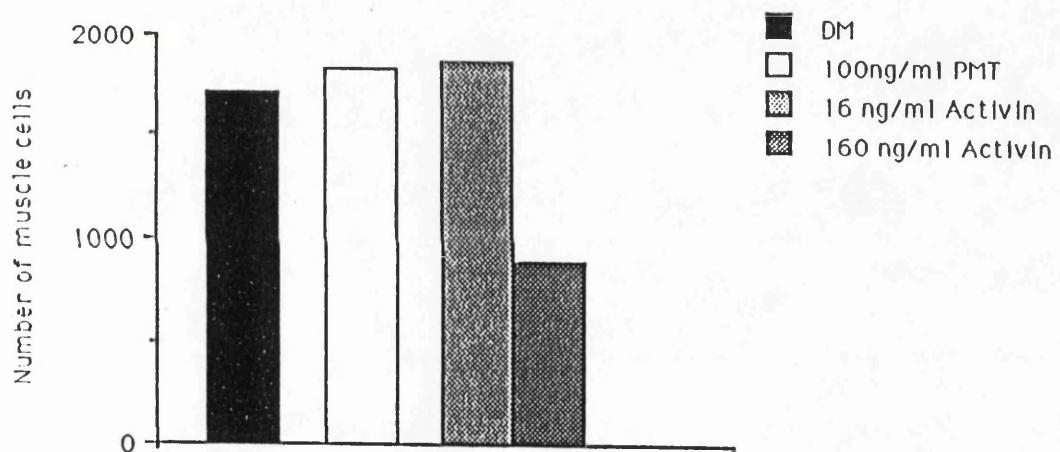
Alcian blue extraction was carried out on 3 day cultures, muscle staining on 2 day cultures and BrdU incorporation on 24 hour-old cultures.

PMT had no effect on cartilage or muscle cell differentiation, however a slight increase in proliferation was observed. Activin produced a dose-dependent stimulation of cartilage differentiation and inhibition of muscle differentiation. A decrease in cell proliferation was also observed at both doses of activin.

Cartilage differentiation



Muscle differentiation



Cell proliferation

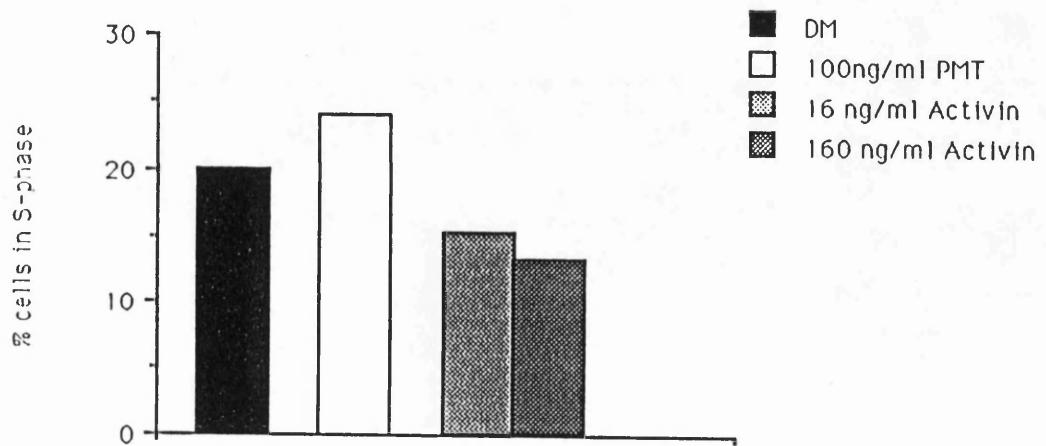


Figure 2.13a

Effect of activin on cartilage differentiation. Alcian blue stained wholemounts of 3 day cultures grown in DM (A), and DM + 160 ng/ml activin (B).

Scale bar = 1mm.

The homogenous sheet of cartilage formed in medium containing 160ng/ml of activin was very similar to that formed in cultures treated with 10ng/ml TGF- β (cf. Fig. 2.02 D,G).

Figure 2.13b

Effect of activin on muscle differentiation. Immunofluorescence micrographs of 2 day micromass cultures stained with myosin heavy chain antibody and FITC-conjugated IgG, grown in (A) DM, (B) DM + 16 ng/ml activin and (C) 160 ng/ml activin.

Scale bar = 200 μ m.

The number of muscle cells which differentiated in medium containing 16ng/ml activin was similar to that in control cultures, although the cells appeared to be smaller with less extensive cell processes. At a dose of 160ng/ml activin the number of differentiated muscle cells had fallen by about 50%, the cells which did differentiate were rounded and did not exhibit cell processes typical of cells in control cultures.

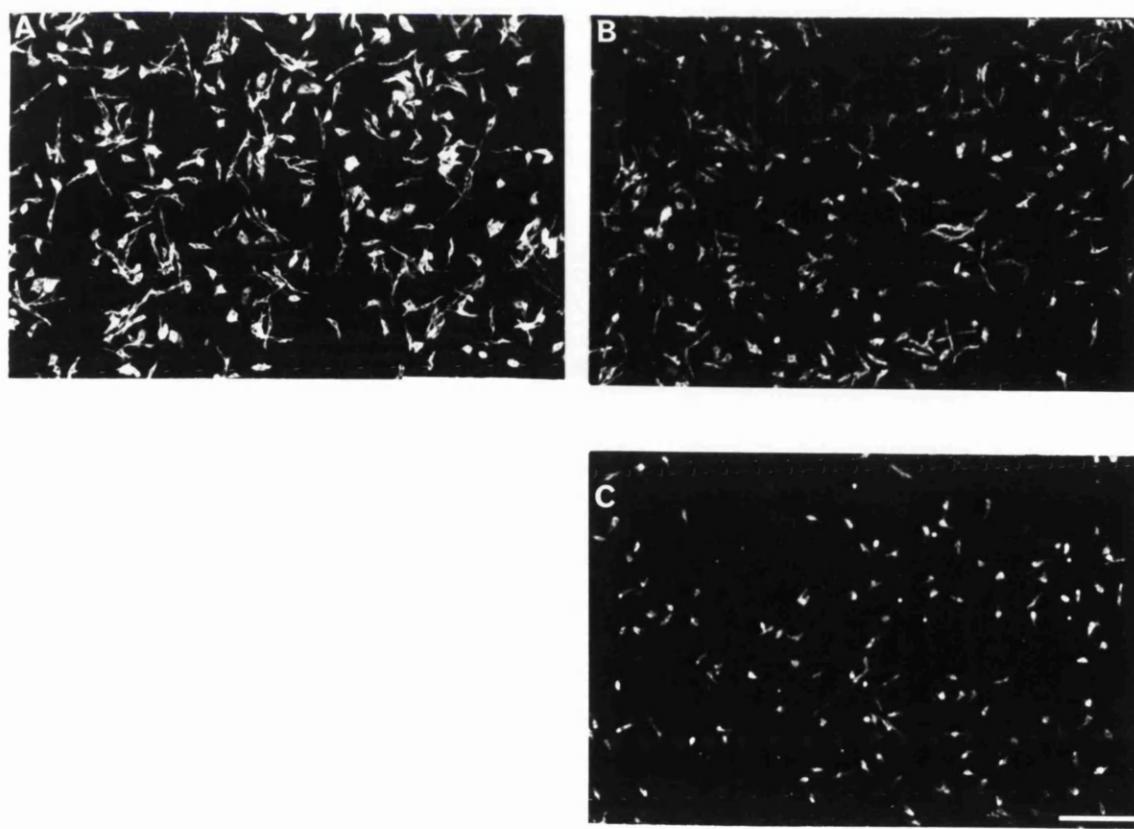
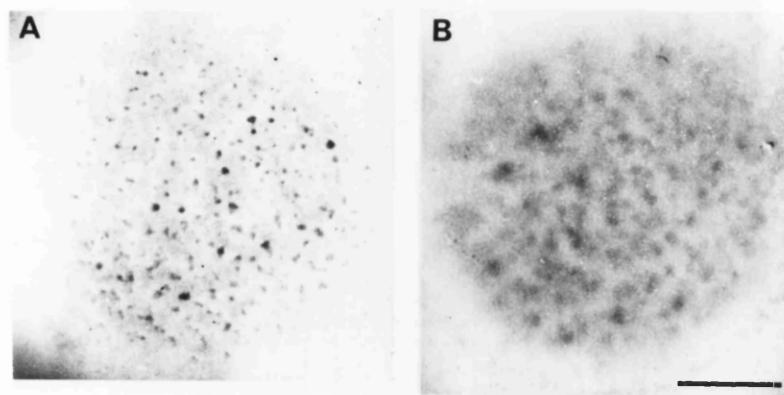


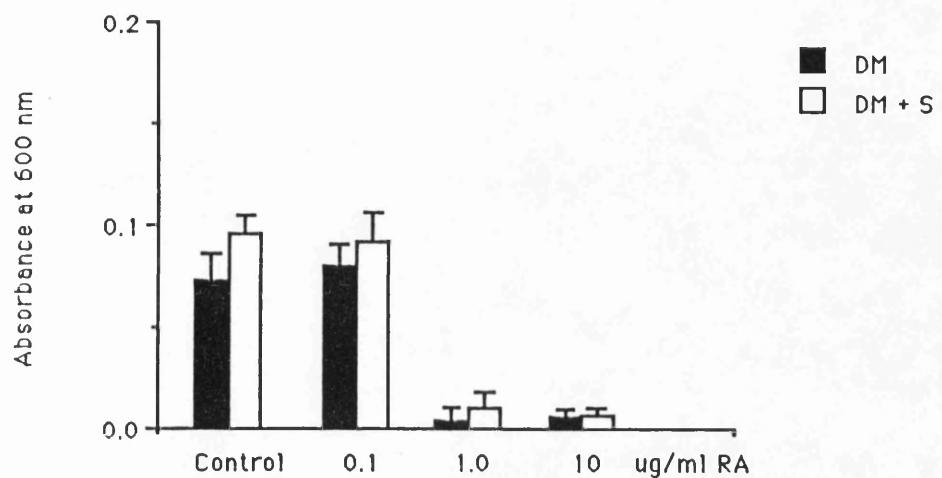
Figure 2.14

Histograms showing the effect of retinoic acid (RA) on cartilage differentiation of distal and proximal micromass cultures. Cultures were grown in DM containing different doses of RA for 3 days before staining with Alcian blue which was then extracted and quantified. Data represent means of 2-4 experiments. Bars indicate standard deviations.

In distal control cultures cartilage differentiation was slightly higher in serum-containing medium. At 0.1 μ g/ml RA cartilage differentiation was slightly enhanced in the absence of serum but remained similar to control cultures in serum-containing medium. At doses of 1 μ g/ml and 10 μ g/ml RA cartilage differentiation was completely inhibited in both the presence and absence of serum.

In proximal control cultures cartilage differentiation was greater in the absence of serum. At 0.1 μ g/ml RA cartilage differentiation was reduced in both serum-containing and serum-free medium, and as with distal cultures, doses of 1 μ g/ml and 10 μ g/ml RA inhibited cartilage differentiation.

RA treatment (cartilage differentiation) - distal cultures



RA treatment (cartilage differentiation) - proximal cultures

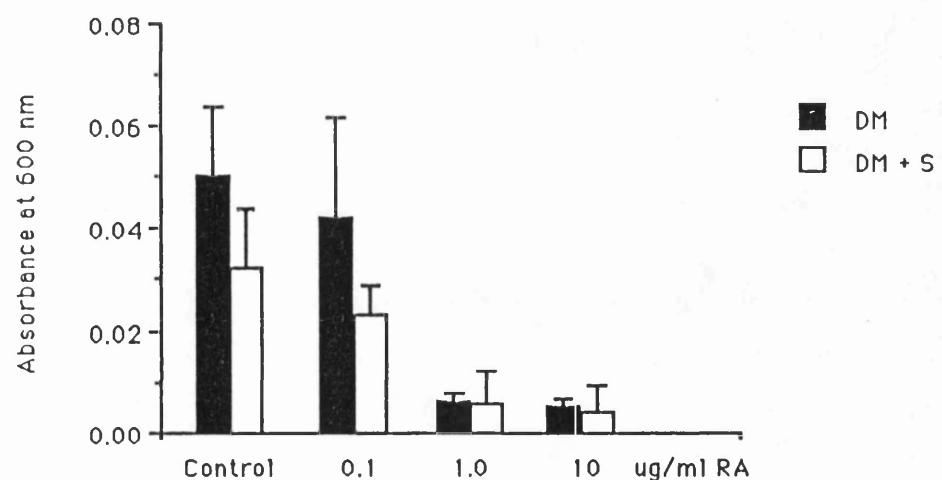


Figure 2.15

Alcian blue stained wholemounts of 3 day distal micromass cultures grown in DM (A,C,E,G) or DM + S (B,D,F,H) with no RA (A,B) or 0.1 $\mu\text{g}/\text{ml}$ RA (C,D), 1 $\mu\text{g}/\text{ml}$ RA (E,F) and 10 $\mu\text{g}/\text{ml}$ RA (G,H).

Scale bar = 1mm.

Distal cultures grown in DM exhibited a nodular pattern of cartilage differentiation, whereas cultures grown in the presence of serum formed a homogenous sheet of cartilage. Addition of 0.1 $\mu\text{g}/\text{ml}$ RA did not significantly alter the amount or pattern of cartilage differentiation seen in the presence or absence of serum. However doses of 1 $\mu\text{g}/\text{ml}$ and 10 $\mu\text{g}/\text{ml}$ RA completely inhibited cartilage differentiation in both the absence and presence of serum.

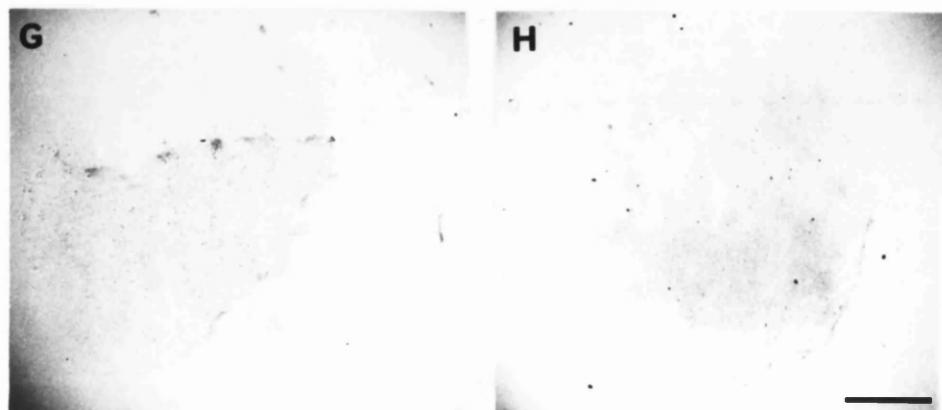
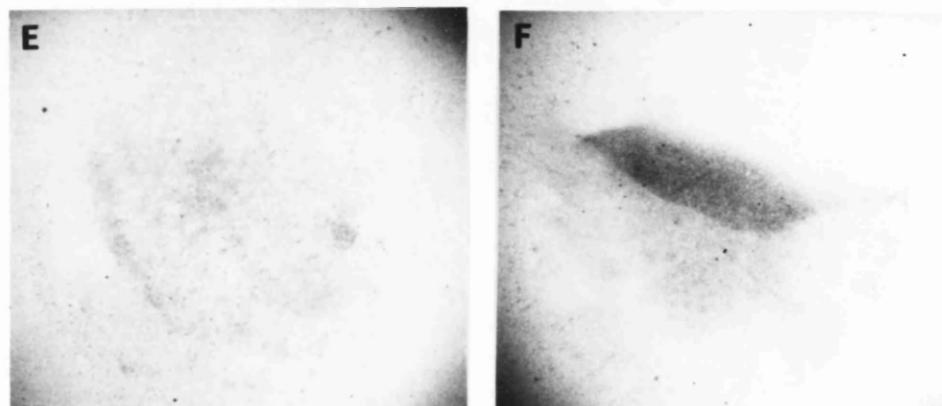
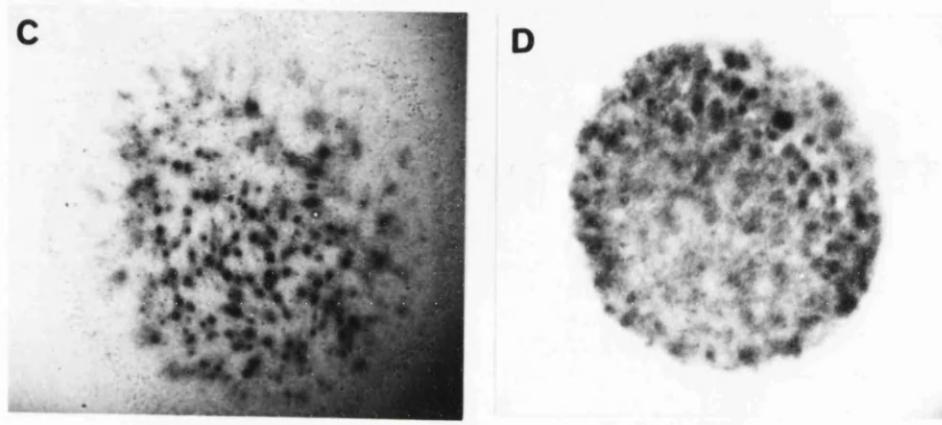
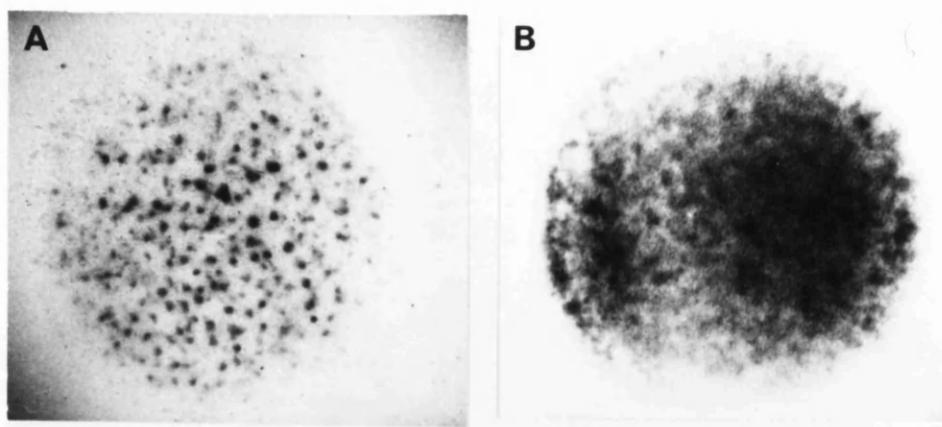


Figure 2.16

Alcian blue stained wholemounts of 3 day proximal cell micromass cultures grown in DM (A,C,E,G) or DM + S (B,D,F,H) with no RA (A,B) or 0.1 μ g/ml RA (C,D), 1 μ g/ml RA (E,F) and 10 μ g/ml RA (G,H).

Scale bar = 1mm.

Proximal cultures grown in DM exhibited an interconnected, nodular pattern of cartilage differentiation throughout the culture, whereas cultures grown in the presence of serum formed a more concentrated pattern of interconnected nodules in the centre of the culture with a larger peripheral sleeve of fibroblast cells. Addition of 0.1 μ g/ml RA reduced cartilage differentiation in both serum-containing and serum-free medium. Addition of 1 μ g/ml or 10 μ g/ml RA inhibited cartilage differentiation.

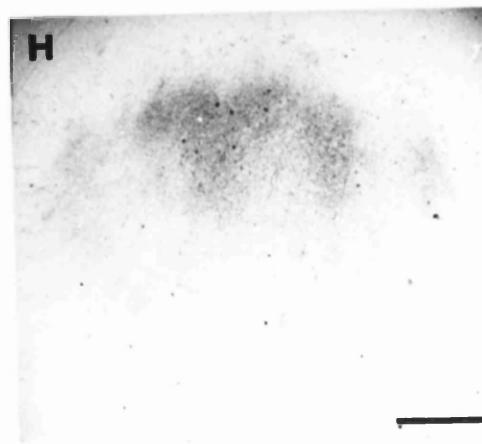
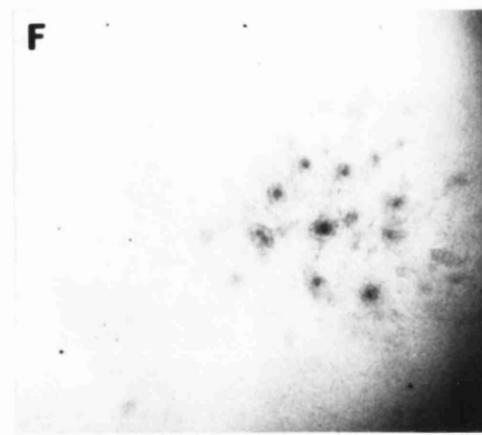
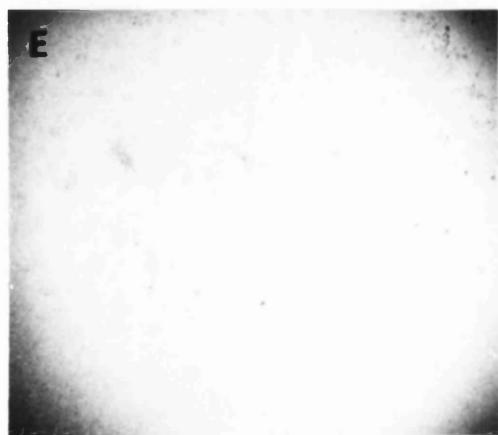
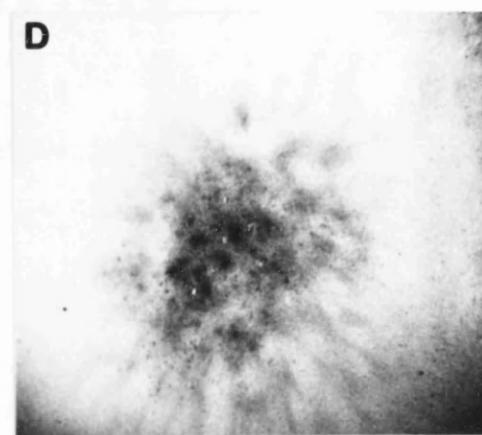
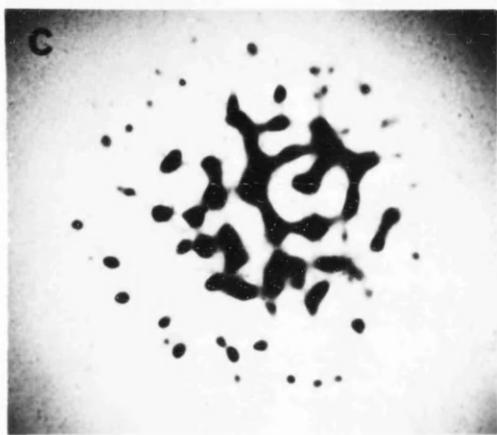
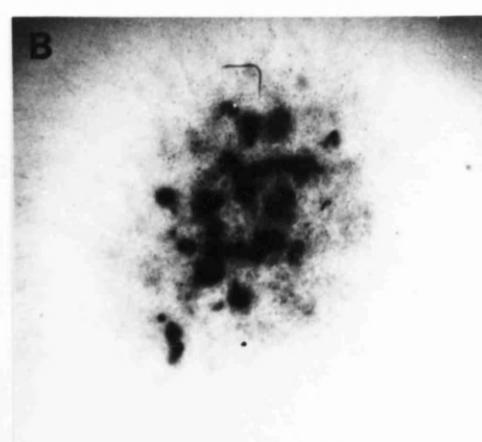
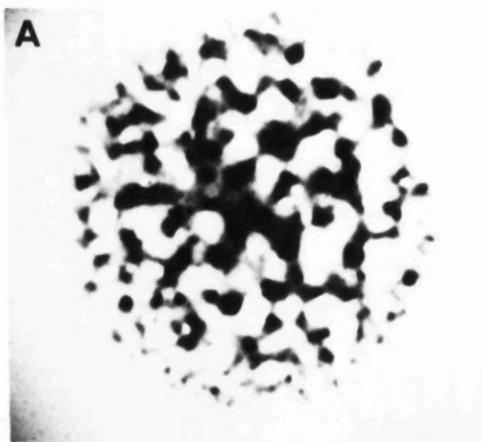


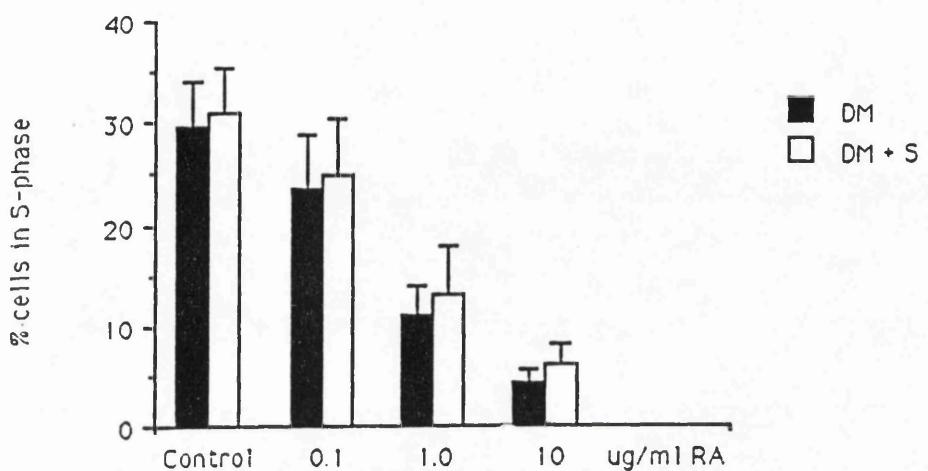
Figure 2.17

Histograms showing the effect of RA on the rate of cell proliferation as measured by BrdU incorporation by 24 hour old distal cell and proximal cell micromass cultures over a two hour period. Data represent the means of 3-5 experiments. Bars indicate standard deviations.

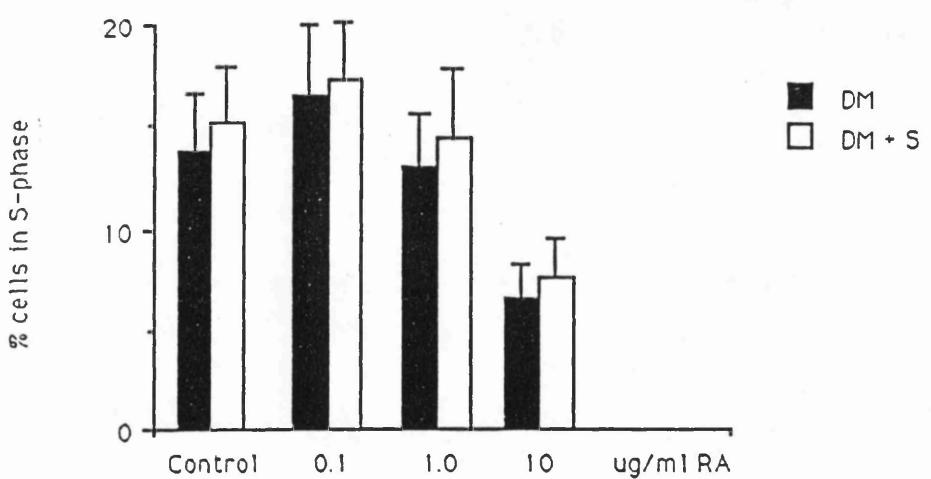
In distal cultures the rate of proliferation was higher in all cases in the presence of serum. A dose of 0.1 μ g/ml RA increased the rate of proliferation slightly. Addition of 1 μ g/ml RA resulted in a similar rate of proliferation to control cultures, whilst addition of 10 μ g/ml RA decreased proliferation.

In proximal cultures the rate of proliferation was only slightly enhanced in the presence of serum. In contrast to distal cell cultures, RA decreased proliferation of proximal cells in a dose-dependent manner.

RA treatment (BrdU incorporation) - proximal cultures



RA treatment (BrdU incorporation) - distal cultures



DISCUSSION

TGF- β 1, TGF- β 2 and bFGF assay

The three growth factors tested, TGF- β 1, TGF- β 2 and bFGF, all stimulated chondrogenesis in a dose-dependent manner. Addition of TGF- β resulted in a sheet of cartilage, whilst bFGF gave a nodular pattern. TGF- β 1 and TGF- β 2 have already been shown to stimulate cartilage-specific gene expression and the formation of cartilage matrix in this system (Kulyk *et al.* 1989). However these results are the first showing the effect of bFGF on distal chick limb micromass cultures.

TGF- β 1 and bFGF added together enhanced cartilage differentiation in an additive manner. There are several precedents for positive interaction between these two factors; for example, Iwamoto *et al.* (1989) showed that TGF- β increased the efficiency of FGF in stimulating the growth of chondrocytes in soft agar, and Kimmelman and Kirschner (1987) showed that the mesoderm-inducing effect of FGF on animal hemisphere cells of *Xenopus* embryos was greatly enhanced by the addition of TGF- β , even though TGF- β alone was unable to induce mesoderm.

Cartilage matrix production was significantly reduced in the absence of insulin, in cultures treated with bFGF and with bFGF in addition to TGF- β . Previous reports of interactions between these factors are limited, however Hiraki *et al.* (1988) found that TGF- β and IGF-I acted additively to stimulate GAG synthesis by growth plate and articular chondrocytes grown in culture. Inoue *et al.* (1989) showed that FGF increased the synthesis of proteoglycans from growth plate chondrocytes. Addition of IGF-I or TGF- β along with FGF enhanced this effect, however addition of all three factors did not result in a further increase in proteoglycan synthesis. Thus these three factors appear to interact in a positive manner in promoting cartilage-specific

gene expression. However it seems likely that the combination of growth factors required for optimal GAG and proteoglycan synthesis may change during the differentiation of cells of the chondrogenic lineage.

The number of differentiated muscle cells was slightly decreased by addition of TGF- β 1 and TGF- β 2 although neither decrease is significant compared to the number that differentiated in DM. Previous reports state that TGF- β inhibits muscle cell differentiation, but since much of this work was carried out on cloned cell lines, these results may not be comparable to those from our primary culture system. However, Massague *et al.* (1986) claimed that myoblasts from day 12 chick embryos were prevented from differentiating by TGF- β .

bFGF caused a more striking effect, progressively reducing the number of differentiated muscle cells with increasing concentration. In addition, the cells which were able to differentiate in bFGF appear rounded and did not exhibit the bipolar morphology with associated cell processes typical of differentiated muscle cells grown without bFGF. These results are consistent with previous findings that FGF inhibited the differentiation of cloned myoblast cell lines (Clegg *et al.* 1987; Lathrop *et al.* 1985; Spizz *et al.* 1986).

Seed and Hauschka (1988) looked at the muscle-colony forming (MCF) potential of stage 23 chick myoblasts and concluded that two subclasses of MCF cells exist. One subclass showed a delay in differentiation in the presence of FGF, but not inhibition. This later group might represent the 20% of myoblasts which were able to differentiate in the presence of 10ng/ml bFGF in this study. In order to determine whether bFGF was delaying rather than inhibiting muscle cell differentiation in these experiments, the culture period was extended to 72 and 96 hours. However, at all time points similar levels of inhibition were seen, the number of differentiated cells began to fall with time rather than increase. Therefore it appears that the

differentiation of muscle cells was inhibited by bFGF in this study. This result supports the earlier findings with cloned cell lines and the recent demonstration that FGF inhibits transcription of the myogenin gene in the BC3H1 myoblast cell line, an effect which is sufficient to cause inhibition of differentiation in these cells (Brunetti and Goldfine, 1990).

Addition of TGF- β to bFGF blocked the inhibitory effect on muscle differentiation, thus restoring both the number and morphology of the muscle cells that differentiated. In a different system, Stocker *et al.* (1991) have recently reported that TGF- β 1 (10ng/ml) blocks the bFGF-induced differentiation of melanocytes from dorsal root ganglion explants of quail embryos. However, the results reported here are difficult to interpret in terms of the current literature, which has shown that FGF and TGF- β both inhibit the differentiation of several myoblast cell lines. Both factors inhibit transcription of the MyoD1 gene (Vaidya *et al.* 1989), although they appear to inhibit terminal differentiation of these cells at a subsequent point, since TGF- β and FGF also inhibit differentiation of transfected cells made to constitutively express a MyoD1 cDNA clone.

The number of muscle cells that differentiated in bFGF-containing medium without insulin was significantly higher than that in insulin-containing medium. This is surprising in light of previous work showing that insulin stimulates myoblast differentiation in culture (Schmid *et al.* 1983; Ewton and Florini, 1981). Thus it would appear that bFGF is incapable of inhibiting muscle differentiation in the absence of insulin. This is interesting since both IGFs and bFGF have been detected in cells of the myogenic lineage throughout their differentiation. Therefore in the early myoblasts these two factors presumably interact to ensure the continued proliferation of myoblasts whilst preventing them from differentiating. In order for myogenic

differentiation to occur at later stages, either an antagonistic factor must act to block this effect or an intrinsic change must occur in the response of the myogenic cells to these two factors.

The rate of cell proliferation, as measured by tritiated thymidine and BrdU incorporation, declined over the 3 day culture period in cultures maintained in DM with the highest level found at 24 hours. Addition of 10 ng/ml bFGF substantially increased the proportion of cells in S-phase at 24 hours, although at 48 and 72 hours the rate of proliferation did not differ markedly from control cultures. This is consistent with the previous finding that FGF (200ng/ml), IGF-I (20ng/ml) and insulin (1 μ g/ml) all stimulate proliferation of mouse limb bud cells in micromass cultures (Kaplowitz *et al.* 1982). Addition of 10 ng/ml TGF- β 1 did not alter the rate of proliferation at any time compared to control cultures. When TGF- β was added along with bFGF the increase in the rate of proliferation at 24 hours seen with bFGF alone was not seen; thus as with the effect upon muscle differentiation, addition of TGF- β blocks the effect of bFGF. These results confirm previous studies (Baird and Durkin, 1986; Frater-Schroder *et al.* 1986) which reported that the proliferative response of endothelial cells to FGF was blocked by the addition of TGF- β . However in cultured growth plate chondrocytes addition of TGF- β together with FGF potentiated the stimulatory effect of FGF, thus resulting in increased DNA synthesis, whereas TGF- β alone decreased DNA synthesis (Hiraki *et al.* 1988).

The fact that the attachment and survival of these cells was extremely poor in the absence of insulin suggests that the cells must possess IGF (or less likely, insulin) receptors at the time they are cultured. In the absence of insulin addition of bFGF prevented cell detachment and retained the healthy morphology of the cells, thus suggesting that these cells also express functional FGF receptors at the time of grafting. This implies that IGFs and bFGF are involved in

the normal physiology of the distal mesenchyme cells *in vivo*. This hypothesis is supported by the finding that addition of insulin and bFGF to cultures of distal mesenchyme cells resulted in the highest rate of cell proliferation and the maximal inhibition of muscle cell differentiation. Both of these results are consistent with a role for these two factors in maintaining distal mesenchyme in a proliferative and undifferentiated state. Further support for this idea comes from the fact that both IGF-II mRNA and IGF peptide have been localised in the distal tip of the developing limb bud. However bFGF has not been specifically localised to the distal tip of the limb, in fact Joseph-Silverstein *et al.* (1989) using an antibody to human bFGF could only detect the protein in myoblasts which were migrating into the limb. However bFGF is known to bind to heparin in the extracellular matrix, and it is possible that in their immunolocalisation study such bFGF was masked from the antibody. In Chapter 1 it was shown that mesenchyme beneath an AER *in vitro* synthesises and accumulates a large amount of heparan sulphate proteoglycan, this would thus sequester any bFGF present. In addition this finding strengthens the view that distal mesenchyme cells are capable of responding to bFGF, since it has been shown that low affinity heparan sulphate binding sites for bFGF are necessary for cells to respond to bFGF, even in the presence of high-affinity FGF receptors. Other work also tends to support a role for bFGF in the distal limb mesenchyme. bFGF has been detected at high levels in stage 18 limb buds (Munaim *et al.* 1988) with levels decreasing by stages 22-4 and onwards, thus correlating with the stage of development when proliferation is the predominant activity occurring in the limb bud. Furthermore distal mesenchyme cells in the developing rat limb have been shown to express the FGF receptor (Wanaka *et al.* 1991) and the secreted factor FGF-4 is synthesised in the posterior portion of the AER in developing mouse limbs (Niswander and Martin,

submitted). bFGF also mimics the effect of posterior polarising region cells by selectively stimulating proliferation of anterior distal cells (Aono and Ide, 1988). Thus during the precondensation stages of limb development (stages 18-22) bFGF and IGFs may be acting to stimulate the proliferation of mesenchyme cells and thus outgrowth of the early limb bud, at the same time as stimulating continued proliferation and inhibiting premature differentiation of myoblasts. The finding that bFGF and insulin did not maintain cells in a continually proliferative and undifferentiated state in these experiments suggests that additional factors are also required in the distal mesenchyme in order to bring about these effects.

It is tempting to suggest that as limb development proceeds, TGF- β is synthesised by cells of the cartilaginous condensation and is able to act in both an autocrine manner, thus stimulating further chondrogenic differentiation of chondroblasts and additionally in a paracrine manner. Thus cells leaving the distal mesenchyme would be proliferating and still undifferentiated due to the combined influences of bFGF and IGFs, as they came into contact with TGF- β this would act to neutralise the stimulatory effect of bFGF on cell proliferation thereby facilitating differentiation of these cells. Myoblasts in the proximal region of the bud, maintained in an undifferentiated state by the combination of IGF and bFGF (which they have been shown to contain), would also come into contact with TGF- β . This would therefore have the effect of neutralising the effects of bFGF and thus allowing differentiation of these cells to occur. This is consistent with the recent findings that both bFGF and IGF peptide can be detected in cells of the myotome as they migrate into the limb bud, but the myogenic regulatory genes MyoD1 and myogenin cannot be detected in the limb buds of mouse embryos before 11.5 days (Sassoon *et al.* 1989). Since bFGF has been shown to inhibit the transcription of both of these genes in

cloned cell lines (Vaidya *et al.* 1989; Brunetti and Goldfine, 1990), this suggests that myoblast cells have to come into contact with an antagonist to bFGF before the transcription of muscle regulatory genes and thus expression of the differentiated myogenic phenotype can occur. The problem with this hypothesis is that TGF- β has not been detected until later stages of limb development when it is found in the perichondrium of developing cartilage skeletal elements. However the distribution of the TGF- β isoforms 3,4 and 5 has yet to be reported. It is also conceivable that BMP-2A, a member of the TGF- β superfamily which is present in the condensing cells of the cartilage blastema, may act in a similar manner to TGF- β .

Activin/PMT assay

Activin exhibited similar effects upon cartilage differentiation to TGF- β , but only at the supraphysiological dose of 160 ng/ml. In addition, activin inhibited muscle differentiation in a dose-dependent manner and reduced cell proliferation, effects which were not seen with TGF- β , at least not at a dose of 10 ng/ml, although these effects have been reported for TGF- β in many other systems. Previously activin has been shown to be 300 times less effective in an assay specific for TGF- β (Smith *et al.* 1990). In the assays described here activin is only about 10 times less effective, and also elicits effects on muscle differentiation and cell proliferation not seen with TGF- β . This raises the possibility that activin is not acting through the TGF- β receptor, but is acting through a receptor for another member of the TGF- β superfamily such as BMP-2A or BMP-4. Since BMP-2A transcripts have been localised to the AER (Lyons *et al.* 1990) and BMP-4 transcripts are present throughout the limb bud (Jones *et al.* 1991), it seems likely that limb bud cells do express receptors for these growth factors.

Pasteurella Multocida toxin (PMT) resulted in a slight elevation

of cell proliferation, but only at the relatively high dose of 100ng/ml. This is considerably greater than the 1-2pM dose that causes half-maximal stimulation of DNA synthesis in 3T3 cells. PMT is thought to cross the plasma membrane and exert its mitogenic effect in the intracellular environment, possibly through the actions of inositol phosphates. The finding that chick limb bud mesenchyme does not respond to PMT suggests that this mechanism of stimulating proliferation does not operate in these cells.

Retinoic acid assay

In these experiments RA inhibited cartilage differentiation in both serum-containing and serum-free defined medium, as has been previously reported. Chondrogenesis was completely inhibited by a dose of 1 μ g/ml. At 100ng/ml cartilage differentiation was not significantly effected although the amount of Alcian blue extracted was slightly decreased in the proximal cultures and increased in the distal cultures.

In proximal cultures RA decreased cell proliferation in a dose-dependent manner in both serum-free and serum-containing medium. In distal cultures the rate of proliferation was slightly increased by a dose of 100ng/ml. 1 μ g/ml RA resulted in similar levels of proliferation to control cultures, whilst a dose of 10 μ g/ml resulted in a lower rate of proliferation. A dose of 10 μ g/ml resulted in decreased rates of proliferation and judging by morphological criteria was probably somewhat toxic to the cells, as has been previously suggested by Biddulph *et al.* (1989).

There are several precedents in the literature which support these results, for example Kistler *et al.* (1985) tested RA on the chondrogenesis of micromass cultures of rat embryo limb buds and found a dose-dependent response, with maximal inhibition at 1 μ M (0.3 μ g/ml).

Biddulph *et al.* (1989) confirmed that doses of 1 $\mu\text{g}/\text{ml}$ RA applied for the initial 24 hours of culture completely inhibited chondrogenesis in micromass cultures of distal mesenchyme from stage 25 chick limb buds. In addition he showed that this inhibition was not caused by decreasing endogenous PGE₂ or cAMP levels, rather it appeared that RA arrests the cells in the mesenchymal state since they still show responsiveness to addition of exogenous PGE₂, a feature shown by mesenchymal cells but lost upon differentiation into chondrocytes. However, Paulsen and Solursh (1988) grew high-density whole limb cultures stage 23-24 in 96-well microtiter plates and found that retinoic acid showed a dose-dependent inhibition of chondrogenesis with maximal inhibition at 75ng/ml. In contrast DNA accumulation was only slightly effected with a dose of 25ng/ml stimulating and a dose of 150ng/ml inhibiting accumulation respectively. The above authors also claimed that addition of 5-10ng/ml RA enhanced matrix production 2-3 fold.

It is interesting that a very high dose of RA is required to significantly effect the differentiation (1 $\mu\text{g}/\text{ml}$) and proliferation (10 $\mu\text{g}/\text{ml}$) of both distal and proximal cells in this system, since retinoids are widely implicated in normal limb development. However it may be the case that RA is not involved with the normal control of proliferation and differentiation of limb mesenchyme, but is rather concerned with the assignment of positional information along the antero-posterior and possibly the proximo-distal axes of embryonic development.

CHAPTER 3

EXPRESSION OF HOX-7.1 AND HOX-8.1 IN THE LIMB BUD AND IN MICROMASS CULTURE

INTRODUCTION

Homeobox-containing genes

The homeobox is a highly conserved 180 base-pair sequence of nucleotides encoding a helix-turn-helix motif, which is involved in DNA-binding. Homeobox genes thus act as transcription factors. Vertebrate homeobox genes were initially isolated on the basis that a significant fraction of the amino acids they encoded were identical to the homeobox of the *Drosophila Antennapedia* gene. Homeobox-containing genes have now been discovered across the whole animal kingdom from yeast to man. Their role in embryogenesis has best been studied in *Drosophila*, where they are responsible for the organisation of the body along the anterior-posterior axis (reviewed by Akam, 1987; Ingham, 1988). The homeogenes interact with each other in both positive and negative regulatory fashion to define the specific identity of each parasegment of the developing thorax and abdomen.

Four homologous clusters of *Antp*-like homeogenes have been identified in the mouse; known as the class I homeogenes they consist of the *Hox-1*, *Hox-2*, *Hox-3* and *Hox-4* complexes, contained on chromosomes 6, 11, 15 and 2 respectively. These clusters are thought to have arisen by duplication during the course of evolution and exhibit a high degree of homology both with each other and with the *Drosophila* homeogenes. As in *Drosophila*, these genes are expressed throughout embryogenesis in a series of spatially restricted domains along the anterior-posterior axis

of the embryo. These domains are established at the primitive streak stage of development, before there is morphological evidence of a developing body plan, and persist throughout subsequent organogenesis, although later expression may be restricted temporally and to different tissue types. In both mouse and *Drosophila* the anterior boundary of expression of each gene corresponds to the relative order of genes in each chromosome, with downstream (3') genes exhibiting the most anterior expression and each upstream (5') genes sequentially exhibiting more posterior expression. The duplication of a single, ancestral homeogene cluster may therefore have enabled the development of increased complexity along the length of the body, since many new anterior boundaries of *Hox* gene expression are created which in turn encode many new positional values (Gaunt, 1991). Within these four gene clusters subfamilies exist, consisting of a gene or pair of genes from each homeogene cluster which are regarded as direct homologues of one of the *Drosophila* genes; the genes within each subfamily are described as paralogues. In the hindbrain 3' paralogous genes often exhibit identical or similar anterior boundaries of expression, which define the boundaries between adjacent rhombomeres. Thus each rhombomere possesses a unique positional identity encoded by the combination of homeogenes that are expressed. It has been suggested that each rhombomere or pair of rhombomeres develops in relative isolation, since the boundaries between them also serve as barriers to cell migration. Thus in this instance vertebrate homeogenes appear to be acting in a similar manner to their *Drosophila* counterparts. However it is more difficult to relate the expression patterns of homeogenes in other parts of developing vertebrate embryos to this lineage compartment model.

In addition to their expression along the main body anteroposterior axis, genes of the *Hox-4* cluster are also strongly

implicated in specifying positional identity along the anterior-posterior axis of the developing limb bud. Initially in the lateral flank tissue, which gives rise to the limb bud, only the most 3' gene *Hox-4.4* is expressed. As limb bud outgrowth continues *Hox-4* genes are sequentially expressed in a 3' to 5' direction in a progressively restricted fashion, such that *Hox-4.4* is expressed throughout the whole limb bud whereas *Hox-4.8* is restricted to the most distal and posterior mesenchyme. Thus each 5' gene is included within the expression zone of the more downstream members of the complex. This expression pattern is the same in both mouse and chick limb development (Dollé *et al.* 1989; Izpisua-Belmonte *et al.* 1991). Polarising region grafts or local application of beads soaked in RA to the anterior margin of chick limb buds cause mirror-image duplications of the digit pattern (Saunders and Gasseling, 1968; Tickle *et al.* 1982). The respecification of wing pattern following RA application becomes irreversible 14-24 hours later (Eichele *et al.* 1985), with the extra digits established sequentially beginning with digit 2. *Hox-4* genes are ectopically expressed in the anterior mesenchyme following polarising region grafts and local RA application. *Hox-4.4* expression in the anterior region is established within 16 hours, *Hox-4.6* within 20 hours and *Hox-4.8* within 24 hours (Izpisua-Belmonte *et al.* 1991). Thus the ectopic expression of members of the *Hox-4* gene complex precedes the appearance of the duplicated digit pattern, strongly suggesting that these genes encode positional information in the limb bud along the anterior-posterior axis.

Additional evidence supporting the crucial role of homeobox genes in development comes from experiments in which the expression of a particular gene is ablated. Targeted disruption of *Hox-1.5* results in a regionally restricted pattern of developmental defects such as loss of the thymus, parathyroid and carotid artery, in addition to abnormalities of other tissues derived from the pharyngeal arches

(Chisaka and Capecchi, 1991).

As previously stated, the homeobox-containing genes encode proteins which act as transcription factors. The full range of the downstream target genes upon which these transcription factors act is not known, although both positive and negative interactions between various homeobox genes has been described in *Drosophila*. Recent work has also shown reciprocal regulatory interactions between homeobox genes and putative growth factors. For example Reuter *et al.* (1990) have shown that the *Drosophila* homeotic gene *Ubx* is required for expression of the TGF- β -like factor *decapentaplegic* (*dpp*) in the central midgut visceral mesoderm of parasegments 6 and 7. In turn *dpp* is required for the expression of the homeotic gene *labial* in underlying endoderm cells (Panganiban *et al.* 1990). In contrast the homeotic gene *abd-A* represses *dpp* expression in the posterior midgut visceral mesoderm independently of its repression of *Ubx* and is also required for the expression of *wingless* (*wg*) in the visceral mesoderm of parasegment 8. *wg* is another putative *Drosophila* growth factor related to the mammalian proto-oncogene *int-1*, a member of the *Wnt* family.

Hox-7.1 and Hox-8.1

Hox-7.1 and *Hox-8.1* are members of a different family of homeobox genes, more closely related to the *Drosophila* gene *msh* (muscle segment homeobox), since *Hox-7.1*, *Hox-8.1* and *msh* show 90% similarity at the amino acid level compared to 65% homology at best with *Antp*-like homeobox genes. Holland (1991) has suggested that duplication of *msh* genes occurred at the onset of vertebrate evolution, since he isolated three *msh* genes in mice and zebrafish but only one in ascidians, which diverged from the chordate lineage before the origin of the vertebrates. In contrast to the *Antp*-like homeobox genes, *Hox-7.1* and

Hox-8.1 are expressed throughout the length of the embryo during gastrulation and in migrating neural crest cells, indeed transcripts for these genes are more widely distributed in anterior regions than other *Hox* genes (Suzuki *et al.* 1991). The expression of these genes does however appear to be restricted laterally, in that transcripts are detectable in lateral plate mesoderm but not in somatic mesoderm.

During organogenesis the patterns of *Hox-7.1* and *Hox-8.1* expression diversify and appear to be controlled by different mechanisms. This has been illustrated by Robert *et al.* (submitted) during development of the limb. In the early stages of limb bud formation (stage 16-17) *Hox-7.1* and *Hox-8.1* transcripts are detected within the entire limb mesoderm, but as development proceeds they become restricted to the mesenchyme beneath the AER (stage 20). Analysis by *in situ* hybridisation of *Hox-7.1* and *Hox-8.1* expression in the chick mutant *limbless* has shown that at early stages both genes were present throughout the limb buds, but by stages 20 and 21 the level of transcripts had significantly decreased. *Limbless* mutants are unable to form an AER and thus, although initial budding and mesenchymal outgrowth occur, the bud later starts to degenerate at stage 19 and disappears by stage 24. The finding that *limbless* stage 22 limbs were still transcribing the β -actin gene suggests that the decrease in *Hox-7.1* and *Hox-8.1* transcripts was not caused by necrosis but was the result of the absence of an AER. Therefore *Hox-7.1* and *Hox-8.1* are induced in response to factors produced by an AER at later stages of limb development, whereas at earlier stages the control of expression is mediated by a different mechanism. This was confirmed by grafting a second AER onto the dorsal surface of stage 22-23 limb buds, which resulted in induction of *Hox-7.1* and *Hox-8.1* expression in mesenchyme which had stopped expressing these genes.

The induction of *Hox-7.1* and *Hox-8.1* expression by the AER was

also shown by the experiments of Davidson *et al.* (1991), who grafted mouse tissue into chick limbs in order to see the effects of position on *Hox-7.1* and *Hox-8.1* expression. Expression of *Hox-7.1* was induced in normally non-expressing mouse proximal tissue when grafted beneath the AER within 5 hours. In large grafts, the part directly under the AER expressed a high level of transcripts, whilst the most proximal part often exhibited no transcripts, thus showing that there was a gradient of expression consistent with the AER as a source of the inducing factor. Normally expressing distal tissue grafted proximally had completely lost expression after 5 hours, thus showing that *Hox-7.1* and *Hox-8.1* expression is position dependent, and suggesting that this is a reaction to a signal produced by the AER. Expression of *Hox-7.1* and *Hox-8.1* is associated with other sites of inductive epithelial-mesenchymal interactions although there does not appear to be any correlation between expression of these genes and any one cellular process. The close relationship between *Hox-7.1* and *Hox-8.1* expression suggests that they are under common control but may exhibit different thresholds of expression, for example both genes are expressed in the AER and subjacent mesenchyme in the developing limb, thus suggesting that both are induced in response to factors produced by the AER.

Yokouchi *et al.* (1991) isolated the chick homologue of the *Hox-8* gene which they referred to as *Msx-1*. Expression of the gene was seen in the presegmented mesoderm, later becoming restricted to the somatic part of the developing mesoderm and the associated overlying ectoderm (i.e; the somatopleure). As the limb bud develops, expression was seen in the anterior mesenchyme and in the AER throughout its length. A small region of expression in the posterior marginal zone was also seen at slightly later stages, although expression in the posterior distal mesenchyme was not seen. Local application of doses of RA to the anterior margin of the bud, which cause mirror-image duplications of

the digit pattern, resulted in a significant downregulation of mRNA levels 12 hours later in the anterior mesenchyme, but did not result in loss of transcripts from the AER. The above authors suggested that *Hox-8* expression in the anterior and posterior marginal zones of the limb bud may be associated with the programmed cell death seen in these areas. They also postulated that application of RA could thus act to release these cells from programmed cell death by inhibiting *Hox-8* expression, thereby enabling the cells to be respecified into forming the new digits. This anterior and distal expression pattern has also been confirmed by Robert *et al.*, who have suggested in light of the above results that transcription of *Hox-8* is repressed in posterior mesenchyme by an endogenous retinoid-based signalling system emanating from the polarising region.

Experimental aims

The experiments described in this chapter have used *in situ* hybridisation to investigate the expression patterns of *Hox-7.1* and *Hox-8.1* in normal mouse limbs, and *Hox-7.1* during chick limb development.

The expression of *Hox-7.1* and *Hox-8.1* in cultured mouse limb bud tips either intact or with the AER surgically removed was investigated and compared with the observed expression patterns of these genes *in vivo*. Expression in distal cell and proximal cell micromass cultures was also investigated, in an attempt to see how accurately this model system reflected *in vivo* limb development.

At a later stage, when the chick *Hox-7.1* probe was cloned, further investigations were carried out in micromass cultures of chick limb tissue. The effect of adding bFGF and RA was investigated since both of these factors are implicated in development of the distal mesenchyme *in vivo* and as stated above, it has been suggested that *Hox-*

s is repressed by RA.

AERs were also grafted onto distal cell cultures in an extension of the experiments described in Chapter 1, to see if the mesenchymal outgrowth produced beneath a grafted AER provides an accurate *in vitro* model of the progress zone.

METHODS

Mouse experiments

Mouse embryos were removed from the uteri of pregnant females (C57 black) in PBS and transferred to minimum essential medium (MEM) where limb buds were excised. In the first set of experiments distal tips were excised and transferred to a droplet of culture medium in a dry 35 mm dish containing a square of millipore filter. This had previously been sterilised in 70% alcohol and then soaked overnight in medium. Tips were orientated on the filter and the dishes placed in a humidified 37°C, 5% CO₂ incubator for 1-2 hours. After this period the dishes were examined and, if the tips had attached to the filters, 1ml of medium was added. On other occasions the apical ectodermal ridge (AER) was removed from the limb buds using iridectomy scissors, distal tips were then excised and grown on millipore filters for 24 hours in culture as before. Tips were maintained in DM (see pp.134) or DM + S.

In the second set of experiments micromass cultures of mouse limb bud mesenchyme were made (as described previously for chick, pp.29). Since the number of embryos was often small, distal cultures were made from 500μm tips of both fore- and hind-limbs. Proximal cultures were made from the proximal-most 500μm of limb tissue. In one set of experiments cultures were made from a 1:1 mixture of distal and proximal cells. In other experiments chick AERs (from stage 22 embryos) were grafted onto 24 hour old mouse distal and proximal cell cultures (as described previously on pp.78). All micromass cultures were grown for 48 hours in DM + S before fixation in fresh 4% paraformaldehyde overnight at 4°C.

Chick experiments

The distribution of chick *Hox-7.1* in the normal limb was first

investigated. Embryos (stages 19, 23 and 25) were removed from the egg and placed in PBS. The amnion and ventral parts of the body were removed and the embryos transferred to fresh 4% paraformaldehyde at 4°C. Embryos were then pinned out on their ventral surfaces such that the limb buds extended laterally from the body wall, and left in fixative at 4°C overnight.

The expression of *Hox-7.1* in micromass cultures of chick limb mesenchyme was then investigated. Proximal (500 μ m) and distal (350 μ m) cultures were prepared as described previously (see pp.29). After counting, cells were resuspended in DM and plated in 35mm culture dishes for 1-1.5 hours. Proximal cultures were grown in DM or DM + S and distal cultures were grown in DM, DM + S, DM + 1 μ g/ml retinoic acid (RA), DM + 10ng/ml bFGF or DM + 1 μ g/ml RA + 10ng/ml bFGF. Cultures were then fixed after varying intervals.

In a further set of experiments, cultures were made from the central region of the proximal bud, excluding the anterior and posterior cells. The expression of *Hox-7.1* was then investigated in these cultures grown in DM, DM + S or DM + 1 μ g/ml RA for varying periods of time.

Finally the expression of *Hox-7.1* in 4 day old distal micromass cultures onto which an AER had been grafted after 24 hours was investigated. These cultures were grown in F12 medium, which promotes formation of a mesenchymal outgrowth, and in DM + S, which does not support such outgrowth (see Chapter 1, section 1.2).

In situ hybridisation

Preparation of probe

cDNA sequences encoding the 3'untranslated portion of the mouse *Hox-7.1* and *Hox-8.1* were cloned into plasmid vectors (Hill *et al.* 1989). Templates were linearised with the appropriate restriction

enzymes at 37°C for 4 hours. The reaction was stopped by addition of 0.5% SDS and 10mM EDTA, and the enzyme removed by phenol extraction, followed by chloroform extraction to remove the phenol. The template was then precipitated in 1/10th vol. sodium acetate and 2.5 vols. absolute ethanol at -70°C and then spun down for 25 minutes in a microfuge (13000 rpm). The template was then washed in 80% and 100% ethanol followed by 2-3 minute spins; the ethanol was allowed to evaporate and the pellet resuspended in Tris/EDTA buffer (50mM Tris, 5mM EDTA, pH 7.2).

A complementary riboprobe was then synthesised by transcription from the cDNA template. The transcription reaction mixture contained 6μl 5X transcription buffer, 1μl 1M dithiothreitol (DTT), 5μl DNA (0.5-1μg/5μl), 3μl ddH₂O (double distilled water), 1.2μl RNase inhibitor, 0.8μl T3 or T7 RNA polymerase and 1μl 10mM thymidine, adenosine and uridine triphosphate with 1μl 50μM cytosine triphosphate and 12μl of high-activity ³⁵S-labelled cytosine triphosphate. The reaction was carried out at 37°C for 60 minutes. DTT is included in this and in subsequent reactions since it protects ³⁵S from oxidation.

The cDNA template was then removed from the reaction mixture by incubation in 1μl RNase-free DNase with 1μl tRNA (10mg/ml) at 37°C for 10 minutes. The reaction was then stopped by the addition of 1μl 200mM EDTA. The volume was then increased to 200μl by addition of Tris/EDTA buffer containing 50mM DTT, the enzyme removed by phenol extraction and the probe precipitated in sodium acetate and ethanol at -70°C. The pellet was then spun down for 25 minutes at 4°C, washed twice in 80% ethanol with 50mM DTT, respun for 3-4 minutes and washed in absolute ethanol. The ethanol was then allowed to evaporate and the probe resuspended in 100μl ddH₂O containing 50mM DTT. A sample of the probe was then counted in a scintillation counter.

The probe was hydrolysed into pieces approximately 150 base pairs

in length in order to penetrate the section adequately and thus gain access to the mRNA. Hydrolysis was achieved by incubating the probe in 95 μ l sodium bicarbonate (80mM NaHCO₃, 120mM Na₂CO₃, pH 10.2) and 5 μ l 50mM DTT at 60°C for 60 minutes. The reaction was then stopped by addition of 100 μ l 6M ammonium acetate, pH 5.2. 1 μ l tRNA was added and the probe precipitated with 1200 μ l absolute ethanol at -70°C. As previously, the pellet was spun for 25 minutes at 4°C, washed in 80% ethanol with 50mM DTT twice, and in absolute ethanol once. The ethanol was evaporated and the probe resuspended in Tris/EDTA buffer containing 50mM DTT. The probe was then counted again and the concentration adjusted to 1x10⁵ dpm/ μ l in 1:9 mixture of Tris/EDTA buffer to hybridisation mixture. Probes were then stored at -70°C.

Processing of specimens

Cultures and embryos fixed in 4% paraformaldehyde in PBS overnight at 4°C were processed the next day according to the following schedule:

PBS	10 mins
0.85% NaCl	15 mins
1:1 0.85% NaCl:alcohol	20 mins
70% alcohol	20 mins
90% alcohol	20 mins
100% alcohol	3 x 20 mins
toluene	2 x 20 mins
wax (at 60°C)	2 x 30 mins

Wax was filtered before use. After embedding, wax blocks were stored at 4°C. 7 μ m sections were then cut on a wax microtome and mounted in sterile ddH₂O. The slides on which sections were mounted were first cleaned and then coated with 3-aminopropyltriethoxysilane

(TESPA) according to the following schedule:

10% HCl in 70% alcohol	10 secs
ddH ₂ O	10 secs
95% alcohol	10 secs
slides were then dried at 150°C	
2% TESPA in acetone	10 secs
100% acetone	10 secs
100% acetone	10 secs
slides were then dried at 42°C overnight	

Pretreatment

Pretreatment is necessary to increase the permeability of the section to the riboprobe. This involves deproteination of the section usually using dilute HCl and proteinase K. In addition, prehybridisation treatments are designed to reduce non-specific binding of the riboprobe to basic residues in the section. This is accomplished by acetylation of these residues with acetic anhydride.

Slides were first dewaxed in xylene (10 mins), then rehydrated through a series of alcohols (absolute, 90%, 70%, 50%, 30%) with 2 minutes in each. Slides were then washed for 5 minutes each in 0.85% NaCl and PBS before fixation for 20 minutes in fresh 4% paraformaldehyde, followed by a further two washes in PBS. Sections were then incubated in 20µg/ml proteinase K in 50mM Tris, 5mM EDTA for 7.5 minutes, followed by washing in PBS and re-fixing with 4% paraformaldehyde for 5 minutes. After brief rinsing in ddH₂O sections were acetylated by incubation for 10 minutes in 0.1M triethanolamine (TEA) with 1/400 vol. acetic anhydride with agitation. Sections were then washed (5 minutes 0.85% NaCl, 5 minutes PBS), dehydrated through alcohols (1 minute in 30%, 50%, 70%, 90%; 5 minutes in 100%) and air

dried.

Hybridisation

Hybridisation is carried out in the presence of 50% formamide, in order to optimise stringency and 10% dextran sulphate, which increases the rate of hybridisation and results in amplification of the signal by an excluded volume effect.

The hybridisation mixture consisted of 1 x "salts" (diluted from 20 x "salts" stock containing 20X Denhardts, 400mM Tris - pH 8.0, 100mM EDTA, 200mM sodium phosphate - pH 8.0), 0.3M NaCl, 50% formamide, 10% dextran sulphate, 50µg/ml yeast tRNA and the riboprobe diluted to a suitable concentration. The hybridisation mixture was spun and vortexed in order to remove air bubbles. The probe was denatured at 80°C for 2-3 minutes and rapidly cooled on ice for 2 minutes. 10µl aliquots of hybridisation mixture were then placed on each strip of sections and covered with a siliconised coverslip (previously prepared by dipping for 10 seconds in 100% ethanol then 20 seconds in silane followed by 10 seconds in 100% ethanol). Hybridisation was carried out overnight at 55°C in a humidified chamber.

Post-hybridisation washing

The purpose of post-hybridisation washing is to remove as much unhybridised probe as possible, thus resulting in a lower background. This involves washing at high stringency, thus breaking the weaker interactions involved in non-specific binding of probe to tissue sections and RNase treatment, which specifically degrades single-stranded RNA.

Coverslips were first removed by incubating for 20 minutes in pre-warmed 5X SSC (sodium chloride/ sodium citrate), 10mM DTT at 55°C. The first wash consisted of 50% formamide, 2X SSC, 10mM DTT at 55°C but

with the temperature increased to 65°C over the 20 minute period. Slides were washed for a further 20 minutes in the same mixture at 65°C and were then washed twice in 0.5M NaCl, 10mM Tris - pH 7.5, 5mM EDTA at 37°C for 15 minutes and then incubated in 40 µg/ml RNase at 37°C for 15 minutes in the same buffer. This was followed by a further stringent wash in 50% formamide, 2X SSC and 10mM DTT for 25 minutes at 65°C and four 10 minute washes at room temperature in decreasing salt concentrations of 2X SSC (twice) and 0.1X SSC (twice). Sections were then dehydrated through alcohols (30%, 50%, 70%, 90%) containing 0.3M ammonium acetate for 1 minute each followed by 5 minutes in absolute alcohol and then allowed to air dry.

Autoradiography, developing and staining

Emulsion was prepared as described previously (see pp.81). Slides were dipped and allowed to dry before packaging with dessicant in light-proof boxes and exposure at 4°C for approximately 4-6 weeks.

Slides were developed in Kodak D19 developer for 5 minutes, rinsed in ddH₂O and fixed in Kodak Unifix (5 minutes). Sections were then stained in methyl green (mouse *Hox-7.1* and *Hox-8.1* experiments), malachite green (chick *Hox-7.1* normal distribution) or haematoxylin and eosin (chick *Hox-7.1* experiments) and were air dried and then coverslipped without dehydration through alcohol, which distorts the emulsion.

RESULTS

Expression of mouse *Hox-7.1* and *Hox-8.1*

Expression in normal limb development

Expression of *Hox-7.1* and *Hox-8.1* in 11 day mouse embryo limb buds is shown in Figure 3.01. *Hox-7.1* transcripts were abundant throughout the distal mesenchyme whereas *Hox-8.1* transcripts were restricted to a smaller area, although transcripts for both genes appeared to be abundant in the surrounding distal ectoderm. In the proximal region of the limb *Hox-7.1* was expressed in anterior and posterior peripheral domains but was absent from the core mesenchyme. *Hox-8.1* was also expressed in anterior and posterior peripheral domains although these were less extensive than the corresponding *Hox-7.1* domains. Thus *Hox-8.1* transcripts in the limb bud mesenchyme appear to be confined to a subset of the tissue that also expresses *Hox-7.1*. Computer digititalised threshold imaging (see Monaghan *et al.* 1991) showed that *Hox-7.1* was mainly expressed in the distal mesenchyme beneath the AER although some transcripts were seen in the AER (Fig. 3.01 F). In contrast *Hox-8.1* transcripts were restricted to a very thin rim of mesenchyme directly beneath the AER but were extremely abundant in the AER.

Distal tips in culture

Distal tips from mouse limbs grown in culture for 24 hours in either DM or DM + S expressed both *Hox-7.1* and *Hox-8.1*. *Hox-7.1* was expressed in a fairly extensive zone directly beneath the AER (Fig. 3.02 B). *Hox-8.1* was expressed in the AER and the directly adjacent distal ectoderm (Fig. 3.02 D). A nearby section hybridised with the control probe is shown in Figure 3.02 C. These expression patterns of

Hox-7.1 and *Hox-8.1* were consistent with the normal expression pattern seen in intact limbs.

Distal tips cultured for 24 hours after removal of the AER in either DM or DM + S continued to express both *Hox-7.1* and *Hox-8.1*. As seen in Figure 3.02 E,F the expression of *Hox-7.1* did not appear to be reduced by removal of the AER and *Hox-8.1* was still expressed in the ectoderm adjacent to the removed AER (Fig. 3.02 H). An adjacent section hybridised with the control probe is shown in Figure 3.03 G.

Expression in micromass culture

Cultures of 500 μ m tips grown in DM + S for 48 hours expressed *Hox-7.1* and *Hox-8.1*. A very high level of *Hox-7.1* transcripts were found in large patches which constituted the majority of the culture (Fig. 3.03 A,B). The distribution of *Hox-8.1* transcripts (Fig. 3.03 D) was similar to *Hox-7.1*, but the transcripts were considerably less abundant, often with labelling at levels just above that of sense-strand controls (Fig. 3.03 C).

Hox-7.1 and *Hox-8.1* were both expressed under the AERs grafted at 24 hours of culture (Fig. 3.03 E-H). The level of transcripts in these regions did not appear to be greater than that in the surrounding culture, although since the whole cultures were expressing *Hox-7.1* and *Hox-8.1* this was very difficult to determine.

Cultures of proximal limb tissue grown in DM + S for 48 hours also expressed *Hox-7.1* and *Hox-8.1* (Fig. 3.04), although at considerably lower levels than the distal cultures. Cultures made from a mixture of 50% proximal cells and 50% distal cells did not appear to express *Hox-7.1* or *Hox-8.1* transcripts at levels above those of the proximal cultures. AERs were grafted onto the proximal cultures at 24 hours but were only probed with *Hox-8.1* (Fig. 3.04 E,F). The level of transcripts was however the same under the AER as in other regions of

the cultures.

Expression of chick *Hox-7.1*

Expression in normal limb development

Hox-7.1 transcripts were found in two regions of developing limb buds. Firstly transcripts were present at high levels in the distal-most mesenchyme of the limb bud and in the overlying AER. At stages 19-23 this distal expression extended approximately 120 μ m from the AER (Fig. 3.05 A-F), whereas by stage 25 transcripts could only be detected within approximately 90 μ m of the AER (Fig. 3.05 G-I). Secondly, *Hox-7.1* transcripts were expressed in peripheral anterior and posterior domains. At stage 19 transcripts were expressed along the entire anteroposterior length of the limb bud extending anteriorally along the flank (Fig. 3.06 A-C). By stages 23 and 25 the anterior domain of expression still extended along the entire anterior boundary of the limb bud and into the flank but the posterior expression had become restricted to the most distal region of the bud and transcripts were no longer present at the posterior junction of the limb with the flank (Fig. 3.06 D-I).

Expression in micromass culture

Comparison of *Hox-7.1* expression in cultures made from proximal cells derived from the entire anteroposterior length of the bud was made with those derived from the central region alone. Expression was quantified by counting the number of silver grains in a given area of 50 μ m²; the results are summarised in Table 3.1. *Hox-7.1* transcripts were detected in cultures made from proximal tissue encompassing the entire anteroposterior region of the limb (Fig. 3.07 A-C). The level of transcripts, as determined by the counting of silver grains, did not

differ substantially between cultures grown in the presence or absence of serum (see Table 3.1). In both cases transcripts were not uniformly expressed but appeared to be confined to distinct patches throughout the culture. In contrast no transcripts were detected in cultures made from the proximal core region (Fig. 3.07 D-F) and grown in the presence or absence of serum. Incorporation of RA into the medium of these cultures did not result in expression of *Hox-7.1* transcripts.

A comparison of *Hox-7.1* expression was also made in distal cell micromass cultures grown in a variety of different media (see Table 3.2). Transcripts were not detected in cultures grown in DM alone or in the presence of 10% serum, 10ng/ml bFGF or 1 μ g/ml RA; however cultures grown in the presence of bFGF and RA did express *Hox-7.1* transcripts (Fig. 3.08). The rate of cell proliferation at 24 hours of culture was determined in four separate trials. Cultures grown in bFGF and RA exhibited a similar rate of proliferation (28.4%) to those grown in bFGF alone (27.8%), therefore the expression of *Hox-7.1* was not influenced by the rate of proliferation, since addition of bFGF alone did not cause expression of transcripts.

Finally the ability of a grafted AER to stimulate *Hox-7.1* expression was determined. AERs were grafted onto 24 hour old distal micromass cultures and incubated for a further 3 days before fixing. As previously shown (Chapter 1), this resulted in the formation of a large mesenchymal outgrowth. *Hox-7.1* transcripts were expressed at very high levels by cells in these mesenchymal outgrowths, but remained undetectable in other regions of these cultures (Fig. 3.09). To determine whether the grafted AER was acting to maintain *Hox-7.1* expression or to induce it *de novo*, some of the cultures were fixed and probed at 24 hours of culture. These cultures did not express *Hox-7.1* transcripts (Fig. 3.09 G-I), therefore it could be concluded that factors produced by the AER induce transcription of the *Hox-7.1* gene,

at least in cells which retain the ability to express this gene. In an additional experiment 4 day old distal cell cultures grown in DM + S, onto which AERs had been grafted after 24 hours of culture, were also examined for *Hox-7.1* transcripts. In four out of four cases no expression of transcripts was found in cells beneath the grafted AER, or in any other regions of these cultures.

Table 3.1 - silver grain counts on proximal cell micromass culture

origin of cells	culture medium	silver grains/ $50\mu\text{m}^2$		
		<i>Hox-7.1</i>	Control	Nett
all prox.	DM	121.3	58.0	63.3
	DM + S	128.7	64.0	64.7
central	DM	77.5	79.0	- 1.5
	DM + S	43.2	57.0	- 13.5
	DM + RA	94.5	78.5	16.0

key: all prox. = cultures derived from the entire anteroposterior extent of proximal $500\mu\text{m}$ of the limb bud

Table 3.2 - silver grain counts on distal cell micromass cultures

culture medium	<i>Hox-7.1</i>	Control	Nett
<hr/>			
DM	66.7	52.0	14.7
DM + S	64.7	58.0	6.7
DM + bFGF	106.3	98.7	7.6
DM + RA	65.0	64.7	0.3
DM + bFGF + RA	198.7	63.3	135.4

Figure 3.01

(A) Diagram of an 11 day mouse embryo hindlimb showing the distal (a) and proximal (b) levels at which sections were taken, and probed for *Hox-7.1* (B,D) and *Hox-8.1* transcripts (C,E). Anterior is at the top of the page. Magnification is approximately x35.

(F) and (G) show computer-digitised threshold images (see Monaghan *et al.* 1991) of *Hox-7.1* and *Hox-8.1* expression respectively, in sections through the distal mesenchyme and AER of 11 day mouse hindlimb buds. The AER is indicated by arrowheads. Magnification is approximately x100.

Hox-7.1 was strongly expressed in the distal mesenchyme beneath the AER and proximally in anterior and posterior peripheral domains.

Hox-8.1 was expressed strongly in the AER and adjacent distal ectoderm, and also in a subset of the distal and proximal regions that expressed *Hox-7.1*.

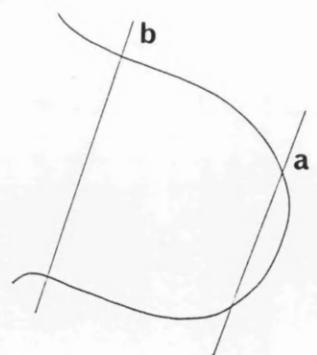
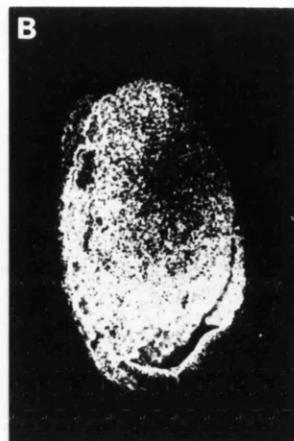
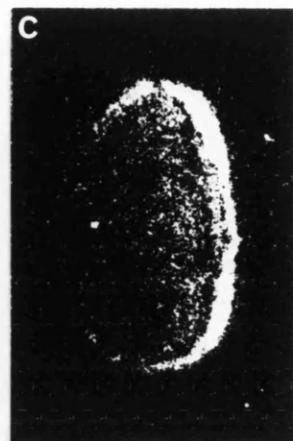
A**B****C****D****E****F****G**

Figure 3.02

(A) Light-field view of a mouse distal limb tip grown in culture for 24 hours. a = AER. (B) Dark-field view of the same section hybridised with the probe for *Hox-7.1* transcripts. (C) Dark-field view of a nearby section of the same tip hybridised with the control probe. (D) Dark-field view of another section of the same tip hybridised with the probe for *Hox-8.1* transcripts.

A high level of *Hox-7.1* transcripts was expressed in a large area of the distal mesenchyme underlying the AER, whilst *Hox-8.1* transcripts were expressed in the AER at a high level and in the distal-most mesenchyme at a lower level.

(E) Light-field view of a mouse distal limb bud tip grown in culture for 24 hours following surgical removal of the AER. (F) Dark-field view of the same section hybridised with the probe for *Hox-7.1* transcripts. (G) Dark-field view of a nearby section of the same tip hybridised with the control probe. (H) Dark-field view of another section of the same tip hybridised with the probe for *Hox-8.1* transcripts. Arrowheads indicate the cut edges of the ectoderm.

A high level of *Hox-7.1* transcripts were still expressed in an extensive area of distal mesenchyme 24 hours after removal of the AER. *Hox-8.1* transcripts were expressed in a small region of the ectoderm adjacent to the removed AER at a high level, and at a lower level in the distal-most mesenchyme of the tip.

Scale bar = 100 μ m.

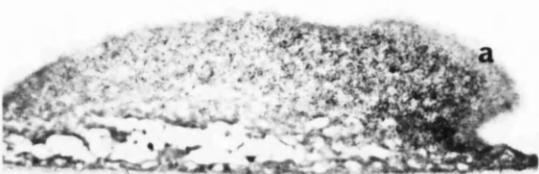
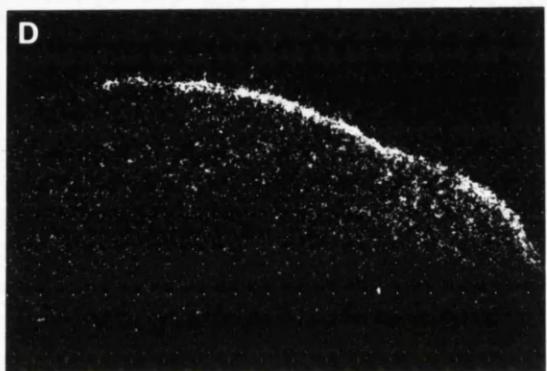
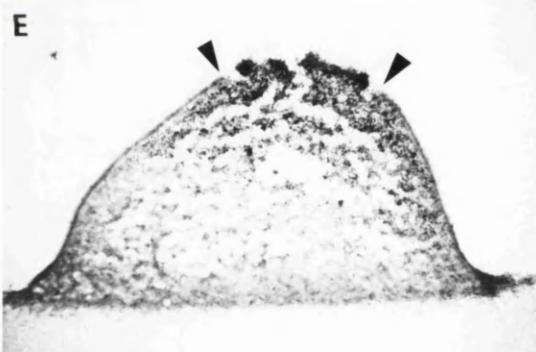
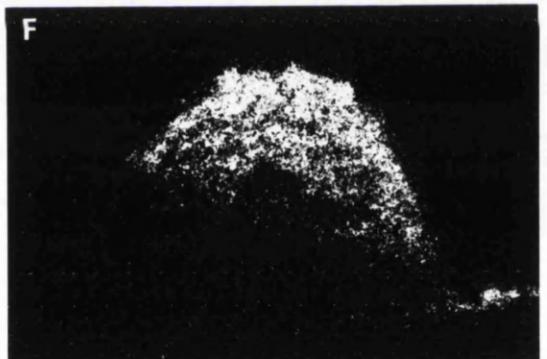
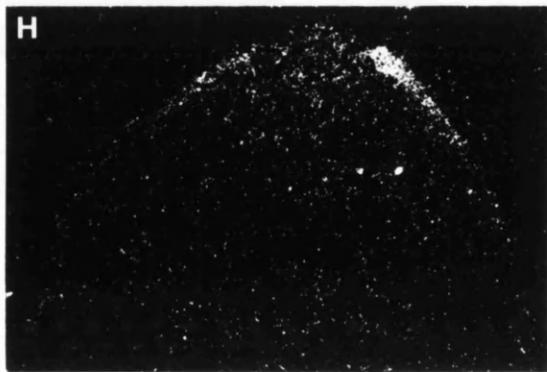
A**B****C****D****E****F****G****H**

Figure 3.03

(A) Light-field view of a section through a 2 day distal cell micromass culture. (B) Dark-field view of the same section hybridised with the probe for *Hox-7.1* transcripts. (C) Dark-field view of a nearby section of the same culture hybridised with the control probe. (D) Dark-field view of another section through the same culture hybridised with the probe for *Hox-8.1* transcripts.

Scale bar = 200 μ m.

A high level of *Hox-7.1* transcripts was expressed throughout the culture. *Hox-8.1* transcripts were also expressed throughout the culture at a relatively low level with occasional patches of increased expression. The level of *Hox-8.1* transcripts was however substantially lower than the level of *Hox-7.1* transcripts expressed in these cultures.

(E) Light-field view of a region of a 2 day distal cell mouse micromass culture onto which a chick AER had been grafted 24 hours previously. a = AER. (F) Dark-field view of the same section hybridised with the probe for *Hox-7.1* transcripts. (G) Dark-field view of a nearby section hybridised with the control probe. (H) Dark-field view of another section hybridised with the probe for *Hox-8.1* transcripts.

Scale bar = 50 μ m.

The mesenchyme underlying the grafted AER expressed a high level of *Hox-7.1* transcripts and a lower level of *Hox-8.1* transcripts. However all regions of the culture expressed these transcripts normally, thus it was impossible to determine whether the level of transcripts was elevated beneath the grafted AER. Note also the highly species-specific nature of these probes, since the grafted chick AER did not label for *Hox-8.1* transcripts.

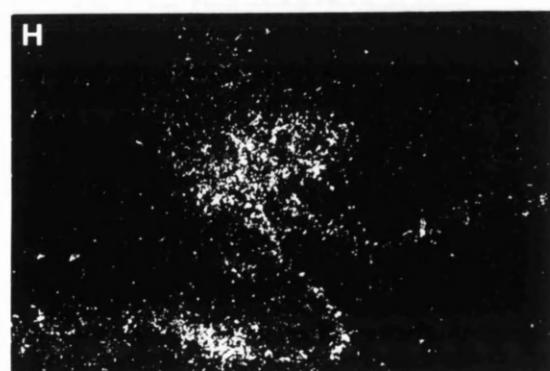
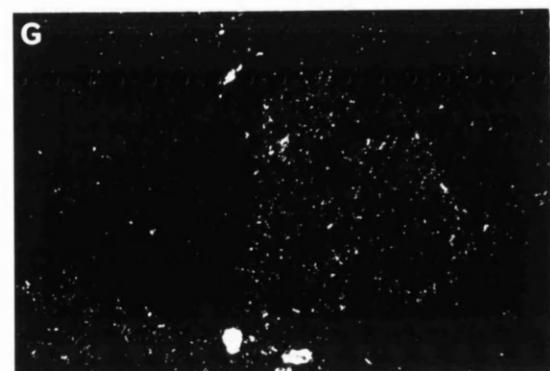
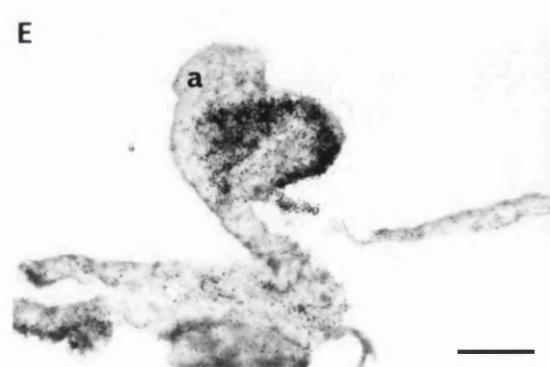
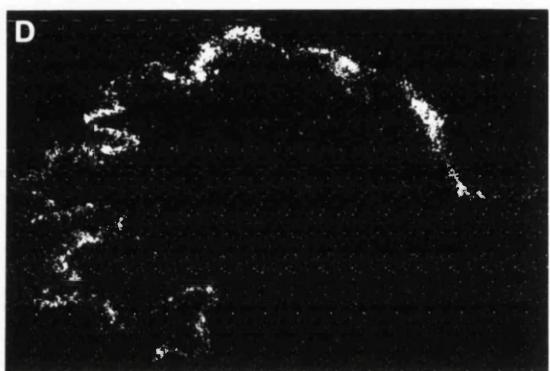
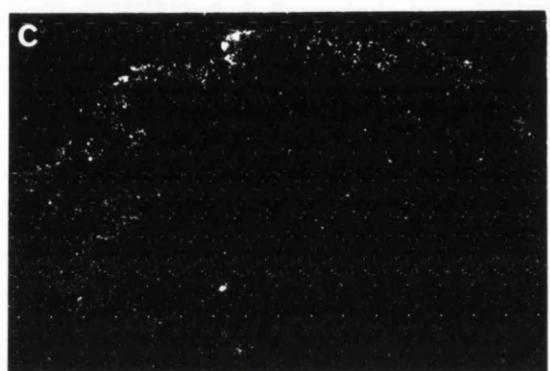


Figure 3.04

Light-field (A,C,E) and corresponding dark-field (B,D,F) views of sections through 2 day proximal cell micromass cultures hybridised with the probes for *Hox-7.1* (A,B), *Hox-8.1* (E,F) or the control probe (C,D). In sections (C-F) a chick AER was grafted onto the cultures 24 hours earlier. a = AER.

Scale bar (A,B), (C-F) = 100 μ m.

Proximal cell cultures expressed *Hox-7.1* transcripts at a level significantly higher than the level of labelling seen with the control probe. In contrast, *Hox-8.1* transcripts were barely detectable above the level of labelling seen with the control probe. The level of *Hox-8.1* transcripts seen beneath the grafted AER did not differ from the level seen in other parts of the same culture. The level of both transcripts seen in proximal cultures was significantly lower than that seen in the distal cultures.

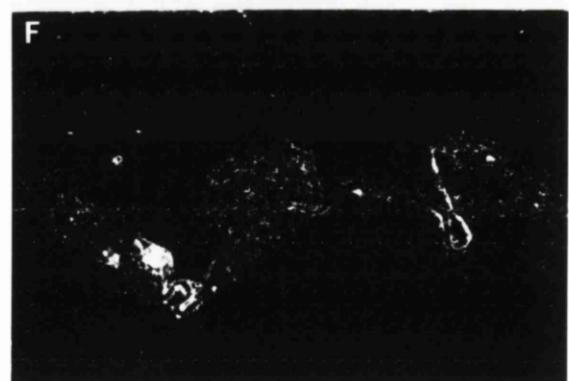
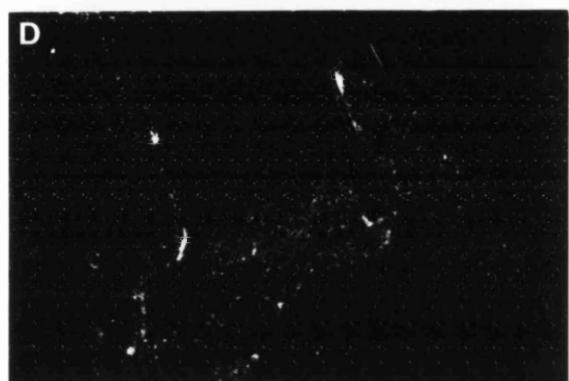
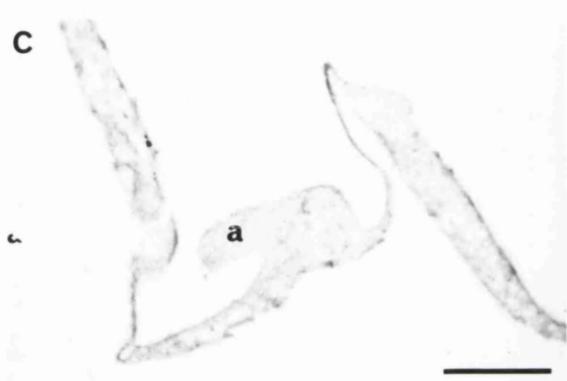
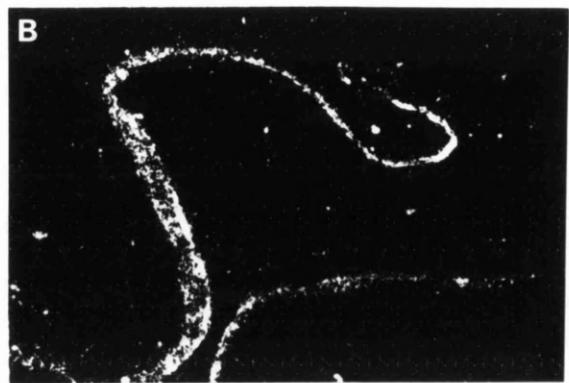


Figure 3.05

Sections through the long axis of the developing chick limb at stages 19 (A-C), 23 (D-F) and 25 (G-I). (A,D,G) show the respective light-field views and (B,E,H) the corresponding dark-field views of sections probed for *Hox-7.1*. (C,F,I) show dark-field views of adjacent sections probed with the control sense probe.

Scale bars = 250 μ m.

At all stages *Hox-7.1* was strongly expressed in the distal mesenchyme beneath the AER and in the AER. At stages 19-23 the limit of expression appeared to be about 120 μ m from the AER. At stage 25 *Hox-7.1* transcripts were also found in the proximal core region in this particular section, which corresponded to the anterior region of expression. Note also the restricted distribution of transcripts in the dorsal part of the neural tube (B).

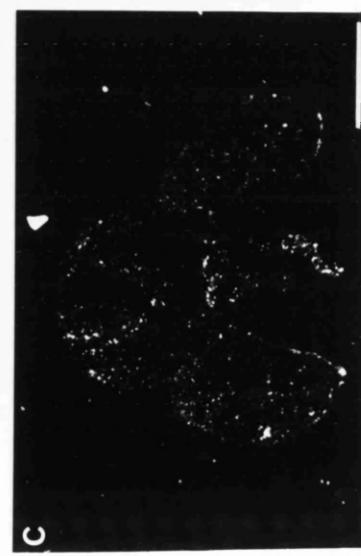
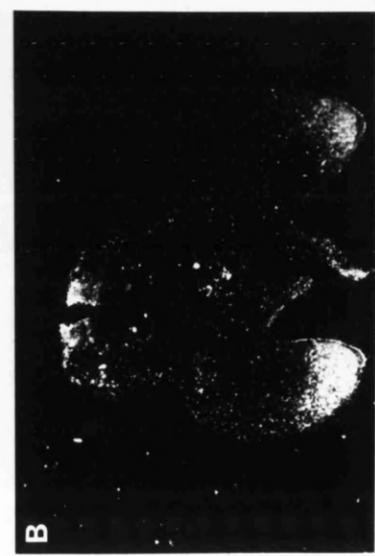
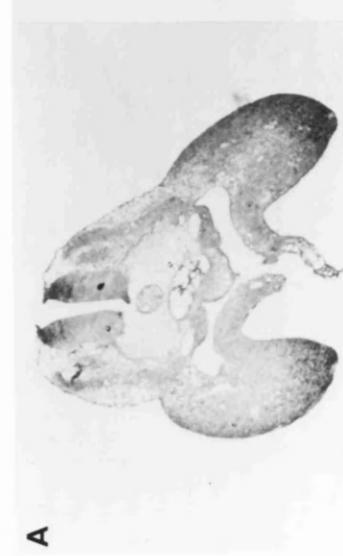


Figure 3.06

Frontal sections through the anteroposterior axis of developing limb buds at stages 19 (A-C), 23 (D-F) and 25 (G-I). (A,D,G) show the respective light-field views and (B,E,H) the corresponding dark-field views of sections probed for *Hox-7.1* transcripts. (C,F,I) show dark-field views of adjacent sections probed with the negative sense control probe. Anterior is at the top of the page.

Scale bar = 500 μ m.

At stage 19 *Hox-7.1* was expressed in the peripheral tissue of the flank anterior to the limb bud and in distal limb tissue across the whole anteroposterior length of the bud. At stages 23 and 25 the anterior domain of expression still extended from the flank tissue anterior to the limb to the tip of the limb. In contrast, the posterior domain of expression was limited to the distal-most part of the bud.

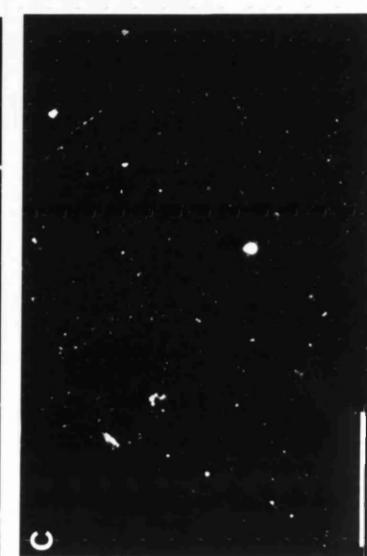
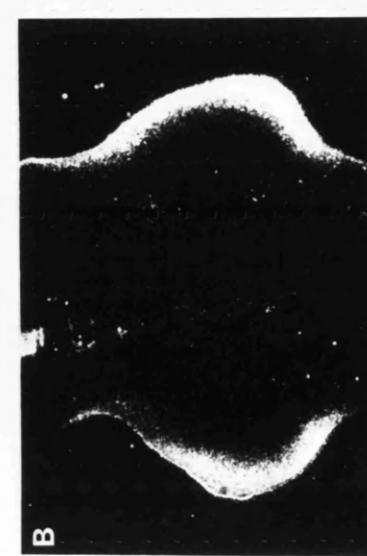
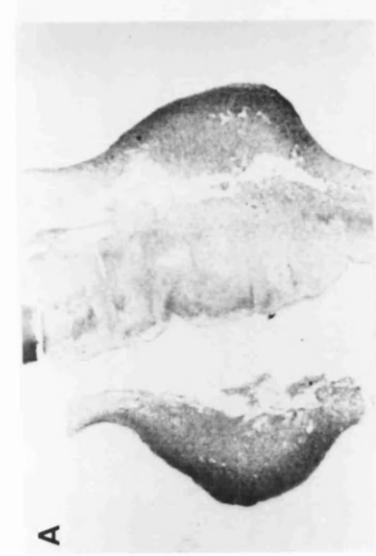
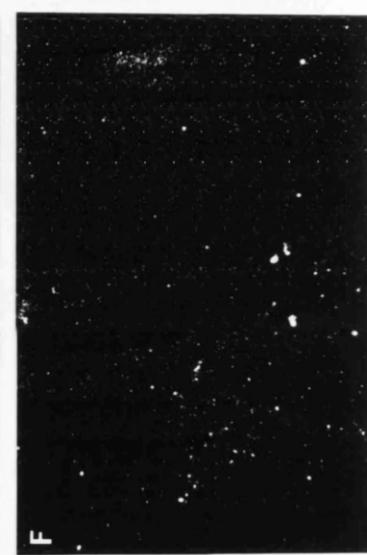
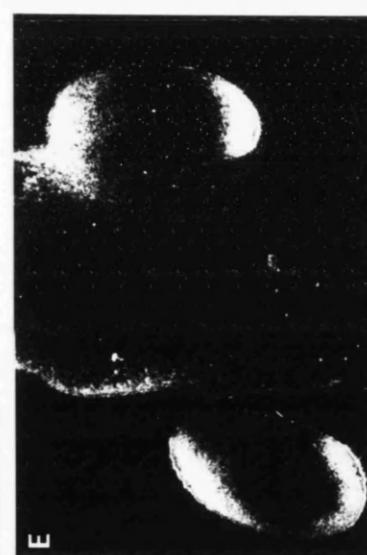
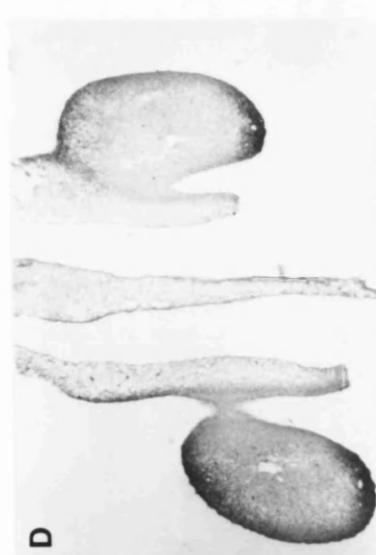


Figure 3.07

Proximal cell micromass cultures made from the whole proximal region (A-C) and the core region (D-F). (A,D) show the respective light-field views and (B,E) the corresponding dark-field views of sections through 3 day old cultures probed for *Hox-7.1* transcripts. (C,F) show dark-field views of adjacent sections probed with the control probe.

Scale bar = 50 μ m.

The cultures made from the whole proximal regions, including the anterior *Hox-7.1*-expressing cells, retained a patchy expression of transcripts, whereas cultures prepared from the core mesenchyme did not express *Hox-7.1*.

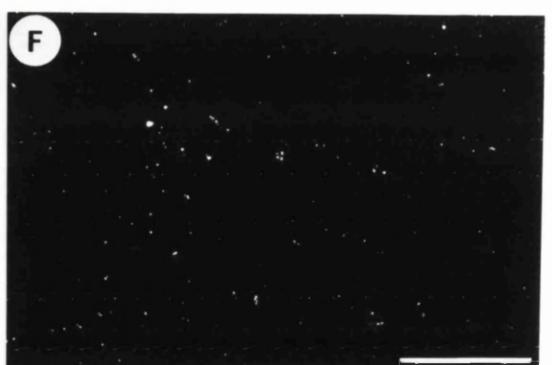
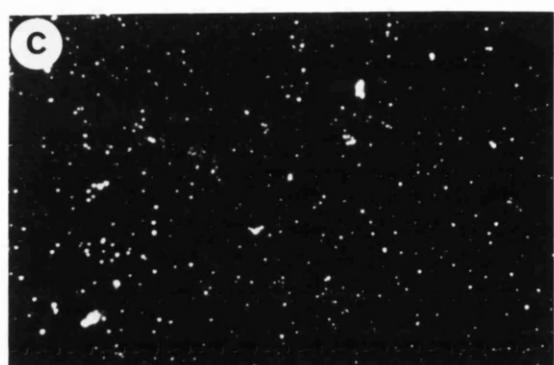
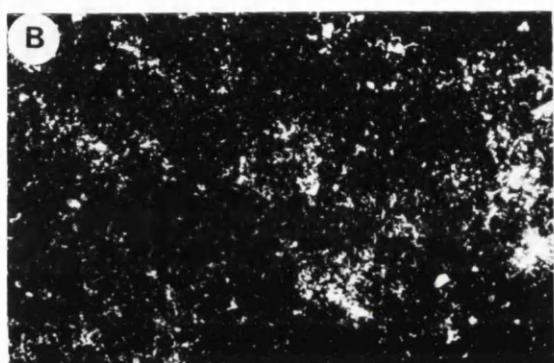
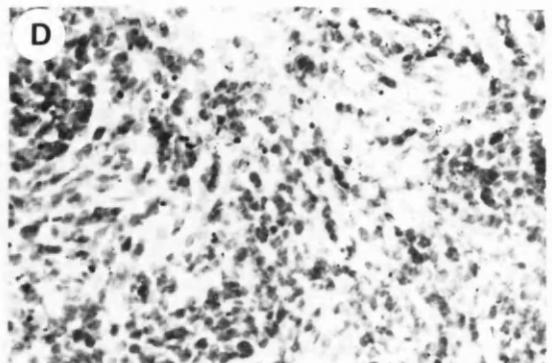
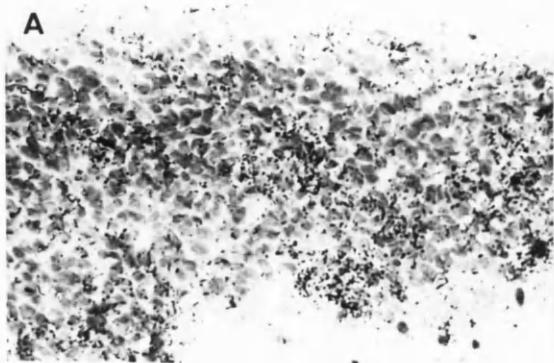


Figure 3.08

Micrographs of sections through 2 day old distal cell micromass cultures grown in DM + S (A-C), DM + 10ng/ml bFGF (D-F) and DM + 10ng/ml bFGF + 1 μ g/ml RA (G-I). (A,D,G) Light-field and (B,E,H) the corresponding dark-field views of sections probed for *Hox-7.1* transcripts. (C,F,I) show dark-field views of adjacent sections probed with the control probe.

Scale bar = 50 μ m.

Cultures grown in DM or in DM + bFGF did not express *Hox-7.1* transcripts as judged by comparison with the number of grains detected in control cultures. Cultures grown in bFGF + RA did express *Hox-7.1* transcripts, however the expression was not uniform since some cells expressed a high level of transcripts whereas others expressed much lower levels.

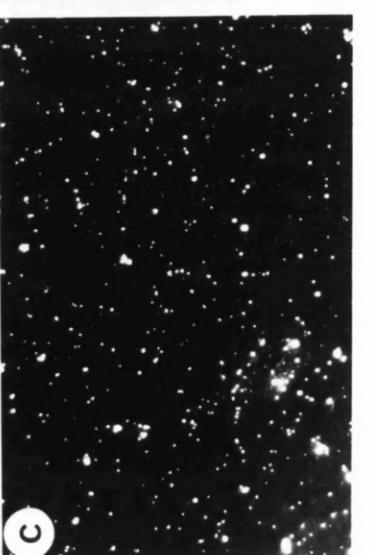
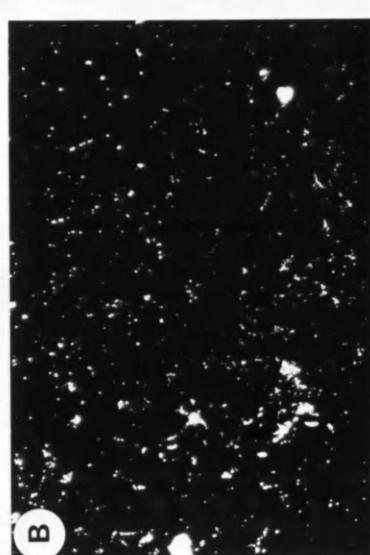
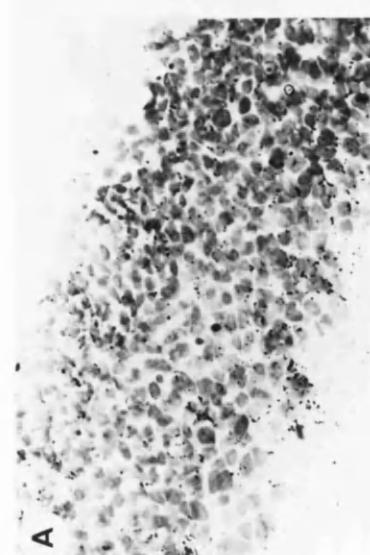
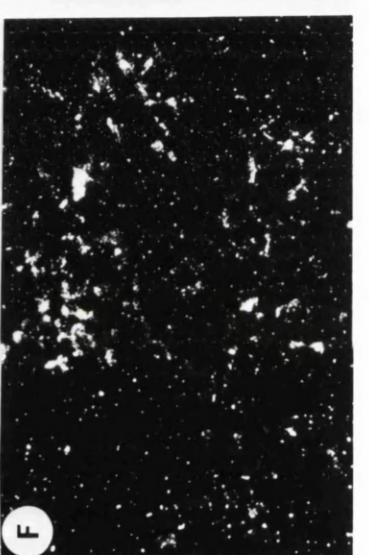
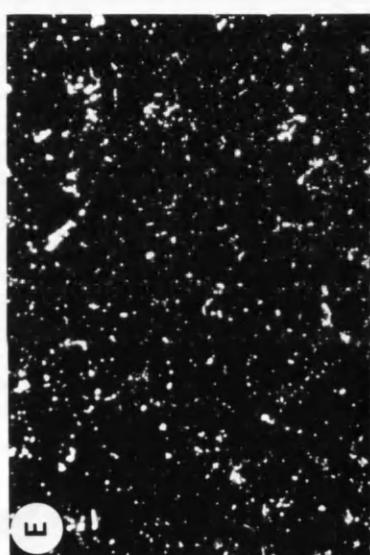
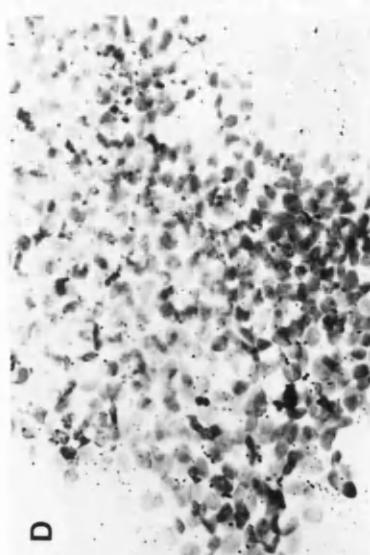
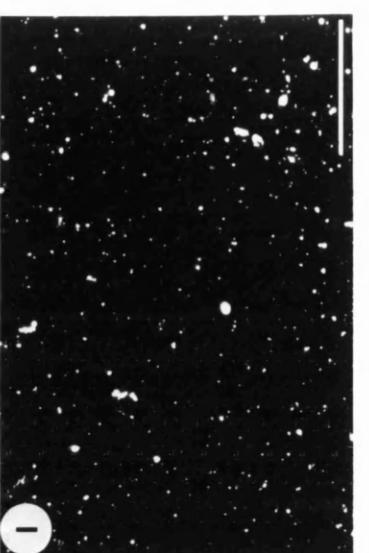
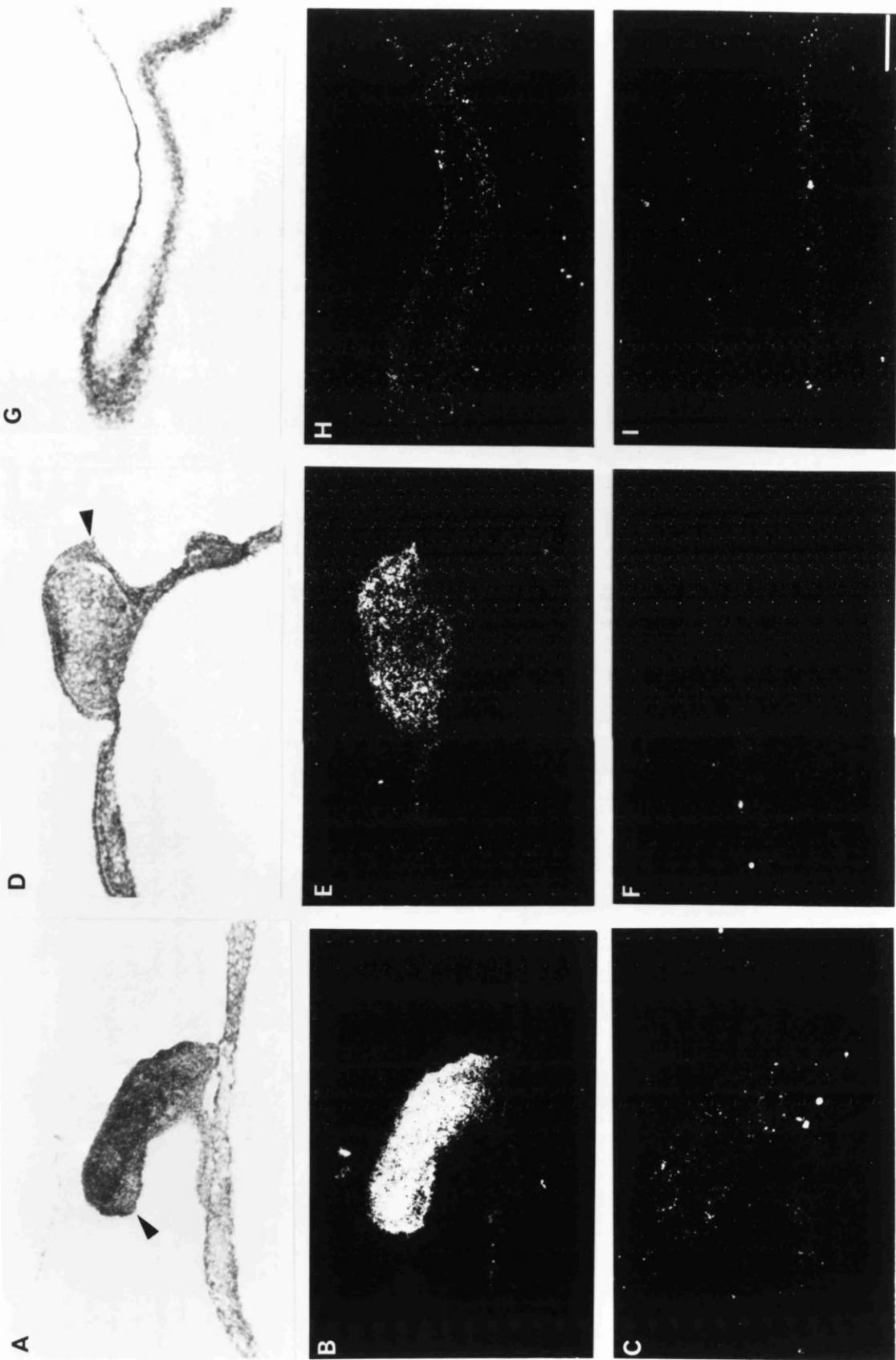


Figure 3.09

(A-F) Micrographs of 4 day old distal micromass cultures onto which AERs had been grafted after 24 hours of culture, and (G-I) 24 hour micromass culture from the same experimental run as (A-C). (A,D,G) show the respective light-field and (B,E,H) the corresponding dark-field views of cultures probed for *Hox-7.1* transcripts. (C,F,I) show dark-field views of adjacent sections probed with the control probe. AERs are indicated by arrowheads.

Scale bar = 100 μ m.

After 24 hours of culture, distal cell micromass cultures were not expressing *Hox-7.1* transcripts. If an AER was grafted onto these cultures and allowed to develop for a further 3 days, a large mesenchymal outgrowth was produced. Cells within this outgrowth expressed a high level of *Hox-7.1* transcripts although cells in the other regions of these cultures did not express transcripts. This shows that factors produced by the AER are capable of switching on *Hox-7.1* expression in cells which are competent to respond.



Expression of mouse *Hox-7.1* and *Hox-8.1*

In 11 day mouse limb buds *Hox-7.1* transcripts were detected in the distal mesenchyme beneath the AER and proximally, in peripheral anterior and posterior domains with a lower level of transcripts detected in the AER. *Hox-8.1* transcripts were most abundant in the AER and were also detected in the distal mesenchyme and in proximal anterior and posterior peripheral domains, however these transcripts were confined to a smaller area than the corresponding expression domains of *Hox-7.1*. These results are consistent with other reports and support the finding that *Hox-7.1* and *Hox-8.1* transcripts are expressed in overlapping domains. These expression patterns are also consistent with the hypothesis that *Hox-7.1* and *Hox-8.1* expression is induced by a signal produced by the AER and the anterior and posterior ectoderm, but that the threshold for *Hox-8.1* expression is greater and thus *Hox-8.1* is restricted to mesenchymal cells closer to the source of the inducer.

The expression of *Hox-7.1* and *Hox-8.1* in distal tips grown *in vitro* in either DM or DM + S for 24 hours is consistent with the expression in the intact limb, in that *Hox-7.1* was expressed in the distal mesenchyme but not in the AER, whilst a high level of *Hox-8.1* transcripts was expressed in the AER and distal ectoderm with a lower level in the distal-most mesenchyme. However 24 hours after removal of the AER *Hox-7.1* transcripts were still abundant in the distal mesenchyme. *Hox-8.1* expression was also maintained in the distal ectoderm adjacent to the removed AER and in the distal mesenchyme. These results do not support the findings of Davidson *et al.* (1991) and Robert *et al.* (submitted), who showed that expression of these two genes was dependent on inducing factors produced by the AER. It is possible that small fragments of AER remained on these limb tips, but

the majority of the AER was removed and could not be identified morphologically in sectioned tips. Another possibility is that *Hox-7.1* and *Hox-8.1* transcripts are relatively stable, although the finding of Davidson *et al.* that transcripts were undetectable in distal tissue grafted to a proximal site after only 5 hours suggests that this is not the case. Alternatively removing the AER may not be sufficient to cause downregulation of the level of transcripts, since the distal cells are still surrounded by distal extracellular matrix which may contain enough of the inducing factor to maintain transcription for a limited period, unlike the grafted distal tissue in the experiments of Davidson *et al.*. Micromass cultures of distal cells also retained expression of *Hox-7.1* and, at a lower level, *Hox-8.1*. Since the production of micromass cultures results in disruption and loss of the extracellular matrix, these results cannot be explained by such a sequestration of AER-produced factors. The finding that micromass cultures of proximal cells expressed *Hox-7.1* and possibly *Hox-8.1* transcripts, although at considerably lower levels than in distal cultures, suggests that either these cells are behaving autonomously in culture or that the culture system and/or factors in the medium are artificially stabilising these transcripts.

Expression of chick *Hox-7.1*

Expression in normal limb development

Hox-7.1 transcripts were expressed in the distal mesenchyme underlying the AER and in the mesenchyme along the entire anterior border of the limb and along the distal part of the posterior border. The finding that *Hox-7.1* expression was restricted to a depth of approximately 120 μ m from the ectoderm may suggest that it is controlled by a factor present in these specific regions of the ectoderm,

including the AER.

The function of *Hox-7.1* in limb development remains unclear, however it has been suggested that expression of *Hox-7.1* may retain cells in an undifferentiated state since *Hox-7.1* transcripts are found in the distal mesenchyme beneath the AER and at later stages in the interdigital mesenchyme, where it may prevent the development of ectopic cartilage between the digits. However *Hox-7.1* transcripts were also found in mesenchyme along the anterior margin of the limb bud where there is no apparent inhibition of differentiation.

Expression in micromass culture

Proximal cultures derived from the entire anteroposterior extent of the limb contain anterior cells which normally express *Hox-7.1*. After 2 days in culture it appears that these cells retained their expression of *Hox-7.1* since transcripts were only detected in isolated patches of cells surrounded by apparently non-expressing cells. Cottrill *et al.* (1987b) have previously shown that some degree of cell sorting occurs in proximal cultures, therefore it seems likely that *Hox-7.1* expressing anterior cells become concentrated in certain regions. In contrast cultures derived from the central region, which does not include the *Hox-7.1*-expressing anterior cells, did not express *Hox-7.1* transcripts.

A large proportion of the cells used to make distal cultures would normally express *Hox-7.1*, however expression was not retained in micromass culture, except in cultures grown in the presence of bFGF and RA. bFGF and RA are both implicated in limb development, especially in the distal-posterior region. This suggests that this combination of factors mimics the distal environment sufficiently to permit at least the maintenance of *Hox-7.1* transcripts.

Expression of *Hox-7.1* was maximally induced in mesenchyme cells

beneath a grafted AER. These cultures did not contain detectable transcripts at 24 hours of culture, when the AERs were grafted, thus this shows that factors produced by the AER are capable of stimulating expression of *Hox-7.1* and that this stimulation is brought about at the level of transcription.

The finding that *Hox-7.1*-expressing proximal cells retain expression of transcripts in culture whereas distal cells do not, suggests that expression of *Hox-7.1* may be under the control of different mechanisms in these two sites. The results reported here show that *Hox-7.1* expression in distal cells is controlled by factors produced by the AER. Potential candidates for such a factor are BMP-2A, which is localised in the AER in developing mouse limb buds (Lyons *et al.* 1989) and *Wnt-5a*, which is expressed in the ventral ectoderm in 9.5 day mouse limb buds and in the AER and distal mesenchyme at 10 days of development. At later stages, expression is maintained in the distal mesenchyme (Gavin *et al.* 1990).

The control of proximal *Hox-7.1* expression is likely to be caused by a different mechanism. In these cells either the transcripts are more stable, or transcription continues in culture, thereby suggesting that these cells are behaving autonomously, i.e. the fate of these cells is already established and involves constant *Hox-7.1* production which is independent of the need for any other stimulatory factor. This could be tested experimentally *in vivo* by grafting this anterior tissue to sites which do not express *Hox-7.1*. If *Hox-7.1* expression is cell autonomous transcripts should be retained in these grafts, whereas if expression is induced by the anterior ectoderm transcripts should disappear from the grafts. If however *Hox-7.1* expression in anterior proximal tissue is induced in response to an ectodermally produced factor, possible candidates include members of the vertebrate *Wnt* family. These are secreted peptide factors which bind strongly to the

extracellular matrix and thus presumably act over short distances. They are the vertebrate homologues of the *Drosophila* putative growth factor *wingless*, which controls the expression of the homeobox gene *engrailed* and thus leads to the delineation of segmental boundaries in the thorax and abdomen. At least three members of this family have been detected in the ectoderm of the developing limb. *Wnt-7a* is expressed in the dorsal apical ectoderm (see Tabin, 1991) and *Wnt-3* is expressed in the ectoderm of the whole bud (Roelink and Nusse, 1991). Since a thorough analysis of the distributions of all members of this family has not yet been performed it remains possible that one gene will be found which is restricted to the ectoderm overlying areas of *Hox-7.1* expression.

Alternatively the expression of *Hox-7.1* in anterior proximal regions may be controlled by the interaction of several factors.

The experiments described here have also shown that transcription of the *Hox-7.1* gene is not upregulated by RA alone, although it appears that either transcript stability or transcription are favoured by addition of RA in the presence of bFGF. This does however suggest that *Hox-7.1* is not inhibited by RA, as appears to be the case for *Hox-8.1* (Yokouchi *et al.* 1991). The ability of distal cells to express *Hox-7.1* in the presence of bFGF and RA suggests that these factors act synergistically and may play an important role in the normal physiology of the progress zone *in vivo*. Retinoids have been identified in distal mesenchyme, although not at the doses used in these experiments; however it should be noted that the effects of more physiological doses were not investigated in these experiments. bFGF has not been identified in the distal mesenchyme, but the secreted factor FGF-4 is synthesised by posterior AER cells and the results presented in Chapter 2 strongly suggest that FGFs are present in the distal mesenchyme.

The ability of cells to express *Hox-7.1* may be correlated with the ability of these cells to respond to factors produced by the AER.

Thus if cells lose the ability to express *Hox-7.1* they may also lose the ability to respond to a grafted AER and thus fail to produce a mesenchymal outgrowth. The failure of distal cultures to produce mesenchymal outgrowths beneath grafted AERs when grown in DM + S (see Chapter 1, section 1.2) might therefore result from the inability of these cells to express *Hox-7.1*. This is supported by the preliminary finding that cells beneath grafted AERs in cultures grown in DM + S did not express *Hox-7.1* transcripts.

This hypothesis can also explain the fact that small regions of proximal cell cultures of all ages were able to respond to and in turn maintain a small fragment of AER, since *Hox-7.1* is expressed in a subset of the cells used to make these cultures. It has been shown that limited cell sorting occurs in proximal cell micromass cultures, and this is supported by results presented here, in that *Hox-7.1* expression in proximal cultures tends to be located in patches. If it is assumed that the other proximal cells used to make proximal cultures have lost the ability to express *Hox-7.1*, then an AER grafted onto a proximal culture would act to stimulate and maintain *Hox-7.1* expression only in cells originally from the anterior portion of the limb. Presumably the AER-mesenchymal interaction would only be successful in terms of inducing a small outgrowth and maintaining the AER in areas of the culture which contained a sufficient proportion of these cells. Since *Hox-7.1* is also expressed by cells of the frontonasal mass (Hill *et al.* 1989) but not in the flank between the limb buds (Davidson - personal communication), this hypothesis also explains the fact that FNM cultures but not flank cultures were capable of responding to, and maintaining parts of, a grafted AER.

These results have shown that the pattern of *Hox-7.1* expression in mouse and chick limb buds is similar although there appear to be some differences. For example *Hox-7.1* was not detected in the AER of

mice and was apparently expressed in proximal posterior tissue, whereas chick *Hox-7.1* was present in the AER and absent from all but the most distal posterior tissue. The expression *in vitro* however was not similar. Micromass cultures prepared from mouse distal limb tissue continued to express a high level of *Hox-7.1* transcripts after 48 hours in culture, whereas chick distal cultures did not contain a detectable level of transcripts after 24 hours. It seems extremely unlikely that the control of *Hox-7.1* expression in the distal limb is substantially different in these two species. This is reflected by the highly conserved nature of vertebrate development including features such as identical patterns of homeobox gene expression and cross-reactivity of mouse tissue in interspecific grafting experiments into chick embryos. Therefore it appears that this contradictory result must have been caused by differences in the behaviour of mouse and chick cells in culture. Since the culture medium used for each species was the same, it appears that *Hox-7.1* transcripts were artificially stabilised in mouse cells. These results suggest that the micromass culture system does not provide an accurate reflection of *in vivo* development of the mouse; however the behaviour of chick cells in micromass culture appears to reflect *in vivo* development more closely, since *Hox-7.1* is not expressed in distal cells in the absence of an AER.

The major finding of this work is that the AER induces *Hox-7.1* expression in cells which had stopped expressing these transcripts. This confirms the findings of Davidson *et al.* (1991) and Robert *et al.* (submitted), and therefore proves that factors produced by the AER induce transcription of the *Hox-7.1* gene. An important continuation of this work will be to discover the minimum amount of time required for *Hox-7.1* transcripts to appear beneath a grafted AER, although the appearance of transcripts within 5 hours in grafts of proximal tissue to the tip of the limb in the grafts of Davidson *et al.* suggests that

the AER-produced factor acts directly on the transcription of the *Hox-7.1* gene rather than through an indirect pathway.

CHAPTER 4

EXPRESSION OF THE RETINOIC ACID RECEPTOR BETA IN LIMB DEVELOPMENT AND IN MICROMASS CULTURE

INTRODUCTION

RA as a morphogen

Retinoic acid (RA) is now becoming strongly implicated as a morphogen in embryogenesis. It has been known for sometime that RA treatment or vitamin A deficiency leads to a wide range of developmental abnormalities (Lammer *et al.* 1985; Tamarin *et al.* 1984); however detailed studies in a variety of developmental systems have confirmed the significance of RA. In addition, the discovery of retinoic acid receptors (RAR) and cytoplasmic binding proteins has established the cellular components capable of mediating the effects of RA.

Nuclear receptors for RA have now been isolated from several vertebrate species. There are three members of the retinoic acid receptor family, designated α , β and gamma. These receptors, which exist in several different isoforms (Ruberte *et al.* 1991), are highly conserved between vertebrate species and appear to utilise RA as their specific ligand. The RARs are all transcription factors and presently little is known of their downstream targets.

Cytoplasmic cellular binding proteins for both RA (CRABP) and its precursor retinol (CRBP) also exist. In embryonal carcinoma cells, increased expression of CRABP results in a desensitising of cells to the effects of RA (Boylan and Gudas, 1991). This supports the view that

the role of CRABP is to sequester RA in the cytoplasm, thus preventing it from interacting with nuclear receptors (Dollé *et al.* 1990). Thus high expression of CRABP may be associated with cells which are sensitive to RA and require very low levels for their normal differentiation and function.

RA has been implicated as the morphogen responsible for establishing positional values along the anteroposterior axis of the embryo and also of the developing limb bud. In chick limb development local application of RA to the anterior margin of the wing bud results in duplication of the digits (Tickle *et al.* 1982), which is preceded by duplication of the *Hox-4* gene pattern (Izpisua-Belmonte *et al.* 1991). This mimics the effect of grafting cells from the posterior polarising region of the limb. Two explanations for this effect have been suggested. The first assumes that RA is the morphogen produced by the polarising region. Locally applied retinoids have been shown to become distributed in a gradient across the anteroposterior axis of the limb (Tickle *et al.* 1985). A gradient of RA could then provide the required positional information if each digit is specified at a particular retinoid concentration. Alternatively RA could induce anterior tissue to become a second polarising region which in turn releases some other morphogen resulting in the duplication of pattern (Wanek *et al.* 1991; Noji *et al.* 1991; Brockes 1991). Endogenous retinoids have been identified in developing wing buds at concentrations which are similar to those required to induce digit duplications (Thaller and Eichele, 1987); with the level of endogenous RA greater posteriorly than anteriorly. Another metabolite of retinol, 3,4-didehydroretinoic acid, has also been isolated from wing buds (Thaller and Eichele, 1990) and is present at levels approximately six times that of RA, whilst having a similar ability as RA to induce digit duplications.

There is less evidence supporting the role of RA as the morphogen

of the main body axis, although RA treatment of *Xenopus* embryos results in the progressive loss of anterior fore- and mid-brain structures with increasing dose. This is accompanied by concomitant expansion of the hindbrain (Durston *et al.* 1989). It has been proposed that anteroposterior pattern is laid down during gastrulation and that involuting mesoderm and overlying neuroectoderm are initially specified with an anterior positional identity. The dorsal lip of the blastopore might then act as the source of a posteriorising substance, possibly RA. The earliest mesoderm to form, which had migrated some distance from the blastopore, would largely escape the influence of RA and would therefore retain an anterior positional value; whereas later mesoderm would be nearer to the blastopore and therefore have a more posterior positional value (Green, 1990). Further support for this hypothesis is the finding that Hensen's node, the chick anatomical equivalent of the dorsal lip of the blastopore, possesses polarising activity when grafted to the anterior margin of the chick wing bud (Hornbruch and Wolpert, 1986), thus mimicking the action of locally applied RA. However this evidence does not rule out the possibility that the endogenous factor responsible for establishing anteroposterior positional identity is not RA, but is mimicked by RA; alternatively RA might result in the induction of synthesis of such a factor when applied locally to certain tissues.

Further support for the role of RA as a morphogen comes from studies showing the interaction of RA and homeobox genes. Homeobox genes are clearly involved in patterning in *Drosophila* embryogenesis (Akam, 1987; Ingham, 1988) and are also strongly implicated in patterning in vertebrate development, partly due to the fact that they are expressed in distinct temporal and spatial domains during the development of a wide range of embryonic structures. In murine development, genes of the *Hox-2* cluster are expressed along the

anteroposterior axis of the CNS with 3' genes exhibiting more rostral boundaries of expression in the myelencephalon and 5' genes exhibiting more caudal boundaries of expression in the spinal cord. Simeone *et al.* (1990) showed that genes of the HOX-2 cluster are sequentially activated in a 3' to 5' direction in the human embryonal carcinoma cell line NT2/D1 following application of RA. 3' genes were maximally induced at RA concentrations of 10^{-8} M, whilst 5' genes were less responsive requiring a dose of 10^{-5} M for maximal expression. Thus differential expression of *Hox-2* genes along the anterior-posterior axis of the CNS could in theory be explained by an anterior-posterior gradient of RA with maximal expression posteriorally. Further support for a central regulatory role for RA are the recent findings that RA has now been shown to induce expression of members of all four class I homeogene clusters in human embryonal carcinoma cells (Boncinelli *et al.* 1991).

Direct evidence for the ability of RA to change the positional identity of cells comes from the urodele regenerating limb (reviewed by Stocum, 1991). Following amputation of the adult limb, the mesodermal cells undergo dedifferentiation beneath a wound epidermis, forming a small population of actively dividing cells known as the regeneration blastema. Positional identity is specified in the blastema such that only the amputated structures are replaced; however if RA is exogenously applied it causes the cells of the blastema to become respecified with more proximal positional identities (Maden, 1982). Thus a whole limb can be made to regenerate from a stump amputated at the level of the wrist. The phenomenon is also dose-dependent, in that increasing doses of RA cause blastemal cells to acquire increasingly proximal positional identities. There is also some evidence that RA may affect positional identity in the anteroposterior and dorso-ventral axes of the blastema. The molecular basis of these effects is as yet

unknown, although the three members of the RAR family (Giguere *et al.* 1989) and CRABP (Keeble and Maden, 1986) have been isolated from regenerating limb cDNA libraries, therefore the machinery responsible for mediating the effects of RA is present, although it has yet to be proved that RA is the endogenous factor responsible for establishing positional identity within the normal urodele limb.

The direct effect of RA on gene expression has best been illustrated in studies using a variety of cell lines, including certain teratocarcinoma cell lines, which can be induced to differentiate by the addition of RA (Strickland and Mahdavi, 1978). Analysis of the effect of RA addition to cultures of these cells has shown that genes encoding transcription factors are among the primary targets of RA action. In F9 teratocarcinoma cells, the levels of transcripts of the homeobox-containing transcription factors *Hox-1.6* and *Hox-1.3* are rapidly induced in response to RA (LaRosa and Gudas, 1988; Murphy *et al.* 1988), as are transcripts of the retinoic acid receptor β (Hu and Gudas, 1990). Many of the genes which exhibit an early response to RA have been shown to possess retinoic acid response elements (RARE). Other genes are affected several days after RA application; these include genes encoding structural proteins and polypeptide growth factors and their receptors (reviewed by Gudas, 1991). The set of transcription factors rapidly induced in response to RA treatment is similar in a variety of cell types; however the secondary target genes which are affected later are different. Thus Gudas (1991) has suggested that the differential accessibility of these secondary target genes to RA in different cell types enables RA to have such a wide variety of effects, and thus in turn lends support to the role of RA as a morphogen which can be used to establish positional fields in different spatial and temporal domains throughout embryogenesis.

Dollé *et al.* (1989) examined the distributions of CRABP and the three RARs during mouse limb development. At early stages when the bud consists of undifferentiated cells, RAR- α and RAR-gamma are uniformly distributed within the limb, whilst CRABP is expressed in a proximo-distal gradient with the highest expression at the distal extremity of the limb. RAR- β transcripts are located in the most proximal bud mesenchyme and the adjacent mesenchyme of the flank. As development proceeds, RAR- α remains uniformly distributed. RAR-gamma becomes localised to developing cartilage and the ectoderm, and is thought to be involved in mediating the effects of RA on these two tissue types. CRABP is excluded from developing cartilage and the ectoderm, and becomes localised at the periphery of the cartilage elements and in the dermis. RAR- β remains restricted proximally but is also strongly expressed in the interdigital mesenchyme and in areas between the ulna and the radius, both areas previously shown to exhibit programmed cell death.

The distributions of RAR- β in chick wing development have been briefly described (Noji *et al.* 1991; Smith and Eichele, 1991). The data of Noji *et al.* (1991) confirm expression in the ectoderm and AER and also the highly restricted proximal expression. However, Smith and Eichele (1991) claimed that transcripts were undetectable in the ectoderm and also that only one isoform of the gene was present at higher levels proximally than distally. Recently Mendelsohn *et al.* (1991) constructed two independent lines of transgenic mice expressing the RAR- β_2 promoter fused to a *lacZ* reporter gene from *E. coli*. The distribution of β -galactosidase activity corresponded to the majority of sites which had previously been shown to express RAR- β transcripts by *in situ* hybridisation. In the limb bud the proximal region stained

strongly at 9.5 and 10.5 days of development. From 11.5 days onwards the apical ectodermal ridge (AER) stained strongly and at later stages staining was seen in the mesenchyme between the ulna and radius, and between the digits.

Experimental aims

In these experiments the distribution of RAR- β in normal limb development was determined by *in situ* hybridisation, in an attempt to confirm previous reports (Noji *et al.* 1991; Smith and Eichele, 1991). Grafting experiments were then performed to see if the proximally restricted expression of RAR- β was autonomous to these cells, or whether it was dependent on position within the limb. Thus normally non-expressing distal tissue was grafted to proximal sites and normally expressing proximal tissue to distal sites and the level of transcripts in the grafts examined after 6 and 24 hours to see if RAR- β expression had changed.

Micromass cultures of both distal and proximal cells grown in a number of different media were also examined for RAR- β transcript expression, in order to see if this culture system accurately reflected the pattern of RAR- β expression in the normal limb.

The RAR- β probe used in this study has been previously characterised (see Rowe *et al.* 1991). It recognises the B,C (DNA binding) and D domains with partial overlap into the A and E (RA binding) domains. Previous work detailed the distribution of this gene in the facial primordia and showed that the expression pattern was changed after treatment with RA, which is known to produce abnormalities of the upper beak (Tamarin *et al.* 1984). The probe recognises all three isoforms of RAR- β (Rowe - personal communication).

METHODS

The normal distribution of RAR- β in the developing chick limb bud was first determined as previously described for *Hox-7.1* (see pp.197).

Grafting

Stage 22 chick embryos were removed from the egg, placed in cold PBS and the amniotic membrane removed with forceps. To obtain distal wing tissue (see Fig. 4.03 A), 250 μ m tips were excised using electrically sharpened tungsten needles and incubated in 2% trypsin in CMF for 30 minutes at 4°C. The trypsin was replaced with F12 medium containing 10% FCS and 1% antibiotic/antimycotic and the ectoderms removed. The remaining mesenchyme was cut into small pieces and kept on ice until grafting.

To obtain proximal tissue, embryos were pushed down on their ventral surfaces and a thin slice of the most proximal limb tissue excised (see Fig. 4.03 B). This was then turned through 90° and the central region dissected free and cut into three pieces. These pieces were kept on ice as described above until grafting.

Host embryos were exposed by tearing the amniotic membrane. Stage 20-21 embryos were used for proximal to distal grafts, and stage 22 embryos for distal to proximal grafts. For distal to proximal grafts, a square was cut in the central proximal region of the limb. For proximal to distal grafts, an incision was made at the tip of the limb bud and extended to the AER which was then pulled out into a small loop. Donor tissue was transferred into the egg with a Pasteur pipette and manoeuvred into position using tungsten needles. Post-operation eggs were resealed and returned to the incubator for 6 or 24 hours. After this time embryos were removed and cleared of amniotic membrane in PBS before being pinned out in 4% paraformaldehyde so that the limbs extended laterally from the body wall.

Micromass culture

Cultures were prepared from distal 300 μ m tips or 500 μ m wide proximal pieces as described previously (pp.29). After counting, the cell suspension was separated. Cells were pelleted and then resuspended and plated in either DM or DM + S. Proximal cultures were then grown in either DM or DM + S. Distal cultures plated in DM alone were then grown in DM, DM + 10 ng/ml bFGF, DM + 1 μ g/ml RA or DM + 10ng/ml bFGF + 1 μ g/ml RA for 48 hours.

Cultures were fixed in 4% paraformaldehyde in PBS, pH 7.2, removed from the culture dish with a rubber policeman and left in fixative overnight at 4°C. Processing of cultures was performed under the dissecting microscope to prevent accidental loss of tissue.

In situ hybridisation

Sections were hybridised with a 35 S-labelled antisense-strand RNA probe that had previously been shown to be specific for chicken RAR- β transcripts (Rowe *et al.* 1991). As a negative control, adjacent sections were hybridised with a 35 S-labelled sense-strand RNA probe.

Since the protocol utilised for detection of RAR- β mRNA is very similar to that described previously for *Hox-7.1* (see Chapter 3) it is only described here in brief.

Preparation of probe

Templates were linearised, phenol and chloroform extracted, precipitated in ethanol and resuspended in double-distilled water (ddH₂O) at approximately 1 μ g/ml. Transcription (involving 5 μ l 5X BRL T7 buffer, 0.5 μ l 1M DTT, 1.2 μ l 10mM GTP, 1.2 μ l 10mM ATP, 1.2 μ l 10mM UTP, 1 μ l 50 μ M CTP, 2-3 μ l DNA, 7 μ l 35 S-CTP, 0.5 μ l RNAsin and 1 μ l T7/T3 RNA polymerase, made up to 25 μ l with ddH₂O) was carried out at 37°C for

40 minutes. 1 μ l of fresh enzyme was then added and the reaction repeated for 30-60 minutes. The template was removed by DNase treatment (0.5 μ RNAsin, 1 μ l 10mg/ml tRNA, 0.5 μ l 1M DTT and 0.5 μ l RNase-free DNase I for 10 minutes at 37°C). The volume was increased to 200 μ l with 5 μ l 4M NaCl, 4 μ l 1M DTT and ddH₂O, and the probe extracted once with an equal volume of water-saturated phenol/chloroform, and once with water-saturated chloroform. The probe was then precipitated in 1/10th vol. 3M sodium acetate (pH 5.2) and 2.5 vols. absolute ethanol for 15 minutes at -70°C. The pellet was spun down, washed with 10mM DTT in 70% ethanol, vacuum dried, resuspended in 50 μ l 50mM DTT in ddH₂O and 0.5 μ l counted in "Ecoscint" (count should be in the region of 5 \times 10⁷ to 1.2 \times 10⁸ counts/min). The probe was then hydrolysed in 50 μ l of 80mM NaHCO₃, 120mM Na₂CO₃ - pH 10.2 and 10mM DTT for 60 minutes at 60°C. The reaction was halted by adding 50 μ l neutralising buffer (0.2M sodium acetate, 1% glacial acetic acid, 10mM DTT). The probe was precipitated in 15 μ l sodium acetate and ethanol, washed in 10mM DTT in 70% ethanol, dried, resuspended and recounted.

Processing

The trunk region of the embryo encompassing the wing buds was dissected and processed according to the following schedule:

PBS, pH7.2	- 10 mins
50% alcohol	- 30 mins
70% alcohol	- 30 mins
90% alcohol	- 30 mins
100% alcohol	- 15 mins
	- 15 mins
	- 15 mins
toluene	- 30 mins

	- 30 mins
paraffin wax	- 60 mins
(60°C)	- 60 mins

The limbs were then correctly orientated in the moulds with a heated tungsten needle and the wax allowed to cool and solidify by placing the mould in cold water. Wax blocks were then stored at 4°C until sectioning.

8 μ m sections were cut on an American 820 Spencer microtome. Limbs were sectioned parallel to their long axis in an anterior to posterior direction. Every 15th section was mounted, dried and baked onto a gelatin-coated slide at 60°C overnight. The following morning these sections were stained with haematoxylin and eosin so that the sections containing the graft could be identified. Such sections were then mounted with sterile distilled water onto slides, which had been precleaned and TESPA-coated as described previously (see pp.200). When dry, the sections were baked onto the slides at 60°C overnight and stored in boxes containing dessicant at 4°C until hybridisation.

Pretreatment

Sections were dewaxed (15 minutes in fresh xylene) and rehydrated (2 minutes each in absolute, 95%, 90%, 80%, 60%, 30% ethanol) to ddH₂O. Sections were then washed twice in ddH₂O (2 mins each) followed by incubation in 1/49 HCl for 20 minutes. After 2 washes in 5X SSC sections were incubated for 10 minutes at 37°C in 5 μ g/ml proteinase K in 100mM Tris - pH 7.5, 50mM EDTA. Sections were then washed in 2mg/ml glycine (to block the action of proteinase K) for 2 minutes and PBS (1 min.) before refixing in 4% paraformaldehyde in PBS. After rewashing in PBS (2 mins.), sections were acetylated for 10 minutes in 0.1M triethanolamine with 1/400 acetic anhydride. Slides were then washed in PBS (5 mins.) and twice in ddH₂O (2 mins.), dehydrated through ethanols

and air dried.

Hybridisation and washing

The probe was then diluted to 5×10^4 to 1×10^5 counts/min/ μ l in the hybridisation mixture containing (1X "salts", 50% formamide, 10% dextran sulphate, 50mM DTT, 500 μ g/ml yeast RNA and 50 μ g/ml poly-A RNA). The mixture was then spun, vortexed, spun again and heated to 80°C for 2-3 minutes before rapid cooling on ice (2 minutes). 10 μ l aliquots of probe were applied to each row of sections and the sections coverslipped. The slides were then hybridised overnight in a humidified chamber at 55°C.

Slides were then washed with agitation in 2X SSC, 50% formamide, 10mM DTT for 15 minutes at 55°C. Slides were transferred to a second, identical wash for 20 minutes, during which time the temperature was increased to 65°C and finally to a third identical wash for 20 minutes at 65°C. Slides were then rinsed twice (15 minutes each) in buffer (500mM NaCl, 10mM Tris - pH 8.0, 1mM EDTA) to remove DTT (which inhibits RNase). Sections were incubated for 30 minutes in 40 μ g/ml RNase A in the same buffer as above at 37°C, rinsed in buffer at 37°C for 15 minutes and washed twice for 20 minutes each in 2X SSC, 50% formamide, 10mM DTT at 65°C and then in 0.1X SSC, 10mM DTT at 65°C for 20 minutes. Finally, slides were rinsed at room temperature for 5 minutes in 0.1X SSC and dehydrated through 300mM ammonium acetate in 70% ethanol, 95% ethanol and absolute ethanol for 2 minutes each before air drying.

Slides were dipped in emulsion, exposed and developed as described previously (see pp.80 and 203). Limb sections were stained in 1% malachite green, whilst cultures were stained with haematoxylin and eosin.

RAR-β transcript distribution in the limb bud

At stage 20, RAR-β transcripts were expressed in the proximal half of the wing bud (Fig. 4.01 A,B). At stage 22, transcripts were still present in the proximal region of the limb but were now restricted to the central core (Fig. 4.01 C,D). By stage 24, as cellular condensation and the first signs of overt differentiation were beginning, RAR-β transcripts were confined to a small area encompassing the most proximal part of the central core region (Fig. 4.01 E,F). At stage 27, the expression of transcripts was still retained in this proximal core region, but now appeared to correspond to the area of the developing shoulder extending distally as far as the head of the humerus (Fig. 4.01 G,H). Throughout all stages described above RAR-β transcripts were expressed in the ectoderm of the limb bud including the AER.

These results suggest that RAR-β is normally expressed in the region of the limb which will give rise to at least part of the shoulder girdle. Figure 4.02 shows a frontal section through a stage 22 limb bud, together with two shoulder fate maps. At stage 22, a high level of RAR-β transcripts were seen in the body wall just proximal to the limb, extending from the anterior region to the posterior angle of the bud (Fig. 4.02 A). According to the fate map of Saunders (1948) (Fig. 4.02 C), this area of expression corresponds to the region destined to form the scapula and also parts of the coracoid bone and the glenoid cavity. This is confirmed by the fate maps of Yander and Searls (1980) (Fig. 4.02 D,E), who showed that two regions, which correspond to areas expressing high levels of RAR-β transcripts in these experiments, are destined to contribute to the formation of both the scapula and the posterior part of the coracoid.

Position-dependent expression of RAR- β transcripts in reciprocal proximodistal grafting experiments

Some of the tissue fragments that were fixed immediately after dissection are shown in Figure 4.04. The proximal pieces (Fig. 4.04 A) retained high levels of RAR- β transcripts compared to controls (Fig. 4.04 B), whereas the distal pieces (Fig. 4.04 C) contained a much lower level of transcripts, which was not significantly above the signal obtained with the control sense probe (Fig. 4.04 D).

Proximal to distal grafts

The results of the proximal to distal grafts are summarised in Table 4.1a. After as little as 6 hours in the distal environment of the limb, RAR- β expression had been totally downregulated in the grafts to the level of the surrounding tissue in 4 out of 6 cases (Fig. 4.05). In total 6 out of 9 grafts showed complete downregulation of RAR- β transcripts. In one case a portion of the graft extended from the dorsal surface of the limb; this portion retained a high level of transcripts whereas the remainder of the graft, which was positioned in the distal tip of the bud, exhibited downregulation of RAR- β expression (Fig. 4.06 A-D). In the remaining two cases, downregulation of RAR- β transcripts occurred in the most distal and peripheral portions of the grafts, however the most proximal region of the grafts appeared to have differentiated into a nodule of cartilage and continued to express a high level of RAR- β transcripts (Fig. 4.06 E-H).

Distal to proximal grafts

Distal to proximal grafts were divided into 2 groups depending on whether the graft was positioned within the highly-expressing central core region of the limb or was dorsal to this, in a more peripheral

position.

The results of grafts contained within the central core are summarised in Table 4.1b. All 6 grafts expressed levels of transcripts above that of the distal region from which they originated, but well below that of the adjacent highly-expressing proximal core tissue.

Figure 4.07 A-D shows a graft fixed 24 hours after grafting which was half in the highly-expressing central core region and half in the dorsal periphery of the limb. The level of transcripts was constant throughout both regions of the graft, being higher than that seen in the distal tip of the bud, approximately equal to that in the adjacent peripheral limb tissue but substantially lower than that in the central core region of the bud.

The results of grafting distal tissue to the periphery of the proximal region are summarised in Table 4.1c. 3 out of 8 grafts did not appear to express RAR- β transcripts, whilst 5 contained low levels of transcripts (Fig. 4.07 E-H), which were approximately equal to the levels seen in the adjacent peripheral limb bud tissue and appeared to be greater than the levels seen in the distal tissue from which the grafts originated.

In one case, the distal graft ended up in the flank of the embryo anterior to the limb. In this case the level of RAR- β transcripts did appear to have been upregulated to the high level seen in adjacent flank tissue, although this graft was performed at an earlier stage than the other grafts. Thus it is possible that there is an inducer of RAR- β expression in the flank, although more experiments would have to be carried out to confirm this.

RAR- β expression in micromass cultures

The proximal tissue used to produce proximal cell micromass cultures would normally include cells expressing high-levels of RAR- β

transcripts. After 2 days of culture, cells grown in both DM and DM + S contained RAR- β transcripts (Fig. 4.08), although cultures grown in the presence of serum contained a higher level than those grown in DM alone (Table 4.2a).

In contrast, the distal tissue used to make distal micromass cultures would not normally express RAR- β transcripts *in vivo*, however after 2 days of culture all distal cell cultures expressed RAR- β transcripts at levels above those of control sections (Fig. 4.09, 4.10). In this case the presence of serum did not result in an increase in the level of transcripts, however cultures grown in the presence of RA and bFGF contained twice as many transcripts as cultures grown in all other media, including cultures grown in bFGF (Table 4.2b).

Table 4.1a - Proximal to distal grafts

RAR- β expression				
time point	number of grafts	off	partially off	on
6 h	6	4	2	0
24 h	3	2	1	0

Table 4.1b - Distal to proximal grafts contained within the central core region of the limb

RAR- β expression (cf. adjacent tissue)			
time point	number of grafts	equal	lower
6 h	3	0	3
24 h	3	0	3

Table 4.1c - Distal to proximal grafts contained within the periphery of the limb

RAR- β expression (cf. distal tissue)			
time point	number of grafts	upregulated	not upregulated
6 h	4	2	2
24 h	4	3	1

Table 4.2a - grain counts of 2 day proximal cultures grown in the presence and absence of serum

medium	RAR-β grains/50µm ²	control grains/50µm ²	nett grains/50µm ²
<hr/>			
DM	418	163	255
DM + serum	618	136	482

Table 4.2b - grain counts of 2 day distal cultures grown in different media

medium	RAR-β grains/50µm ²	control grains/50µm ²	nett grains/50µm ²
<hr/>			
DM	439	135	304
DM + serum	421	123	298
DM + bFGF	422	78	344
DM + bFGF + RA	789	104	685

Figure 4.01

Dark-field micrographs showing the distribution of RAR- β transcripts (A,C,E,G) in transverse sections through the long axis of the limb bud, and adjacent sections hybridised with the sense-strand probe (B,D,F,H). RAR- β transcripts were initially present in the proximal half of the limb bud at stage 20 (A,B), becoming restricted to the central core of the proximal region at stage 22 (C,D) and stage 24 (E,F). At stage 27 (G,H) a high level of transcripts was present in the central region proximal to the condensed mesenchyme of the humerus (c).

Scale bar = 250 μ m.

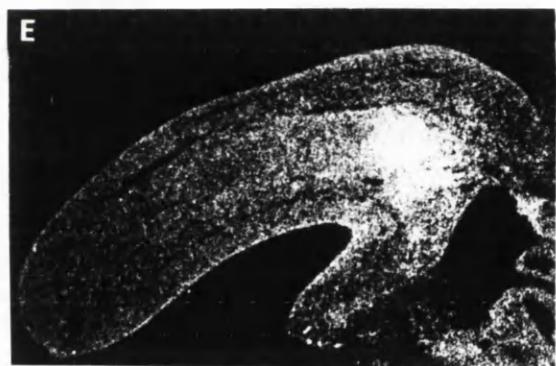
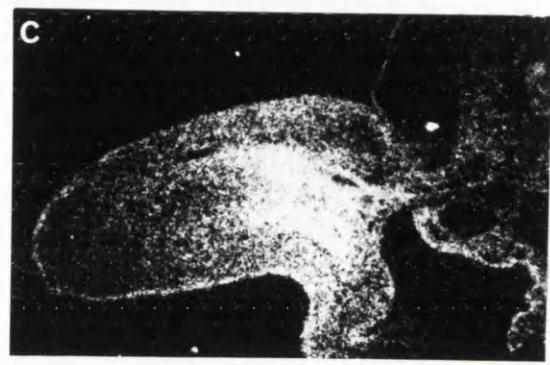
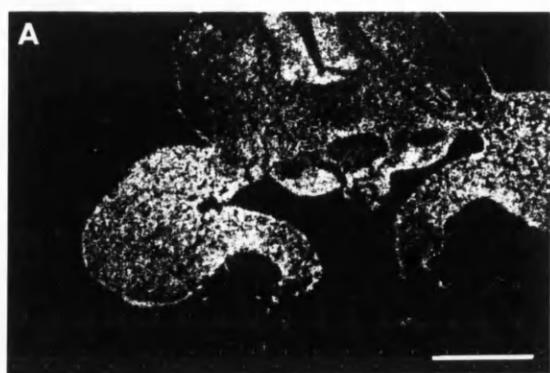
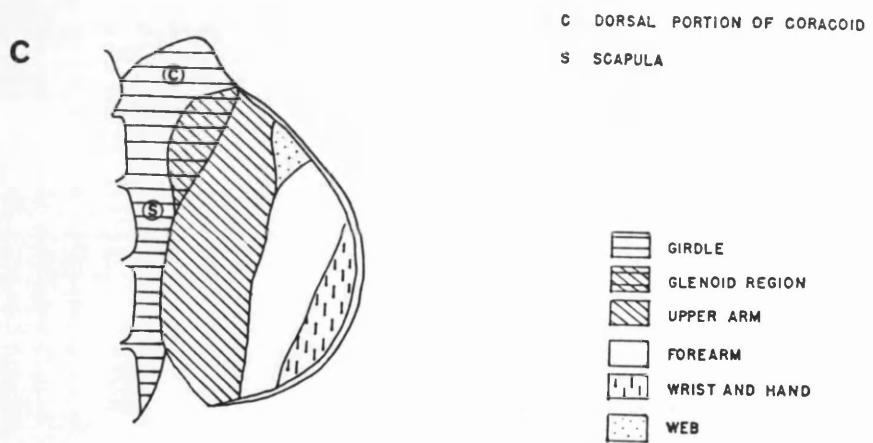
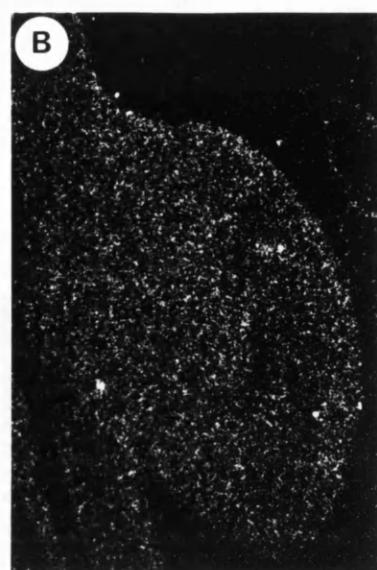
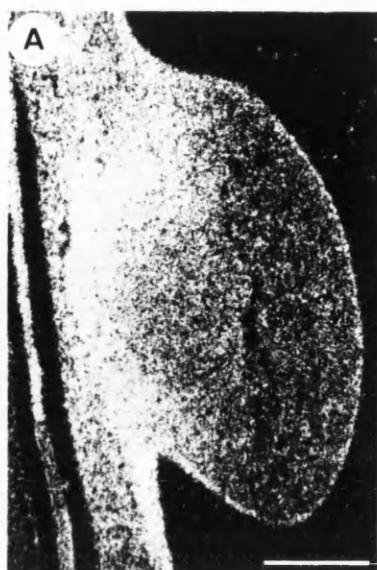


Figure 4.02

Dark-field views of alternate frontal sections through the anterior-posterior axis of a stage 22 limb bud hybridised with anti-sense (A) and sense-strand (B) RAR- β probes. a = anterior, p = posterior.

Scale bar = 250 μ m.

The proximal region which expressed high-levels of transcripts corresponds to regions which give rise to the scapula, dorsal portion of the coracoid and the glenoid cavity, according to the fate map of Saunders 1948, a copy of which is shown in part (C). This is confirmed by the fate maps of Yander and Searls (1980), copies of which are shown in part (D) and (E). The figures show dorsal views of two embryos, with anterior to the top of the page and posterior to the bottom. The hatched areas represent labelled grafts made into a stage 21 (D) and stage 22 (E) limb. The right hand drawing in each figure part illustrates the final location of these labelled cells in the embryos fixed at stage 27 (D) and 28 (E) respectively. In part (D) the graft contributes to the posterior part of the scapula and associated posterior tissues. In part (E) the graft gives rise to parts of the scapula and coracoid and the intervening connective tissue.



C DORSAL PORTION OF CORACOID
S SCAPULA

- █ GIRDLE
- █ GLENOID REGION
- █ UPPER ARM
- █ FOREARM
- █ WRIST AND HAND
- █ WEB

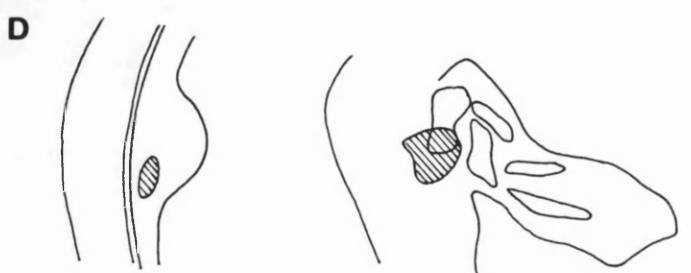
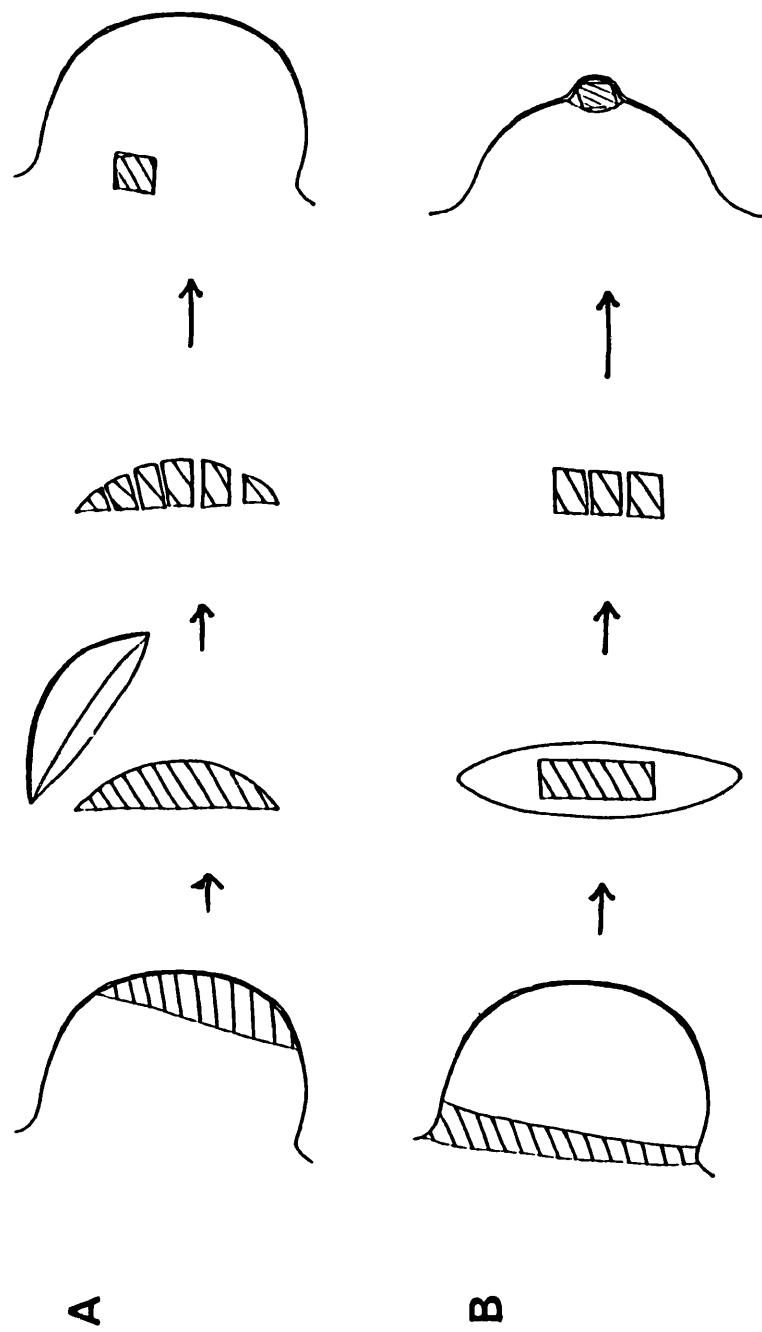


Figure 4.03

Drawing to show the regions of stage 22 limb buds that were dissected to provide the distal (A) and proximal (B) tissue used in the grafting experiments. (A) 250 μ m distal tips were cut with tungsten needles, trypsinised and the ectodermal hulls removed. Tips were then cut into smaller pieces which were grafted to an anterior, proximal site. (B) A thin strip of the most proximal limb tissue was removed, turned through 90°C and the central portion dissected free. This tissue was then cut into three pieces which were grafted to the distal tip of stage 20-21 wing buds.



B

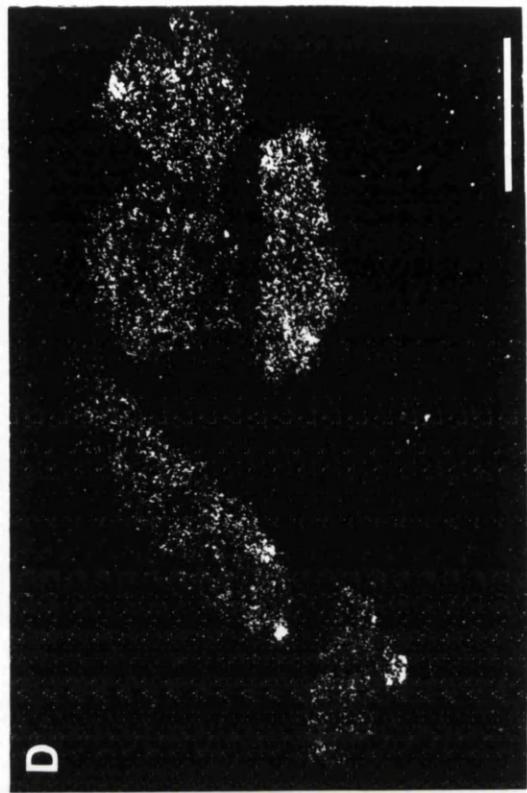
Figure 4.04

Dark-field micrographs of tissue pieces dissected to provide tissue for grafting experiments, fixed shortly after dissection. Proximal pieces (A,B), distal pieces (C,D). Sections were hybridised with anti-sense (A,C) and sense (B,D) RAR- β mRNA probes. Scale bar = 250 μ m.

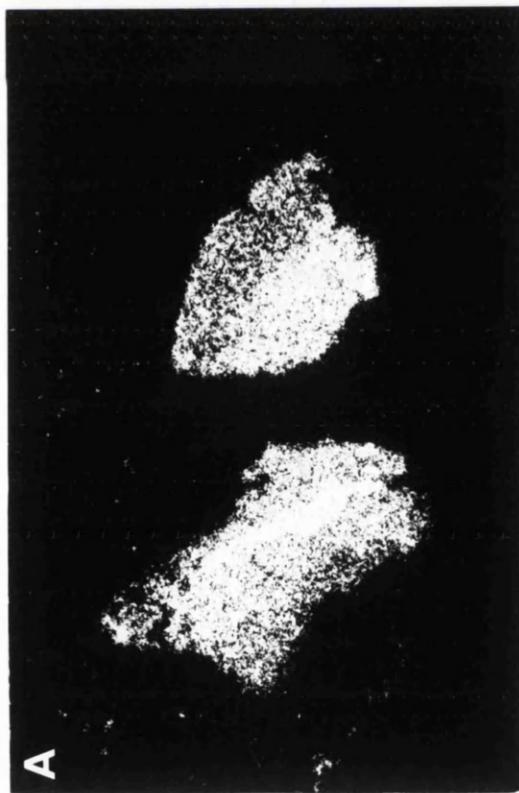
Proximal pieces retained a high-level of RAR- β transcripts (A), whereas distal pieces (C) appeared to express a very low level, the labelling being just higher than that seen with the control probe (D). These tissue pieces would have been cut into much smaller pieces before grafting.



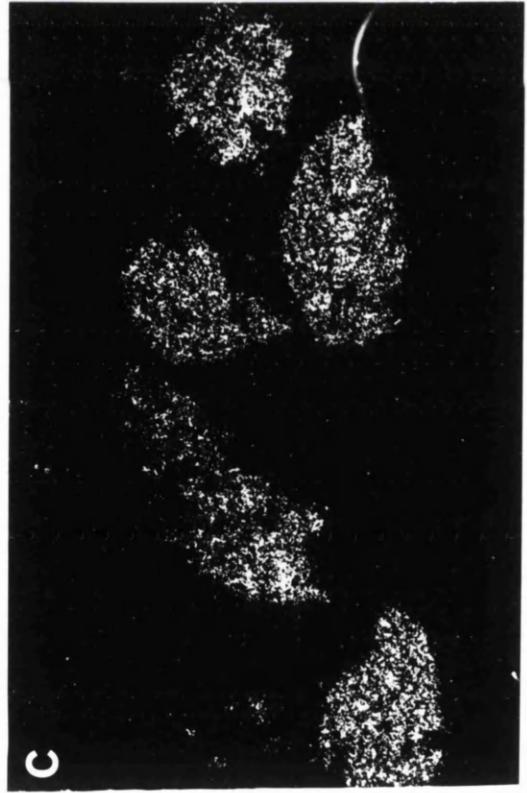
B



D



A



C

Figure 4.05

Two examples of cases in which proximal tissue was grafted to the distal tip of the wing buds of stage 21 host embryos. Both embryos were fixed 6 hours after grafting. (A,E) Haematoxylin and eosin stained sections showing the position of the graft (indicated with arrowheads). (B,F) adjacent sections hybridised with anti-sense RAR- β probe, with higher magnification views in parts (C,G). (D,H) adjacent sections hybridised with the control, sense strand probe.

Scale bar (A,B,D,E,F,H) = 250 μ m, scale bar (C,G) = 100 μ m.

As can be clearly seen, the level of transcripts within the grafts was completely downregulated to the level seen in the surrounding distal mesenchyme.

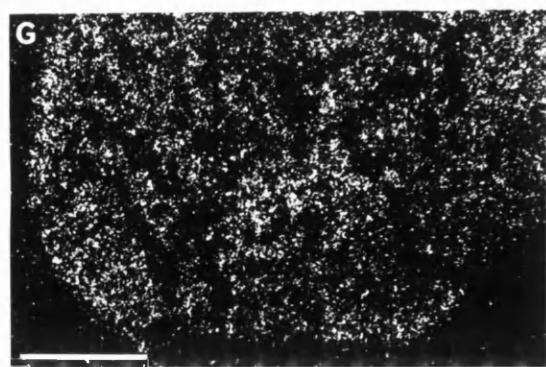
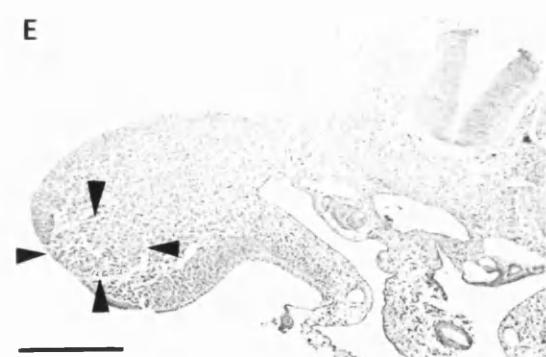
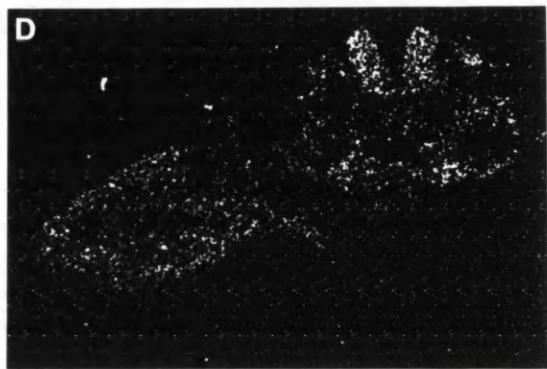
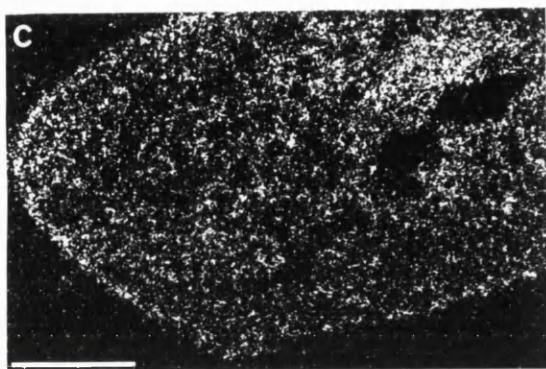
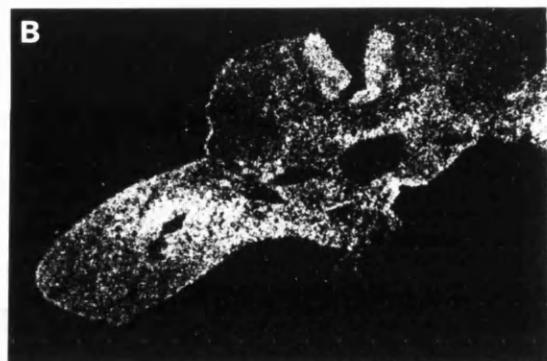


Figure 4.06

Two examples of cases in which proximal tissue was grafted to a distal site; embryos were fixed 24 hours after grafting.

(A) Haematoxylin and eosin stained section showing a graft positioned within the distal tip of the limb but which protruded from the dorsal surface. The boundaries of the graft are indicated with arrowheads. (B) Dark-field view of an adjacent section hybridised with the probe for RAR- β transcripts, with a higher magnification view in part (C). (D) Dark-field view of an adjacent section hybridised with the control sense strand probe.

Scale bar (A,B,D) = 250 μ m, scale bar (C) = 100 μ m.

The portion of the graft contained within the distal environment of the limb exhibited a downregulation in the level of RAR- β transcripts, however the portion extending from the dorsal surface retained a high level of transcripts.

(E) Haematoxylin and eosin stained section of a large graft at the dorsal surface of the limb; the boundaries of the graft are indicated with arrowheads. (F) Dark-field view of an adjacent section hybridised with the probe for RAR- β transcripts, shown at higher magnification in (G). (H) Dark-field view of an adjacent section hybridised with the control sense strand probe.

Scale bar (E,F,H) = 250 μ m, scale bar (G) = 100 μ m.

The part of the graft adjacent to the dorsal surface exhibited partial downregulation of transcripts, however the deeper, more proximal part of the graft retained a high level of transcripts. This region consisted of condensed mesenchyme with a very similar appearance to the condensed mesenchyme of the developing cartilage element (c), whereas the superficial portion of the graft consisted of loosely packed mesenchyme, which is more characteristic of peripheral limb tissue.

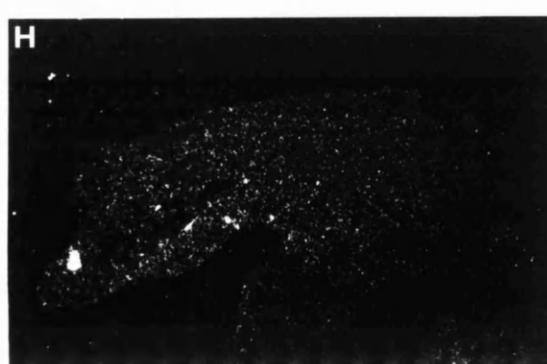
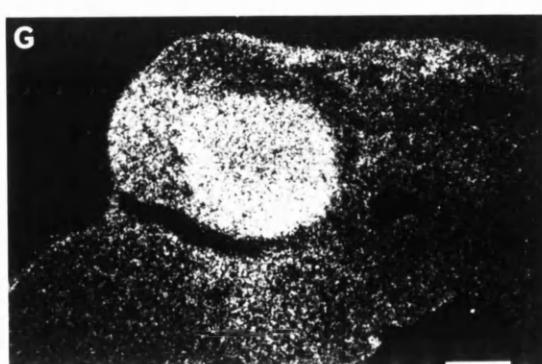
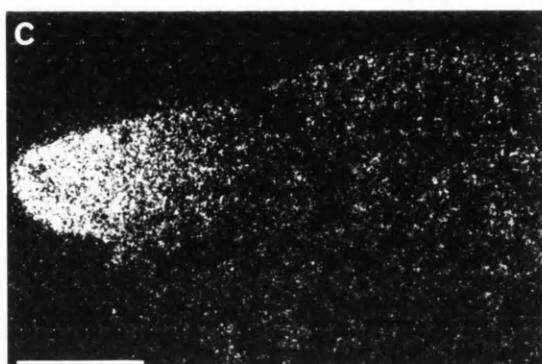
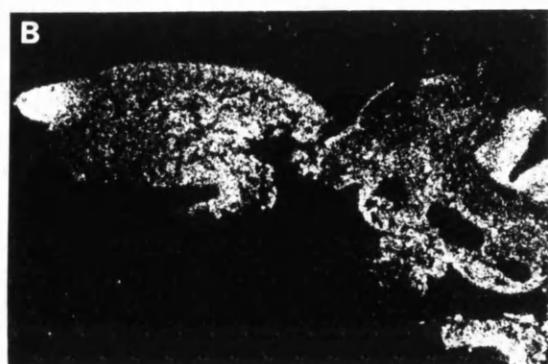


Figure 4.07

Two examples of limbs in which distal tissue was grafted to a proximal site.

(A) Haematoxylin and eosin stained section of a graft fixed 24 hours after grafting. The graft was contained within the dorsal periphery (large arrowhead) and the central cartilage element (small arrowhead). (B) Dark-field view of an adjacent section hybridised with the probe for RAR- β transcripts, with a high magnification view shown in part (C). (D) Dark-field view of an adjacent section hybridised with the control sense strand probe.

Scale bar (A,B,D) = 250 μ m, scale bar (C) = 100 μ m.

The graft contained a detectable level of transcripts, but this did not approach the high level of transcripts expressed by the central, proximal condensed mesenchyme.

(E) Haematoxylin and eosin stained section of a limb fixed 6 hours after grafting. The boundaries of the graft are indicated by arrowheads. (F) Dark-field view of an adjacent section hybridised with the probe for RAR- β transcripts, with a higher magnification view in part (G). (H) Dark-field view of an adjacent section hybridised with the control sense strand probe.

Scale bar (E,F,H) = 250 μ m, scale bar (G) = 100 μ m.

The graft was expressing RAR- β transcripts at a slightly higher level than the distal mesenchyme, however this level was still substantially lower than the level of transcripts expressed by the surrounding proximal mesenchyme.

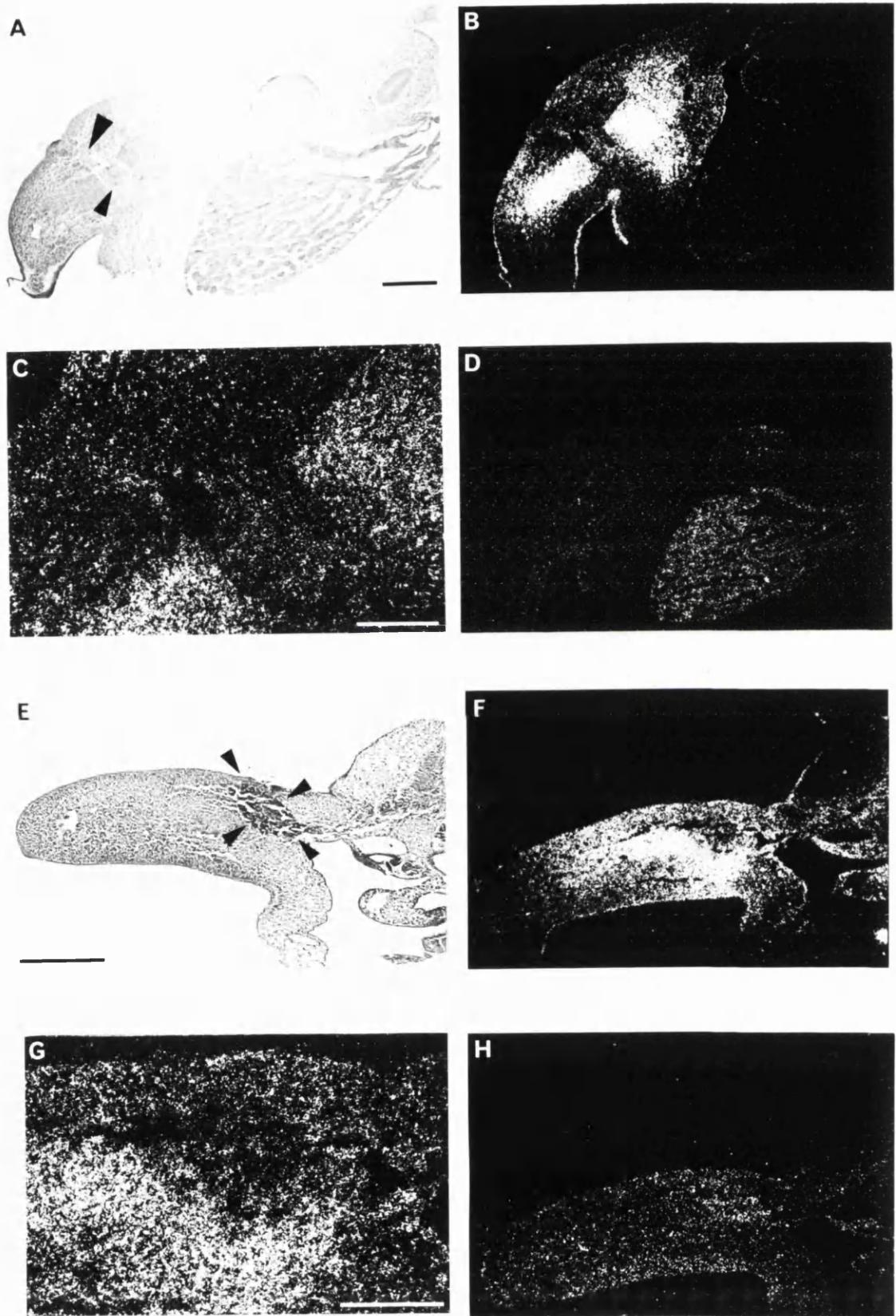


Figure 4.08

Light-field (A,C,E,G) and corresponding dark-field (B,D,F,H) views of sections of proximal cell micromass cultures grown in DM (A-D) or DM + serum (E-H). Sections were hybridised with probe for RAR- β transcripts (A,B,E,F) or the control sense strand probe (C,D,G,H). Scale bar = 50 μ m.

Cultures grown in both media were expressing RAR- β transcripts relative to the labelling seen with the control probe; although the number of grains per unit area was slightly higher in cultures grown in the presence of serum (Table 4.2a).

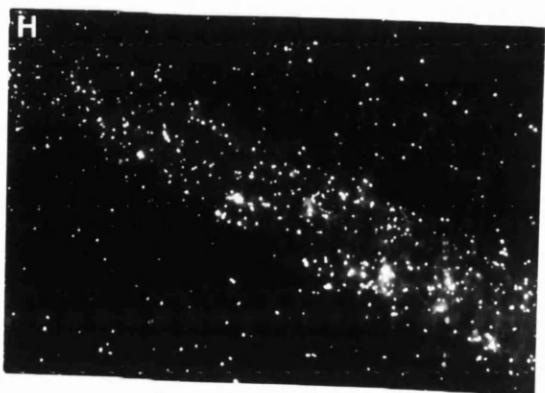
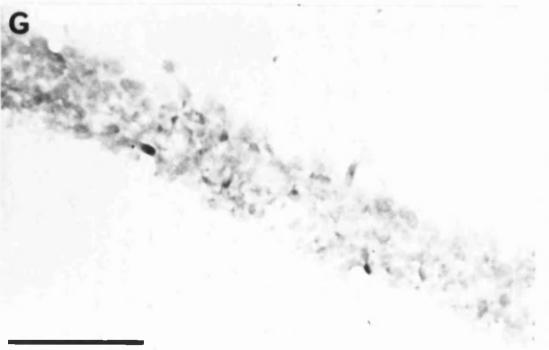
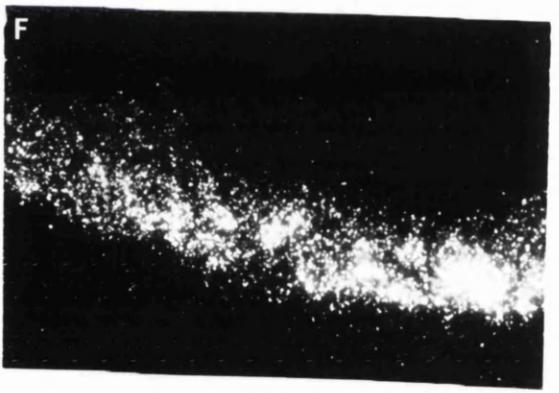
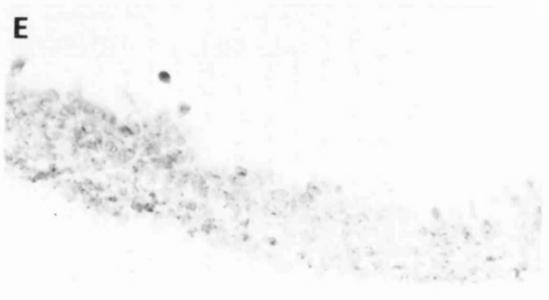
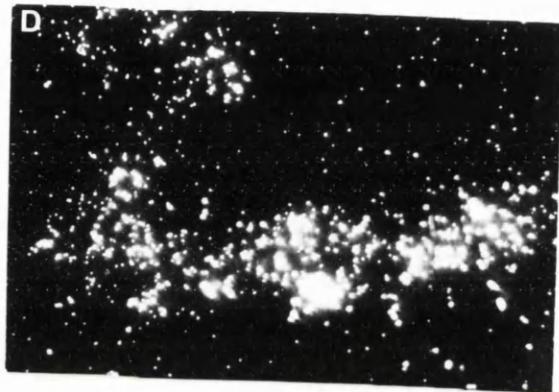
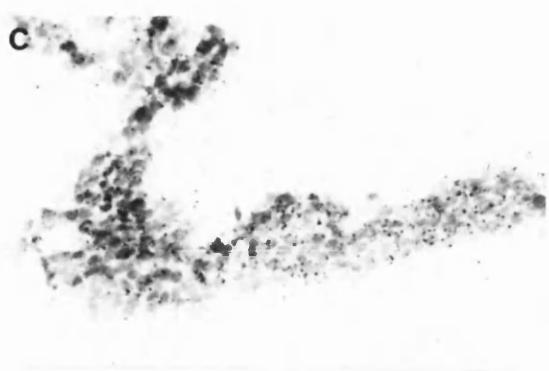
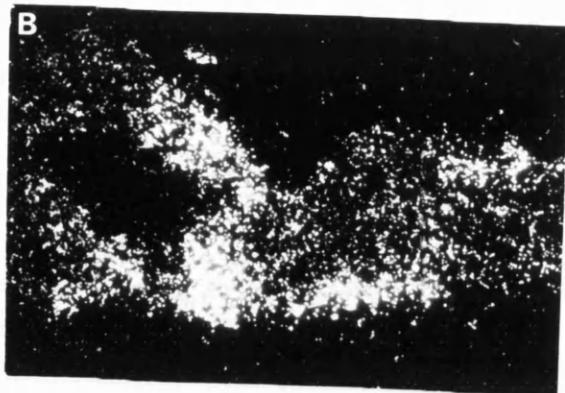


Figure 4.09

Light-field (A,C,E,G) and corresponding dark-field (B,D,F,H) views of sections of 2 day distal cell micromass cultures grown in DM (A-D) or DM + serum (E-H). Sections were hybridised with the probe for RAR- β transcripts (A,B,E,F) or the negative control probe (C,D,G,H).

Scale bar = 50 μ m.

Cultures grown in both media were expressing RAR- β transcripts relative to the labelling seen with the control probe. The number of silver grains per unit area in the two different media was not significantly different (see table 4.2b).

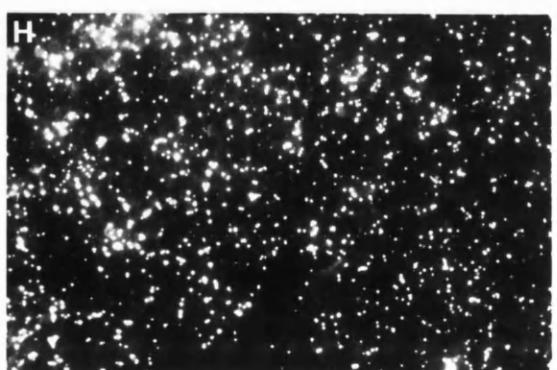
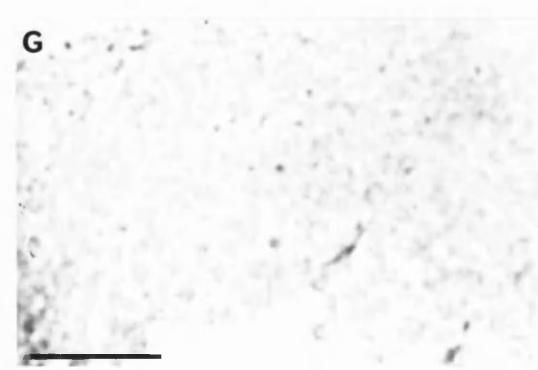
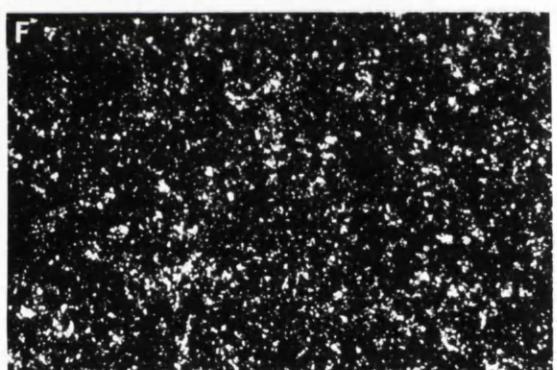
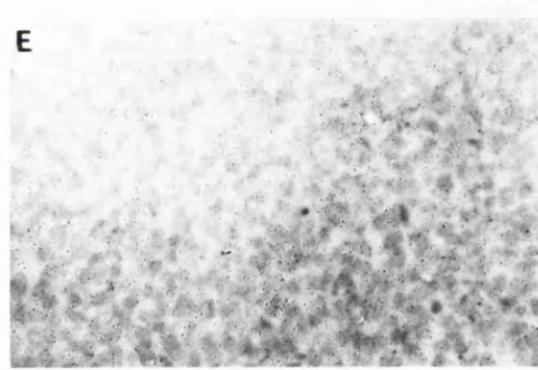
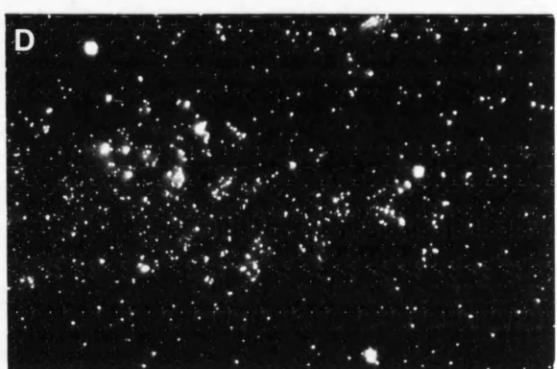
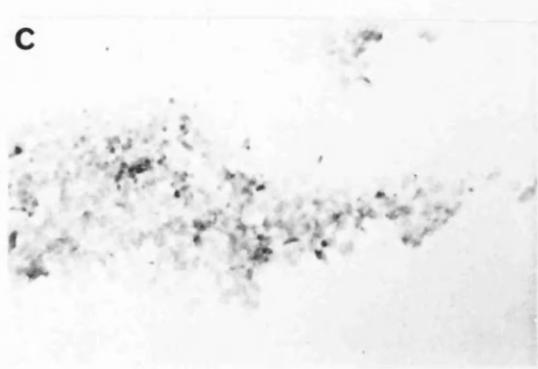
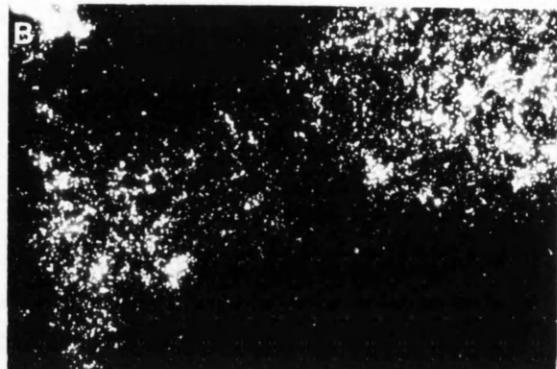
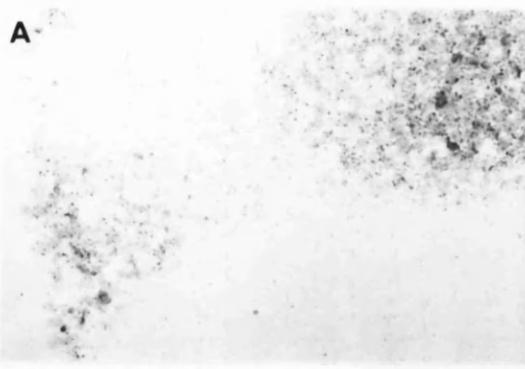
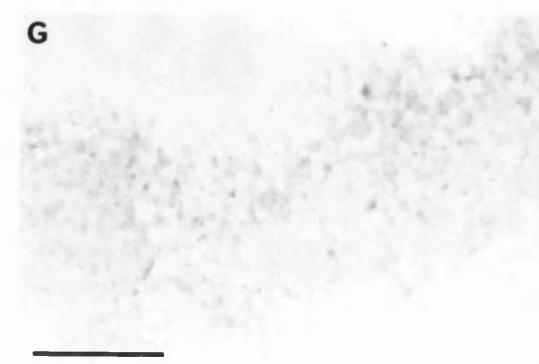
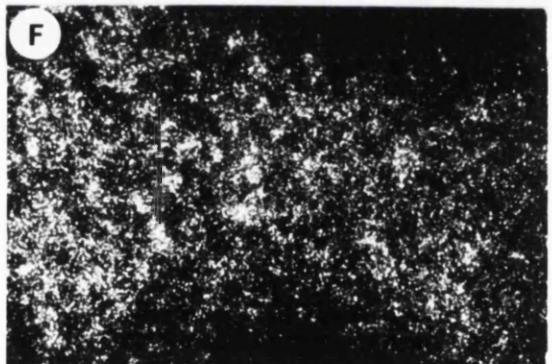
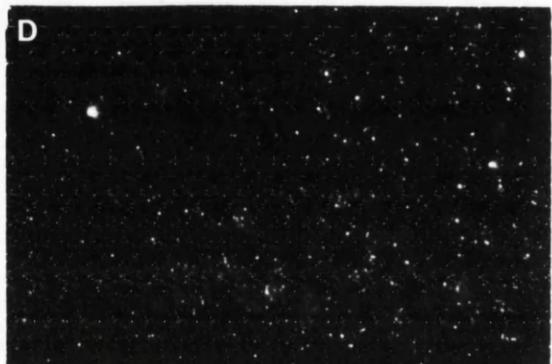
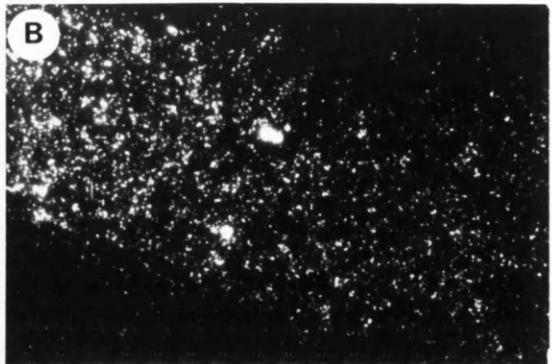
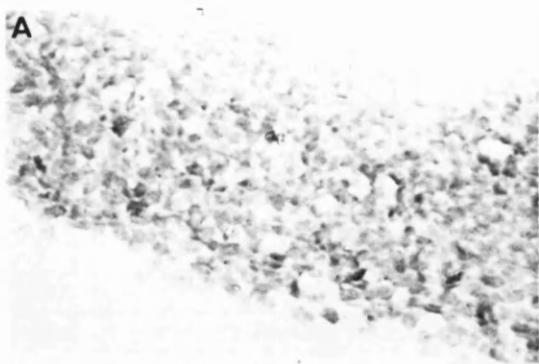


Figure 4.10

Light-field (A,C,E,G) and corresponding dark-field views (B,D,F,H) of sections of 2 day distal cell micromass cultures, grown in DM + 10ng/ml bFGF (A-D) or DM + 10ng/ml bFGF + 1 μ g/ml RA (E-H). Sections were hybridised with the probe for RAR- β transcripts (A,B,E,F) or the control sense strand probe (C,D,G,H).

Scale bar = 50 μ m.

Cultures grown in both media expressed transcripts relative to labelling with the control probe. However the number of silver grains per unit area was substantially higher in cultures grown in the presence of RA (see Table 4.2b).



DISCUSSION

Normal distribution of RAR- β transcripts

The results presented here show that RAR- β transcripts are present in the proximal region of the early wing bud at stages 20-22. As outgrowth of the bud progresses, RAR- β transcripts become restricted to the central most proximal core region of the limb, and remain undetectable in the distal tissue and in cells leaving the progress zone. This region of proximal expression corresponds to the region destined to form part of the shoulder, according to the fate maps of Saunders (1948) and Yander and Searls (1980). The fact that at stage 27 RAR- β transcripts appear to be expressed in the shoulder developing proximally to the humerus confirms these findings and therefore suggests that RAR- β is involved in the normal development of the shoulder region. It appears from this limited analysis that RAR- β expression is associated with proximal cartilage, perhaps corresponding to the scapula; however, Dollé *et al.* (1989) reported that RAR- β is excluded from differentiating cartilage, which characteristically expresses RAR-gamma. Therefore in this study the region of RAR- β expression may correlate with the soft tissues of the shoulder region, although further experiments are necessary to clarify this point. RAR- β transcripts were also found in the ectoderm, including the AER of the wing bud throughout the developmental stages considered here, although previously Rowe *et al.* (1991) using the same probe as in this study, did not detect transcripts in the ectoderm of the facial primordia.

The pattern of expression reported here agrees with that shown by Noji *et al.* (1991) in the chick wing and with that of Dollé *et al.* (1989) in the mouse limb; however it differs from that shown by Smith and Eichele (1991), who claimed that only one isoform of RAR- β exhibited higher expression proximally than distally, whilst the others

appeared to be uniformly expressed in stage 22 limb buds. They also found that the ectoderm and AER did not contain detectable levels of transcripts. The proximally restricted expression of transcripts detected by *in situ* hybridisation also correlates with the area of β -galactosidase activity in transgenic mice containing the RAR- β 2 promoter fused to the *lacZ* reporter gene (Mendlesohn *et al.* 1991). In addition, staining of the AER was detected in these mice from 11.5 days onwards. In later limbs (12.5 and 14.5 days) Dollé *et al.* (1989) found that RAR- β transcripts were expressed in discrete patches between the ulna and the radius, and in the mesenchyme between the digits. Staining was also found in these areas of the RAR- β 2 promoter-containing transgenic mice. These areas both undergo programmed cell death, thus RAR- β may be involved in this process; although closer examination of the interdigital region in transgenic mice revealed that the RAR- β 2 promoter was activated most strongly in the loose connective tissue forming around the digits, whilst the central interdigital region (which was shown to exhibit necrosis by Nile blue staining) contained a much lower level of X-gal reaction product. The above authors therefore suggested that the primary function of RAR- β may be to inhibit cartilage differentiation in this region, rather than to cause cell death. This may also be the primary function of the RAR- β transcripts expressed in the mesenchyme between the ulna and radius. Further evidence suggesting that RAR- β may not be primarily involved in cell death is that RAR- β is not expressed in the other regions of programmed cell death within the limb, namely the anterior and posterior necrotic zones.

Since the RAR- β gene contains a retinoic acid response element (RARE) in its promoter region, it is possible that the expression of RAR- β transcripts, and β -galactosidase activity in Mendlesohn's transgenic mice, could be interpreted as evidence for the presence of

free endogenous RA in those regions of the limb. Thus it would be concluded that RA is absent from areas which lack RAR- β transcripts and β -galactosidase activity, such as the distal tip of the limb. However this assumption seems unlikely since RA treatment of transgenic mouse embryos did not result in β -galactosidase activity in distal tissue, although local application of beads soaked in RA to the anterior margin of chick wing buds resulted in substantial expression of RAR- β transcripts within distal tissue (Noji *et al.* 1991). However local application of RA to the facial primordia of chick embryos did not result in a uniform upregulation of RAR- β transcripts, although changes in some areas were seen (Rowe *et al.* 1991). The finding that transcripts for the CRABP II gene, which is also induced by RA, are present in this region of mouse limb buds (Dollé *et al.* 1989) also suggests that RAR- β expression can not be considered as an accurate reporter for endogenous RA.

In the transgenic mice of Mendlesohn, RA treatment at 11.5 days increased β -galactosidase staining in the AER, and the region of staining in the core mesenchyme was more extensive. Such treatments resulted in truncation of the limbs caused by loss of tissue from the radius and ulna. The proximal domain of expression was not altered and subsequently abnormalities in this region were not seen. Interestingly local application of beads soaked in RA to the anterior humeral region of stage 20 embryos results in abnormalities of the shoulder girdle which increase with increasing dose of RA (Oliver *et al.* 1990). It seems likely that in these cases RAR- β levels are further increased since RAR- β is induced by RA and is normally expressed in this region. These observations suggest that the overexpression of RAR- β is associated with retinoid-induced limb abnormalities. The findings that RAR- β transcripts are continually expressed in sites which are known to give rise to parts of the shoulder girdle, and that the development of

this region is sensitive to application of exogenous RA therefore suggests that RAR- β plays an important role in the normal development of the shoulder region.

Proximodistal grafting

The pieces of proximal tissue grafted were from the central core region and were expressing a high level of RAR- β transcripts. In 6 out of 9 grafts of proximal tissue to distal limb the grafts integrated fully with the local environment, no cartilage was formed and the expression of RAR- β was downregulated within 6 hours. In 3 out of 9 grafts a portion of the graft remained too far proximally to be affected by the distal environment. This tissue appeared to be differentiating into cartilage and retained a high level of RAR- β expression, whilst the portion of these grafts most distally and beneath the dorsal ectoderm integrated with the surrounding tissue. In these regions the expression of RAR- β was downregulated. These results suggest that RAR- β expression is a characteristic of the initial cartilage to form in the most proximal part of the limb. If this tissue is grafted to another site in which it is prevented from differentiating into cartilage, the expression of RAR- β is lost. If however the tissue is grafted to a site where cartilage differentiation can occur, then the expression of RAR- β is maintained. It should be emphasised that RAR- β is only expressed by the most proximal cartilage of the limb, although this is contrary to previous reports that RAR- γ is the RA receptor which becomes associated with differentiating cartilage throughout the body (Dollé *et al.* 1989).

These results show that expression of RAR- β transcripts is consistently repressed in proximal tissue grafted to the distal tip of the limb bud. The speed with which RAR- β transcripts disappear from the

grafts suggests that repression is a direct consequence of the cell's new position. This repression could involve either a decrease in the efficiency with which the RAR- β gene is transcribed or a decrease in the stability of RAR- β transcripts. The source of such a repressor is probably the AER, however it was noted that the parts of grafts directly beneath the dorsal ectoderm also exhibited a degree of transcript downregulation although they had come to lie some distance from the AER. Thus the repressor may be produced by a larger region of the distal ectoderm than the AER alone.

It is also interesting to compare these results with the similar grafting experiments performed by Davidson *et al.* (1991) using probes specific for *Hox-7.1* and *Hox-8.1*. They found that transcripts for both genes were rapidly upregulated in normally non-expressing proximal tissue grafted beneath the AER. Their experiments showed that a clear gradient of transcripts existed within the grafts, with expression being highest immediately beneath the AER and decreasing proximally. Additionally in large grafts, the portion furthest from the AER did not exhibit upregulation of transcripts. These results are consistent with *Hox-7.1* and *Hox-8.1* expression being controlled by a factor produced by the AER which diffuses proximally into the limb, thus establishing a concentration gradient. A possible candidate factor being BMP-2A which has been shown to be localised to the AER during the early stages of mouse limb development (Lyons *et al.* 1990). The decrease in RAR- β transcripts reported here occurred within a larger area of the distal limb and thus is probably affected by a different factor which would appear to be more widespread within the distal mesenchyme. However it is clear that whatever the nature of the repressor, it can be overridden by addition of endogenous RA since Noji *et al.* (1991) showed that local application of beads soaked in RA or in various retinoid-analogues, resulted in the rapid upregulation of RAR- β transcripts within the

distal part of the limb.

6 distal to proximal grafts were partially contained in the highly expressing central core region of the limb. However the level of RAR- β transcripts expressed in these grafts never approached those found in the adjacent proximal core tissue even after 24 hours, when it appeared that the graft was differentiating into cartilage. This suggests that the proximal core tissue which is expressing high-levels of RAR- β transcripts is incapable of inducing a similar high level of transcripts in adjacent tissue. It is debateable whether or not the portion of the graft which differentiates into cartilage and becomes incorporated into this skeletal element is respecified in terms of its position or is merely affected by local factors which cause its differentiation into cartilage. Kieny (1977) claimed that a high degree of regulation was found when distal limb bud tissue was grafted onto proximal stump tissue of a different age; however Summerbell and Lewis (1975) found the opposite, in that proximo-distal positional identity was determined at an early stage and little if any regulation occurred at the boundary of tissue of different proximo-distal identities. The results presented here also tend to suggest that proximodistal identity is established early, and distal tissue moved to a proximal site is not respecified with the positional value of the surrounding proximal tissue. Thus the graft is influenced by local cues to form cartilage and is incorporated into the cartilage element, however it has not been respecified as proximal cartilage since it does not express the high-levels of RAR- β transcripts that these results suggest are a specific characteristic of this tissue.

The regions of grafts contained within the dorsal periphery of the limb differentiate as loose connective tissue. In 6 out of 9 cases these grafts were expressing RAR- β transcripts at levels somewhat higher than in the distal tissue from which they originate, and

approximately equal to the surrounding host peripheral tissue. In the remaining 3 grafts there was no detectable increase in the level of transcripts. The finding that the level of transcripts increases within distal tissue moved to a proximal site is consistant with the presence of a repressor in the distal tip of the limb, since following removal from this environment the inhibition of RAR- β transcription is also removed. These results indicate that RAR- β gene expression in the wing bud is not cell autonomous but is position-dependent, with expression being repressed at the distal tip of the bud and permitted in the proximal region. However RAR- β expression is not induced in distal tissue grafted proximally, therefore it would appear that expression of high levels of RAR- β transcripts in the proximal core tissue of the limb is not maintained by the presence of an inducer. Rather it is a characteristic of this tissue throughout its development. Other limb tissue is unable to express these levels of RAR- β in the absence of exogenous inducers at these early stages of limb development. The only other limb tissue which is known to express high levels of RAR- β transcripts are the cells between the ulna and radius and between the digits. In these areas RAR- β may be involved in programmed cell death or inhibition of cartilage differentiation as discussed previously. Since RAR- β expression is retained throughout the development of cells destined to form the shoulder, and since local application of high doses of RA, which is known to increase RAR- β levels, to this site result in a high incidence of shoulder girdle abnormalities (Oliver *et al.* 1990), RAR- β is likely to be important in the normal development of this region.

RAR- β expression in micromass culture

Having established that the expression of RAR- β is position-

dependent in that expression is repressed at the distal tip but permitted in the proximal region, and that high levels of transcripts are only seen in cells destined to form parts of the shoulder girdle or in cells exposed to application of exogenous RA, it was then possible to observe the behaviour of RAR- β expression in micromass culture. This permitted the examination of RAR- β behaviour outside of the controlling factors present *in vivo* and also gave some indication of how accurately this system mimics limb development.

Proximal cultures grown in the presence or absence of serum both expressed RAR- β transcripts, with cultures grown in the presence of serum expressing higher levels than those grown in DM alone. These findings are consistent with the behaviour of RAR- β expression *in vivo*, since when cultured the cells are not prevented from differentiating into cartilage, in fact the culture system favours cartilage differentiation especially in the presence of serum. Also serum is known to contain RA which also increases RAR- β transcript levels.

Distal cell cultures also expressed RAR- β transcripts. Transcript levels were not enhanced by addition of serum although they were doubled by addition of 1 μ g/ml RA with 10ng/ml bFGF. Addition of bFGF alone did not cause this increase therefore it is presumably the result of adding RA, although a synergistic action of bFGF and RA cannot be ruled out. These findings are also consistent with *in vivo* expression, in that upon removal from the inhibitory influence of the AER, the cells are able to differentiate and also to express RAR- β transcripts. Addition of exogenous RA also doubled the number of transcripts although serum had no effect. The reason why serum did not increase RAR- β transcript levels in distal cultures may be that distal cells are unresponsive to such low levels of RA. This may be due to the fact that they contain high levels of CRABP, although it has not been shown that they retain expression of high levels of this protein when removed from

the limb and cultured. It is also possible that the proximal cultures grown in serum were not responding to RA in the serum, but retained higher levels of transcripts because a greater proportion of the original RAR- β expressing cells were able to differentiate into cartilage.

The fact that these results are consistent with the distribution and position-dependent behaviour of RAR- β transcripts *in vivo* therefore suggests that this culture system provides a fairly accurate reflection of *in vivo* behaviour.

An *in vitro* model of the progress zone?

One of the recurring themes of this study has been investigation of various aspects of the progress zone.

The experiments described in Chapter 2 illustrated that newly prepared distal mesenchyme cells require insulin or bFGF for survival and attachment in culture. This strongly implies that these cells possess functional FGF and IGF receptors at the time of plating. The finding that bFGF in the presence of insulin also resulted in the highest rate of proliferation after 24 hours supports this view and thus suggests that FGF and IGF are involved in the normal physiology of this region *in vivo*. This is further supported by the identification of IGF peptide (Ralphs *et al.* 1990) and IGF-I mRNA (Stylianopoulou *et al.* 1988) in the distal mesenchyme of limb buds, and the finding that FGF receptor transcripts are present in distal mesenchyme cells (Wanaka *et al.* 1991) and the secreted factor FGF-4 is produced by cells of the posterior AER (Niswander and Martin, submitted).

AERs grafted onto one day old distal cell micromass cultures stimulated proliferation in the underlying mesenchyme and resulted in formation of an outgrowth. This effect was not seen in 2 or 3 day old distal cultures, nor in one day cultures grown in a richer medium (DM + S). It appeared that these cells had progressed too far down the pathway of chondrogenic differentiation, and had thus lost the ability to respond to a grafted AER.

In cultures which were able to respond to a grafted AER the rate of proliferation within the outgrowth was consistently higher than that in other regions of the cultures, especially 72 hours after grafting when cell differentiation in these other regions was well advanced. The

slight decline in the rate of proliferation of sub-AER cells reflected a similar decline *in vivo*. When these cultures were examined by *in situ* hybridisation, the cells within the outgrowth expressed a high level of *Hox-7.1* transcripts. Cells beneath the adjacent non-ridge ectoderm and in the rest of the culture did not contain transcripts. Since transcripts were also undetectable in the same cultures after 24 hours when the AERs were grafted this suggests that the AER produces a factor which stimulates transcription of the *Hox-7.1* gene. This result therefore confirms the previous reports of Davidson *et al.* (1991) and Robert *et al.* (submitted).

The implication of these results is that AERs grafted onto distal cultures result in outgrowths which mimic the *in vivo* progress zone. As such this system could prove useful in determining the complex interactions which occur in this region. The finding that cells within these outgrowths elaborate an extracellular matrix rich in fibronectin, collagen type I, chondroitin sulphate and heparan sulphate therefore suggests that this is a characteristic of the progress zone. These components may function to trap growth factors produced by the AER and the progress zone cells, thus restricting their activity to this region. This function has been shown for ECM components in other regions, as discussed in Chapter 1. The localisation of *Hox-7.1* to other sites of inductive epithelial-mesenchymal interactions together with these findings raises the possibility that genes encoding extracellular matrix structural components may be downstream targets of *Hox-7.1*.

One way of using this experimental system to identify which factors are responsible for mediating the effects of the AER would be to inject antibodies or sense oligonucleotides specific for various factors or their mRNAs respectively beneath a grafted AER, and to see which of these were sufficient to prevent the *Hox-7.1*-inducing and

outgrowth promoting effects. The difficulty with this approach is that it may be difficult to ensure that the function of a particular factor is completely ablated. Injection of *Hox-7.1* sense-strand oligonucleotides into this system might also give some insight into its function. For example if such treatment prevented outgrowth formation it would suggest that *Hox-7.1* was involved in the stimulation or at least maintenance of proliferation in these cells.

A possible approach to determining which growth factors are produced by progress zone cells would be to make use of the polymerase chain reaction (PCR). This technique involves the amplification of specific DNA sequences and thus could enable the identification of growth factors from small numbers of cells. The disadvantage of this is that the exact DNA sequence encoding the factor must be known, therefore this technique would only allow identification of previously characterised factors.

Another way of identifying growth factors produced by cells in different regions of the limb, would be to make cell lines from transfected mouse embryos and isolate factors from conditioned media. The micromass culture system would provide an excellent assay upon which to initially test cell clones for activity and eventually to test conditioned medium and the purified factor. The growth factors responsible for mediating the effect of the AER could thus be determined using the expression of *Hox-7.1* and the rate of proliferation as assays for AER-like activity. Obvious initial candidate factors include BMP-2A and *Wnt-5a*.

Similarly this approach could also be adopted to isolate the anti-chondrogenic factor produced by non-ridge ectoderm. The investigation of the ectoderm-induced anti-chondrogenic effect reported here revealed that it was not mediated by changes in the extracellular matrix components fibronectin, heparan sulphate or hyaluronate.

Similarly addition of TGF- β for 24 hours prior to ectoderm grafting was insufficient to prevent the resulting inhibition of cartilage differentiation, despite the fact that TGF- β is a potent stimulator of chondrogenesis in this experimental system (Chapter 2; Kulyk *et al.* 1989). This, together with the finding of Gregg *et al.* (1989) that type II collagen transcripts were downregulated in cells beneath a grafted ectoderm after as little as three hours, suggests that this effect is mediated directly by a growth factor produced by the ectoderm. A possible candidate is the *Wnt-3* gene product, transcripts of which are restricted to the entire wing bud ectoderm but are absent from the mesenchyme (Roelink and Nusse, 1991); however other as yet uncharacterised members of this growth factor family, or indeed other growth factor families, may also share this distribution.

Myogenic differentiation

Another interesting finding of this work was that bFGF inhibited muscle differentiation, although this effect was only seen in the presence of insulin. However both bFGF and IGFs have been localised in cells of the myogenic lineage from their origin in the myotome, throughout their subsequent development and in the terminally differentiated myotubes. If the combination of these two factors has the same effect *in vivo* as in this culture system, then some further antagonist of bFGF must block this inhibition in order to allow muscle cell differentiation to occur. In this system addition of TGF- β blocked both the inhibitory effect of bFGF on muscle cell differentiation and the stimulatory effect on cell proliferation. *In vivo* TGF- β is first detectable in the perichondrium of the developing cartilage elements, although the distribution of the TGF- β isoforms 3-5 has yet to be described, however BMP-2A is present at earlier stages in the condensing cartilage blastema. Since this is also a member of the TGF- β

superfamily it is conceivable that it would also mimic this antagonistic action of TGF- β on bFGF. Since TGF- β is a secreted protein it could act in both an autocrine manner, on cells of the developing chondrogenic lineage, and in a paracrine manner, upon the adjacent cells in the condensing dorsal and ventral muscle masses. Thus the inhibitory effect of bFGF would be alleviated and muscle differentiation could therefore commence. This model is consistent with the observation that transcripts for the muscle regulatory genes MyoD1 and myogenin are detected in myoblasts in the proximal regions of 11 day old mouse embryo limb buds, but cannot be detected at earlier stages when proliferation is the predominant activity in the developing limb.

This observation would be difficult to confirm *in vivo*, however one possible approach would be to use antibodies, especially antibodies to TGF- β receptors; the theory being that these would bind to TGF- β receptor sites on myoblast cells thus preventing these cells from responding to any TGF- β which might be present. If these cells subsequently failed to differentiate this would provide strong evidence supporting the need for a bFGF antagonist in order to allow muscle differentiation to proceed.

RAR- β

The proximally restricted distribution of RAR- β transcripts reported here suggests that RAR- β may be involved in the normal development of the shoulder region. It has been shown that local application of retinoic acid to the humeral region of stage 20 limb buds results in abnormalities of the scapula and coracoid bones (Oliver *et al.* 1990). Since RAR- β is induced in response to RA, it appears that overexpression of this receptor may be responsible for mediating these effects.

In reciprocal proximal-distal grafting experiments the expression of RAR- β was shown to be position dependent. When proximal tissue, which originally expressed a high level of transcripts, was grafted to the distal tip of the bud transcripts rapidly disappeared within 6 hours. This effect only occurred in the portions of the grafts which came to lie beneath the distal ectoderm. Parts of grafts which lay outside of this area, such as exposed from the dorsal surface of the limb or placed too far proximally did not exhibit a downregulation of transcripts. This suggests that transcription of the RAR- β gene or possibly the stability of RAR- β transcripts is repressed by a factor produced by the distal ectoderm. These results also support the earlier hypothesis that RAR- β is involved in the formation of the shoulder, in that the fate of the proximal tissue used in these experiments appeared to be to form cartilage. If the graft was placed in a region where cartilage differentiation was inhibited then the level of transcripts in the graft decreased whereas when placed in more proximal regions, where cartilage differentiation was permitted, the level of transcripts remained unchanged.

The results of grafting distal tissue to a proximal site, showed that upon removal from the repressor at the tip of the limb, the level of transcripts within the grafts increased; however if placed in the middle of the central cartilaginous core of the bud, the level of transcripts within the graft never approached the high level seen in the adjacent proximal core tissue, despite the fact that the graft appeared to be differentiating into cartilage. These results are thus consistent with the existence of a RAR- β repressor in the distal ectoderm, and also suggest that high levels of RAR- β expression may be a characteristic of proximal core cartilage.

There are several approaches which could be used in an attempt to understand the role of RAR- β in limb development. It would be

interesting to see the effects of perturbing RAR- β function by injecting oligonucleotides at various levels and stages of developing limbs. It would also be interesting to map the availability of free RA within the developing limb. A suitable technique might be implantation of cells containing a RA-activated promoter fused to a reporter gene, such as *lacZ*. If clones of different sensitivities could be developed, then different levels of free RA could be detected. However, as with all of the transcription factor-encoding genes that have been identified in the developing limb, identification of their downstream targets will be required before the molecular mechanisms controlling limb development can be elucidated.

Identifying the downstream targets of genes is however difficult. Possible strategies include transfecting cells with constructs containing the gene in question under the control of an constitutive promoter. Differential screening of cDNA libraries from cells induced to express the gene and non-expressing cells would thus identify some of the downstream targets. The major disadvantage of this procedure is that some of the target genes may be highly tissue specific and thus may not be open to transcription in cloned cell lines.

This method could also be used *in vivo* to give an indication of the function of specific genes. Thus limb bud cells could be transfected *in vivo* with retroviruses such that they are made to constitutively express a specific gene. The changes to normal development that this causes would therefore give an indication of the normal function of these genes. For example, if limb bud cells were made to constitutively express *Hox-7.1* would the whole bud remain in an undifferentiated, proliferative progress zone-like state?

The developing vertebrate limb bud is one of the most fully characterised systems in developmental biology. The identification of the spatially restricted synthesis of growth factors in the developing

limb has suggested the means by which cell differentiation may be controlled. The identification of spatially restricted expression patterns of homeobox-containing genes has provided a potential mechanism for the establishment of positional identity at the molecular level and the discovery of receptors and cytoplasmic binding proteins for retinoic acid has established the presence of an intracellular machinery capable of mediating the diverse effects of this potential morphogen. The challenge for the future is to understand the complex interactions of all of these elements and thus eventually to identify the molecular mechanisms involved in the development of the limb.

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