~~STUDIES ON~~ STUDIES GENE EXPRESSION IN FIRST TRIMESTER EMBRYOGENESIS

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Abstract

The expression of genes of the type β transforming growth factor (TGF-β) and retinoic acid receptor (RAR) gene families was investigated in human first trimester development using the techniques of *in situ* hybridization and reverse transcription polymerase chain reaction (RT-PCR).

The TGF-β1 gene was found to be expressed in choriocarcinoma cells, but not in teratocarcinomas. In human embryos, TGF-β1 RNA was detected in regions of ossification, endothelia and in haematopoietic progenitor cells. TGF-β2 RNA had a much wider pattern of expression, both temporally and spatially, including respiratory and sensory epithelia, certain neuroepithelia, prechondrogenic scleroblastemae and the growth plates of the cartilage models of the long bones. TGF-β3 RNA was primarily expressed in mesenchyme, notably the intervertebral discs, but was also found in respiratory epithelia, where it had a different distribution to TGF-β2 RNA, and in mesothelia.

The RAR-β gene was expressed in zones of chondrogenesis, the stroma of the metanephros and kidney, and in the mesenchyme of the developing mediastinum. RAR-β RNA was also detected in the oral epithelium and in the ventral mantle zone of the spinal cord. RAR-gamma RNA was detected in mature cartilage, and was found extensively in the mesenchyme.

These results are discussed in the light of *in vitro* data, and speculations are made regarding the possibilities of interaction within and between these two gene families in human development, and also on the implications of the data for the theory of evolution of gene families.

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This thesis is dedicated to my parents.

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Note. Some of the work presented in this thesis has already been published. Reprints are bound inside the back cover.

Abbreviations

ag	attogram (10^{-18}g)
ATP	adenosine triphosphate
BSA	bovine serum albumin
CTP	cytosine triphosphate
DNA	deoxyribonucleic acid
cDNA	complementary deoxyribonucleic acid
DNase	deoxyribonuclease
DPP-C	decapentaplegic complex
DTT	dithiothreitol
EGF	epidermal growth factor
fg	femtogram (10^{-15}g)
FGF	fibroblast growth factor
Fig.	Figure
GTP	guanosine triphosphate
hCG	human chorionic gonadotropin
ICM	inner cell mass
IVF	*in vitro* fertilization
kb	kilobase pairs
kDa	kilodalton
LMP	last menstrual period
ml	millilitre
mM	millimolar
MOPS	morpholinopropanesulphonic acid

nM	nanomolar
PBS	phosphate buffered saline
p.c.	*post-coitum*
PCR	polymerase chain reaction
pers. comm.	personal communication
pg	picogram (10^{-12}g)
RAR-α	type alpha retinoic acid receptor
RAR-β	type beta retinoic acid receptor
RAR-gamma	type gamma retinoic acid receptor
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcriptase poymerase chain reaction
SDS	sodium dodecyl sulphate
TE	trophectoderm
TEMED	tetramethylethylenediamine
TGF-β	type beta transforming growth factor
TTP	thymidine triphosphate
U	International unit of enzyme
UTP	uracil triphosphate
TESPA	3-aminopropyltriethoxysilane

Chapter 1

Introduction

1.1 The use of human embryological material for the molecular analysis of development

In recent years, the mouse has become the most popular organism for studies in mammalian molecular developmental biology.

The reasons for this are mostly of a practical nature. The mouse has a short generation time, is relatively inexpensive to breed and easy to handle. Murine biology is fairly typical of the *Mammaliae* as a phylogenetic class, and, due to the activities of Victorian "mouse fanciers", many genetic mutant strains had already been isolated by the early part of this century. However, the medical importance of human biology requires that general conclusions regarding mammalian development be confirmed using human embryological specimens.

Most available human embryological and fetal material falls into three general classes. Firstly, there are prostaglandin-induced pregnancy terminations, which range from 18 to 24 weeks *post-coitum* (*p.c.*). These are generally intact but are too advanced to be of interest with respect to organogenesis. Curettage terminations are performed between 10 and 18 weeks *p.c.*. However, these are frequently damaged, and soft tissues are rarely available in a condition suitable for molecular analysis.

The RNA, for example, is usually found to be degraded. Nevertheless, some investigators have been able to detect specific RNA species in such material by Northern blotting (Scott *et al.* 1985) or *in situ* hybridization (Pfeifer-Ohlsson *et al.* 1985, Hopkins *et al.* 1987, Han *et al.* 1987, Brice *et al.* 1989). A few preimplantation embryos (usually referred to as pre-embryos) are also available, and have been used for *in situ* hybridization studies on Y chromosomal DNA (Jones *et al.* 1987), ribosomal RNA and ribosomal RNA genes (Tesarik *et al.* 1986, 1987) and mRNA (Bonduelle *et al.* 1988). More recently, PCR technology has been used for experiments aimed towards prenatal diagnosis (Coutelle *et al.* 1989, Monk and Holding 1990) and fetal sexing (Handyside *et al.* 1989). However, pre-embryos may not be grown beyond 14 days *in vitro*, due to ethical and legal considerations.

A new source of intact embryological material aged between 4 and 9 weeks of gestation has been provided by clinical trials on terminations of pregnancy using the progesterone-analogue RU486. Since the clinical procedure does not involve surgery, this material is usually undamaged, and of a suitable stage to be of interest in the study of organogenesis. These embryos are also small enough to be fixed rapidly in their entirety without the need for prior dissection. This technique was developed by Rodger and Baird (1987).

The problem of obtaining good quality human embryonic tissues suitable for molecular analysis places limits on the types of study that can be performed. In this thesis, three independent, but inter-related, studies on gene expression in human embryos have been attempted. The first study attempted to address the question of when lineage-specific gene expression is first established in the human pre-embryo. This was approached by using *in situ* hybridization to cellular RNA encoding β-human chorionic gonadotropin (hCG-β) (reviewed by Pierce and Parsons 1981). hCG-β is one of the earliest genes expressed by the zygotic genome (Fishel *et al.*

1984) and, as a trophoblast marker (Gaspard *et al.* 1980), its expression should be limited to trophectoderm-derived tissue, thus allowing the examination of differential gene expression between inner cell mass (ICM) and trophectoderm (TE).

As will be discussed later, this first project presented many technical problems which became insurmountable when combined with scarcity of embryonic material. The second two projects examined the expression of two gene families encoding proteins predicted to be of central importance in early development. Information gained from these studies has contributed to our knowledge of the role of these genes in development and demonstrates the similarity, at the molecular level, in developmental mechanisms between man and mouse.

1.2 Development of the in situ hybridization technique

In situ hybridization was developed in the late 1960s by two research groups (John *et al.* 1969, Gall and Pardue 1969), initially for application in the field of cytogenetics. This was adapted for use in the detection of viral RNA in cells by Brahic and Haase (1978). The use of *in situ* hybridization to study tissue-specific gene expression was pioneered by Hudson *et al.* (1981) and Venezky *et al.* (1981). These investigators used double-stranded DNA probes, which were denatured prior to hybridization. Modern riboprobe methodology dates from Cox *et al.* (1984). Most investigators in the field of mammalian embryology follow the protocol of Wilkinson *et al.* (1987). Other current methodologies include the use of biotin-labelled probes (Singer and Ward 1982), single-stranded DNA probes (Akam 1983) and oligonucleotides (Beck *et al.* 1987).

Riboprobes (Melton *et al.* 1984) have become the method of choice for molecular embryologists for a variety of reasons. They can be labelled to a very high specific activity; single-stranded DNA probes become unstable at comparable levels (Akam 1983). Riboprobes can also be alkaline-digested to a size of 50 to 150 nucleotides allowing greater penetration of the target tissue (Cox *et al.* 1984). Unlike double-stranded DNA probes, they have no competing complementary strand with which to reassociate. The RNA-RNA duplexes which are formed on binding of the riboprobes to their target RNA have melting temperatures 10 to 15°C higher than corresponding DNA-RNA duplexes (Wetmur *et al.* 1981, Gray *et al.* 1981).

The sensitivity of *in situ* hybridization is debatable. Some investigators have claimed to be able to detect as few as 10 to 1000 (Berger 1986), or 10 to 20 (Bandtlow *et al.* 1987), target RNA molecules per cell. However, other investigators (Duprey *et al.* 1985, Akhurst *et al.* 1988, Millan *et al.* 1991) have presented data suggesting that *in situ* hybridization is less sensitive than Northern blotting.

1.3 Lineage specification in the human preimplantation embryo

1.3.1 Historical background

Detailed morphological study of the murine preimplantation embryo began with the work of Sobotta (1903), and manipulative embryology with that of Tarkowski and Wroblewska (1967). Subsequently, techniques of cell biology and molecular biochemistry have resulted in a vast increase in knowledge of the early murine embryo. Very little, by comparison, is known concerning pre-implantation human development (reviewed by Tesarik 1988). This field has become clinically important

due to the development of techniques of *in vitro* fertilization (IVF). The success rate for IVF, in terms of number of patients who become pregnant, is 40% (Winston *et al.* 1989). Medical science could therefore benefit from a more detailed investigation of the biology of the human pre-embryo.

1.3.2 The development of the blastocyst

The eight identical blastomeres of the 2.5 day *p.c.* murine embryo divide to generate two cell populations, named the "inside" and "outside" cells on account of their relative positions within the embryo (Tarkowski and Wroblewska 1967, Barlow *et al.* 1972). By 3.5 days *p.c.*, the embryo has 64 to 128 cells in two highly distinct groups, the inner cell mass (ICM) and the trophectoderm (TE). The TE is the progenitor of the chorion, ectoplacental cone and the trophoblast giant cells. The ICM gives rise to the embryo and the remaining extra-embryonic structures. Just prior to implantation, there is a second differentiation event when the inner cell mass divides into primitive endodermal and ectodermal populations

It has been found that both the TE and ICM are not "tissues" in the sense of being developmentally committed, but are capable of extensive pluripotency and possibly even exchange of lineage (Dyce *et al.* 1987). The mechanism by which the inner and outer cell populations give rise to the ICM and TE is a central theme in pre-embryology. (reviewed by Graham and Deussen 1978).

The ultrastructure of isolated blastomeres from eight-cell embryos (1/8 cells) was studied in detail by Dulcibella and Anderson (1975) and Handyside (1980), who observed them to be polar, with microvilli on the external surface. Johnson and Ziomek (1981a,b) observed that isolated 1/8 cells will divide to give two 1/16 cell blastomeres by one of two processes. In over 80% of cases the plane of cleavage

segregates the villous surface to only one of the progeny, giving one polar and one non-polar cell. In the remaining 20% of cases, the plane of division is perpendicular to the villous surface, thus generating two polar 1/16 cells. These two forms of division are designated "differentiative" and "conservative", respectively. Johnson *et al.* (1988) postulate that the choice between differentiative and conservative division is a result of an interaction between the relative rates of loss of polarity and rapidity of mitosis. At the next two cell divisions there is some "internal allocation" of cells from the outer cell lineage into the inner lineage (Balakier and Pedersen 1982, Pedersen *et al.* 1986, Dyce *et al.* 1987).

1.3.3 Activation of the pre-embryonic genome

At fertilization the oocyte contains a great quantity of RNA. This "maternal RNA" is a heterogeneous mixture of mRNA and other RNA species in a variety of stages of processing (reviewed by Davidson 1985). Braude *et al.* (1979) used two dimensional (2-D) polypeptide gels to compare the cell-free translation products of oocyte RNA with the proteins of intact oocytes and two-cell embryos. The *in vitro* translated oocyte RNA generated a larger range of proteins than those detectable *in vivo*. This suggests that the availability of maternal mRNA for translation is subject to control. 2-D gels of zygotes and unfertilized oocytes of similar ages have shown that the processes of translation, and post-translational modification, of protein encoded by maternal message are initially independent of whether the egg has been fertilized or not (Van Blerkom 1981). This implies that a considerable proportion of the developmental programme of the pre-embryo has already been set in motion prior to fertilization.

The murine zygotic genome is active at the one-cell pronuclear phase as measured by ^{3}H-adenosine uptake (Clegg and Pikó 1982), producing large, heterogeneous, non-polyadenylated RNA. There is also a *de novo* cytoplasmic polyadenylation of stored maternal RNA at this stage. Flach *et al.* (1982) used the transcriptional inhibitor, α-amanitin, to detect two bursts of embryonic transcription at 18-21 and 26-29 hours *p.c.*, flanking the S phase of the second cell cycle. The first of these produces a 67 kilodalton (kDa) set of polypeptides, and the second a more extensive group. The 67 kDa protein group consists mainly of the murine HSP 69 and HSP 70 heat shock proteins (Bensaude *et al.* 1983). It had previously been shown by Sawicki *et al.* (1981) that a paternal isoform of β2-microglobulin was present on 2-D gels at the two-cell stage, but the precise age of the embryos in hours was not specified. Handyside and Johnson (1978) used 2-D gels to show that the morphological differentiation at the 4th cell division is accompanied by changes in the protein synthetic profiles of the two resulting cell populations. However, the possibility of lineage crossing at the next two divisions (Balakier and Pedersen 1982, Dyce *et al.* 1987) suggests that these changes are reversible.

Braude *et al.* (1988) used similar techniques to show that the corresponding activation of the human genome occurs at the four-cell stage. This is supported by the findings of Tesarik *et al.* (1986) that ^{3}H-uridine is first incorporated by the human pre-embryo at the four-cell stage. In the sheep pre-embryo the genome is activated at the eight-cell stage (Crosby *et al.* 1988). The significance, if any, of this heterogeneity between species is unknown.

The first project was designed to elucidate whether the first transcription of the hCG-β gene occurred at the stage of activation of the embryonic genome, or one cell-cycle later at the division of the embryonic and trophectodermal lineages. The possibility of

repression of transcription in cells crossing from the trophectodermal to the embryonic lineage was also borne in mind.

1.4 The Type β Transforming Growth Factor Gene Family

1.4.1 Historical perspective on growth factor research

In the late 19th century, the increasing economic and technical development of the Western World resulted in a great decline in the epidemic diseases which had previously been the major health problem of civilized communities. One consequence of this was that the focus of medical research tended to switch from scourges such as typhoid and cholera to the medical problems of a materially developed society. Two fields of interest which expanded rapidly at this time were embryology, with its obvious application to congenital malformations, and cancer research.

The growth of biochemistry in the early part of the 20th century, with the improved ability to purify biological molecules, stimulated an effort to isolate factors which might be involved in the control of development and/or tumorigenesis.

Rous and Murphy (1911) found that a filtered extract of a connective tissue tumour from chickens could transmit the tumour when injected into another chicken. The active agent in this case was Rous sarcoma virus (RSV). This discovery stimulated a whole school of cancer research, in which the technique of tumour transplantation was very prominent. Bueker (1948) was the first to demonstrate that peripheral nervous system proliferation occurs in the region of tumour transplantation. This was

confirmed by Levi-Montalcini and Hamburger (1951) who coined the term "nerve growth factor" to describe the active agent.

The first polypeptide "growth factor" to be satisfactorily purified was epidermal growth factor (EGF) (Cohen 1962). By the late 1970s and early 1980s, techniques of cellular subfractionation by chromatography, electrophoresis and column separation had advanced sufficiently to allow isolation of cellular sub-fractions, and ultimately proteins, which had effects on the phenotype of the cell with respect to growth and differentiation.

1.4.1.1 The discovery of TGF-β

In 1978, DeLarco and Todaro reported the isolation of a novel growth factor from murine sarcoma virus (MuSV)-transformed mouse fibroblasts, which they termed "sarcoma growth factor" (SGF). SGF induced a transformed phenotype in normal rat kidney (NRK) fibroblasts. The transformed phenotype was defined by loss of density-dependent growth inhibition, and acquisition of anchorage-independent growth, thus resulting in colony formation. This effect was immediately reversible on withdrawal of the factor. SGF was shown to be antigenically distinct from epidermal growth factor (EGF), but nevertheless was a competitor for the EGF receptor (Todaro *et al.* 1980). Similar factors were subsequently isolated from a variety of virally and chemically-transformed cell lines (Roberts *et al.* 1980), and from human tumour cell lines (Todaro *et al.* 1980). It was Roberts *et al.* (1980) who coined the term "transforming growth factor" (TGF) in preference to SGF.

Moses *et al.* (1981) detected two kinds of TGF in chemically-transformed fibroblasts, as did Roberts *et al.* (1982) in virally-transformed fibroblasts. Only one of these

competed for the EGF receptor. The competing and non-competing fractions were termed TGF-α and β respectively. The original SGF was re-isolated by Anzano *et al.* (1983) and shown to be a mixture of TGF-α and TGF-β.

A large amount of data has since accumulated indicating that the function of TGF-β is growth-inhibitory in most systems (reviewed by Sporn and Roberts 1988). Even in fibroblasts it can be antiproliferative under some circumstances (Roberts *et al.* 1985, Kimura *et al.* 1988). This has resulted in attempts to introduce less specific terminology (*eg.* Hanks *et al.* 1988). However, the name "transforming growth factor" has endured, and it seems unlikely that it will be superseded at this stage.

Prior to the realization that TGF-β proteins were encoded by a gene family, the term TGF-β was used as if it referred to a unique protein. Although inexact, this usage persists in some quarters to this day. Where the term TGF-β is used without qualification, it must be assumed to refer to a mixture of the various TGF-β isoforms.

1.4.2 The TGF-β gene superfamily

The cloning of a human cDNA for TGF-β (Derynck *et al.* 1985), now designated TGF-β1, revealed several interesting facts concerning the molecule. Firstly, the mature protein only represents a small carboxy-terminal fraction, consisting of 112 amino acids which is cleaved from a much larger precursor polypeptide. As expected for a secreted molecule, a sequence encoding a signal peptide (reviewed by Walter and Lingappa 1986) was present at the N-terminus of the precursor. Other members of the TGF-β gene family possess this general structure, although each has its own variations in sequence and size, for example chick TGF-β4 has no signal peptide (Jakowlew *et al.* 1988c).

As TGF-β1 genes began to be isolated from other species, it became apparent that the bioactive 112 amino acid mature monomer of TGF-β1 is almost totally conserved across species boundaries (Derynck and Rhee 1987, van Obberghen-Schilling *et al.* 1987, Sharples et al 1987, Kondaiah *et al.* 1988, Jakowlew *et al.* 1988a). The most divergent TGF-β1 isolated is that from the mouse (Derynck *et al.* 1986) which has only a single amino acid change from the human sequence in the mature portion.

Wrann *et al.* (1987) isolated a 12.5 kDa protein from a human glioblastoma cell line that had 8 out of 20 N-terminal amino-acids identical to the corresponding region of the mature TGF-β1 monomer. This second molecular species was also found in a human prostatic adenocarcinoma cell line (Ikeda *et al.* 1987, Marquardt *et al.* 1987), and named TGF-β2. The isolation of TGF-β2 cDNA clones (De Martin *et al.* 1987, Madisen *et al.* 1988) revealed heterogeneity in the TGF-β2 precursor protein. The clone of De Martin *et al.* (1987) predicted a 414 amino acid precursor, whereas that of Madisen *et al.* (1988) coded for a 442 amino acid polypeptide. The larger species had a 29 amino acid insertion in the amino terminal region of the precursor, in the place of a single asparagine residue in the shorter form. Webb *et al.* (1988) re-isolated both species from human and simian cDNA libraries, and proposed that the difference was due to alternative splicing of the TGF-β2 mRNA. Hanks *et al.* (1988) isolated a cDNA clone for the growth inhibitor protein from the simian BSC-1 kidney epithelial cell line (Holley *et al.* 1980, Tucker *et al.* 1984a). This corresponded to the shorter form of TGF-β2. Webb *et al.* (1988) proposed that the shorter and longer forms be designated TGF-β2a and TGF-β2b respectively.

TGF-β2 was also isolated from porcine platelets (Cheifetz *et al.* 1987), and murine TGF-β2 has now been cloned (Miller *et al.* 1990, Millan *et al.* 1991). TGF-β1 and β2

can exist as a heterodimer, TGF-β1.2, which is biologically active (Cheifetz *et al.* 1987, 1988) and present in several human cell lines (Danielpour *et al.* 1989).

A cDNA clone for a third family member, TGF-β3, was isolated from a human rhabdomyosarcoma cell line (ten Dijke *et al.* 1988). This also had a 112 amino acid mature monomer. Porcine TGF-β3 was cloned by Derynck *et al.* (1988), chick TGF-β3 by Jakowlew *et al.* (1988b) and mouse TGF-β3 by Denhez *et al.* (1990). All these proteins demonstrate great inter-specific conservation of mature protein-coding sequences.

TGF-β4 cDNA has been obtained from chick embryo chondrocytes (Jakowlew *et al.* 1988c), and TGF-β5 from *Xenopus* (Kondaiah *et al.* 1990). TGF-β4 is unique in the gene family in that it does not possess a signal peptide and is thus presumed to be totally intracellular, or secreted by an alternative pathway. Three additional TGF-β genes have been cloned recently from *Xenopus* (Thomsen and Melton 1990). Therefore, the immediate gene family now comprises at least 8 members, of which only 3 have been found in mammals.

Whereas the immediate members of the TGF-β gene family described above generally have at least 70% amino acid homology to the TGF-β1 polypeptide, there exist a number of genes which are more distantly related. It is notable that most of these genes are involved in a variety of developmental processes.

Inhibins and activins (Mason *et al.* 1985, Forage *et al.* 1986, Ling *et al.* 1986, Vale *et al.* 1986) are gonadal hormones which act upon the mammalian pituitary to inhibit or stimulate the release of follicle-stimulating hormone (FSH), respectively. The structure of inhibin is a heterodimer of an α and either of two β monomers, thus represented as $\alpha\beta_a$ or $\alpha\beta_b$. The β chains are the most closely related to TGF-β at

46% and 38% homology respectively. The α chain is rather more distantly related. Activin molecules are homodimers of either of the two β chains of inhibin, $\beta_a\beta_a$, or $\beta_b\beta_b$. The inhibin/activin polypeptide chains are cleaved from a longer precursor in the same manner as TGF-β. Activin A ($\beta_a\beta_a$) is identical to erythroid differentiation factor (Murata *et al.* 1988). Activin B ($\beta_b\beta_b$) has been shown to be the mesoderm-inducing factor (MIF) (ie. the "second signal" of Slack *et al.* 1989) from the *Xenopus* XTC cell line (Smith 1987, reviewed by Smith 1989, Green *et al.* 1990). Exogenous TGF-β2 can act as a mesoderm-inducer on *Xenopus* animal caps cultured *in vivo* (Rosa *et al.* 1988). TGF-β1 can also act in this manner but requires the additional presence of bFGF (Kimelman and Kirschner 1987, Slack *et al.* 1987, 1988). However, no endogenous TGF-β1 nor TGF-β2 is found in *Xenopus* embryos at this stage (Kimelman *et al.* 1988, Slack and Isaacs 1989).

Mullerian inhibitory substance (MIS) (Cate *et al.* 1986) is responsible for the regression of the female reproductive tract during the development of male embryos. Its mature protein sequence is 31% homologous to that of TGF-β1. The role of MIS in the regression of the female reproductive tract in male embryos is complex (Behringer *et al.* 1990).

Another related sub-group of the TGF-β gene superfamily is found in non-mammalian systems, and is also apparently involved in the regulation of development. The prototype of this group is the Drosophila *decapentaplegic* gene (*dpp*) (Padgett *et al.* 1987), which is the coding sequence within the larger *Decapentaplegic* complex of mutations (*DPP-C*), involved in the specification of embryonic pattern formation. *dpp* is 36% homologous in its C-terminal region to TGF-β1 and is particularly interesting from an evolutionary point of view in that it demonstrates that the origin of the TGF-β gene superfamily pre-dates the divergence of the arthropod and vertebrate lineages.

In *Xenopus*, a maternal mRNA species, encoded by the *Vg1* gene, and localized to the vegetal hemisphere of the oocyte, is also closely related to *dpp* (Weeks and Melton 1987, Tannahill and Melton 1989). The mammalian homologue of *Vg-1*, named *Vgr-1* (Lyons *et al.* 1989a), has a specific pattern of expression during embryogenesis (Lyons *et al.* 1989b).

Also within this sub-group are the bone morphogenetic proteins (BMPs) (Wozney *et al.* 1988), which are the nearest mammalian homologues of *dpp.* These also exhibit temporal and spatial specificity of expression in murine development (Lyons *et al.* 1989b, 1990).

1.4.3 Latent TGF-β

As mentioned in the previous section, the isolation of a TGF-β1 cDNA clone by Derynck *et al.* (1985) revealed that the mature polypeptide chain represented a carboxy-terminal fragment of a rather longer precursor protein. Assoian *et al.* (1983) isolated the active form of TGF-β from human platelets using acid extraction. The active TGF-β molecule was shown to be a dimer of 12.5 kDa subunits. The monomer was biologically inactive. This molecular weight of 12.5 kDa matches exactly that predicted from the TGF-β1 cDNA clone (Derynck *et al.* 1985).

TGF-β exists both in an active form and in a latent precursor form requiring activation *in vitro* by heat or acidification (Lawrence *et al.* 1984, 1985). The latent form consists of the TGF-β dimer, uncleaved from its precursor and complexed with another protein (Wakefield *et al.* 1988). It is biologically inactive (Pircher *et al.* 1986), and incapable of binding to any TGF-β receptor (Wakefield *et al.* 1988).

Miyazono *et al.* (1988) employed a delicate procedure to isolate latent TGF-β from human platelets, minimizing activation during preparation. The latent form thus purified had three components; a 25 kDa disulphide-bonded dimer corresponding to mature TGF-β, an 80 kDa disulphide-bonded dimer that is the precursor protein without the signal peptide, known as the latency-associated peptide (LAP) and a third, TGF-β-binding protein (TGF-β-BP) sized at 125 to 160 kDa. The LAP dimer was also disulphide-bonded to TGF-β-BP. This last protein has been cloned and sequenced (Kanzaki *et al.* 1990). The predicted core protein was 151 kDa, and contained 16 EGF-like repeats. In studies on recombinant TGF-β1 genes in CHO cells, Gentry *et al.* (1987,1988) saw no larger protein as part of the precursor complex. In view of this, Miyazono *et al.* (1988) suggested that the TGF-β-BP may be a protease co-isolated in the process of cleaving the precursor dimer. However, the TGF-β-BP cDNA sequence (Kanzaki *et al.* 1990) had no homology to any known protease, and purified TGF-β-BP had no protease activity *in vitro*.

Lyons *et al.* (1988) demonstrated that some proteases could activate latent TGF-β activity from conditioned media. Miyazono and Heldin (1989) demonstrated that specific removal of N-linked polysaccharides from the precursor is sufficient to result in the release of TGF-β in the active form.

Analysis of platelet-rich serum revealed the presence of an additional latent form consisting of a 25 kDa dimer in association with α-2-macroglobulin (O'Connor-McCourt and Wakefield 1987). This complex was readily created *in vitro* (Huang *et al.* 1988). Wakefield *et al.* (1988) suggested that this form was a clearance complex.

1.4.4 The TGF-β receptor

The method of radioactively labelling TGF-β with ^{125}I was used by a number of investigators to study TGF-β-receptor interaction by Scatchard analysis. Additionally, the binding of ^{125}I-TGF-β to the receptor enabled complexes to be isolated and studied by polyacrylamide gel electrophoresis. Frolik *et al.* (1984) carried out a Scatchard analysis on NRK cells, finding that high affinity binding sites, by inference receptors, were present at around 17,000 per cell. This experiment was repeated by Tucker *et al.* (1984b) and Massagué and Like (1985), who extended the analysis to a variety of normal and transformed cell lines. Binding tended to be less efficient to chemically-transformed or tumour cell lines. Wakefield *et al.* (1987) studied the widest variety of cells, finding a range in receptor number from 600 per cell in stimulated human tonsillar T-lymphocytes, up to 81,000 per cell in murine embryonic 3T3 fibroblasts. Essentially, TGF-β receptors can be considered to be ubiquitous, although there are some cases where failure to respond to TGF-β has been correlated with an inability to bind TGF-β on the cell surface, for instance in retinoblastoma cell lines (Kimchi *et al.* 1988).

Other studies on TGF-β receptor proteins suggested that there were a variety of cell-surface proteins binding TGF-β with varying affinity under different circumstances (Massagué and Like 1985, Massagué 1985, Cheifetz *et al.* 1986, Segarini *et al.* 1987, Cheifetz *et al.* 1988, Segarini *et al.* 1989). The three principal TGF-β-binding moieties were sized at 280 kDa, 85 kDa and 65 kDa (Massagué and Like 1985, Massagué 1985). The relative efficiency of binding of TGF-β1, TGF-β2 and the TGF-β1.2 heterodimer varies between receptors (Segarini *et al.* 1987, 1989). The largest binding species is a dimeric proteoglycan (Massagué 1985, Segarini and Seyedin 1988), named betaglycan, which can also exist in a soluble form (Andres *et*

al. 1989) and may be merely a binding protein with no receptor activity (Cheifetz *et al.* 1988, Boyd and Massagué 1989). The two lower molecular weight receptors are essential for the cellular response to TGF-β (Boyd and Massagué 1989, Segarini *et al.* 1989, Laiho *et al.* 1990), but their relative importance is still unclear. None of the genes for receptor proteins have yet been isolated. Andres *et al.* (1989) speculated that betaglycan is a membrane bound form of TGF-β-BP (Miyazono *et al.* 1988). However, Kanzaki *et al.* (1990) demonstrated that the two proteins have different structures and properties.

1.4.5 Possible molecular mechanisms of action of TGF-β

Numerous studies have been carried out on the effects of TGF-β on cells *in vitro* (reviewed by Lyons and Moses 1990, Moses *et al.* 1990). In general, TGF-β acts on mesenchymal cells to stimulate their growth, and on epithelial cells as a growth inhibitor. In some systems, TGF-β can act as either a growth stimulator or inhibitor depending on its concentration (Battegay *et al.* 1990). Experiments *in vitro* have suggested that the effects of TGF-β may be due to its ability to regulate synthesis of the extracellular matrix.

Roberts *et al.* (1986) showed that collagen secretion by fibroblasts is elevated by TGF-β *in vitro*. Ignotz and Massagué (1986) studied the effects of TGF-β on a chick embryonic fibroblastic cell line, finding that immunoprecipitable fibronectin, pro-α-1 collagen and pro-α-2 collagen were elevated. This effect can be disrupted by actinomycin D, indicating an involvement of transcription, and, in the case of collagen, is dependent on a mechanism mediated by nuclear factor type 1 (NF-1) (Rossi *et al.* 1988). Similar experiments have demonstrated that TGF-β can stimulate the production in fibroblasts of type II collagen (Seyedin *et al.* 1985), type III

collagen (Varga *et al.* 1987), tenascin (Pearson *et al.* 1988) and thrombospondin (Pentinnen *et al.* 1988). Wikner *et al.*(1988) showed that TGF-β increases fibronectin secretion by keratinocytes. In human MRC-5 fibroblasts, TGF-β decreases transcription of collagenase. Levels of collagen and fibronectin are unaffected (Edwards *et al.*1987) and there is an increase in transcription of tissue inhibitor of metalloproteinase (TIMP). Other degradative enzymes are suppressed by the action of TGF-β, including elastase (Redini*et al.*1988), transin/stromelysin (Machida *et al.*1988), plasminogen activator (Lund *et al.*1987) and major secreted thiol protease (Chiang and Nilsen-Hamilton 1986). Concurrent with this down-regulation of proteases, protease inhibitors are stimulated. These include plasminogen-activator inhibitor (Lund *et al.*1987) and tissue inhibitor of metalloproteinases (Edwards *et al.*1987).

Summarizing these data, TGF-β has a tripartite effect on extracellular matrix, through the stimulation of synthesis of matrix components, inhibition of those enzymes which degrade these components and stimulation of factors which inhibit the degradative enzymes. Postlethwaite *et al.*(1987) demonstrated that TGF-β can also exert a chemotactic effect on fibroblasts.

Leof *et al.* (1986) analysed the proliferative action of TGF-β on AKR-2B fibroblasts. TGF-β first increases the level of c-*sis* mRNA and PDGF protein, and secondly of c-*myc*, c-*fos* and c-*jun* mRNA. The last two may be a consequence of the autocrine action of PDGF. The complexity of this chain of events means that TGF-β-induced proliferation only occurs in this system after a 24 hour delay (Shipley *et al.*1985). The observations of Leof *et al.*(1986) have been further investigated by Makela *et al.*(1987), who found that, in leukaemia cells, TGF-β increases transcription of PDGF-A chain only. However, in endothelial cells, TGF-β induces the transcription of

both chains (Daniel *et al.*1987, Starksen *et al.*1987). Antibodies directed against the PDGF-AA homodimer can reverse TGF-β induced DNA synthesis in aortic smooth muscle cells demonstrating that the proliferative action in this case acts through induction of PDGF-AA homodimer (Battegay *et al.*1990).

The mechanism by which TGF-β inhibits cell proliferation has been investigated in the skin keratinocyte system by Pietenpol *et al.* (1990,a,b). Once again, the action of TGF-β is indirect, acting through retinoblastoma protein (pRb) to inhibit c-*myc* transcription.

Ignotz and Massagué (1987) showed that TGF-β1 and β2 both increase the level of cell adhesion protein receptors on the surface of a variety of cell lines. This case is particularly interesting in that it showed that this is effected by stimulation at both the transcriptional and post-transcriptional levels, and it also implies that the effects of TGF-β extend to the expression or activity of proteins that regulate the kinetics of polypeptide processing.

1.4.6 Regulation of expression of TGF-β

Danielpour *et al.* (1989) detected TGF-β1 and TGF-β2 immunologically in a variety of cell lines, demonstrating that relative levels of the two isoforms vary widely. This implies that the regulation of each gene is independent. Van Obberghen-Schilling *et al.* (1988) showed that TGF-β1 can positively regulate its own gene expression *in vitro*. Bascom *et al.* (1989b) extended these observations to the other TGF-β isoforms, finding that TGF-β1 does not induce production of TGF-β2 nor TGF-β3. TGF-β2, by contrast, induced production of all three isoforms.

Kim *et al.* (1989a,b) identified the 5′-transcriptional start sites for the human TGF-β1 gene and defined the regions of the promotor responsible for the autoregulatory response in A549 lung adenocarcinoma cells. There are two major transcriptional start sites located 273 nt apart, between which there is a weak promotor site. A two to threefold stronger promotor is located between 454 and 323 nt upstream of the first start site. The consensus sequence for the binding of nuclear factor-1 (NF-1) located at –260 to –240 nt (van Obberghen-Schilling *et al.* 1988) does not appear to be involved, suggesting that NF-1 is not a transcription factor in TGF-β autoinduction. Gel retardation assays demonstrated a range of protein-DNA complexes in the TGF-β1 promotor region as defined by Kim *et al.* (1989a,b), suggesting that transcriptional regulation of TGF-β1 is complex.

In contrast, a NF-1 site is involved in the stimulation of the α2(I)-collagen promotor by TGF-β (Rossi *et al.* 1988). This sequence is around –300nt in the α2(I)-collagen gene, and when placed upstream of the SV40 early transcriptional start site renders it TGF-β-inducible. Evidently, the route by which TGF-β activates genes can be different depending on the system and the gene involved.

Northern blotting has been used to give some indication of the patterns of expression of TGF-β genes *in vitro*. Derynck *et al.* (1985) probed polyadenylated RNA from a variety of epithelial and mesenchymal cell lines, finding that all expressed a 2.5kb TGF-β1 mRNA. A slightly smaller species was also found in lymphomatous B lymphoblasts. A larger species of 4.2 to 4.5kb was also detected by Akhurst *et al.* (1988) following tumour promotion in mouse skin, and also in several transformed cell lines (ten Dijke *et al.* 1988).

TGF-β1 can undergo alternative splicing. In addition to the production of a mRNA encoding the mature protein product, there is also an alternatively spliced mRNA

which results in the deletion of two exons and leads to a frame-shift. The putative precursor protein translated from this transcript has only 256 amino acids (Kondaiah *et al.* 1988). When proteolytically cleaved, this would release C-terminal peptides of either 39 or 45 amino acids, depending on the choice of cleavage site. However, these peptides have never been detected *in vivo,* and the function, if any, of this alternative product is unclear.

TGF-β2 has three major mRNA species of 4.1kb, 5.1kb and 6.5kb (Madisen *et al.* 1988). The relationship of these to the two kinds of TGF-β2 as defined by Webb *et al.* (1988) is uncertain. An oligonucleotide probe for the region present only in the 5′-region of the longer TGF-β2b cDNA species detects only the 5.1kb mRNA. The 4.5kb TGF-β2a mRNA does not have the extra sequences present in the 3′-untranslated region of the 6.5kb TGF-β2b mRNA and the 5.1kb TGF-β2b mRNA. Adenocarcinoma cells contain all species of TGF-β2 mRNA, whereas BSC-40 cells contain only TGF-β2b species. Unlike the alternative splice in TGF-β1 mRNAs, the mature TGF-β2 protein is unaffected. However, the differences may be important in regulation of TGF-β2 translation.

TGF-β3 encodes a single mRNA species of between 3.2 and 3.5kb (ten Dijke *et al.* 1988, Derynck *et al.* 1988), which is present in a variety of mesenchymal cell lines comparable to those expressing TGF-β1, but is absent from all epithelial lines tested (Derynck *et al.* 1988). So far there is no evidence of alternative splicing for TGF-β3.

1.4.7 TGF-β in mammalian embryogenesis

This project was planned in the light of the studies of Lehnert and Akhurst (1988) and Wilcox and Derynck (1988) on the expression of TGF-β1 RNA in murine

embryogenesis. In summary, TGF-β1 mRNA was localized in liver megakaryocytes, ossifying bone, whisker pads, salivary glands, tooth buds and the mesenchyme of the lung, gut and kidney. It was also detected in the endocardial cushions, where it persists at least to the neonatal period (Akhurst *et al.* 1990a,b). TGF-β1 RNA seems to be common at sites where tissue remodelling is taking place, for example in heart, bone and tooth development, but is also detectable in the more developmentally static mesenchyme of the gut, kidney and lung.

In preimplantation mouse embryos, Rappollee *et al.* (1988) amplified TGF-β1 RNA , using the reverse transcription polymerase chain reaction (RT-PCR) technique. Studies on human embryos were confined to the work of Sandberg *et al.* (1988a,b), who detected TGF-β1 mRNA in 16.5 week *p.c.* human fetal bones.

During the progress of the project, Pelton *et al.* (1989) published results of *in situ* hybridizations carried out on murine embryos using a human TGF-β2 riboprobe. Some discrepancies were found between the work of Pelton *et al.* (1989) and that presented in this thesis. These are detailed in the Discussion.

1.5 Morphogenesis and retinoic acid

1.5.1 Historical background

The search for a "morphogen", an agent capable of directing embryonic developmental pattern specification by its relative concentrations (Turing 1952, Slack 1987), was one of the major research efforts of developmental biology in the middle part of the 20th century. Lack of success, and the advent of models for development based on gene interaction rather than the dominating influence of a single factor (*eg.*

Wolpert 1969, Davidson and Britten 1971), resulted, despite occasional encouraging data (*eg.* Schaller and Bodenmuller 1981), in the relative decline of the field. However, it was revived in 1982 with the discovery that all-*trans*-retinoic acid was capable of stimulating the development of digit pattern duplication if applied to an avian limb bud at an appropriate stage of development (Tickle *et al.* 1982, Summerbell 1983). Retinoic acid is a member of the retinoid family, a group of compounds also including retinol (Vitamin A) and several natural and synthetic derivatives. Retinoic acid was also found to stimulate regrowth of amphibian embryonic digits which had been amputated (Maden 1982). Its mode of action in these cases is to reform the apical ectodermal ridge (AER), although this occurs in a somewhat haphazard fashion.

Further investigation of endogenous retinoic acid levels *in vivo* revealed that it exists as a gradient *in vivo* in the developing wing bud, with the highest concentrations found at the position of the zone of polarizing activity (ZPA) (Thaller and Eichele 1987). The effect of retinoic acid on the developing limb is identical to that produced by a graft of the ZPA to the same location. Neither retinol nor retinal have this effect, nor do they exist in any gradient of concentration in the developing limb.

1.5.2 Biological effects of retinoic acid

1.5.2.1 Retinoic acid and teratogenesis

Teratogenesis is the process by which progeny of abnormal morphology are born to normal mothers as a consequence of the administration of a toxic compound to the mother during pregnancy. It is generally assumed that the cause of the deformity in the offspring is perturbation of normal development by the teratogenic agent.

In 1953, Cohlan observed the teratogenic effects of vitamin A (retinol) on pregnant rats. This was also found by Kalter and Warkany (1961) in inbred strains of mice. Hypervitaminosis A produces a wide range of developmental defects in the offspring including limb deformities, central nervous system aberrations and facial abnormalities including cleft palate. Similar teratogenic effects have been reported in human pregnancies where vitamin A analogues had previously been administered therapeutically to the mothers for various reasons (reviewed by Rosa *et al.* 1986). The majority of these cases had central nervous system defects and approximately half also exhibited heart abnormalities.

Subsequent work has focussed on the mechanism by which retinoic acid produces these malformations. For instance, in retinoic acid-induced cleft palate of the mouse, an alteration in EGF receptor expression in medial epithelial cells results in the failure of programmed cell death (Abbott *et al.* 1988).

1.5.2.2 Retinoic acid and differentiation

Retinoic acid induces differentiation in embryonal carcinoma cells (Strickland and Mahdavi 1978, Andrews 1984, Williams and Napoli 1985, Lee and Andrews 1986), in a manner which involves transcriptional induction of embryonal carcinoma-derived growth factor (ECGDF), basic fibroblast growth factor (bFGF), cytokeratins A and B, laminin, major histocompatibility complex (MHC) genes and plasminogen activator. There is a simultaneous down-regulation of c-*myc*, platelet-derived growth factor A chain (PDGF-A), and the zinc finger-containing gene, *rex-1*. Several homoeobox genes are also induced (Malvilio *et al.* 1988, LaRosa and Gudas 1988, Simeone *et al.* 1990). Vasios *et al.* (1989) identified the retinoic acid response element (RARE) in the 5′-region of the laminin B1 gene. Given that the principal action of retinoic acid

in EC cells is wide-ranging transcriptional activation, it seems likely that this is also the mechanism of action of retinoic acid in its other roles as morphogen and teratogen.

1.5.3 Retinoic acid receptor genes

1.5.3.1 Retinoic acid and the steroid hormone receptors

The biochemical study of steroids and related molecules began in the early part of the present century (Kendall 1915), and receptor proteins for thyroid hormone and some steroids had been identified by the early 1970s (reviewed by Jensen and DeSombre 1972). Members of the steroid receptor family are unusual among hormone and growth factor receptors in that they are not localized, on the plasma membrane, but in the cell nucleus. The steroid, or retinoid, passes through the cell and nuclear membranes before binding to the receptor.

The prototype of the steroid receptor gene family (reviewed by Evans 1988, O'Malley 1990) is the glucocorticoid receptor, genetically isolated by Hollenberg *et al.* (1985). This receptor contains specific DNA-enhancer-binding and ligand-binding domains (Hollenberg *et al.* 1985, Umesono and Evans 1989, Danielson *et al.* 1989). Having bound to its ligand, the receptors then dimerize and bind to the DNA to stimulate gene transcription (reviewed by Green and Chambon 1988, Evans 1988). Each receptor has its own specific DNA target sequence (Umesono and Evans 1989). A palindromic consensus sequence for these response elements (the steroid response elements, SREs) has been identified (Umesono *et al.* 1988, Glass *et al.* 1989). The DNA-binding domain of the receptor consists of two "zinc fingers" (Miller *et al.* 1985), which differ from the protoype zinc finger, originally isolated in the *Xenopus* TFIIIA

gene, in that the zinc molecule is bound by four conserved cysteines, instead of two cysteines and two histidines.

The gene family also includes thyroid hormone receptor (Weinberger *et al.* 1986, Thompson and Evans 1989) and the H-2 region II binding protein (H2-RIIBP) (Hamada *et al.* 1989). There are also several "orphan receptors" (reviewed by Evans 1988, Mangelsdorf *et al.* 1990) which have been isolated on the basis of homology to other members of the steroid hormone receptor family, but for which no ligand is known. The *seven-up* gene of *Drosophila*, which is involved in the developmental specification of the compound eye, has also been found to be a member of the gene superfamily (Mlodzik *et al.* 1990).

1.5.3.2 Gene structure

The three human RAR genes have also now been cloned (Petkovich *et al.* 1987, Giguère *et al.* 1987, Benbrook *et al.* 1988, Brand *et al.* 1988, Krust *et al.* 1989), and found to be almost 100% identical at the amino acid level to those of the mouse (Zelent *et al.* 1989). Related receptors have been found in newt (Ragsdale *et al.* 1989), indicating a conserved function throughout vertebrate evolution. The RAR-α, RAR-β and RAR-gamma genes are 462, 448 and 454 amino acids long respectively. Each receptor has a highly conserved DNA-binding domain containing two zinc-fingers. There is also a highly conserved ligand-binding region. The former region is 97% homologous within the RAR gene family and the latter is similarly about 80% homologous. On either side of the ligand-binding domain are divergent regions which are member-specific.

The other genes in the steroid hormone receptor family are rather more divergent from the RAR genes; for instance, the human thyroid hormone receptor-β gene (hTRβ) has 62% amino acid homology to hRAR-α in the DNA-binding region, dropping to 35% homology in the ligand-binding region.

1.5.4 Cellular retinoic acid and retinol-binding proteins

Spatial and temporal variation in the expression of different retinoic acid receptors in development could allow a considerable flexibility in the response of the embryo to retinoic acid. The response to retinoic acid can be further regulated by proteins that are capable of sequestration of retinoic acid and its precursor retinol. These proteins are the cellular retinoic acid and retinol-binding proteins.

Cellular retinoic acid-binding protein (CRABP) is a 15.6 kDa high-affinity retinoic acid-binding protein (Eriksson *et al.* 1981, Chytil and Ong 1984), which has a wide distribution in adult mammalian tissues (Ong *et al.* 1982) and in the developing embryos of the mouse and chick (Momoi *et al.* 1988, Perez-Castro *et al.* 1989, Vaessen *et al.* 1989, Dollé *et al.* 1989, 1990, Maden *et al.* 1988, 1989a,b, 1990). CRABP is a good candidate for a regulator of response to retinoic acid action. This theory is based on the high specificity of the CRABP-RA interaction (Saari *et al.* 1982), and the ability of CRABP to transfer retinoic acid to specific-binding sites in nuclei (Takase *et al.* 1986). The corresponding high-affinity binding protein for retinol, cellular retinol-binding protein (CRBP) is also found in adult tissues (Ong *et al.* 1982) and in embryos (Perez-Castro *et al.* 1989, Maden *et al.* 1990). For both CRABP and CRBP, the pattern of distribution of RNA and protein is identical, suggesting that the their expression is transcriptionally regulated. A second form of CRABP, named CRABP II has been isolated (Kitamoto *et al.* 1988, Bailey and Siu

1988), and there is some evidence that its pattern of expression in embryogenesis may be different to that of CRABP I (Maden *et al.* 1990).

Dollé *et al.* (1990) studied the distribution of CRABP and CRBP transcripts in the mouse embryo. CRABP has the more restricted distribution of the two, being confined to the mesenchymal components of the gut, skin, and kidney, and the developing retina. CRBP has a far wider pattern of expression, including most areas which do not express CRABP.

The function of CRABP may be to titrate levels of retinoic acid in the limb, in order to ensure that the gradient is correctly maintained throughout limb development (Smith *et al.* 1989, Maden *et al.* 1990). This model involves the slow release of retinoic acid from cells expressing high levels of CRBP. This retinoic acid would then be sequestered by cells expressing high levels of CRABP. Thus, a gradient of CRABP could create a converse gradient of free retinoic acid, available for binding to the RARs in regions where they were expressed. Such gradients of CRABP have been observed in the anteroposterior axis of the developing mouse (Perez-Castro *et al.* 1989) and chick (Maden *et al.* 1988) limbs, although Dollé *et al.* (1989) reported that the gradient was in the proximo-distal axis. It is interesting, in this respect, that in the developing mammalian limb, CRABP is expressed in those areas which are devoid of RAR-β and RAR-gamma (Dollé *et al.* 1989). In the murine embryo as a whole, RAR-β and CRABP transcription areas overlap to a certain extent, but, in structures where both are expressed, CRABP transcripts are preferentially superficial (Dollé *et al.* 1990). In later embryogenesis zones of CRABP transcription are absent from areas expressing CRBP, RAR-β or RAR-gamma.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

Substance	Supplier
acetic acid	May and Baker
acetic anhydride	Sigma
acrylamide	BDH
ATP	Pharmacia
agarose (for preparative gels)	Seakem
agarose (for analytical and blot gels)	Sigma or BRL
α-^{35}S uridine triphosphate	Amersham
α-^{32}P uridine triphosphate	Amersham
3-aminopropyl-triethoxy-silane (TESPA)	Sigma
ammonium persulphate	BDH
ampicillin	Sigma
Bacto agar	Difco

Bacto tryptone	Difco
Bacto yeast extract	Difco
bis-acrylamide	BDH
bovine serum albumin powder	Sigma
BSA (DNase-free)	Gibco BRL
BSA (DNase, RNase-free)	Pharmacia
bromophenol blue dye	Sigma
caesium chloride	BDH
chloroform	May and Baker
chromic potassium sulphate (chrome alum)	BDH
citric acid, AR	BDH
CTP	Pharmacia
Decon 90 detergent	Decon
d-ATP	Pharmacia
d-CTP	Pharmacia
d-GTP	Pharmacia
d-TTP	Pharmacia
dextran sulphate, sodium salt	Pharmacia
diethyl-pyrocarbonate (DEPC)	Sigma
dimethyl-dichloro-silane (Repelcote)	BDH
dimethyl formamide (DMF)	BDH
dithiothreitol	Sigma
eosin, yellowish	BDH
ethanol	Glasgow University chemical store

ethylene-diamine-tetra-acetic acid (EDTA)	BDH
ethidium bromide	Sigma
Ficoll	Pharmacia or Sigma
formamide	Fluka
gamma-^{32}P ATP	Amersham
gelatine powder, biochemical	BDH
glucose (dextrose)	Formachem
guanidine isothiocyanate	Fluka
GTP	Pharmacia
Histoclear	National Diagnostics
hydrochloric acid	May and Baker
Hybond N	Amersham
8-hydroxy-quinoline	BDH
K5 nuclear track emulsion	Ilford
isoamyl alcohol	Sigma
magnesium sulphate heptahydrate, AR	Koch-Light
Methylene blue dye	BDH
morpholino-propane-sulphonic acid MOPS, sodium	Sigma
nitrocellulose	Sartorius
NuSieve low melting point agarose	FMC Bioproducts
Orange G dye	BDH
Paraffin wax	BDH
paraformaldehyde	Sigma
Phenisol developer	Ilford

phenol	Rathburn
poly-vinyl-pyrolidone	Sigma
potassium acetate, AR	BDH
potassium chloride	Hopkin and Williams
potassium hydrogen phosphate	May and Baker
potassium hydroxide	Koch-light
rubidium chloride	Sigma
Sephadex G50	Pharmacia
7X detergent	Sterilin
silica gel	BDH
sodium acetate, anhydrous	Sigma
sodium carbonate	Sigma
sodium dodecyl sulphate, AR	BDH
sodium hydrogen carbonate	Koch-Light
sodium hydroxide, AR	BDH
sodium phosphate, dibasic	Sigma
sodium thiosulphate, technical	BDH
Standard agar	Merck
tetra-ethyl-methyl-ethylene-diamine (TEMED)	BDH
TTP	Pharmacia
toluene	May and Baker
triethanolamine	Sigma
Tris base	Sigma
trisodium citrate	BDH
urea	Fisons

UTP-S	New England Nuclear
Vaseline pure petroleum jelly	Vaseline
X-gal	Northumbria
xylene	May and Baker
Xylene cyanol dye	Sigma

2.1.2 Biological reagents

Substance	Supplier
Alkaline phosphatase (calf intestinal)	Boehringer
DNase I (RNase-free)	Pharmacia
1 kb DNA ladder	Gibco BRL
Klenow fragment (DNA pol I)	Northumbria
proteinase K	Boehringer
Random primed DNA labelling kit	Boehringer
restriction enzymes	Gibco BRL or Northumbria
RNase A	Sigma
RNA guard (placental RNase inhibitor)	Pharmacia
ribosomal RNA *E.Coli*	Boehringer
salmon sperm DNA	Sigma
SP6 RNA polymerase (cloned, FPLC pure)	Pharmacia
spermidine	Sigma
Taq DNA polymerase	Cetus
tRNA	Boehringer

T4 DNA ligase (cloned, FPLC pure)	Pharmacia
T4 polynucleotide kinase	Pharmacia or Stratagene
T3 RNA polymerase (cloned, FPLC pure)	Pharmacia
T7 RNA polymerase (cloned, FPLC pure)	Pharmacia

2.2 Methods

2.2.1 Solutions

2.2.1.1 Preparation of solutions and glassware

All solutions for work involving RNA, including solutions used in the prehybridization and hybridization steps of the *in situ* hybridization process, were treated in the following manner to remove RNase. The solutions were filtered through 0.45µm Millipore filters, and treated with diethyl-pyrocarbonate (DEPC) at approximately 10µl of DEPC per 500 ml bottle for a minimum of 20 minutes, and autoclaved. Solutions containing Tris degrade DEPC (Maniatis *et al.* 1985) and therefore this last step was omitted for all these solutions. The DEPC destroys any protein molecules which may have survived the Millipore filtration process. The DEPC was then destroyed by autoclaving.

Glassware was washed thoroughly in millipore-filtered water, and then baked at 180°C overnight to destroy RNase adhering to the surface of the glass.

Plastic ware was treated by soaking in DEPC solution (a few drops per litre, well mixed) overnight. The plastics were then drained, packaged in suitable containers, autoclaved and dried in an oven.

2.2.1.2 General laboratory stocks

Ethanols

The ethanol was filtered through Whatman number 4 filter paper. 95%, 90%, 80%, 70%, 50% and 30% ethanol solutions were made up with milli-Q H_2O.

Phenol

Phenol was equilibrated with salt solutions to either pH 5.0 (for RNA work) or pH 8.0 (for DNA). For phenol pH 5.0, 3M sodium acetate pH 4.8 was used, and for phenol pH 8.0, 1M Tris pH 8.0 was used. Both types of phenol were stored protected from light at 4°C, under a tenfold dilution of the solution used for equilibration. In some batches, a few grains of 8-hydroxy-quinoline were added as a preservative.

L-Broth

10g of bactotryptone, 10g of yeast extract, 5g of NaCl dissolved in 1 litre of milli-Q H_2O and autoclaved immediately. For selection of bacteria containing Bluescribe or Bluescript plasmids, ampicillin was added to the cooled L-broth to a final concentration of 0.1 mg/ml.

L-plates

As for L-broth, but with the addition of 15g per litre of Bacto-agar. Ampicillin was added to a final concentration of 0.1mg/ml when the agar reached 50°C. The agar was poured into sterile Petri dishes and left to set, then stored until use at 4°C.

2.2.2 Gene cloning techniques

DNA techniques were taken from the standard works of Maniatis *et al.* (1985) and Sambrook *et al.* (1989).

2.2.2.1 Bacterial strains and plasmids

E. Coli DH5α, genotype *supE*44 δ*lac*U169(Φ80*lacZ*δM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thy*-1 *relA*1 (Hanahan 1983) (Gibco BRL) was used for all work involving recombinant DNA. Its genome contains the sequences necessary to α-complement the amino-terminus of the β-galactosidase gene of pUC-based cloning vectors. Therefore, it was possible to use blue/white colour selection for subcloning of fragments into these vectors. These vectors also have an ampicillin-resistance gene, enabling ampicillin selection to be used, and the T7 and T3 promotors flanking a multiple cloning site. Thus, radioactive RNA probes (riboprobes) can be made from any gene cloned into Bluescribe or Bluescript.

2.2.2.2 "Maxiprep" preparation of large quantities of plasmid DNA

DH5α *E. Coli* were streaked out from glycerol stocks on LB plates containing appropriate selective antibiotic. These were then cultured at 37°C overnight. Single colonies from the plates were picked using sterile wooden toothpicks, and used to

inoculate 500ml of L broth in two litre flasks. These were then cultured in a shaking incubator overnight at 37°C. The bacteria were harvested by centrifugation at 7000 rpm for 10 minutes at 4°C in a HS-4 rotor. The bacterial pellet was resuspended in 1/50 volume solution I (50mM glucose, 10mM EDTA, 20mM Tris pH 8.0, autoclaved). This solution had lysozyme added at a final concentration of 2 mg/ml. 1/25 volume solution II (0.2M NaOH, 1% SDS, freshly prepared) was then added to complete the cell lysis and denature the bacterial genomic DNA. This was then mixed by gentle inversion until the solution clarified. After 5 minutes on ice, 3/100 volume solution III (3M sodium acetate pH 4.8, autoclaved) was added to neutralize the solution, and to precipitate proteins and denatured genomic DNA. This was mixed gently to prevent fractionation of genomic DNA and left on ice for one hour. The mixture was centrifuged at 7000 rpm in an HS-4 rotor for 30 minutes to pellet the bacterial genomic DNA. The plasmid-containing supernatant was filtered through gauze and 0.6 volumes of isopropanol were added. The plasmid DNA was precipitated at –20°C for one hour, and harvested by centrifugation at 7000 rpm for 30 minutes at 4°C. The pellet was drained and resuspended in 10 ml of TE buffer (10mM Tris pH 8.0, 1mM EDTA), containing 1µg/ml RNase A, and incubated for 15 minutes at 65°C. The plasmid solution was then phenol-extracted twice, Sevag-extracted and ethanol precipitated. The pellet was washed in 70% ethanol, dried and taken up in 1ml of TE buffer (10mM Tris pH 8.0, 1mM EDTA). DNA concentration was determined by spectrophotometry at 260nm and by quantification on an agarose gel.

2.2.2.3 Preparation of plasmid DNA for digestion

Plasmid DNA from the maxiprep was phenol-extracted 3 times, extracted further with chloroform/isoamyl (24:1 v/v) alcohol twice and then precipitated by the addition of 1/9 volume of 3M sodium acetate pH 6.8 and then 2.5 x the total volume ethanol. This mixture was then incubated at –20°C overnight or on dry ice for 15 minutes. The DNA was pelleted by centrifugation in a bench-top microcentrifuge for 15 minutes. The pellet was washed in 70% ethanol, dried and taken up in H_2O and dissolution assisted by heating to 65°C for 5 minutes.

2.2.2.4 Restriction endonuclease digests

The restriction endonuclease digests were set up as follows. An appropriate quantity of plasmid DNA, usually between 5 and 50µg, was incubated in a total volume of 20 to 200µl with final concentrations of x1 reaction buffer, 1 mM spermidine, 0.1 mg/ml BSA, 1 unit of enzyme per µg of DNA to be digested, but the glycerol concentration was not allowed to exceed 5%. Digests were incubated at 37°C for 20 to 30 minutes. The reaction was stopped by two phenol extractions of the sample followed by a chloroform/isoamyl alcohol (24:1 v/v) extraction and ethanol precipitation. The precipitated digested plasmid DNA was taken up in 20 µl of H_2O.

2.2.2.5 Gel electrophoresis of DNA

Electrophoresis of DNA was carried out in agarose gels containing 1µg/ml ethidium bromide. The concentration of the gel varied according to the size range of the fragments of interest. In general, for the visualization of fragments greater then 1kb, a gel of 1% was used. For smaller fragments a 1.5% gel was preferred. Gels were made either in TBE (90mM Tris-borate, 2mM EDTA pH 8.3) or TAE (40mM Tris-

acetate, 1mM EDTA pH 8.0) buffer. A one-tenth volume of gel loading buffer (0.25% Bromophenol blue, 0.25% xylene cyanol in 30% glycerol in milli-Q H_2O, stored at 4°C) was added to the sample prior to loading. Gels were always run in the same buffer in which they were made, again with the addition of 0.5µg/ml ethidium bromide. "Minigels" were run for 20 to 30 minutes at 100V, and larger gels for several hours at 150 V or overnight at 50 to 75 V. Exact times and voltages varied according to the sizes of the bands of interest.

2.2.2.6 Recovery of DNA fragments by "genecleaning"

To obtain pure preparations of specific fragments of plasmid DNA, the digested sample was subjected to electrophoresis in a low melting point agarose gel (0.8 to 1.0%). The migration of bands was observed using a hand-held long-wave UV lamp held at least 20 cm from the gel to mimimize damage to the DNA molecules. When good separation of the band of interest had been achieved, the band was cut out of the gel using a sterile scalpel blade.

DNA was eluted from gel fragments using the "Geneclean" kit method. The gel slice was dissolved in 2.5 volumes of saturated sodium iodide solution and then incubated with an appropriate quantity of "glass milk". The glass milk was then centrifuged to a pellet and washed several times in the wash mixture provided in the kit. The pellet was then resuspended in TE (10mM Tris pH 8.0, 1mM EDTA) buffer to release the DNA from the glassmilk, and the glassmilk was pelleted with a further centrifugation. An aliquot containing the purified DNA was gel electrophoresed to check for adequate yield and purity of the fragment of interest.

The reference quoted in the product literature of the "geneclean" kit is Wilson (1988).

2.2.2.7 Ligations

For ligations, insert and vector DNA were mixed at a molar ratio of at least 9:1, at a vector concentration of 5µg/ml in 50mM Tris pH 7.6, 10mM $MgCl_2$, 1mM ATP, 1mM DTT, 0.1 mg/ml BSA and 2 U T4 ligase at 16°C overnight. Extra ligase was added at this stage and the reaction incubated for a further 4 hours.

2.2.2.8 Preparation of competent cells

Competent DH5α cells were generated by the method of Hanahan (1983). DH5α from glycerol stocks were streaked onto L-plates with no antibiotic selection. A single colony was used to inoculate 5ml L-broth and incubated overnight at 37°C in a shaking incubator. This culture was then transferred to 100 ml of L-broth in a 500ml flask and then grown for approximately four hours at 37°C until the OD_{550} reached 0.48. The cells were harvested at 3500rpm for two minutes at 4°C, and resuspended in 40 ml of TfbI (30mM potassium acetate, 100mM $RbCl_2$, 10mM $CaCl_2$, 50mM $MnCl_2$, 15% glycerol, pH 5.8 with 0.2M acetic acid, filter sterilized). This was then left on ice for 90 minutes, and then repelleted at 4500rpm. The pellet was resuspended in 4ml of pre-cooled TfbII (10mM MOPS sodium salt, 75mM $CaCl_2$, 10mM $RbCl_2$, 15% glycerol, pH 6.5 with acetic acid, filter sterilized). The competent cell suspension was then aliquoted, using pre-cooled pipette tips, into pre-cooled Nunc cryotubes which were then snap-frozen in a dry ice/ethanol mixture and stored at –70°C. Alternatively, DH5α subcloning-efficiency competent cells were purchased form BRL and processed according to the manufacturers instructions.

2.2.2.9 Transformation of competent DH5α

Transformation of competent DH5α with plasmid DNA was performed by mixing 20µl of the ligation reaction with 100µl of competent cells, and leaving to incubate on ice for 45 minutes. Cells were heat-shocked at 42°C for 90 seconds, and kept on ice for a further two minutes. Up to 4 volumes of L-broth, without antibiotic, were added, and the culture was incubated with shaking for one hour at 37°C, at 225 rpm. The cells were then plated on L-plates with antibiotic selection, and incubated overnight at 37°C. Approximately 200µl of cells were used per plate, thus generating about five plates per ligation reaction. Plates contained 0.1 mg/ml ampicillin, to select for the successfully transformed cells, and 50µg/ml X-gal to differentiate between plasmids with, and without, insert. Appropriate controls were included in each transformation experiment, including transformation of uncut Bluescribe plasmid to determine transformation efficiency, and transformation of religated vector to determine relative efficiency of ligation. This method generally gave at least 10^7 colonies per µg transformed DNA.

2.2.2.10 Small-scale preparation of plasmid DNA

This follows the method of Serghini *et al.* (1989). 5ml cultures of recombinant *E. Coli* were grown overnight at 37°C with appropriate antibiotic selection. 1.5 ml of the 5ml culture were added to Eppendorf tubes and pelleted by microcentrifugation for 1 min. The bacterial pellet was drained, and resuspended in 50µl of STE buffer (100mM NaCl, 10mM Tris pH 8.0, 1mM EDTA). 50µl of a 25:24:1 phenol/chloroform/isoamyl alcohol mixture was added, and the cells denatured by vigorous vortexing. The cellular debris was then pelleted by microcentrifugation for 5 minutes, and the

supernatant transferred to a fresh tube. 20µl of 7.5M ammonium acetate was added (or an appropriate volume to give a final concentration of 2M). Two volumes of ice-cold ethanol were then added, followed by a 15 min precipitation on dry ice. Plasmid DNA and RNA were pelleted by microcentrifugation for 15 minutes. The pellet was drained and washed in 70% ethanol, vacuum-dried and resuspended in 25µl of TE buffer (10mM Tris pH 8.0, 1mM EDTA) containing 1µg/ml of RNase A. Aliquots of this sample could be restriction-digested to confirm that the correct plasmid DNA had been harvested. The remainder of the overnight bacterial culture could then be used to inoculate a 1 litre overnight culture, or used to make a glycerol stock in the following manner. 900µl of overnight bacterial culture were mixed with 900µl of sterile glycerol in a Nunc cryotube. These tubes could then be stored indefinitely at –20°C.

2.2.3 RNA samples

2.2.3.1 Gel electrophoresis of RNA

RNA gel electrophoresis was performed using a 1.5% agarose gel containing 1/6 volume formaldehyde and 1 x MOPS buffer (20mM morpholino-propane-sulphonic acid, sodium salt (NaMOPS), 8mM sodium acetate. 1mM EDTA pH 7.0 with acetic acid). After the addition of one-tenth volume of RNA loading buffer (0.25% Bromophenol blue, 0.25% xylene cyanol dissolved in 30% glycerol, 50% formamide, 25mM EDTA, stored at 4°C), samples were denatured at 55°C for 10 minutes. After electrophoresis, the formaldehyde was eluted in milli-Q H_2O for one hour, and the gel was stained with ethidium bromide at 1µg/ml in milli-Q H_2O for 15 minutes, and

washed in milli-Q water for 60 minutes (twice). The RNA bands were visualized under ultraviolet light.

2.2.3.2 RNA extraction from tissue samples

RNA was extracted according to the method of Chomczynski and Sacchi (1987). Fresh tissue samples were rapidly dissected and snap-frozen in liquid N_2. They were then ground to a powder using a mortar and pestle cooled to –70°C, and resuspended in 2ml of denaturing solution (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7.0, 0.5% sarkosyl, 0.1M β-mercaptoethanol). This was then transferred to a clean 15ml polypropylene tube. 0.2ml of 2M sodium acetate pH 4.0 were added, followed by 2ml of water-saturated phenol and 0.4 ml of Sevag. The mixture was thoroughly mixed after the addition of each reagent. The final suspension was vortexed for 10 secs and kept on ice for 15 minutes, followed by centrifugation at 6500 rpm for 20 minutes at 4°C. The aqueous phase, containing the RNA, was removed to a fresh tube, 2ml of isopropanol were added and the sample was left at –20°C for 1 hour, or for 15 minutes on dry ice to precipitate the RNA. The RNA was pelleted by centrifugation at 6500rpm at 4°C for 20 minutes. The pellet was drained, vacuum dried and resuspended in 0.3 ml of denaturing solution. The RNA was reprecipitated by the addition of an equal volume of isopropanol, and the precipitation, centrifugation, vacuum-drying procedure was repeated. This time the pellet was washed thoroughly in 75% ethanol prior to vacuum drying, and then resuspended in 0.5% SDS in RNase-free H_2O. Redissolution was assisted by briefly heating to 65°C. The concentration and purity of the RNA preparation was checked by spectrophotometry of a 100-fold dilution at OD_{260} and OD_{280}. If the protein content was too high, the sample was re-extracted with phenol pH 5.0, Sevag and

then reprecipitated. The integrity of the isolated RNA was checked by electrophoresis of 2µg of the total RNA on a denaturing gel.

2.2.4 Nucleic acid blotting techniques

2.2.4.1 Southern gels

After electrophoresis, DNA gels were denatured for 45 minutes in 0.1M NaOH, 1.5M NaCl, and neutralised in 1M Tris pH 7.5, 1.5M NaCl for one hour. The gel was then trimmed and the portion containing the lanes of interest was blotted onto Hybond-N filters (previously wetted in H_2O) in 20 x SSC (3M NaCl, 0.3M tri-sodium citrate pH 7.0), in a plastic tray overnight. The filter was then washed briefly in 2 x SSC and then baked at 80°C for two hours to bond the DNA, and was stored between filter papers at room temperature until use.

2.2.4.2 Preparation of probe for Southern hybridization

Oligo-labelled DNA probes were used for hybridization to the Southern filters. ^{32}P-labelled deoxynucleotides were incorporated to give a rapid autoradiographic signal. A Boehringer oligo-labelling kit was used and the insert of interest was gel purified. The purified insert was diluted to a concentration of 50ng/µl, denatured by boiling for 10 minutes, cooled rapidly on ice to prevent renaturation, and centrifuged. 1µl of this denatured DNA was mixed with the deoxynucleotide solutions, buffer and random-priming oligo fragments provided in the kit, and left at 37°C for 5 minutes to allow hybridization of the oligo fragments to the DNA. Then 2 U of Klenow enzyme and 50µCi of ^{32}P-dCTP were added and the reaction left for at least two hours.

The method of Feinberg and Vogelstein (1984) was used and the insert of interest was gel-purified

Unincorporated nucleotide was removed by the use of a Sephadex G50 spin column as previously described, and the eluate from the column was ethanol precipitated. The precipitated probe was pelleted by centrifugation, vacuum dried and taken up in H_2O at a concentration of 10^8dpm/ml.

2.2.4.3 Prehybridization and hybridization

Prehybridization was performed in 5 x SET buffer (1 x SET is 0.15M NaCl, 10mM Tris pH 8.0, 1mM EDTA), 5 x Denhardt's solution (20 x Denhardt's is 0.1% (w/v) bovine serum albumin powder, 0.1% (w/v) Ficoll and 0.1% (w/v) polyvinylpyrolidone), 20mM sodium phosphate pH 6.8, 50µg/ml denatured salmon sperm DNA, 10µg/ml polyA, 50% formamide and 0.1% SDS. The filter was wetted in 2 x SSC and incubated in the prehybridization solution for at least two hours, and preferably overnight, at 42°C. For hybridization, the prehybridization solution was poured off and replaced with hybridization solution. This was identical to prehybridization solution with the addition of dextran sulphate to a final concentration of 10%. The probe was added to the hybridization mixture at a concentration of 10^6dpm/ml. Prior to this the probe was denatured by boiling for 10 minutes, quenched on ice and centrifuged briefly. Hybridization was carried out at 42°C, overnight.

2.2.4.4 Post-hybridization washes

The filter was removed from the hybridization bag, and incubated in 50% formamide, 2 x SSC, 0.1% SDS for two hours. The filtered was then monitored to assess background hybridization. Further washes can be carried out using decreasing salt concentrations, down to 0.1 x SSC. When background emission was at a

satisfactory level (ie. tending to zero), the filter was wrapped in clingfilm and exposed with autoradiographic film at –20°C for one day or more.

2.2.5 Embryology

2.2.5.1 Pre-implantation embryology

Both mouse and human pre-implantation embryos were used in experiments. The mouse embryos were obtained in the following manner.

3 week old virgin female mice of the strains NIH, Parkes, C57Bl/6, or crosses of the above, were obtained from the National Institute of Medical Research (NIMR), and superovulated by the injection of 5U of pregnant mare serum, followed by 5U of chorionic gonadotropin 46 hrs later. The females were caged individually or in pairs with males of the strains Parkes or NIH. Fertilization was ascertained by the presence of a vaginal plug the next morning. For the purposes of staging the embryos, fertilization was assumed to have taken place at midnight. Pregnant females were then sacrificed at the following times: 0.5 days for fertilized oocytes, 1.5 days for two-cell embryos, 2.5 days for 8 or 16 cell morulae, and 3.5 days for blastocysts. Uteri were dissected and placed in pre-warmed PB1 medium (Gibco). Embryos were flushed out of the oviducts with PBS (0.8% NaCl, 0.02% KCl, 0.29% $Na_2HPO_4.12H_2O$, 0.02% KH_2PO_4), washed several times in PBS and then fixed in ice cold 4% paraformaldehyde or 1% glutaraldehyde in PBS. The fixed embryos were washed again in PBS.

2.2.5.2 Preparation of whole mount embryos

The fixed and washed embryos were dehydrated through an ethanol series of 30%, followed by 60% ethanol, and then pipetted onto sterile, subbed slides. After a brief drying, the slides were stored at –70°C until use.

These slides were subbed in TESPA or poly-L-lysine.

2.2.5.3 Preparation of preimplantation embryos for sectioning

The fixed and washed embryos were pipetted into molten agarose of a variety of concentrations ranging from 1.0% to 2.5%. The agarose was left to solidify. Blocks were then cut of approximately 3mm square containing the embryos as close to the centre of the block as possible. The agarose blocks were then dehydrated through an ethanol series to 100% ethanol, cleared in xylene and embedded in paraffin wax for sectioning. In an alternative method, the washed and fixed embryos were initially placed in molten low melting point agarose. These blocks were then remelted in a polythene electron microscopy embedding capsule ("BEEM" capsule). This allowed the embryo to sink to the tip of the conical area of the capsule. The agarose was then recooled, the capsule peeled off, and a block was cut containing the embryo at its tip. This block was then dehydrated and embedded in the manner described above. Sections were cut to a nominal thickness of 7μ and stored with dessicant at –70°C.

2.2.5.4 Human preimpantation embryology

Human preimplantation embryos were obtained as a by-product of a clinical IVF programme, and work was approved by the Voluntary (now Interim) Licensing

Authority. The embryos were processed for sectioning and whole-mounting in the same manner as the mouse embryos.

2.2.5.5 Human post-implantation embryos

Intact human embryos were kindly provided by Prof. D Baird of the Centre for Reproductive Biology at the University of Edinburgh. Embryos of 32-57 days post-conception (*p.c.*) were obtained following therapeutic abortion using mifepristone and gemeprost prostaglandin pessaries. These were processed for *in situ* hybridization according to the protocol of Wilkinson *et al.*. (1987) (see Histological Techniques, 2.2.6). Sex was determined by karyotype analysis carried out in the Centre for Reproductive Biology. Embryonic stage was estimated by crown-rump length, embryonic weight and morphological appearance; the estimated date of the last menstrual period was found to be less reliable. This assessment was carried out by Prof. Baird and colleagues. Supplementary material of 10 to 12 weeks gestational age (mainly limbs) were collected by suction termination and similarly processed. These samples were kindly provided by Dr M Whittle of the Queen Mother's Hospital in Glasgow, Drs. J Kingdom and R Chatfield of the Samaritans Hospital in Glasgow, and Dr L Wong of the Royal Marsden Hospital, London.

Staging relative to mouse embryos was very difficult since different organ systems develop at different rates in the two species. However, approximate correspondences are given in Rugh (1990).

The embryos obtained were as follows:

RU113: 44 days post-last menstrual period (LMP). No chromosome analysis was performed on this embryo. Crown-rump length could not be measured due to

distortion. The embryonic weight was 0.025g. This embryo had a well developed liver. This organ begins to proliferate at 9.5 days *p.c.* in the mouse and 22 days *p.c.* in the human embryo. The rudiment of the hind limb-buds was visible. This demonstrates that the embryo was older than 28 days *p.c.*. The hind limb-bud is first visible in the murine embryo at 10.5 days *p.c.*. The heart was in the process of septation. The gut and mesonephros were distinguishable but the lung-buds could not be identified. It was concluded that the embryo was aged about 32 days*p.c.* (Prof. DT Baird pers. comm.). Rugh (1990) gives an equivalent age of 11 to 11.5 days p.c. for the murine embryo.

RU88: 37 days post-last menstrual period (LMP). A chromosomally normal male fetus assessed as about 38 days of gestation. The crown-rump length was 8 mm and the fetal weight was 0.0672g. Limb buds were present, but without any morphological differentiation. The pharyngeal arches were very prominent. Rugh (1990) gives an equivalent age of 13 to 13.5 days *p.c.* for the murine embryo.

RU74: Assessed chromosomally as Y-negative and morphologically as 43 days gestation. The crown-rump length was 12 mm and the weight was 0.2131g. The tail was torn off, and the embryo had already been sectioned sagittally for histological purposes and was therefore lacking its left side to a thickness of about 1/4 of its total. Lung-buds were visible and the limb-buds were well developed though there were no presumptive digits. The lens vesicle was closed and separate from the surface. Rugh (1990) gives an equivalent age of 13.5 to 14 days *p.c.* for the murine embryo.

RU108: this embryo was assessed as the same age as RU74. No further information was available.

RU118: 58 days post-LMP. No chromosomal analysis was performed on this embryo, assessed as 47 days *p.c.* on the basis of fetal morphology. Crown-rump length was 19mm and the weight was 0.614g. The lungs, limbs and eyes were noticeably more advanced than in RU74. The tongue was readily distinguishable, although the pinnae were scarcely developed. Rugh (1990) gives an equivalent age of 14.5 days *p.c.* for the murine embryo.

RU59: 71 days post-LMP. A chromosomally normal male fetus assessed as 57 days of gestation on the basis of morphology. The gut was herniated and there was a small degree of abrasion on the skin in many areas. Weight was 1.411g and the crown-rump length was 25 mm. The fingers were separated distally, very primitive toothbuds were visible, and intramembranous ossification had begun. However, the eyelids and palate were unfused. Rugh (1990) gives an equivalent age of 15 to 15.5 days *p.c.* for the murine embryo.

2.2.6 Histological Techniques

2.2.6.1 Fixation and embedding of embryos

The embryos were processed for *in situ* hybridization following the methodology of Wilkinson *et al.* (1987). The embryo was washed briefly in PBS followed by fixation overnight in 4% paraformaldehyde in PBS at 4°C. The embryo was then washed in ice-cold PBS for two hours, the PBS solution being changed every 30 minutes. The embryo was then transferred to 70% ethanol for storage at 4°C until embedding. Embryos were not kept in 70% ethanol for more than two weeks. The embedding process was as follows; the embryo was transferred through 90% and 95% ethanol,

being kept for 30 minutes in each, and was then transferred to 100% ethanol for 1 hour, the ethanol being changed after 30 minutes. Clearing was in Histoclear for a maximum of 30 minutes. Once the embryo begins to take on a transparent appearance, the clearing process was complete. The embryo was then transferred to paraffin wax at 55°C for one hour. The wax was changed every twenty minutes. The block of wax containing the embryo was then allowed to harden and was stored at 4°C until sectioning.

2.2.6.2 Preparation of microscope slides

TESPA (3-aminopropyl triethoxy silane) coated slides were prepared according to the method of Rentrop *et al.* (1986). Slides were cleaned in 10% Decon detergent overnight at 65°C and then washed under hot running tap water for at least 4 hours. This was followed by extensive rinsing in milli-RO H_2O and then milli-Q H_2O. The slides were sterilized by baking overnight at 180°C. After cooling, the slides were coated by dipping in 2% TESPA in acetone. The slides were washed in at least 5 changes of acetone, or 3% acetic acid in ethanol, followed by at least 3 washes in milli-Q H_2O. The slides were dried in an oven at 60°C and stored in boxes at room temperature until use.

2.2.6.3 Sectioning

Sections were cut from the embedded embryo at a nominal thickness of 7µ, and collected on microscope slides coated with 3-aminopropyl-triethoxy-silane (TESPA). They were left to dry at 42°C overnight, then stored at 4°C in boxes until use for *in situ* hybridization.

2.2.7 Probes

2.2.7.1 Sources of DNA

Full length cDNA probes encoding human TGF-β1 (Derynck *et al.*. 1985), TGF-β2 (Madisen *et al.*. 1988) and TGF-β3 (ten Dijke *et al.* 1988) were kindly supplied by Dr GI Bell (Howard Hughes Institute, Chicago), Dr AF Purchio (Oncogen, Seattle) and P ten Dijke (Oncogene Science, Manhasset) respectively. A probe for human cardiac actin (Sassoon *et al.* 1988) was kindly provided by Dr P Barton (Heart and Lung Institute, London). Dr JC Fiddes (California Biotechnology Inc.) supplied the human chorionic gonadotropin-β (Fiddes and Goodman 1980) cDNA gene probe. Dr P Chambon (CNRS, Strasbourg) kindly provided the cDNA clones for the human α, β and gamma retinoic acid receptor (RAR) genes (Petkovich *et al.* 1987, Giguère *et al.* 1987, Benbrook *et al.* 1988, Brand *et al.* 1988, Krust *et al.* 1989). Dr F Jacob (Pasteur Institute, Paris) kindly provided the mouse B2 repetitive sequence DNA clone (Brulet *et al.* 1985).

Because both TGF-β and RAR genes were members of gene families with extensive homology between members, it was necessary to construct subclones which were less likely to cross hybridize with the other family members than the full-length cDNA clones. The regions of the genes chosen for these subclones are illustrated in Fig. 1. The strategies employed were as follows.

For the TGF-β genes, gene-specific probes were generated by subcloning sequences from the non-conserved 5′ non-coding or precursor coding regions into Bluescribe or Bluescript plasmid vectors. The gene-specific TGF-β1 sub-clone was a 712 nucleotide EcoRI-PstI fragment from the 5′ non-coding region, inserted antisense

with respect to the T3 promotor. This probe recognizes both differentially-spliced forms of TGF-β1 (Kondaiah *et al.* 1988). The gene-specific TGF-β2 subclone was a 450 nucleotide *Ava*I- *Hae*II fragment spanning amino acid residues 61 to 211, inserted antisense with respect to the T3 promotor. This probe recognizes both differentially-spliced forms of TGF-β2 (Madisen *et al.* 1988, Hanks *et al.* 1988). The gene-specific TGF-β3 sub-clone was a 463 nucleotide fragment spanning from 16 nucleotides upstream of the translation initiation site to amino acid 149, and inserted antisense with respect to the T7 promotor. This latter subclone also contained the conserved 30 amino acid coding region found at the N terminus of the TGF-β precursor polypeptides. The TGF-β1-specific subcloning was carried out specifically for the purposes of this project, and the TGF-β2 and β3 subclonings by Dr RJ Akhurst and P ten Dijke, respectively.

RAR genes can be divided into 6 structural domains (Brand *et al.* 1988, Zelent *et al.* 1989), named A to F. The F region is the most divergent and therefore the most suitable for the construction of gene-specific riboprobe templates. The adjacent E region is the second most conserved area of the gene, and therefore should be avoided where possible. RAR subclones were constructed as follows. The RAR-α specific subclone was a 288 nucleotide *Sma*I fragment spanning all of the F region and a small portion of the E region. The RAR-β specific subclone was a 140 nucleotide *Eco*RI – *Bam*HI fragment spanning the F region and an area of the 3′ untranslated sequence. The RAR-gamma specific subclone was a 430 nucleotide *Pst*I – *Eco*RI subclone spanning the F region and flanking sequences on both sides (see Fig. 1). Therefore, it does not differentiate between RAR-gamma-1 and RAR-gamma-2 isoforms, nor any of the other five differentially-spliced RAR-gamma RNA species (Krust *et al.* 1989, Giguère *et al.* 1990, Kastner *et al.* 1990). The full-length RAR-gamma riboprobes were transcribed from the original RAR-gamma-1 cDNA.

However, these would probably detect both isoforms of RAR-gamma given the relatively small size of the differentially-spliced segment compared to the total length of the cDNA (153nt in 1.5kb). Exactly the same case applies to the RAR-α and RAR-β gene-specific and full-length riboprobes.

Human chorionic gonadotropin cDNA probe, kindly supplied by J.C. Fiddes (Fiddes & Goodman, 1980), was subcloned as follows. A 490 nt *Sma*I fragment, covering the entire coding sequence, but omitting the poly(dA).poly(dT) region, was subcloned into the Bluescribe vector and the T7 promotor was used to generate antisense RNA. This was used as the experimental probe in the studies on the human preimplantation embryos, and as a negative control probe for the other *in situ* hybridization experiments. Alternatively, sense strand RNA was generated from the opposite strands of the TGF-β specific subclones.

For TGF-β family genes, both full-length cRNA probes and gene-specific subclone cRNA probes were utilized. No difference was found between these two categories, indicating that the *in situ* protocol was stringent enough to avoid cross-hybridization within the gene family.

As a positive control, a human cytoskeletal β-actin (Gunning *et al.* 1983) was used. Due to its evolutionary conservation, this was used on both murine and human embryos. For murine embryos alone, the B2 repetitive sequence, which is known to be expressed in murine preimplantation embryogenesis (Vasseur *et al.* 1985), was utilized. In the post-implatation embryos, the positive control was human cardiac actin (Sassoon *et al.* 1988), kindly provided by Dr P Barton. This hybridized to all areas of developing skeletal muscle.

2.2.7.2 Probe synthesis

^{35}S-labelled single-stranded riboprobes were generated to a specific activity of 8 X 10^8 disints min^{-1} ug^{-1} using the Bluescribe or Bluescript T3 and T7 transcription systems (Vector Cloning Systems). To make the template, the DNA was linearized by restriction endonuclease digestion at a site in the polylinker.

Radiolabelled cRNA probes were generated to high specific activity (8 x 10^8 dpm/µg). 0.5 µg of linearized DNA template was incubated in a 20µl reaction mixture containing 1mM ATP, 1mM CTP, 1mM GTP, 5mM DTT, x1 reaction buffer (40mM Tris pH 8.0, 20mM $MgCl_2$ for T7; 40mM Tris pH 8.0, 50mM NaCl, 8mM $MgCl_2$, 2mM spermidine for T3), 7.5 µM UTP-S (unlabelled), 75pM α-^{35}S-UTP (75µCi), 0.3 mg/ml BSA, 1 U RNA guard and about 10 U of the relevant RNA polymerase. The reaction mixture was incubated at 37°C for 90 minutes. The specific activity of the resulting radiolabelled RNA was calculated as 8 x 10^8 dpm/µg of RNA.

The transcription reaction was followed by a DNase I digestion, designed to remove the DNA template, which might impair the hybridization reaction. The 10µl of transcription reaction was briefly spun in a microcentrifuge, and made up to a volume of 100µl in 0.1M sodium acetate pH 6.0, 5mM $MgSO_4$, 10mM DTT, 0.1 mg/ml carrier rRNA *E.Coli*, 1 U RNA guard and 2 U DNase I (RNase free). The reaction was incubated at room temperature for 30 minutes. This reaction was stopped by phenol extraction, Sevag extraction and ethanol precipitation.

Probes were digested to an average length of 100 – 150 nucleotides by controlled alkaline hydrolysis (Cox *et al.*. 1984) in 60 mM $NaHC0_3$, 40mM $NaCO_3$ pH 10.2,

10mM DTT, 60°C. The length of the incubation was determined by the following equation:

$$t = \frac{L_o - L_f}{k\, L_o\, L_f}$$

t = time of incubation

k = a constant 0.11

L_o= original length

L_f= final length ie 0.1 or 0.15 kb

The alkaline digestion was terminated by the addition of 10µl of 5% acetic acid, buffered by the addition of 10µl of 1M sodium acetate pH 6.0. 10µg of carrier rRNA was added and the mixture was added to a Sephadex G50 spin column.

2.2.8 Polyacrylamide gel electrophoresis

Aliquots from the riboprobe synthesis mixture were taken prior to alkaline digestion and at the end of the procedure. These aliquots were run on polyacrylamide/urea gels to determine the sizes of the RNA synthesized and check for degradation. The gels were 5.7% acrylamide, 0.3% *bis*-acrylamide, 50% (w/v) urea and x1 TBE buffer. The gel was polymerized by the addition of 500µl of 10% ammonium persulphate, and 50µl of TEMED, and poured between 1.0 mm spacers. The gel was pre-run vertically in x1 TBE buffer at 250 V for about 30 minutes, the samples were then added and the gel was run for approximately 60 minutes. PA91, a pBR322-derived plasmid, digested with *Hinf*I and denatured by boiling was used as a size marker.

The results of a typical polyacrylamide gel are shown in Fig. 4. The probes analyzed were both orientations of the hCG gene and the cytoskeletal actin riboprobe. This illustrates the extent to which controlled alkaline digestion produces shortened riboprobes.

2.2.9 In situ hybridization

2.2.9.1 Prehybridization treatments

In situ hybridizations were performed essentially according to the protocol of Wilkinson *et al.* (1987) except that the hybridizations were carried out at 52°C. Probe concentration was adjusted to 2 to 5 x 10^4 dpm ul^{-1}. Higher probe concentrations were found to increase background hybridization and "edge effects". Sections were dewaxed in Histoclear, 10 minutes (twice), washed in 100% ethanol (twice) for two minutes, and rehydrated through an ethanol series (95%, 90%, 80%, 70%, 50%, 30%). The slides were washed twice in PBS for 5 minutes, and fixed in 4% paraformaldehyde in PBS for 20 minutes. Following fixation, the slides were washed twice in PBS for 5 minutes and placed in a 60µg/ml solution of proteinase K (Boehringer) in 50mM Tris pH8.0, 0 5mM EDTA pH 7.5 for 7.5 minutes at room temperature. The slides were washed again for 5 minutes in PBS and refixed in 4% PFA for 5 minutes. The PFA was washed off with PBS and the slides acetylated for 10 minutes in 0.75% (v/v) solution of acetic anhydride in 0.1M triethanolamine, pH 8.0. Following a further PBS wash, the slides were dehydrated through the ethanol series and left to dry before hybridization. Wherever possible, hybridization was performed as soon as possible after the conclusion of the prehybridization steps.

2.2.9.2 Hybridization

Hybridization was carried out using the following: 53% formamide, 0.3 M NaCl, 20 mM Tris pH 8.0, 20mM sodium phosphate buffer pH 8.0, 5mM EDTA, 10% dextran sulphate, 1 x Denhardt's solution, 0.3 mg/ml tRNA, 10mM DTT. Probes stocks of 3 to 5 x 10^5 dpm/µl were diluted 10-fold in this mixture. The sections were overlaid with this mixture (5 to 100 µl, depending on area), and covered with sterile, siliconized coverslips. Hybridization was carried out in a sealed plastic box, containing filter paper soaked in 50% formamide and 2 x SSC to preserve humidity. The temperature of incubation was 52°C, maintained either in an incubator or in a waterbath. In the latter case, the plastic box was sealed in a plastic bag.

2.2.9.3 Post-hybridization washes

The next day, slides were washed briefly in 4 x SSC, 0.1% β-mercaptoethanol at 50°C to remove the coverslips. The slides were then put through the following washes.

Firstly, 2 x SSC, 50% formamide, 0.1% β-mercaptoethanol at 65°C for 20 minutes. This was followed by 0.5M NaCl, 10 mM Tris pH 8.0, 5mM EDTA at 37°C for 10 minutes at 37°C, 4 times. An incubation in the same buffer was then carried out with the addition of 20µg/ml RNAse A. This reaction was for 30 minutes at 37°C. The slides were then returned to 50% formamide, 2 x SSC, 0.1% β-mercaptoethanol for 20 minutes. The final two washes were in 2 x SSC for 10 minutes, and 0.5 x SSC for 10 minutes, at room temperature. The slides were then dehydrated through an ethanol series, dipped in 0.1% gelatine, 0.01% chromic potassium sulphate (BDH) in PBS (this solution ensures even spreading of the autoradiographic emulsion on the slide, and prevents growth of bacteria during long periods of exposure), redried and dipped in emulsion.

2.2.9.4 Autoradiography

This follows the methodology of Rogers (1979).

Ilford K5 or Amersham LM-1 nuclear track emulsion was diluted on the day of use by melting about 6ml of emulsion shreds at 42°C with an equal volume of 2% glycerol in H_2O. Each experimental slide was dipped into the emulsion, wiped clean on the reverse side and then left to dry horizontally in a large light-tight box, for two hours or overnight. Slides were then sealed in light-tight boxes and stored at 4°C to expose.

Autoradiographic exposure times were between 1 and 6 weeks. The extremes of variation were between the actin positive controls, which could be developed after 3 or 4 days, and the RAR probes which required 4-6 weeks exposure. The slides were developed in 20% Phenisol developer for 2.5 minutes, neutralized in 2% acetic acid for 30 secs, washed in tap water, and fixed in 30% sodium thiosulphate. The slides were then washed in tap water for at least two hours. After development, slides were counterstained in haematoxylin and eosin, dehydrated through an ethanol series, cleared in Histoclear and mounted in Neutral Mounting Medium or Paramat. Where appropriate, cartilage was identified by staining with alcian green. Examination was by means of an Olympus B2 microscope equipped with dark field optics and a manual C35DA-2 35 mm camera. Photomicrography was performed using Kodak Panatomic X film (iso 32) and development was in Kodak D76 developer for 4.5 minutes at 20°C. Dark-field images were photographed using a 6-8 fold underexposure and bright-field images were exposed at the normal setting, using a LBD-2N blue filter.

2.2.10 Polymerase chain reaction (PCR)

2.2.10.1 Choice of oligonucleotides for PCR

Oligonucleotide primers were chosen from areas within the TGF-β1, β2 and β3 genes which were known to be the most divergent within the gene family, namely the area encoding the non-conserved portion of the precursor protein, just 5′ to the mature protein sequence (see Fig. 2). This was done in an attempt to ensure maximum specificity of the amplification reactions.

When performing reverse transcription-PCR, it is necessary to guard against false positive results produced by amplification of contaminating genomic DNA. Therefore, the oligonucleotides were chosen from different exons. This means that amplification of genomic DNA will produce bands of a larger size, which will not be confused with bands produced by RT-PCR amplification of spliced mRNA. The position of the intron-exon boundaries are known for TGF-β1 (Derynck *et al.* 1987) and TGF-β3 (Derynck *et al.* 1988). They are highly conserved and are assumed to be homologous for TGF-β2. The exon from which each oligo primer is taken is specified below. PCR products spanning more than one exon were also chosen as a means of detecting possible alternative splicing of the transcripts. The T_m for each primer was estimated according to the rule of Thein and Wallace (1986).

The oligonucleotides were as follows;

TGF-β1:

left oligo: 5′- CTG GCA CCC AGC GAC TCG CCA –3′

Exon 3 pos. 1403 to 1423 (Derynck *et al.* 1985)

Est. T_m = 72°C.

right oligo: 5′- AAT GTA CAG CTG CCG CAC GCA –3′

Exon 6 pos. 1744 to 1724 (Derynck *et al.* 1985)

Est. T_m = 66°C.

TGF-β2:

left oligo: 5′- CAT AAA GAC AGG AAC CTG GGA –3′

Exon 3/4 boundary pos. 721 to 741 (Madisen *et al.* 1988)

Est. T_m = 62°C.

right oligo: 5′- AAT GTA AAG TGG ACG TAG GCA –3′

Exon 6 pos. 1056 to 1036 (Madisen *et al.* 1988)

Est. T_m = 59°C.

TGF-β3:

left oligo: 5′- AGA GAG TCC AAC TTA GGT CTA –3′

Exon 3/4 boundary pos. 905 to 925 (ten Dijke *et al.* 1988)

Est. T_m = 59°C

right oligo: 5′- AAT GTA GAG GGG GCG CAC ACA –3′

Exon 6 pos. 1228 to 1208 (ten Dijke *et al.* 1988)

Est. T_m = 66°C

All primers thus have a T_m within the prescribed range of 55°C to 80°C.

It is thus also possible to detect the presence of alternative splicing of the mRNA. Should exon 5 be missing from TGF-β2 and β3, or exons 4 or 5, or both, from TGF-β1, the RT-PCR will result in the amplification of smaller bands.

Oligonucleotides were chosen without the aid of any computer analysis, but bearing in mind the need for a roughly equal GC:AT ratio and the need to avoid potentially palindromic sequences which might cause secondary structures to arise in the oligonucleotides. The need to avoid complementarity between the oligonucleotides was also considered.

2.2.10.2 The polymerase chain reaction

The PCR reaction was carried out in a standard manner as follows, although variations were subsequently introduced in attempts to optimise the process (these are discussed further in the Results and Discussion sections).

A 100µl reaction was set up containing 1µl sample, 5µl of each oligonucleotide primer 20µM, 4µl of each 1.25mM dNTP, 10µl 10xPCR buffer (100mM Tris, 0.5M KCl, 15mM $MgCl_2$, 0.1% gelatin) and 2.5 U of *Taq* polymerase. The basic thermal cycle program was as follows:

1 min at 95°C, 2 minutes at 55°C, 3 minutes at 70°C. After 30 cycles, a final step of 5 minutes at 70°C to ensure that all amplified molecules were double-stranded.

PCR products were visualized by electrophoresis on 1.5 to 2% agarose gels, or Southern blotted.

2.2.10.3 Reverse transcription polymerase chain reaction (RT-PCR)

RNA samples were incubated in a final volume of 20µl containing 1µl of the sample, 1µl carrier RNA, 4µl reverse transcriptase 5 x buffer (BRL), 1µl each 10mM dATP, dCTP, dGTP, dTTP, 1µl RNA guard (Pharmacia), and 1µl (10 U) MMLV reverse transcriptase (BRL), and incubated at 37°C for 90 minutes.

The reverse transcription reaction was diluted to a final volume of 100µl, containing the ingredients for a PCR reaction described above, with the exception of the deoxynucleotides, which were were already present in the reverse transcriptase reaction.

Chapter 3

Results

3.1 Optimization of *in situ* hybridization protocol

At the time of commencement of this project (1986), *in situ* hybridization for the detection of cellular RNAs in tissues was not a routine technique. Several other laboratories had reported the use of this technique in a variety of experimental systems. However, there was great variation in the protocols employed by different investigators, and optimization of the procedure for use on mammalian embryos was necessary.

3.1.1 Optimization of microscope slide adhesion

Initially, one of the most troublesome aspects of the procedure was the production of microscope slides to which sections would adhere throughout the various treatments. This problem was particularly acute for pre-implantation embryos because of the very small surface area of the tissue for adhesion. In the first experiments, sterile microscope slides were "subbed" by incubation for 60 minutes in 0.1 mg/ml poly-L-lysine (Sigma) in 10mM Tris, pH 8.0 (Cox *et al.* 1984). The slides were then air-dried and stored at –20°C with dessicant prior to use. This procedure had the

disadvantage that the slides thus prepared had to be used within two weeks. Poly-L-lysine was also an expensive reagent. Furthermore, the ability of the slides firmly to attach sections was not great, and a considerable loss of material was experienced initially.

An effort was made to find a superior reagent for the subbing of slides. Following the recommendations of other investigators, 1 x Denhardt's reagent (Brahic and Haase 1978), 1% gelatin (Gall and Pardue 1969), 10% albumin (Brigati *et al.* 1984) and 0.1 mg/ml lysine (Koopman *et al.* 1989) were all tested and not found to be superior. However, the treatment of Berger (1986), which involved coating slides in 1% TESPA (Sigma) in toluene at 90°C for one hour, gave substantially better results, with little or no loss of material from the slides. This also had the advantage that the slides thus prepared could be stored at room temperature for an indefinite period. The final modification followed Rentrop *et al.* (1986). Slides were coated in 2% TESPA in acetone for 30 secs at room temperature. Although this involved the use of twice the quantity of TESPA, it had the advantages of speed and safety, and gave equally satisfactory results. A subsequent modification, coating in only 0.3% TESPA in 3% acetic acid in ethanol, despite its greater safety in avoiding the use of organic solvents, was found to be less satisfactory in terms of adhesiveness.

3.1.2 Optimization of prehybridization treatments

in situ hybridization to tissue sections requires that the cellular RNA be rendered accessible to the riboprobe. Sections must therefore be treated to increase permeability to the probe while preserving the integrity of the target RNA, and morphology of the tissue sample. Most protocols include a step designed to specifically degrade cellular proteins, thus rendering the cellular RNA more available

for hybridization. Many protocols have included pronase for this purpose (Hafen *et al.* 1983). Brahic and Haase (1978) introduced the use of Proteinase K, which is less likely to be contaminated with RNase, and gives a more specific reaction than pronase.

For the purposes of this study, an *in situ* optimization experiment was carried out using a human cardiac actin (Sassoon *et al.* 1988) probe, hybridized to sections from a 57 day *p.c.* human embryo. This is a strong positive control, which gives a hybridization signal visible after overnight autoradiographic exposure. Several examples of this hybridization were carried out, using varying concentrations of proteinase K in the prehybridization treatments, ranging from zero to 200µg/ml Sections not proteinase-treated (Fig.7 A,B) and those treated with the standard (Cox *et al.* 1984) 20µg/ml proteinase K (Fig.7 C,D), had a signal which was noticeably lower than that of sections treated with the newer standard (Wilkinson *et al.* 1987) concentration of 60µg/ml (Fig.7 E,F). At 200µg/ml proteinase K, the signal is even stronger (Fig.7 G,H). However, in the interests of standardization of protocol and, not less, of economy, it was decided that a concentration of 60µg/ml should be adopted. Furthermore, since slides were initially subbed with poly-L-lysine, the sections began to be lost from the slides at concentrations above 60µg/ml, although, on Tespa-coated slides, this was not a problem.

Hafen *et al.* (1983) proposed that treatments with 0.2M HCl at room temperature for 30 minutes, and 2 x SSC at 70°C for 30 minutes, enhanced the degradation of any cellular proteins or polysaccharides which might interfere with the ability of the cellular RNA to hybridize to the riboprobe. However, these steps were found to greatly increase the chances of loss of sections from poly-L-lysine-coated slides (also found by Godard 1983) and were therefore discontinued. Their effect on the *in situ*

hybridization procedure was examined. Neither treatment appreciably alone nor in combination increased the intensity of hybridization (Fig.7 I,J,K,L,M,N) as compared with the standard treatment (Fig.7 E,F).

Formamide is a denaturing solvent which can be used to increase the stringency of nucleic acid hybridization by lowering the T_M (Thomas *et al.* 1976, Casey and Davidson 1977). Standard *in situ* protocols (Cox *et al.* 1984, Wilkinson *et al.* 1987) include 50% formamide in the hybridization mixture, in addition to 0.3M NaCl, 10mM Tris pH 8.0, 1mM EDTA, 0.1 mg/ml rRNA, 0.1 mg/ml polyA, 1 x Denhardt's solution, 10mM DTT and 10% Dextran sulphate. It was endeavoured to maximize this concentration of formamide to as near 60% as possible by omitting poly A from the hybridization mixture, and using high concentrations of DTT stock solution. A formamide concentration of 58% could be achieved without omission of any other components of the mixture. Control hybridizations, performed with standard prehybridization but only 30% formamide in the hybridization mixture (Fig.7 O,P), gave stronger signals than those with standard prehybridization and 50% formamide (Fig.7 E,F). However, it was decided to use a maximal concentration of formamide in the interests of specificity of hybridization, given that there is a potential for cross-hybridization within gene families. Experiments carried out by other investigators confirmed that 52°C is the optimal temperature for hybridization (Cox *et al.* 1984, R J Akhurst, pers. comm.).

Bandtlow *et al.* (1987) speculated that background hybridization could be increased by the formation of disulphide bonds between sulphydryl groups on the section and sulphur atoms on the riboprobes, and therefore recommended that a prehybridization mixture be used to incubate the sections prior to application of the probe. This mixture consisted of 50% deionized formamide, 0.3M NaCl, 10mM Tris pH 7.5, 1mM

EDTA, 5 x Denhardt's solution, 20mM β-mercaptoethanol, 500μM-α-thio-UTP, and 300μg/ml each of polyA, polyC and rRNA. However, in my hands, this was not found to improve hybridization in any way (data not shown) and was thus abandoned.

3.2 *in situ* hybridization on preimplantation embryos

3.2.1 Optimization of preimplantation embryo sectioning techniques

At the commencement of this work (1986), very little molecular biology had been carried out on preimplantation mammalian embryos. The advent of PCR technology made it feasible to carry out studies on gene expression in small numbers of preimplantation embryos (*eg.* Rappollee *et al.* 1988, Brenner *et al.* 1989). However, prior to this development, *in situ* hybridization represented the only means of studying gene expression in small quantities of embryonic material.

It was attempted to use sections of preimplantation human embryos in order to study the temporal and spatial expression of the gene encoding human chorionic gonadotropin β (hCG-β) chain (Fiddes and Goodman 1980) from oocyte to blastocyst, to determine when the gene was first expressed and when trophoblast-specific gene expression could first be observed. Bonduelle *et al.* (1988) used *in situ* hybridization to show that hCG-β was expressed in tripronuclear (by inference triploid) human pre-embryos. However, in that study, only three embryos out of 13 examined, were scored as positive. Furthermore, although tripronuclear embryos can survive to term (Van Blerkom *et al.* 1987), it is unclear if gene regulation in such embryos is normal at the stages in question. Therefore, in this study normal embryos

from an *in vitro* fertilization (IVF) programme were utilized. It was also envisaged that a study could be made of growth factor gene expression over a similar time course.

3.2.1.1 Media for embedding of embryos

The use of agar for the embedding of preimplantation embryos dates back to Samuel (1944) who developed the technique of pipetting embryos into molten agarose and leaving to set. The block of agarose containing the embryo was then embedded like a larger piece of tissue. In this study, the same technique was used with the substitution of agarose for agar. Initial difficulty was encountered in obtaining sections from embedded preimplantation embryos due to detachment of the embryos from the blocks during sectioning. A number of alternative embedding materials were tested in an attempt to find one which would retain the embryo better than agarose. Gelatin was utilized in concentrations up to 10%. However, at these concentrations the gelatin block was prone to hardening during the embedding procedure. Lower concentrations of gelatin did not solidify as well as the agarose. Bovine serum albumin, at concentrations of up to 10%, or egg white, solidified well on fixation and did not harden during embedding. However, it was difficult to pipette the embryo into these media, and then fix them without washing out, or otherwise losing, the embryo. Hogan *et al.* (1986) suggested embedding in a length of washed, fresh mouse oviduct or uterus. However, this was manipulatively difficult, and was inappropriate for use on human embryos in a clinical setting.

A solution to this problem was suggested by Dr T Fleming (Dept. of Biological Sciences, Southampton), which involved the use of an electron microscope embedding capsule for remelting the agarose (low melting point agarose was used in these cases) after the addition of the embryo. This allowed dispersal of any buffer

pipetted into the agarose along with the embryo, which was reckoned to be a major cause of embryo instability within the block, and ensured that the embryo was surrounded by an even concentration of agarose. The capsule could then be peeled away. Agarose blocks prepared in this way could then be dehydrated and embedded in the normal manner.

3.2.1.2 Whole mounting of embryos

An alternative involved the use of the technique of whole mounting of embryos (Brulet *et al.* 1985). Embryos were fixed, dehydrated and pipetted onto sterile microscope slides. This technique had previously been used (Vasseur *et al.* 1985, Duprey *et al.* 1985, Brulet *et al.* 1985) to study gene expression in preimplantation mouse embryos, and has also been widely used in the study of *Drosophila* embryogenesis (Baumgartner *et al.*1987)

3.2.2 Heterogeneity of results experienced with pre-embryos

Whole-mounted preimplantation embryos, although easier to prepare, exhibited extensive background hybridization on both control and experimental preparations. An example is shown in Fig.3. It was therefore decided to section the embryos. Sectioning had the advantage that a greater number of experiments could be performed per single embryo. However, sections of preimplantation embryos also exhibited the same problems with background hybridization of the negative control probes. Furthermore, the positive control and experimental probes gave hybridization of variable intensity to the embryos, ranging from strong hybridization to negative, even within the same experiment. This was not due to genuine differential

gene regulation according to stage, since each embryonic stage exhibited heterogeneity of hybridization.

The mouse embryo cannot be used as a control for the binding of hCG-β antisense riboprobe, since rodents do not have a homologous gene to hCG (Wurzel *et al.* 1983, Carr and Chin 1985). The conclusion of this study was that the *in situ* hybridization procedure was not appropriate for the study of human preimplantation embryonic development. In view of the limited quantity of human preimplantation material, it was decided that it was preferable to study human preimplantation embryos using the RT-PCR technology which was then becoming available (Rappollee *et al.* 1988).

3.3 Study of TGF-β gene expression in human preimplantation embryos by reverse transcription polymerase chain reaction

The RT-PCR technique has been used to study the expression of the genes for TGF-β1 and other growth factors in preimplantation murine embryogenesis (Rappollee *et al.* 1988, Brenner *et al.* 1989). Thus, it was attempted to reproduce this data in human preimplantation embryos, and to extend it to other members of the TGF-β gene family. RT-PCR also has the advantage that it can, by the use of oligo primers from different exons, detect alternative splicing of mRNA (Ffrench-Constant and Hynes 1989). This is relevent to the study of the TGF-β genes, since it has been shown that alternative splicing to produce alternative polypeptide moieties occurs in TGF-β1 (Kondaiah *et al.* 1988) and TGF-β2 (Webb *et al.* 1988). As explained in the Materials and Methods (2.2.10.1), the oligo primers were designed in order that

should exon 5 be missing from TGF-β2 and β3, or exons 4 or 5, or both, from TGF-β1, the RT-PCR will result in the amplification of smaller bands.

Human preimplantation embryos are a very rare resource. Therefore, it was desirable to optimize RT-PCR techniques to a level where the reaction was capable of detecting very few target molecules of RNA. Initial experiments thus focussed on the sensitivity of the PCR reaction.

3.3.1 Optimization of PCR technology

Theoretically, it is possible to detect a single molecule of DNA or RNA using this method (Saiki *et al.* 1985, Mullis *et al.* 1986, Mullis and Faloona 1987). The positive control amplification was a gel-purified 2.1kb human TGF-β1 cDNA insert. Negative controls were either Bluescribe vector DNA or mouse α-actin DNA. One femtogram of target DNA was considered to represent 500 molecules since the molecular weight of a 2.1kb double-stranded DNA is around 1.4×10^6g.

The first optimization reaction (Fig.5 A) utilized three aliquots of target DNA, containing 1 picogram, 1 femtogram, and 1 attogram of DNA (10^{-12}, 10^{-15} and 10^{-18} g) respectively. The reactions were amplified utilizing the TGF-β1 specific oligonucleotide primers, and the following program: 1 minute at 95°C, 2 minutes at 55°C, 3 minutes at 70°C. After 30 cycles, a final step of 5 minutes at 70°C to ensure that all amplified molecules were double-stranded (see Materials and Methods 2.2.10.2). The protocol is basically that of Innis and Gelfand (1990), with slight modifications as recommended in the Cetus Corp. technical literature, and by other investigators.

A clear band of the correct size (342 nt) is produced by 1 picogram, but not 1 femtogram or 1 attogram, of target DNA (Fig.5 A). The limit of sensitivity of the original protocol was therefore determined to be between 432 and 4.32×10^5 target molecules. It was therefore decided to increase the number of cycles of amplification

PCR reactions were set up with 1pg, 1fg and 1ag of target DNA (Fig.5 B). The samples were given 30, 41 and 44 cycles of amplification respectively. The 1pg sample amplified well, although there were now some bands of higher molecular weight. The other lanes had no amplified bands, but merely a smear of primers similar to that found in the negative control lane.

In a further effort to increase sensitivity, the cycle parameters were altered to the following; 94°C for 1 minute, 37°C for 1 minute and 72°C for 3 minutes. The lowered annealing temperature was designed to increase the likelihood of hybridization between the oligos and the target DNA (Saiki *et al.* 1988). The number of cycles was increased to 99 to maximize the quantities of product generated. Some authors do not recommend this (*eg.* K. Mullis quoted in Innis and Gelfand (1990)). However, this was included to ensure that the problem was not merely one of insufficient cycles of amplification. Additionally, the target DNA samples were boiled for 5 minutes prior to addition to the amplification mixture, to ensure that all DNA was single-stranded and available for hybridization. The final polymerization step was lengthened to 7 minutes in an effort to ensure that all amplified product was double-stranded and of full-length. 100fg of target DNA amplified well, but the lower concentrations were negative (Fig.5 C). The negative control appears to have been contaminated.

It has been reported that the sensitivity of the PCR reaction, can depend on the concentration of magnesium in the buffer (Saiki *et al.* 1988). The standard buffer contains 1.5 mM $MgCl_2$. High and low Mg^{2+} buffers were made by using

concentrations of $MgCl_2$, of 0.5 mM and 4 mM. It was decided also to raise the annealing temperature slightly, but to use an annealing time of 4 minutes. This gives the primers a greater time in which to anneal to the target DNA. The program used was as follows: 94°C for 1 minute, 45°C for 4 minutes and 72°C for 3 minutes. 60 cycles were run.

High (4mM) and low (0.5mM) salt buffers were compared for reactions containing 100fg and 1fg of target DNA. The high salt buffer gives many bands (Fig.5 D), making it impossible to decide if the reaction has worked or not, and the low salt buffer does not suffice for activity of the polymerase. Therefore, the standard medium $MgCl_2$ buffer was preferable to the two extreme concentrations.

It was decided to utilize a standard buffer concentration but to maintain a lengthened annealing time. The program adopted was as follows: 94°C for 1 minute, 48°C for 4 minutes and 72°C for 2 minutes, for 60 cycles.

The reaction containing 10pg of target DNA gave a band of the correct size (Fig.5 E). However, the sensitivity was no greater than the previous reaction which had utilized an annealing step of 37°C for 1 minute. Furthermore, all lanes, including the negative control, contain numerous small bands, both larger and smaller than the specific band. Therefore, increasing annealing time results in no increase in sensitivity, and a decrease in specificity.

The general conclusions from the optimization experiments were as follows.

1) Increasing the duration of the annealing time decreased the specificity of the reaction.

2) Lowering the temperature of annealing while maintaining a short duration resulted in no improvement in sensitivity, but no drop in specificity.

3) Magnesium concentration was also influential, a moderate concentration giving better results than the two extremes.

4) The number of cycles had to be at least 60 if very small quantities of DNA were to be amplified to the point where they could be visualized by ethidium bromide staining.

In view of the complexity of the parameters involved in the amplification process, it was resolved to proceed directly to reverse-transcribed PCR and optimize the process as required for that system.

3.3.2 Optimization of RT-PCR reactions

In order to establish that the reverse transcription-polymerase chain reactions (RT-PCR) were functional, full length TGF-β1 cRNA, synthesized using the riboprobe protocol (see Materials and Methods) was used as a positive control. A trace (10μCi) of α^{35}S-UTP was added to quantify the RNA produced. An aliquot of 10ng of synthetic RNA was precipitated and added to a reverse transcriptase reaction. 5% of the reverse transcriptase reaction was added to a PCR reaction mixture and amplified using the following program: 1 minute at 95°C, 2 minutes at 55°C, 3 minutes at 70°C. After 30 cycles, a final step of 5 minutes at 70°C to ensure that all amplified molecules were double-stranded

In addition to synthetic TGF-β1 RNA, cellular RNAs from BeWo (choriocarcinoma), NTera2 (teratocarcinoma) and HeLa cell lines were also tested. These were of

interest in view of the possible role of TGF-βs in the regulation of embryogenesis and the transformed phenotype. The positive control TGF-β1 full-length synthetic RNA gave the expected band (Fig.6 A). The other cellular RNA samples gave a variety of bands. The predicted band was also found in the BeWo sample. A smaller major band was found in the BeWo, HeLa and NTera2 samples at around 154 nt. None of these bands, apart from that due to the primers, could be seen in the negative control lane. Comparison with an aliquot of BeWo RNA indicates that these bands were PCR products and not due to 2° structural effects of the original RNA sample, and could therefore be the products of alternative splicing of the TGF-β1 mRNA in these cell lines, such as had already been demonstrated for TGF-β1 by Kondaiah *et al.* (1988).

In an effort to establish if these bands were genuine products of alternatively spliced RNA, the samples were Southern blotted, and hybridized with TGF-β1 oligo-labelled DNA probe (Fig.6 B). Unfortunately, a low stringency wash only revealed two bands, one in the positive control lane and another in the BeWo lane. The other bands seen on the gel were therefore not the products of genuine alternative splicing, but artefacts of the reverse transcription or PCR processes.

Following the advice of Dr J Gow (Ruchill Hospital, Glasgow) a shortened amplification procedure specially designed for RT-PCR, was adopted as follows: 94°C for 1 minute, 50°C for 1 minute and 72°C for 1.7 minutes. This short cycle with relatively high annealing temperature, is designed to minimize the possibility of artefacts arising in the RT-PCR procedure.

The RT-PCR experiment was repeated, using this protocol, with TGF-β1 synthetic RNA as a positive control, and *E.Coli* rRNA and antisense synthetic RNAs as negative controls. The products were Southern Blotted and probed using TGF-β1

oligo-labelled DNA probe (Fig.6 C). TGF-β1-containing plasmid DNA, digested with *Eco*RI, hybridizes strongly to the TGF-β1 probe, as does the same plasmid digested with *Ava*I. In contrast, a plasmid containing the full-length TGF-β2 cDNA also digested with *Ava*I, was negative, indicating that the signal on the blot was TGF-β1 specific. The first four samples have several strong bands hybridizing to TGF-β1. These are the positive control TGF-β1 synthetic RNA and the chorion and BeWo RNA samples. Unfortunately, the negative control rRNA sample also has a strong specific band. The NTera2 sample is negative.

No firm conclusion can be drawn from this experiment due to the presence of a TGF-β1 specific band in the negative control lane. However it can be said that the multiplicity of bands which can be created by a RT-PCR experiment must be blotted to check which are genuine and which artefactual. The bands which hybridize to the probe on Southern blots appear to be identical to the band found in the positive control lane, indicating that the lower molecular weight bands do not occur due to alternative splicing within the area amplified by this set of oligo primers. The possible exception appears to be the BeWo RNA, which has a band of approximately 250nt which hybridized almost as strongly as the main band.

In order to investigate if there was a problem of contamination in the cellular RNA samples utilized for RT-PCR, it was attempted to amplify any contaminating DNA by PCR. None of the cellular RNA samples produced any amplification (Fig 6 D,E), indicating that the bands observed in the RT-PCR experiments on the chorion and BeWo RNA are the products of reverse transcription, and therefore represent TGF-β1 RNA within these cells.

In this section, it was attempted to create a system for the study of the expression and possible alternative splicing of TGF-β RNA in preimplantation embryos. Due to

the scarcity of human pre-embryonic material, it was decided to perform preliminary experiments on RNA from various cell lines. RT-PCR resulted in the production of several amplified DNA species. Southern blotting of the products demonstrated that the majority of these fragments were not homologous to TGF-β1. Those that were homologous were of the larger size, indicating that alternative splicing did not occur to a level which could be detected within the sensitivity of the technique. Therefore, it is necessary to exercise caution in the interpretation of RT-PCR results, confirmation, by Southern blotting, of the specificity of PCR products being advisable in every case.

The difficulties with optimization of the sensitivity of the PCR method, presented in the previous section, were also borne in mind. Since it was not possible to detect TGF-β1 cDNA at levels of less than 1fg per 100μl, it is possible that levels of TGF-β1 RNA were present in the cell lines studied at levels which were below the limits of sensitivity of the technique.

TGF-β1 RNA was amplified and its specificity confirmed by Southern blotting in BeWo cells and in human chorion. The latter experiment could be called into question by the presence of a band in one of the negative control lanes. However, it was demonstrated that contamination with TGF-β1 cDNA had occured in the negative control DNA used for this experiment, and not in the chorion RNA, thus confirming that the result was genuine. This sporadic contamination of samples with TGF-β1 cDNA was a feature of the project which made it very difficult to state definitively that any result obtained was conclusive. In view of this, it was decided that the preimplantation embryo RNA samples should not be used until a more reliable technique was available.

3.4 Expression of TGF-β genes in post-implantation embryogenesis

3.4.1 TGF-β gene expression in the developing liver

All blood cells are derived from haematopoietic progenitors. Haematopoiesis takes place during embryonic development in the yolk sac, liver, bone marrow and Hassal's corpuscles, and TGF-β is detectable in these sites during embryogenesis (Ellingsworth *et al.* 1986, Heine *et al.* 1987, Wilcox and Derynck 1988, Lehnert and Akhurst 1988) but see Hyldahl *et al.* (1990) for a negative result for TGF-β1 expression in the yolk sac.

In this study, TGF-β1 was the only member of the TGF-β gene family to be expressed at high level within the human embryonic liver (Fig.8 A,B,). At 43 and 57 days *p.c.*, intense expression of TGF-β1 RNA was seen in single cells and cell clusters. Morphology at higher power magnification suggested that the expressing cells were megakaryocytes (Fig.8 E,F,G,H) and other cells of the haematopoietic lineage which were tentatively identified as type B normoblasts. These cells had not appeared at 32 days *p.c.*. Primitive parenchymal cells were not found to express the gene at any time, and TGF-β2 (Fig.8 C,D) and TGF-β3 (data not shown) were negative in the embryonic liver at all stages examined.

3.4.2 TGF-β gene expression in the cardiovascular system

Expression of TGF-β1 in the mouse cardiovascular system has been extensively studied by Akhurst *et al.* (1990a), who observed TGF-β1 RNA in early endothelial cells and areas associated with septation and valve formation within the heart. In this study, TGF-β1 RNA expression was also detected in human fetal cardiac valve

endothelia at 43 days *p.c.* (Fig.9 C,D) at a time when human heart development was virtually complete. In the earliest embryo examined (32 days *p.c.* which was equivalent to an 11.5 day *p.c.* mouse embryo), no expression of TGF-β1, 2 or 3 were seen. This was probably due to poor preservation of the RNA, since the relative level of expression of TGF-β1 RNA in the murine embryonic heart was low (Akhurst *et al.* 1990a), and there was variation in the speed at which fetuses were processed for *in situ* hybridization following their collection.

TGF-β1 RNA was also seen in the endothelia of large arteries (Fig.9 E,F), whereas TGF-β3 was expressed in the tunica intima underlying these endothelia (Fig.9 G,H).

3.4.3 TGF-β expression in epithelia

In previous studies TGF-β1 RNA was detected in the epithelial component of the developing tooth, hair follicle, salivary gland and secondary palate of the mouse (Lehnert and Akhurst, 1988, Akhurst *et al.* 1990b; Fitzpatrick *et al.* 1990). The equivalent stages of development of these structures were not available for study here, so no conclusions regarding epithelial TGF-β1 expression can be made.

3.4.3.1 Detection of TGF-β RNA in the embryonic lung

The lungs are formed by the proliferation and branching of the lung buds into the splanchnic mesoderm from the fifth week of development. The development of the lung occurs relatively late in gestation and, at the early stages examined in this study, the pulmonary epithelial cells are all columnar in morphology, the transition to a simple cuboidal cell occurring much later.

In the 43 day *p.c.* embryo TGF-β2 RNA could be found in the primitive lung epithelia (Fig.10 C,D). By 47 days *p.c.* expression of TGF-β3 transcripts were detectable in the submucosa of the proximal bronchi, and in the terminal bronchial epithelium (Fig.10 E,F). By 57 days *p.c.*, the process of branching is fairly well advanced and only the growing tips of the developing bronchioles expressed TGF-β2 RNA in their epithelia (Fig.10 I,J). In contrast, TGF-β3 RNA showed a more widespread pattern of expression in the lung at this stage (Fig.10 G,H). As found at 47 days *p.c.*, the RNA was seen submucosally in the proximal respiratory tract from the presumptive larynx downwards to a point which we believe represents the boundary between bronchi and bronchioles. Distal to this it had an epithelial expression pattern in the linings of the bronchioles but was also co-expressed with TGF-β2 in the terminal growing end buds.

3.4.3.2 TGF-β gene family expression in sensory epithelia

Intense epithelial expression of TGF-β2 was seen in the sensory epithelium, but not the simple cuboidal epithelium, of the developing inner ear at 47 and 57 days *p.c.* (Fig.12 G,H,I,J) and in a number of epithelial structures within the developing eye. At 43 days *p.c.*it was expressed in all the cells of the lens vescicle and the inner layer of the optic cup, which gives rise to the retina (Fig.12 A,B,C,D). By 57 days *p.c.* TGF-β2 RNA has become limited to the anterior germinal epithelium of the lens and was also seen in the most anterior part of the inner layer of the optic cup, which was destined to become the muscular iris (Fig.12 E,F).

3.4.4 Expression of TGF-β2 in the nervous system

There was no obvious expression of TGF-β1 or β3 in any neuronal tissue in the embryos examined in this study, although the brain tissue tended to be less well preserved. However, the TGF-β2 probe hybridizes strongly with the neuroepithelium in the ventral region of the spinal cord, demonstrated in a para-sagittal section (Fig.11 A,B). This would be in agreement with the observations of Millan *et al.* (1991) who saw TGF-β2 expression in the ventral horns of the spinal cord in the mouse embryo at 12.5 days *p.c.*.

3.4.5 TGF-β expression in chondrification and ossification

Sandberg *et al.* (1988a,b) demonstrated that TGF-β1 was expressed at high levels in osteoblasts, osteocytes and osteoclasts in areas of intramembramous ossification of 17 week *p.c.* human fetuses. In the present study, it was found that this pattern of expression was visible at sites of endochondral ossification as early as 12 weeks *p.c.* (Fig.13 M,N). TGF-β2 (Fig.13 O,P) and TGF-β3 (Fig.13 Q,R), by contrast, were not expressed in the zone of osteogenesis. It was also confirmed that one of the most intense sites of TGF-β1 RNA expression was in areas of intramembranous ossification. This was observed in the 57 day *p.c.* embryo in the maxillary and palatine bones, and in the mandible (Fig.14 A,B). There is no endochondral ossification at 8 weeks *p.c.*. Hypertrophic cartilage cells are visible in the centres of the long bone cartilages, but do not hybridise with any of the three TGF-β gene probes (Fig.13 G,H).

Pelton *et al.* (1989) observed expression of TGF-β2 in osteoblasts and endothelial cells during endochondral, periosteal and intramembramous ossification in the

mouse. However, in the present study, although the TGF-β1 probe was seen to hybridise intensely to these sites, the TGF-β2 and TGF-β3 probes generated no autoradiographic signal (compare Figs.14 A,B with 14 C,D and 14 G.H). TGF-β2 RNA expression in the limb was, however, seen at the limb bud stage (43 days *p.c.*), in areas of mesenchyme which are morphologically indistinguishable from the surrounding tissue (Fig.13 A,B,C,D,E,F). These areas are also negative on staining with 1% alcian green (Fig.24), which was specific for cartilage. It was assumed that these areas are early pre-cartilaginous blastemae.

At later stages of limb development TGF-β2 RNA expression becomes limited to the actively proliferating chondroblast zone at the diaphyseal/epiphyseal junction (Fig.13 G,H). There was also intense expression, at 57 days *p.c.*, in tendinous material, surrounding the digits and bones of the feet (Fig.13 I,J,K,L), as observed by Pelton *et al.*(1989) in the murine embryo.

The earliest embryonic expression of TGF-β3 RNA seen in this study was in the intervertebral disc anlagen at 43 days *p.c.* (Fig.15 C,D) and 47 days *p.c.* (Fig.15 G,H). This pattern persists at least to 57 days *p.c.* (Fig.15 I,J). At 57 days *p.c.*, TGF-β3 RNA is also expressed in the perichondria of cartilage models associated with the vertebral column, in particular the ribs (Fig.15 O,P), but not those associated with the long bones of the limbs. A low level of expression is seen in the tracheal cartilage rings (Fig.15 K,L), and in the perichondria of non-ossifying cartilage, such as Meckel's cartilage, although there appears to be more TGF-β3 RNA in the surrounding mesenchyme than in the perichondria *per se* (Fig.14 G,H).

3.4.6 TGF-β gene family expression in other mesenchyme and mesothelia

3.4.6.1 Detection of TGF-β RNA in human embryonic mesenchymal and mesothelial tissues

TGF-β2 and β3, unlike TGF-β1, show widespread mesenchymal expression at the stages examined. In fact, the earliest expression of any of the TGF-βs detected in this study was that of TGF-β2 in the somatic mesoderm surrounding the liver at 32 days *p.c.*(Fig.16 A,B). TGF-β3 was also expressed in mesenchymal tissue, particularly those surrounding perichondria (Fig,14 G,H), and in the non-muscular part of the centre of the tongue (Fig.16 I,J). More striking, however, was the expression of this gene in mesothelia surrounding all the major organ systems, including the pericardium, diaphragm and viscera (Fig.16 E,F,G). In the diaphragm, the use of the human cardiac actin probe showed that TGF-β3 RNA was associated only with the mesothelial and not the muscular layers (Fig.16 H).

TGF-β2 was also expressed extensively in mesenchymal areas at 43 and 57 days *p.c.*, particularly in the non-ossifying regions of the head (Fig.14 C,D,I,J), and neck (Fig.15 A,B). Hybridization with a human cardiac actin gene probe (kindly supplied by P Barton), which hybridizes to fetal skeletal and cardiac actin (Sassoon *et al.* 1988), indicates that TGF-β2-positive tissue was adjacent to, but does not generally overlap, regions of myogenesis (data not shown). This is interesting in view of the apparent importance of TGF-β to myogenesis *in vitro* (Florini *et al.* 1986, Massague *et al.* 1986, Olson *et al.* 1986, Allen and Boxhorn 1987, Ewton *et al.* 1988).

3.4.6.2 Detection of TGF-β2 RNA in human embryonic skin

Over the period of development examined in this study the skin was at a very immature stage, the epidermis being only one cell thick. At 57 days *p.c.*, TGF-β2 RNA was seen in the hypodermal mesenchyme immediately beneath the skin (Fig.16 C,D). This is similar to the observations in the murine embryo by Pelton *et al.* (1989).

3.4.7 Summary of TGF-β gene expression in embryogenesis

TGF-β genes displayed a wide range of sites of expression in the embryo. TGF-β1 was found to be expressed in haematopoietic tissue, in endothelia and osteogenic cells. The patterns of expression of TGF-β2 and TGF-β3 were more extensive. TGF-β2 was expressed in various mesenchymal areas, including precartilaginous tissue. Additionally, it exhibited expression in a variety of epithelia and in the central nervous system. TGF-β3 was also predominantly detected in mesenchyme, with minor areas of expression in terminal bronchial epithelia and mesothelia (See **Table** 3.6).

3.5 Expression of retinoic acid receptor genes in embryogenesis

3.5.1 Expression of RAR-α

RAR-α riboprobes, whether gene-specific or full-length were found to hybridize strongly to all tissues examined at all stages. These results were initially interpreted

as background hybridization, and are not depicted photographically. However, the similar results of Dolle *et al.* (1990) in the murine embryo suggest that the decision to abandon further study of the RAR-α gene was incorrect (see Discussion), but that, indeed, the RAR-α gene is ubiquitously expressed in mammalian embryos.

3.5.2 RAR gene expression in the developing limb.

As mentioned in the Introduction, retinoic acid has major effects in the developing limb as a morphogen and, in amphibians, as an inducer of regeneration. The limb is also a major target for retinoic acid-induced teratogenesis. The expression of RAR types α, β and gamma was studied in the murine embryo by Dolle *et al.* (1989). The earliest limb stage examined in this study was that of the 43 day *p.c.* embryo. At this stage, the lower limb is more developed than the upper, which is still a very primitive mesenchymal outgrowth. In the upper limb at this stage, RAR-β was found to be expressed in a region in the centre of the limb bud (Fig.17 E,F). RAR-β was also found to be expressed in the prechondrogenic scleroblastemae of the developing lower limb (Fig.17 I,J). This was the same area of the lower limb in which TGF-β2 was detected (compare Fig.13 E,F). RAR-gamma was expressed more proximally (Fig.17 A,B,C,D), and appeared to be negative in the prechondrogenic scleroblastemae at that stage. By 47 days *p.c.*, the prechondrogenic blastemae have begun to differentiate into cartilage. The first cartilage appears in the inner part of the scleroblastemae. This area was found to be negative for RAR-β by 47 days *p.c.* (Fig.17 M,N). However, the outer, undifferentiated part of the scleroblastema was still positive for RAR-β RNA. In the 57 day *p.c.* embryo, the cartilage models of the long bones are fully formed. At this stage, RAR-gamma was found to be expressed

in the cartilage model (Fig 17 Q,R), with RAR-β being confined to the perichondrium and the mesenchyme surrounding the cartilage (Fig.17 O,P,S,T).

3.5.3 RAR-gamma expression in the developing axial skeleton

The principal manifestation of segmentation in the developing human embryo is the condensation of the paraxial mesoderm into a series of paired, lateral segmental blocks, the somites. The somite soon differentiates into three components, the dermatome, the sclerotome and the myotome. These three tissue types give rise to integumentary tissues, connective tissue and bone, and muscle, repectively. Although initially paired, medial migration of somitic cells results in fusion around the notochord. It has been reported that the administration of retinoic acid during pregnancy can affect the axial segmented, and unsegmented, mesoderm, causing rib and vertebral malformations (Marin-Padilla 1966).

By 38 days *p.c.*, the somites are visible as mesenchymal condensations anterior to the neural tube. These condensations were found to express the gene for RAR-gamma (Fig.18 C,D). By 43 days *p.c.*, the posterior sclerotomal portions of the somites have begun to differentiate into condensed cartilaginous structures, which will become the intervertebral discs, while the less condensed anterior part becomes the progenitor of the vertebral bodies. RAR-gamma was found to be expressed in the intervertebral discs at this stage (Fig.18 G,H). This pattern of expression was partially overlapping with that of TGF-β3 (Fig.15 C,D), with the difference that the area expressing TGF-β3 tended to be more medial. This correspondence between TGF-β3 and RAR-gamma was maintained at 57 days *p.c.*, where both were found to be expressed in the intervertebral discs (Fig.15 I,J and Fig.18 I,J). Neither area of

expression overlapped with that of cardiac actin (Fig.18 E,F), indicating that neither gene was likely to be involved in myogenesis.

3.5.4 Expression of RAR genes in chondrogenesis

Expression of the RAR gene family is detected throughout the process of chondrogenesis. Expression of RAR-β and RAR-gamma is found throughout the process of limb development as described above (3.5.2). RAR-gamma was also found to be expressed extensively in other cartilaginous areas which are well developed by 57 days *p.c.*. These included the developing pelvis (Fig.19 E,F), the thyroid and hyoid cartilages (Fig.19 A,B), rib cartilages (Fig.19 C,D) and those of the digits (Fig.19 G,H). In the digits, transverse sections revealed that RAR-gamma RNA was expressed in the outer parts of the cartilage and the perichondria, rather than throughout the thickness of the cartilage. In this respect, the digit cartilages differed from the rib cartilages, which appeared to express RAR-gamma throughout their thickness.

3.5.5 RAR gene expression in the developing respiratory and digestive systems

By 38 days *p.c.*, the respiratory system has begun to develop from the median ventral diverticulum of the foregut tube. The mouth is not formed at this stage, but is merely an opening, the stomatodaeum, between the pharyngeal arches and the head. RAR-β is expressed at this stage in the mesenchyme surrounding the developing gut and respiratory tubes. Expression was found to extend from the area inferior to the third pharyngeal arch (Fig.20 I,J), continuing inferiorly and posteriorly to the primitive lung sac (Fig.20 K,L). RAR-β RNA was detected in the superior part

only of the lung sac. Expression was not found in the epithelia of either respiratory or digestive systems at this stage.

RAR-gamma was also found to be expressed in the mesenchyme of the pharyngeal arches. In the most medial sections, the first three pharyngeal arches expressed RAR-gamma RNA (Fig.20 C,D). However, in more lateral sections, it was found that the mandibular and hyoid arches were negative, the positive signal being restricted to the third pharyngeal arch (Fig.20 E,F). RAR-gamma was not expressed in the more inferior parts of the digestive and respiratory tracts at 38 days *p c.*.

By 43 days *p.c.*, RAR-β was detectable in the epithelium of the glottal region overlying the developing epiglottis and arytenoid (Fig. 21 A,B), and in the outer mesenchyme surrounding the developing oesophagus (Fig.20 M,N). The visceral mesenchyme of the upper two-thirds of the oesophagus differentiates into striated muscle. However, there is no expression of cardiac actin visible at this stage. RAR-gamma was also found to be expressed in the area immediately anterior to the developing trachea. (Fig.20 O,P). This area is presumptive tracheal cartilage.

At 47 days *p.c.*, the area expressing RAR-gamma, anterior to the trachea, could be seen to be developing tracheal cartilage (Fig.20 S,T). This expression was found to extend inferiorly beyond the bifurcation of the trachea and into the bronchial cartilage (Fig.20 U,V). Bt this stage, the expression of RAR-β had weakened in the mesenchymal area surrounding the oesophagus (Fig.20 Q,R).

In the 57 day *p.c.* embryo, the RAR-β signal in the area surrounding the oesophagus had considerably declined. However, the expression of RAR-gamma in the cartilage of the trachea and bronchii continued strongly (Fig.20 W,X,Y,Z)). RAR-β expression

had by this stage become intense in the oral epithelium (Fig.21 A,B,E,F), including the tooth buds (Fig.21 I,J), and also in the centre of the tongue (Fig.21 G,H).

3.5.6 Expression of RAR genes in the mesenchymal regions of the face

Retinoic acid-induced teratogenesis in mammals can cause facial abnormalities, including cleft palate. In the chick embryo, the frontonasal mass mesenchyme is one of the targets for retinoid action (Wedden 1987). RAR-β and gamma were both found to be expressed diffusely in the mesenchyme of the face and jaw at 57 days *p.c.* (Fig.21 I,J,K,L,M,N). In general, RAR-gamma had a more extensive pattern of hybridization than RAR-β. In the lower jaw, the mesenchymal patterns of expression of each gene were more readily definable, both being detected in the area antero-superior to Meckel's cartilage (Fig.21 I,J,K,L).

3.5.7 RAR-β expression in the central nervous system

The central nervous system is one of the areas most affected in retinoic acid-induced teratogenesis in humans (reviewed by Rosa *et al.* 1986). The cellular retinoic acid and retinol-binding proteins, CRABP and CRBP, have been detected in the developing murine spinal cord and brain (Perez-Castro *et al.* 1989). In the 43 days *p.c.* embryo, transverse sections of the spinal cord showed areas of expression of RAR-β in the mantle layer (Fig.22 A,B). The mantle eventually becomes the grey matter of the central nervous system, and at this stage is composed of rounded, apolar neuroblasts and the precursors of the glial cells. These areas expressing RAR-β were not apparently different in histological structure to the surrounding negative areas (Fig.22 C,D). High power magnification showed that both neuroblasts

and gliablasts were positive, and confirmed that there is no obvious difference between expressing and non-expressing neural cell populations. Transverse sections from the levels of the upper and lower limb buds were examined in the 43 day *p.c.* embryo.

By 57 days *p.c.*, sagittal sections demonstrated that a column of cells hybridizing to the RAR-β riboprobe could be found extending from the hindbrain to the sacral region (Fig.22 E,F). This was assumed to be a longitudinal view of the area of expression viewed transversely at 43 days *p.c.*.

3.5.8 RAR-β expression in the developing kidney

The kidney differentiates from the metanephros. At 43 days *p.c.*, the metanephric mesenchyme was found to be positive for RAR-β (Fig.23 A,B). The metanephric duct epithelium was negative.

By 57 days *p.c.*, RAR-β was detected in the mesenchymal component of the developing kidney (Fig.23 C,D). The glomeruli were found to be negative, as were the remains of the mesonephros located inferior to the developing gonad.

3.5.9 Summary of RAR gene expression in human embryogenesis

RAR-α was expressed ubiquitously in the human embryo at all stages studied. Both RAR-β and RAR-gamma were mostly expressed in mesenchymal tissues of the embryo. RAR-gamma was exclusively mesenchymal, expressed principally in developing cartilage and the associated perichondria and mesenchyme. RAR-β was most prominently associated with prechondrogenic tissue and was also found in

other mesenchymal areas, including perichondria. Additionally, RAR-β had a unique pattern of expression in oral epithelia and the ventral spinal cord (See **Table** 3.6).

3.6 TABLE

Differential localization of RNAs encoding TGF-β1 (a), TGF-β2 (b), TGF-β3 (c), RAR-β (d) and RAR-gamma (e) during human embryogenesis.

	a	b	c	d	e
Haematopoietic tissue	+	–	–	–	–
Epithelia					
Oral epithelium	–	–	–	+	–
Early tooth bud	–	–	–	+	–
Bronchial epith.	–	+	+	–	–
Otic epithelium	–	+	–	–	–
Lens epithelium	–	+	–	–	–
Retina	–	+	–	–	–
Immature epidermis	–	–	–	–	–
Cartilage and Bone					
Precart. blastema	–	+	+(iv)	+	+[#]
Growth zone of long bone	–	+	–	–	–
Perichondria	–	–	+	+	+
Mature/hypertrophic cartilage	–	–	+(iv)	–	+
Osteogenic cells	+[1]	–	–	–	–
Cardiovascular system					
Endothelium	+	–	–	–	–
Tunica intima	–	–	+	–	–
Neuronal tissue					
Ventral spinal cord	–	+	–	+	–
Mesothelia	–	–	+	–	–
Mesenchyme	–	+	+	+	+

+, denotes that RNA is detectable at some stage between 32 and 57 days *p.c.*.
–, is below the detection level of *in situ* hybridization.
[1], observations from 12 week *p.c.* material.
iv, denotes intervertebral disc anlagen.
[#], not exactly the same distribution as TGF-β2 and RAR-β.

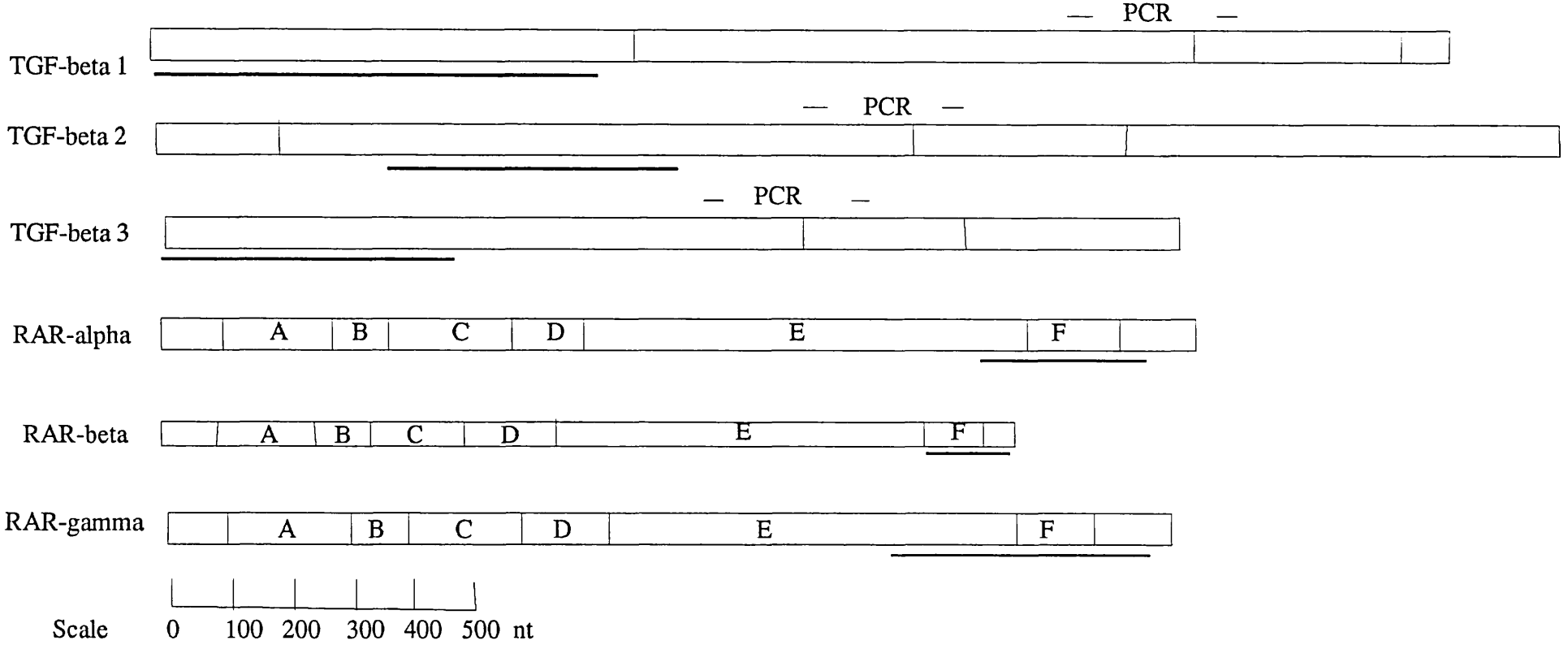


Fig. 1: Diagram of areas selected for gene-specific subclones, and regions selected for PCR amplification.

The bold lines ——— indicate the extent of the subclones.

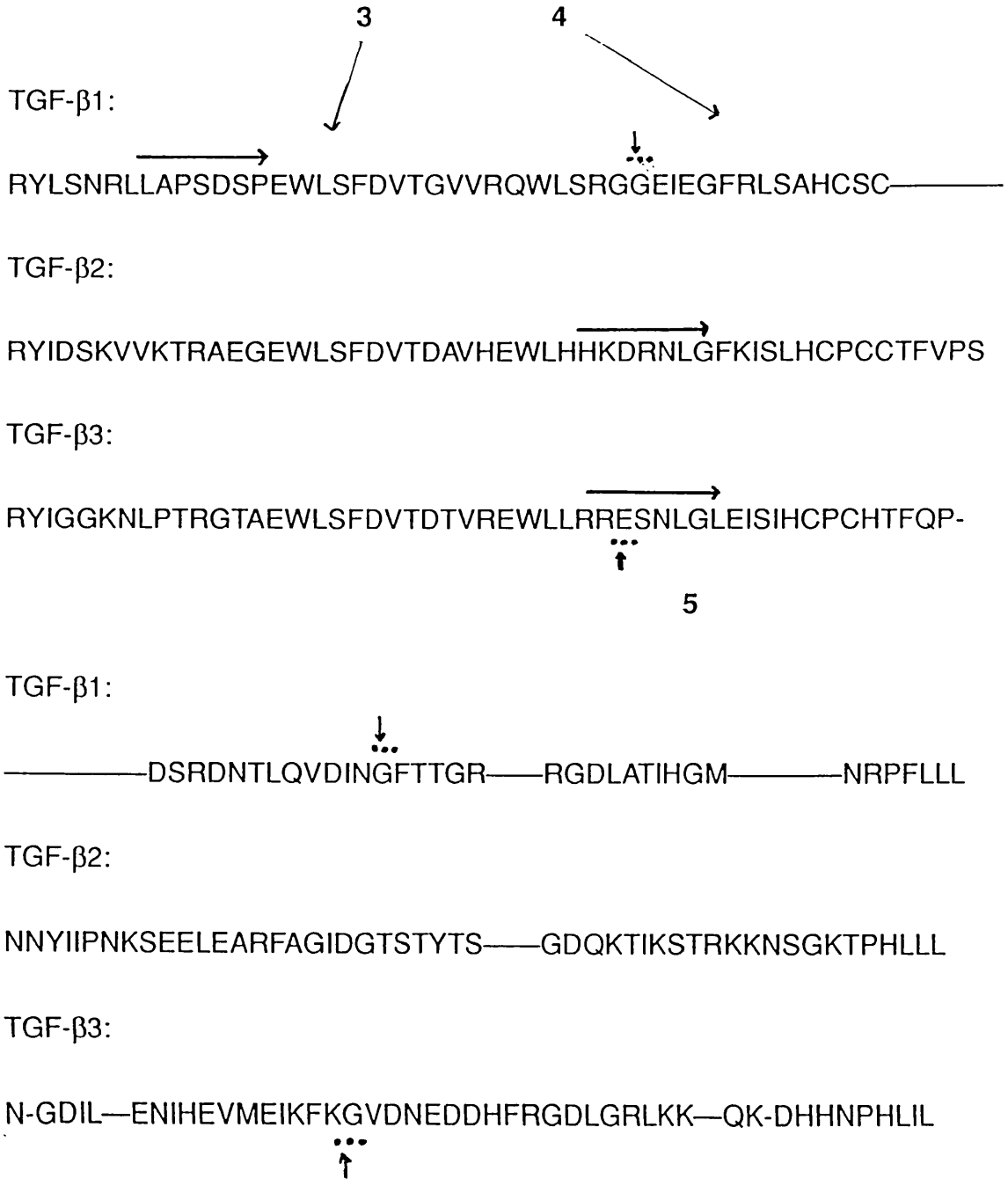
_ PCR __ indicates the location of the regions chosen for amplification (TGF-beta genes only).

The transverse lines across the bars | | indicate the boundaries of the coding sequences.

The RAR coding sequences are further subdivided into the six regions designated A to F.

The regions of the TGF-beta genes encoding the mature bioactive portion are shaded.

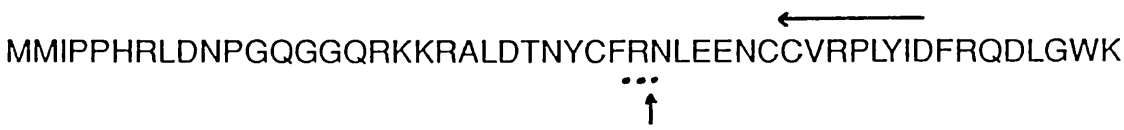
Figure 2: Amino acid sequences of TGF-β genes (from Derynck *et al.* 1988), showing location of oligonucleotides relative to the intron-exon boundaries (known for TGF-β1 and TGF-β3 only, Derynck *et al.* 1987,1988). The standard single-letter amino-acid code is used and the artificial gaps inserted to create maximum homology are indicated by ——-.



TGF-β1:

TGF-β2:

TGF-β3:



6

TGF-β1: WIHEPKGYHANFCLGP

TGF-β2: WIHEPKGYNANFCAGA

TGF-β3: WVHEPKGYYANFCSGP

The arrowed lines above the sequences indicate the position of the oligonucleotides. The arrowhead indicates the 3′ end.

∴ indicates the position of the intron-exon boundaries.

The exons are numbered **3**, **4**, **5** and **6**.

Figure 3: Whole-mounted human blastocyst. A surplus human blastocyst from an IVF programme was prepared for whole-mount according to the protocol of Brûlet *et al.* (1985). Light-field (left) and dark-field (right) views are shown. Background hybridization covers the whole embryo, although heavy staining of the inner cell mass (ICM) obscures the grains over that area. Inner cell mass (ICM). Trophectoderm (TE). The diameter of the blastocyst is approximately 150μm.

The probe utilized was antisense hCG.

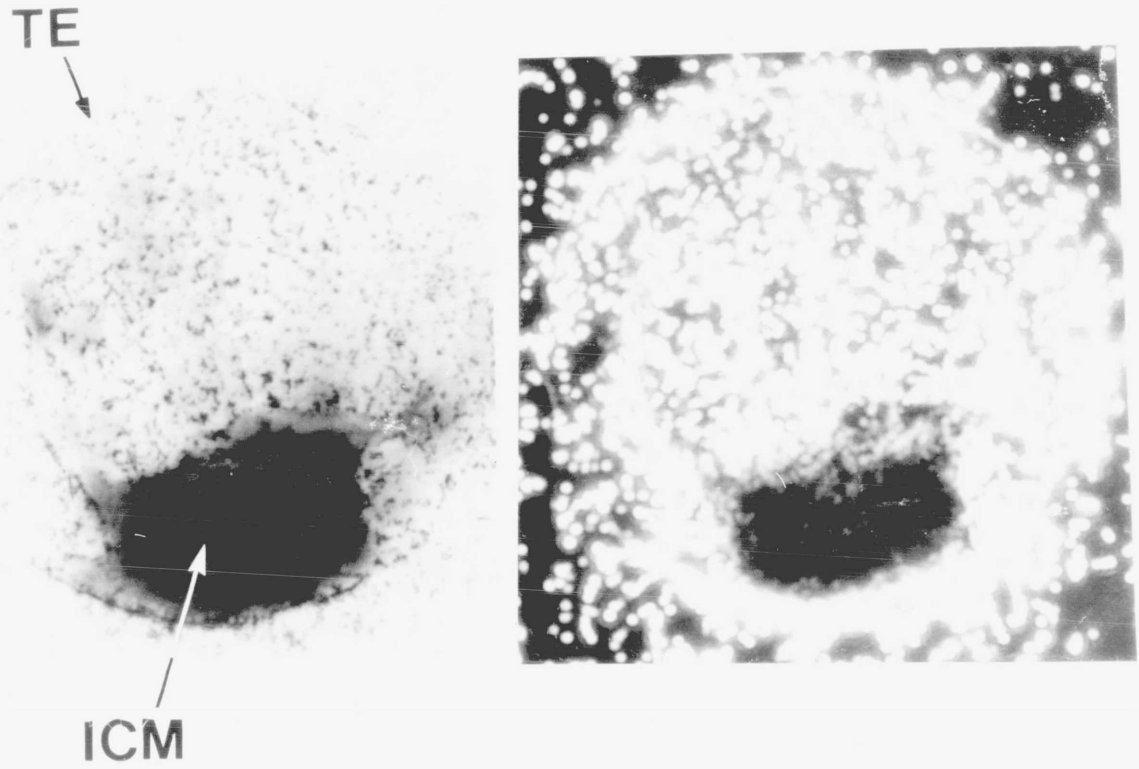

TE
ICM

Figure 4: Polyacrylamide gel electrophoresis of synthetic RNA probes. Riboprobes prepared essentially according to the protocol of Cox *et al.* (1984), were electrophoresed on a 6% polyacrylamide denaturing gel. Antisense riboprobe (+). Sense riboprobe (-).

Lane 1 and Lane 9: Size marker, end-labelled plasmid DNA digestion products . This DNA is boiled to denature it before loading, resulting in the appearance of extra bands due to partially denatured products. However, the characteristic pattern of fully denatured products is easily recognizable.

Lane 2: undigested human β-actin riboprobe
Lane 3: alkaline-digested β-actin riboprobe
Lane 4: undigested hCG antisense riboprobe
Lane 5: alkaline-digested hCG antisense riboprobe
Lane 6: blank
Lane 7: undigested hCG control sense riboprobe
Lane 8: alkaline-digested hCG control sense riboprobe

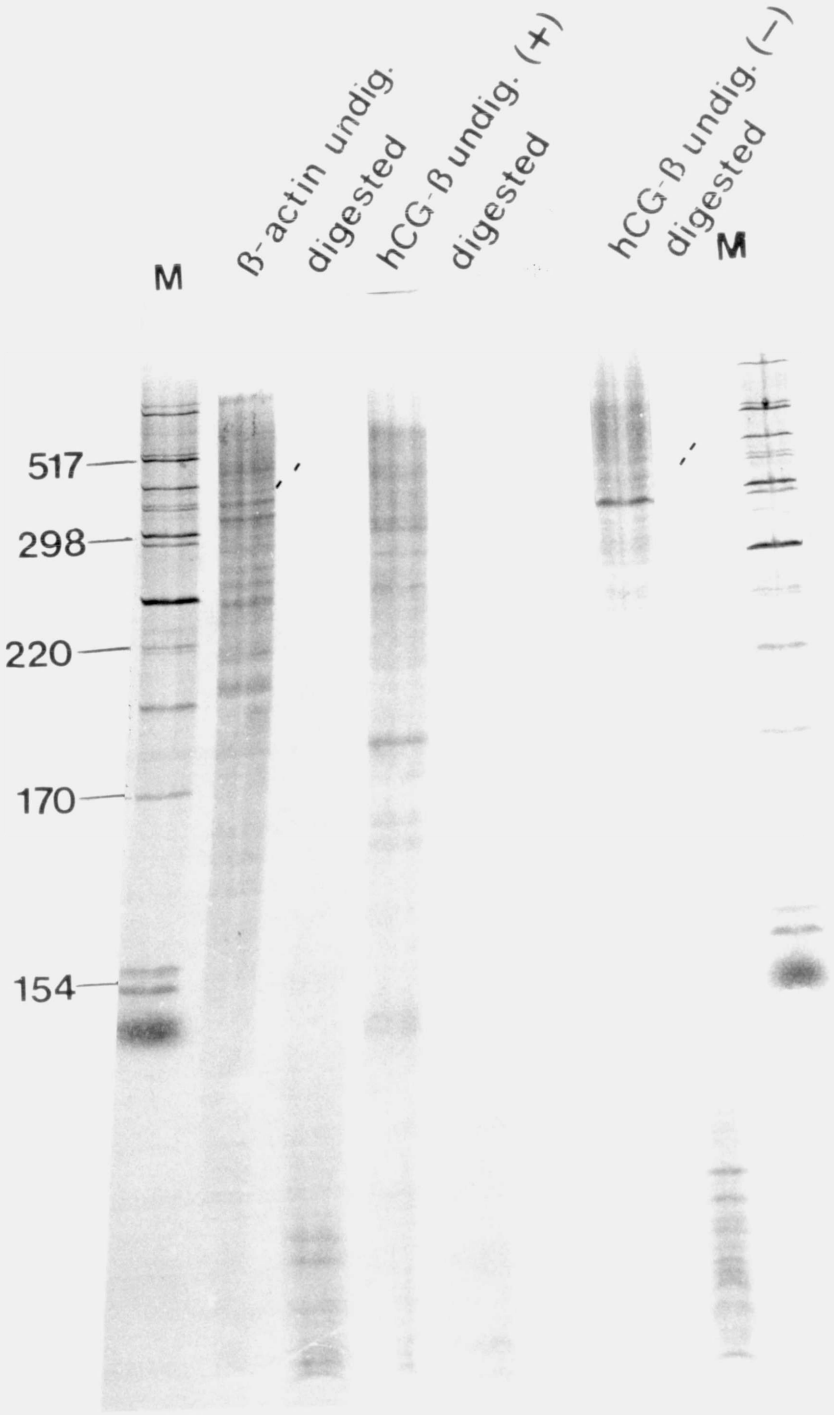

M
β-actin undig.
digested
hCG-β undig. (+)
digested
hCG-β undig. (−)
digested
M
517
298
220
170
154

Figure 5: PCR optimization experiments.

A) Different quantities of human TGF-β1 cDNA were amplified using a standard PCR protocol (1 min at 95°C, 2 mins at 55°C, 3 mins at 70°C. After 30 cycles, a final step of 5 mins at 70°C to ensure that all amplified molecules were double-stranded), in order to establish the sensitivity of the technique.

Lane 1 and Lane 8: 1kb ladder marker; the sizes of the bands are indicated. Lanes 2, 3 and 4: The indicated quantities of TGF-β1 cDNA were amplified. Lane 7: negative control.

B) PCR amplification of TGF-β cDNA using extended number of cycles. A greater quantity of reaction products were loaded than in gel A.

Lane 1 and Lane 8: 1kb ladder marker. Lanes 2, 4 and 6: The indicated quantities of TGF-β1 cDNA were amplified. Lane 7: negative control.

C) PCR amplification using lowered annealing temp and higher no. of cycles (1 min at 94°C, 1 min at 37°C, 3 mins at 72°C. After 99 cycles, a final step of 7 mins at 72°C to ensure that all amplified molecules were double-stranded).

Lane 1 and Lane 8: 1kb ladder marker. Lanes 2 to 6: The indicated quantities of TGF-β1 cDNA were amplified. Lane 7: negative control.

D) Effect of $MgCl_2$ concentration on PCR amplification. The PCR program was adjusted to nearer the previous standard protocol (1 min at 94°C, 4 mins at 45°C, 3 mins at 72°C. After 60 cycles, a final step of 7 mins at 72°C to ensure that all amplified molecules were double-stranded).

Lane 1 and Lane 8: 1kb ladder marker. Lanes 2 to 7: The $MgCl_2$ concentration of the buffers used in each reaction are indicated:

The following quantities of TGF-β1 cDNA were amplified:

Lanes 2 and 3 100fg TGF-β1.
Lanes 4 and 5 10fg TGF-β1.
Lanes 6 and 7 negative control.

E) Effect of extended annealing time. Standard concentration buffer was used with an extended annealing time (1 min at 94°C, 4 mins at 48°C, 2 mins at 72°C. After 60 cycles, a final step of 7 mins at 72°C to ensure that all amplified molecules were double-stranded).

Lane 1 and Lane 8: 1kb ladder marker. Lanes 2 and 3: The indicated quantities of TGF-β1 cDNA were amplified. Lane 7: negative control.

A
1016
517
398
344
298
221
M
1pg
1fg
1ag
-ve
M
B
M
1pg
1fg
1ag
-ve
M
C
M
100fg
10fg
1fg
100ag
10ag
-ve
M
D
M
4mM
0.5mM
4mM
0.5mM
4mM
0.5mM
M
E
M
10pg
1fg
-ve
M

Figure 6: Optimization of reverse transcription-polymerase chain reaction (RT-PCR).

A) RT-PCR reactions were carried out on the following samples:

Lane 2: 10ng TGF-β1 synthetic RNA.
Lane 3: BeWo RNA (approx. 1μg).
Lane 4: HeLa RNA (approx. 1μg).
Lane 5: NTera2 RNA (approx. 1μg).
Lane 6: negative control (no RNA added to RT-PCR reaction).
Lane 7: BeWo RNA sample (approx. 1μg) not processed by RT-PCR.

B) Southern blot of samples processed by RT-PCR probed with oligo-labelled TGF-β1 DNA. The sizes of a non-radioactive 1 kb DNA ladder are indicated.

Lane 1: negative control (no RNA added to RT-PCR reaction).
Lane 2: TGF-β1 synthetic RNA.
Lane 3: HeLa.
Lane 4: BeWo.
Lane 5: NTera2.

C) Further RT-PCR blot probed with oligo-labelled TGF-β1 DNA.

Lane 1: TGF-β1 plasmid DNA digested with *Eco*RI. The 2.1kb TGF-β1 insert is indicated.
Lane 2: TGF-β1 plasmid DNA digested with *Ava*I. This gives a range of size markers, at sizes indicated, which hybridize to oligo-labelled TGF-β1.

Lane 3: TGF-β2 plasmid DNA digested with *AvaI*. This is a negative control, showing that neither TGF-β2 DNA fragments, nor the Bluescribe vector in which they were cloned, hybridize to oligo-labelled TGF-β1 DNA. The following RNA samples were processed by RT-PCR.

Lane 4: TGF-β1 synthetic RNA.

Lane 5: rRNA *E.Coli*.

Lane 6: chorion (approx. 1μg).

Lane 7: BeWo (approx. 1μg).

Lane 8: NTera2 (approx. 1μg).

D and E) PCR carried out on RNA stocks for the purpose of detecting any TGF-β1 plasmid contamination.

Lane D2: TGF-β1 cDNA PCR (positive control).

Lane D3: negative control PCR.

Lane D4: BeWo RNA (approx. 1μg).

Lane D5: NTera2 RNA (approx. 1μg).

Lane D6: chorion RNA (approx. 1μg).

Lane D7: mouse placental RNA (approx. 1μg).

Lane E2: TGF-β1 cDNA PCR (positive control).

Lane E3: HeLa RNA (approx. 1μg).

Lane E4: TGF-β1 cDNA PCR (positive control).

Lane E5: TGF-β1 synthetic RNA RT-PCR.

Lane E6: NTera2 RNA (approx. 1μg).

Lane E7: negative control PCR.

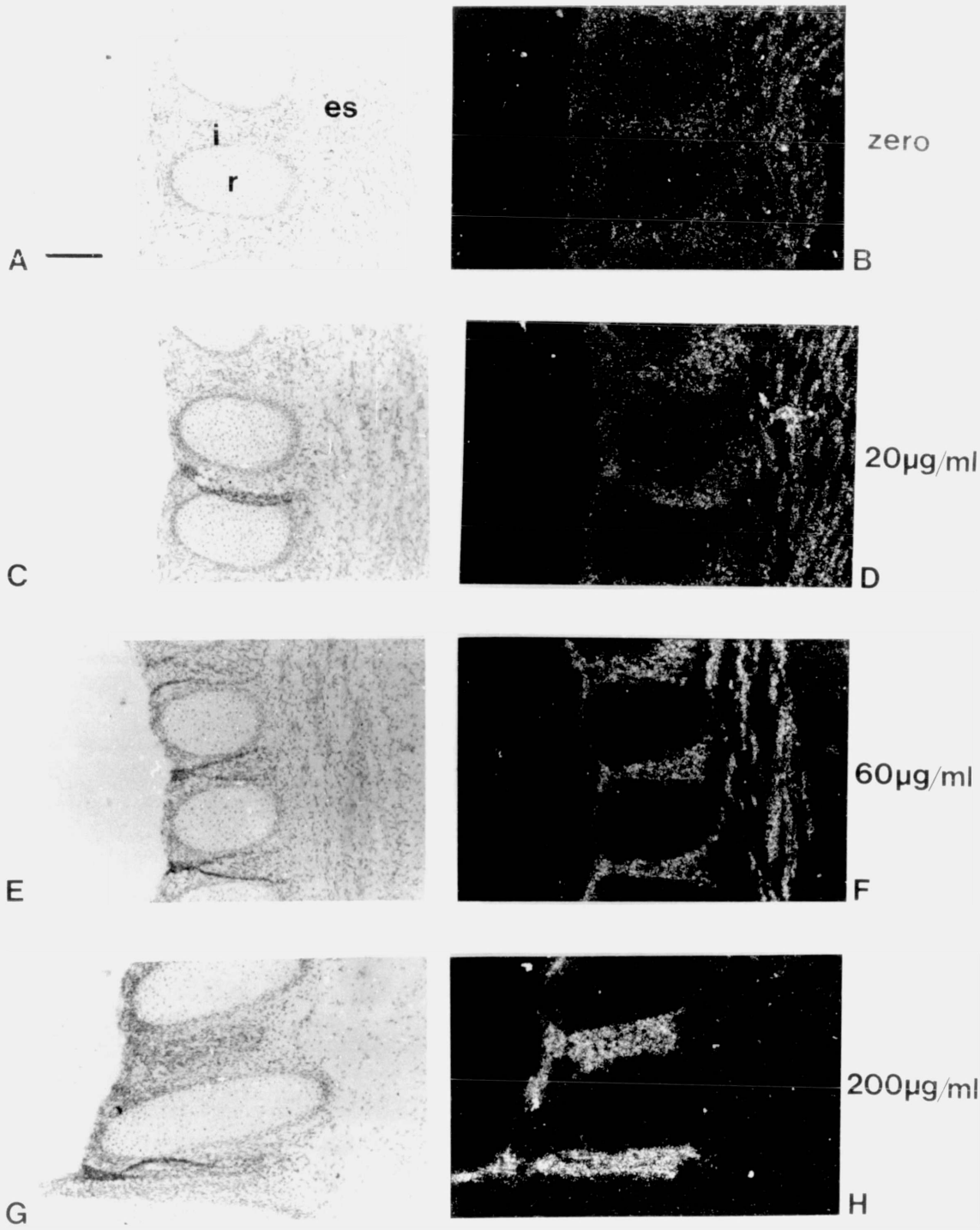
A
M
+ve
BeWo
HeLa
NTera2
-ve
RNA
M
B
1016
517
398
344
298
220
-ve
+ve
HeLa
BeWo
NTera2
C
2138
518
432
300
248
TGF-ß1 EcoRI
TGF-ß1 AvaI
TGF-ß2 AvaI
+ve
-ve
chorion
BeWo
NTera2
D
M
+ve
-ve
BeWo
NTera2
chorion
placenta
M
E
M
+ve
HeLa
+ve
+ve RT-PCR
NTera2
-ve
M

Figure 7: Optimization of *in situ* hybridization. Human cardiac actin riboprobe was hybridized to sagittal sections through the most medial parts of the rib cartilage models (r) of a 57 day *p.c.* human embryo. The cardiac actin probe is a marker for myogenesis in the embryo, and in this experiment hybridized to the developing intercostal (i) and erector spinae (es) muscles. Scale bar represents 200μm.

Proteinase K concentration in the prehybridization treatments were as follows:

A,B) no proteinase K treatment.
C,D) 20μg/ml proteinase K.
E,F) 60μg/ml proteinase K (the standard concentration of Wilkinson *et al.* (1987)).
G,H) 200μg/ml proteinase K.

The intensity of hybridization improves with increasing concentration of proteinase K.


Prot. K
conc.
es
i
r
zero
A
B
20µg/ml
C
D
60µg/ml
E
F
200µg/ml
G
H

Standard prehybridization (with 60µg/ml proteinase K) was also carried out with the addition of the the following extra treatments:

I,J) 2 x SSC at 70°C for 30 mins.

K,L) 0.2M HCl at room temperature for 30 mins.

M.N) Both of the above.

These can be compared to the standard treatment (E,F).

O,P) These samples were processed with a standard prehybridization treatment. However, the hybridization mixture contained only 30% formamide. This can also be compared to (E,F).

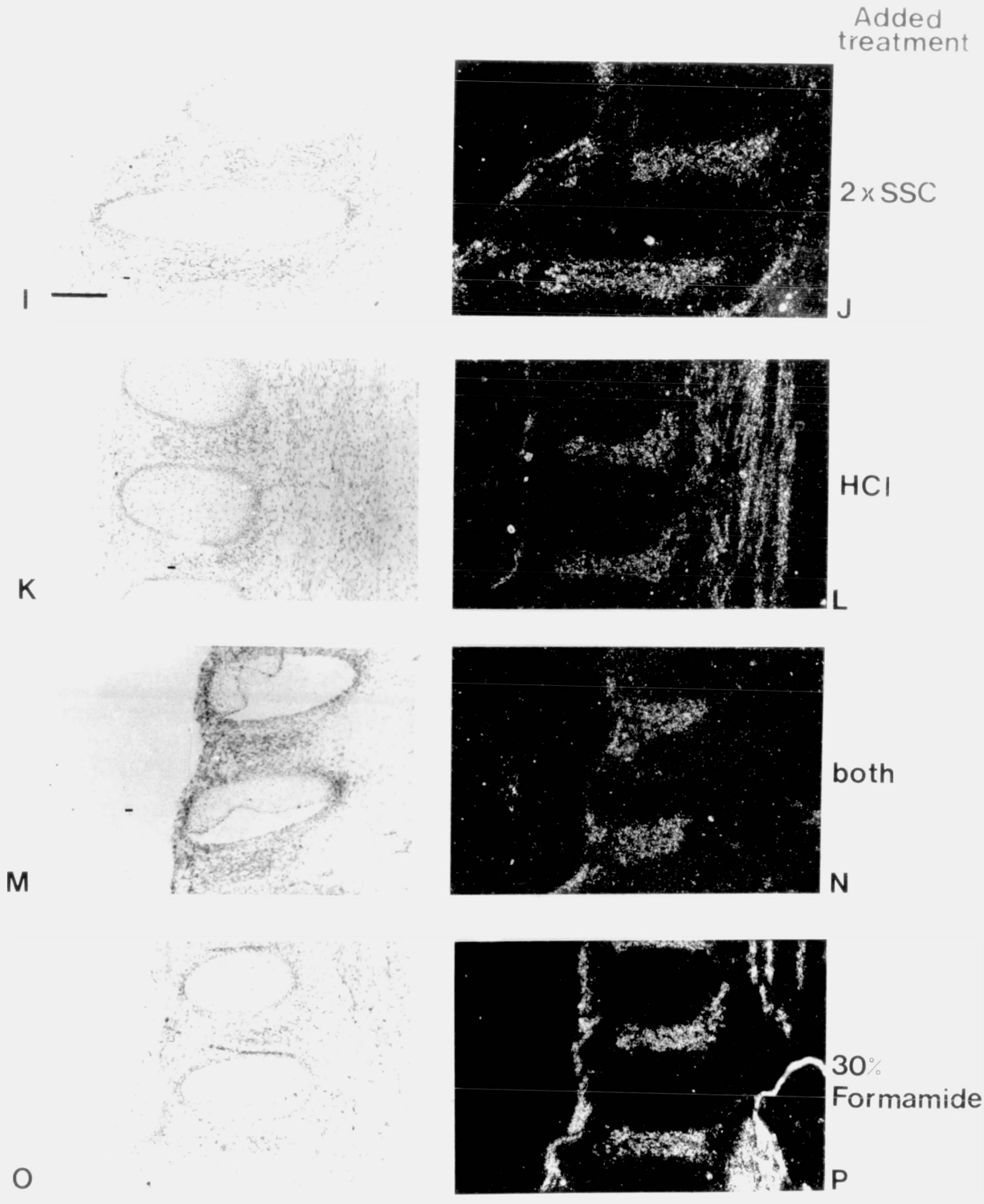

Added treatment
2 x SSC
I
J
HCl
K
L
both
M
N
30% Formamide
O
P

Figure 8: Expression of TGF-β1 RNA in fetal liver detected by *in situ* hybridization to sagittal sections through 57 day *p.c.* liver. Liver (l). Diaphragm (d). Scale bar represents 200μm (A,B,C,D) or 20μm (E,F,G,H).

A,B) TGF-β1-specific riboprobe hybridized to megakaryocytes (arrowed).

C,D) TGF-β2 specific riboprobe did not hybridize to any cells in the liver at 57 days *p.c.*.

E,F,G,H) Higher power magnifications focussed at different levels to show morphology (E,G) and silver grains (F,H). The positive cells were megakaryocytes (white arrow). Liver parenchymal progenitor cells (dark arrow) were negative.

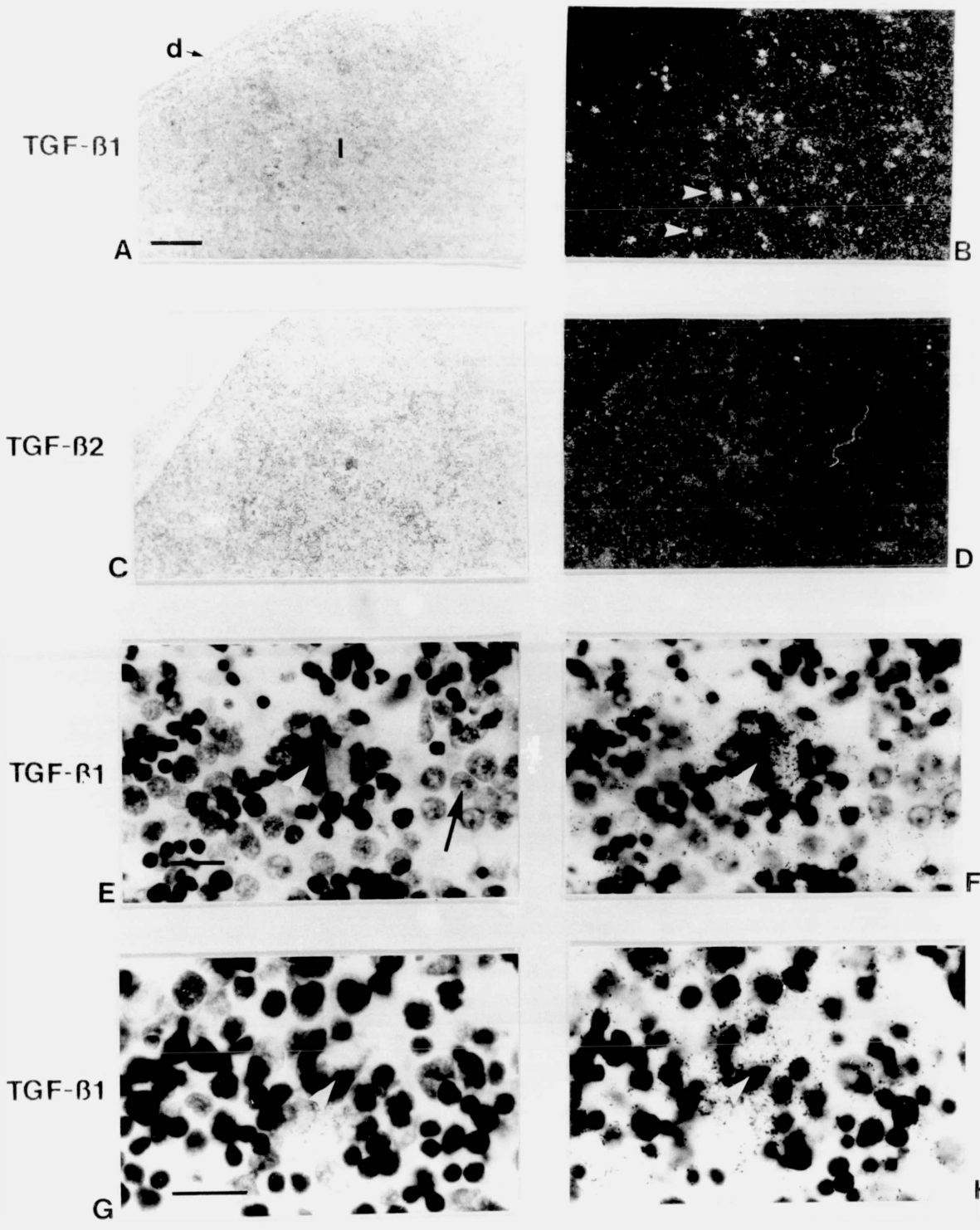

d
TGF-ß1
l
A
B
TGF-ß2
C
D
TGF-ß1
E
F
TGF-ß1
G
H

Figure 9: Expression of TGF-β1 and TGF-β3 RNAs in embryonic endothelia, detected by *in situ* hybridization. Scale bar represents 200μm.

A,B) Transverse section through the mitral valve (v) of a 43 day *p.c.* embryo, hybridized with a negative control riboprobe. c: cut edge of embryo (previously sagittally sectioned).

C,D) Adjacent section to A,B, hybridized with TGF-β1-specific riboprobe. The mitral valve endothelium (v) was positive.

E,F) Sagittal section through the femoral artery of a 57 day *p.c.* embryo, hybridized with a TGF-β1 specific riboprobe. The endothelium (e) of the artery was positive. Note the nucleated pro-erythroblasts within the lumen of the artery.

G,H) Nearly adjacent section to E,F, hybridized with TGF-β3-specific riboprobe. In this case the positive signal was over the tunica intima (ti) of the artery underlying the epithelium.

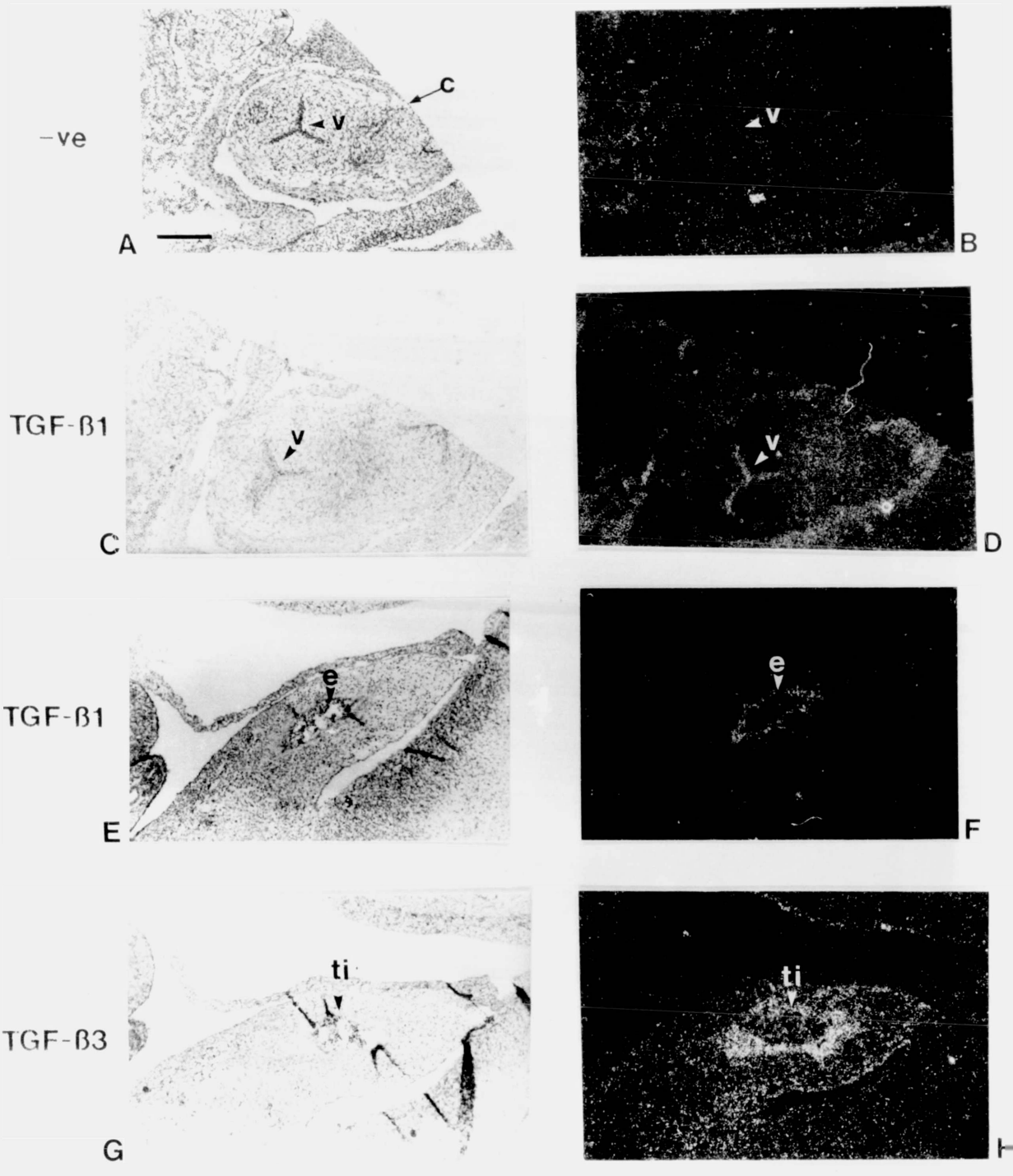

-ve
c
v
A
B
TGF-ß1
v
C
v
D
TGF-ß1
e
E
e
F
TGF-ß3
ti
G
ti

Figure 10: Expression of TGF-β2 and TGF-β3 RNAs in the embryonic respiratory system detected by *in situ* hybridization. Scale bar represents 200μm.

A,B) Transverse/coronal section through the lung bud (lb) of a 43 day *p.c.* embryo, hybridized with a negative control riboprobe. Heart (h), bronchioles (b), mesonephric ridge (m).

C,D) Adjacent section to A,B, hybridized with a TGF-β2-specific riboprobe. The epithelium of the terminal parts only of the bronchioles (b) were positive.

E,F) Transverse section through the lung bud of a 47 day *p.c.* embryo hybridized with a TGF-β3-specific riboprobe. The submucosa of the proximal bronchus (b) was positive, and the epithelium of the more distal bronchi was positive.

G,H) Sagittal section through the lung of a 57 day *p.c.* embryo, hybridized with a TGF-β3-specific riboprobe. The epithelium of the terminal bronchioles (tb) was positive. More proximally, it appeared that only the submucosa of the bronchioles are positive. However, this was an artefact caused by the dark staining of the nuclei. It was concluded that both the submucosa and the overlying epithelium were expressing TGF-β3 at this stage.

I,J) Similar section to G,H, hybridized with TGF-β2-specific riboprobe. The hybridization was confined to the epithelia of the most terminal bronchioles (tb).

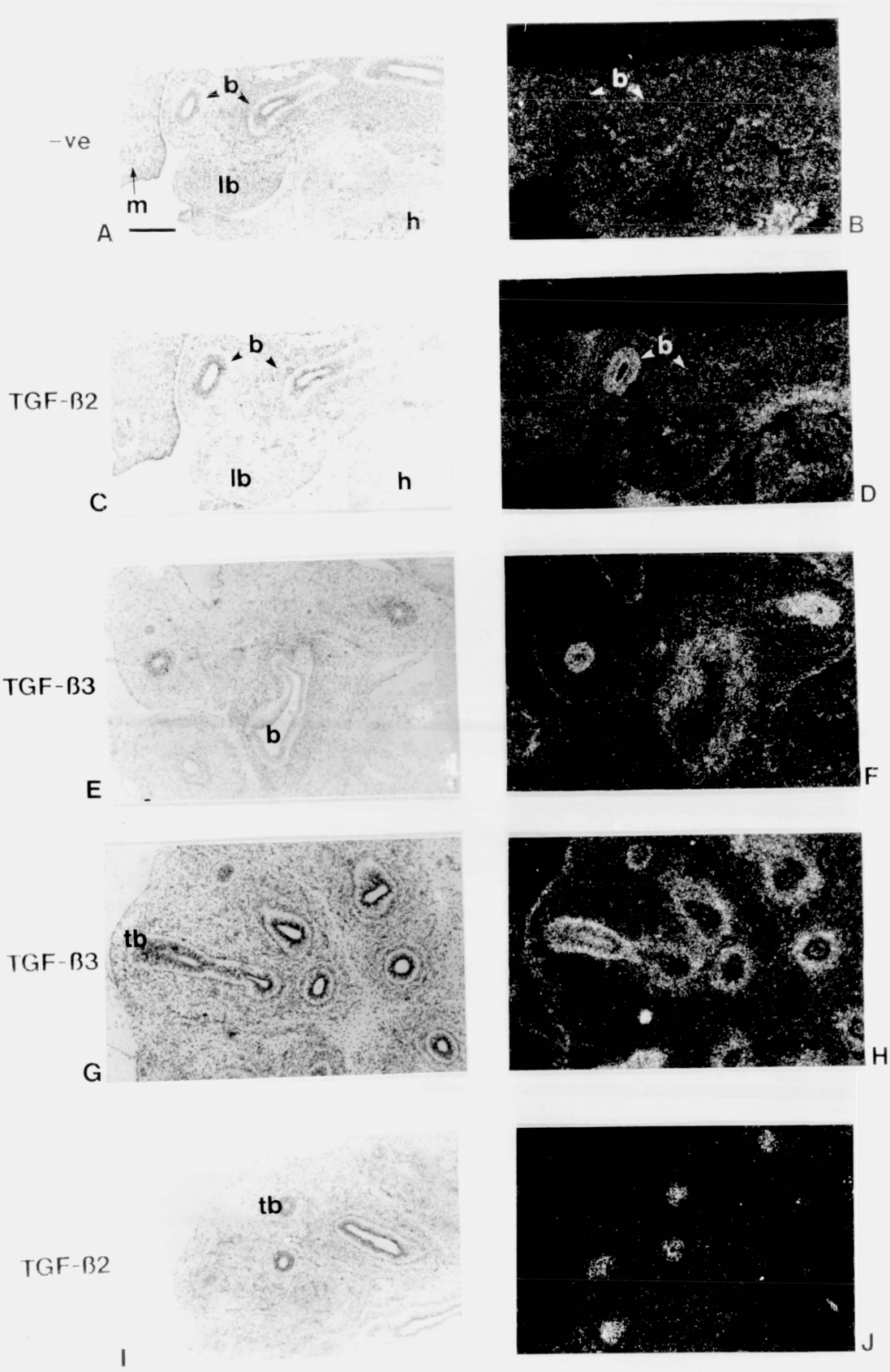
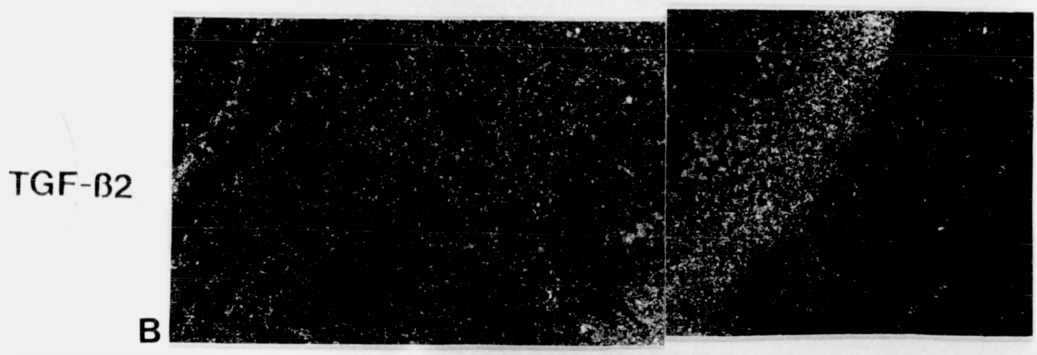

-ve
b
lb
m
h
A
B
TGF-ß2
C
D
TGF-ß3
E
F
tb
G
H
I
J

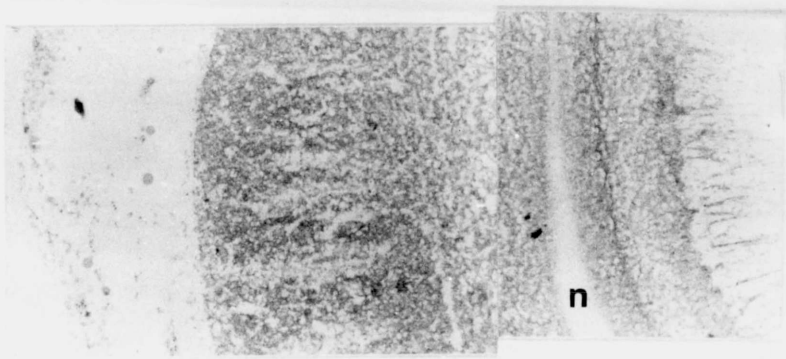
Figure 11: Expression of TGF-β2 RNA in the embryonic spinal cord detected by *in situ* hybridization. Scale bar represents 200μm.

A,B) Sagittal section through the spinal cord of a 57 day *p.c.* embryo, hybridized to TGF-β2-specific riboprobe. Hybridization was detected in the area of neuroepithelium (e) anterior to the neural canal (n).

C,D) Similar section to A,B hybridized with a negative control riboprobe.


e
n
A


TGF-ß2
B


n
C

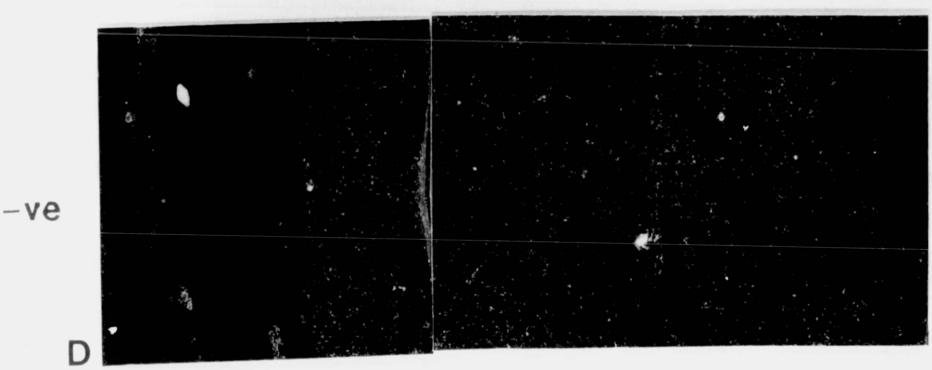

-ve
D

Figure 12: Expression of TGF-β2 RNA in the developing sensory epithelia detected by *in situ* hybridization. Scale bar represents 200μm.

A,B) Sagittal section through the developing eye of a 43 day *p.c.* embryo, hybridized with a negative control riboprobe.

C,D) Similar section to A,B, hybridized to a TGF-β2-specific riboprobe. A positive signal was observed on the lens vesicle (lv), and in the anterior part of the inner layer of the optic cup, which is the presumptive retina (r).

E,F) Sagittal section through the developing eye of a 57 day *p.c.* embryo, hybridized with a TGF-β2-specific riboprobe. Positive signal was observed on the anterior part of the retina (r) and on parts of the lens (l), which is decended from the optic vesicle.

G,H) Coronal section through the otic epithelium of a 47 day *p.c.* embryo, hybridized with a TGF-β2-specific riboprobe. The otic epithelium (o) was positive where the cells were stratified, but negative in the simple epithelial portions. The localized thickening arise in relation to the ingrowth of nerve fibres from the auditory (vestibulocochlear) nerve.

I,J) Sagittal section through the otic epithelium of a 57 day *p.c.* embryo, hybridized with a TGF-β2-specific riboprobe. The signal was weaker over the otic epithelium (o) at this stage than at 47 days *p.c.*. The vestibulo-cochlear nerve (vc), which supplies the areas of thickened epithelium in which TGF-β expression was detected, were found to be negative.

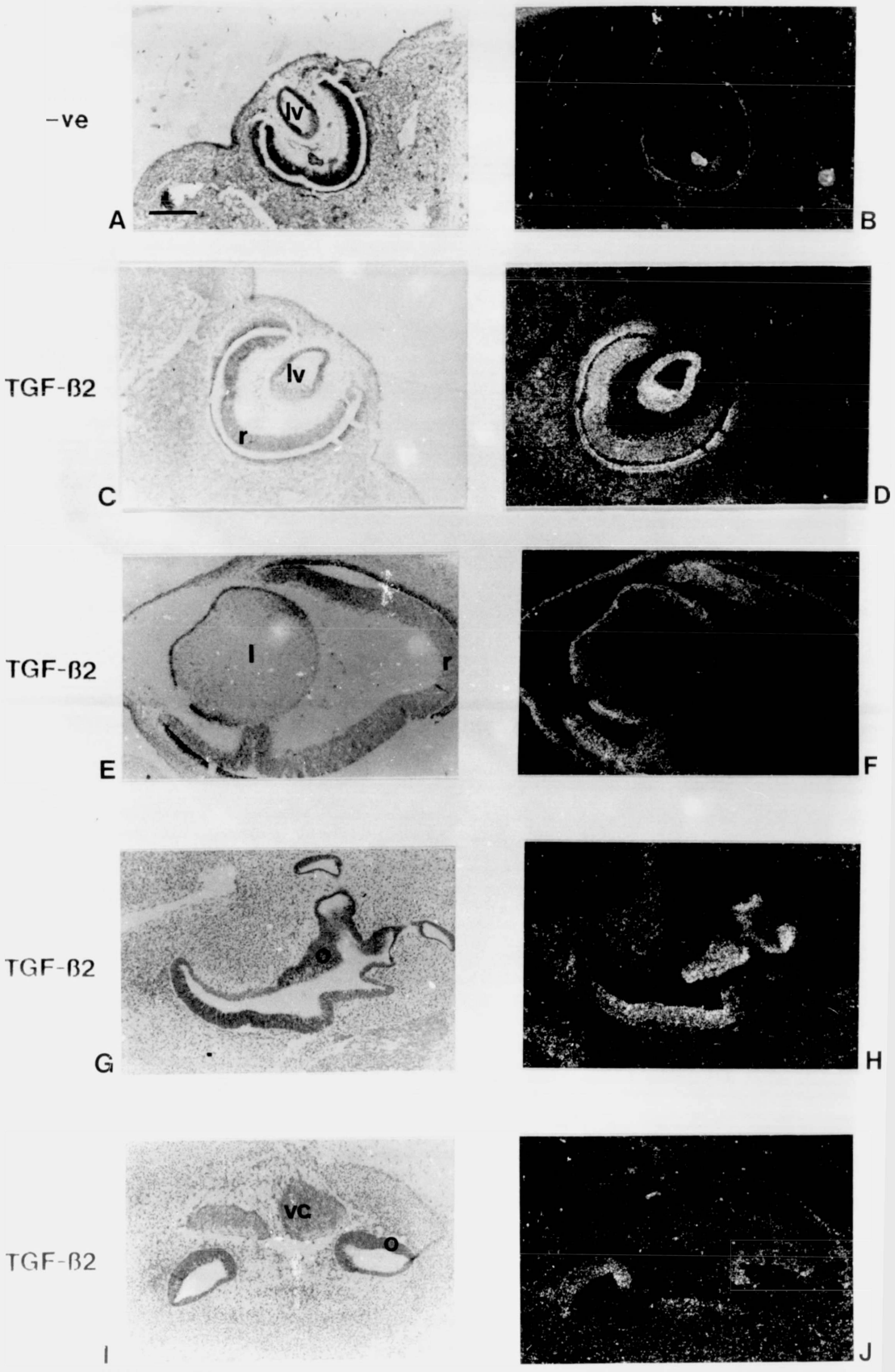

-ve
lv
A
B
TGF-ß2
lv
r
C
D
TGF-ß2
l
r
E
F
TGF-ß2
o
G
H
TGF-ß2
vc
o
I
J

Figure 13: Expression of TGF-β RNAs in limb development detected by *in situ* hybridization. Scale bar represents 200μm.

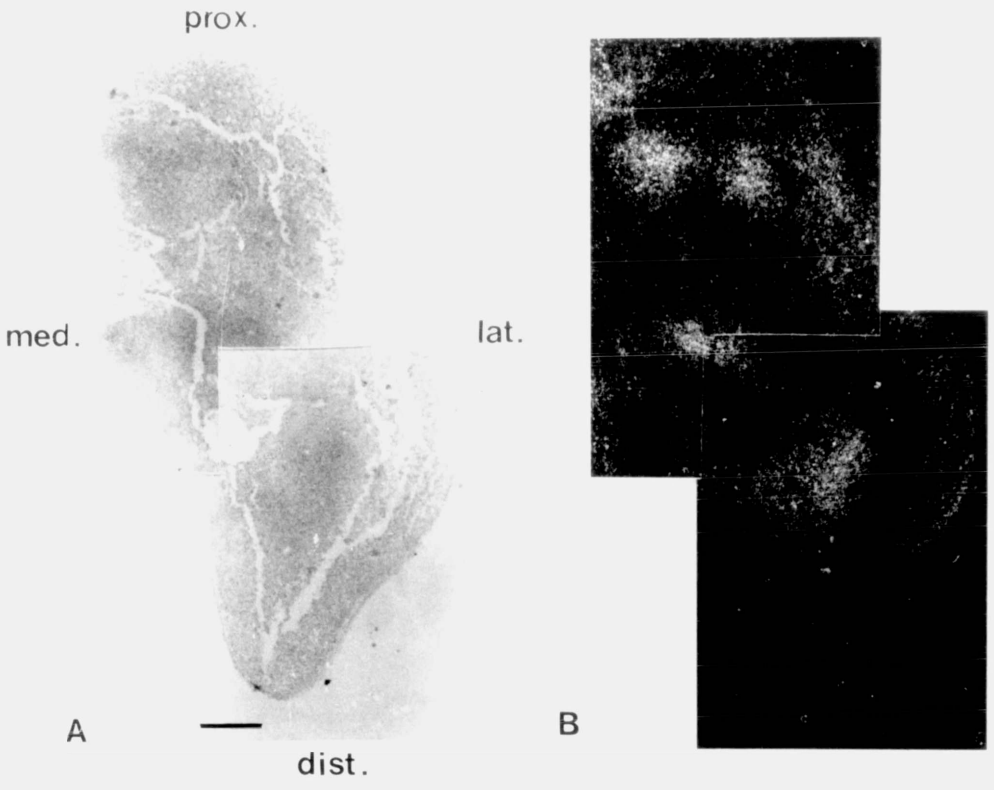
A,B) Composite picture of a section through a lower limb bud from a 43 day *p.c.* embryo, transverse/coronal with respect to the trunk, hybridized with a TGF-β2-specific riboprobe. Discrete areas of hybridization were seen in both the upper and lower parts of the limb bud.

C,D) Composite picture of a section of an upper limb bud from a 43 day *p.c.* embryo, transverse/coronal with respect to the trunk hybridized with a TGF-β2-specific riboprobe. The upper limb bud is less well developed than the lower at this stage.

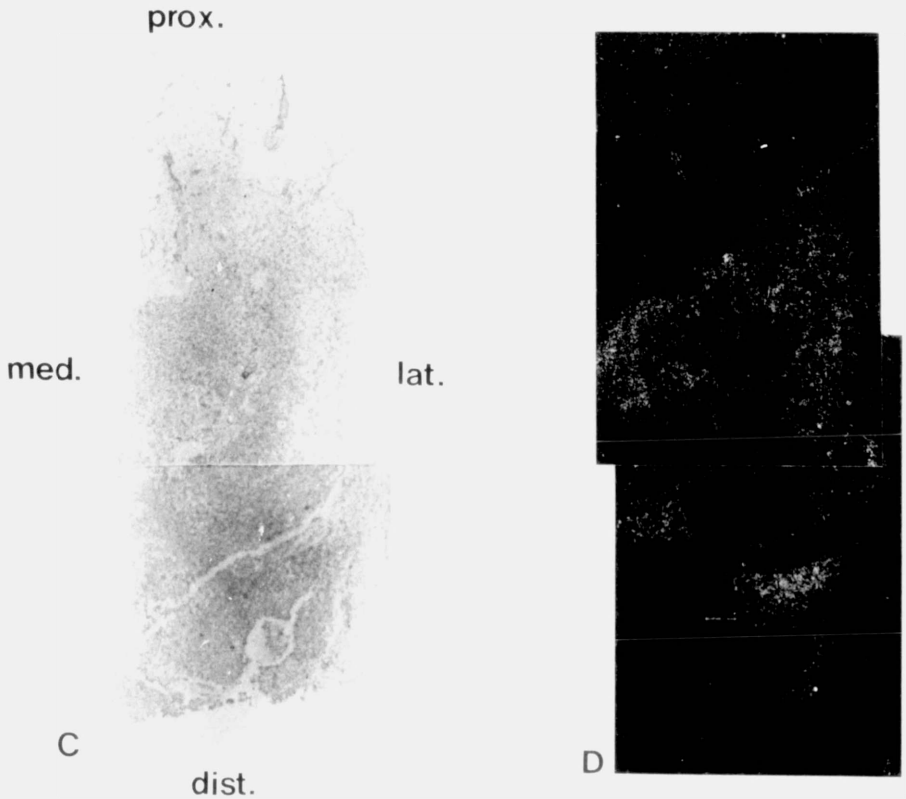
E,F) Section through a region of the lower limb bud of a 43 day *p.c.* embryo, transverse/coronal with respect to the trunk hybridized with a TGF-β2-specific riboprobe. This section is at a more inferior level than that of A,B. Expression was detected in the prechondrogenic scleroblastemae (s), presumably corresponding to the tibia and fibula.

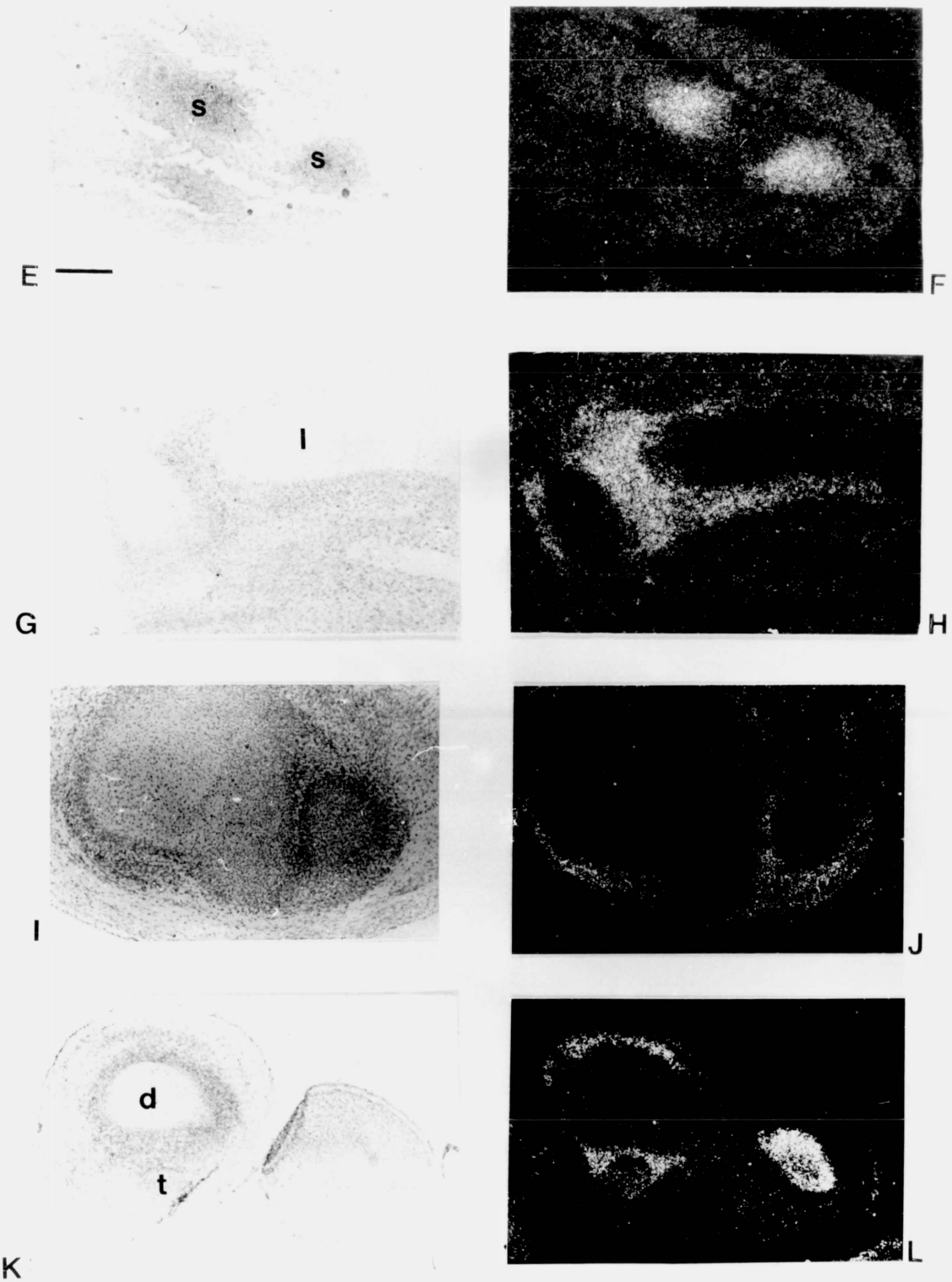
G,H) Longitudinal section through the developing upper limb of a 47 day *p.c.* embryo, hybridized with a TGF-β2-specific riboprobe. By this stage, the cartilage models of the long bones of the arm (l) are visible. TGF-β2 was found to be expressed in the perichondria surrounding the cartilage model and the region of the diaphyseal/epiphyseal junction.

I,J) Sagittal section through the calcaneus cartilage model of a 57 day *p.c.* embryo, hybridized with a TGF-β2-specific riboprobe. Expression is confined to the perichondrium.



BOTH
TGF-ß2





ALL TGF-β2

M,N) Longitudinal section through the long bone of a 12 week *p.c.* fetus, hybridized with a TGF-β1-specific riboprobe. The mature cartilage (c) did not express TGF-β1, which was confined to the zone of ossification (zo).

O,P) Similar section to M,N, hybridized with TGF-β2-specific probe. TGF-β2 was not found to be expressed in the limb at this stage.

Q,R) Similar section to M,N, hybridized with a TGF-β3 full-length riboprobe. Like TGF-β2, TGF-β3 was found to be negative in all tissues of the limb at this stage.

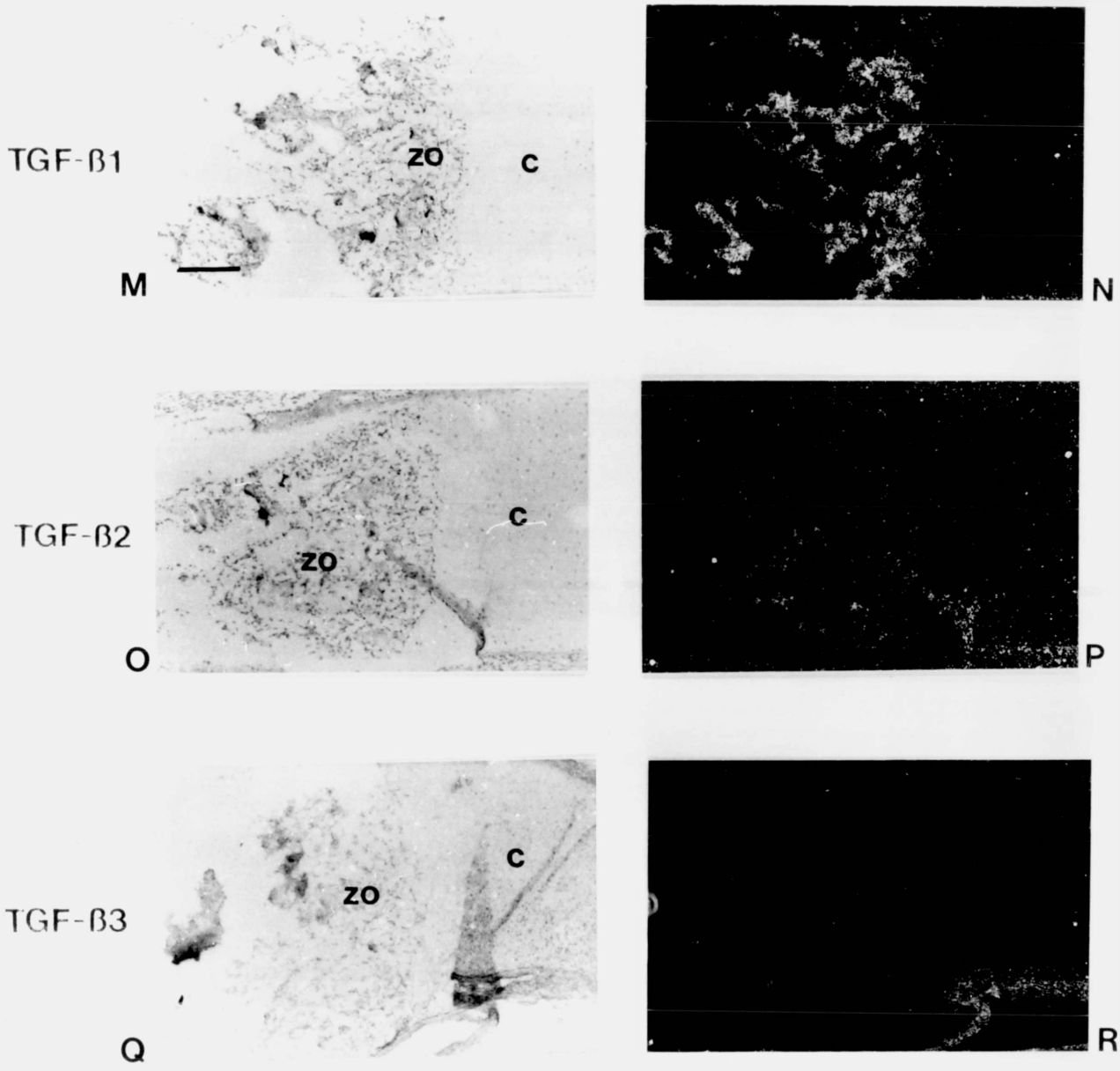

TGF-ß1
ZO
C
M
N
TGF-ß2
C
ZO
O
P
TGF-ß3
C
ZO
Q
R

Figure 14: Expression of TGF-β RNAs in the developing face detected by *in situ* hybridization. Scale bar represents 200μm.

A,B) Sagittal section through the developing jaw of a 57 day *p.c.* embryo, hybridized with a TGF-β1-specific riboprobe. Meckel's cartilage (m) was found to be negative, as was the primitive tooth bud (t). Expression of TGF-β1 was confined to the zone of intramembranous ossification (zo).

C,D) Similar section to A,B, hybridized with a TGF-β2-specific riboprobe. Meckel's cartilage (m) and the zone of ossification (zo) were negative. Expression of TGF-β2 was seen in the mesenchymal portion of the developing jaw surrounding the cartilage and developing bone.

E,F) Adjacent section to C,D, hybridized to a negative control riboprobe, demonstrating that the diffuse hybridization of TGF-β2 seen in the jaw is not a background hybridization effect.

G,H) Similar section to A,B, hybridized with a TGF-β3 full-length riboprobe. The zone of ossification (zo), tooth bud (t), and Meckel's cartilage (m) were negative. TGF-β3 expression was confined to the area of mesenchyme posterior to the above structures, and was generally less diffuse than that of TGF-β2.

I,J) Sagittal section through the maxillary portion of the developing face of a 57 *p.c.* embryo. The mesenchyme of the developing maxilla (mx) had a diffuse expression of TGF-β2 which paralleled that observed in the mandibular mesenchyme. However, the most anterior parts of the developing mandible (md) were seen to be negative.

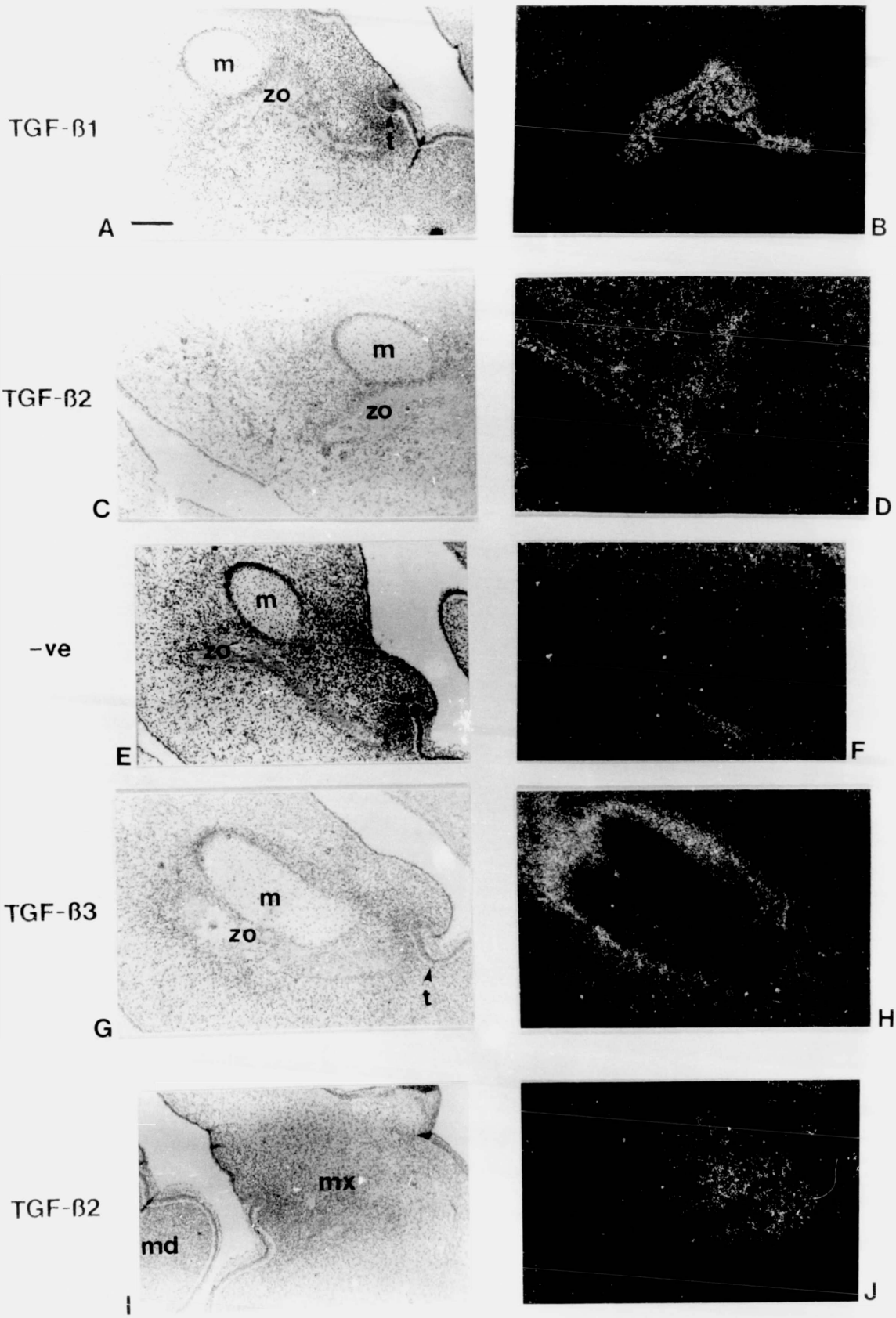

TGF-ß1
m
zo
t
A
B
TGF-ß2
m
zo
C
D
-ve
m
zo
E
F
TGF-ß3
m
zo
t
G
H
TGF-ß2
mx
md
I
J

Figure 15: Expression of TGF-β RNAs in other regions of chondrogenesis as detected by *in situ* hybridization. Scale bar represents 200μm.

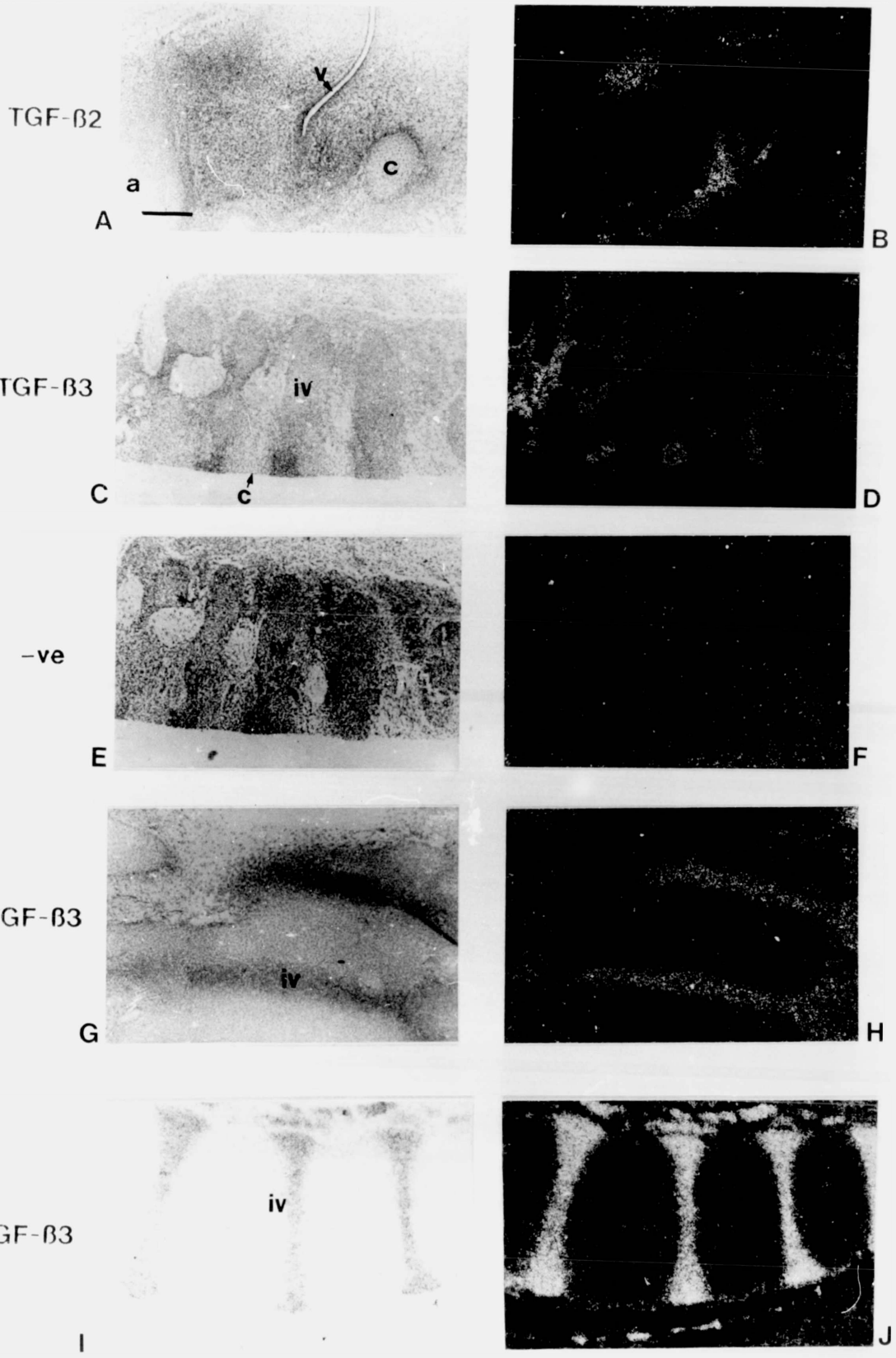
A,B) Sagittal section through the glottal region of a 57 day *p.c.* embryo, hybridized with a TGF-β2-specific riboprobe. TGF-β2 expression was seen in mesenchymal areas superior to the arytenoid cartilage (a), and posterior to the cricoid cartilage (c). Vallecula (v).

C,D) Transverse/coronal section through the vertebral column of a 43 day *p.c.* embryo, hybridized with a TGF-β3-specific riboprobe. The medial part of the intervertebral disc anlagen (iv) was found to be positive. Medial cut edge of the embryo (c).

E,F) Adjacent section to that of C,D, hybridized with a negative control probe.

G,H) Coronal section through the vertebral column of a 47 day *p.c.* embryo, hybridized with a TGF-β3-specific riboprobe. the intervertebral disc anlagen (iv) was found to be expressing TGF-β3.

I,J) Sagittal section through vertebral column of 57 day *p.c.* embryo, hybridized to a TGF-β3-specific riboprobe. Expression of TGF-β3 was found to continue strongly through all stages of formation of the intervertebral disc.


TGF-ß2
v
c
a
A
B
TGF-ß3
iv
C
c
D
-ve
E
F
TGF-ß3
iv
G
H
TGF-ß3
iv
I
J

K,L) Sagittal section through the upper part of the developing trachea of a 57 day *p.c.* embryo, hybridized with a TGF-β3-specific riboprobe. Expression was seen in the developing tracheal cartilages (t).

M,N) Sagittal section through the cartilage models of the ribs of a 57 day *p.c.* embryo, hybridized with a negative control riboprobe.

O,P) Adjacent section to K,L, hybridized with a TGF-β3-specific riboprobe. Expression was seen in the perichondria of the cartilage models of the ribs (r).

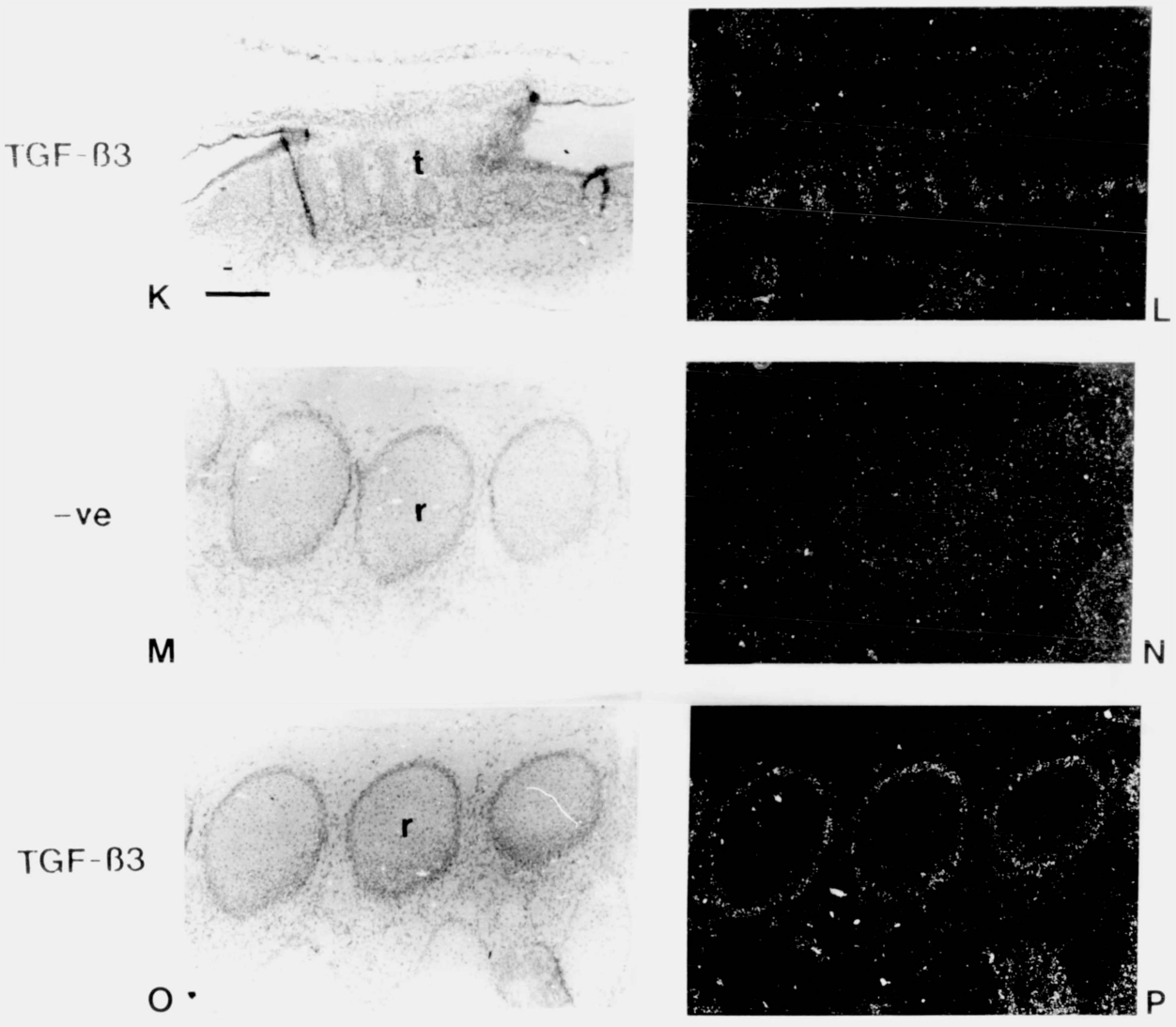

TGF-ß3
t
K
L
-ve
r
M
N
TGF-ß3
r
O
P

Figure 16: TGF-β RNA expression in other mesenchymal and mesothelial tissues as detected by *in situ* hybridization. Scale bar represents 200μm.

A,B) Coronal section through the abdomen of a 32 day *p.c.* embryo, hybridized with a TGF-β2-specific riboprobe. Expression was seen in the mesenchyme surrounding the liver (lv) and foregut (f), and also in part of the mesentery (m) of the midgut. The lower limb bud (lb) was negative at this stage.

C,D) Sagittal section through the skin in the rump region of a 57 day *p.c.* embryo, hybridized with a TGF-β2-specific riboprobe. Positive signal was found over the mesenchymal area (m) below the surface of the developing skin, whereas the thin integumentary layer (i) was negative.

E,F) Sagittal section through the diaphragmatic region of a 57 day *p.c.* embryo, hybridized with a TGF-β3-specific riboprobe. Expression was seen in the diaphragm (d) and the mesothelium of the heart (h). The liver (l) was negative.

G) High power view of the diaphragmatic region shown in E, which demonstrated that TGF-β3 expression is in the outer parietal mesothelial layer (m), and not in the underlying muscular diaphragm. Scale bar represents 200μm.

H) Adjacent section to G, hybridized with cardiac actin riboprobe. The actin was expressed in the muscular layer of the diaphragm (d) and not in the overlying parietal mesothelium (p).

I,J) Sagittal section through the tongue (t) of a 57 day *p.c.* embryo, hybridized with a TGF-β3-specific riboprobe. The central mesenchymal area of the tongue was found to express TGF-β3.

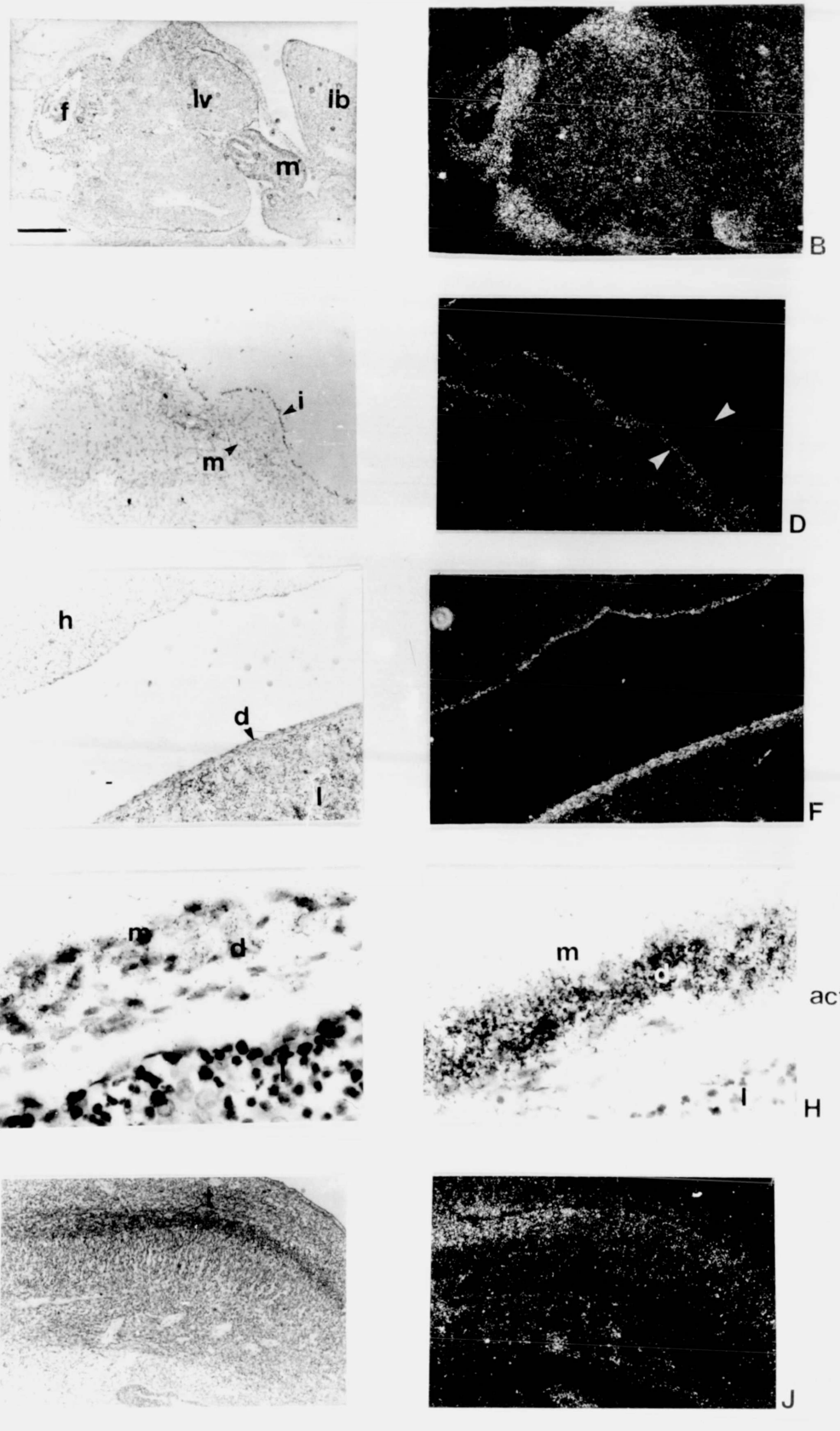

TGF-ß2
f
lv
lb
m
A
B
TGF-ß2
i
m
C
D
h
TGF-ß3
d
l
E
F
m
d
TGF-ß3
G
m
actin
l
H
TGF-ß3
I
J

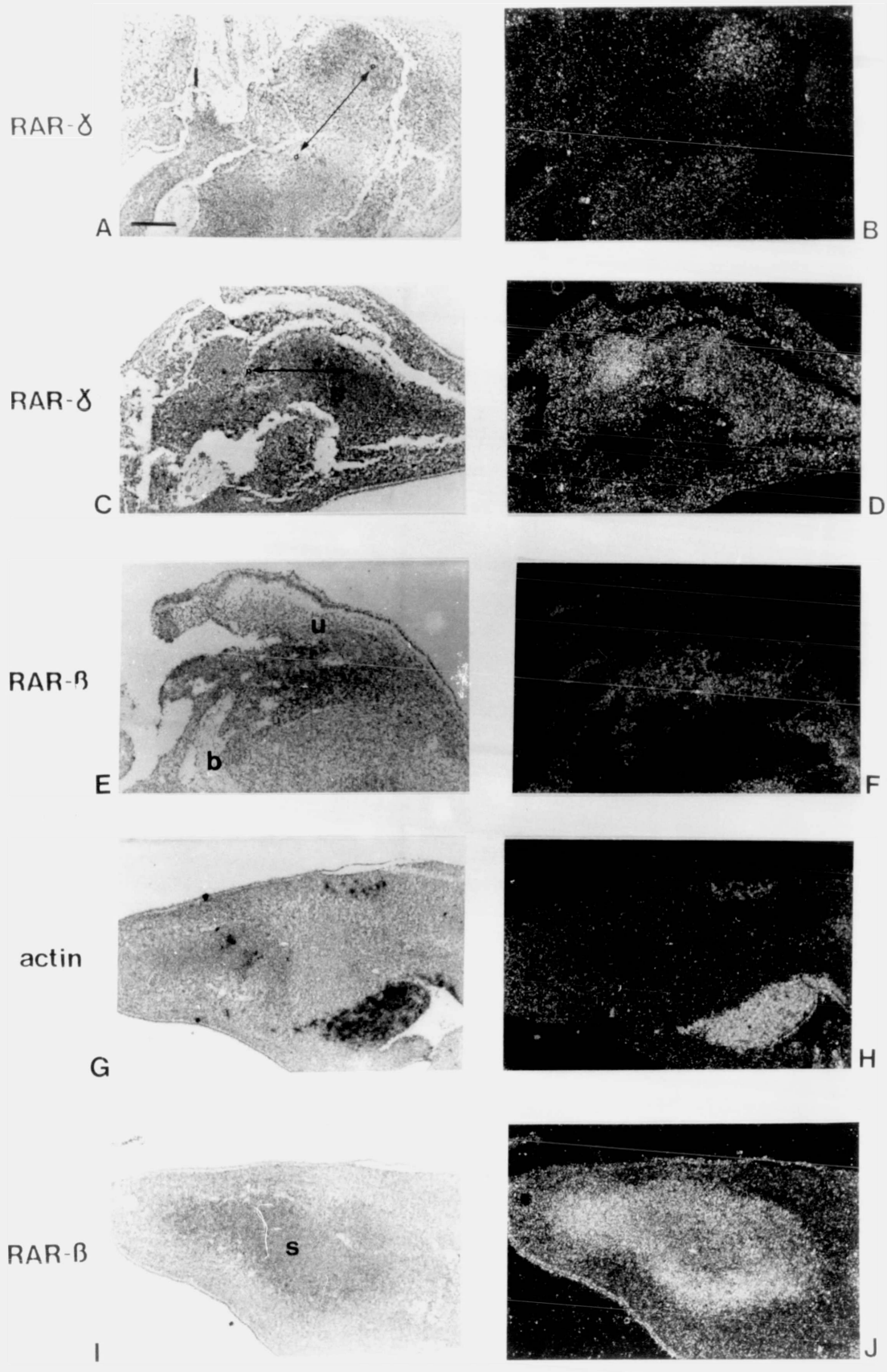
Figure 17: Expression of RAR RNAs in the developing limb as detected by *in situ* hybridization. Scale bar represents 200μm.

A,B) Section through the proximal part of the lower limb bud of a 43 day *p.c.* embryo, transverse/coronal with respect to the trunk, hybridized with a RAR-gamma-specific riboprobe. Expression was seen in the mesenchyme in the proximal part of the limb bud. The medial lumbo-sacral plexus (l) was negative. The arrow p to d orients the proximo-distal axis of the developing limb.

C,D) Section through the more distal part of the lower limb bud of a 43 day *p.c.* embryo, transverse/coronal with respect to the trunk, hybridized with a RAR-gamma-specific riboprobe. The arrow p to d orients the proximo-distal axis of the developing limb. Hybridization was seen in the mesenchyme of the limb bud. However, this region was proximal to the prechondrogenic scleroblastema (s) which had been seen to express TGF-β2 (compare with Fig. 11 A,B).

E,F) Section through the upper limb bud (u) of a 43 day *p.c.* embryo, transverse/coronal with respect to the trunk, hybridized to a RAR-β full-length riboprobe. The proximal regions of the limb bud were found to be positive. The medial brachial plexus (b) was negative.

G,H) Longitudinal section through the lower limb bud of a 43 day *p.c.* embryo, hybridized with a cardiac actin riboprobe. Developing muscle was identified medially.


RAR-δ
l
A
B
RAR-δ
C
D
RAR-β
u
b
E
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actin
G
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RAR-β
s
I
J

I,J) Longitudinal section through the lower limb bud of a 43 day *p.c.* embryo, hybridized with a RAR-β full-length riboprobe. The prechondrogenic scleroblastema (s) was found to be strongly positive.

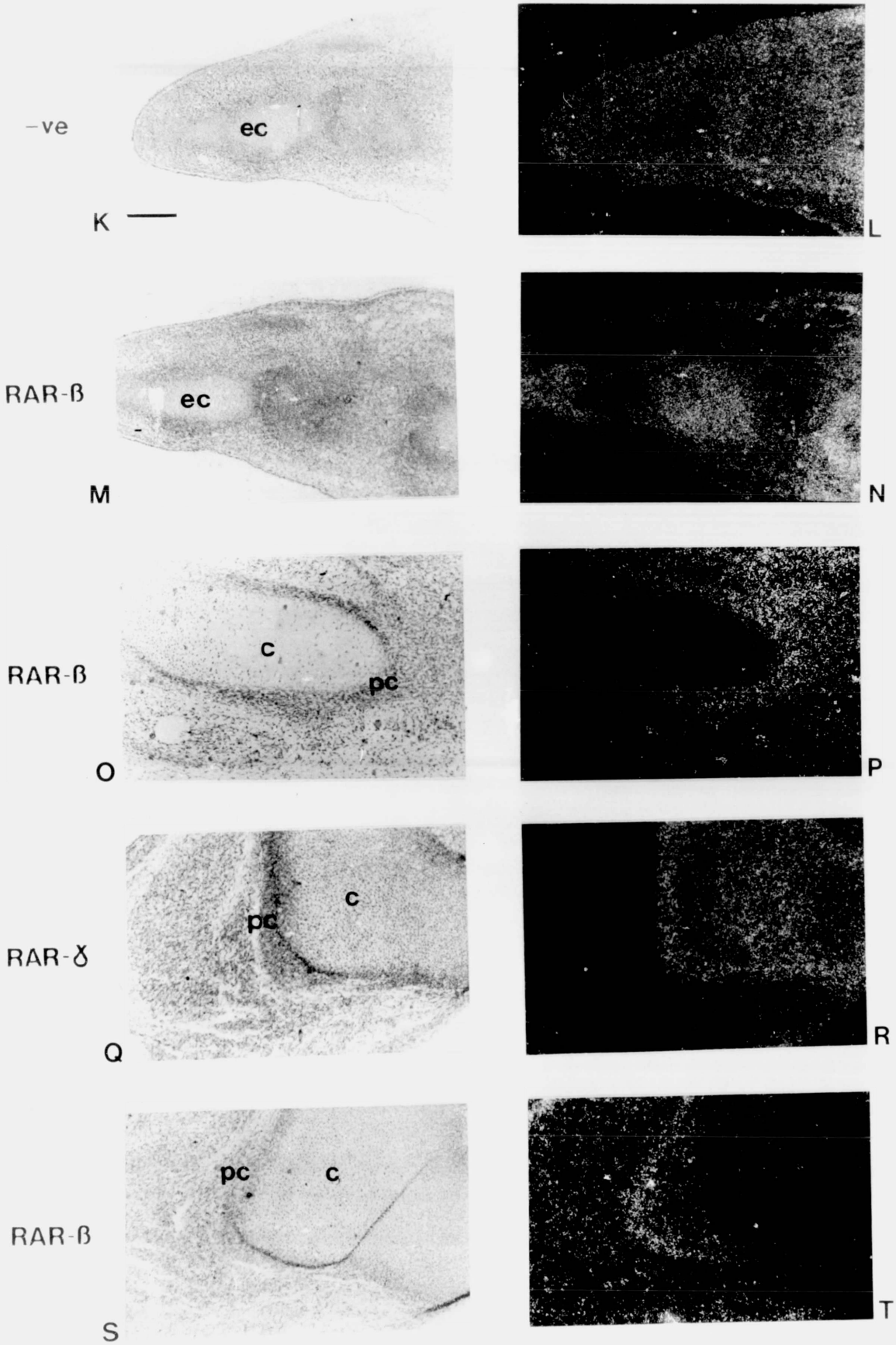
K,L) Longitudinal section through the upper limb bud of a 47 day *p.c.* embryo, hybridized with a negative control riboprobe.

M,N) Longitudinal section through the upper limb bud of a 47 day *p.c.* embryo, hybridized with a RAR-β full-length riboprobe. By this stage early cartilage cells (ec) could be seen in the centre of the prechondrogenic scleroblastema. These were seen to be negative, while the external, less differentiated cells continued to express RAR-β.

O,P) Sagittal section through the lower limb bud of a 47 day *p.c.* embryo, hybridized with a RAR-β-specific riboprobe. The cartilage model (c) is fully developed by this stage and was found not to express RAR-β. Expression was confined to the perichondrium (pc).

Q,R) Sagittal section through the hind limb of a 57 day day *p.c.* hybridized with RAR-gamma full-length riboprobe. Expression was seen in the cartilage model (c) and perichondrium (pc).

S,T) Adjacent section to Q,R, hybridized with a RAR-β full-length riboprobe. Expression was confined to the perichondrium (pc).


-ve
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ec
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N
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pc
O
P
RAR-ß
pc
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Q
R
RAR-ß
pc
c
S
T

Figure 18: Expression of RAR RNAs in the developing spinal column as detected by *in situ* hybridization. Scale bar represents 200μm.

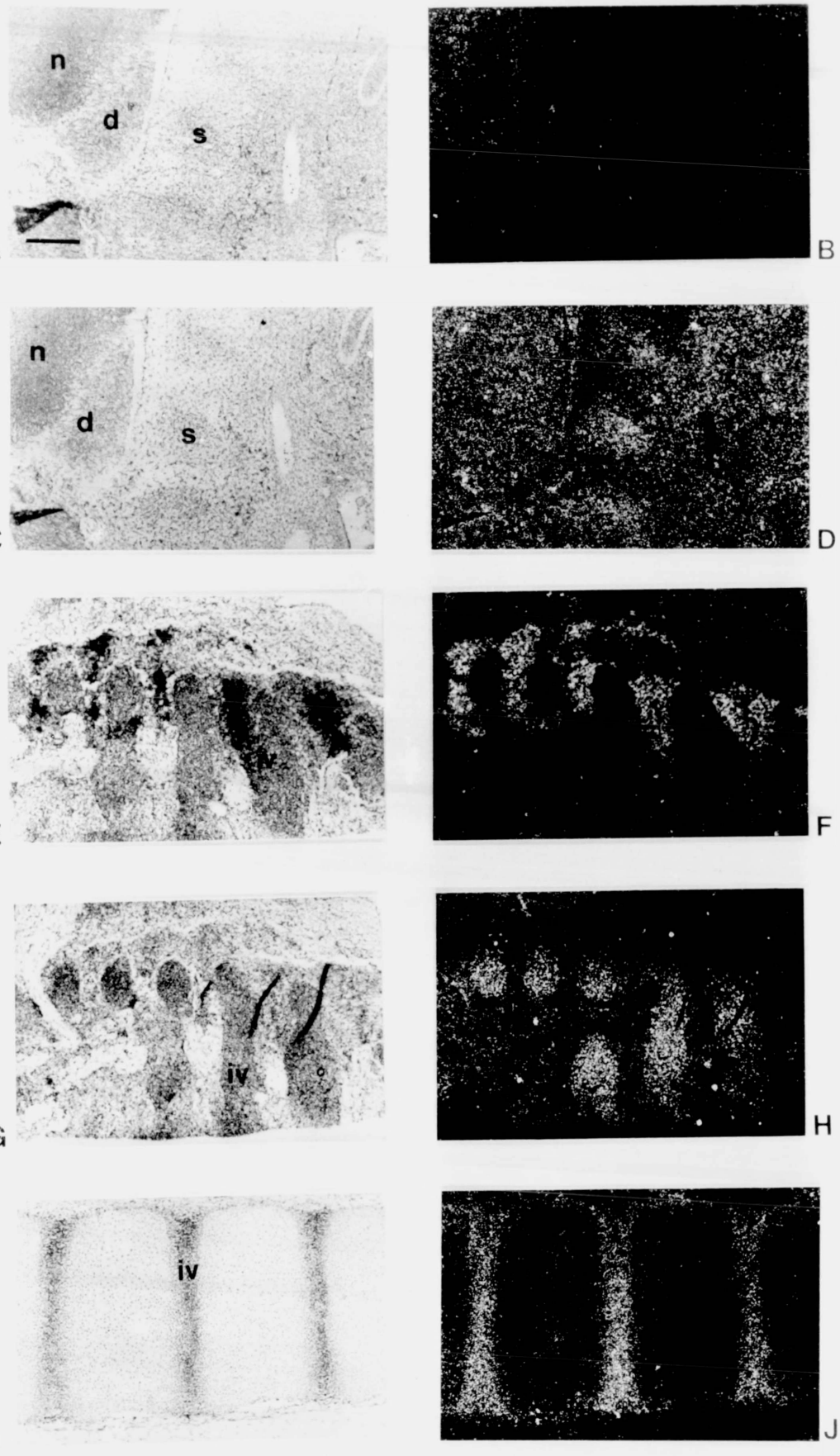
A,B) Sagittal section through the inferior part of a 38 day *p.c.* embryo, hybridized with a negative control riboprobe. Somite (s), neural tube (n), dorsal root ganglion (g).

C,D) Adjacent section to A,B, hybridized with a RAR-gamma-specific riboprobe. The somites (s) were found to be positive. The adjacent neural tube (n) and dorsal root ganglion (d) exhibited background hybridization only (compare with A,B above).

E,F) Transverse/coronal section through the spinal column of a 43 day *p.c.* embryo, hybridized with a cardiac actin probe. Regions of developing muscle were identified, lateral to the intervertebral disc anlagens (iv).

G,H) Similar section to E,F, hybridized with a RAR-gamma-specific riboprobe. Expression was detected in the intervertebral disc anlagen (iv). This expression was seen to be generally just lateral to that of TGF-β3 at the same stage (compare Fig. 13 C,D).

I,J) Sagittal section through the vertebral column of a 57 day *p.c.* embryo, hybridized with a RAR-gamma-specific riboprobe. Expression was seen to continue in the intervertebral disc anlagens (iv).


n
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s
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A
B
n
d
s
RAR-δ
C
D
actin
E
F
iv
RAR-δ
G
H
iv
RAR-δ
I
J

Figure 19: Expression of RAR RNAs in other areas of chondrogenesis, as detected by *in situ* hybridization. Scale bar represents 200μm.

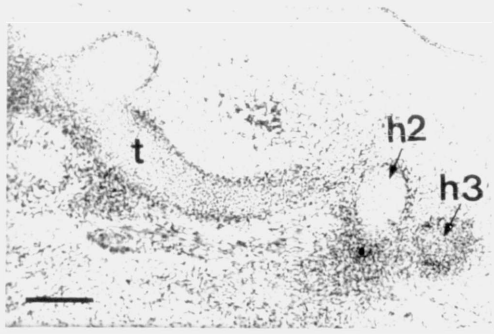
A,B) Sagittal section through the neck of a 57 day *p.c.* human embryo, hybridized with a RAR-gamma-specific riboprobe. Expression was detected in the thyroid cartilage (t), and anteriorly in the portion of the hyoid cartilage (h3) derived from the 3rd pharyngeal arch. The part of the hyoid (h2) derived from the 2nd pharyngeal arch is negative.

C,D) Sagittal section through the ribs of a 57 day *p.c.* embryo, hybridized with a RAR-gamma-specific riboprobe. The cartilage model of the ribs (r) expressed strongly.

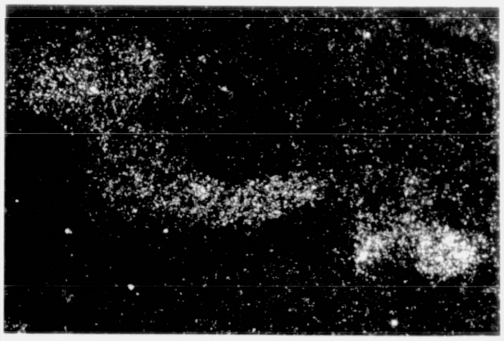
E,F) Transverse section through the pelvic region of a 43 day *p.c.* embryo, hybridized with a RAR-β full-length riboprobe. The bladder (b) and genital tubercle (g) are visible. Hybridization was observed in the area of mesenchyme which becomes the cartilage model of the pelvic girdle. The hybridization to the integument is an "edge effect" artefact.

G,H) Transverse section through the finger cartilage models (f), hybridized with a RAR-gamma-specific riboprobe. The outer, denser, part of the cartilage was seen to be positive. The tendon (t) was negative.

RAR-δ

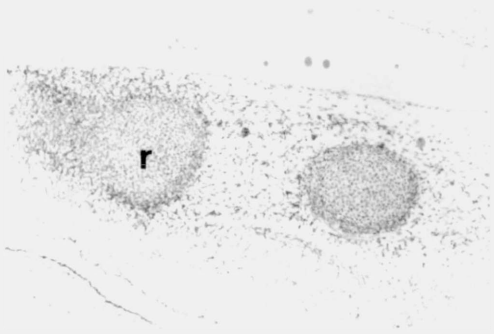


A

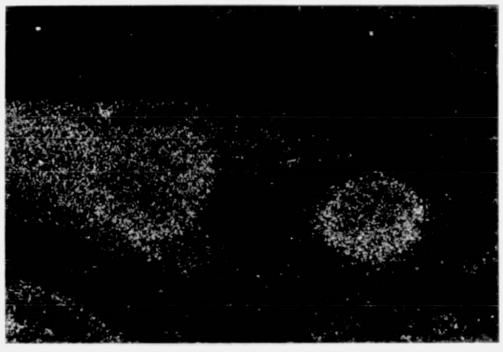


B

RAR-δ

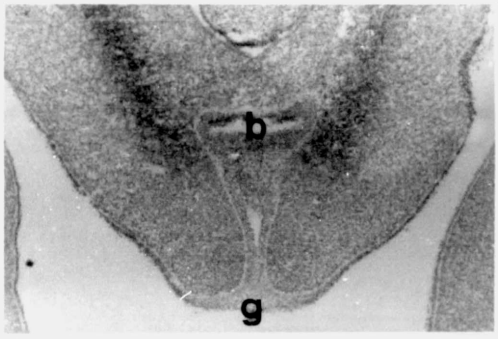


C



D

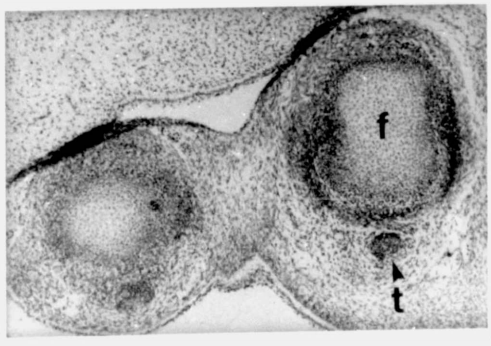
RAR-β



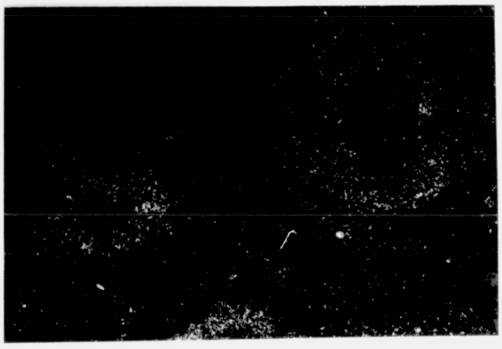
E

F

RAR-δ



G



H

Figure 20: RAR RNA expression in the developing digestive and respiratory systems, as detected by *in situ* hybridization. Scale bar represents 200μm.

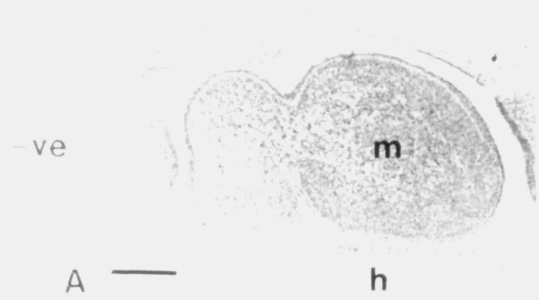
A,B) Sagittal section through the pharyngeal arches of a 38 day *p.c.* embryo, hybridized with a negative control riboprobe.

C,D) Adjacent section to A,B, hybridized with a RAR-gamma-specific riboprobe. The mesenchyme of the mandibular arch (m) and the two more posterior arches hybridized strongly. The epithelium (between arrow heads) was negative, as was the heart (h).

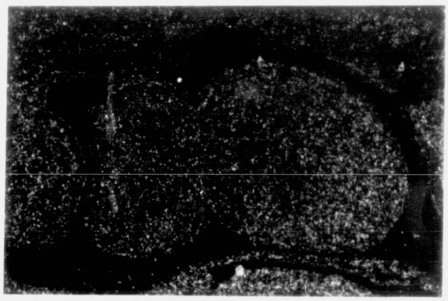
E,F) More lateral sagittal section through the pharyngeal arches of a 38 day *p.c.* embryo, hybridized with a RAR-gamma-specific riboprobe. The field of view is rotated 90° with respect to C,D, so anterior is now at the bottom of the photograph. Slightreflection fromblood cells was observed in the heart (h). The main area of hybridization was observed to be the 3rd pharyngeal arch (3rd).

G,H) Sagittal section through the upper part of the thorax of a 38 day *p.c.* embryo, hybridized with a negative control riboprobe. This is at the point inferior to the glottis where the stomatodeum (s), which will become the mouth, narrows to the presumptive larynx, trachea and oesophagus.

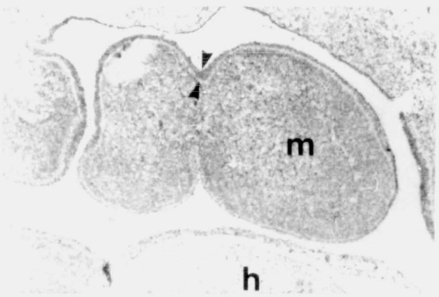
I,J) Adjacent section to G,H, with slightly rotated field of view, hybridized with a RAR-β-specific riboprobe. Expression was seen in the mesenchyme of the upper part of what will become the mediastinum.



A



B



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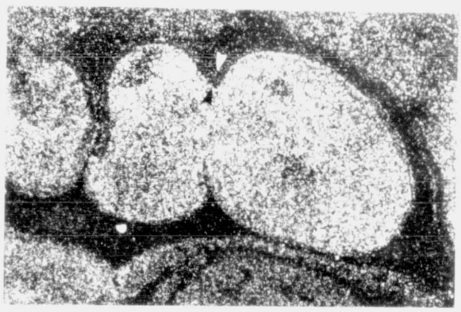

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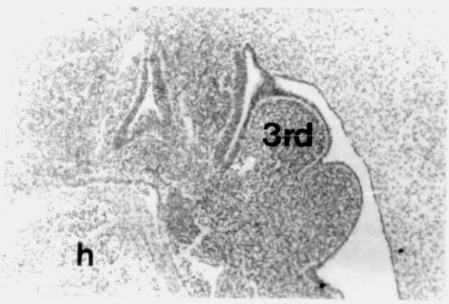

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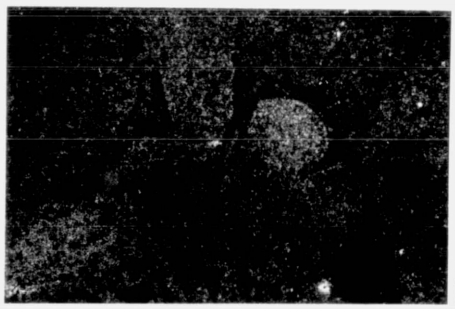
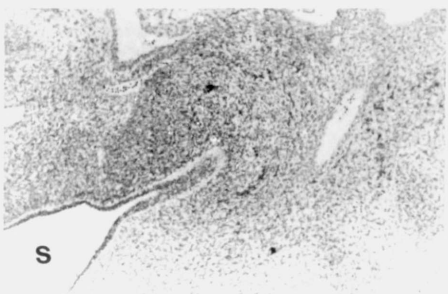
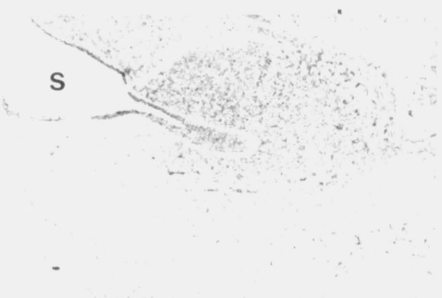


F

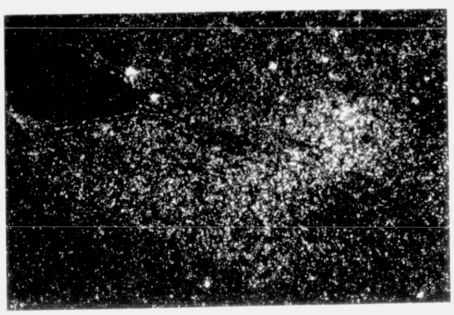


G

H



I



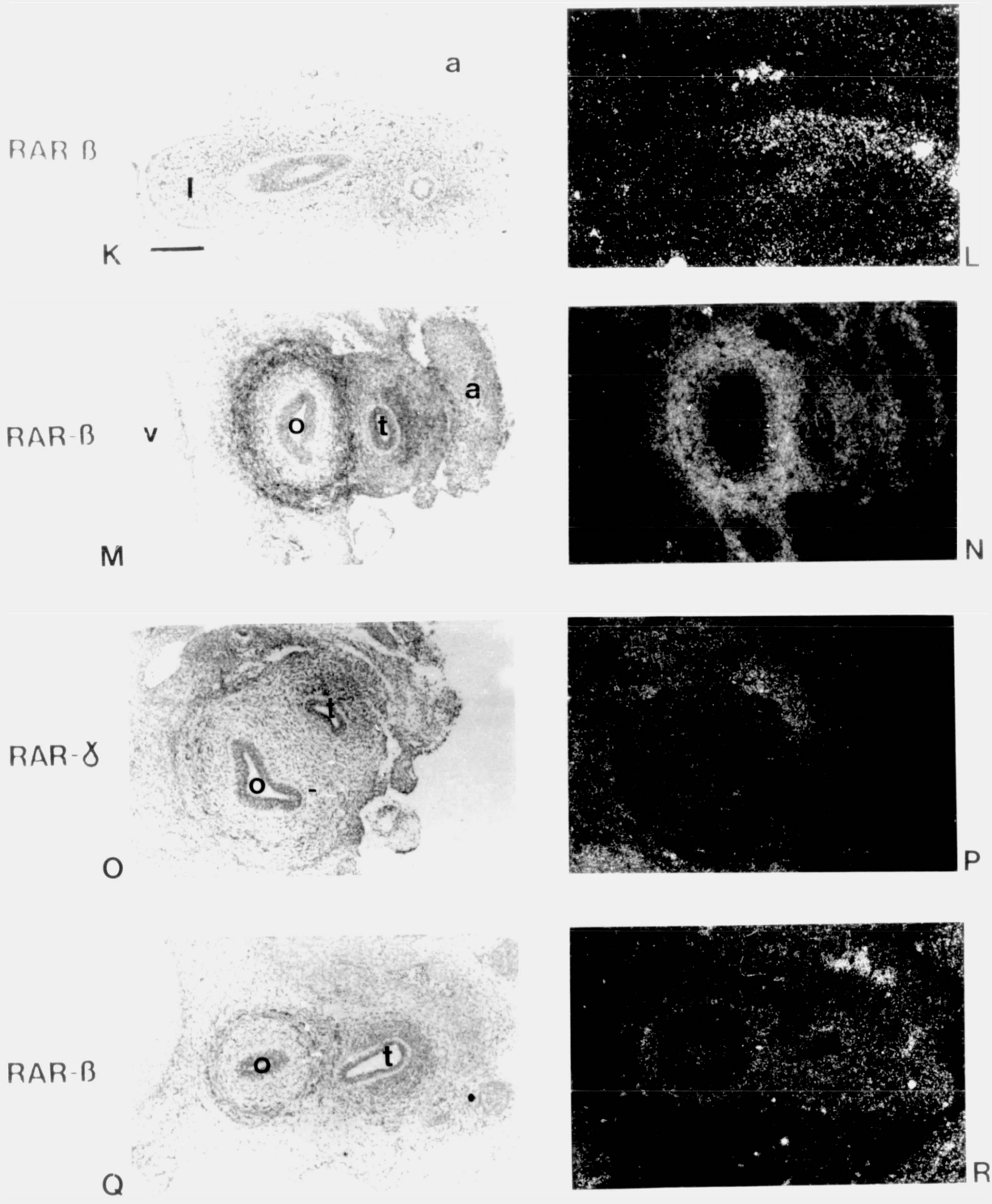
J

K,L) Sagittal section through the lung bud (l) of a 38 day *p.c.* embryo, hybridized with a RAR-β-specific riboprobe. The descending aorta (a) can be observed posteriorly. Expression was seen in the upper part of the stroma of the developing lung bud.

M,N) Transverse section through the oesophagus (o), trachea (t) and ascending aorta (a) of a 43 day *p.c.* embryo, hybridized with a RAR-β full-length riboprobe. Expression was observed in the mesenchyme surrounding the oesophagus. The vertebral body (v) was negative.

O,P) Similar section to M,N, hybridized with a RAR-gamma full-length riboprobe. The scleroblastema of the tracheal cartilage was seen to be positive.

Q,R) Transverse section through the oesophagus (o) and trachea (t) of a 47 day *p.c.* embryo, hybridized with a RAR-β full-length riboprobe. By this stage, the strong signal around the oesophagus in the 43 day *p.c.* embryo was found to have declined considerably.


a
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K
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v
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t
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M
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RAR-δ
t
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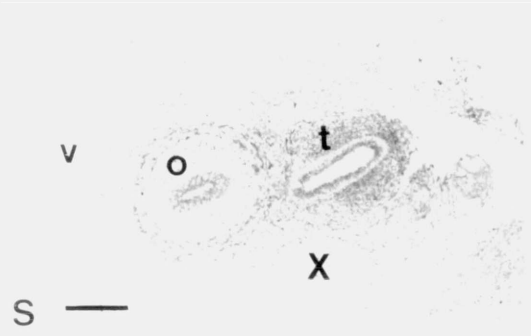
P
RAR-ß
o
t
Q
R

S,T) Transverse section through the oesophagus (o) and trachea (t) of a 47 day *p.c.* embryo, hybridized with a RAR-gamma full-length riboprobe. The tracheal cartilage was found to continue its strong expression. The lateral vagus nerve (X) was negative. Vertebral body (v).

U,V) Transverse section through the oesophagus (o) and bronchi (b) of a 47 day *p.c.* embryo, hybridized with a RAR-gamma full-length riboprobe. The expression of RAR-gamma in the tracheal cartilage was found to continue into the presumptive bronchial cartilage.

W,X) Sagittal section through the trachea of a 57 day *p.c.* embryo, hybridized with a RAR-gamma-specific riboprobe. Both the tracheal (t) and the arytenoid (a) cartilages were observed to be positive.

Y,Z) Sagittal section through the lung of a 57 day *p.c.* embryo, hybridized with a RAR-gamma-specific riboprobe. Reflection can be seen posteriorly in the blood cells of the ascending vena cava (v). RAR-gamma was found to be expressed in the cartilage of the larger bronchi (b).



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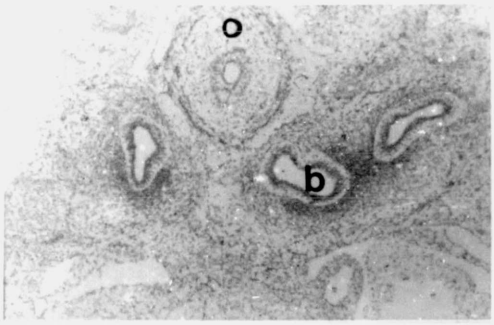

U

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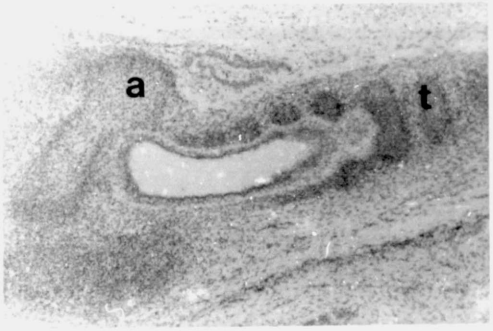
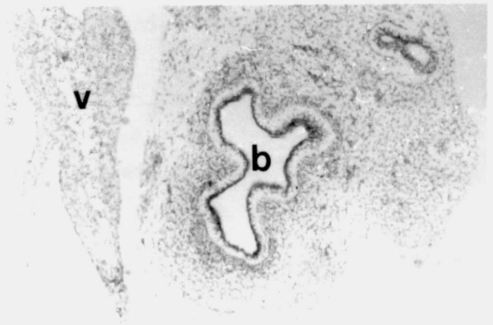


W

X



Y

Z

ALL RAR-δ

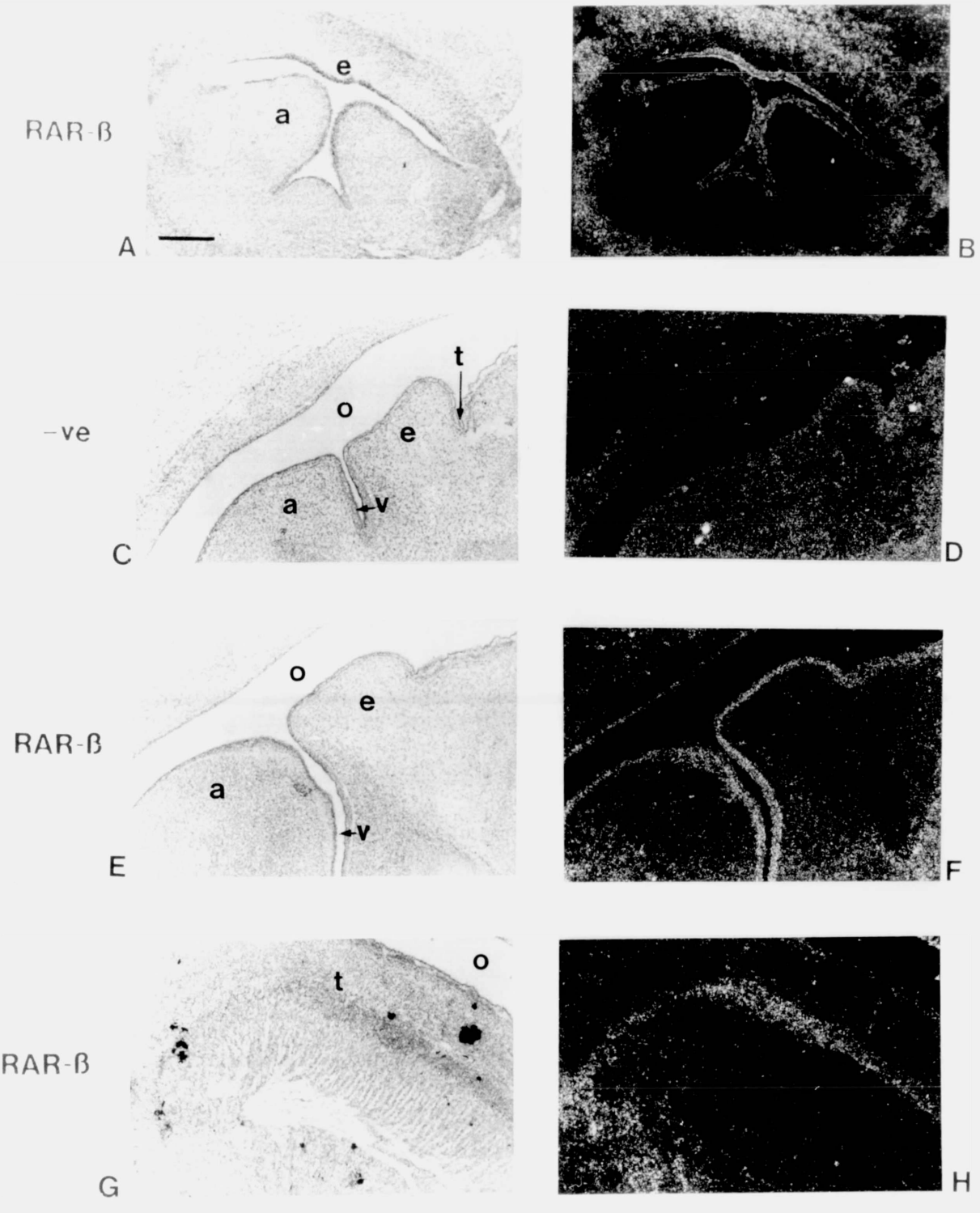
Figure 21: RAR RNA expression in the developing mouth. Scale bar represents 200μm.

A,B) Transverse section through the glottal region of a 43 day *p.c.* embryo, hybridized with a RAR-β-specific riboprobe. Expression was found in the epithelium of the epiglottis (e) and the arytenoids (a), and also in the mesenchyme anterior to the epiglottis.

C,D) Sagittal section through the mouth of a 57 day *p.c.* embryo, hybridized with a negative control riboprobe. Arytenoid (a), epiglottis (e), oral cavity (o), thyroglossal pit (t), vallecula (v).

E,F) Similar section to C,D. hybridized with a RAR-β full-length riboprobe. The epithelium of the oral cavity (o) was found to express RAR-β.

G,H) Sagittal section through the tongue (t) of a 57 day *p.c.* embryo, hybridized with a RAR-β full-length riboprobe. The centra; part of the tongue was found to express RAR-β. This was very similar to the area expressing TGF-β3 (compare Fig. 14 I,J).


RAR-ß
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RAR-ß
t
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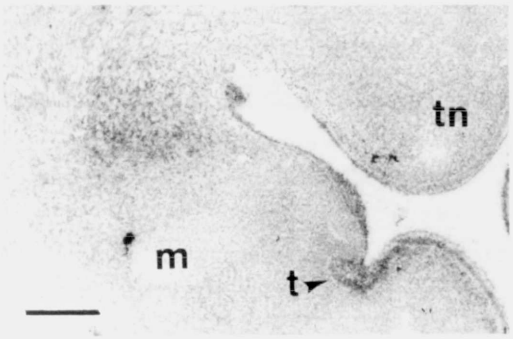
G
H

I,J) Sagittal section through the tongue (t) and jaw of a 57 day *p.c.* embryo, hybridized with a RAR-β full-length riboprobe. Expression was found in the epithelium of the tongue, and the tooth bud (t), and also in the mesenchymal area superior to Meckel's cartilage (m).

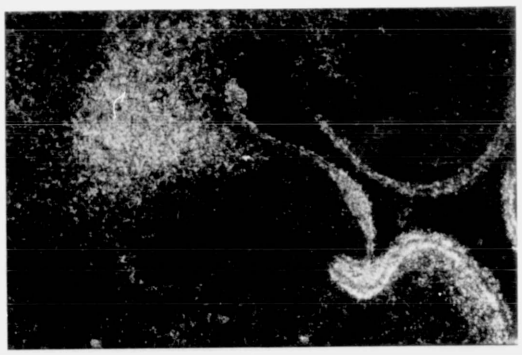
K,L) Sagittal section through the jaw of a 57 day *p.c.* embryo, hybridized with a RAR-gamma-specific riboprobe. The region superior and anterior to Meckel's cartilage (m) was found to be positive. The zone of ossification (zo) was negative. This has similarity to the region of the developing jaw found to express TGF-3 (compare Fig. 12 G,H).

M,N) Sagittal section through the maxilla (m) of a 57 day *p.c.* embryo, hybridized with a RAR-gamma-specific riboprobe. Expression was found in the mesenchyme around the nasal epithelium (n), above the oral cavity (o).

RAR-ß



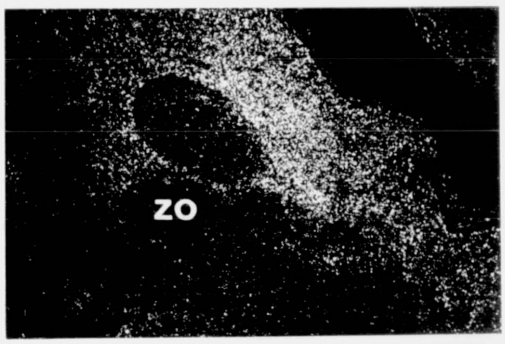
I



J

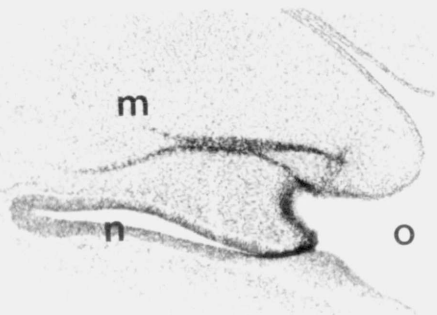
RAR-δ

K



L

RAR-δ



M

N

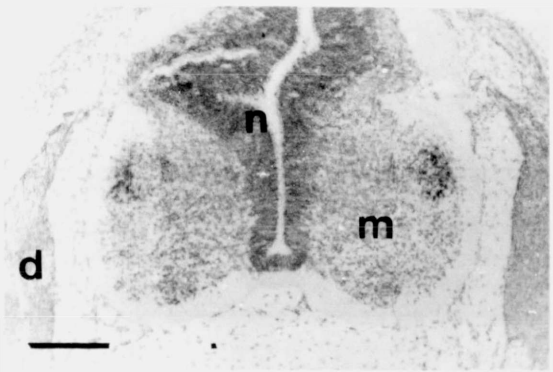
Figure 22: RAR-β RNA expression in the central nervous system. Scale bar represents 200μm (A,B,C,E,F) or 20μm (D).

A,B) Transverse section through the spinal cord of a 43 day *p.c.* embryo at approximately the lower cervical or upper thoracic level, hybridized with a RAR-β full-length riboprobe. Signal was detected in the basal plate of the mantle layer (m). The neuroepithelium (n) and the dorsal root ganglion (d) were found to be negative.

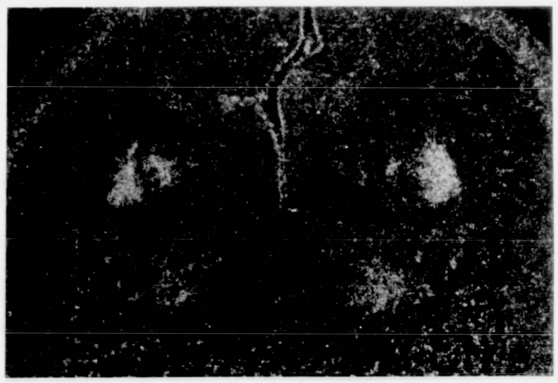
C) Adjacent section to A,B, without hybridization of riboprobe. The inset shows the field of view of D.

D) High power view of the inset of C. This demonstrates that the area found to express RAR-β consists of apolar neuroblasts, and that these expressing neuroblasts are not histologically distinct from the other neuroblasts of the mantle.

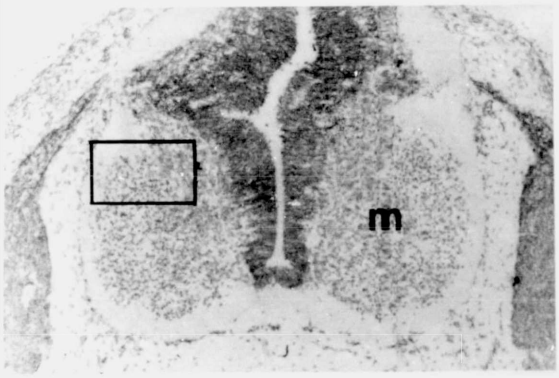
E,F) Sagittal section of the spinal cord (s) of a 57 day *p.c.* embryo, hybridized with a RAR-β full-length riboprobe. Anterior is at the bottom of the picture. Expression was detected in a column of cells running from the floor of the hind-brain to the sacral region. It was assumed that this was equivalent to a longitudinal section of the cells expressing RAR-β shown in A,B,C,D.



A



B



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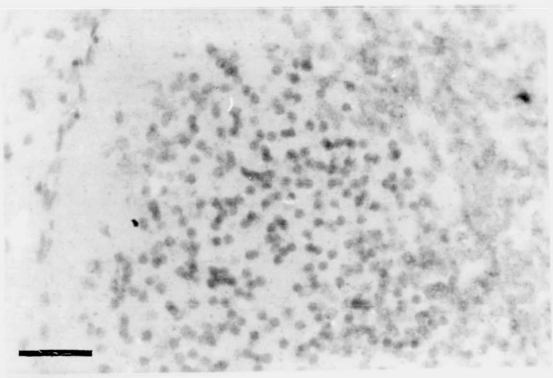
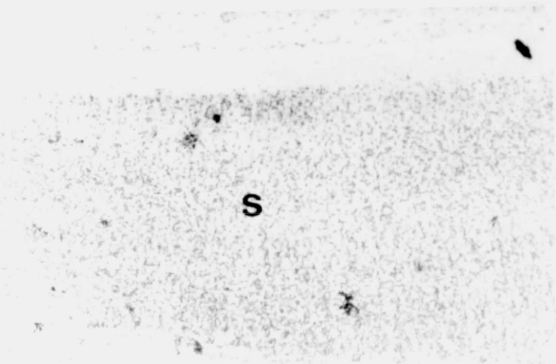
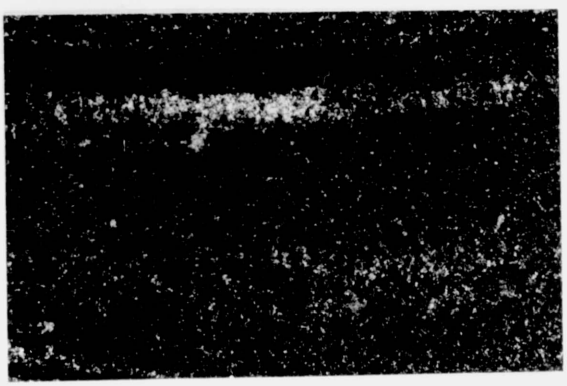


D



E



F

ALL RAR-ß

Figure 23: RAR-β RNA expression in the developing kidney. Scale bar represents 200μm.

A,B) Transverse section through the developing metanephros of a 43 day *p.c.* embryo, hybridized with a RAR-β full-length riboprobe. The metanephric epithelium (m) was found to be negative, as were the adjacent lumbosacral plexus (p) and the aorta (a). Expression was confined to the mesenchyme medial and anterior to the metanephros.

C,D) Sagittal section through the developing kidney of a 57 day *p.c.* embryo, hybridized with a RAR-β full-length riboprobe. Expression was found in the mesenchyme of the kidney. The glomeruli (gl, and arrowed), the suprarenal (sr) gland and the gonad (gd) were negative.

E,F) Similar section to C,D. hybridized with a negative control riboprobe.

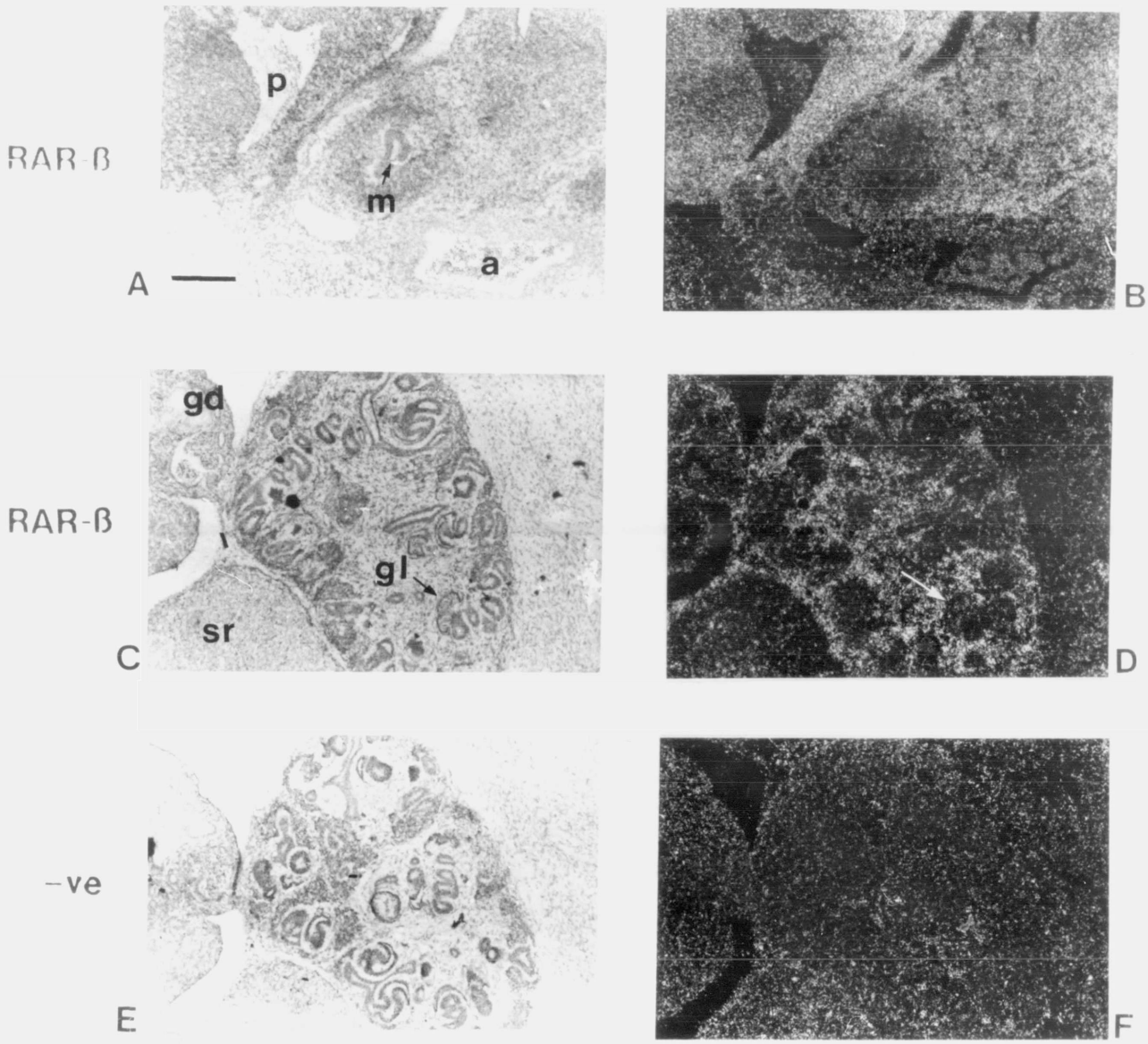
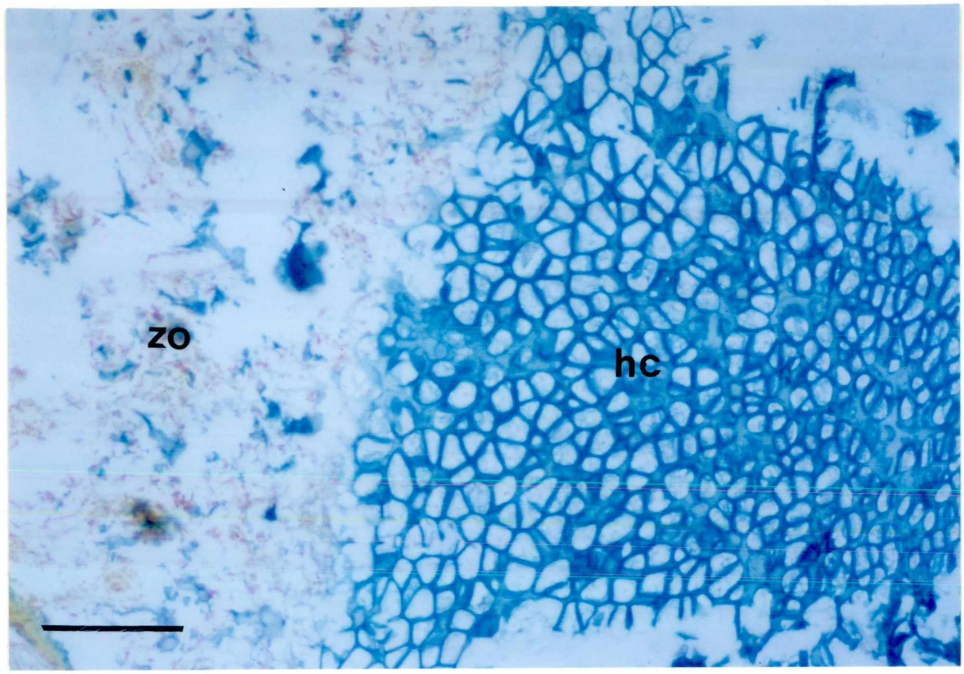

RAR-ß
p
m
a
A
B
RAR-ß
gd
gl
sr
C
D
-ve
E
F

Figure 24: Detection of cartilage by use of 1% alcian Green. Scale bar represents 200µm.

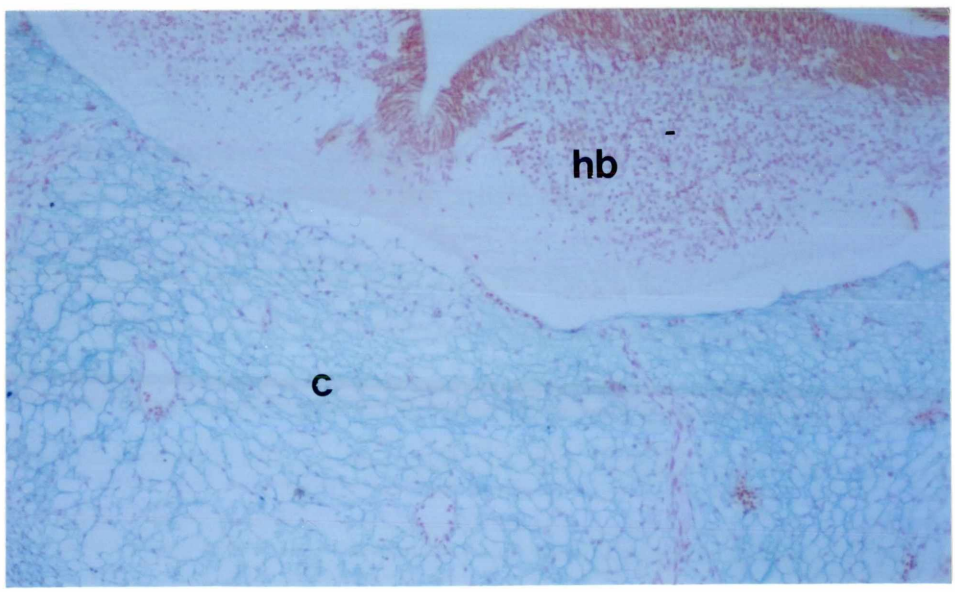
A) Longitudinal section through the cartilage model of a long bone of a 12 weeks *p.c.* embryo. Hypertrophic cartilage (hc) stains intensely blue-green. zone of ossification (zo).

B) Oblique transverse section through the occipital region of a 43 day *p.c.* embryo. Cartilage (c) of an earlier developmental stage, stains less strongly. hind-brain (hb).

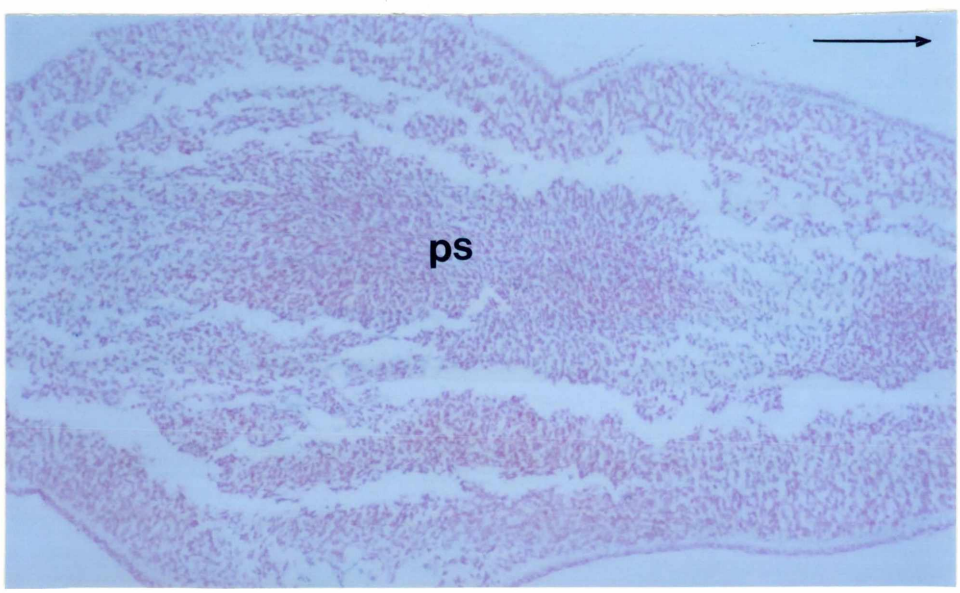
C) Transverse section across the lower limb bud of a 43 day *p.c.* embryo. Absence of any blue-green staining in the area of the prechondrogenic scleroblastema (ps) indicates that there is no cartilage deposition in the limb bud at this stage. The arrow points anteriorly.



A



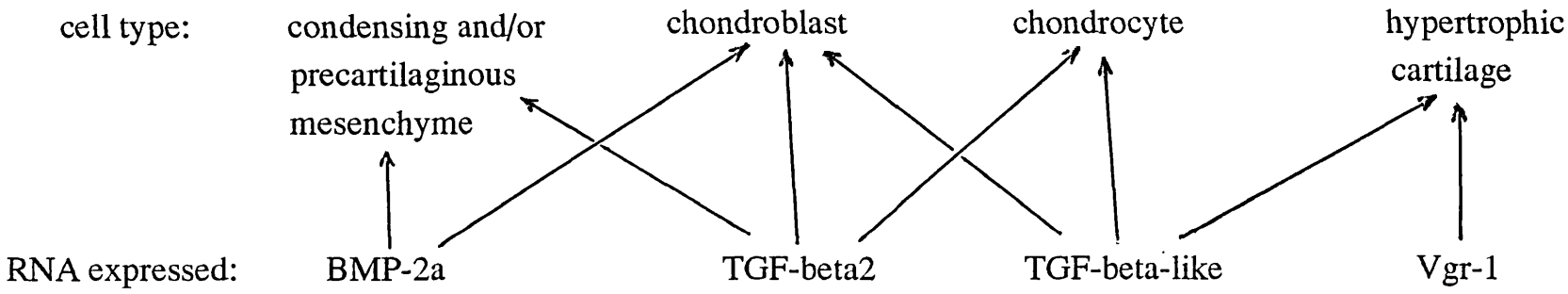
B



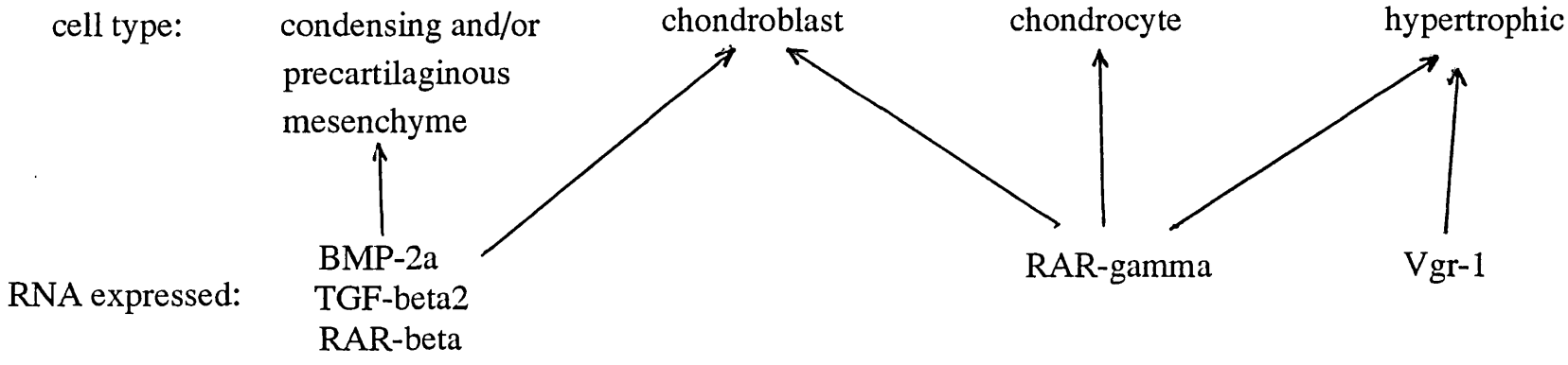
C

Figure 25: The model given in Lyons et al. (1989b) compared to a revised model based on the work of this thesis, and that of Millan et al. (1991), Ruberte et al. (1990) and Dolle et al.(1989,1990).

Lyons et al. (1989b):



New alternative model:



Chapter 4

Discussion

4.1 Limitations of the in situ hybridization technique

All hybridization techniques rely on the assumption that the probe will hybridize only to its homologous DNA or RNA sequences. TGF-β and RAR genes are members of gene families (see Introduction 1.4.2 and 1.5.3), and regions of considerable sequence homology exist between each of the genes. Therefore, there is an increased danger of cross-hybridization. To guard against this, the *in situ* hybridization protocol used in this study included stringent conditions for both hybridization and washing. Where less stringent conditions have been employed to study members of the TGF-β gene family (Pelton *et al.* 1989), results have been obtained which are at variance with those produced using more stringent hybridization conditions (*eg.* Millan *et al.* 1991, this thesis, McMaster *et al.* 1991). In Northern and Southern blots, cross-hybridization within the TGF-β and RAR gene families does not occur under standard conditions, even using full-length cDNA probes (Derynck *et al.* 1985, Petkovich *et al.* 1987, Giguère *et al.* 1987, Benbrook *et al.* 1988, Brand *et al.* 1988, Webb

et al. 1988, ten Dijke *et al.* 1988, Akhurst *et al.* 1988, Krust *et al.* 1989, Zelent *et al.* 1989). However, blot hybridization cannot be directly compared to hybridization *in situ*, since the conditions of the target nucleic acids bound to filters and in tissue sections are considerably different. Perhaps the strongest argument in favour of the specificity of the hybridization process in this study is that each gene examined has a unique spatial and temporal pattern of expression.

Differential splicing of TGF-β1 (Kondaiah *et al.* 1988) and TGF-β2 (Hanks *et al.* 1988, Webb *et al.* 1988) RNA has been detected. The alternative isoforms of TGF-β2 differ only in the precursor sequence, whereas the alternatively-spliced form of TGF-β1 generates a completely altered protein, due to a frameshift. (see Introduction 1.4.6). While the work presented in this thesis was in progress, Giguère *et al.* (1990) detected the existence, in the mouse, of a novel isoform of RAR-gamma, designated RAR-gamma-B, which differed in the extreme N-terminal region of the protein, having a stretch of 51 amino acids replacing the first 62 amino acids of the originally cloned isoform, now known as RAR-gamma-A. The two alternative N-termini were shown to be only 15% homologous in amino acid sequence. Kastner *et al.* (1990) isolated five other minor isoforms which only differ in their 5′-untranslated sequences. These authors also demonstrated that the isoforms were generated by the alternative splicing of seven exons. The function of these novel isoforms is unclear. No studies have yet been carried out on the question of alternative splicing of RAR or TGF-β genes in mammalian embryogenesis.

For the genes studied in this thesis, riboprobes made from non-conserved parts of the genes were used, as well as those made from full-length gene templates. None of the gene-specific probes were constructed from regions known to be involved in alternative splicing (see Materials and methods 2.2.7.1 for details). The patterns of expression were identical regardless of whether the gene-specific or full-length probes were used. In addition to confirming the specificity of the hybridization procedure, this suggests that, if alternative splicing of TGF-β or RAR transcripts occurs in development, no RNA species exclude those areas spanned by the gene-specific probes, since these would be detected by the full-length riboprobes alone. A fuller investigation of potential alternative splicing would require the use of multiple short probes, or of reverse transcription PCR (RT-PCR) with multiple dispersed pairs of primers.

The level of RNA transcribed from TGF-β genes in any tissue does not give any information on the levels of TGF-β protein synthesized there, since post-transcriptional control of TGF-β has been observed in several systems (Hennings *et al.* 1987, Assoian *et al.* 1987, Kehrl *et al.* 1986, Colletta *et al.* 1991). Furthermore, it is unjustified to assume that any translated TGF-β protein will be in an active configuration, since there is extensive evidence of TGF-β in inactive states, both in the latent precursor form and in the mature form bound to other proteins (Introduction 1.4.3).

In situ hybridization alone cannot give any information regarding putative mode of action. For example, TGF-β proteins may exert their activity in a paracrine manner. This has been demonstrated for TGF-β1 in murine embryos, where RNA patterns (Lehnert and Akhurst 1988, Wilcox and

Derynck 1988) and protein patterns (Heine *et al.* 1987, 1990) are distinct but complementary. It has not been possible to extend the studies of Lehnert and Akhurst (1988) and Heine *et al.* (1987, 1990) to other members of the TGF-β family in the murine embryo due to a lack of satisfactory isoform-specific antibodies raised against TGF-β2 or TGF-β3. However, it is possible that TGF-β2 and TGF-β3 may act in a similar paracrine manner.

4.2 Co-ordinate expression of TGF-β genes

The results presented in this thesis represent the first comparative study of the expression patterns of TGF-β1, TGF-β2 and TGF-β3 in mammalian embryogenesis. They indicate that expression of the TGF-β gene family is extensive and, by inference, important in human embryonic development. The study on TGF-β2 RNA expression was the first to be carried out without the use of cross-specific probes, and the data concerning expression of TGF-β3 RNA represent one of the first comprehensive surveys of the expression of this gene in embryogenesis (Gatherer *et al.* 1990) in any species. A similar study on murine embryos (Pelton *et al.* 1990) was published simultaneously.

The developmental patterns of expression of different members of the human TGF-β gene family sometimes overlap (see **Table** 3.6). This suggests that co-ordinate expression of TGF-β genes may be essential to precise control of growth, differentiation and morphogenesis of several tissues and organs. The respiratory system and the processes of

chondrification and osteogenesis are systems where co-ordinate expression of TGF-β genes could serve such a role, and where the *in vivo* biological activity data are available with which to compare *in situ* hybridization data, assisting interpretation of the biological roles of the genes. A few speculations may also be made regarding the action of TGF-β genes in parts of the embryo which cannot be so readily compared with *in vitro* systems.

4.2.1 TGF-β expression in the respiratory system

In the human embryo, TGF-β2 RNA was found to be expressed in the terminal bronchial epithelium at all stages studied. TGF-β3 RNA was also expressed in this epithelium, although not until a slightly later stage of development, and was additionally found in the submucosal mesenchyme of more proximal bronchi (see Results 3.4.5.1 for details). This data can be partially interpreted in the light of previous studies on the function of TGF-β in respiratory cells *in vitro*.

TGF-β isoforms have been generally found to be strong inhibitors of proliferation of epithelial cells *in vitro* (Holley al 1983, Nakamura *et al.* 1985, Jetten *et al.* 1986, Masui *et al.* 1986, Kurokowa *et al.* 1987, McKeehan and Adams 1988, Barnard *et al.* 1989, Daniel *et al.* 1989, Bascom *et al.* 1989a). This is particularly true for respiratory epithelia, in which induction of differentiation also occurs. The BSC-1 kidney epithelial cell growth inhibitor (Holley *et al.* 1980), now known to be an alternatively-spliced form of TGF-β2 (Hanks *et al.* 1988), has marked growth-inhibitory effects on CCL64 mink lung epithelial cells (Holley *et al.* 1983). Growth of

normal human bronchial epithelial (NHBE) cells and tracheal epithelial cells is inhibited irreversibly on treatment with TGF-β (Masui *et al.* 1986, Jetten *et al.* 1986). NHBE cells, thus treated, undergo differentiation to a squamous phenotype. Like and Massagué (1986) used TGF-β to inhibit the mink lung epithelial cell line Mv1Lu, finding that TGF-β induced a similar squamous phenotype, with associated increase in fibronectin in the extracellular matrix (see 1.4.5). Inhibition of proliferation by TGF-β in this cell type is thus associated with the onset of the differentiated state. A recent comparative study (Cheifetz *et al.* 1990) on the differential effects of TGF-β isoforms on lung epithelia *in vitro* demonstrated that all three TGF-β isoforms studied had anti-proliferative effects, but that TGF-β3 was the most potent.

Masui *et al.* (1986) found that TGF-β does not influence the levels of cyclic AMP (cAMP), suggesting that TGF-β acts at a level distal to the receptors for growth-activating factors and their immediate second messengers. Jetten *et al.* (1986) were able to define the differentiative response as consisting of two steps. Firstly, there is the terminal cell division stimulated by TGF-β, and secondly the differentiation process. The second step can be prevented by retinoic acid even when the first has taken place. This implies that the lung epithelium expresses a retinoic acid receptor. This could be RAR-α, since this species is expressed ubiquitously (see 3.5.1 and Dollé *et al.* 1990). In humans, RAR-β and RAR-gamma, although expressed extensively in the development of the respiratory system (see 3.5.5), are absent from the developing epithelia of the lung. However, Dollé *et al.* (1990) detected RAR-β RNA in the

respiratory epithelia of murine embryos, so this question remains unresolved.

These papers suggest that the effect of TGF-β on the respiratory system is primarily to inhibit growth and induce a terminal division, using pathways which are independent of those utilized by the growth-stimulatory molecules. Once this has been achieved, the cells are primed to undergo a differentiation event, which may be controlled by TGF-β or some factor induced by TGF-β.

This theory requires explanation of the paradox of why antiproliferative agents which promote terminal differentiation are present in a tissue at a time when the cells are rapidly proliferating. It may be necessary to control the rate at which the epithelia proliferate, in order to prevent uncontrolled growth. TGF-β genes would therefore fulfil a homoeostatic function with regard to the speed of epithelial proliferation. Alternatively, the TGF-β gene transcripts may not be translated until much later, or may produce protein which would remain latent until required. A third possibility is that the protein may be synthesized in the developing epithelia, but act in a paracrine fashion on the underlying mesenchyme. Consideration of these last two alternatives would be greatly assisted by data on the distribution of TGF-β2 and TGF-β3 proteins in the embryo, for which satisfactory antibodies are not currently available.

The first of the three above alternatives may be further considered. Given that TGF-β2 and TGF-β3 have essentially the same qualitative effect on respiratory cells (Cheifetz *et al.* 1990), the differing spatial distributions of TGF-β2 and TGF-β3 RNAs, presented in this thesis and confirmed in

murine embryos by Millan *et al.* (1991), suggest that these genes may be involved in differences in the relative rates of differentiation of the epithelia in the proximal and distal parts of the respiratory tree. Since TGF-β3 has the most potent antiproliferative effect (Cheifetz *et al.* 1990) it may be that expression of TGF-β3 in the terminal end buds of the developing lung is a principal factor in inducing those epithelial cells to assume a differentiated state. The expression of TGF-β2, in this system the weaker antiproliferative factor, in the same subset of epithelial cells may be necessary to modulate the action of TGF-β3, thus preventing too rapid an onset of differentiation. The submucosal expression of TGF-β3 in the more proximal parts of the respiratory tree (see 3.4.5.1) may be associated with proliferation of the submucosal mesenchyme, although it may effect differentiation of the overlying epithelia in a paracrine manner. The mesenchymal expression of TGF-β1 (Lehnert and Akhurst 1988, see also 4.3) may be involved in the proliferation of the lung stroma, and modulation of the extracellular matrix, with no effect on the epithelial differentiation.

4.2.2 Expression of TGF-β2 in the sensory epithelia

The work presented in this thesis and elsewhere (Lehnert and Akhurst 1988, FitzPatrick *et al.* 1990, Millan *et al.* 1991) demonstrates that members of the TGF-β gene family are expressed extensively in epithelia, endothelia and mesothelia during development. The case of the respiratory epithelia has been discussed above, but, in general, the other areas of expression of TGF-β genes in human embryonic epithelia do not

relate to systems studied extensively *in vitro*. The human embryonic material utilized for this study was of a limited number of developmental stages. This meant that the extensive transient epithelial expression of TGF-β1 RNA, seen by Lehnert and Akhurst (1988) and FitzPatrick *et al.* (1990) in morphogenically active organ systems, could not be compared with the human data. In all epithelia studied *in vitro*, TGF-β is growth inhibitory (reviewed by Moses *et al.* 1990, Lyons and Moses 1990), but controversy exists as to whether it is a universal differentiation inducer.

In the human embryo, TGF-β2 appears to be the most widely expressed isoform in epithelia. TGF-β2 RNA was the only isoform detected in the sensory epithelia and the neuroepithelium of the central nervous system. Given the potent antiproliferative effects of TGF-β on epithelia *in vitro*, (Holley al 1983, Masui *et al.* 1986, Jetten *et al.* 1986, McKeehan and Adams 1988, Nakamura *et al.* 1986, Kurokowa *et al.* 1987, Barnard *et al.* 1989, Daniel *et al.* 1989, Bascom *et al.* 1989a), it is likely that TGF-β2 functions to control the proliferation of the sensory and neural epithelia. It is also notable that the areas of the otic epithelium which express TGF-β2 RNA are those receiving innervation from the vestibulo-cochlear nerve, and that TGF-β2 RNA was also detected in the developing retina, innervated by the the optic nerve. This was pointed out by Millan *et al.* (1991), who raised the possibility that TGF-β2 is in some way connected with the process of innervation, possibly by regulation of the rate of growth of the axons into the epithelium.

4.2.3 Expression of TGF-β genes in embryonic endothelia

The expression of TGF-β1 and TGF-β3 RNA in different layers of the developing blood vessels may be important in control of their relative rates of proliferation and differentiation, in a similar way to the expression of TGF-β2 and TGF-β3 RNA in different parts of the developing bronchial tree, as discussed above. There is, however, a basic difference between the two systems, in that TGF-β2 and TGF-β3 RNA overlap in expression in certain areas of the developing lung, whereas in the developing blood vessels, TGF-β1 and TGF-β3 RNA expression are strictly mutually exclusive. TGF-β3 RNA is confined to the mesenchyme underlying the endothelium, leaving TGF-β1 RNA as the sole endothelial species. This suggests that the nature of the interaction between TGF-β2 and TGF-β3 in the lung and that of TGF-β1 and TGF-β3 in the developing blood vessel may be different. The possibility of the formation of TGF-β heterodimers (Cheifetz *et al.* 1987, 1988) would obviously depend on co-expression of different isoforms in the same cell.

In vitro studies have shown that TGF-β exerts a strong inhibitory effect on endothelial cell proliferation (Heimark *et al.* 1986, Baird and Durkin 1986, Frater-Schroder *et al.* 1986, Takehara *et al.* 1987). However, Madri *et al.* (1988) showed that this is dependent on the method by which the endothelial cells are grown *in vitro*; in three-dimensional cultures of endothelial cells, TGF-β mimicked angiogenesis and had no antiproliferative effect. The implication of this study is that it is difficult to predict the *in vivo* effect of TGF-β in endothelial cells without a thorough knowledge of the environment of those cells. A comparative study

(Cheifetz *et al.* 1990) of the effects of purified TGF-β isoforms on endothelial cells momlayers *in vitro* revealed that the growth-inhibitory effect of TGF-β2 was 50-fold less than that of TGF-β1 and TGF-β3. The expression of the more potent growth-inhibitory isoforms in this system, suggests that there is a requirement for tight control of both endothelial and tunica intima proliferation during development of the blood vessels.

4.2.4 Expression of TGF-β genes in embryonic mesothelia

The mesothelium covers the parietal surfaces of the pleural and abdominal cavities, and surrounds the organs within. TGF-β is mitogenic to mesothelia *in vitro* (Gabrielson *et al.* 1988). TGF-β3 RNA was detected in all mesothelia in the 57 day *p.c.* human embryo. There does not appear to be any involvement of other TGF-β genes in this system. The expression of TGF-β2 in the sensory epithelia, and that of TGF-β3 in the mesothelia seem to be examples of systems where only a single TGF-β isoform is expressed. As referred to above (4.2.1), co-expression of more than one isoform of TGF-β in developing lung could be a possible means of modulating the rate at which the epithelium differentiates. According to this hypothesis, epithelia which only express one TGF-β isoform would lack this putative regulatory mechanism. However, it is possible that human homologues of TGF-β4 (Jakowlew *et al.* 1988c), TGF-β5 (Kondaiah *et al.* 1990) and the additional TGF-β genes of *Xenopus* (Thomsen and Melton 1990), should they exist in mammals, may be co-expressed.

Further elucidation of these issues may not be possible until it is possible to manipulate TGF-β genetic activity *in vivo* (see 4.4).

4.2.5 TGF-β genes in chondrogenesis and ossification

The work presented in this thesis demonstrates that the TGF-β genes are expressed extensively in different stages of human bone and limb morphogenesis, ranging from the prechondrogenic stage to the stage of ossification. These findings can be considered in the light of a large body of previous research carried out on a variety of species.

Cartilage-inducing factors A and B (CIF-A and B), isolated from bovine bone by Seyedin *et al.* (1985), are now known to be TGF-β1 and β2 respectively (Seyedin *et al.* 1986, 1987). These factors were isolated on the basis of their ability to stimulate collagen and fibronectin synthesis, and ultimately the appearance of a chondrocyte morphology in undifferentiated rat muscle mesenchymal cells. This suggests that the expression of TGF-β2 RNA in the prechondrogenic scleroblastema (3.4.7 and Millan *et al.* 1991) is a major factor in the histogenesis of the cartilage model. TGF-β also has an effect on the differentiated chondrocyte cell, promoting proliferation of growth plate chondroblasts, and inhibiting proliferation of articular cartilage chondrocytes (Rosen *et al.* 1988, O'Keefe *et al.* 1988). The expression of TGF-β2 RNA in the growth plate zones of human embryos (3.4.7) is thus probably involved in the continued proliferation of chondroblasts in that area.

Ellingsworth *et al.* (1986) prepared antisera against a synthetic peptide of the first thirty amino acids of TGF-β1. Using these antibodies, immunohistochemical studies on 6 month *p.c.* fetal bovine bone revealed that TGF-β1 protein was present in osteocytes in both cancellous and cortical bone, in chondrocytes associated with the articular region, but not chondrocytes from the region of the growth plate. Heine *et al.* (1987) performed the same study on murine embryos. In the 15 day *p.c.* embryo, the calcifying parts of long bones and membranous bones are intensely stained. These represent an area where TGF-β1 appears to act in an autocrine, rather than paracrine fashion. Lack of specific antibodies against TGF-β2 and TGF-β3 means that the results obtained on these isoforms cannot be extended to their protein distributions.

Sandberg *et al.* (1988a,b) used *in situ* hybridization on 16 week *p.c.* human fetuses to detect TGF-β1 RNA, also localised in regions of ossification, specifically osteoblasts and osteoclasts of the growth plate and adjacent periosteal fibroblasts. Northern blots demonstrated the presence of TGF-β1 mRNA in calvariae of the equivalent developmental stage, but these were not studied by *in situ* hybridization. In the mid-gestation murine embryo, TGF-β1 is found in the osteogenic cells of the zone of ossification (Lehnert and Akhurst 1988). These results are supported by the results presented in this thesis.

A discrepancy is found between the human embryos and the murine embryos studied by Pelton *et al.* (1989, 1990), who demonstrated expression of TGF-β2 and TGF-β3 RNA, in addition to TGF-β1 RNA, in the zones of ossification. However, the murine embryos studied by Millan *et*

al. (1991) had a pattern of TGF-β gene family expression which exactly matched that reported in this thesis. The general differences between the work of Pelton *et al.* (1989, 1990) and those of other authors (Gatherer *et al.* 1990, Millan *et al.* 1991, McMaster *et al.* 1991), is probably due to the low stringency of hybridization and the cross-specific probes used by the former authors.

Lyons *et al.* (1989b, 1990) found RNA for *Vgr-1* (Lyons *et al.* 1989a) and BMP-2a (Wozney *et al.* 1988), both members of the TGF-β superfamily, in developing cartilage cells, suggesting that the TGF-β gene superfamily is widely involved in the development of the skeletal system. Lyons *et al.* (1989b) proposed a model (see Fig.25) for the regulation of long bone development from precartilagenous mesenchyme to hypertrophic cartilage, involving genes of the TGF-β superfamily. This model involved the sequential activation of BMP-2a, TGF-β2, another unidentified TGF-β gene and finally *Vgr-1*. This model does not fit with the data obtained on TGF-β2 RNA in this thesis nor with that by Millan *et al.* (1991), nor McMaster *et al.* (1991). It also suffers from the fact that the postulated additional TGF-β gene has not yet been isolated. Based on the subsequent data of Millan *et al.* (1991) and Pelton *et al.* (1990), as well as the work presented in this thesis, this hypothetical gene cannot be TGF-β3.

Functional studies on TGF-β in bone have fallen into two classes; those in which fragments of bone have been placed in "whole organ" culture, and those in which cell lines derived from fetal bone have been used as *in vitro* models.

Tashjian *et al.* (1985) cultured the whole calvariae of neonatal mice. It was found that TGF-β stimulated synthesis of prostaglandin E2 (PGE-2) and consequent bone resorption. This effect was inhibited by indomethacin, the anti-inflammatory drug, indicating that PGE-2 is the principal mediator of this effect. In a similar culture system, Pfeilschifter and Mundy (1987) showed that conditioned medium from the calvariae contained its own TGF-β, which was elevated in a dose-dependent manner by osteotropic (bone resorption) factors. These include Vitamin D_3, parathyroid hormone (PTH) and interleukin-1 (IL-1). Conversely, calcitonin, an anti-osteotropic hormone, decreased TGF-β activity. This allows a model to be formed in which TGF-β stands between the osteotropic hormones and intracellular PGE-2 in a chain of actions leading to bone resorption.

However, different circumstances appear to apply to long bones from those apparent in calvariae. Pfeilschifter *et al.* (1988) found that TGF-β inhibits bone resorption in a rat fetal long bone culture. This effect is found even at 100-fold higher concentrations than those used in Tashjian *et al.* (1985), but takes three hours to become observable. In this system, TGF-β also inhibits the effects of exogenous IL-1 and Vit. D_3.

Chenu *et al.* (1988) used human bone marrow cultures to study this effect in more detail. In bone marrow the progenitors of osteoclasts are large multinucleated cells (MNCs) which can be counted microscopically. TGF-β decreases the numbers of these cells in bone marrow culture and counteracts the effects of those agents which stimulate their formation, namely IL-1, TGF-α and PTH. The authors attempt to account for the discrepancy between the purely osteotropic effect of TGF-β in calvariae

and its more modulatory function in long bone, by postulating that TGF-β might potentiate the action of mature osteoclasts but inhibit the formation of new ones.

These papers suggest that the expression of TGF-β1 in zones of ossification (3.4.5 and Sandberg *et al.* 1988, Lehnert and Akhurst 1988) may have a more complex function. The solution to this problem is not yet clear, but some suggestions may be found in the work of Robey *et al.* (1987), who studied the effects of TGF-β on a fetal bovine cell line derived from collagenase-treated sub-periosteal bone fragments. TGF-β was capable of stimulating growth of these cells in sub-confluent cultures only. Therefore, the density of the cells may be an important factor in the effect exerted by TGF-β. However, Centrella *et al.* (1987) found the opposite effect on their osteoblast-enriched calvaria-derived cell line, in which TGF-β was inhibitory to sparse cell cultures and exerted its most marked effect on confluent cells. At all densities, type I collagen mRNA is produced, whereas Robey *et al.* (1987) found no collagen production.

ten Dijke *et al.* (1990) demonstrated that TGF-β3 is more potent than TGF-β1 at promoting all activities associated with osteogenesis *in vitro*. However, TGF-β1 is the sole isoform expressed in zones of ossification in human (see 3.4.7) and murine embryogenesis (Lehnert and Akhurst 1988, Millan *et al.* 1991). This implies that ossification does not occur as quickly as possible, but that it is allowed to proceed slowly under the influence of the more weakly osteogenic TGF-β1.

Based on the work presented in this thesis, and that of Millan *et al.* (1991), an alternative model to that of Lyons *et al.* (1989b) can be proposed (see

Fig.25), in which the first members of the TGF-β superfamily to be expressed in the prechondrogenic scleroblastema are TGF-β2 (Millan *et al.* 1991) and BMP-2a (Lyons *et al.* 1989b, 1990). These genes would be down-regulated in maturing cartilage, and TGF-β1 would be activated in the invading osteogenic precursors. This would eliminate the necessity to invoke a novel TGF-β gene in the mature chondrocyte. However, it is possible that mammalian homologues, should they exist, of some of the TGF-β genes isolated from *Xenopus* (Kondaiah *et al.* 1990, Thomsen and Melton 1990) and chicken (Jakowlew *et al.* 1988c) could fulfil this role.

4.3 Comparison of TGF-β gene expression in human and murine embryogenesis

The work presented in this thesis was planned in the light of the studies on the expression of TGF-β1 RNA (Lehnert and Akhurst 1988, Wilcox and Derynck 1988) and protein (Heine *et al.* 1987) in the mid-gestation murine embryo. Concurrently with the work reported in this thesis, other investigators were studying the expression of other members of the TGF-β gene superfamily in murine embryogenesis.

Rappollee *et al.* (1988) utilized RT-PCR to detect expression of TGF-β1 RNA in the murine blastocyst. A similar study was planned to be carried out on human preimplantion embryos (see Introduction), but was abandoned due to technical problems (see Results 3.2.2). However, TGF-β1 RNA was detected by RT-PCR in BeWo (choriocarcinoma) cells. NTera2 cells, which share many characteristics with early preimplantation

embryonic cells (reviewed by Andrews 1984), did not contain sufficient TGF-β1 RNA to be detected by RT-PCR. The expression of TGF-β1 RNA in the human embryo reported in this thesis matches well with that reported by Lehnert and Akhurst (1988), where comparable organs and tissues can be identified.

Pelton *et al.* (1989) examined the distribution of TGF-β2 RNA in the post-implantation mouse embryo, utilizing *in situ* hybridization with a human TGF-β2 probe, and a low stringency hybridization protocol. Pelton *et al.* (1989) detected TGF-β2 RNA in mesenchymal tissues, including perichondria and skin. The results presented here are not strictly comparable with those of Pelton *et al.* (1989) due to a dissimilarity of the embryonic stages examined. However, where the murine and human embryos correspond, there remain discrepancies between reports. For instance, neither TGF-β2 nor TGF-β3 RNA were detectable in the human embryo in zones of ossification, whereas Pelton *et al.* (1989, 1990) detected RNA for all three TGF-β isoforms in these areas. Conversely, Pelton *et al.* (1989) did not detect TGF-β2 RNA expression in the epithelia of the lung, the sensory epithelia, nor the ventral neuroepithelia of the spinal cord, when it is clearly expressed at high levels in these tissues in human embryos.

Pelton *et al.* (1989) detected TGF-β2 RNA in the dermis of the 15.5 day *p.c.* mouse embryo, but by 18.5 days *p.c.* it had become localized to the epidermis. In the human embryo, TGF-β2 RNA was only found in the hypodermal mesenchyme at 57 days *p.c.*, the latest stage examined. However, this stage of skin development is very immature in the human embryo and does not correspond to any of the stages investigated by

Pelton *et al.* (1989). It is difficult to draw comparisons concerning gene expression between human and murine skin development, since the human embryos frequently suffer surface damage during retrieval. This can lead to an increase in background hybridization over the skin, and make interpretation of results very difficult.

The differences between the work presented here and that of Pelton *et al.* (1989) may indicate that the pattern of expression of TGF-β2 RNA differs between the two species. Alternatively, and more likely, the differences may be due to the fact that Pelton *et al.* (1989) used a less stringent hybridization protocol, and a non-homologous TGF-β2 probe. These factors could result in cross-hybridization with other members of the TGF-β gene family. Millan *et al.* (1991) and McMaster *et al.* (1991) studied murine embryonic TGF-β2 expression using the homologous murine riboprobe, giving a closer correspondence to the results presented here from human embryos.

TGF-β3 RNA expression in murine embryogenesis has been studied using the homologous murine TGF-β3 riboprobe (FitzPatrick *et al.* 1990, Pelton *et al.* 1990, Millan *et al.* 1991, McMaster *et al.* 1991). TGF-β3 RNA also has a specific pattern of expression in the murine embryo, being detected in the submucosa of the developing lung epithelia, in some perichondria, in the tunica intima of large arteries, and most strikingly in the intervertebral disc anlagen, where it has a similar pattern of expression to the *Pax-1* homoeogene (Deutsch *et al.* 1988). These results agree substantially with those presented here.

Due to the relative inferiority of state of preservation of RNA, results obtained in murine embryos were not always confirmed in human material. In the murine embryo, TGF-β1 RNA is expressed transiently in association with active epithelial morphogenesis in the tooth, salivary gland, hair follicle and secondary palate (Lehnert and Akhurst 1988, Akhurst *et al.* 1990b, FitzPatrick *et al.* 1990). For example, in the developing murine secondary palate, medial edge epithelium expresses TGF-β1 RNA only during a 24 hour period. The inadequacy in coverage of all developmental stages of human embryonic material used here make it unlikely that any such transient expression of any RNA would be detected. Expression of TGF-β1 RNA was detected in the mitral valve of the human embryo, but expression of TGF-β1 and TGF-β2 RNA in the developing heart did not reach the levels reported in the murine embryo by Lehnert and Akhurst (1988), Akhurst *et al.* (1990a), and Millan *et al.* (1991). This is probably a reflection of the poor state of preservation of the cellular RNA of human embryonic material, relative to that of freshly fixed mouse embryos.

These studies on the murine embryo strongly suggest that the expression of the TGF-β gene family is the same in both murine and human embryogenesis, thus suggesting that the function of TGF-β genes is conserved across evolutionary distances within the mammalian class. In a clinical context, these conclusions are significant, since they demonstrate an example of conservation of molecular developmental mechanisms between man and mouse, suppporting the use of the latter as a model for the study of human embryogenesis.

4.4 Differential function of TGF-β isoforms in embryogenesis

It was recently demonstrated that several mutants, associated with developmental defects, genetically map near members of the TGF-β gene superfamily (Dickinson *et al.* 1990). However, the consequences for development of a mutation within the coding sequence of a TGF-β gene remain to be demonstrated. The data presented in this thesis, along with the corresponding studies in the murine embryo (Lehnert and Akhurst 1988, FitzPatrick *et al.* 1990, Millan *et al.* 1991), suggest that mutations in different TGF-β genes would produce effects in different sets of tissues, and, therefore, presumably distinct mutant phenotypes. It is equally likely that all the mutations would result in embryonic lethality. If the morphogenetic mutants quoted by Dickinson *et al.* (1990) are found to correspond to mutant TGF-β genes, the implication will be that the functions of TGF-β, although important for development, can be omitted without the production of a lethal phenotype in the organism. Alternatively, these mutants may be in the regulation of TGF-β genes, producing abberrations in expression during embryogenesis while preserving essential functions. A precedent for such a phenomenon in the TGF-β gene superfamily exists in the *DPP-C* complex of *Drosophila*, where *shortvein* mutants, which have breakpoints in a non-coding region of the complex, result in partial expression of the *dpp* gene which still permits normal gastrulation and dorsal-ventral pattern formation (Panganiban *et al.* 1990).

The study of gene expression in the embryogenesis of murine mutants is complicated by the difficulty in obtaining enough embryos, of the correct genotype, in mutant strains which may be either sub-fertile or only in existence as heterozygous stocks. Of course, the findings of Dickinson *et al.* (1990) may be due to mere coincidence, given the density of mutant gene loci in the murine genome. The solution to this problem may be to screen, using PCR, the TGF-β superfamily genes of mutant strains for deviations from the published sequences. This methodology was used by Balling *et al.* (1988) to identify the *undulated* mutation as a defect in the *Pax-1* gene.

It is, as yet, not totally clear whether TGF-β isoforms are functionally interchangeable in different biological contexts, or if they have separate functions *in vivo* (reviewed by Roberts and Sporn 1990). Since the isoforms of TGF-β are 70% identical in the amino acid sequence of their bioactive domains, it seems unlikely that they carry out qualitatively different functions. The earliest comparative study (Cheifetz *et al.* 1987) found that TGF-β1 and TGF-β2 were essentially functionally equivalent. However, Ohta *et al.* (1987) found that TGF-β1 was a 100-fold more effective inhibitor of colony formation by haematopoietic progenitor cells than TGF-β2, but this result was not confirmed by Sing *et al.* (1988), who assessed both isoforms as functionally equivalent in the same assay. Later comparative studies (Jennings *et al.* 1988, Graycar *et al.* 1989, ten Dijke *et al.* 1990, Cheifetz *et al.* 1990) have shown that in some systems there are appreciable functional differences, but that these are quantitative rather than qualitative. For example, Jennings *et al.* (1988) found that TGF-β1 was 600-fold more efficient than TGF-β2 in the inhibition of

endothelial cell proliferation. The most durable piece of evidence for qualitatively different properties for TGF-β isoforms is that of Rosa *et al.* (1988), who demonstrated that only TGF-β2 can act as an exogenous mesoderm-inducer in *Xenopus*. TGF-β3 has been studied less extensively with respect to differential function than TGF-β1 and TGF-β2, but where differences have been found (ten Dijke *et al.* 1990, Cheifetz *et al.* 1990), they are purely quantitative.

Two *a priori* observations could possibly argue in favour of the existence of some qualitative functional specificities. Firstly, there is extreme phylogenetic conservation in the amino acid sequence of the mature bioactive portion of each TGF-β isoform. Amino acid divergence between gene family members within any species is much greater than the divergence of individual family members between species. This suggests that each TGF-β gene has a strictly conserved function across large expanses of evolutionary time, and such strict conservation in any gene would tend to imply a unique function which cannot be carried out by another gene product. Secondly, the conservation of expression patterns between murine and human embryogenesis also implies that there has not been much scope for alteration of function in the preceding 100 million years.

Clarification of this issue could be assisted by the use of transgenic technology. A variety of approaches could be used. Constructs composed of TGF-β genes and heterologous promotors would yield information on the consequences of tissue-specific or generalized over-expression of TGF-β isoforms. Approaches to deactivation of the genes

are more technically problematic. These could include overexpression of an inactive mutant TGF-β monomer, which would form inactive dimers when associated with the normal bioactive monomer, or overexpression of antisense RNA to prevent translation of the normal mRNA. Site-directed mutagenesis of the coding sequence, to produce a nonsense or missense mutation, in embryonic stem cells is also now a possibility. Lastly, it might be possible to switch promotors between TGF-β genes and express the constructs in transgenic mice. This is complicated by the need to characterize fully the relevant promotor sequences for each gene, which has been partially completed for TGF-β1 alone (Kim *et al.* 1989a,b).

It is tempting to predict the phenotypes which may result from the perturbation of TGF-β function *in vivo*. Based on the results presented in this thesis, loss of TGF-β1 function in the embryo would presumably lead to defects in ossification, cardiac valve and endothelial defects, and haematopoietic abnormalities. Presuming that the findings of Lehnert and Akhurst (1988) and FitzPatrick *et al.* (1990) also apply to human embryos, there would also be defects in palatogenesis and development of tooth buds, hair follicles and salivary glands. Assuming that TGF-β1 functions as an anti-proliferative agent in epithelia and endothelia (see 4.2), the postulated defects might take the form of over-proliferation of these tissues, or failure to correctly co-ordinate development of complex ectodermal derivatives such as the tooth bud.

Loss of TGF-β2 or TGF-β3 function in embryogenesis would presumably lead to very wide ranging defects in the development of the mesenchyme, and the respiratory epithelium. In the case of TGF-β2, the sensory

epithelia would also be affected. The paradigmatic studies of site-directed mutagenesis *in vivo* in mammals are those of Wolgemuth *et al.* (1989) and Balling *et al.* (1989) on the production of embryos with defects in homoeobox-containing genes. These embryos have interesting phenotypes which do not merely correspond to developmental failure of the areas normally expressing the genes. Discussion of homoeobox genes is outside the scope of this thesis, but these studies raise the possibility that phenotypes of artificial TGF-β mutants will be more complex than thse predicted above.

4.5 Comparison of patterns of expression of RAR-β and RAR-gamma in human and murine embryogenesis

The study on RAR-β and gamma genes presented here was designed to complement previous work on the expression of RAR-gamma in murine embryogenesis (Ruberte *et al.* 1990), and also work on the RAR gene family in murine limb development (Dollé *et al.* 1989). RAR-α RNA was found to be expressed ubiquitously at all stages examined. This is supported by the work of Dollé *et al.* (1990) who found a similar situation in murine embryogenesis, with only slightly lower levels of expression in the liver and CNS. The implication of these findings is that all cells of the embryo are capable of responding to retinoic acid. It is perhaps significant that the affinity of RAR-α for a range of target sequences is influenced by multiple cell type-specific proteins, referred to as RAR coregulators (Glass

et al. 1990). Although RAR-α RNA is expressed in most cells, the RAR coregulators would permit the response of RAR-α to retinoic acid to vary considerably. This variability of response might be a more sensitive and rapid regulatory mechanism for RAR-α than induction and repression of transcription.

The results obtained on expression of RAR genes in the human limb agree with those of Dollé *et al.* (1989) on the developing murine limb, and those obtained on RAR-gamma RNA expression in human embryos match closely those of Ruberte *et al.* (1990) on murine embryos. In a recent, more extensive study, Dollé *et al.* (1990) detected similar patterns of expression of all three RAR genes, in the murine embryo, to those presented here for the human embryo. In general Dollé *et al.* (1990) detected RAR gene expression in a wider variety of tissues. This is again probably a reflection of the ability of these investigators to obtain fresher murine embryonic material. RAR-α RNA was also expressed ubiquitously in the murine embryo, although slightly less so in the liver and central nervous system. Strong expression of RAR-β RNA was detected in the tracheobronchial, intestinal and genital epithelia, which were all negative in the human embryos investigated. Another discrepancy was in the expression of RAR-β RNA in the oral epithelium. In the human embryo, all the oral epithelium, including that of the tongue, expressed RAR-β RNA, but Dollé *et al.* (1990) found that only that derived from the endoderm was positive in the murine embryo. However, expression of RAR-gamma and RAR-β RNA in the frontonasal mesenchyme, expression of RAR-gamma RNA in chondrogenesis, and expression of RAR-β RNA in the stroma of the kidney were identical. Dollé *et al.* (1990) also detected expression of

RAR genes in organs not available for study in the human embryos, such as tooth buds and salivary glands.

4.6 Retinoic acid and teratogenesis

Retinoic acid has long been known to be a teratogen, ie. an agent which perturbs embryonic development if administered to the mother during pregnancy (reviewed by Geelen 1979, Howard and Wilhite 1986, Rosa *et al.* 1986), with a predominance of malformations in the craniofacial area (especially cleft palate), brain, vascular system and limbs. The authors of these studies speculated that the target of the retinoic acid in the embryos was the neuroepithelium and neural crest-derived structures. This was supported by the finding that exogenous retinoic acid tends to accumulate in these structures (Dencker 1987). More recently, retinoic acid has also been found to cause caudalization of the developing nervous system in *Xenopus* (Durston *et al.* 1989) and spina bifida aperta in embryonic mice (Alles and Sulik 1990). However, this latter effect seems to be mediated by excessive cell death during gastrulation, rather than any subtle alteration of developmental regulation.

Dencker *et al.* (1990) examined the distribution of exogenous radiolabelled retinoic acid and derivatives administered throughout early murine embryogenesis. Prior to 10.5 days *p.c.*, the neural plates and neural crest are the principal areas of accumulation. This area was found to express RAR-α RNA in human embryos (see 3.5.1), but RAR-α RNA was rather lower, and demonstrated a specific pattern, over the CNS in the murine

embryo (Dollé *et al.* 1990). At slightly later stages, up to 11.5 days *p.c.*, the fronto-nasal area, mantle layer of the neural tube, and to a lesser extent, visceral arches and limbs, also start to accumulate the exogenous retinoids. The area of the visceral arches, and the fronto-nasal mesenchyme strongly express RAR-gamma RNA in human embryogenesis (see 3.5.6). Some cells of the mantle layer of the neural tube express RAR-β RNA (see 3.5.7). This implies that the effects of exogenous retinoic acid on development are mediated via all three of the RARs studied.

Dencker *et al.* (1990) also utilized immunohistochemistry to study the distribution of CRABP. This was found to be essentially similar to the areas accumulating the exogenous retinoids. The increased sensitivity of the immunohistochemical method also revealed areas of CRABP expression in peripheral nerves and early ventral somites. This evidence suggests that the teratogenic effects of exogenous retinoids are mediated through CRABP, and act upon those tissues in which the retinoids accumulate. To date, the expression of RAR genes and CRABP have only been investigated in normal murine and human embryos. It is not known if the embryos express RAR genes in a normal manner during periods of retinoid overdose.

The human embryos studied were generally of stages slightly later than those murine embryos of Dencker *et al.* (1990). However, the earliest human embryo studied is similar to the 11.5 day *p.c.* murine embryo. The expression of RAR-β RNA in the mantle cells of the neural tube of the 44 day *p.c.* human embryo was a little more spatially restricted than the

generalized accumulation of retinoids in that area found by Dencker *et al.* (1990). Similarly, the RAR-β RNA detected in the 44 day *p.c.* limb bud was generally confined to the prechondrogenic scleroblastema, whereas retinoid accumulation is diffuse throughout the entire limb bud. By contrast, there is almost exact correspondence between the accumulation of exogenous retinoids in the visceral arches and the fronto-nasal area and the expression of RAR-gamma RNA in these areas. The lack of any expression of RAR-β or RAR-gamma RNA in the neural crest and the hind-brain and mid-brain suggests that the effects of retinoids on those regions are mediated by other members of the RAR gene family.

Some regions of the embryo expressing RAR genes are apparently unaffected by exogenous retinoic acid. These include the mesenchyme of the kidney, and areas of chondrogenesis in the pelvis and ribs. The apparent insensitivity of these areas to retinoids may indicate that some other factor is required for teratogenic effects on a developing tissue. The work of Dencker *et al.* (1990) suggests that this may be CRABP, since this gene has a distribution which matches, more closely than any of the RAR genes studied, the areas affected by exogenous retinoic acid.

4.7 Implications of the results for the regulation of RAR genes

The promotor of the RAR-β gene contains a retinoic acid response element (RARE) (de The *et al.* 1989, Sucov *et al.* 1990). This consists of a GTTCAC dimer separated by five nucleotides. This implies that any

treatment with retinoic acid, either exogenously or as part of an endogenous retinoic acid gradient will produce transcription of RAR-β. Since the pattern of expression of RAR-β in mouse (Dollé *et al.* 1989, 1990) and human embryogenesis is very specific, the implication is that there are transcriptional repressors of RAR-β which prevent expression from becoming generalized over the area of the limb. By contrast, RAR-α, which has a far more extensive pattern of expression in embryogenesis (Dollé *et al.* 1990, Osumi-Yamashita *et al.* 1990), has no RARE and is less reponsive than RAR-β to retinoic acid induction by an order of magnitude (Martin *et al.* 1990, Hu and Gudas 1990). RAR-gamma is also non-responsive to retinoic acid (Hu and Gudas 1990). However, Song and Siu (1989) found that both RAR-α and RAR-β were elevated by retinoic acid in a mouse embryonal cell line. Since embryonal carcinoma cells may correspond to those of the very early embryo, it is conceivable that the retinoid-inducibility of RAR-α, and possibly other genes, may vary at different stages of development.

4.8 Speculations on the interaction of these gene families in development

Although knowledge of TGF-βs and RARs is rapidly advancing, there is still little information on the ways in which these categories of developmentally important molecules may interact. It is important to remember that RAR genes encode nuclear receptors whose primary effect is transcriptional activation, whereas TGF-β genes encode extracellular

signal molecules interacting with a complex receptor on the plasma membrane. Little can be inferred by extrapolation from *Drosophila*, since that organism does not have large TGF-β and RAR gene families such as occur in mammals. However, in mammalian embryos, the following cases have been described.

In vitro, some of the effects of exogenous retinoic acid on keratinocytes can be blocked by antibodies to TGF-β2 (Glick *et al.* 1989). Murine suprabasal keratinocytes express both TGF-β2 and RAR-gamma RNA (Pelton *et al.* 1989, Ruberte *et al.* 1990, Millan *et al.* 1991). The distribution of RAR-β RNA in mammalian limb morphogenesis (Dollé *et al.* 1989, and 3.5.2) is closely parallel to that of TGF-β2 (3.4.7 and Millan *et al.* 1991), and that of TGF-β3 RNA in the developing spinal column (3.4.7 and Millan *et al.* 1991) is similar to that of RAR-gamma RNA (Ruberte *et al.* 1990 and 3.5.3). The expression of RAR-gamma RNA, and to a lesser extent RAR-β RNA, in the frontonasal mesenchyme (Ruberte *et al.* 1990, Dollé *et al.* 1990, and 3.5.6) overlaps with the less extensive expression of TGF-β2 and TGF-β3 RNA in that area (3.4.8.1 and Millan *et al.* 1991). The limbs and the frontonasal area are two regions where exogenous retinoids can produce severe abnormalities in human embryos (reviewed by Rosa *et al.* 1986). These examples of co-expression of RAR genes and TGF-β genes suggest that one of the ways in which RAR proteins may regulate morphogenesis in these regions is by the selective activation of the multifunctional TGF-β genes. However, RAREs have not yet been identified upstream of any TGF-β genes.

4.9 Implications of this study for the theory of evolution of gene families

Neo-Darwinian evolutionary theory requires that all genes represent a monophyletic heirarchy, presumably descended from a hypothetical original gene in a primordial organism or "progenote". On a slightly less grand scale, it is hypothesized that gene families, such as the steroid/thyroid hormone receptor gene superfamily, of which the RAR genes are a constituent family, and the TGF-β gene superfamily, have evolved from single progenitor genes. The phylogenetic distribution of TGF-β gene superfamily members (Weeks and Melton 1987, Derynck *et al.* 1988, Gelbart 1989, Thomsen and Melton 1990) suggests that the hypothetical original TGF-β gene probably existed in a very primitive eukaryote, prior to the divergence of the deuterostome and protostome lineages. The recent finding of RAR-related genes in *Drosophila* (Oro *et al.* 1988, Mlodzik *et al.* 1990) suggests that the same is true for the steroid hormone receptor superfamily.

The evolutionary dynamics of gene family evolution were discussed at length by Ohno (1970). The initial step in the formation of a new gene was taken to be the duplication of an existing gene to generate two copies, either in a tandem array or at different locations in the genome. In most cases, the new gene copy would presumably be surplus to the requirements of the organism. Under such circumstances of redundancy, there would be no selective pressure to retain two copies of the gene and one of the two would accumulate deleterious mutations, eventually

becoming a pseudogene. In order for such gene duplications to persist, the new gene would have to fill a role granting a selective advantage. The most simple possibility is that this advantage could be gained from an up-regulation of the gene product. This is the most probable explanation for the evolution of very large, repeated gene families such as rRNA and the histones. However, for the TGF-β and RAR gene families this presumably cannot be the case.

A second possibility is that the presence of two copies of a gene allows a permanent heterozygous advantage (Koehn and Rasmussen 1967). The extra gene copy has a selective advantage, which prevents its loss by accumulation of deleterious mutations. It is then free to slowly undergo a process of mutation to another function. It is difficult to judge whether this may have been the case for members of the TGF-β and RAR gene families since no information is available on the existence of allelic forms of any TGF-β or RAR gene in either mouse or human populations.

TGF-β1 and TGF-β2 can form heterodimers (Cheifetz *et al.* 1987, 1988). RAR-α can also form a heterodimer with thyroid hormone receptor (Glass *et al.* 1989). It is possible that, in both the TGF-β and RAR gene families, the heterodimers are functionally superior, by some criterion, to either homodimer. Alternatively, some members of the gene families could form a permutational series of heterodimers, allowing a greater variety of dimeric receptor or growth factor types to be created. Under these conditions, there might also be selection for novel variations on the original TGF-β or steroid receptor.

Any one of these models could explain how the original primordial TGF-β and steroid hormone receptor genes was able to give rise to the current complexity of the TGF-β and steroid/retinoid receptor superfamilies. However, there is an additional possibility, essentially derived from, but not specifically considered by Ohno (1970), which is more attractive, in this case, since it also provides a tentative explanation for the differences in expression pattern of the various members of the TGF-β and RAR gene superfamilies. All the above models have a common tenet which is that,in order to survive, the duplicated gene would have to fulfill a specific role, different to that of the progenitor gene, which also conferred a selective advantage. One possibility is that the duplicated copy could be transposed to a region of the genome in which it would be under the influence of regulatory sequences dissimilar to those of the area in which the original gene was located. The new gene could thus exhibit a new pattern of expression. In the majority of cases, this would probably be selectively disadvantageous. However, if advantageous, the new arrangement would become established in the population. The gene would then accumulate mutations which increased its adaptation to its new role, thus diverging from its progenitor. This scenario would fit the evolution of the TGF-β and RAR gene families. The work presented in this thesis, and that of other investigators on TGF-β isoforms (Wilcox and Derynck 1988, Lehnert and Akhurst 1988, Rappollee *et al.* 1988, Pelton *et al.* 1989, FitzPatrick *et al.* 1990, Pelton *et al.* 1990, Millan *et al.* 1991) and members of the *dpp* gene family (Lyons *et al.* 1989b, Lyons *et al.* 1990, Jones and Hogan 1990) demonstrates that it is a characteristic of these families that each member has a different spatial and temporal pattern of

expression in embryogenesis. The same is true for RAR genes (Dollé *et al.* 1989,1990, Ruberte *et al.* 1990). These differences may have been the very reason that the gene families were able to proliferate successfully.

This model does not comment upon the question of whether pattern of expression, rather than function, is the most important distinction between members of the gene families. It merely requires that each individual member should be different in some respect, and is compatible with both the regulatory differences and functional similarities, as thus far elucidated, within the TGF-β and RAR gene superfamilies.

The prediction of this hypothesis is that any other mammalian TGF-β genes, which may exist as the homologues of the TGF-β 4, 5, 6, 7, and 8 genes (Jakowlew *et al.* 1988c, Kondaiah *et al.* 1990, Thomsen and Melton 1990), or further members of the RAR gene family should all exhibit distinct patterns of embryonic expression, different to those of other members of their respective gene superfamilies already studied.

4.10 Concluding remarks

This thesis presented data on the expression of the TGF-β and RAR genes in early human embryogenesis. The proposed *in situ* hybridization experiments on preimplantation embryos were confounded by the lack of embryos of a suitable condition. Polymerase chain reaction technology is a better method for the study of gene expression where material is scarce. However, preliminary experiments revealed problems with the sensitivity

of the procedure. Nevertheless, it was possible to detect TGF-β1 RNA in cultured cells using the RT-PCR technique.

On a more positive note, *in situ* hybridization proved to be ideal for the study of TGF-β and RAR RNA in post-implantation embryos. The patterns of expression obtained suggest strongly that both TGF-β and RAR genes are important in the regulation of development in humans.

A revised model was presented for the action of TGF-β and RAR genes in the process of chondrogenesis in the developing limb. This model takes account of the work of other investigators, as well as that presented in this thesis. It has the advantage of simplicity over the older model, and provides a framework for the further investigation of gene expression in the developing limb.

The differential patterns of expression of TGF-β and RAR genes were proposed to be an important feature in the evolution of the gene families, since a novel pattern of expression gives a newly duplicated gene a property distinct from its parent gene, which, if not detrimental to the organism, protects the duplicated gene from slow mutational decay.

The results do not help to answer the question of whether different gene family members are likely to have different qualititative or quantitative properties. However, the specificity of the patterns of expression, and their conservation between man and mouse, suggests that any functional differences, even if purely quantitative, are important.

The molecular investigation of gene expression in mammalian embryogenesis has generated, over the last five or six years, an extensive

volume of data on a wide variety of genes ranging from homoeogenes through growth factors to receptor molecules. This is merely the tip of the iceberg of data which will arise in the future. The ultimate aim of "molecular anatomy" is to characterize the expression of all genes at all stages of an organism's existence. This is an intrinsically valuable task, which may yield many incidental insights into *Entwicklungsmechanik*. However, a fuller understanding of the processes of mammalian development relies on the investigation of the perturbation of gene function *in vivo.* This has begun to be accomplished for a few homoeobox genes, and will be the major thrust of research, into the function of gene families in development, in the next decade.

Chapter 5

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TRANSFORMATING GROWTH FACTOR BETAS IN MAMMALIAN EMBRYOGENESIS

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Type β transforming growth factors (TGFβs) are members of a large superfamily of related proteins, each of which plays a pivotal role in embryonic processes. The TGFβs per se *are at least five in number, though only three isoforms have been identified in mammals. Here we will review the evidence, taken from* in vitro *studies on bioactivity and histochemical localization of RNAs and encoded proteins* in vivo, *that TGFβ1, β2 and β3 are involved in several mammalian developmental processes, including control of growth, differentiation, tissue inductions and morphogenesis.*

Key words: Transforming growth factor β, embryogenesis, cardiogenesis, epithelial-mesenchymal interactions, morphogenesis, extracellular matrix.

INTRODUCTION

It has become widely accepted that growth factors are not only involved in the regulation of cellular mitogenesis, but also in the modulation of a variety of biological processes at the cellular and tissue organizational levels. The transforming growth factor βs (TGFβ) are an excellent example. These growth factors can influence cell growth, differentiation, inductive interactions, transdifferentiation and cell lineage pathways, as well as modulate the biosynthesis of the extracellular matrix, all of which are of central importance in embryogenesis.

The *in vitro* biological activities of TGFβ are variable, depending on cell type, culture conditions and the presence of other growth factors. These have recently been

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extensively reviewed elsewhere [1–3], and thus will only be mentioned in the context of their relevance to specific development processes.

THE TGFβ SUPERFAMILY

TGFβ1 was identified and isolated around a decade ago, on the basis of its ability to induce anchorage-independent growth of non-transformed cells [4–6]. The mature growth factor was shown to be a 25 kDa homodimeric protein. Once the gene for TGFβ1 was cloned [7], it became clear that many molecules, from a diverse range of species, were related to TGFβ1. These include the *Decapentaplegic (dpp)* gene of *Drosophila*, which is involved in dorso-ventral patterning of the fly [8] and the amphibian mesoderm-inducing factors, Vg1 and MIF-XTC (or activin B) [9,10] and also the mammalian proteins; Mullerian inhibitory substance, which aids the regression of male Mullerian duct during development [11], the inhibins and activins [12,13], and the bone morphogenetic proteins (BMPs) [14], which include Vgr-1 [15]. A striking observation is that each of these proteins serves a fundamental function(s) in embryonic processes, which led researchers to investigate a potential role for TGFβ *per se* in development of the embryo.

It is beyond the scope of this article to review the role of each of the superfamily members in embryonic development. In the interest of brevity we will therefore restrict ourselves to the function of the TGFβs *per se*, and focus on their role in *mammalian* development.

THE TGFβ FAMILY

All TGFβs are translated as large precursor proteins which are subsequently processed by the removal of an *N*-terminal signal sequence and cleavage of the larger precursor domain (latency-associated peptide, LAP) from the adjacent *C*-terminal active polypeptide. A dimer of LAP remains associated with the bioactive TGFβ homodimer, thus keeping it in a biologically latent form. Although factors capable of activating TGFβ *in vitro* have been identified, it remains unknown how TGFβ activation occurs *in vivo* (see [2]).

To date, five members of the TGFβ family have been reported. These are characterized by sharing 70% amino acid identity within the bioactive *C*-terminal portion of the polypeptide (in contrast, the superfamily members are only 30–40% homologous). TGFβ1, β2 and β3 have been isolated from various mammalian and avian sources, but, as yet, no mammalian homologues of TGFβ4 or β5 have been found. The latter two have only been isolated from chick and frog, respectively [16,17]. In contrast to the intraspecies divergence of family members, the interspecies amino acid sequence conservation of any individual TGFβ gene is virtually complete. This has raised the discussion of whether multiple TGFβs have arisen to serve distinct biological functions *in vivo*, or whether the purpose of the gene family is to provide expression of proteins of identical function in diverse tissue contexts.

Comparative biological activity studies *in vitro* suggest that TGFβ1, β2 and β3 have similar qualitative effects on keratinocytes, fibroblasts, osteoblasts and endothelial cells, but that these activities vary quantitatively [18–20]. In yet other biological

systems, the TGFβs clearly have distinct roles. As an example, mammalian TGFβ2 can induce the formation of mesoderm from *Xenopus* ectodermal explants. In contrast, TGFβ1 does not possess this bioactivity, but can synergize with fibroblast growth factor (FGF) in this process [21]. It thus appears more likely that the TGFβs do indeed possess functional specificity *in vivo*.

Since the bioactive coding portions of TGFβs are so highly conserved, it is thought that biological specificity may be conferred by the precursor region, either in differential targeting of the latent complex to its site of action, binding to cell surface receptors or activation of the latent forms. The precursor domains exhibit conservation of certain structural moieties, but also possess distinct intermolecular differences. TGFβ2, for example, unlike other isoforms, lacks a potential integrin-binding site [2], whereas TGFβ4 lacks a signal peptide [16].

STUDIES ON MAMMALIAN EMBRYOS

As yet there is no definitive evidence that any of the TGFβs are endogenous regulators of mammalian embryonic processes. Most of the information implicating TGFβs as embryonic modulators comes from descriptive studies on a) the ability of early embryos and embryonal stem (ES) cells to secrete bioactive TGFβs, b) immunohistochemical localization of TGFβs in mouse sections, c) detection of TGFβ RNAs by Northern blot or polymerase chain reaction (PCR) of total RNA, or by *in situ* localization in embryo sections. From this data, together with the extensive knowledge of the *in vitro* bioactivities of TGFβs gained from cell or organ cultures, various inferences may be made. Some elegant *in vivo* studies have also implicated TGFβ as important in morphogenesis of the mammary gland, but, as yet, it has not been demonstrated that any of the TGFβs occur endogenously within this developing organ [22].

Most of the published work relevant to the functions of TGFβ in embryonic processes has been gained from immunohistochemical protein localization studies [23,24] and *in situ* RNA localization studies [25–31]. However, conclusions drawn from these studies must be made with caution for a number of reasons. Firstly, the similarity of TGFβ isoforms at both nucleic acid and polypeptide levels has made the preparation of isoform-specific reagents exceedingly difficult. This is particularly true for the generation of antibodies. Cross-reactivity may not occur in one assay, but be extensive in another. Furthermore, antibodies raised against identical peptides can recognize different epitopes, possibly as a result of conformational changes in the protein following its translation, secretion and activation [32,33].

The two most extensively utilized polyclonal antibodies against TGFβ1 are the so called LC and CC reagents. These were raised against the same 30 amino acid peptide, yet show different staining patterns in immunolocalization studies, the former recognizing intracellular TGFβ1 and the latter staining extracellularly [32–34]. It has been postulated that these antibodies recognize the precursor and mature forms of TGFβ1, since tissues staining with the LC antibody are normally identical to, or in close proximity to those staining with the CC antibody [33,34]. Discrepancies do, however, occur, for example in the adult heart, intense LC staining is observed in the myocardium whereas CC staining occurs only in the valve leaflets [34].

The design of gene probes for *in situ* hybridization studies is less of a problem, but a

judicious choice of probes is warranted, since cross-reactivity can occur [26,31]. In general, the results of TGFβ1 RNA localization studies are in agreement with the immunolocalization of protein. Nevertheless, there are inconsistencies. The spleen, for example, is know to be the richest source of TGFβ1 RNA in the adult [35,36], nevertheless, no protein can be detected with the LC or the CC antibodies [34]. Also in the heart, the intensity of LC staining of the myocardium does not correlate with RNA levels determined by Northern blot or *in situ* hybridization [34,35,37]. These may represent examples of extreme post-transcriptional control of protein production. Finally, in the embryonic mouse lung, staining for pro-TGFβ1 is observed in the early bronchial epithelium [24], whereas no TGFβ1 RNA is detectable by *in situ* hybridization [26,31]. TGFβ3 RNA is, however, detected in the early pulmonary epithelium [31], opening up the possibility that the pro-TGFβ1 antibody also detects pro-TGFβ3.

Another limitation of descriptive studies is the inability to determine what percentage RNA is translated and what portion of the immunohistochemically detectable protein is biologically active and available to the responding cell. Although the half-life of TGFβ in serum is exceedingly short ($t_{1/2}$ = 2.5min), TGFβs can bind to the extracellular matrix, which could either act as a pool of the latent growth factor, readiness for future use, or as a sink for spent protein.

Finally, the presence of the growth factor does not necessarily imply that cells in its vicinity can respond. Although it has been widely reported that TGFβ receptors are ubiquitous on normal cells [38], alterations in ligand-binding have been observed during differentiation of cells in culture [39]. A clear example of the differential responsiveness of similar cell types to TGFβ *in vivo* comes from work on the mammary gland. In this system, TGFβ-impregnated plastic implants potently inhibit DNA synthesis in pubertal ductal epithelial cells of the growing end buds, but have no effect on 'maintainence' DNA synthesis in lumenal epithelial cells nor on the rapidly growing secretory alveolar epithelial cells of the pregnant animal [22].

TGFβs IN PRE- AND PERI-IMPLANTATION EMBRYOS

Much of the evidence implicating TGFs as important in pre-implantation development has come from the study of embryos *per se*, or of embryonal carcinoma (EC) or embryonal stem (ES) cell lines, which are thought of as models of the pre-implantation embryo [39–42]. Several years ago it was shown that EC cells release, but do not respond to, TGFs, whereas their differentiated derivatives are TGF-responsive [40]. This was correlated with the novel appearance of high affinity receptors of TGFβ on the cells following induction of differentiation [39]. Extrapolating to the *in vivo* situation, it has been predicted that TGFβ might act in a paracrine manner, whereby the undifferentiated cells of the inner cell mass are the source of TGFβ which modulates growth and/or differential of the endodermal derivatives. Indeed, TGFβ1 is synthesized by pre-implantation embryos, though with no obvious regional distribution [42,43].

More recently it has been shown that TGFβ2 is also synthesized in pre-implantation embryos, though in this case endodermal outgrowths of the blastocyst, rather than undifferentiated inner cell mass cells, are the source of growth factor [41].

The exact function of the TGFβs in peri-implantation development remains

debatable. They could be involved in autocrine or paracrine control of cell growth/differentiation, elaboration of the extracellular matrices synthesized by the implanting embryo [41] or in interactions with maternal tissue, for example in inducing angiogenesis at the site of implantation [42].

HAEMATOPOIESIS

The first evidence of high concentrations of TGFβ1 RNA within the murine conceptus occurs within the extraembryonic blood islands at 7 days *post-coitum* (*p.c.*) [37]. At this early stage it is impossible to distinguish between haematopoietic and endothelial cell precursors, though we believe that both cell types express TGFβ1. The blood islands are the major sites of haematopoiesis in the early embryo. At later stages, haematopoietic stem cells migrate to the liver, and finally populate the adult spleen and bone marrow. In all of these organs, the haematopoietic progenitors are marked by high levels of expression of TGFβ1 RNA. This is particularly pronounced in proerythroid progenitors and megakaryocytes [26,27,30]. Since TGFβ1 is known to be a potent inhibitor of haematopoiesis [44], it is likely that this growth factor acts as an autocrine negative regulator of cell growth. Its presence at a time of rapid cell division may be necessary for controlled proliferation and/or differentiation.

Neither TGFβ2 or β3 RNAs have been detected in haematopoietic tissue of mouse or man [30,31].

VASCULARIZATION AND ANGIOGENESIS

TGFβ elicits an angiogenic response *in vivo* when injected subcutaneously [45], though it is not known whether this is a direct response of the endothelial cells, or secondary to events initiated in other cell types, such as macrophages [46]. Clearly, *in situ* hybridization studies would suggest that TGFβ1 is not only an endogenous autocrine regulator of vascularization and angiogenesis, but that this growth factor may serve such a role prior to the appearance (and thus involvement) of other cell types. Expression of TGFβ1 RNA is seen in endothelial cells of the blood islands as the extraembryonic blood vessels are laid down, and in the cardiogenic plate of the embryo proper at 7.5 days *p.c.* [37]. This expression is detectable very soon after the appearance of the three definitive germ layers and before overt differentiation of cell types, implicating this growth factor as a very early modulator of vascularization.

During vascularization and angiogenesis, endothelial cells perform several functions, all of which may be modulated by TGFβ. These include cell proliferation, tube formation, protease production and extracellular matrix production. Studies of TGFβ1 bioactivity on endothelial cells *in vitro* have been confused, since the cellular response depends on the precise culture conditions. In general, it can be summarized that TGFβ1 inhibits endothelial cell proliferation in a 2 dimensional (2-d) but not in a 3-d culture system, suggesting that TGFβ1 is an endogenous autocrine negative regulator of endothelial cell growth. In addition, in the 3-d culture system TGFβ1 promotes capillary-like tube formation [47,48].

The endothelial cell response to TGFβ is clearly isoform-specific *in vitro*. Though TGFβ1 is a potent growth inhibitor of this cell type, at physiological concentrations, TGFβ2 shows no such activity [49]. In this respect, it is notable that, of the three TGFβ

isoforms, *only* TGFβ1 RNA is detectable in endothelial cells [30,31]. This may give support to the theory that differential expression patterns within the embryo are related to functional specificity *in vitro*.

CARDIAC DEVELOPMENT

Progenitor cells of the mammalian heart appear in the splanchnopleuric mesoderm very early in embryogenesis, immediately following gastrulation. These cells become organized into two primitive tissue types, the endocardial tube which is encapsulated by the myocardial mantle. Initially, the endothelial and myocardial layers are separated by an extensive acellular basement membrane, the cardiac jelly. However, there is an early regional differentiation of the endothelium, such that some cells overlying areas which will give rise to the future septae and valves undergo a phenotypic transformation and infiltrate the cardiac jelly to form mesenchymal cushion tissue.

The mechanism of endothelial–mesenchymal transformation in chicks has been extensively studied by Markwald and his collaborators using an *in vitro* culture system [50–52]. It has been shown that a signal, arising from the myocardium, induces the overlying endothelium to undergo the transformation event. The inducing signal is produced in a regional manner ie. only from the myocardium of the atrioventricular (av) junction region and outflow tract. Furthermore, only the endothelia of the av junction and outflow tract, but not the atrial or ventricular endothelia, are competent to respond to this signal. Various lines of evidence suggest that TGFβs may be central to the events leading to the formation of mesenchymal cushion tissue and subsequent cardiac morphogenesis.

Potts and Runyan [53] performed tissue recombination experiments with dissected embryonic chick hearts. They demonstrated that, although ventricular myocardium could not induce mesenchymal transformation of av endothelium, if TGFβ was added to the assay the transformation proceeded. Additionally, they showed that antibodies which block the activity of TGFβs could also prevent the *in vitro* transformation of av endothelial cells induced by av myocardium. Thus, it appears that one of the TGFβs was, at least partly, involved in this regional transformation event.

Immunolocalization and *in situ* localization of murine TGFβ proteins and RNAs has also shown that both TGFβ1 and β2 are localized within tissues which contribute to endocardial cushion tissue formation [23, 26, 31, 37] (summarized in Fig. 1). TGFβ1 RNA is expressed ubiquitously in endocardial cells at early stages. This expression subsequently becomes restricted to endocardial cells which overly cushion tissue, and this restricted pattern persists in the cardiac valve leaflets until 1 week post-natally [37].

TGFβ1 expression is not limited to the period of cushion tissue induction and is thus unlikely to be involved in the inducing event *per se*. Its expression appears to be correlated with morphogenetic movements of the endothelium and mesenchyme. Endothelially-synthesized TGFβ1 protein is mainly localized in the underlying cushion tissue [23, 37], and this is correlated with the localization of extracellular matrix proteins, such as tenascin [37]. the function of TGFβ1 may, therefore, be to regulate growth of the endothelium in the vicinity of the heart valves, and to modulate production of extracellular matrix in cardiac cushion tissue, thus favouring tissue movements essential to remodelling of the heart.

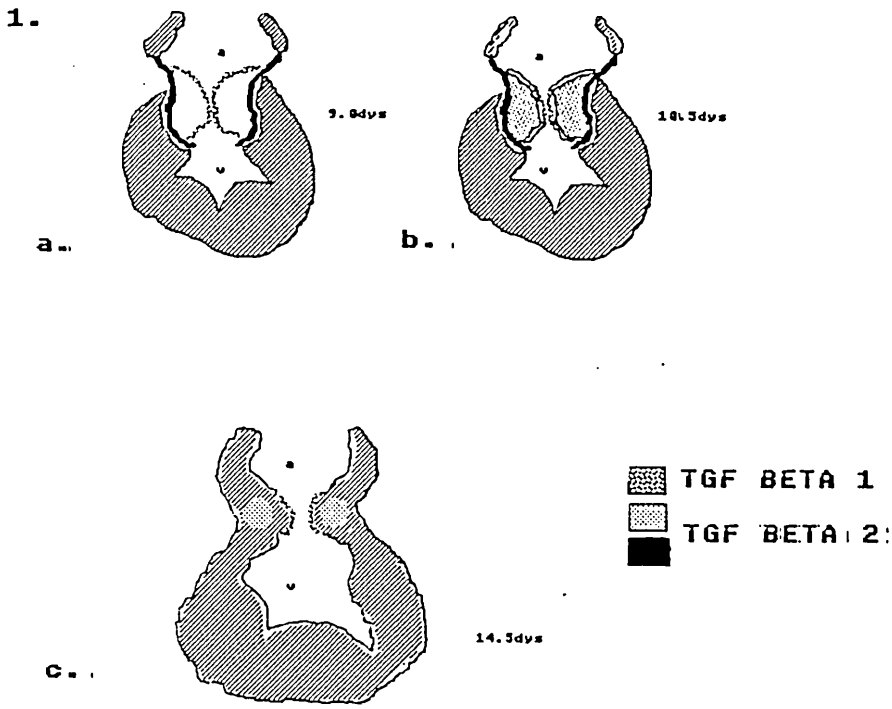

FIGURE 1. Schematic representation of the developing heart showing the pattern of expression of TGFβ1 and β2 during atrioventricular valve (av) formation. As early as 9.5 days *p.c.*, before overt appearance of mesenchymal cushion tissue, there is a regionally restricted distribution of the TGFβ2 transcripts within the myocardium underlying the av valve. TGFβ1 transcripts are localized to the endothelium (part a). The peak of cardiac TGFβ2 RNA occurs at 10.5 days *p.c.*, with a clear spatial restriction pattern to myocardium underlying mesenchymal cushion tissue (part b). By 14.5 days *p.c.* there is no expression of TGFβ2 in the myocardium surrounding the av valve, transcripts being restricted to the mesenchyme of the cushion tissue (part c). TGFβ1 RNA is still expressed in the endothelium at this stage and up to at least one week after birth. a = atria; v = ventricle.

In situ localization of murine TGFβ2 RNA, however, makes it a strong candidate for a component of the inducing signal. This gene is expressed transiently, and in a regionally-restricted manner, in the myocardium underlying the av junction and outflow tract at the time of cushion tissue formation. To a lesser extent, it is also expressed in the cushion tissue *per se* [31].

Since we have shown that av endothelium is a source of TGFβ1, it is surprising that there is a requirement for additional TGFβ in the bioassay used by Potts and Runyan [53]. This might be explained by species differences between mammals and avians, latency of the TGFβ1 protein, requirements for a certain threshold level of TGFβ or requirements for specific isoforms and/or cross-induction of the different TGFβs [2, 54].

SKELETAL DEVELOPMENT

TGFβ is known to have multiple biological effects on mesenchymal cells, chondroblasts, osteoblasts and osteoclasts, affecting cell proliferation, movement, differentiation, secretion of extracellular matrix molecules and even influencing developmental fate of osteoblast progenitor cells [55–57]. Furthermore, adult bone is one of the richest sources of this growth factor. It is, therefore, not surprising that all three TGFβ isoforms, plus the BMP family members [58], are expressed in areas of chondrification and ossification. Expression of each isoform is, however, very specific, both with respect to the cell type, its state of differentiation and body site (see Table 1).

TABLE 1. Differential localization of RNAs encoding TGF β1, β2 and β3 during murine embryogenesis.

	β1[1]	β2[4,5]	β3[4,5]
Haematopoietic tissue	+	−	−
Endothelia	+	−	−
Thyroid	+	−	−
Parathyroid	+	−	−
Thymus	+	−	−
Epithelia			
Whisker follicles	+	+	+
Salivary gland	+	+	−
Tooth bud	+	+	−
Secondary palate[2]	+	−	+
Bronchial epithelium	−	+(s)	+(c)
Optic epithelium	−	+	−
Olfactory epithelium	−	+	−
Lens epithelium	−	+	−
Retina	−	+	−
Hyperplastic nodules	−	+[2]	−
Suprabasal keratinocytes	−	+[3]	−
Cartilage and bone			
Pre-cartilaginous blastema	−	+(limb)	+(iv)
Growth zone of long bone	−	+	−
Perichondria	−	−	+
Hypertrophic cartilage	−	−	−
Osteoblasts, osteoclasts	+	−	−
Cardiac tissue			
Pre-valvular endothelium	+	−	−
Pre-valvular myocardium	−	+	−
Neuronal tissue			
Ventral spinal cord	−	+	−
Ventral fore-brain	−	+	−
Muscle	−	(+)[6]	(+)[6]
Mesothelia	−	−	+
Mesenchyme	−	+	+

Notes; + denotes that RNA is abundant at some stage between 9.5 days *p.c* and birth, though expression may be transient. − is below the detection level of *in situ* hybridization.

[1] Data taken from [25–27]. [2] Data taken from [29]. [3] Data taken from [28]. [4,5] Data taken from [30,31]. [6] Low levels of hybridization are seen in some muscular tissues at some stages, but this is not abundant or ubiquitous.

c = cuboidal; s = squamous; iv = intervertebral disc anlagen.

TGFβ2 and β3 appear to be involved in early stages of formation of the skeletal system, including the condensation of mesenchymal cells to form cartilage precursors [30, 31]. For example, both of these genes are expressed in pre-cartilaginous blastemae; TGFβ2 RNA is seen in the limb buds, whereas TGFβ3 is expressed in the intervertebral disc anlagen [30, 31]. In this respect, it is interesting that both TGFβ2 and, to a lesser extent TGFβ3, are expressed at mesenchymal sites involved in cell proliferation, migration and condensation, such as facial and palatal mesenchyme [29–31]. TGFβ is known to stimulate proliferation and chemotaxis of mesenchymal cells in culture [59, 60] and, indeed, TGFβ1 and β2 were earlier isolated as factors that could induce the formation of chondroblasts from ~~muscle from~~ muscle mesenchymal cells in culture [61].

In contrast, TGFβ1 expression is associated with more overtly differentiated cell types in areas of ossification, namely osteoblasts, osteocytes and osteoclasts [25, 26, 30]. It has been reported that TGFβ2 is also expressed in these cell types [28], though this has been questioned by others [30, 31]. Unlike TGFβ2 and β3, TGFβ1 is, thus, more likely to be involved in control of osteoblast/osteoclast function, including bone remodelling which continues in the adult, and is influenced by osteotropic hormones [62].

TGFβS IN EPITHELIA AND IN EPITHELIAL–MESENCHYMAL INTERACTIONS

It has long been recognized that TGFβ1 is growth inhibitory to most epithelial cell types *in vitro* and *in vivo* [22, 63, 64]. This observation has now been extended to include TGFβ2 and β3, which are, in fact, more potent negative regulators of epithelial cell growth than TGFβ1 [18]. Additionally, some epithelial cell types are induced to differentiate by TGFβ [65–67]. It is, therefore, an interesting observation that all three TGFβ RNAs are expressed at high levels in various embryonic epithelia, often at times of rapid epithelial cell growth (see Table 1) [26, 28–31]. This has raised the question of whether epithelially-synthesized TGFβs are involved in regulation of growth and/or differentiation of the epithelium *per se*, or in paracrine modulation of the adjacent stroma.

Epithelial–Mesenchymal Interactions

TGFβ1 was the first of the isoforms reported to be synthesized in epithelial cells *in vivo* [68]. Surprisingly, the RNA is transiently detected, at high levels, in several specialized epithelia at times of most rapid cell division. Two circumstantial lines of evidence suggest, however, that the major function of the encoded protein is in paracrine interactions with the adjacent mesenchyme. Firstly, the RNA is only seen in epithelia that are actively involved in morphogenetic interactions with the adjacent mesenchyme, such as the salivary gland, tooth bud, whisker follicle and secondary palate [26, 29] (see Fig. 2). Secondly, in each of these situations the encoded protein is localized in the adjacent mesenchyme [23].

Bearing in mind the caveats with interpretation of *in situ* localization and immunolocalization data already discussed, bioactive TGFβ1 may act on the underlying mesenchyme in a variety of ways. It can induce proliferation, differentiation

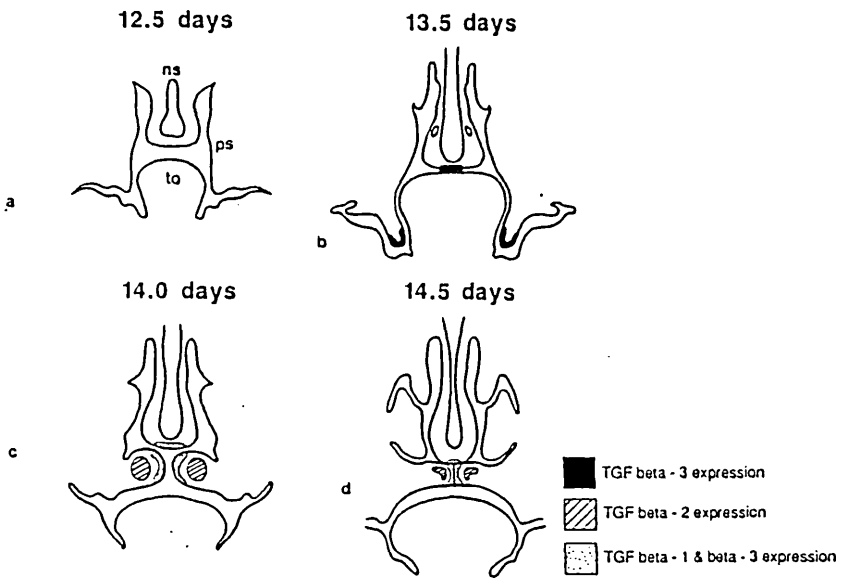

FIGURE 2. Diagrammatic representation of the role of the TGFβ family in palatogenesis. The line drawings represent coronal sections through the midpalate at the gestations (in days indicated above the drawings. The diagrams show the palatal shelves (ps) growing down beside the tongue (to) and then elevating to fuse between the tongue and nasal septum (ns). The filled black areas illustrate TGFβ3 expression, diagonal striping for TGFβ2 and stippled areas for the combined expression of TGFβ1 and β3.

and chemotaxis of mesenchymal cells [56, 59, 60], each of which occurs during morphogenesis. Secondly, it has a major effect on the elaboration of the extracellular matrix (ECM) and on the synthesis of cell surface receptors for ECM components (reviewed in [1]).

It is widely accepted that ECM composition can influence cell-cell and cell-substratum interactions, resulting in differences in cell migration or stabilization of organ structure. Studies in organ culture have emphasized the importance of ECM composition on branching morphogenesis of lung and salivary gland. It has been shown that collagen type III, for example, is essential to cleft formation in the developing salivary gland [69, 70].

Subsequent to studies on TGFβ1, it has been shown that, in each of the cases where this gene is activated in embryonic epithelia, the RNA co-localizes with that of TGFβ2 and/or β3 [29–31] (see Table 1). A good example is the transient epithelial expression of TGFβ1 and β3 during formation of the murine secondary palate [29] (Fig. 2.). Both of these genes are transcribed in the medial edge epithelium of the palatal shelves, as they form, elevate and fuse, and also in the epithelium of the anterior nasal septum, which is destined to fuse with the palate. No information exists on the localization of the corresponding TGFβ3 protein, but one might assume that the two isoforms serve similar paracrine functions within the palatal mesenchyme, stimulating cell proliferation and tissue migration [29].

As in the aforementioned cases (Table 1), epithelially-synthesized TGFβ1 protein co-localizes in the mesenchyme with the ECM component, tenascin, which is one of the many ECM-encoding genes which is transcriptionally up-regulated by TGFβ[71]. This

observation is particularly significant since tenascin is known to disrupt epithelial sheet continuity [72] and to promote the mobility of neural crest-derived cells [73]. Each of these bioactivities is obviously important in morphogenesis of the palate, salivary gland, tooth and whisker follicle.

The Lung and Mammary Gland

The lung and mammary gland represent two further organs in which TGFβs might play a central role in morphogenesis. Heine *et al.* [24], on finding co-localization of extracellular TGFβ1 protein with collagens I and III, fibronectin and proteoglycans in the embryonic lung, have suggested that the ECM-directed branching of this organ is controlled, in part, by epithelially-synthesized TGFβ1. Although TGFβs are most probably involved in lung morphogenesis, the cellular source of the growth factor(s) and the isoform involvement may be debatable.

No expression of TGFβ1 RNA has been detected in bronchial epithelia, though widespread expression is seen in the stroma [26, 31]. In contrast, both TGFβ2 and TGFβ3 RNAs are expressed in bronchial epithelia, albeit in different cell types (Fig. 3).

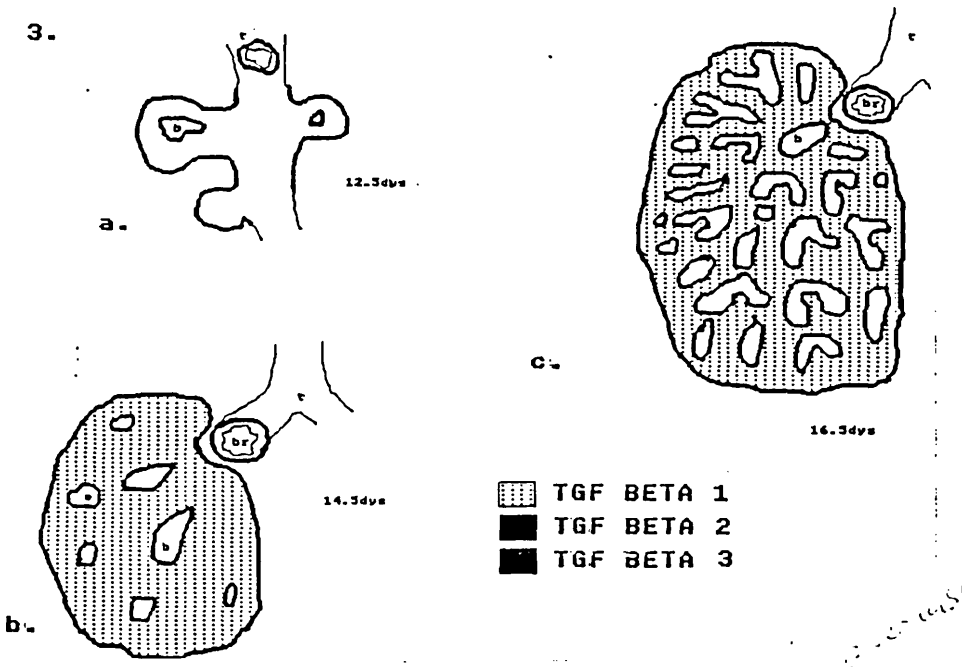

FIGURE 3. Schematic representation of the role of TGF beta isoforms during lung development. The drawings represent sagittal sections through lungs from 12.5 days *p.c.* to 16.5 days *p.c.* TGFβ1 RNA is restricted to the mesenchymal cells of the lung most notably at 14.5 days *p.c.* when the lung is not fully mature. TGFβ3 RNA is seen submucosally in the trachea and proximal bronchi at 12.5 days *p.c.* Simultaneously, intense expression is seen in the immature columnar epithelial cells of the growing bronchioles (a). As lung outgrowth proceeds, TGFβ3 RNA expression remains restricted to the columnear epithelial cells of the proximal respiratory tree. No TGFβ3 expression is seen in the simple cuboidal epithelia of the growing terminal end buds (b). TGFβ2 is restricted entirely to the simply cuboidal cells of the terminal end buds (b), and thus by 16.5 days *p.c.*, expression seems widespread due to the highly differentiated state of the lung (c). t = trachea; b = bronchiole; br = bronchi; e = terminal end bud.

TGFβ3 RNA expression is limited to the immature columnar epithelia cells of the proximal respiratory tract in the early mammalian lung [30, 31]. In contrast, TGFβ2 is seen in the cuboidal epithelium of the growing terminal end buds and in the differentiating alveolar epithelium [31].

Since TGFβ is a potent inducer of bronchial epithelial cell differentiation [66], it is tempting to speculate that this is the endogenous role of TGFβ2 in the differentiating alveoli. However, each of the three isoforms might also be required for control of epithelial growth or branching morphogenesis. At present, no protein localization data exist for TGFβ2 or β3, and no studies on embryonic pulmonary TGFβ function have been reported *in vivo* or in organ culture.

In the mammary gland, a different experimental approach has been taken. As described earlier, using plastic implants impregnated with active TGFβ *in vivo*, it has been found that this growth factor has profound effects on terminal end bud growth, which could not only affect extent of growth, but also the branching pattern of the organ [22]. The response of the epithelial cell was dependent on the exact cell type and position within the mammary gland. As yet, however, the endogenous isoform distribution of TGFβ RNAs or proteins has not been mapped for this developing organ.

Auto-Regulation of Epithelial Growth and Differentiation

Several circumstantial lines of evidence suggest that TGFβ2 might be endogenous auto-regulator of epithelial growth and/or differentiation. Glick *et al.* [74] have demonstrated that TGFβ2 levels are elevated *in vitro* and *in vivo* in keratinocytes treated with retinoic acid or calcium ions, which induce growth arrest and differentiation, respectively. Secondly, in the embryo, TGFβ2 RNA levels are very high in the differentiating epithelial component of several established structures. These include epithelia of the sense organs, lung alveolar epithelium [30, 31], hyperplastic nodules (rugae) of palatal oral epithelia [29], and supra-basal keratinocytes [28] (see Table 1). Furthermore, where it is possible to distinguish the differentiation and dividing cell compartments, it appears that the differentiated cells are the source of this growth factor. Bearing in mind the strong negative growth regulation exerted by TGFβs on epithelial cells, one might imagine a situation where TGFβ2, produced from differentiated epithelial cells, acts on the dividing cell compartment in a paracrine feedback loop, as suggested previously for TGFβ1 [68]. The localization of TGFβ2 protein within the epithelium *per se*, following retinoic acid treatment of adult mouse skin, would give support to this theory [74].

NEURONAL DEVELOPMENT

The presence of TGFβ2 RNA within the sensory epithelia of the ear, eye and nose (Table 1) could be correlated with differentiation of the epithelium, as discussed above, or be associated with the innervation of the epithelium. It is interesting that TGFβ2 RNA is expressed transiently in the ventral nervous system of the early mammalian embryo [30, 31], and that TGFβ2 and β3 proteins have been detected immunohistochemically in central and peripheral nervous systems [75]. Flanders *et al* [75] suggested that these growth factors are important in the regulation of neuronal cell migration and

differentiation and of glial cell proliferation and differentiation.

CONCLUSIONS AND PROSPECTS

In summary, it can be stated that, *in vitro*, each of the mammalian TGFβs possesses quantitative, if not qualitative, functional specificity, and, *in vivo*, that each isoform has a distinct temporal and spatial pattern of expression throughout mammalian embryogenesis. The extreme amino acid sequence conservation of individual isoforms between vertebrates, as compared with isoform variation with individual species, coupled with the conservations of embryonic expression patterns within mammals [30], would suggest that, indeed, each isoform has a distinct function *in vivo*.

To test this proposition, it is essential that more functional studies are carried out, for example using organ culture to examine epithelial–mesenchymal interactions, or transgenic mice to manipulate isoform expression or isoform function.

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The Role of TGFβ in Mouse Development[a]

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INTRODUCTION

The development of a multicellular organism from a single fertilized egg cell involves many biological processes including cell growth, migration, differentiation, inductive interactions and transdifferentiation, as well as synthesis and modulation of the extracellular matrix. All of these are strictly regulated within space and time. Transforming growth factor-β (TGFβ) is known to play a central role in each of these processes in many cell types *in vitro*. All five TGFβ family members, which have been molecularly cloned, are known to be expressed at some stage during embryogenesis. The widespread embryonic and fetal expression of TGFβ has been reported extensively.[1–6] We have also observed widespread expression of TGFβ2 during mouse embryogenesis (unpublished data). TGFβ3 RNA is present in human umbilical cord.[7] TGFβ3 and TGFβ4 are expressed in embryonic chick cells *in vitro*[8,9] and TGFβ5 is present in frog oocytes and early embryos.[10] These observations suggest that this family of growth factors is important in modulating embryonic processes.

In nonmammalian organisms, TGFβ-related molecules are known to function in major morphogenetic events. In *Drosophila* the decapentaplegic gene product is essential for the establishment of dorsoventral pattern[11] and the induction of mesoderm in *Xenopus* is thought to involve at least one TGFβ-related molecule. A mesoderm-inducing factor isolated from *Xenopus* XTC cells shows amino acid homology to the TGFβs.[12] and another *Xenopus* gene product, Vg1, which shows expression restricted to mesoderm-inducing tissues, is TGFβ-related.[13] Furthermore, mesoderm induction in the frog system can be blocked by antibodies raised against mammalian TGFβ2.[14] This growth factor can, in fact, induce mesoderm formation in this heterologous system,[14] whereas mammalian TGFβ1 can only enhance the mesoderm-inducing activity elicited by fibroblast growth factor.[15]

In mammals, in addition to the TGFβ family *per se*, there are several more distantly related molecules, some of which are known to regulate late developmental processes. Mullerian inhibitory substance, for example, actively aids the regression

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of oviduct precursors in male fetuses.[16] This involves cellular transformation from epithelial to mesenchymal cells and could be likened to mesoderm induction during early embryogenesis.

Until recently, our understanding of the role of the TGFβ in mammalian embryogenesis has mainly been gained from studies on the effects of TGFβ on isolated cell types in culture. More recently this information has been augmented by molecular studies on the expression patterns of TGFβ genes[3-6] and their protein products[1,2] *in vivo* both in human and mouse embryos. In the mouse the TGFβ1 gene is activated soon after fertilization[6] and, together with TGFα, could be responsible for the TGF-like bioactivity detected in the culture medium of preimplantation embryos.[17] Studies by *in situ* hybridization and immunohistochemistry show that this gene then remains active during postimplantation development, exhibiting a distinct spatial and temporal pattern of expression, giving clues as to its functional role in embryogenesis.[1-5]

In this paper we review the patterns of expression of the TGFβ1 gene, which we have observed by *in situ* hybridization during murine embryogenesis,[5-18] and relate these findings to other studies of both a functional and descriptive nature.

HEMATOPOIESIS, VASCULARIZATION, AND ANGIOGENESIS

Heine *et al.* have previously demonstrated that in midgestation mouse embryos the TGFβ1 gene is transcribed to give a 2.5 kb RNA.[2] To investigate the tissue localization of expression of these transcripts, we performed *in situ* hybridization to 5 μm tissue sections of mouse embryos from 6.5 to 16.5 days *post coitum* (p.c.) using a cDNA probe specific for TGFβ1.[19] To avoid the possibility of crosshybridization between the closely related members of the TGFβ gene family, we subcloned a portion of the full length cDNA from within the precursor region of the protein (encoding amino acids 68 to 268) to generate a gene-specific probe.[18]

We have not unequivocally demonstrated TGFβ1 levels in pre-implantation embryos by *in situ* hybridization due to the limits of the sensitivity of the technique. Rapolee *et al.* used the polymerase chain reaction to amplify cDNA complementary to RNA from preimplantation embryos and have shown that the TGFβ1 gene is, in fact, activated very soon after fertilization.[6] Thereafter the gene is probably transcriptionally active to some extent in all tissues. Certainly most cell lines *in vitro* express at least a low level of TGFβ1 transcripts, and the general intensity of *in situ* hybridization over most embryonic tissues is slightly higher with the anti-sense probe to TGFβ1 than it is with a sense probe.

The first appearance of high concentrations of TGFβ1 RNA within the embryo proper occurs at 7 days p.c. when the blood islands within the yolk sac show intense hybridization to the anti-sense probe (FIG. 1). A similar observation has been made by Wilcox and Derynck,[4] examining mouse embryos at 9.5 days p.c. The blood islands are the major sites of hematopoiesis in the early embryo. Hematopoietic stem cells colonizing the body of the late embryo first appear in the fetal liver at midgestation and later populate the adult spleen and bone-marrow. At 7 days p.c. the allantois also possesses high levels of TGFβ1 RNA, probably being related to active vascularization (FIG. 1).

At 9.5 to 10.5 days p.c. the liver begins to show high levels of TGFβ1 expression, which is correlated with the time of appearance of hematopoietic stem cells in this organ (FIG. 1). Over the next few days of development large isolated cells with lobular nuclei, which we identify as megakaryocytes, appear within the fetal liver. These cells

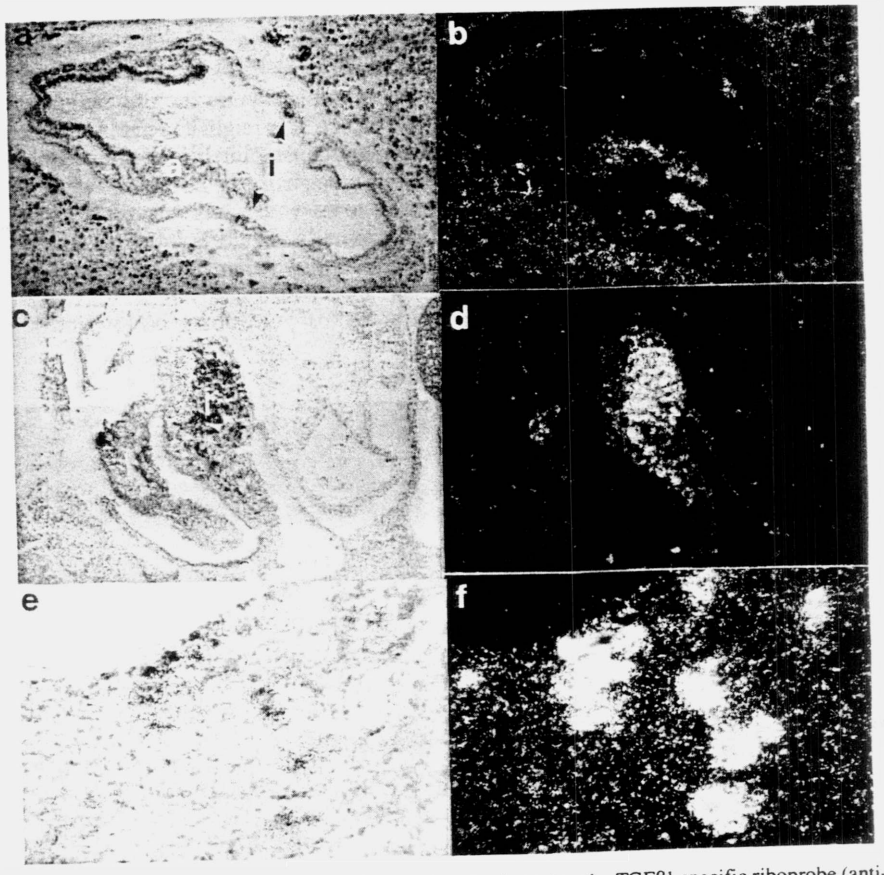

FIGURE 1. TGFβ1 in hematopoiesis. *In situ* hybridization using the TGFβ1-specific riboprobe (anti-sense), (a,c,e) bright field; (b,d,f) dark field. (a,b) 7.5 day p.c. embryo; (c,d) 10.5 day p.c. liver; (e,f) adult spleen. a, allantois; i, blood island; l, liver. (Magnification ×37.5, reduced by 20%.)

hybridize intensely with the anti-sense probe, being the most abundant source of TGFβ1 RNA throughout the development of the mouse (FIG. 1). The hybridization intensity of these cells is an order of magnitude higher than that to fetal bone. Again our results confirm those of Wilcox and Derynck[4] and of Ellingsworth *et al.*,[1] who have shown immunolocalization of TGFβ to megakaryocytes of fetal liver and bone marrow. The results are also predictable since a major source of TGFβ is the blood platelets.

The adult spleen, like the fetal liver, shows similar high levels of TGFβ1 expression (FIG. 1). This growth factor is clearly involved in hematopoiesis although it is expressed at a time of rapid cell division, when it is known to be suppressive to growth of hematopoietic stem cells. It is also inhibitory to megakaryocytopoiesis, suggesting that it may play an important role as a negative autocrine regulator of these cells.

In addition to the obvious sites of hematopoiesis, TGFβ1 RNA is seen in areas of the embryo undergoing vascularization and angiogenesis. An early site of TGFβ1

expression is in the presumptive heart mesoderm during a time when the primitive vascular system is forming (7 days p.c.).[18] In the 8.5 day p.c. embryo the head mesenchyme, which is enriched in capillaries, is also positive (FIG. 2). Later in embryogenesis TGFβ1 expression is seen in certain areas of mesenchyme, such as the gut submucosa and the stroma of the kidney and lung (FIG. 2). This might be related to regions undergoing active angiogenesis. The resolution of the *in situ* hybridization technique does permit us to identify precisely which cell types express TGFβ.

It is well documented that TGFβ is a potent angiogenic factor[20] and has multiple effects on both vascular and capillary endothelial cells in culture.[21,22] Presumably it acts in an autocrine manner on these cell types during mammalian embryogenesis.

Finally we have also observed positive hybridization to macrophage particularly

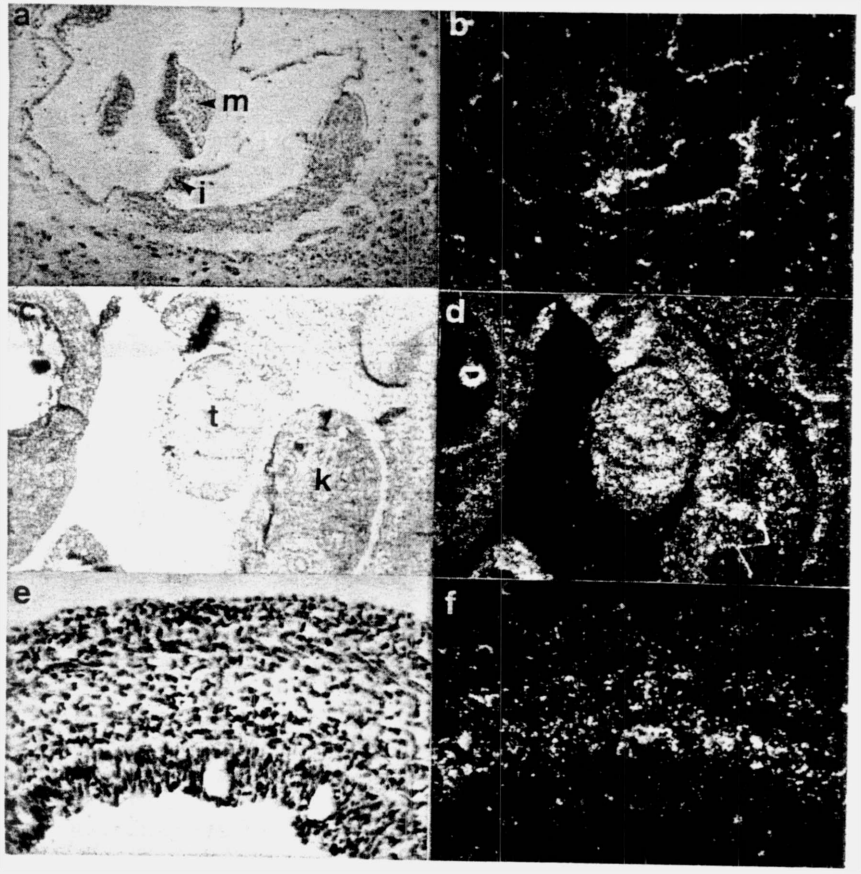

FIGURE 2. TGFβ1 in mesenchymal tissue. *In situ* hybridization with anti-sense TGFβ1-specific riboprobe. (a,c,e) bright field; (b,d,f) dark field. (a,b) 8.5 day p.c. embryo; (c,d) 14.5 day p.c. kidney and testis. (Magnification ×37.5, reduced by 20%.). (e,f) 14.5 day p.c. gut. (Magnification ×150, reduced by 20%.) **m**, head mesenchyme; **t**, testis; **k**, kidney; **i**, blood island.

within the dermis. Again, macrophage is a well established source of TGFβ; this cell type acts as a delivery system for growth factors to sites of wounding. TGFβ is also an autocrine macrophage deactivator.[23]

TGFβ DURING SKELETAL DEVELOPMENT

A striking observation in our studies was the strong hybridization of the TGFβ antisense probe to developing bone tissue (FIG. 3). This is seen both during endochondral ossification, as in the ribs and limb bones, and during intramembranous ossification, as in the developing skull and mandibular bone of the 14.5 to 16.5 day p.c. embryo. Hybridization was restricted to the perichondral envelope and the diaphyses of the

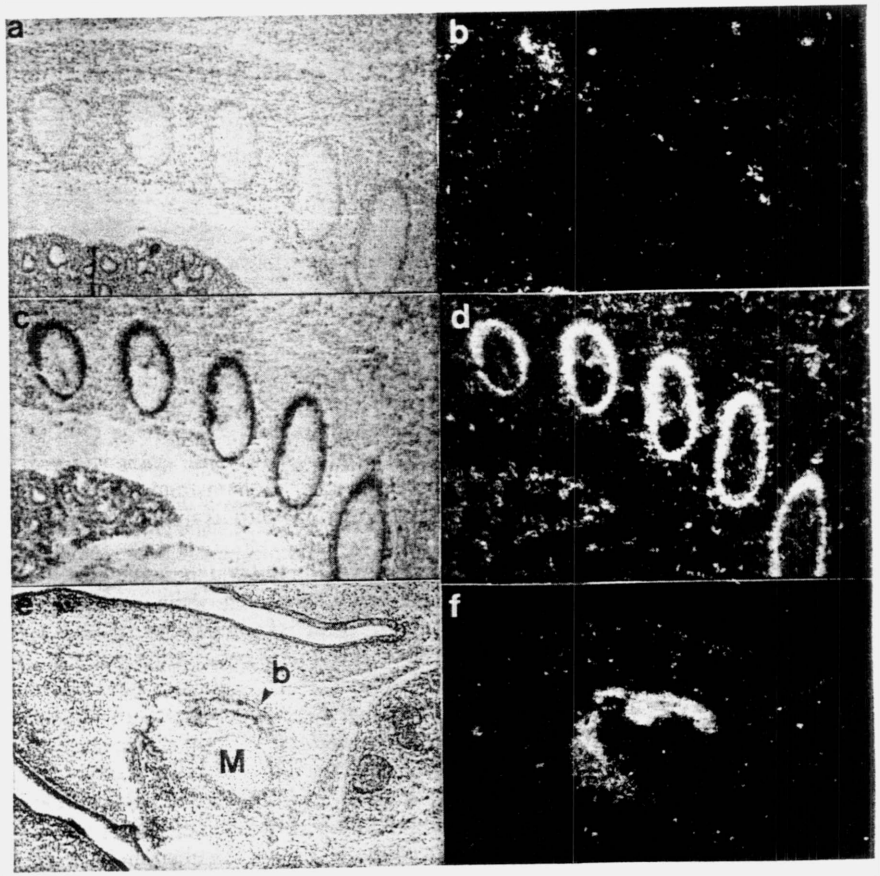

FIGURE 3. TGFβ1 in bone. *In situ* hybridization with antisense (c–f) and sense (a,b) TGFβ1 riboprobe. (a,c,e) bright field; (b,d,f) dark field. (a–d) ribs of 14.4 day p.c. embryo; (e,f) membranous bone in lower mandible of 14.5 day p.c. embryo. **M**, Meckel's cartilage; **b**, bone. (Magnification ×37.5, reduced by 20%.)

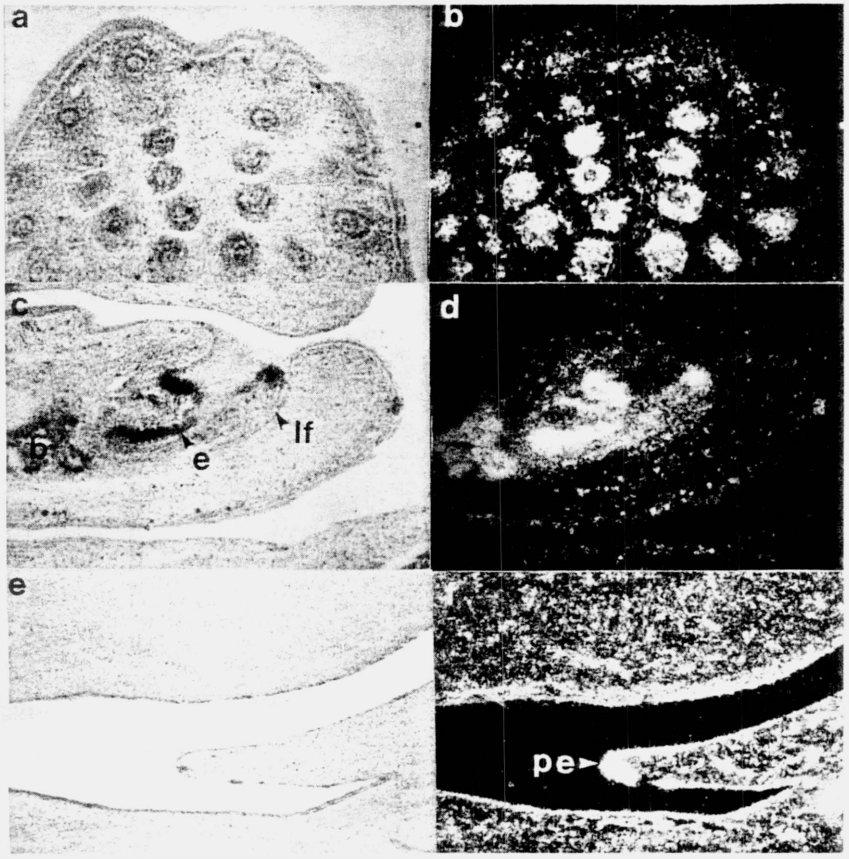

FIGURE 5. TGFβ1 in embryonic epithelia. *In situ* hybridization using anti-sense TGFβ1 riboprobe. (a,c,e) bright field; (b,d,f) dark field. (a,b) whisker pad (14.5 days p.c.); (c,d) tooth (15.5 days p.c.); (e,f) secondary palate (15 days p.c.). **b**, bone; **lf**, lip furrow; **e**, dental epithelium; **pe**, palatal epithelium. (Magnification ×37.5, reduced by 20%.)

in each of these cases their immunohistochemical data show that the protein is localized in the adjacent mesenchymal tissue. The absence of TGFβ1 protein in a tissue expressing high levels of the RNA may be explained. It is known that the TGFβ1 precursor protein is secreted in a biologically inactive complex[33] and it is possible that the latent precursor form of TGFβ1 is not recognized by the antibody used by Heine *et al.* or that the newly synthesized protein is rapidly transported from the epithelial cells preempting the intracellular accumulation of protein. The published immunolocalization data might thus characterize sites of mature TGFβ1 protein rather than sites of synthesis which, in areas of epithelial/mesenchymal interaction appear to be distinct. A precedent for this differential localization of transcripts and protein is set by nerve growth factor (NGF). NGF RNA is localized in the epithelium of its target tissue, whereas the protein is present in the mesenchyme and nerves themselves.[34]

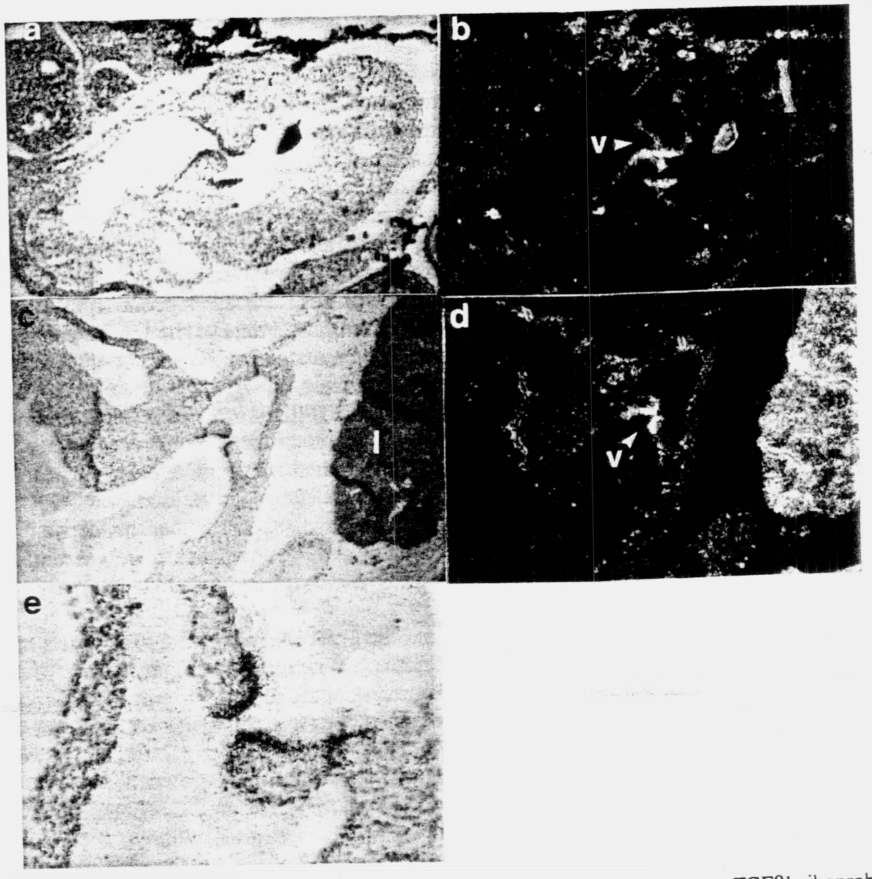

FIGURE 6. TGFβ1 expression in the heart. *In situ* hybridization using anti-sense TGFβ1 riboprobe. (a,c,e) bright field; (b,d) dark field. (a,b) section through atrioventricular valve region of 12.5 day p.c. heart; (c–e) section through valve of outflow tract of 16.5 day p.c. heart. **v**, valve; **l**, liver. (a–d) (Magnification ×37.5, reduced by 20%.) (e) (Magnification ×150, reduced by 20%.)

An alternative explanation, which we cannot yet exclude, is that the antibody used by Heine *et al.* recognizes related members of the TGFβ family. We favor the former explanation and thus propose that TGFβ1 is synthesized in morphogenetically active epithelia and the protein acts in a paracrine manner on the underlying mesenchyme.

Examples of the differential localization of TGFβ RNA and protein include all the above mentioned tissues, and in each case is correlated with regions where epithelial/mesenchymal interactions are of central importance and with periods of intense epithelial growth, cell migration, and mesenchymal condensation. A most likely role for epithelially derived TGFβ1 in organogenesis is in elaboration of the extracellular matrix produced by the mesenchymal cells,[22,35–37] and in induction of synthesis of cell surface receptors for components of the extracellular matrix.[38,39] The composition of the extracellular matrix can influence cellular phenotype in several manners, having

effects on cell growth, differentiation, and migration, not only of the mesenchymal cells[37] but also of the overlying epithelium.[40] TGFβ1 elevates the transcription of many genes encoding extracellular factors[35,36] and their cell surface receptors[39] and it inhibits the production of degradative enzymes such as plasminogen activator, whilst activating the production of inhibitors of these enzymes.[41-43] It could thus extensively influence the remodeling of organ systems via this matrix. The developmental distribution of several extracellular molecules that are modulated by TGFβ has been examined, including specialized proteins such as tenascin,[40] SPARC,[44] and TIMP.[45] Most striking is the embryonic pattern of tenascin distribution, which is known to be synthesized in response to TGFβ. This protein shows a remarkable codistribution with TGFβ1 polypeptide including expression in bone, teeth, whisker follicles,[40] secondary palate, and heart mesenchyme (Akhurst and Faissner, unpublished data). Though we cannot yet prove a causal relationship, it is tempting to speculate that the effects of TGFβ1 are partially mediated by molecules such as tenascin.

It is also possible that epithelially synthesized TGFβ1 is a negative regulator of the epithelium *per se*, controlling proliferation and differentiation. We have previously demonstrated that keratinocytes stimulated to divide and differentiate *in vivo* by topical application of 12-tetradecanoyl-phorbol-13-acetate (TPA) show induction of TGFβ1 RNA in the differentiating cell compartment.[46] We have suggested that this acts as a natural regulator of epithelial homeostasis. Manipulative experiments are currently underway to examine the role of TGFβ in epithelial growth control and epithelial/mesenchymal interactions.

It should be emphasized that not all embryonic epithelia synthesize high levels of TGFβ1. We have not observed RNA in the epithelial component of gut, kidney, and lung, nor in the pituitary gland or sensory epithelium of the embryonic ear. Most strikingly the interfollicular embryonic epidermis does not express significantly elevated levels of TGFβ1 RNA during its critical stages of growth and differentiation, as we had initially expected since this gene is activated in differentiating keratinocytes following stimulation with TPA.[46] It remains to be seen whether other TGFβ genes may be involved in regulation of growth and differentiation of these epithelia.

CARDIAC DEVELOPMENT

The mammalian heart develops as a double tube consisting of an inner endocardium and outer epimyocardium, both derived from the splanchnic mesoderm. Differential growth rates along this tube lead to folding and the formation of sulci, as the heart begins to show regionalization into four chambers. Septation of the heart and major blood vessels is completed by the formation, swelling, and fusion of the endocardial cushion tissue. This is a mesenchymal mass, embedded in an elaborate extracellular matrix, and derived from epithelial/mesenchymal transformation of the endothelium in a regionally specific manner. The heart valves are also derived from the endocardial cushion tissue.[47]

TGFβ1 RNA is observed in the presumptive heart mesoderm as early as 7 days p.c. At 8.5 days, when the myocardium is quite distinct from the endocardium, expression is clearly restricted to the endothelial cells. This expression pattern becomes regionalized by 10.5 days p.c., becoming limited only to endothelia overlying the cushion tissue. This includes the cushion tissue of the atrioventricular region and of the bulbar ridges within the bulbus arteriosus, which contributes to the pulmonary and aortic

valves. As development proceeds, TGFβ1 expression persists in the cardiac epithelium, localized specifically in areas of endocardial cushion tissue and resultant valves (FIG. 6). Not until 7 days after birth, as the valves attain the gross morphology of the adult, does TGFβ1 expression cease.[18]

Similar to the situation seen in other embryonic epithelia expressing TGFβ1 RNA, the polypeptide appears restricted entirely to the underlying mesenchyme of the cushion tissue and valves,[2] suggesting that TGFβ plays a paracrine role in formation of heart valves and septa, possibly mediating its effects via the extracellular matrix.

Recently it has been demonstrated by Potts and Runyon[47] that TGFβ plays an essential function in the epithelial/mesenchymal transition essential for formation of the atrioventricular cushion tissue. A TGFβ-blocking antibody can inhibit this process in explants of chick heart *in vitro*. Furthermore, TGFβ1 can, together with an unidentified component of ventricular myocardium, induce the epithelial/mesenchymal transformation of atrioventricular endocardium *in vitro*. In the absence of TGFβ, ventricular myocardium cannot support this activity. Clearly in the chick, TGFβ can influence the processes involved in septation and valve formation in the heart. The presence of TGFβ1 RNA in the presumptive heart valves of the mouse strongly implicates this growth factor as playing a major role in this important developmental process.

GONADAL EXPRESSION OF TGFβ1

In addition to expression of TGFβ1 during the various developmental processes listed above, we have observed significant expression of TGFβ1 RNA in the gonads. At 14.5 day p.c. TGFβ1 RNA is prevalent in the testis cords (FIG. 2). At this stage in development the spermatogonia and Sertoli cells are morphologically indistinguishable. Benahmed *et al.*[48] have shown that TGFβ is secreted by immature Sertoli cells in culture. It is possible that the growth factor in this case is involved in control of steroidogenesis. TGFβ1 RNA expression is also seen in the stroma of the 14.5 day p.c. ovary (data not shown).

CONCLUSIONS

All of the data on RNA distribution patterns must be interpreted with caution since TGFβ1 is known to exhibit extreme control by post-transcriptional processes in certain tissues. Even when examining both RNA and protein patterns during embryogenesis, one has to consider rates of secretion, activation of the latent form, stability of the protein, and ability of the target cell to respond to the signal.

We, and others, have shown that TGFβ1 RNA is expressed at high levels in tissues undergoing osteogenesis, hematopoiesis, vascularization, and angiogenesis. Together with the protein distribution studies of Ellingsworth *et al.*[1] and Heine *et al.*,[2] and the known biological activities of TGFβ1 in these tissues, we would propose that TGFβ1 acts in an autocrine mode on these cell types.

Our data together with the data of Heine *et al.*[2] would suggest that epithelially synthesized TGFβ1 acts in a paracrine manner on the underlying mesenchyme, possibly acting there to modulate mesenchymal condensation and elaborate the extracellular matrix during peaks of morphogenetic activity. In addition, this growth factor may be an autocrine regulator of epithelial growth and differentiation.[46] It will now be es-

sential to test these various ideas by manipulating levels of TGFβ expression *in vivo* to examine biological effects.

It is clear that TGFβ1 is a multifunctional regulator of embryonic development, modulating several processes some of which are of major clinical relevance, such as cardiac and craniofacial development. With this gene family ever increasing in size as new members are molecularly cloned, it remains to be seen what other developmental functions may be regulated by the new TGFβs.

ACKNOWLEDGMENTS

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Expression of TGF-β isoforms during first trimester human embryogenesis

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Summary

We have studied the expression of the genes encoding transforming growth factors (TGFs) β1, β2 and β3 in human embryos ranging from 32 to 57 days *post-coitum*, using *in situ* hybridization. The spatial and temporal pattern of expression of each gene is distinct, though each occasionally overlaps. TGF-β1 is expressed in haematopoietic, endothelial and osteogenic tissues. TGF-β2 and TGF-β3 are expressed in a wide variety of mesenchymal tissues including areas of chondrogenic activity. TGF-β2 is also found in several epithelia and in the ventral nervous system. The differential transcript distributions are broadly similar to those seen in mouse embryos suggesting that there is conservation of TGF-β gene regulatory sequences and developmental function across this species boundary.

Key words: TGF-β, *in situ* hybridization, human embryo, gene expression.

Introduction

The first member of the TGF beta family to be molecularly cloned was human TGF-β1 (Derynck *et al.* 1985), which has also been the most intensively studied in terms of biological function *in vitro* (Roberts and Sporn, 1990). This was followed fairly shortly by the cloning of TGF-β2 (Madisen *et al.* 1988) and TGF-β3 (ten Dijke *et al.* 1988). The gene family possesses many related members, including the chick and amphibian TGF-βs 4–8 (Jakowlew *et al.* 1988*a*; Melton, 1990), and the more distantly related mammalian proteins, such as the inhibins/activins (Mason *et al.* 1985) and Müllerian inhibitory substance (Cate *et al.* 1986). In human, no more than three true TGFβs have been identified to date.

The interspecies amino acid sequence conservation within the active domain of TGF-β1 is virtually complete (Derynck *et al.* 1985, 1987; Sharples *et al.* 1987; Van Obberghen-Schilling *et al.* 1987; Jakowlew *et al.* 1988*b*). The processed TGF-β1 proteins of human, bovine, chicken, porcine and simian are identical, and, between man and mouse, there is only one amino acid substitution in this region (Derynck *et al.* 1986). The same high degree of conservation is found for TGF-β2 and TGF-β3 (de Martin *et al.* 1987; Madisen *et al.* 1988; Cheifetz *et al.* 1987; Seyedin *et al.* 1985; Derynck *et al.* 1988; ten Dijke *et al.* 1988). Intraspecies divergence between the different TGF-β family members is much greater (70%) (Madisen *et al.* 1988; ten Dijke *et al.* 1988). On the basis of these observations, it is reasonable to suppose that the functions of each individual TGF-β are identical across the range of vertebrate species that possess them, and possibly, that each family member might be functionally distinct. However, there may be interspecies variation in the regulation and differential utilization of members of the TGF-β family during embryogenesis.

Interest in the role of TGF-β genes in mammalian development has been stimulated by homologies with developmentally important genes such as mammalian Müllerian inhibitory substance (Cate *et al.* 1986), the *Decapentaplegic* (DPP-C) gene of *Drosophila* (Padgett *et al.* 1987) and the *Vg1* cDNA clone of *Xenopus* (Weeks and Melton, 1987), and by the observation that exogenous mammalian TGF-β proteins can stimulate mesoderm induction in *Xenopus* tissue (Kimelman and Kirschner, 1987; Rosa *et al.* 1988). Furthermore, TGF-β has been detected in embryonal carcinoma cell lines, which have common features with early mammalian embryos (Rizzino *et al.* 1983, Rizzino, 1987; Mummery *et al.* 1990).

Expression of TGF-βs during murine embryogenesis has been studied immunohistochemically (Heine *et al.* 1987), by RNA gel blot analysis (Miller *et al.* 1989*a*,*b*; Denhez *et al.* 1990), by *in situ* hybridization (Wilcox and Derynck, 1988; Lehnert and Akhurst, 1988; Pelton *et al.* 1989; Akhurst *et al.* 1990*a*; Fitzpatrick *et al.* 1990) and by polymerase chain reaction (Rappolee *et al.* 1988). These studies have revealed that the expression patterns of TGF-β1, TGF-β2 and TGF-β3 RNA are distinct and that, in some cases, there is variation

between the localization of TGF-β1 RNA and its mature protein. Both paracrine and autocrine modes of action of TGF-β1 and TGF-β2 have been proposed based on these models (Lehnert and Akhurst, 1988; Pelton *et al.* 1989). There is very little published data on murine TGF-β3 gene expression (Miller *et al.* 1989*a*; Denhez *et al.* 1990; Fitzpatrick *et al.* 1990) and data on the expression of TGF-β genes in human embryogenesis is limited to the work of Sandberg *et al.* (1988*a*,*b*), who examined TGF-β1 transcript distribution in the process of endochondral and intramembramous ossification of second trimester human fetuses.

An understanding of the molecular mechanisms controlling mammalian development is fundamental to our comprehension of the basis of congenital malformation. In order to validate the use of other mammalian species as models for human embryogenesis it is essential to determine whether key molecular developmental processes are similar across species barriers. The work presented in this paper is an investigation of the expression of TGF-β1, TGF-β2 and TGF-β3 in human embryogenesis using *in situ* hybridization. The results are discussed in the light of results of similar studies which have been performed on mouse embryos (Wilcox and Derynck, 1988; Lehnert and Akhurst, 1988; Pelton *et al.* 1989; Akhurst *et al.* 1990*a*,*b*; Fitzpatrick *et al.* 1990; Millan *et al.* 1990).

Materials and methods

Embryology

Intact human embryos 32–57 days post-conception (*p.c.*) were obtained following therapeutic abortion using mifepristone and gemeprost prostoglandin pessaries. These were processed for *in situ* hybridization according to the protocol of Wilkinson *et al.* (1987). Sex was determined by karyotype analysis. Embryonic stage was estimated by crown–rump length, embryo weight and morphological appearance; the estimated date of the last menstrual period was found to be less reliable. Staging relative to mouse embryos is difficult since different organ systems develop at different rates in the two species, however, we attempted to do this according to the observations of Rugh (1968). Supplementary material of 10 to 12 weeks gestational age (mainly limbs) were collected by suction termination and similarly processed.

The embryos obtained were as follows:

RU113: 44 days post-last menstrual period (LMP). No chromosome analysis was performed on this embryo, assessed as about 32 days *p.c.* on the basis of morphology. Crown–rump length could not be measured due to distortion. The embryonic weight was 0.025 g. This embryo had a well-developed liver and the rudiments of limb buds. The heart was in the process of septation. The gut and mesonephros were distinguishable but the lung buds could not be identified. This is *approximately* equivalent to a 10 to 10.5 day *p.c.* mouse embryo.

RU74: Assessed chromosomally as Y-negative and morphologically as 43 days gestation. The crown–rump length was 12 mm and the weight was 0.213 g. The tail was torn off, and the embryo had already been sectioned sagittally for histological purposes and was therefore lacking its left side to a thickness of about 1/4 of its total. Lung buds were visible and the limb buds were well developed though there were no presumptive digits. The lens vesicle was closed and separate from the surface. This embryo is *approximately* equivalent to an 11 to 11.5 day *p.c.* mouse embryo.

RU118: 58 days post-LMP. No chromosomal analysis was performed on this embryo, assessed as 47 days *p.c.* on the basis of fetal morphology. Crown–rump length was 19 mm and the weight was 0.614 g. The lungs, limbs and eyes were noticeably more advanced than in RU74. The tongue was readily distinguishable, although the pinnae were scarcely developed. This gives an *approximate* correspondence to a 12 to 13 day *p.c.* mouse embryo.

RU59: 71 days post-LMP. A chromosomally normal male fetus assessed as 57 days of gestation on the basis of morphology. The gut was herniated and there was a small degree of abrasion on the skin in many areas. Weight was 1.41 g and the crown–rump length was 25 mm. The fingers were separated distally, very primitive toothbuds were visible, and intramembranous ossification had begun. However, the eyelids and palate were unfused. This is *approximately* equivalent to a 14 to 15 day *p.c.* mouse embryo.

A summary of the biological material used is shown in Table 1.

Table 1. *Quantitative characteristics of embryos studied*

Gestational age (days post-ovulation)	Crown–Rump Length (mm)	Embryonic wet weight (mg)
32	5*	25
43	12	213
47	19	614
57	25	1410

* Estimated crown–rump length, according to Hamilton and Mossman (1972).

Probes

Full-length cDNA probes encoding human TGF-β1 (Derynck *et al.* 1985) and TGF-β2 (Madisen *et al.* 1988) were kindly supplied by Dr G. I. Bell (Howard Hughes Institute, Chicago) and Dr A. F. Purchio (Oncogen, Seattle) respectively. A probe for human cardiac actin was kindly provided by Dr P. Barton (Heart and Lung Institute, London), and Dr J. C. Fiddes (California Biotechnology Inc.) supplied the human chorionic gonadotropin gene probe. The TGF-β3 probe was described by ten Dijke *et al.* (1988).

Gene-specific probes were generated by subcloning sequences from the non-conserved 5′ non-coding or precursor coding regions into Bluescribe or Bluescript plasmid vectors. The gene-specific TGF-β1 subclone was a 712 nucleotide *Eco*RI–*Pst*I fragment from the 5′ non-coding region, inserted antisense with respect to the T3 promoter. The gene-specific TGF-β2 subclone was a 450-nucleotide *Ava*I–*Hae*II fragment spanning amino acid residues 61 to 211, inserted antisense with respect to the T3 promoter. The gene-specific TGF-β3 subclone was a 463 nucleotide fragment spanning from 16 nucleotides upstream of the translation initiation site to amino acid 149, and inserted antisense with respect to the T7 promoter. This latter subclone also contained the conserved 30 amino acid coding region found at the *N*-terminus of the TGF-β precursor polypeptides.

Probe synthesis

^{35}S-labelled single-stranded riboprobes were generated to a specific activity of 8×10^{8} disints min^{-1} μg^{-1} using the Blue-

scribe or Bluescript T3 and T7 transcription system (Vector Cloning Systems). Probes were digested to an average length of 100 nucleotides by controlled alkaline hydrolysis (Cox *et al.* 1984). For the negative control probe, a human chorionic gonadotropin cDNA probe, kindly supplied by J.C. Fiddes (Fiddes and Goodman, 1980), was subcloned into the Bluescribe vector and the T7 promoter was used to generate antisense RNA. Both full-length cRNA probes and gene-specific subclone cRNA probes were utilized. No difference was found between these two categories, indicating that the *in situ* protocol is stringent enough to avoid cross-hybridization within the gene family.

In situ *hybridization*

In situ hybridizations were performed essentially according to the protocol of Wilkinson *et al.* (1987), except that the hybridizations were carried out at 52°C. Probe concentration was adjusted to 2 to 5×10^4 disints min^{-1} μl^{-1}. Autoradiographic exposure times were between 1 and 2 weeks. After development, slides were counterstained in haematoxylin and eosin and examined using an Olympus BK2 microscope. Photomicrography was performed using Kodak Panatomic X film.

Results

TGF-β gene expression in the developing liver

TGF-β1 is the only member of the TGF-β gene family to be expressed at high level within the embryonic liver (Fig. 1 A,B). At 43 and 57 days *p.c.*, intense expression of TGF-β1 RNA is seen in megakaryocytes (Fig. 1E–H) and, to a lesser extent, in certain cell clusters that are probably haematopoietic progenitors. These cells had not appeared at 32 days *p.c.* Primitive parenchymal cells do not express the gene at any time (Fig. 1E,F).

Chondrification and ossification

Sandberg *et al.* (1988*a*,*b*) demonstrated that TGF-β1 is expressed at high levels in osteoblasts, osteocytes and osteoclasts in areas of endochondral and intramembramous ossification of 17 week *p.c.* human fetuses. We have also confirmed that one of the most intense sites of TGF-β1 RNA expression is in areas of intramembranous ossification. This is observed in the 57 day *p.c.* embryo in the maxillary and palatine bones, and in the mandible (Fig. 2A,B). There is no endochondral ossification as early as 8 weeks *p.c.*, although hypertrophic cartilage cells, which do not hybridize with any of the three TGF-β gene probes, are visible in the centres of the long bone cartilages (Fig. 2M,N). We have, however, seen expression of TGF-β1 in invading osteogenic cells at sites of endochondral ossification in the long bones of limbs from 10 to 12 week *p.c.* fetuses (data not shown).

Pelton *et al.* (1989) observed expression of TGF-β2 in osteoblasts and endothelial cells during endochondral, periosteal and intramembramous ossification in the mouse. In our study, although the TGF-β1 probe was seen to hybridize intensely to these sites, the TGF-β2 probe generated no autoradiographic signal (e.g. compare Fig. 2A,B and 2C,D). TGF-β2 RNA expression in the limb is, however, seen at the limb bud stage (43 days *p.c.*) in areas of mesenchyme that are morphologically indistinguishable from the surrounding tissue (Fig. 2K,L). We assume that these are early precartilaginous blastemae.

At later stages of limb development, TGF-β2 RNA expression becomes limited to the actively proliferating chondroblast zone at the diaphyseal/epiphyseal junction (Fig. 2M,N,O,P). There is also intense expression surrounding the digits at 57 days *p.c.* (Fig. 2O,P), in tendinous material, as observed by Pelton *et al.* (1989).

The earliest embryonic expression of TGF-β3 RNA seen in this study is in the intervertebral disc anlagen at 43 days *p.c.* (data not shown). This pattern persists at least to 57 days *p.c.* (Fig. 2G,H). TGF-β3 RNA is also expressed in the perichondria of cartilage models associated with the vertebral column, in particular the ribs (data not shown), but not those associated with the long bones of the limbs. A low level of expression is seen in the perichondria of non-ossifying cartilage, such as Meckel's cartilage and the tracheal cartilage rings, though there appears to be more TGF-β3 RNA in the surrounding mesenchyme than in the perichondria *per se* (Fig. 2E,F,I,J).

The cardiovascular system

Expression of TGF-β1 in the mouse cardiovascular system has been extensively studied by Akhurst *et al.* (1990*a*), who observed low levels of RNA in early endothelial cells and, at later stages, those specifically associated with septation and valve formation within the heart. TGF-β1 RNA expression is also detected in human fetal cardiac valve endothelia at 43 (Fig. 3A,B) and 57 days *p.c.* (data not shown), at a time when human heart development is virtually complete. Due to lack of suitably preserved specimens from very early embryos, we can make no comment as to the cardiac expression of TGF-β RNAs prior to 43 days *p.c.* Expression of TGF-β2 and TGF-β3 RNA in the heart at the later stages was not obvious, although there was some mesenchymal expression of TGF-β3 in the regions of the heart valves at 57 days *p.c.* (data not shown).

TGF-β1 RNA is also seen in the endothelia of large arteries (Fig. 3C,D), whereas TGF-β3 is expressed in the tunica intima underlying these endothelia (Fig. 3E,F).

TGF-β gene family expression in mesenchyme and mesothelia

TGF-β2 and TGF-β3, unlike TGF-β1, show widespread mesenchymal expression at the stages examined. In fact, the earliest expression of any of the TGF-βs detected in this study is that of TGF-β2 in the somatic mesoderm surrounding the liver at 32 days *p.c.* (Fig. 4A,B). TGF-β2 is also expressed extensively in mesenchymal areas at 43 and 57 days *p.c.*, particularly in the non-ossifying regions of the head. Hybridization with a human cardiac actin gene probe (kindly supplied by Dr P. Barton), which hybridizes to fetal skeletal and cardiac actin (Sassoon *et al.* 1988), indicates that TGF-

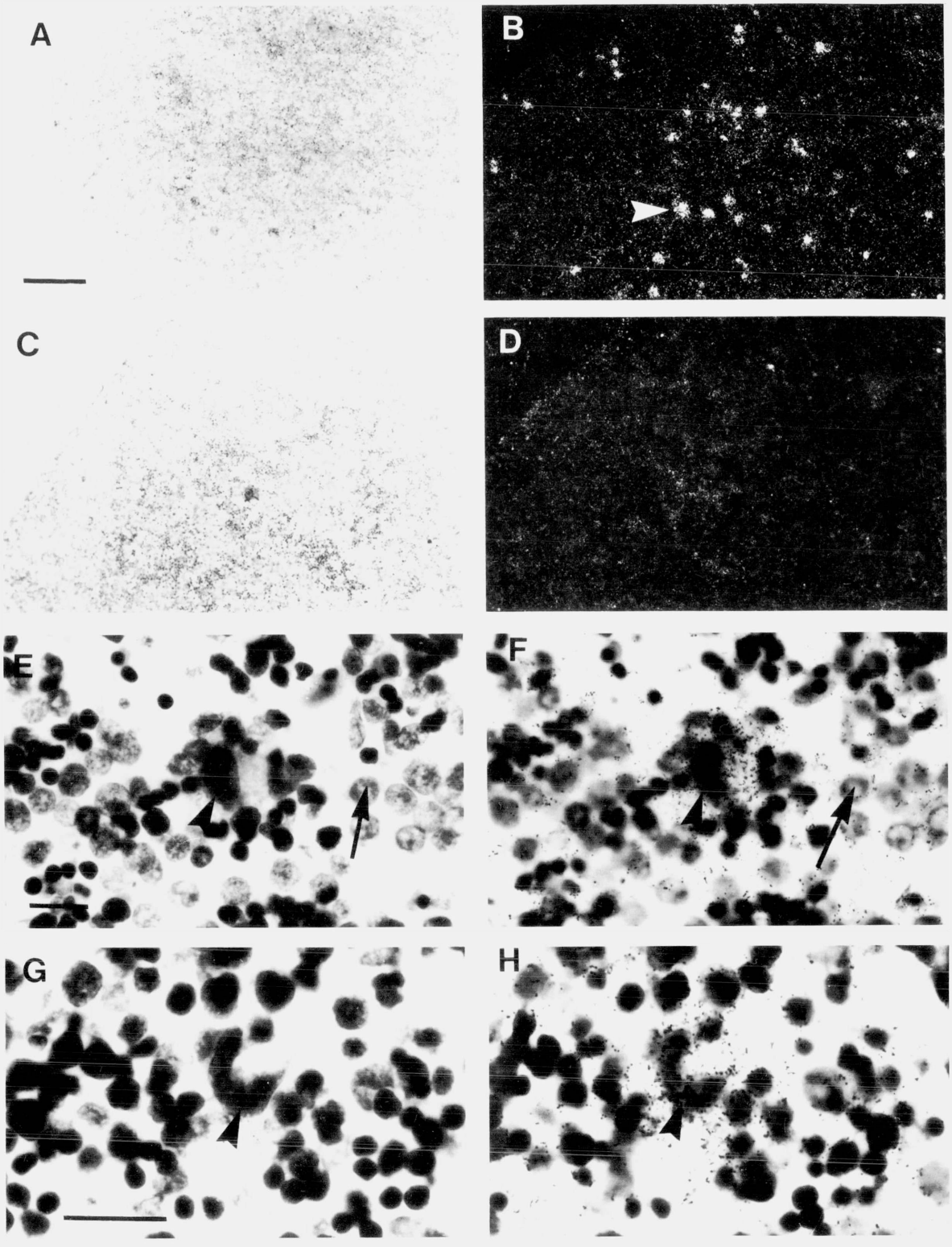


Fig. 1. TGF-β expression in the fetal liver. 57 day *p.c.* fetal liver hybridized with the TGF-β1 specific (A,B,E,F,G,H) and TGF-β2 specific (C,D) probes. (A,C) Bright-field and (B,D) corresponding dark-field images. E and F (also G and H) show the same field in a different plane of focus to show cellular morphology (E,G), and autoradiographic silver grains above the cells (F,H). Arrowheads indicate positively hybridizing megakaryocytes and arrows indicate non-hybridizing parenchymal cells. Scale bar represents 200 μm (A,B,C,D), or 20 μm (E,F,G,H).

β2-positive tissue is adjacent to, but does not generally overlap, regions of myogenesis (data not shown).

Over the period of development examined in this study, the skin is at a very immature stage. At 47 days *p.c.* TGF-β2 RNA is present in the dermatome and by 57 days *p.c.* it is seen in the dermis immediately beneath the epidermis (Fig. 4C,D), as has been observed in the mouse by Pelton *et al.* (1989).

TGF-β3 is also expressed in mesenchymal tissue, particularly those surrounding perichondria (Fig. 2E, F,I,J). More striking, however, is the expression of this gene in mesothelia surrounding all the major organ systems, including the pericardium, diaphragm and viscera (Fig. 4E,F). In the diaphragm, the use of the human cardiac actin probe showed that TGF-β3 RNA is associated both with the mesothelial and the muscular layers (Fig. 4G,H).

Lung development

The lungs are formed by the proliferation and branching of the lung buds into the splanchnic mesoderm from the fifth week of development. The development of the lung occurs relatively late in gestation and, at the early stages examined in this study, the pulmonary epithelial cells are all columnar in morphology, the transition to a simple cuboidal cell occurring much later.

In the 43 day *p.c.* embryo, TGF-β2 can be found in the primitive lung epithelia (Fig. 5A,B). By 57 days *p.c.*, the process of branching is fairly well advanced and only the growing tips of the developing bronchioles express TGF-β2 in their epithelia. (Fig. 5C,D). TGF-β3 shows a different pattern of expression in the lung. The RNA is seen submucosally in the proximal respiratory tract from the presumptive larynx downwards to a point that we believe represents the boundary between bronchi and bronchioles. Distal to this it has an epithelial expression pattern in the linings of the bronchioles but is also co-expressed with TGF-β2 in the terminal growing end buds (Fig. 5E,F,G,H).

TGF-β gene family expression in other epithelia

In previous studies, TGF-β1 RNA was detected in the epithelial component of the developing tooth, hair follicle, salivary gland and secondary palate of the mouse (Lehnert and Akhurst, 1988; Akhurst *et al.* 1990*b*; Fitzpatrick *et al.* 1990). The equivalent stages of development of these structures were not available for study here, so no conclusions regarding epithelial TGF-β1 expression can be made.

Intense epithelial expression of TGF-β2 was seen in the sensory epithelium, but not the simple cuboidal epithelium, of the developing inner ear at 47 and 57 days *p.c.* (Fig. 5I,J) and in a number of epithelial structures within the developing eye. At 43 days *p.c.*, it is expressed in all the cells of the lens vesicle and the inner layer of the optic cup, which gives rise to the retina (Fig. 5K,L). By 57 days *p.c.*, TGF-β2 RNA has become limited to the anterior germinal epithelium of the lens and is also seen in the most anterior part of the inner layer of the optic cup, which is destined to become the muscular iris (Fig. 5M,N).

Expression of TGF-β2 in the nervous system

There is no obvious high level expression of TGF-β1 or TGF-β3 in neuronal tissue in the embryos examined in this study, although the brain tissue tended to be less well preserved. However, the TGF-β2 probe hybridizes strongly with the ventral region of the nervous cord, demonstrated in a para-sagittal section in Fig. 6. This would be in agreement with the observations of Millan *et al.* (1990) who saw TGF-β2 expression in the ventral horns of the spinal cord in the mouse embryo at 10.5 days *p.c.*

Discussion

In this study, we report the spatial distribution of RNA transcripts for TGF-β1, TGF-β2 and TGF-β3 during early human embryogenesis. The results are summarised in Table 2.

We have made two assumptions in the interpretation of our data. First, that the gene-specific probes, when used for *in situ* hybridization, recognise *bona fide* mRNAs for the genes they represent. In general we have found a good correlation between our *in situ* data and RNA gel blot analysis (e.g. Akhurst *et al.* 1988). Furthermore the gene-specific probes used in this study do recognise transcripts of the predicted size for each

Table 2. *Differential localisation of RNAs encoding TGF-β1, TGF-β2 and TGF-β3 during human embryogenesis*

	β1	β2	β3
Haematopoietic tissue	+	−	−
Endothelia	+	−	−
Epithelia			
Early tooth bud	−	−	−
Bronchial epithelium	−	+	+
Otic epithelium	−	+	−
Lens epithelium	−	+	−
Retina	−	+	−
Immature epidermis	−	−	−
Cartilage and Bone			
Precartilaginous blastema	−	+ (limb)	+ (iv)
Growth zone of long bone	−	+	−
Perichondria	−	−	+
Hypertrophic cartilage	−	−	−
Osteoblasts, osteoclasts	+[1]	−	−
Cardiovascular system			
Valvular endothelium	+	−	−
Tunica intima of aorta	−	−	+
Neuronal tissue			
Ventral spinal cord	−	+	−
Mesothelia	−	−	+
Mesenchyme	−	+	+

+, denotes that RNA is abundant at some stage between 32 and 57 days *p.c.*

−, is below the detection level of *in situ* hybridization.

[1] observations from 12 week *p.c.* material.

iv, intervertebral disc anlagen.

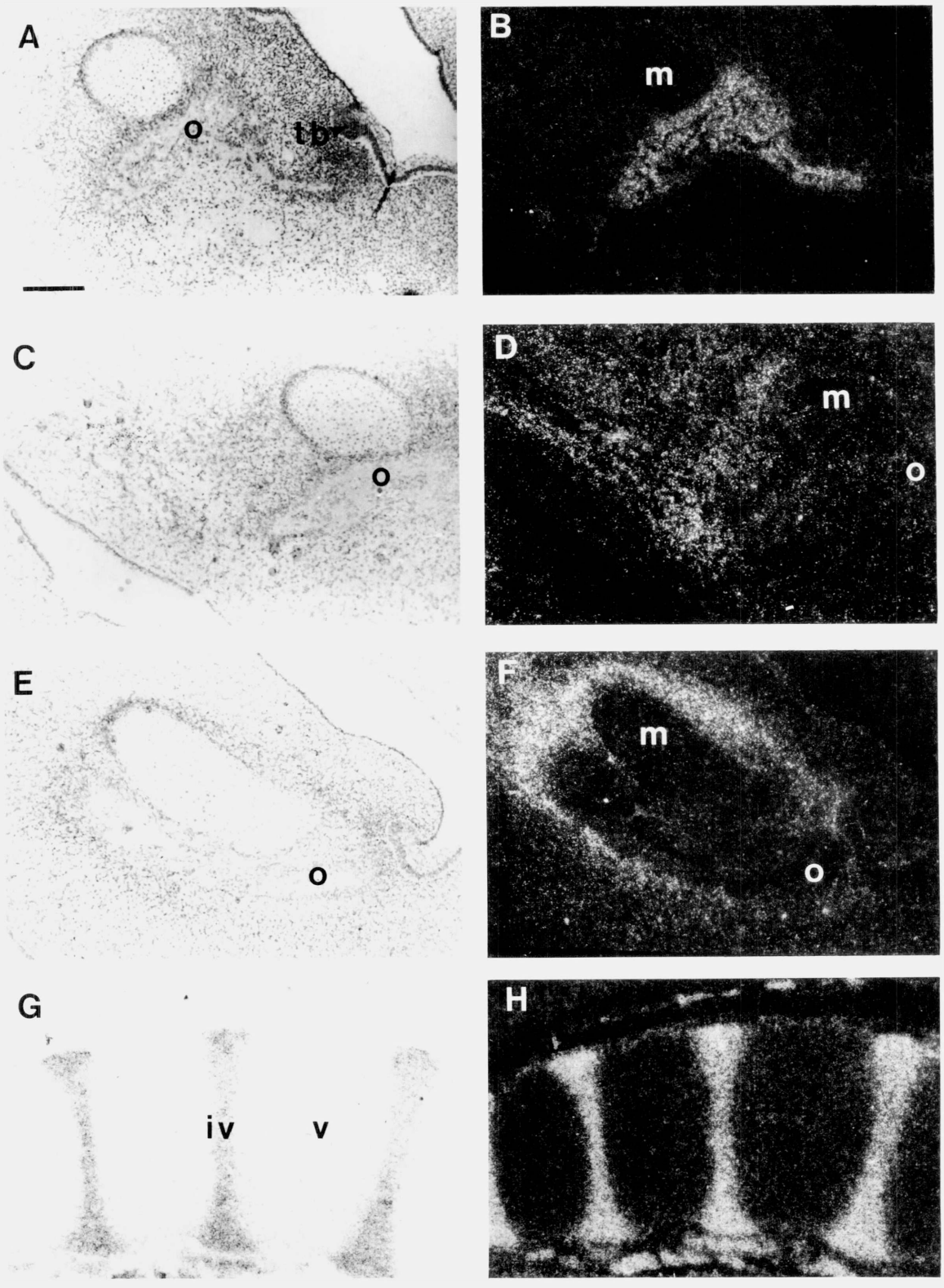
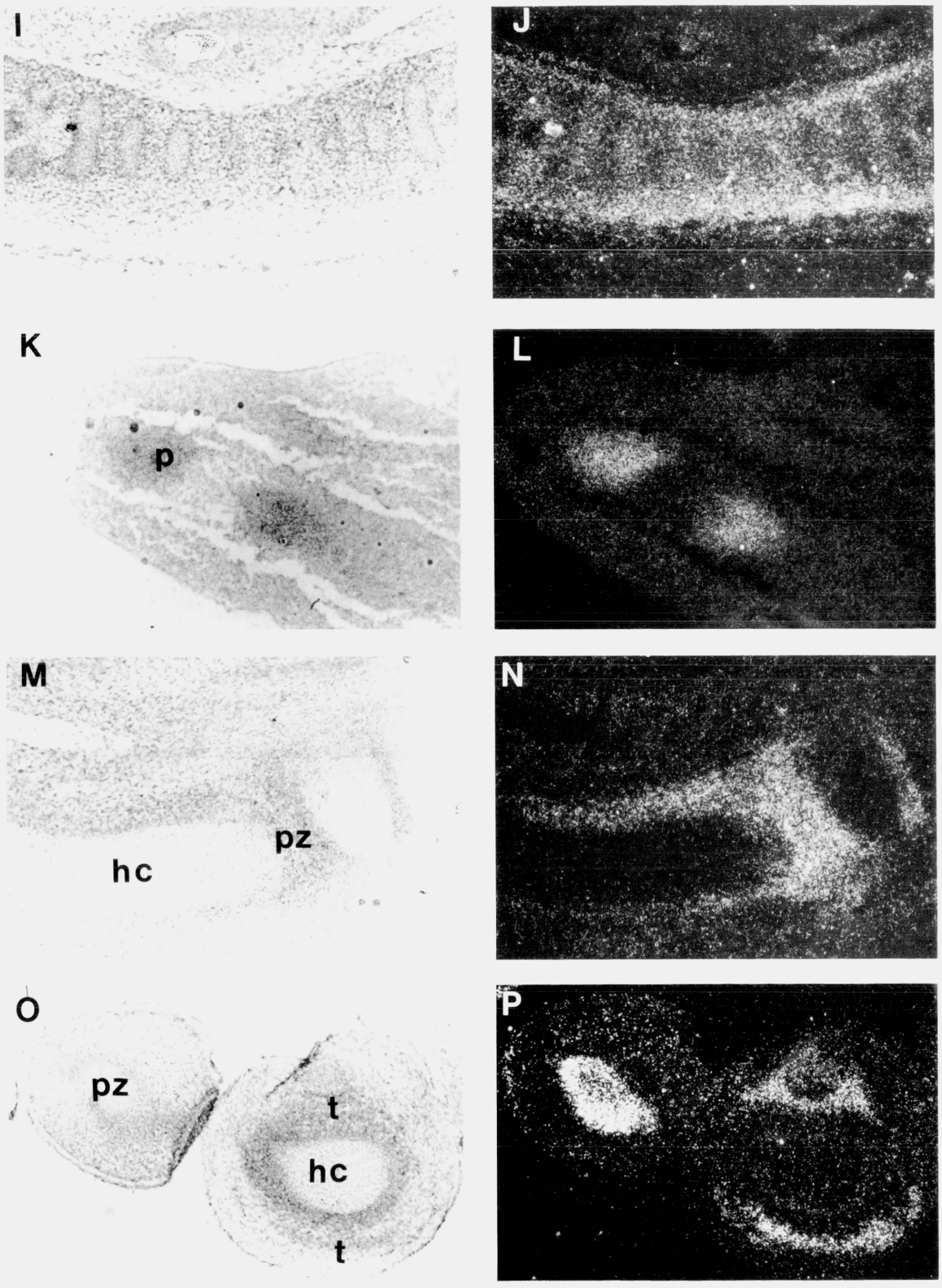


Fig. 2. TGF-βs in cartilage and bone. (A,C,E,G,I,K,M and O) Bright-field images and (B,D,F,H,J,L,N and P) corresponding dark-field images. (A–F) Sagittal section through the mandible of a 57 day *p.c.* fetus hybridized with the TGF-β1 specific (A,B), TGF-β2 specific (C,D) and TGF-β3 full-length (E,F) probes. (G,H) Sagittal section through the spinal column of a 57 day *p.c.* fetus hybridized with a TGF-β3 specific probe. (I,J) Trachea of a 57 day *p.c.* fetus hybridized with a TGF-β3 full-length probe. (K,L) Limb bud of a 43 day *p.c.* fetus hybridized with a TGF-β2 specific



probe. (M,N) Longitudinal section through a long bone of a 57 day *p.c.* fetus hybridized with a TGF-β2 specific probe. (O,P) Cross section through the digits of a 57 day *p.c.* fetus hybridized with a TGF-β2 specific probe. m, Meckel's cartilage; o, intramembranous ossification; tb, tooth bud; iv, intervertebral disc anlagen; v, vertebral anlagen; p, precartilaginous blastema; hc, hypertrophic cartilage; pz, proliferating chondroblast zone; t, tendon. Scale bar represents 200 μm.

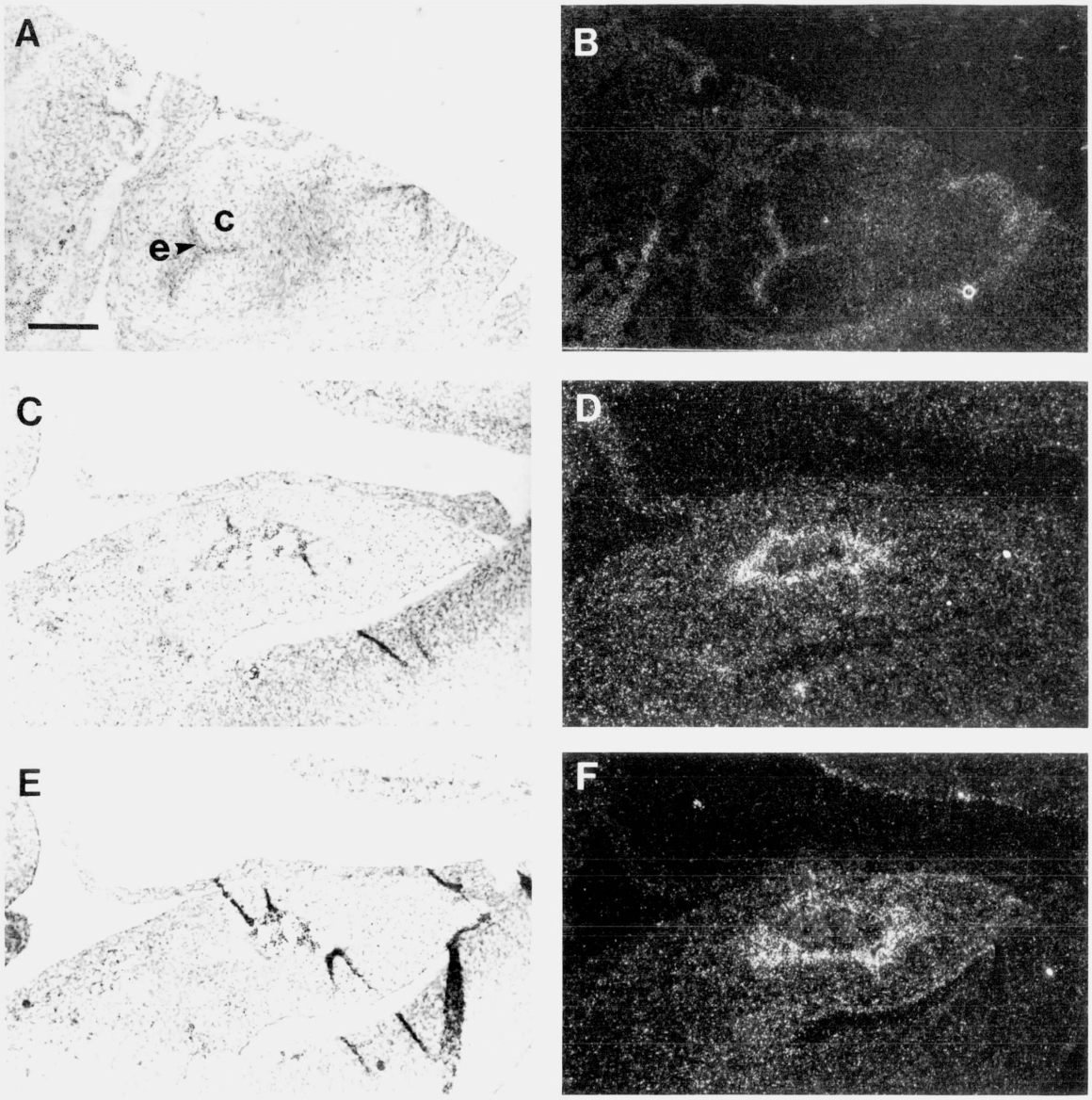


Fig. 3. TGF-β in the cardiovascular system. (A,C,E) Bright-field and (B,D,F) corresponding dark-field images. (A,B) Transverse section through the semilunar valves of an artery of a 43 day *p.c.* fetus, hybridized with the TGF-β1-specific probe. (C,D,E,F) Cross section through a blood vessel hybridized with the TGF-β1 specific (C,D) and TGF-β3 specific (E,F) probe. e, endothelium; c, cushion tissue. Scale bar represents 200 μm.

gene and show no cross-reactivity on Northern blots (ten Dijke *et al.* 1988 and unpublished data). Specificity of hybridization is reinforced by the contrasting expression patterns of the three genes and the cross-species conservation of these expression patterns between mouse and man (see below). The second assumption, that the RNA is translated into biologically active protein, is discussed in detail by Millan *et al.* (1990).

It has been shown that both TGF-β1 and TGF-β2 show differential splicing in primate and porcine tissues. Alternative splicing of TGF-β2 is not reported to alter the nature of the mature TGF-β2 protein product (Webb *et al.* 1988), whereas, in the case of TGF-β1, the translation reading frame is shifted (Kondaiah *et al.* 1988). The probes used in this study, when used for *in situ* hybridization, would not differentiate between the various spliced forms. However, the alternatively spliced TGF-β1 and TGF-β2 transcripts are only minor components of the total TGF-β mRNA (Webb *et al.* 1988; Kondaiah *et al.* 1988); therefore, although we cannot exclude the possibility that the transcripts detected in this study are non-translatable, we believe that this is unlikely, in the majority of cases.

Differential TGF-β isoform distribution is conserved between man and mouse

Extensive accounts of differential gene expression patterns for TGF-β1 (Lehnert and Akhurst, 1988;

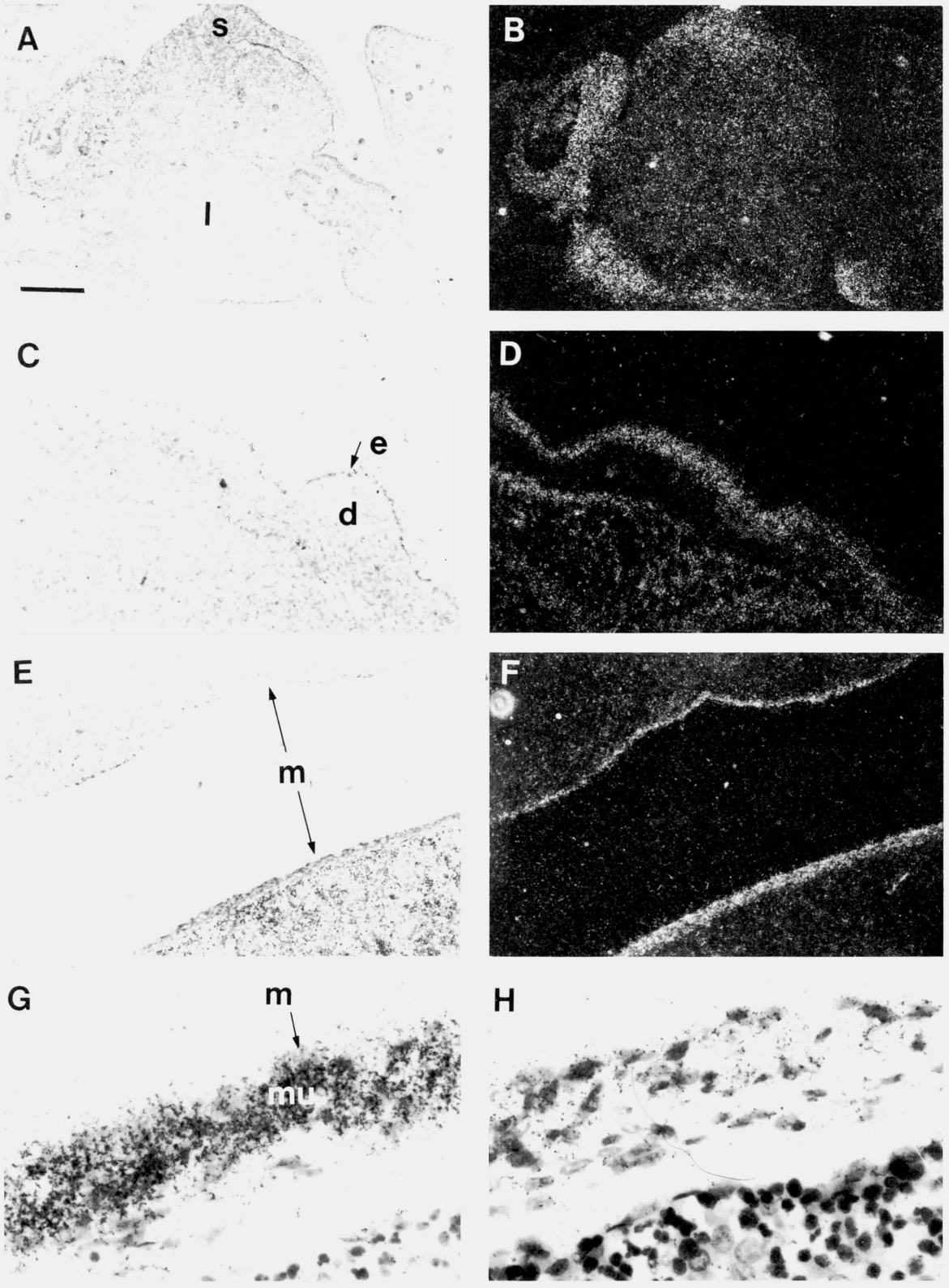


Fig. 4. Mesenchymal and mesothelial expression of TGF-βs. (A,C,E,G,H) Bright-field image and (B,D) corresponding dark-field images. (A,B) Coronal section through a 32 day *p.c.* fetus hybridized with a TGF-β2 specific probe. (C,D) Cross section through the skin of a 57 day *p.c.* fetus hybridized with a TGF-β2 specific probe. (E,F) Section through the heart and liver of a 57 day *p.c.* fetus hybridized with the TGF-β3 full-length probe. (G,H) Section through the diaphragm and adjacent liver hybridized with a cardiac actin probe (G) or the TGF-β3 full-length probe (H). e, epidermis; d, dermis; h, heart; m, mesothelium; l, liver; mc, muscle; s, somatic mesoderm. Scale bar represents 200 μm (or 50 μm in G and H).

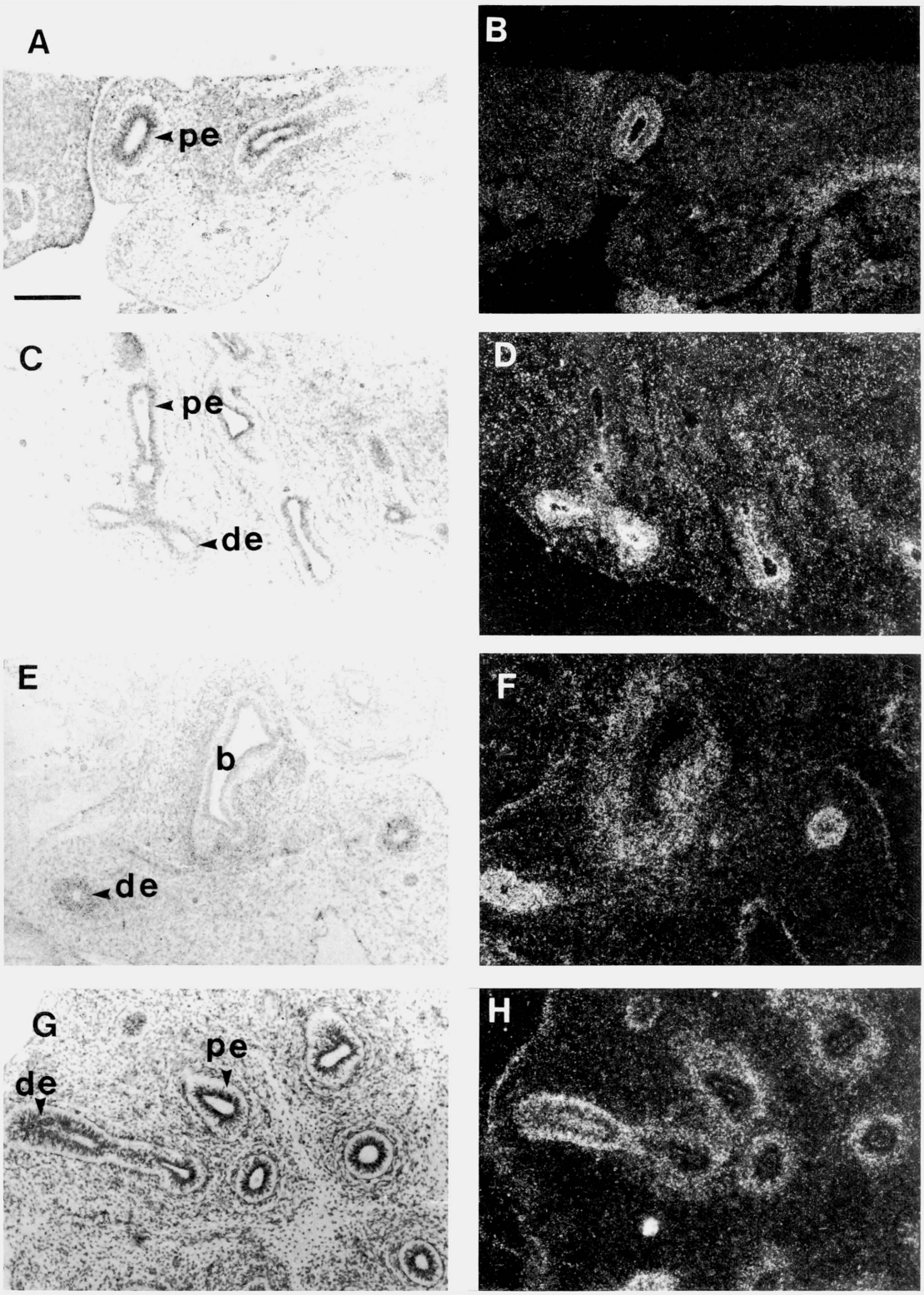
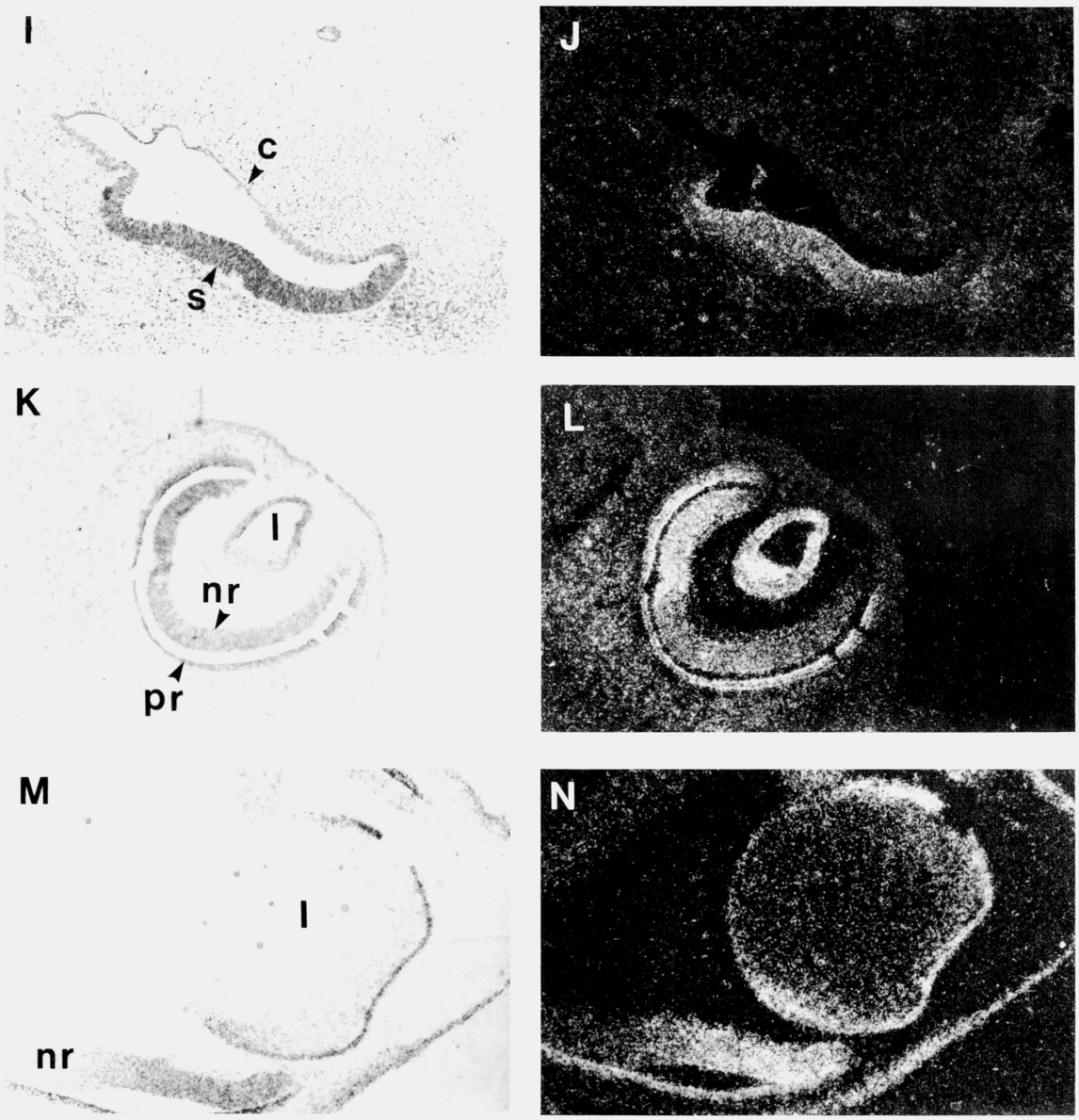


Fig. 5. Epithelial expression of TGF-βs. (A,C,E,G,I,K,M) Bright-field images and (B,D,F,H,J,L,N) corresponding dark-field images. (A,B) 43 day *p.c.* fetal lung and (C,D) 57 day fetal lung hybridized with a TGF-β2 specific probe. (E,F) 47 day fetal lung hybridized with a TGF-β3 specific probe. (G,H) 57 day fetal lung hybridized with TGF-β3 full-length probe. (I,J) 57 day inner ear hybridized with a TGF-β2 specific probe. (K,L) 43 day and (M,N) 57 day fetal eye hybridized with a TGF-β2 specific probe. pe, proximal bronchial epithelium; de, distal bronchial epithelium; b, bronchus; s, sensory epithelium; c, simple cuboidal epithelium; l, lens; pr, pigmented retina; nr, neural retina; c, cornea. N.B. pigmented retina gives an artefactual dark-field signal due to reflection from the pigment granules. Scale bar represents 200 μm.



Wilcox and Derynck, 1988; Akhurst *et al.* 1990*a*,*b*; Fitzpatrick *et al.* 1990; Rappolee *et al.* 1988) and, to a lesser extent, for TGF-β2 (Pelton *et al.* 1989) have been reported during murine embryogenesis. It is therefore possible to compare interspecies divergence in expression patterns of these developmentally important genes. As yet, no extensive reports of embryonic expression of murine TGF-β3 have been published, but we have compared the data presented here with that of Millan *et al.* (1990).

In this study, no epithelial expression of TGF-β1 was observed. This reflects the inherent difficulties of working with human embryonic material. There is not an unlimited supply of well-preserved tissue and it can be difficult to obtain material covering critical developmental events. In the mouse, epithelial expression of TGF-β1 is associated with active morphogenesis, such as during the development of the tooth, salivary gland, hair follicle and secondary palate (Lehnert and Akhurst, 1988; Akhurst *et al.* 1990*b*; Fitzpatrick *et al.* 1990) and expression of this RNA is very transient. During murine secondary palate development, for example, medial edge epithelial expression of TGF-β1 is restricted to only a 24 h time period (Fitzpatrick *et al.* 1990). In the current study, the equivalent stages of development in the human were not available for study.

In general, the results presented in this paper are in broad agreement with those obtained from studying mouse embryogenesis (Heine *et al.* 1987; Wilcox and Derynck, 1988; Lehnert and Akhurst, 1988; Pelton *et al.* 1989; Akhurst *et al.* 1990*a*). Discrepancies between the data presented here and those of Pelton *et al.* (1989) include their report of TGF-β2 expression in areas of ossification, and an absence of this RNA in the epithelia of the lung and other organs. However, our data are entirely consistent with published reports on TGF-β1 RNA distribution in murine embryogenesis, and with our own studies on TGF-β2 and TGF-β3 transcripts

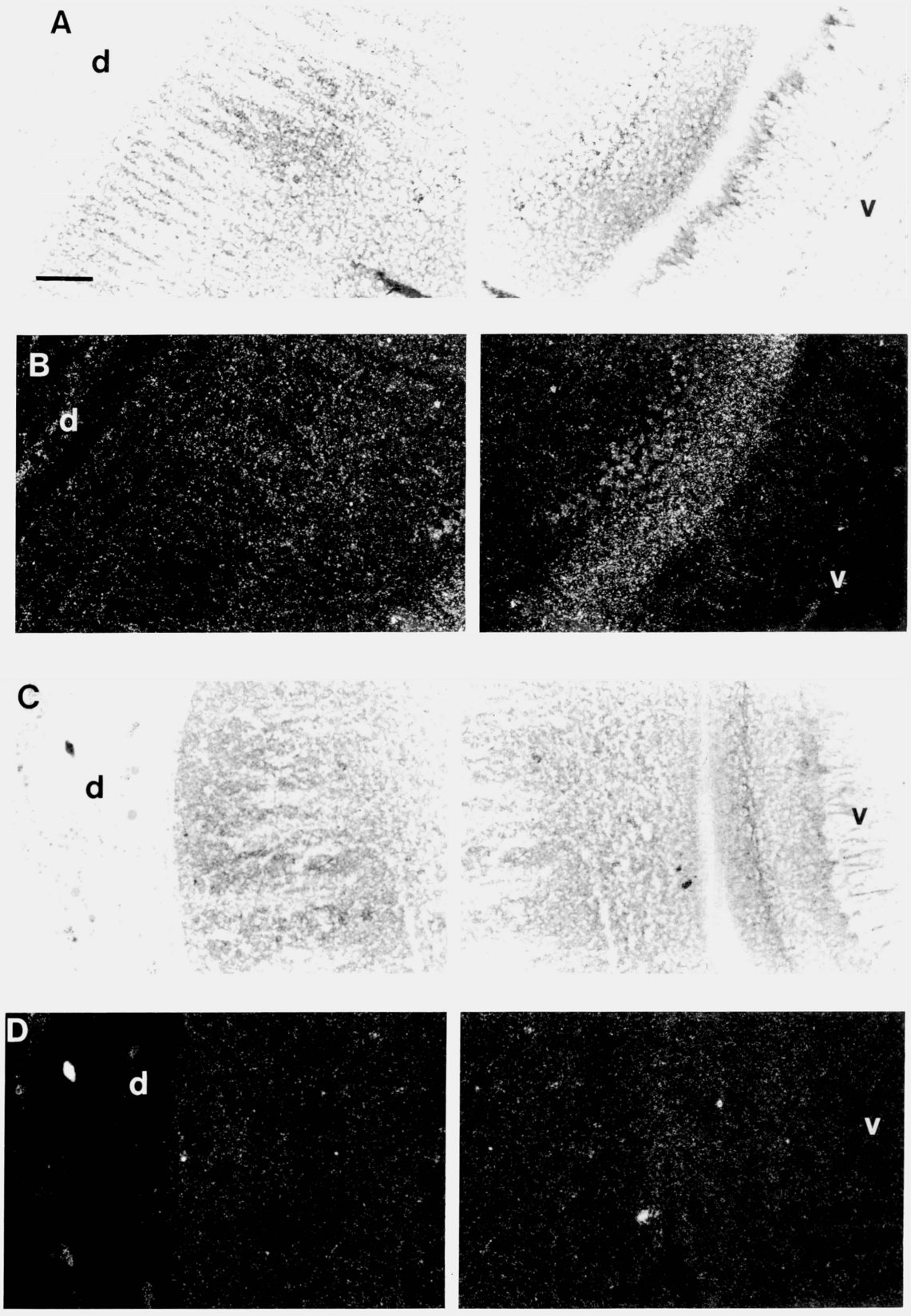


Fig. 6. Neuronal expression of TGF-β2. (A,C) Bright-field and (B,D) corresponding dark-field images. (A,B,C,D) Parasagittal section through the spinal cord of a 57 day *p.c.* fetus, hybridized with TGF-β2 specific (A,B) or TGF-β3 specific (C,D) probe. d, dorsal; v, ventral. Scale bar represents 200 μm.

(Millan *et al.* 1990), at least for the organ systems that were examined. Very minor discrepancies are apparent, which could probably be explained by differential rates of development of different organ systems between mouse and man.

In a clinical context, these conclusions are significant, since it demonstrates a conservation of, at least some, molecular developmental mechanisms between mouse and man, thus reinforcing the use of the former as a suitable model for studying human embryogenesis.

Differential function of TGF-β isoforms in embryogenesis

It has been debated whether the different TGF-β proteins have different functions *in vivo* or whether they are interchangeable in different biological contexts (Roberts and Sporn, 1990). The conservation of the differential expression patterns of these genes during embryogenesis between man and mouse would argue in favour of the functional diversity of members of this gene family. This would also be supported by the fact that interspecies amino acid sequence conservation of individual genes between man and chick is much greater than intraspecies sequence conservation of gene family members (Jakowlew *et al.* 1988*a*; Wilcox and Derynck, 1988).

An alternative interpretation is that each gene encodes a protein of very similar function, but that the presence of several family members may have evolved to assist in modulation of gene regulation during development, where very precise temporal and spatial gene activity is required. Conservation of differential gene expression patterns would then reflect evolutionary pressures on maintenance of accurate gene regulatory machinery.

Comparative biological activity studies have mainly been restricted to analysis of TGF-β1 and TGF-β2. Indeed, it has been shown that, in several biological systems, not only are these proteins functionally distinct (Rosa *et al.* 1988; Jennings *et al.* 1988; Ohta *et al.* 1987), but their receptor-binding activities also vary (Segarini *et al.* 1987; Ohta *et al.* 1987; Cheifetz *et al.* 1988). Recent comparative biological activity studies on TGF-β1, TGF-β2 and TGF-β3, show that all three proteins have qualitatively similar, but quantitatively different, biological activities on keratinocyte, fibroblast, osteoblast and endothelial cells (Grayar *et al.* 1989; ten Dijke *et al.* 1990; Cheifetz, S., Hernandez, H., Laiho, M., ten Dijke, P., Iwata, K. K. and Massague, J. unpublished data).

The only member of the TGF-β gene family expressed at high levels in human endothelial and haematopoietic tissue is TGF-β1, which is entirely consistent with the TGF-β1 expression pattern of the mouse (Wilcox and Derynck, 1988; Lehnert and Akhurst, 1988; Akhurst *et al.* 1990*a*). This observation is particularly significant since comparative studies on the biological activities of TGF-β1 and TGF-β2 at physiological concentrations show that only the former is growth inhibitory to endothelial cells (Jennings *et al.* 1988). Controversial reports on the differential growth inhibitory activities of TGF-β1 and TGF-β2 on early haematopoietic progenitor cells have, however, been made (Ohta *et al.* 1987; Sing *et al.* 1988). Nevertheless, at least for the endothelial cell system, our data provides evidence that differential expression patterns within the embryo are related to functional specificities within this growth factor family. It also indicates that, *in vivo*, TGF-β1 is an endogenous autocrine growth regulator of both endothelial and haematopoietic cells.

The *in vitro* biological activity data, together with the possibility that the precursor domains of each TGF-β may confer biological specificity in tissue-targeting, receptor-binding and/or activation of the latent form (Roberts and Sporn, 1990), would support the idea that there is indeed functional specificity of members of the TGF-β family. Only when it is possible to switch gene promoters between the various TGF-β genes *in vivo* using animal models, will it be possible to unequivocally establish whether this is correct.

Possible interaction of TGF-βs with other developmental control genes

Glick *et al.* (1989) showed that some of the biological affects of retinoic acid on keratinocytes *in vivo* are mediated by TGF-β2, since they can be blocked by antibodies to this protein. In this context, it is a rather intriguing observation that the transcript distribution of the gamma retinoic acid receptor gene (Dollé *et al.* 1989; Ruberte *et al.* 1990) shows considerable overlap with those of TGF-β2 and TGF-β3 (Pelton *et al.* 1989; Millan *et al.* 1990 and this report). This includes not only suprabasal keratinocytes (Pelton *et al.* 1989), but head mesenchyme, precartilage condensations, such as the intervertebral disc anlagen and blastemae of the long bones, and perichondria of ossifying and non-ossifying cartilage. It is tempting to speculate that the TGF-βs might be natural mediators of some of the biological effects of endogenous retinoic acid during development.

The predominant mesenchymal localisation of transcripts for TGF-β2 transcripts has been previously demonstrated by Pelton *et al.* (1989). In the current study, we have shown that, at the later stages of embrogenesis examined, the mesenchymal expression of TGF-β2 and TGF-β3 tends to be localised to specific structures, such as precartilaginous blastemae, tendons and proliferating chondroblasts. However, at earlier stages, a specific association with identifiable structures is less clear. The high transcript prevalence of TGF-β2 in head mesenchyme and somatic mesoderm might suggest a correlation with areas of mesenchymal cell proliferation and migration, both of which are known to be modulated by TGF-βs (Moses *et al.* 1985; Postlethwaite *et al.* 1987). Expression of TGF-β2 in the fronto-nasal mesenchyme of the mouse (Millan *et al.* 1990) and of TGF-β2 and TGF-β3 in condensing mesenchyme of the limbs and vertebral column would support such an hypothesis. If, indeed, some of the effects of retinoic acid were mediated by TGF-βs, one could easily imagine how deformities in facial structure

might arise by administration of this substance at teratogenic doses.

Another gene that exhibits an overlapping expression pattern with one of the TGF-βs is *Pax-1*, a paired box-related gene of the mouse which is expressed specifically in the intervertebral disc anlagen in a very similar manner to TGF-β3 (Deutsch *et al.* 1988). *undulated*, a recessive mutation of the *Pax-1* gene, leads to vertebral column deformities (Balling *et al.* 1988). The idea that centrally important developmental genes, such as *Pax-1* or the HOX genes, are regulated by growth factors is not novel (Ruiz i Altaba and Melton, 1989). The correlation between TGF-β1 and HOX 7 gene expression during murine cardiac heart valve development has already been noted as one such possible interaction (Robert *et al.* 1989).

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