

EXPRESSION
THE STRUCTURE AND ORGANISATION OF THE
CHICKEN *BONE MORPHOGENETIC PROTEIN-2* GENE
~~(BMP-2) GENE IN CHICK LIMB DEVELOPMENT~~

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A thesis submitted for the degree of Ph.D.

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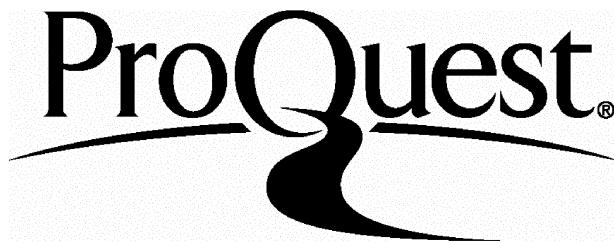
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ABSTRACT

The aim of the research described in this thesis was to investigate the structure and function of the *bone morphogenetic protein-2* gene in the developing chick limb.

A chicken *Bmp-2* genomic clone was isolated. The intron-exon structure was analysed and a portion of the 5' coding region, that was missing from cDNA clones previously isolated in our laboratory, was sequenced and analysed. This 5' sequence and the intron-exon structure were found to be highly conserved with other members of the TGF- β superfamily, and *Bmp* genes from other species. Preliminary evidence indicates that the *Bmp-2* gene has multiple 5' non-coding exons, like *Bmp-4* and *dpp*, but unlike the other TGF- β superfamily genes.

In order to analyse the 5' promoter region of the *Bmp-2* gene, a novel system was developed for transfecting primary chick mesoderm cells isolated from embryonic limb buds. This transfection system provides as valuable model system in which to analyse the regulation of patterning genes expressed in the limb, since it appears to mimic various aspects of the developing limb, as shown by positional and temporal expression of various marker genes tested in the system. This is particularly useful since there are at present no established chick cell lines. However, we were unable to generate any promoter constructs of the putative promoter region of the *Bmp-2* gene, possibly because of a region of instability in the 5' region of the gene.

The role of the *Bmp-2* gene in development was therefore further investigated by attempting to inhibit *Bmp-2* gene function using an antisense RNA strategy. Antisense constructs containing various different regions of the chicken *Bmp-2* gene were generated in a retroviral vector, RCAS(BP), which can be targeted to specific areas of the developing chick embryo, by injection or grafting of infected cells. Stage 16-21 embryonic chick limb buds were infected with these antisense *Bmp-2* constructs. In preliminary experiments, these retroviral constructs appear to be expressed at relatively high levels in the limb, and to inhibit levels of endogenous *Bmp-2* transcripts, as shown by *in situ* hybridisation. A possible phenotypic effect of this inhibition was shown to be a fusion between the metatarsals of digits III and IV in the wing.

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ABBREVIATIONS

9-*cis*-RA: 9-*cis*-retinoic acid

AER: apical ectodermal ridge

A-P: anterior-posterior

β -gal: β -galactosidase

BMP: bone morphogenetic protein

bp: base pair

CAT: chloramphenicol acetyl transferase

CEF: chick embryonic fibroblast

CNS: central nervous system

HPLC: high pressure liquid chromatography

RAR: retinoic acid receptor

dpp: *decapentaplegic*

ds: double-stranded

D-V: dorso-ventral

FGF: fibroblast growth factor

GDF: growth/differentiation factor

hh: *hedgehog*

Hox/HOX: homeobox containing gene or protein

kb: kilobase

LTR: long terminal repeat

M-MuLV: Moloney leukaemia virus

nM: nanomolar

p.c.: post coitum

P-D: proximo-distal

PR: polarising region

PZ: Progress Zone

RA: retinoic acid

RARE: retinoic acid response element

RSV: Rous Sarcoma virus

RXR: retinoid X receptor

Shh: sonic hedgehog

ss: single-stranded

TGF- β : transforming growth factor- β

ZPA: zone of polarising activity

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CHAPTER 1

INTRODUCTION

The aim of the work described in this thesis was to investigate the structure and regulation of expression of the chicken *bone morphogenetic protein-2 (Bmp-2)* gene and its role in the development of the chick limb.

In this introduction, I shall first describe the developing chicken limb, before giving a brief description of the BMP family and their potential roles in development.

1.1 Embryonic Development:

During development, an embryo changes from a mass of undifferentiated cells, to a highly complex organism, made up of many different cell types, organised into functional tissues. A fundamental problem in developmental biology is understanding how these patterns of differentiated cells are established.

The first sign of this structured differentiation occurs at gastrulation, when the embryo forms three distinct spatially organised layers: ectoderm, mesoderm and endoderm. These three layers then signal to one another and interact to form the organs (Figure 1.1). This occurs because certain cell populations produce signals which influence the development of other cells, switching functions on or off, and committing the cells to a particular developmental path.

Identifying these signalling molecules and their modes of action may help us to understand how the embryo develops.

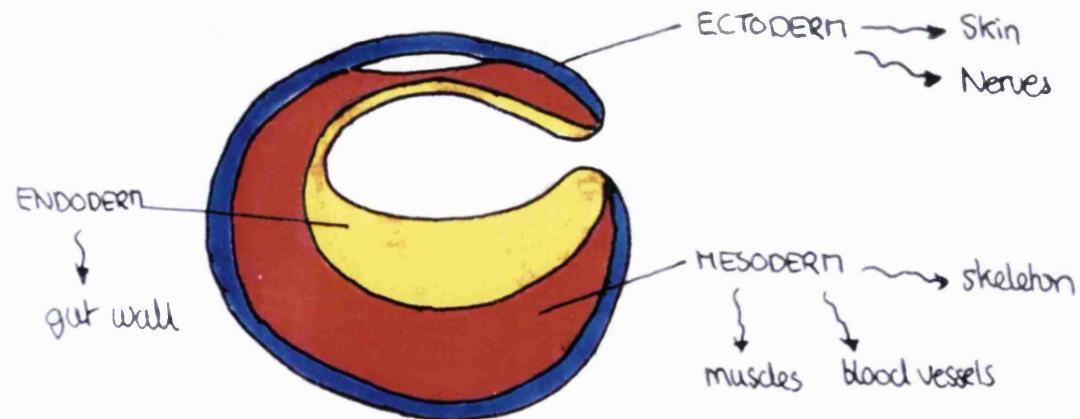


Figure 1.1: The first sign of organised differentiation occurs at gastrulation.

At gastrulation, the embryo forms three distinct spatially organised layers. These three layers then signal to one another and interact to form organs.

1.2 The Developing Chick Limb as an Experimental System:

The chick limb bud provides an excellent model system for studying pattern formation in vertebrates (Figure 1.2).

A primary benefit of working with chick embryos is that the embryo is easily accessible during development. A small hole can be opened in the shell of the chicken egg, giving access to the growing embryo and allowing experimental manipulations to be performed *in ovo*, during early development, when patterning is occurring. The hole can then be sealed, and the embryo can be left to develop normally, without the need for a culture system, and be harvested and analysed at later stages. This makes the chick embryo a good system for experimental manipulations such as grafting.

In addition, the chick limb has the advantage that it is possible to accurately identify any duplicated or experimentally produced structures, because the digits have a specific anatomical identity based on the number and shape of the cartilage elements, both within a limb, and between wing and leg. Toe digits can be differentiated from wing digits by the long thin metatarsals of digits II, III and IV, and by the more numerous phalanges; also, at later stages, feather germs form on wing digits and scales form on the toes (Honig, 1981).

An understanding of the processes involved in the development of the limb may also throw light on the patterning mechanisms used in other, less amenable, regions of the embryo.

Therefore, despite the limitations of chicken genetics, compared to mice or *Xenopus*, such as the difficulty of micro-injecting or making transgenic animals and the lack of transformed cell lines (Love *et al*, 1994, Bosselman *et al*, 1989, Perry *et al*, 1988, Mitrani and Eyal-Giladi, 1982, Petitte and Eches, 1988), a number of tissue interactions and signalling pathways have been identified that lead to the formation and patterning of the complex array of skeletal elements found in the adult limb.

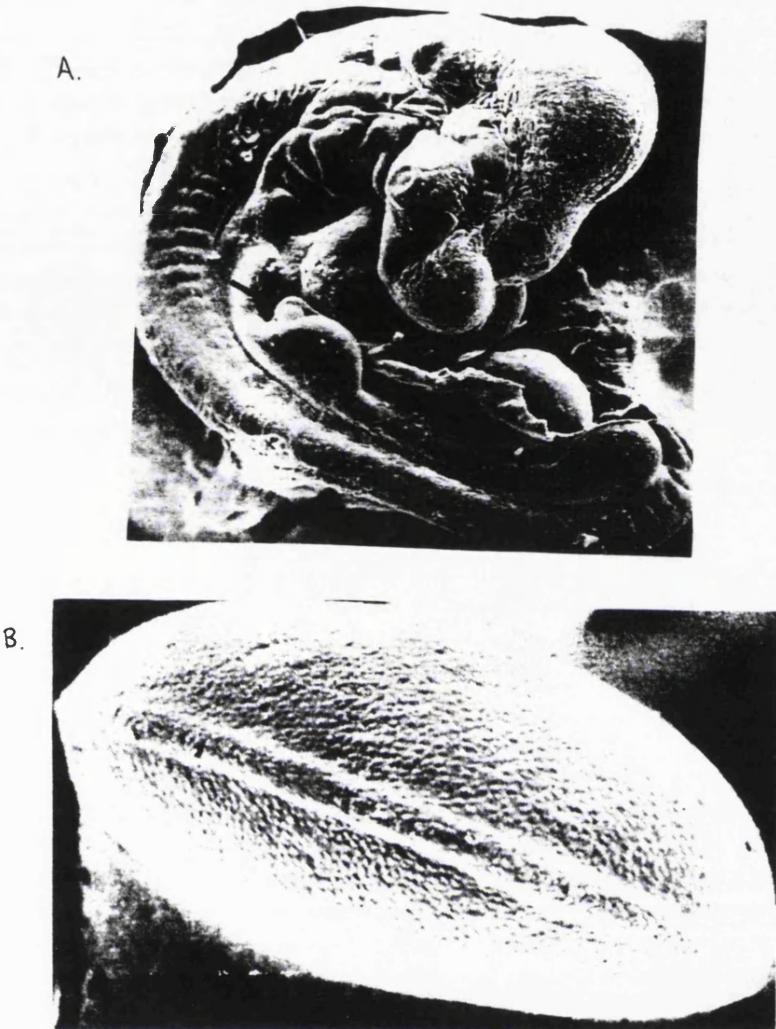


Figure 1.2: The embryonic chick limb.

- a) Scanning electron micrograph of the whole embryo showing the limb buds.
- b) High power scanning electron micrograph of the limb bud, seen end on, showing the apical ectodermal ridge.

(Taken from Brickell & Tickle, 1989)

1.3 Limb Development:

In chicks, the limbs develop from small buds that are first visible as outgrowths from the body wall at stages 16-17 (Hamburger and Hamilton, 1951). The early limb bud consists of undifferentiated mesoderm cells encased in an ectodermal shell. Once the buds have formed, they grow rapidly at the tip, and mesenchyme cells in the remainder of the bud start to differentiate into the various tissues of the limb (Zwilling, 1961; Hornbruch and Wolpert, 1970; reviewed by Tickle and Eichele, 1994). Mesenchyme cells in the limb, originally derived from lateral plate mesoderm, condense to form cartilage and connective tissues, and mesodermal cells from the somites migrate into the limb to give rise to the muscles (Figure 1.3).

The limb's structure can be described in terms of 3 axes: a proximodistal axis that extends between the shoulder and the tips of the digits; an antero-posterior axis that extends across the digits; and a dorsoventral axis, that runs between the back face of the limb and the front (Figure 1.4). All three axes are intimately linked during limb outgrowth and patterning.

Various specialised areas of the chick limb are thought to produce signals which establish the limb axes (reviewed by Tabin, 1991; and Laufer, 1995) (Figure 1.4). The two main regions are a group of mesenchymal cells at the posterior margin of the limb bud, known as the Polarising Region (PR) or Zone of Polarising Activity (ZPA), and the Apical Ectodermal Ridge (AER), a thickened area of epithelium at the tip of the bud. The dorsal ectoderm is also thought to play a role in establishment of the dorsoventral axis (Parr and McMahon, 1995; Niswander, 1995).

a) The Apical Ectodermal Ridge:

The AER is required for outgrowth of the limb: if it is removed experimentally, the limb is truncated (Saunders, 1948; Summerbell, 1974), and if a supernumerary AER is grafted onto a limb, an extra limb forms (Saunders and Gasseling, 1968).

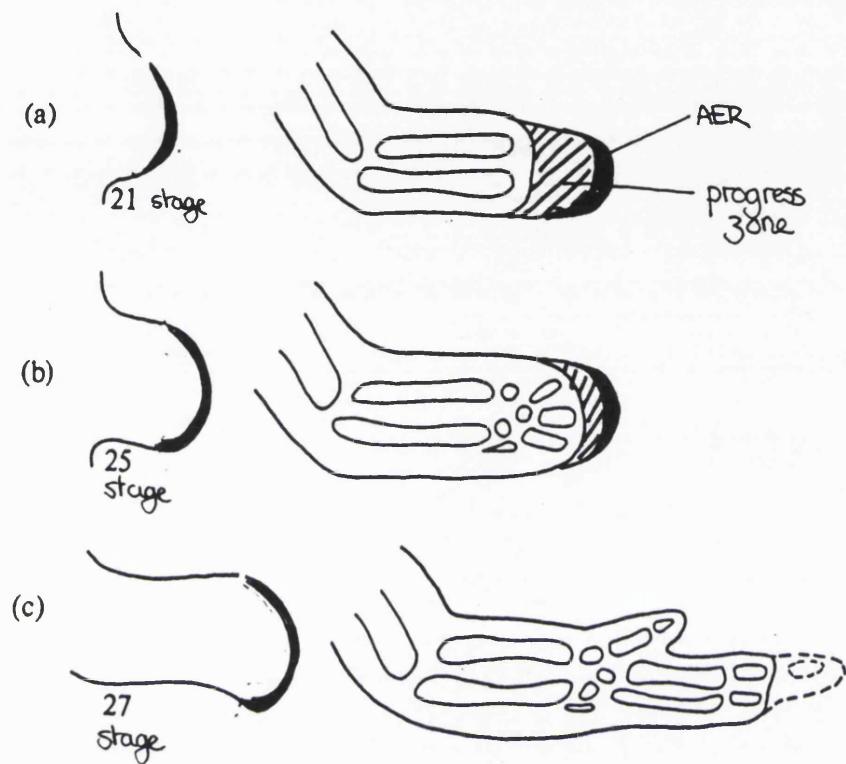


Figure 1.3: Development of the limb across time.

This diagram shows how cells differentiate according to their proximo-distal position over time.

(Adapted from Hinchliffe & Johnson, 1980)

Signals from the AER are thought to affect cells in an area immediately below the AER, called the Progress Zone, signalling them to proliferate and maintaining them in an undifferentiated state (Rowe *et al*, 1982). The Progress Zone extends about 350 µm into the limb from the tip of the AER (Saunders, 1948) (Figure 1.4). Since all the cells in the Progress Zone are dividing, as the limb grows, cells continually leave the zone, and they differentiate according to the length of time they or their ancestors have spent in the Progress Zone: cells that have spent a short time in the zone form proximal structures, and cells that have spent longer there form distal structures (Summerbell *et al*, 1973; Wolpert, 1978). The only spatial signal needed is one to mark out the extent of the Progress Zone, and no long-range signals are required. Evidence for this comes from the fact that cells of the Progress Zone continue to develop autonomously if grafted onto new hosts, with duplications or truncations occurring, depending on the relative stages of the graft and host. There is therefore no signal from the host stump involved, only signals from the AER (Summerbell *et al*, 1973).

However, the signals required from the AER for outgrowth and patterning are permissive, not instructive. The type of limb (i.e., fore or hind limb) and the type of structures formed by the mesoderm cells is controlled by the mesoderm, not the AER, as shown by experiments swapping AERs from wings and legs, and from limbs at different stages (Zwilling, 1955; Rubin and Saunders, 1972). For example, if non-limb ectoderm is grafted onto a limb which has had the AER removed, the limb fails to develop, but swapping leg ectoderm onto wings, and vice-versa, has no effect on development: the limb develops as the mesoderm would normally (Zwilling, 1955). The AER loses its inductive capacity after stage 29, regardless of the age of the mesoderm, but, until that stage, an AER of any age can induce outgrowth of limb parts in the correct proximo-distal sequence. Since the AER signal is therefore constant from stage to stage, the information for the specific order of patterning in the limb must be programmed intrinsically in the mesoderm, rather than specified by the AER (Rubin and Saunders, 1972).

b) The Zone of Polarising Activity:

Signals from the ZPA are involved in the specification of the antero-posterior axis of the limb (Figure 1.4). Grafts of this region of the posterior part of the limb to the

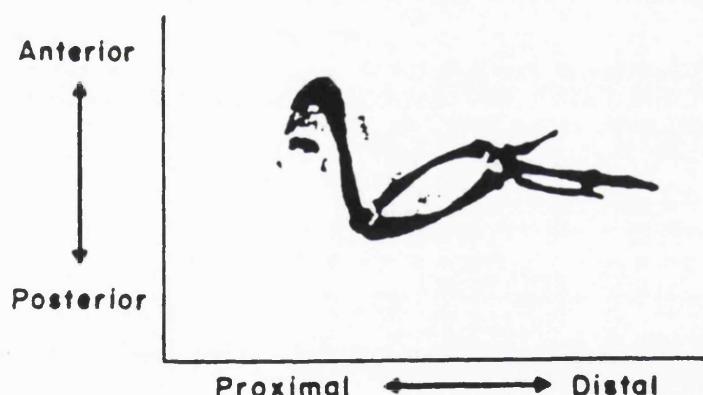
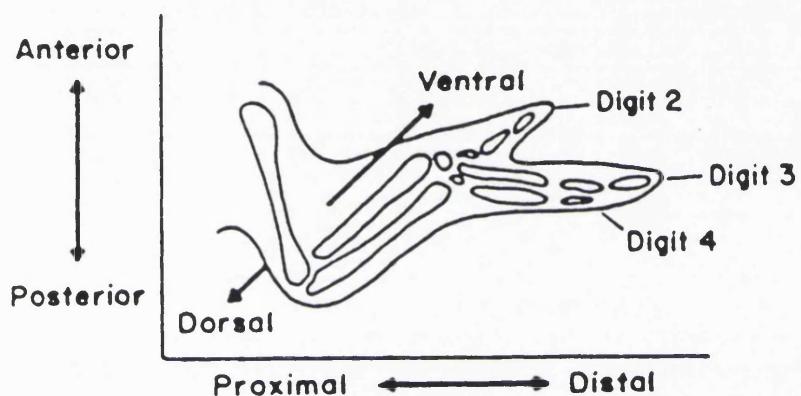
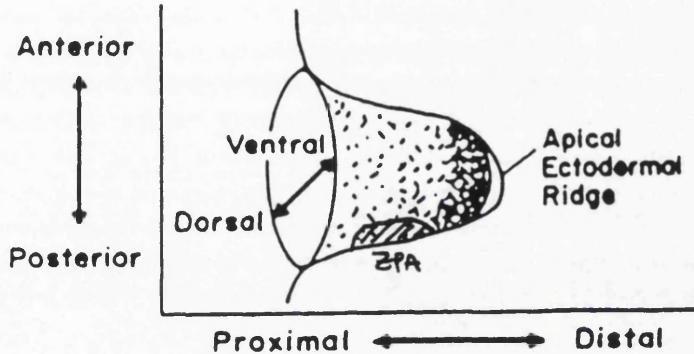


Figure 1.4: Structure of the developing limb.

These drawings show the limb axes, the positions of the main areas thought to be involved in specifying the axes, and the skeletal pattern.

(Adapted from Tabin, 1991).

anterior edge, under the ridge, result in complete mirror-image duplication of the digits, respecifying cells such that anterior cells form posterior structures (Saunders and Gasseling, 1968; Tickle *et al.*, 1975) (Figure 1.5).

Polarising cells cannot be identified histologically, but their distribution in limb buds has been mapped by cutting out small blocks of tissue and grafting these anteriorly in host wing buds to assay for digit-inducing activity (Maccabe *et al.*, 1973; Honig and Summerbell, 1985; Hinchliffe and Sansom, 1985). Polarising activity is first detected at stage 15-16 at low frequency, and is well established by stage 17, when it remains at a high level until stages 27 and 28, when it decreases markedly and is essentially absent by stage 29. The ZPA also shifts in position during development: at stage 17-22 it extends both into the wing bud and posteriorly into the adjacent body wall, whereas by stage 23, it lies entirely in the limb bud, and, by stage 27, it is only found near the apex of the bud in the handplate-forming region (Maccabe *et al.*, 1973). Dissociated ZPA cells can induce digit duplications as well, when grafted anteriorly, so intact cell-cell relationships are not required for signalling (Honig, 1983). However, the mapping experiments demonstrate potential rather than actual ZPA activity, because signals from the AER are also required for ZPA function, and the putative polarising cells were grafted to a permissive location (Charité *et al.*, 1994).

The signalling effect of the ZPA is not species-specific, and quail, mouse, hamster or reptilian ZPA cells can induce extra digits in the chick (Tickle *et al.*, 1976; MacCabe and Parker, 1976; Iten, 1982). This suggests that the signal used by the ZPA is the same in all these species, and it has been proposed that the same signal is used in all vertebrates (Tickle *et al.*, 1976). Other regions of the embryo also have polarising activity and can induce duplicated wing patterns in the chick when grafted there. Examples include Hensen's node and the primitive streak of the quail, chick (Hornbruch and Wolpert, 1986; Stocker and Carlson, 1990) and mouse (Hogan *et al.*, 1992; Izpisúa-Belmonte *et al.*, 1992a), the notochord, and the floorplate, a group of epithelial cells located at the ventral midline of the developing central nervous system (Wagner *et al.*, 1990). There is, however, some debate about whether removal of the ZPA causes complete loss of pattern, because of uncertainty over the precise timing of patterning events and the exact extent of the ZPA. (Fallon and Crosby, 1975; Summerbell, 1979; Saunders and Gasseling, 1963; reviewed by Slack, 1993).

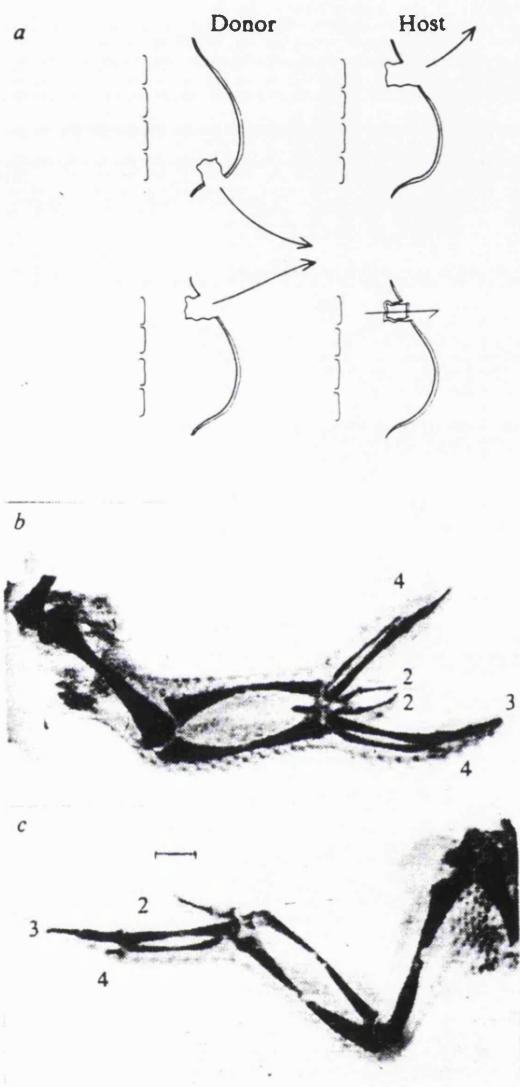


Figure 1.5: Digit duplication caused by ZPA grafts.

- This diagram shows the graft site on the host and the origin of the ZPA graft.
- Typical results of a ZPA graft at 10 days of development.

c) **Dorso-ventral patterning:**

Dorso-ventral identity is specified by ectodermally derived signals. Evidence for this comes from experiments where grafting ectoderm from right to left wing buds and inverting it dorso-ventrally leads to the production of limbs that are also inverted, at least distally (Pautou and Kieny, 1973; MacCabe *et al.*, 1974).

1.4 Models Describing Limb Formation:

The current paradigm explaining how cells are able to form a well-defined spatial pattern during development, is that the cells are assigned specific states depending on their position in the developing system (Wolpert, 1969, 1971). A cell's positional value is related to its position within the system, and it is as though there is a co-ordinate system with respect to which the cells have their position specified. This information, together with the cell's genome, is then used to specify the differentiation of the cell, and the cell thus interprets its positional value by developing in a particular way.

Unlike a prepattern, which provides a model for the final developmental pattern, such as a homunculus, the pattern of specification of the positional information need not resemble the final pattern, but rather the final pattern depends on the rules for interpretation. In this way, the same primary positional information can be used to generate various different patterns. This interpretative system provides flexibility since a change in pattern, such as might occur during evolution, can be achieved by a simple shift in the rules governing interpretation, rather than a shift in the entire pattern. Wolpert (1969, 1971) suggested that there could be a universal co-ordinate system for the whole organism with similar interpretational rules and mechanisms for the different fields rather than individual mechanisms for the development of each structure.

Positional information could be assigned in many ways, such as a linear gradient with fixed boundary levels with rules governing the interpretation of the thresholds (the Morphogen Gradient model), two gradients with rules for interpreting the ratio between them, interactions between stationary and diffusible inhibitors and activators, or the

time differences or phase shift between two periodic signals. There are currently two main models that attempt to explain limb development.

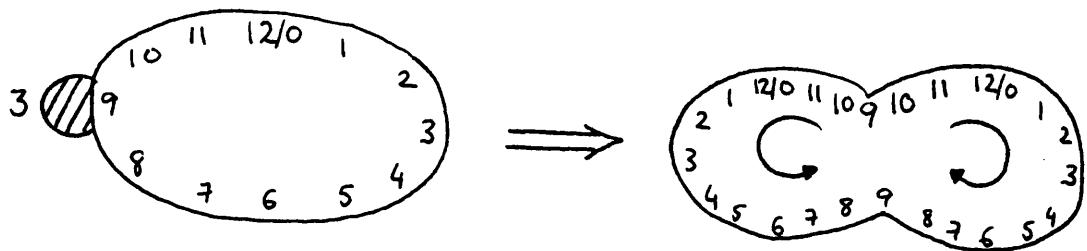
a) The Polar Co-ordinate Model:

The Polar Co-ordinate model is not a detailed molecular model, but instead considers how the regulative behaviour of tissues can be explained in terms of rules for the behaviour of individual cells (French *et al*, 1976). French *et al* (1976) suggest that positional information is better represented by polar co-ordinates (position on the perimeter of a circle, like the hours on a clock-face, and the radius position, or distance out from the centre of the circle) than by orthogonal axes (Figure 1.6). The outer circle represents the proximal boundary of limb field and the field centre is the distal tip of the limb. The theory postulates that by grafting ZPA cells that possess very posterior positional values to the anterior bud margin, a discontinuity in positional values is created. Cells then respond by dividing and acquiring new positional values so as to generate the missing values and fill in the discontinuity. The circular sequence is continuous and the shortest route is chosen. In addition, the circular sequence at any particular level can transform to more central (distal) positional values, but only if complete sequence of positional values is exposed or generated by intercalation, or if the intercalated value that a cell would take is already present in adjacent cells, forcing the cell to take up a more distal value (Bryant *et al*, 1981).

The best evidence for the polar co-ordinate model comes from grafting experiments in insects such as cockroaches (reviewed by French *et al*, 1976). Removing strips of cuticle from the limbs or grafting extra bits leads to intercalary growth, and if the grafts are rotated, extra limbs are formed. This theory can also be used to explain the development of grafts of imaginal disks in *Drosophila*, and the development of regenerating amphibian limbs, particularly the formation of supernumerary limbs upon rotation of grafts (reviewed by French *et al*, 1976).

In the chick limb, this model can also be used to explain the results of apical and posterior-to-anterior grafts, and this theory is supported by the fact that an increase in cell proliferation has indeed been detected in anterior cells as early as 4 hours after a

A.



B.

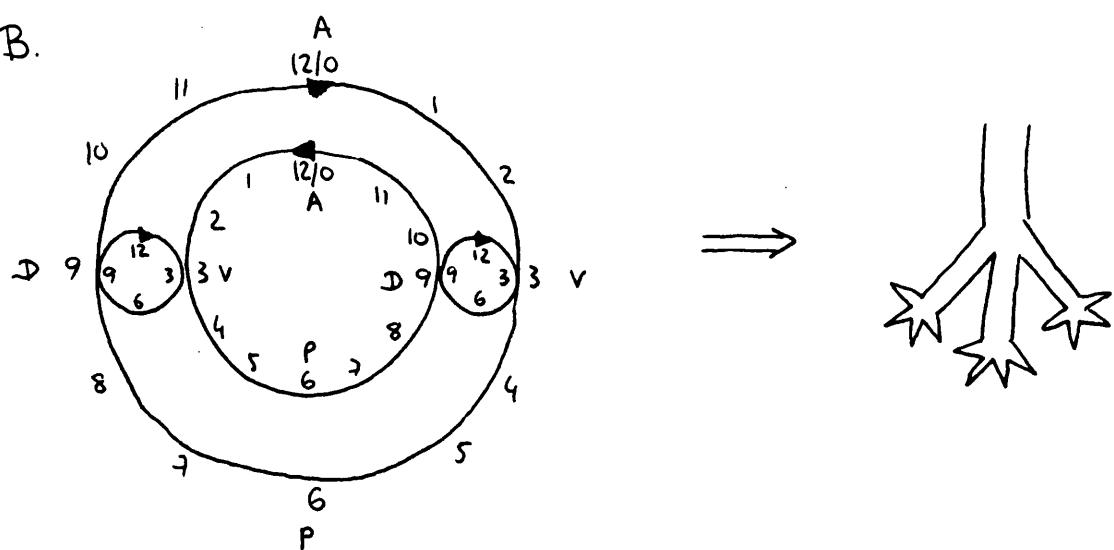


Figure 1.6: The Polar Co-ordinate Model.

- Apposition of cells with disparate positional identities leads to digit duplications through full circular intercalation.
- Where the full circle of values is generated, for example by rotating grafte tips of amphibian limbs, supernumerary limbs form by distal transformation.

ZPA graft (Cooke and Summerbell, 1980). However, according to the predictions of this theory, grafts of anterior tissue to posterior regions of the limb should be as effective at inducing extra digit formation as grafts of posterior ZPA cells. This is not the case in the chick limb, although various conflicting results have been achieved by grafting mouse and quail anterior cells, both freshly dissected and after culture, which show that under some conditions, anterior cells can induce the formation of rods and nodules of cartilage and sometimes even digits (Iten and Murphy, 1980; Wanek and Bryant, 1991; Stocker and Carlson, 1988). There is however, some doubt as to whether these grafts are inducing host tissue to develop, or whether the observed structures are merely the result of differentiation of the graft tissue (Carlson, 1984).

b) The Morphogen Gradient Model:

The Morphogen Gradient Model proposes that positional information is provided by a monotonic decrease in the concentration of a chemical - a morphogen. The concentration of the morphogen at any point then provides a scalar measure of the distance from the boundary, and the slope of the gradient provides polarity. The clearest example of this model is the establishment of the anterior-posterior axis in *Drosophila* using a gradient of the gene *bicoid* (reviewed in Akam, 1989).

This model assumes that, in the chick limb, the polarising region produces a morphogen, a molecule which is distributed in a graded manner across the limb, that specifies, in a concentration-dependent fashion, the fate of a group of limb bud cells and hence the type of digit these cells will form (Wolpert, 1969, 1971) (Figure 1.7). It postulates that there are distinct thresholds for each digit; low levels of morphogen specify a digit 2 and increasing concentrations specify digits 3 and 4, respectively (Tickle *et al*, 1975; Wolpert, 1989). Therefore, according to this model, cell fate at different distances from the ZPA is determined by the local concentration of the morphogen, with specific thresholds of the morphogen inducing successive structures.

The morphogen gradient postulated by this model is thought to arise by simple diffusion, rather than directed signalling (Crick, 1970). The process of diffusion is very simple: all that is needed is a source and a sink, and diffusion between them produces a

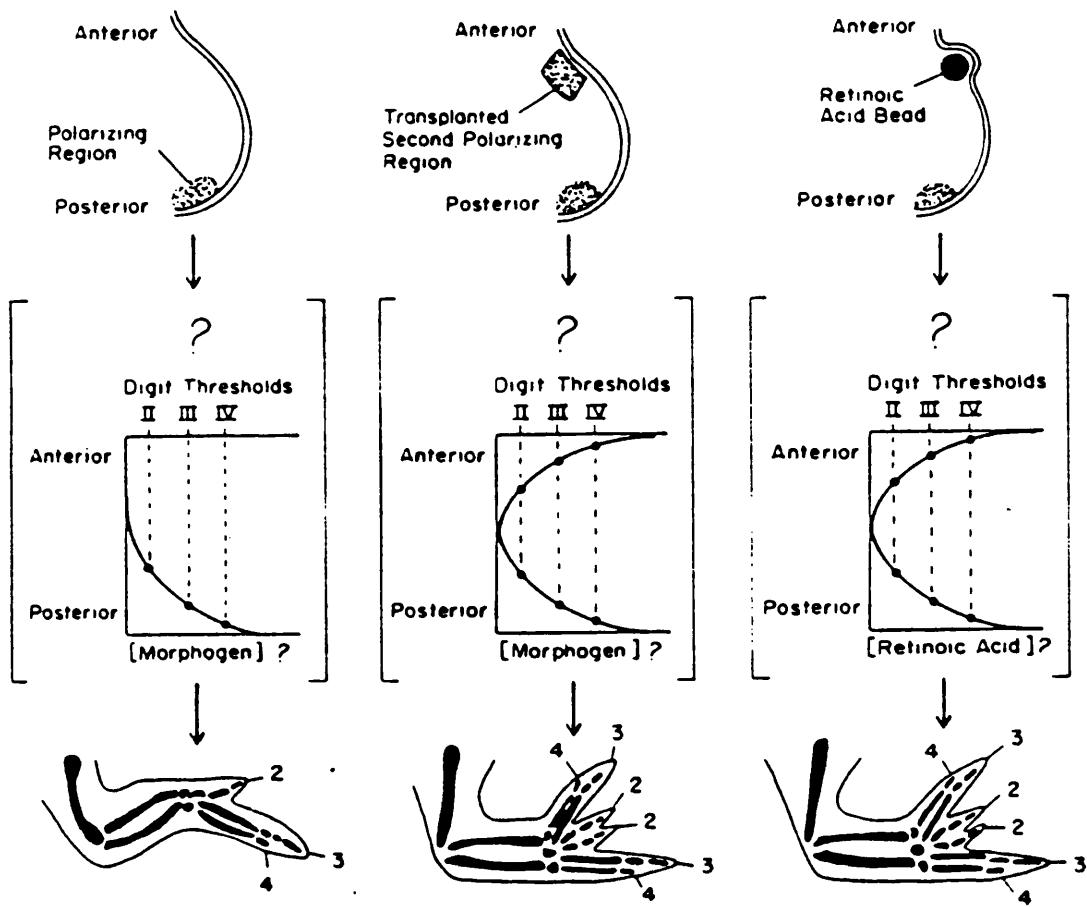


Figure 1.7: The Morphogen Gradient model.

A normal limb may pattern its antero-posterior axis by producing a diffusible morphogen in the ZPA, whose gradient decreases anteriorly. As the concentration falls through certain threshold levels, the limb bud tissue is instructed to form particular digits. Grafting a second ZPA at the anterior edge would be postulated to create a second source of the morphogen, so that each morphogen threshold would be reached twice in a symmetrical pattern. Implanting a retinoic acid bead has the same effect as a transplanted ZPA, consistent with RA being, or inducing the expression of the proposed morphogen.

(From Tabin, 1991)

gradient. The theory assumes that the morphogen is broken down at a rate proportional to its concentration, creating an exponential concentration gradient with the high point at the posterior margin. For this gradient to be set up through diffusion, the morphogen would need to be small and probably rather hydrophobic, to pass across cells (Crick, 1970).

Wolpert (1969) calculated that the morphogen field for positional information probably has to be less than 100 cells, and preferably less than 50, and that at least 10 hours would be needed to set up the gradient by simple diffusion. Smith (1980) showed that grafted quail ZPA cells require at least 15 hours to induce additional host structures, and previous experiments rotating the limb tip or putting a tantalum foil barrier suggested that at least 10-12 hours were required (Saunders and Gasseling, 1963): this is ample time for a morphogen gradient to form. The ZPA can also induce the formation of extra host digits through a "barrier" of leg tissue, which does not contribute to the duplicate wing digits (Honig, 1981). This effect of the ZPA works over 20 cell diameters (200 μm) but not more than 300 μm . This is approximately the size of the initial presumptive limb field (Honig, 1981).

There may be some element of prepatternning occurring in tandem with the morphogen gradient signal (Wolpert, 1991). This could take the form of an alternating wave to generate repeated structures such as digit or bone condensation, with a signal applied over the top of this to specify the different identities (Wolpert, 1989). Prepatternning requires only a reaction-diffusion equation with a single threshold, to generate a periodic signal which could then be used to generate repeated structures (Wilby and Ede, 1975).

One difference between the morphogen gradient theory and the cell growth intercalation theory lies in whether a grafted ZPA can alter existing positional values and the distance over which it exerts its influence. Both theories can be used to explain apical and anterior-to-posterior grafts, but it is difficult to explain the digit pattern when two polarising regions are grafted to the limb bud on the basis of an intercalation model (Wolpert and Hornbruch, 1981). If two ZPA grafts are made, one apically and one anteriorly, the intercalation model would predict that the effect would be the sum of the positional values, resulting in a digit pattern of 4322344 between the two grafts, no

matter where the middle graft is placed. The morphogen gradient theory predicts different results based on how close the grafts are placed: if the two grafts are too close, it predicts a digit pattern of 4334 because there would not be sufficient space for a full fall in the gradient level to the threshold of digit 2. The actual results show that distance is needed between the grafts to form a digit 2, consistent with the predictions of the morphogen model (Wolpert and Hornbruch, 1981).

In summary, the most important distinction between a morphogen-based model and the polar co-ordinate model is that, in the former model, cell fate determination occurs by long-range signalling encompassing several cell diameters, whereas, in the latter model, position is specified purely on the basis of short-range cell-cell interactions. Furthermore, only in the first model does the ZPA play any special role as a signalling centre.

1.5 Candidate Signalling Molecules:

a) RA, a Candidate Morphogen:

The effect of the ZPA can be mimicked by implanting a retinoic-acid (RA) soaked bead into the anterior edge of the chick limb (Tickle *et al*, 1982; 1985; Summerbell, 1983; Summerbell and Harvey, 1983; reviewed by Tickle and Brickell, 1991; and Hofmann and Eichele, 1994) (Figure 1.7). Retinoic acid was the first chemically-defined, naturally-occurring molecule to be identified which profoundly affects the developing and regenerating limb.

Various pieces of evidence suggested that RA could be the ZPA signal and it was postulated that it was the morphogen responsible for antero-posterior limb patterning, required by the Morphogen Gradient Theory (Tickle *et al*, 1985). Application of increasing concentrations of RA (1-25 nM) leads to the formation of increasingly complete digit duplications: 2234, 32234, and 432234 (Tickle *et al*, 1985). There is no effect if the RA-soaked bead is placed posteriorly, except at very high doses of RA (above 0.05 mg/ml), when the number of digits decreases, until, at 5 mg/ml, no digits form (Tickle *et al*, 1985; Summerbell, 1983) (Figure 1.8). The contralateral limb never shows any effects, despite the presence of a substantial amount of RA in the blood

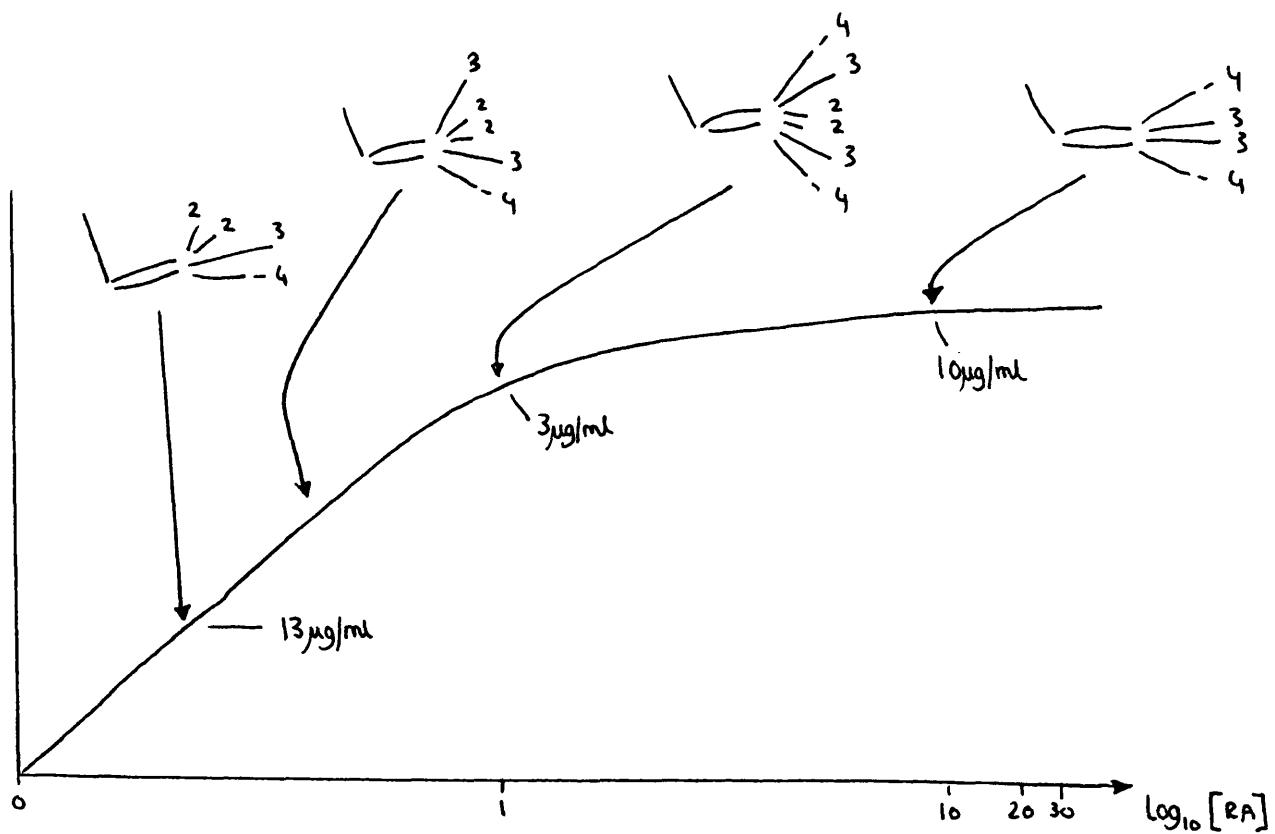


Figure 1.8: RA dose response curve and corresponding digit duplications.

(Adapted from Hofmann & Eichele, 1994 and Tickle *et al*, 1985)

(Tickle *et al*, 1985). An initial priming phase of 8 hours exposure to RA is required to get full duplications; no effects are seen during these first 8 hours (Eichele *et al*, 1985). Additional digits are then formed in a defined temporal sequence, with digit II formed first and IV last, with each digit taking approximately 1-2 hours to form or promote (Eichele *et al*, 1985). It does not appear that an active derivative is made during the priming phase, but rather that the time is required to establish a gradient, because if the RA bead is removed, the source is removed and the gradient will level off. Evidence for this comes from the fact that when beads soaked in 10 times the concentration needed to get full digit duplications are placed in the limb and then removed, there is no effect (Eichele *et al*, 1985).

Retinoic acid is a small hydrophobic molecule (300 MW), the ideal size for a diffusible morphogen (Crick, 1970; Tickle *et al*, 1985). This means, however, that the RA distribution cannot be visualised by *in situ* hybridisation, but application of radioactive RA to the limb has shown that a near-exponential anterior-posterior gradient of RA of the shape predicted by the Morphogen Gradient model forms after 6 hours, and is stable over time (Wolpert 1969, 1970; Tickle *et al*, 1985).

Studies using HPLC and sectioning in chick and mouse limbs have shown that all-*trans* RA and its precursor, all-*trans* retinol, are present endogenously in limb buds and are enriched in the posterior region that contains the ZPA (average concentration = 25 nM, 50 nM posteriorly and 20 nM anteriorly) (Thaller and Eichele, 1987, 1988; Satre and Kochhar, 1989). Importantly, the dose of RA required to induce a full set of digits yields a concentration of applied RA in the limb bud tissue that is in the same range as the endogenous RA concentration (Thaller and Eichele, 1990; Thaller *et al*, 1993). Thaller and Eichele (1990) predict that the tissue concentrations needed for an extra digit to form will be in the low nanomolar range: 0.9 nM for an extra digit 2, 2.5 nM for 3, and 25 nM for 4. The concentrations of RA are highest in the undifferentiated limb bud and fall off as differentiation ends, slightly preceding the loss of polarising activity (Thaller and Eichele, 1990; Honig and Summerbell, 1985).

Thaller and Eichele (1987) showed that limb bud cells can synthesise RA from its precursor, all-*trans* retinol, which is very abundantly present in the limb, providing an abundant pool. Thus a steady state of synthesis during development would produce the

steady state gradient predicted by the Morphogen Gradient model. It is not known, however, whether the ZPA is a high point of RA production, although Hensen's node, another polarising tissue, can act as a source of RA, by synthesising it from precursors (Hogan et al, 1992; Chen et al, 1992). Thaller *et al* (1993) showed that 9-*cis* RA can also produce duplications when applied to the limb bud: in fact it is 25 times more potent at inducing duplications than all-*trans* RA. They found that applied all-*trans* RA is converted to 9-*cis* in the wing-bud, and vice-versa, therefore all-*trans* RA could be the precursor of the actual active species in the limb.

The morphogen model can explain the digit additions, but not the reductions (Summerbell, 1983). They could be due either to a switch to a toxic effect or growth-differentiation inhibition by RA at high levels, or it could be due to an "enhancement" effect, where if the activity is above that normally experienced by the ZPA, nonsense positional values are generated.

Limb buds have also been shown to express all the constituents required for an intact retinoid signalling pathway at the time when the pattern of digits is specified (Thaller and Eichele, 1988; Maden *et al*, 1988). RA activates its target genes by binding and activating nuclear receptors, which then act as transcription factors (Giguere *et al*, 1987; Evans, 1988). Two families of retinoid receptors have been identified which mediate the action of RA, the retinoic acid receptors (RAR α , β , and γ), which bind either all-*trans*-retinoic acid or 9-*cis*-retinoic acid, and the retinoid-X-receptors (RXR α , β and γ), which only bind 9-*cis*-retinoic acid (reviewed by Mangelsdorf *et al*, 1994). Each class also has different isoforms, due to variation at the amino terminus. These retinoid receptors can form heterodimers or homodimers, which then have different specificities for target genes, allowing fine control over the effects of RA (reviewed by Linney, 1992).

Recently, however, doubts have been cast on whether RA is in fact involved *in vivo* and there is good evidence to suggest that RA does not act as the endogenous morphogen (Brockes, 1991b; Wanek *et al*, 1991; Noji *et al*, 1991; Tamura *et al*, 1993). One suggestion is that local RA instead induces neighbouring tissue to become a ZPA, which then determines the AP axis by some mechanism not involving the graded activity of RA. Implantation of an RA-soaked bead causes neighbouring cells to gain polarising

activity after 16 hours (Wanek *et al*, 1991), whereas, transplanted ZPA cells do not induce a new ZPA in the host (Smith, 1979). Wanek *et al* (1991) suggested that the graded response to endogenous RA may therefore reflect a variation in the number of cells induced to become polarising, rather than a dose-dependent effect of the RA itself (Tickle, 1981).

In addition, Noji *et al* (1991) showed that exogenous RA placed anteriorly or posteriorly activates the chicken RAR β promoter RARE, unlike ZPA grafts (de Thé *et al*, 1990; Sucov *et al*, 1990; Rossant *et al*, 1991). Activation of the RARE can be detected after 4 hours by *in situ* hybridisation. The RAR β gene is normally expressed in the proximal region of the limb, but not distally and not around the ZPA, possibly due to concentration effects (Rossant *et al*, 1991; Noji *et al*, 1991). These experiments do not rule out possible effects of endogenous RA though, and experiments done on expression of RAR β in the chick facial primordia show that its induction by RA is complex: in some places RAR β is strongly induced and in others it is not induced at all by an equal concentration of RA (Wedden *et al*, 1987; Rowe *et al*, 1991; reviewed by Tickle and Brickell, 1991).

RA also has effects in limb regeneration in urodele amphibians, but there is no evidence for an RA signalling centre in this system (Brockes, 1991a and 1991b). In amphibian limb regeneration, RA resets the axial specification: it proximalises the blastema in a dose- and time-dependent, but not position-dependent manner: after RA treatment, extra elements appear in the proximo-distal axis, and complete limbs can be re-grown from the amputation plane (Brockes, 1991; Maden, 1982, 1983, 1985). In frogs and toads, it is also possible to get anterior-posterior duplications, similar to those induced in chickens, by systemic application of RA (Niazi and Saxena, 1978; Maden, 1983, 1985). In this system, RA initially inhibits cell division and cartilage matrix breakdown and cell aggregation, and the patterning effect only happens after the RA is removed (Maden, 1985).

b) Sonic hedgehog:

The product of the *Sonic hedgehog* gene has also been implicated as the ZPA morphogen. In 1993, Riddle *et al* isolated *Sonic hedgehog* (*Shh*), a vertebrate homologue of the *Drosophila* segment polarity gene *hedgehog*. The sequence of this gene is very highly conserved evolutionarily, suggesting that it may play an important role in development. There are two other similar genes found in mice, *desert hedgehog* and *Indian hedgehog*: *desert hedgehog* is not expressed in embryonic limb buds (Echelard *et al*, 1993).

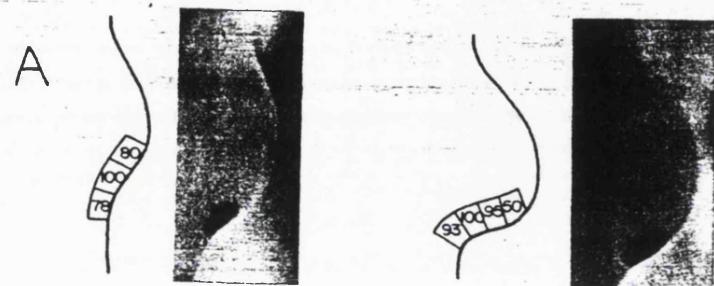
Riddle *et al* (1993) found that expression of *Shh* co-localises with the mapped domain of the ZPA (Figure 1.9). *Shh* is also expressed strongly in other tissues capable of polarising activity, such as Hensen's node, the notochord and the posterior fin mesoderm in the zebrafish (Krauss *et al*, 1993), but it is not expressed in presumptive limb bud cells along the flank before limb bud formation, an area which can nevertheless induce digit duplication if grafted anteriorly. Most convincingly, ectopic expression of *Shh* anteriorly, using grafts of cells expressing recombinant *Shh*, or SHH protein on beads, can also cause mirror-image skeletal duplications (Riddle *et al*, 1993).

However, there is no gradient of *shh* mRNA in the limb, and antibody studies on the localisation of the protein have shown that, although SHH is a secreted protein, the active region of the processed protein does not diffuse very far from its site of production at the posterior margin of the limb (Bumcrot *et al*, 1995; López-Martínez *et al*, 1995).

However, RA induces cells to express *Shh*, suggesting that RA may possibly act by inducing *Shh* expression, but it is not known if RA is normally involved in regulating *Shh* expression.

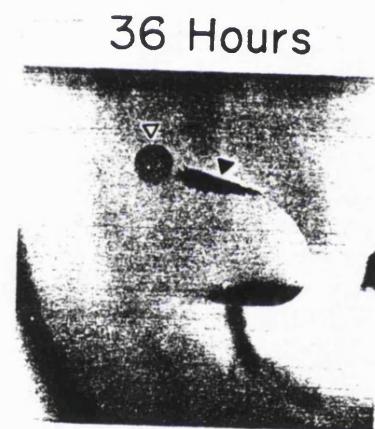
c) Putative AER signals: the Fibroblast Growth Factors:

Fibroblast growth factors appear to be key players in signalling by the AER (reviewed by Laufer, 1995).



Stage 19

Stage 21



B

Figure 1.9: Expression domain of *Sonic hedgehog* in the developing limb.

- Normal expression domain of *Shh* in the developing limb.
- Ectopic induction of *Shh* expression caused by RA, 36 hours after implantation.

(Taken from Riddle *et al*, 1993)

The fibroblast growth factor family comprises at least nine members, FGF-1-9 (Miyamoto *et al*, 1993; reviewed by Burgess and Maciag, 1989; Baird and Klagsbrun, 1991). In culture, FGFs influence the proliferation capacity of the majority of mesoderm- and neuroectoderm-derived cells. FGF-1, 2 and 9 lack classical consensus signal peptide sequences, but are still found extracellularly, secreted in some unknown way (Abraham *et al*, 1986a and 1986b; Burgess and Maciag, 1989; Miyamoto *et al*, 1993). The other FGFs are processed and secreted normally (Delli-Bovi *et al*, 1988).

Fgf-4 is expressed in the posterior half of the AER in the mouse, from E10 to E12, after which expression is no longer detected, although the AER is still present (Niswander and Martin, 1992; Suzuki *et al*, 1992). No expression is found in the mesenchyme. In the chick also, expression is confined to the posterior half of the AER (Niswander *et al*, 1994). Although FGF-4 is a secreted protein, it is thought likely that it will act locally, because it interacts strongly with cell surface and extracellular matrix molecules (reviewed by Rifkin and Moscatelli, 1989; Ruoslahti and Yamaguchi, 1991) and matrix binding of FGF-4 may provide a reservoir of growth factor.

In short-term organ cultures of mouse limb buds stripped of the AER, FGF-4 can increase proliferation of cells in the bud that would otherwise have effectively stopped growing (Niswander and Martin, 1993a), and application of FGF-4-soaked beads simultaneously to the apex and the posterior margin of the bud can rescue chick limb buds which have had the AER removed, stimulating local proliferation and allowing quasi-normal limb development, including maintenance of the expression of marker genes such as *Evx-1*, a gene found in the posterior progress zone mesenchyme shortly after the formation of the AER and which switches off when the ridge regresses (Dush and Martin, 1992; Niswander *et al*, 1993; Niswander and Martin, 1993b). However, in these experiments, the digits were bunched, probably because the ridge also performs a mechanical role, keeping the limb bud flattened. Thus FGF-4 can fully substitute for the ridge-derived signals required to maintain limb outgrowth, but cannot entirely replace the ridge.

FGF-2 protein is also found throughout the AER in the chick limb (Savage *et al*, 1993). It is also found in the dorsal surface ectoderm and a 2-3-cell-thick layer of subectodermal mesenchyme, from the initiation of limb budding at stage 16, throughout

differentiation. There are three different isoforms of *Fgf-2* all of which are expressed in the same places and at similar levels in the chick limb (Dono and Zeller, 1994).

FGF-2 also permits cell survival and stimulates proliferation of limb bud mesenchyme in culture (Savage *et al*, 1993), and like FGF-4, FGF-2 can maintain outgrowth *in vivo* after ridge removal (Fallon *et al*, 1994). However, it also causes the formation of extra cartilage when applied in bead form, and the duplication of anterior limb bones and digits, including the proximal humerus, radius and digits II and III, when over-expressed in limbs infected with retroviral vectors expressing *Fgf-2* (Riley *et al*, 1993). It is not known whether FGF-4 also has this effect. It has however been proposed that this effect arises by a stimulation of proliferation of skeletal precursor cells rather than by a respecification of anterior limb bud cells (Riley *et al*, 1993; Dono and Zeller, 1994; Laufer, 1995). This is supported by the fact that posterior duplications were never seen.

Most of the FGF signal from the AER appears to be FGF-2, not 4, since addition of monoclonal antibodies specific to FGF-2 block the FGF activity of limb cells by 94-97% (Fallon *et al*, 1994). However, FGF-2 lacks a consensus signal sequence, and its mechanism of release from the limb cells is unknown (reviewed by Rifkin and Moscatelli, 1989), although forced secretion by fusing FGF-2 to the immunoglobulin signal peptide leads to correct processing and secretion (Rogelj *et al*, 1988).

FGFs may also be involved in the initial outgrowth of the limb bud. Beads soaked in FGF-1, 2 or 4, placed in the flank, can induce the formation of ectopic limbs, which then develop into complete limbs (Cohn *et al*, 1995). These have a polarising region, and an AER and express the appropriate marker genes, such as *Hox* genes (see below). The whole flank, between the limbs, can form buds, but generally wings form anteriorly, and legs posteriorly, and the limbs have mirror-image reversed polarity. Muscles, tendons and nerves all form, and the dorso-ventral axis is normal. This suggests that normal limb outgrowth is triggered by a local source of FGF. The endogenous FGF is probably FGF-8, because it is the only FGF that is expressed in the correct place and time (between embryonic days 9.25-13.5 in the mouse) (Ohuchi *et al*, 1994; Heikinheimo *et al*, 1994, Crossley and Martin, 1994).

Fgf-5 is also expressed in the mouse limb in a patch of cells near the base of each limb, between E12 and 14.5 (Haub and Goldfarb, 1991) and *Fgf-7* is expressed in subsets of mesenchymal cells at later stages.

So far 4 receptors for FGFs have been identified (Lee *et al*, 1989; Mansukhani *et al*, 1990; reviewed by Johnson and Williams, 1993). They are high affinity receptor tyrosine kinases. Heparin and heparin sulphate, low affinity FGF receptors, are also needed for high affinity binding to FGF receptors (Yayon *et al*, 1991). Both FGF-2 and 4 can bind to the same receptor, FGFR-1 (Mansukhani *et al*, 1990), which is expressed diffusely in the mesenchyme of mouse limb buds (Peters *et al*, 1992; Noji *et al*, 1993); FGFR 2 is found in the epithelium of the limb bud, including the AER (Peters *et al*, 1992). There are also multiple mRNAs of the receptors produced by alternative splicing: the different splice variants have different ligand and tissue specificities (Johnson and Williams, 1993).

However, other factors are expressed in AER too, such as *Bmp-2* and *4*, which may play a role in signalling by the AER.

d) Dorso-ventral Signals:

The signal mediating dorso-ventral axis specification may be *Wnt7a*, a member of the *Wnt* family of signalling molecules, which is expressed in the ventral ectoderm (Parr *et al*, 1993; Dealy *et al*, 1993; Parr and McMahon, 1995). There are 10 *Wnt* genes in the mouse, and 8 in the chick (3, 3a, 4, 5a, 5b, 7b, 8b and 10) (McMahon, 1992). *Wnt-1* is the homologue of the *Drosophila* gene, *wingless* (reviewed by McMahon, 1992; Nusse and Varmus, 1992). The *Wnt* gene products are secreted glycoproteins but the protein appears to be quite tenaciously associated with the extra-cellular matrix or the cell surface, and is therefore not freely diffusible.

Mutant mice which lack *Wnt-7a* expression have dorso-ventral transformations in the limbs, with ventral structures replaced by dorsal ones (Parr and McMahon, 1995). However, only the mesoderm is affected and other markers, such as *Bmp-2* are still correctly expressed in the ectoderm. Therefore *Wnt-7a* may normally act to block the

formation of ventral structures, rather than as a dorsalising signal. However, the effects are more severe distally than proximally, so there are probably other mechanisms involved. Many of these transgenic mice also lack posterior digits, suggesting that *Wnt-7a* plays some role in anterior-posterior patterning, suggesting that normal limb development requires interactions between the signalling systems for these two axes.

Other *Wnt* genes are also expressed in the limb, and these may well interact with *Wnt-7a*. In the mouse, *Wnt-3, 4, 6* and *7b* are expressed uniformly in the limb ectoderm (Roelink and Nusse, 1991), *5a* is distributed in a proximal to distal gradient through the limb mesenchyme, with highest expression in the Progress Zone, and in the AER and ventral ectoderm (Gavin *et al*, 1990; Parr *et al*, 1993; Dealy *et al*, 1993). *Wnt-5a* is expressed in the mesenchyme in three domains along the proximo-distal axis in mesoderm which may correspond to the regions which will give rise to the three distinct proximo-distal segments of the limb: the autopod, zeugopod and stylopod (Dealy *et al*, 1993). Expression ceases upon differentiation.

Wnt-1 is not normally expressed in the limbs, but if *Wnt-1* is ectopically expressed in the limbs, the hemizygote mice show extensive distal truncations of skeletal elements, skeletal fusions and interdigital webbing (Zákány and Duboule, 1993). All four limbs are affected, and the paws are all rotated 90° and are shorter. The limb plate is larger and round and no cell death occurs. This is thought to arise because the retarded mesenchymal condensations are replaced by highly proliferative cells in the limb bud. Interruption of the condensation causes the limbs to be shorter and lack digits and phalanges and no cell death occurs.

d. Co-ordination of the three axes:

All three axes must act in co-ordination to specify growth and patterning of the limb, and there are various interactions between the putative signalling areas and candidate molecules that may act to achieve this.

For example, the AER is required for effective signalling of ZPA cells (Tickle, 1981); in the absence of the ridge, polarising activity is reduced (Vogel and Tickle, 1993), but

can be restored to normal levels by applying FGF-4-soaked beads. FGF-4 can also maintain polarising activity in culture (Vogel and Tickle, 1993), as can FGF-2 (Anderson *et al*, 1993). In turn, the polarising activity is needed to maintain the progress zone in conjunction with the AER to allow proper proximodistal patterning.

Niswander *et al* (1994) showed that there is a positive feedback loop between FGF-4 in the ridge and *Shh* in the underlying mesenchyme: *Fgf-4* expression can be induced in the anterior ridge by *Shh*-expressing cells or in response to RA application, and in turn, the ridge or FGF-4 is required to maintain *Shh* expression. In addition, *Shh* expression in the mesenchyme can be activated by FGF-4 but only in conjunction with RA. However, once induced, *Shh* expression can be maintained by FGF-4 alone (Niswander *et al*, 1994).

Temporal studies indicate that, following RA application to the anterior limb bud, *Fgf-4* is induced within 18 hours and *Shh* is induced about 6 hours later (Niswander *et al*, 1994). Thus, Niswander *et al* (1994) have proposed that RA acts to respecify the anterior limb bud mesenchyme by first inducing FGF-4 in the anterior ridge. Then FGF-4 from the AER, in combination with RA, induces *Shh* expression in the anterior mesenchyme underlying the ridge. Then the positive feedback loop acts to maintain both *Shh* and *Fgf-4* expression, and RA is no longer needed, because the changes induced by ectopic RA are irreversible about 16 hours after treatment (Eichele *et al*, 1985). This all acts to establish and maintain a new ZPA.

In this way, via a simple positive feedback loop, proximo-distal outgrowth and antero-posterior patterning can be co-ordinately regulated.

Signals from the ectoderm overlying the mesenchyme are also required for antero-posterior patterning, in addition to its role in dorso-ventral patterning. The dorsal ectoderm is necessary to maintain *Shh* expression and posterior skeletal formation. It has been shown that *Wnt7a* must act in conjunction with the ridge or FGF-4 in the regulation of *Shh* expression (Niswander *et al*, 1995).

Thus, all three axes act in co-ordination to specify growth and patterning of the limb.

1.6 Secondary Response Genes:

a) The Homeobox genes:

A number of homeobox-containing genes have been found to respond to these various signalling molecules in the developing limb (reviewed in Lobe, 1992).

The homeobox domain is a conserved protein-coding sequence, found in genes in both invertebrates and vertebrates (McGinnis *et al*, 1984a and 1984b; Colberg-Poley *et al*, 1985). It codes for a highly conserved helix-turn-helix DNA-binding protein domain (Levine and Hoey, 1988; Hoey and Levine, 1988; reviewed in Lewis, 1989; Scott *et al*, 1989). The products of *Hox* genes are therefore thought to be transcription factors, which act to stimulate the transcription of other genes in a developmental cascade (Hoey and Levine, 1988).

In *Drosophila*, mutations in the homeotic genes cause defects where one body structure is replaced by another, which is normally located elsewhere. There are two complexes: the ANT-C complex, which is responsible for segmental identity in the posterior head and thorax, and BX-C, responsible for the posterior thoracic and abdominal segments (Lewis, 1989; reviewed in Akam, 1989).

There are 38 *Hox* genes in the genome of higher vertebrates, all of which encode proteins related to the *Drosophila* homeotic genes (reviewed in Hunt and Krumlauf, 1992; Lewis, 1989; McGinnis and Krumlauf, 1992). The *Hox* genes are organised into 4 clusters (*HoxA*, *B*, *C* and *D*; previously known as *Hox-1*, *2*, *3* and *4*) whose organisation is closely related to that of their *Drosophila* counterparts (Boncinelli *et al*, 1988; reviewed by Hunt and Krumlauf, 1992). In fact, several of the mammalian *Hox* genes can be identified as individually homologous to specific *Drosophila* genes, and are more closely related to them than to their chromosomal neighbours (Lewis, 1989). The mouse and *Drosophila* gene complexes can therefore be aligned based on their relative position and sequence identity (Graham *et al*, 1989) (Figure 1.10).

Thus the organisation of the genes within the clusters is highly evolutionarily conserved and may play a role in the control of expression: genes at the 3' end of each

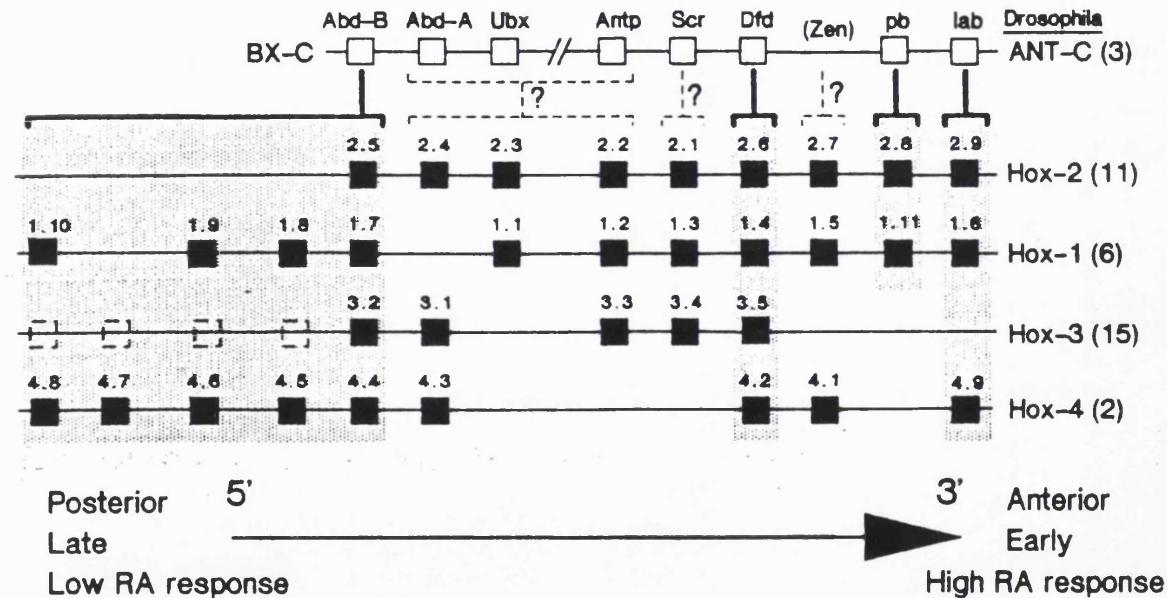


Figure 1.10: Alignment of the *Hox* genes

Alignment of the *Drosophila HOM-C* homeotic complex (top) and the four mouse *Hox* clusters based on regions of homology. The columns of solid filled boxes represent related mouse genes that form paralogous groups, and the brackets above each column align them with the closest *Drosophila* counterpart(s). The dashed brackets and (?) indicate that it is difficult to identify individual homologs. The arrow at the bottom indicates the relationship between gene order, expression boundaries, temporal order and RA responsiveness.(From Graham *et al*, 1989).

cluster are expressed prior to those at the 5' end and are expressed in domains that extend more anteriorly than those of 5' genes. Thus, the further a gene lies from the beginning of the gene cluster, the more posterior the domain to which its expression is restricted (reviewed in Lewis, 1989). For example, in vertebrates, the *Hox* genes are expressed in different overlapping domains along the antero-posterior axis of the body, in the prevertebral column and the central nervous system, with more 3' genes being expressed more anteriorly (Gaunt *et al*, 1988; Graham *et al*, 1989) (Figure 1.11).

As in *Drosophila*, over-expressing or knocking out expression of *Hox* genes in vertebrates leads to changes in the development of a wide variety of organs, including the prevertebrae of the trunk, the branchial region of the head and the rhombomeres, segmental structures that make up the neural tube of the hindbrain (Kessel *et al*, 1990; Kessel and Gruss, 1991; Conlon and Rossant, 1992; Marshall *et al*, 1992; Sundin and Eichele, 1992; reviewed in Hunt and Krumlauf, 1992). Knocking out *Hoxa-2*, for example, results in the transformation of structures of the second branchial arch into structures typical of the first arch (Gendron-Maguire *et al*, 1993; Rijli *et al*, 1993).

For these reasons, it is thought that expression of the *Hox* genes, possibly in some coded combination, may be involved in specifying cell identity along the antero-posterior body axis of vertebrates (Gaunt *et al*, 1986).

b) Expression of homeobox genes in the developing limb:

Several *Hox* genes are also expressed in the developing limb bud at the time when limb pattern is being specified (reviewed by Izpisúa-Belmonte and Duboule, 1992). The expression domain of a given gene is generally contained within the domain of the gene located 3' to it, therefore successively more 5' located genes have progressively smaller expression domains (Figure 1.12).

Expression of the three genes, *Hoxa-10*, *a-11* and *a-13*, is switched on postero-distally, and subsequently expands anteriorly so that the final boundaries are perpendicular to the proximo-distal limb axis (Yokouchi *et al*, 1991; Haack and Gruss, 1993). The

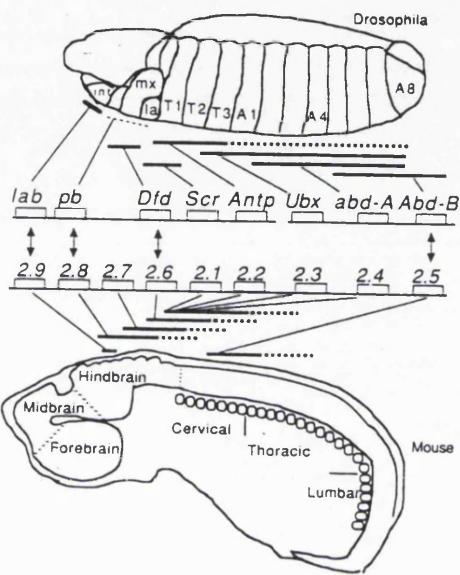


Figure 1.11: Expression of *Hox* genes along the main body axis.

The upper part of the figure contains a diagram of a 10 hour *Drosophila* embryo, showing the approximate extents of the epidermal expression domains of the *HOM-C* genes. The lower half of the figure shows a schematic diagram of a 12 day mouse embryo, with the approximate extents of *Hoxb* expression domains in the CNS indicated by the horizontal bars. The dotted extensions indicate that these expression domains extend in overlapping fashion to posterior regions of the CNS. The various *Hox* genes expressed in the hindbrain have subtly different boundaries in the posterior regions of the hindbrain; for simplicity, their expression domains are represented together.

(From Akam, 1989)

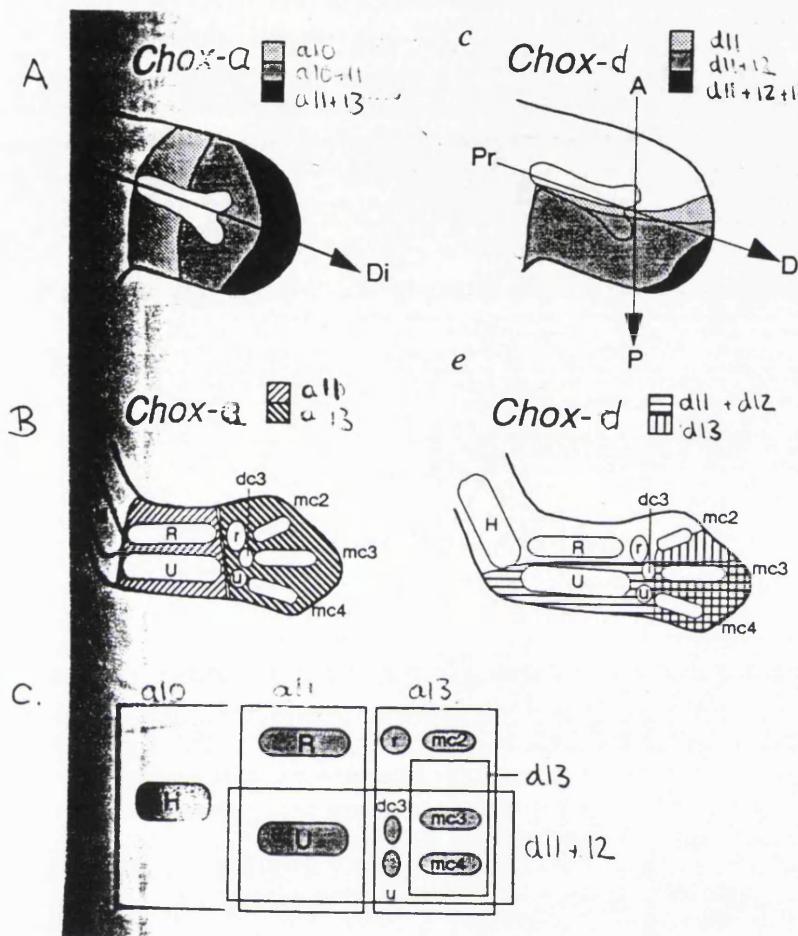


Figure 1.12: Expression domains of the *Hox* genes in the developing limb.

a) Schematic view of the expression patterns of the *Hox* genes in the stage 24 limb bud.

(Dorsal view, at the plane around the central region.)

b) Correlation of the domains of *Hox* expression with chondrogenic pattern in the stage 28 limb.

c) Scheme of superimposition of boundaries of *Hox* gene expression giving "map" of cartilage elements.

(From Yokouchi *et al*, 1991b)

proximal boundaries of these three genes are staggered so that *Hoxa-10* has its boundary most proximally and *Hoxa-13* most distally.

Members of the *Hoxd* cluster are also expressed in the limb in both the mouse and the chick (Oliver *et al*, 1989; Dollé *et al*, 1989; Izpisúa-Belmonte *et al*, 1991; Yokouchi *et al*, 1991; Nohno *et al*, 1991; reviewed in Izpisúa-Belmonte and Duboule, 1992). In the chick, *Hoxd-9* is expressed first at stage 17, and *Hoxd-13* is the last one to be expressed, just before stage 18. Their domains of expression overlap. Starting with the 3'-most gene, *Hoxd-9*, each gene is first expressed at the posterior side of the limb bud, and its expression spreads anteriorly as the limb grows. Each successive gene is thus sequentially activated in an increasingly restricted posterior region, forming a nested set (reviewed in Tabin, 1991). The zone of maximum overlap of all the *Hoxd* genes occurs at the ZPA. They are not expressed in the AER. Later in development, *Hoxd* genes are expressed in the perichondrial areas of the digits.

Hoxc genes are also expressed in the limb but little is known about their role, except that they are possibly involved in the specification of fore- and hind-limb differences (Oliver *et al*, 1988; Tabin, 1989).

It has been suggested that the *Hox* genes directly specify positional values in the limb, possibly involving precise combinations of gene expression (Dollé *et al*, 1989). For example, a comparison between the patterns of digits and of gene expression suggests that the formation of particular digits depends on which *Hoxd* genes are expressed (Dollé *et al*, 1989). However both digits III and IV would have the same code (Izpisúa-Belmonte *et al*, 1991). In addition, the expression domains of the *Hoxa* genes correspond to the cartilage condensations along the proximo-distal axis (Yokouchi *et al*, 1991). In this way, superimposing the expression domains of the *Hoxa* and *Hoxd* genes would give rise to a "map" of the cartilage elements in the chick limb, with expression of *Hoxa* genes being responsible for subdividing the limb along the proximodistal axis, and *Hoxd* gene expression exerting influence on the branching of cartilage primordia that eventually composes the pattern along the antero-posterior axis (Yokouchi *et al*, 1991). This idea that a particular combination of *Hoxd* genes could encode positional information is in part supported by experiments where *Hoxd-11* is ectopically over-expressed throughout the limb bud using replication-competent retroviral vectors

(Morgan *et al*, 1992; reviewed by Tickle, 1992). In these experiments, the domain defined by the co-expression of *Hoxd-9*, *d-10*, and *d-11*, which normally encompasses the primordia of digit II, was expanded anteriorly to encompass the digit I primordia. So, if the combinatorial *Hox* code is correct, only digits I and II should alter. In the leg bud, in 30% of cases, digit I is homeotically transformed into a digit II. However, in the wing bud, there is no change in digit identity, rather an additional digit 2 forms. This may be because the anterior region is normally specified for cell death, since there is not normally a digit I in the wing.

The correlations between the *Hox* gene expression domains and the regions of the limb buds that will give rise to specific skeletal structures are apparent prior to any overt sign of differentiation and persist into the period in which differentiation of skeletal elements is occurring. This supports the idea that *Hox* gene expression domains represent an early indication of "determination" in terms of the positional identity of cells in the limb bud (Hayamizu *et al*, 1994; Yokouchi *et al*, 1991). However, the protein expression domains of these genes may be different to the RNA domains.

Specification by *Hox* genes is abstract in the sense that *Hox* genes do not directly define where a particular final structures will form. The *Hox* genes are presumably involved in setting up the framework within which those processes that actually delineate the final morphogenesis can occur. For example, expression of the *Hox* genes may regulate expression of molecules that alter cell adhesion (Yokouchi *et al*, 1991).

c) Effects of RA and the ZPA on homeobox gene expression:

Grafts of polarising tissues to the anterior limb bud margin and application of RA have both been shown to induce ectopic anterior expression of *Hoxd-10* to *13* genes, and this correlates with the subsequent development of mirror-image patterns of digits (Izpisúa-Belmonte *et al*, 1991, 1992a; Nohno *et al*, 1991). Ectopic induction of *Hoxd* gene expression takes place in the same 3' to 5' sequence as during normal development, and the patterns follow their normal nested patterns, except they are mirror-image reversed. Expression is irreversible: if the RA bead is removed, the *Hox* genes stay on (Izpisúa-Belmonte *et al*, 1991). In all cases, all the induced genes are on after 24 hours: *Hoxd-9*

comes on after 16 hours, and *d-11* after 20 hours. This length of exposure to RA suggests that RA may not be acting directly on the posterior genes. The ectopic expression domains of *Hoxd* genes suggest that each member of the complex may respond to different concentrations of RA, with 3' genes being more sensitive than 5' genes. Alternatively, the spatial pattern could depend on the time that the genes are switched on and interactions between them. In anterior expression, the rule seems to be that cells cannot express upstream members without first expressing downstream ones. This may be important in the ordering of digits and may account for why digit II is never found next to digit IV, even in limbs treated with RA.

In embryonal teratocarcinoma cells induced to differentiate with RA, the *Hox* genes also show this same sequential activation, differential sensitivity, and delay in RA response (Colberg-Poley *et al*, 1985; Breier *et al*, 1986; Deschamps *et al*, 1987; Mavilio *et al*, 1988; reviewed by Boncinelli *et al*, 1988; Simeone *et al*, 1990, 1991). Very high doses of RA, however, inhibit expression of the 5' genes in the cluster.

While activation of early responding genes appears to be regulated primarily at the transcriptional level, synthesis of short half-life proteins is needed to maintain the timing and polarity of expression (Simeone *et al*, 1991). There are apparently a multiplicity of promoters in each *Hox* cluster: there is an individual promoter upstream of each coding region plus a master or major upstream promoter for each cluster (Simeone *et al*, 1988; Cho *et al*, 1988). Boncinelli *et al* (1988) has suggested that RA affects the proximal promoter but that the master promoter is needed for the correct timing and spatial expression of the genes. However, deletion promoter constructs have been made using the individual promoters of various *Hox* genes which show that the individual gene promoter elements are capable of driving correct spatial and temporal expression in the absence of the entire cluster (Püschel *et al*, 1990, 1991; Whiting *et al*, 1991).

Ectopic over-expression of *Shh* also induces the *Hoxd* genes. Riddle *et al* (1993) found that RA induces *Shh*, which then activates *Hoxd-11* expression, as does RA alone. Based on this finding, they have proposed that RA first induces cells to express *Shh* and in turn *Shh* activates *Hoxd* genes. However, both RA and *Shh* take about the same length of time to induce *Hoxd-11* (20 compared with 24 hours). This, together with the finding that RA takes 24 hours to induce *Shh*, argues against the sequential operation of

RA and *Shh* in activating *Hoxd* genes and suggests that different pathways may be taken, with the intervening steps being as yet unknown.

Signals from the AER are also required for both normal and RA-induced expression of *Hoxd* genes: if the AER is removed during normal development, or after implantation of a RA bead, *Hoxd* genes are not expressed (Izpisúa-Belmonte *et al* 1992b; Hayamizu *et al*, 1994). However, although the presence of the AER is required for elaboration of the pattern of expression of the *Hoxd* genes, once the genes are switched on, activation cannot be reversed by removal of the AER. (Izpisúa-Belmonte *et al*, 1992b). These findings can either be explained in terms of an AER signalling molecule which directly co-operates with retinoic acid in inducing *Hoxd* gene transcription, or, alternatively, the ridge could play a more passive role and merely maintain cells in the Progress Zone in a proliferative state that permits *Hox* gene expression.

Dollé *et al* (1989) have suggested that, while RA or a morphogen from the ZPA gradient could switch on *Hox* genes in a threshold-dependent manner, each gene may only be accessible and "open-for-business" at a certain time. Hence at the time when a given gene becomes available for transcription, it will be activated, or its transcripts stabilised, only in the region where the local concentration of morphogen is above threshold, and to an extent directly related to this concentration. Izpisúa-Belmonte and Duboule (1992) have expanded on this model, suggesting that *Hox* genes are activated following a temporal sequence which progresses in parallel with the limb's proximo-distal morphogenetic sequence. In this instance, 3' to 5' colinear opening of the complexes is postulated to occur in the Progress Zone, because only these dividing cells are able to reinitiate *Hox-d* gene transcription following the correct progression. This opening process would therefore depend on the presence of the AER, maintaining proliferation. As cells leave the PZ, the opening process is interrupted and cells are determined to express a particular combination of *Hox* genes: the code is therefore fixed by the length of time the cells spend in the PZ. Therefore "opening" of genes and initiation of transcription only occurs, in the presence of the AER, in actively dividing PZ cells, but transcription of genes can be maintained outside these areas. In this way, growth of the limb, proximo-distal and antero-posterior patterning are all linked in one system. The temporal activation of *Hox* genes thus provides continuous, region-specific information for local growth, and the *Hox* code therefore reflects relative position within a time

controlled ontogenetic sequence rather than position in a spatial field. The *Hox* genes are therefore molecular clocks. This system would be very flexible and provides a simple way to simply effect a wide variety of changes during evolution, by affecting the relative timing of expression of *Hox* genes rather than the number of genes.

Support for this model comes from a transgenic *Hoxd-13* loss-of-function mouse (Dollé *et al*, 1993). These mutant mice have skeletal alterations along all the body axes, supporting the existence of a general multi-axial patterning system. In the limbs, the mutant mice show a reduction in the length of some bony elements, loss of phalanges, bone fusions and the presence of an extra element, a rudimentary posterior digit, in both fore and hind limbs. Dollé *et al* (1993) suggest that these defects are due to a local retardation in limb development, giving rise to neoteny, rather than a combinatorial homeotic transformation. This is supported by the fact ossification is very delayed and in some elements does not occur.

d) Other secondary response genes in the limb:

Other putative secondary response genes include *Msx-1* and *2*, related to the *Drosophila Msh* genes (reviewed by Davidson and Hill, 1991). *Msx-1* is expressed in the AER and distal mesenchyme corresponding to the Progress Zone at early stages of limb development, stage 18-21, and then in necrosing cells in the interdigital region at stage 28 (Yokouchi *et al*, 1991; Suzuki *et al*, 1991; Robert *et al*, 1989, 1991; Davidson *et al*, 1991; Brown *et al*, 1993). *Msx-2* is expressed in similar regions in the mouse and chick (Davidson *et al*, 1991; Robert *et al*, 1991). It has been suggested that these genes are in some way involved either in specifying the Progress Zone, or in defining areas which will undergo programmed cell death at a later stage. These genes might therefore also be responsible for linking growth to patterning (Izpisúa-Belmonte and Duboule, 1992).

Davidson *et al* (1991) showed that a signal from the AER is involved in controlling *Msx-1* and *2* expression. If mouse limb mesoderm is grafted to the chick wing bud in a distal area which expresses these genes, both genes are rapidly activated, whereas if they are placed in a non-expressing region proximally, expression is switched off in the grafts. It has been suggested that this signal is *Bmp-2* or *4* (Davidson and Hill, 1991)

(see below). RA beads also suppress the expression of *Msx-1* in the chick limb after 12 hours exposure (Yokouchi *et al*, 1991).

Thus, a complex cascade of genes is involved in controlling the establishment of the axes and the further development of the limb. We are interested in the possible roles of the bone morphogenetic proteins in this signalling cascade.

1.7 The Bone Morphogenetic Proteins:

Bone Morphogenetic Proteins (BMPs) are proteins expressed during development that can induce cartilage and bone formation (reviewed by Wozney and Rosen, 1993).

They were first discovered in 1965 (Urist, 1965), when it was observed that pieces of demineralised bone, or extracts from osteosarcomas and certain epithelia could induce ectopic cartilage and bone formation when implanted subcutaneously or intramuscularly in rats (Figure 1.13). They are, to date, the only known factors that can direct the *de novo* synthesis of cartilage and bone *in vivo* (reviewed by Rosen and Thies, 1992).

After BMPs are implanted ectopically, mesenchymal cells are recruited to the site of implantation, where they proliferate and differentiate into chondroblasts. The cartilage formed then hypertrophies, calcifies and is eventually replaced by bone, complete with a functional marrow cavity (Reddi, 1981). This sequence of events closely resembles endochondral bone formation as it occurs during embryogenesis and in fracture healing, and suggests that the proteins responsible for bone induction in this assay may be the natural bone inductive factors.

Biochemical analysis of this activity led to the isolation of a family of related evolutionarily conserved proteins, called BMPs (Urist *et al*, 1979, 1984, 1987). The cDNAs of these BMPs were then isolated using synthetic oligonucleotides derived from the amino-acid sequences of the proteins (Wozney *et al*, 1988; Wang *et al*, 1988). To date, 8 members of the family have been described in the literature, but there is reason to think that there may be more to be found (Wozney, personal communication).

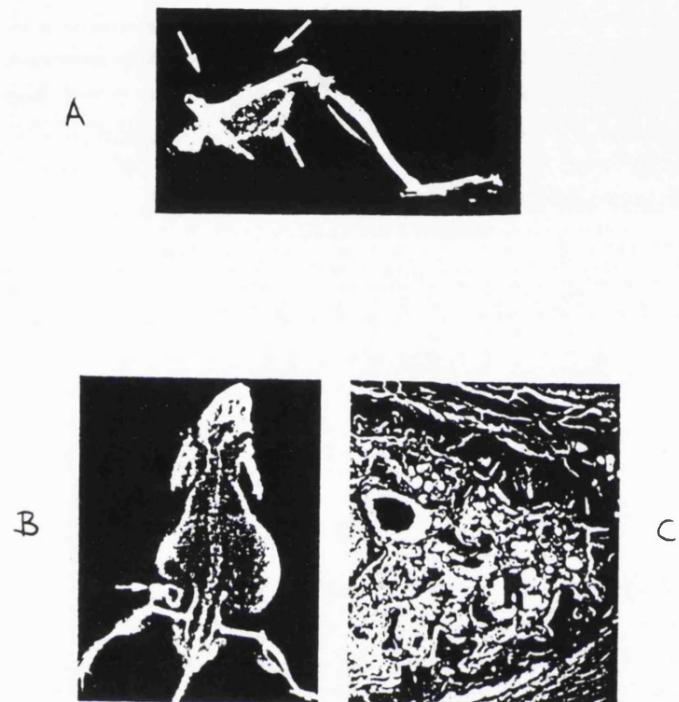


Figure 1.13: Ectopic bone assay.

A. and B. Roentgenograms of deposits of bone formed after ectopic implantation of BMPs in mouse thigh muscles.

C. Photomicrograph of a cross-section of the deposit shown in B., showing the marrow cavity.

(From Urist *et al*, 1984)

Bmp-2-8 are structurally related to one another and are members of the Transforming Growth Factor- β (TGF- β) superfamily of secreted growth factors (reviewed by Massagué *et al*, 1994), whereas *Bmp-1* is structurally unrelated.

a) **BMP-2-8:**

i) **Protein Structure:**

The BMPs, like the TGF- β s, are synthesised as precursor glycoproteins with an amino-terminal signal sequence and a pro-domain of varying size (Israel *et al*, 1992; reviewed by Wall and Hogan, 1994). The precursor protein is then proteolytically cleaved to release a 30 kDa mature carboxy-terminal segment which is then secreted (Gentry *et al*, 1989; Lyons and Moses, 1990) (Figure 1.14). The active signalling molecule is made up of dimers of this carboxy-terminal segment. The BMPs can form homodimers, or heterodimers with other BMPs, and possibly with other members of the TGF- β superfamily (Sampath *et al*, 1990).

The pro-domain shows limited sequence homology between different family members, but is often well conserved for a particular protein across different species. It appears to be required for normal synthesis, processing and secretion of protein (Gray and Mason, 1990; Hammonds *et al*, 1991; Thomsen and Melton, 1993). In TGF- β , it can also remain associated with the carboxy-terminal signalling fragment to produce an inactive complex (Gentry and Nash, 1990). This inactive form of the protein can accumulate in the extracellular matrix, and be activated by proteolysis or low pH at a later time. However, so far this has only been shown to be true for TGF- β , and is not thought to be true for BMPs, because the propeptide lacks the cysteine required to form a covalent complex with the mature form (Hammonds *et al*, 1991). However, the BMPs have been shown to associate with the extra-cellular matrix, and this may form a store of active protein (Israel *et al*, 1992).

The mature region is much more highly conserved and contains most of the sequence landmarks by which new family members are recognised, such as the characteristic 7-9 conserved cysteine residues in the pro-region, used for dimerization.

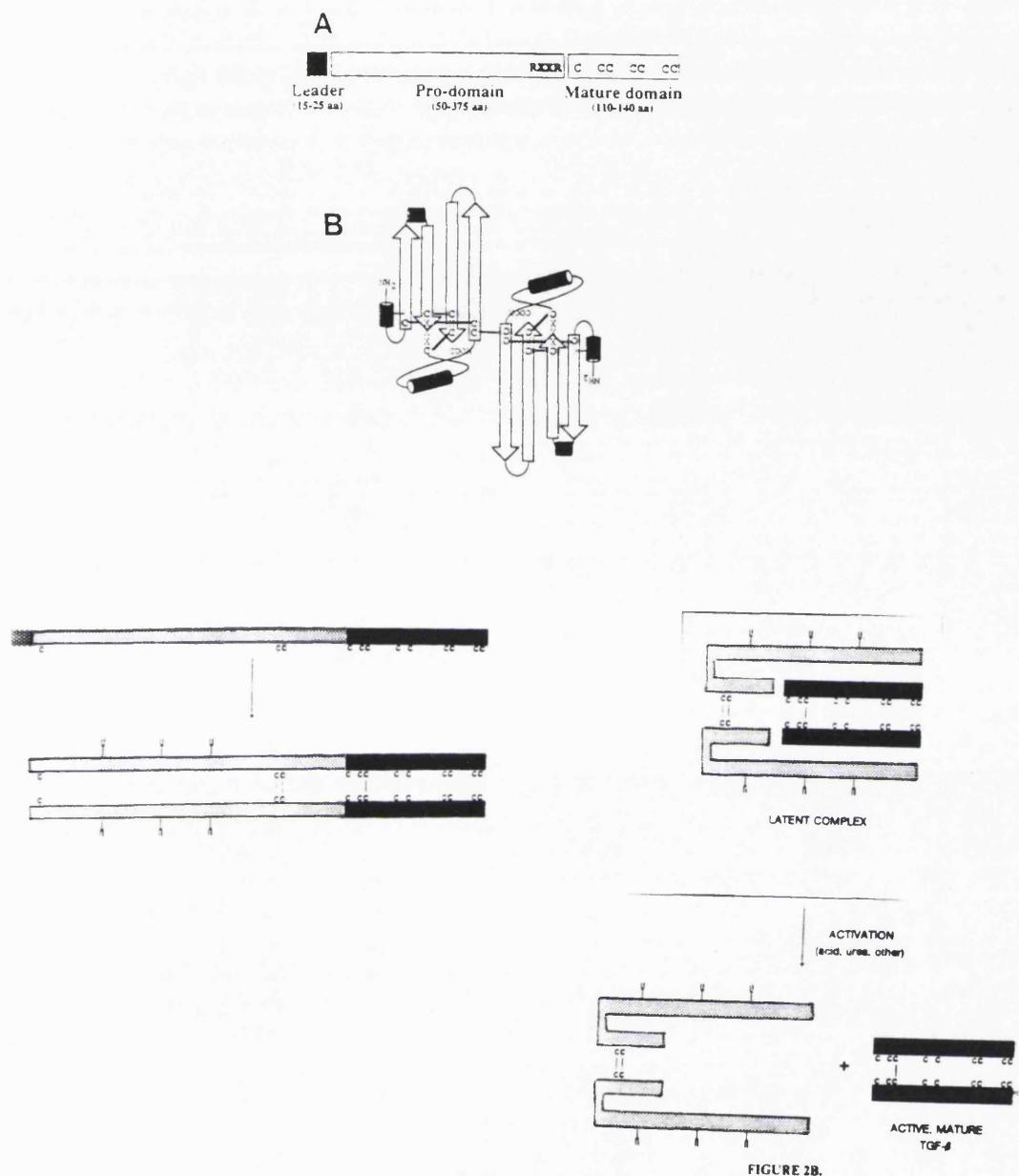


Figure 1.14: TGF- β protein structure and processing.

- a) Map of the structure of the TGF- β superfamily precursor proteins.
- b) Crystal structure of the mature region of the TGF- β proteins, showing the characteristic cystine knot. (From Kingsley, 1994a)
- c) Synthesis and processing of TGF- β proteins, showing dimerisation and structures of the latent and activated forms. (From Wozney, 1989).

The crystal structure of TGF- β 2 revealed a characteristic structure called a cystine knot, formed by 7 of the conserved cysteines (Schlunegger and Grütter, 1992; Daopin *et al*, 1992; McDonald and Hendrickson, 1993; reviewed by Massagué, 1994). This three-dimensional structure is likely to be shared by all members of the family (Schlunegger and Grütter, 1992; Daopin *et al*, 1992).

b) Subfamilies within the TGF- β Superfamily:

To date, 74 TGF- β -like sequences have been identified in different species, representing 23 distinct genes: the evolutionary conservation of these genes across species provides support for their importance in normal growth and development(Burt and Law, 1994). These have a remarkable range of activities (reviewed by Ying, 1989; Massagué, 1990; Lyons *et al*, 1991; Sporn and Roberts, 1992). The molecules can be grouped into distinct subfamilies with highly related sequences (Kingsley, 1994) (Figure 1.15). The large number of similar family members suggests the possibility of heterodimer formation or functional redundancy (Lyons *et al*, 1991). Within the TGF- β superfamily, *Bmp-2-8* are most closely related to those molecules thought to be involved in determination of cell fate during development, such as *dpp*, activin, and the GDFs, and these comparisons may give some insight into the possible roles of the *Bmp* genes in development (Figure 1.16).

Burt and Paton (1992) have suggested that all the TGF- β genes evolved from a single ancestral gene that originated before the time of arthropod and chordate divergence (approx. 300 million years ago). This is supported by the fact that there are only 3 TGF- β genes in *Drosophila*, *dpp*, *60A* and *screw*, compared to the large number of vertebrate genes. Also, the chromosomal locations of the genes are diverse, suggesting that the genes evolved by a series of duplications followed by separation by translocation (Fujii *et al*, 1986; Tabas *et al*, 1991; Burt and Paton, 1992). This is supported by sequence comparisons (Lyons *et al*, 1991). Possible phylogenetic trees relating all these groups are shown in Figure 1.17.

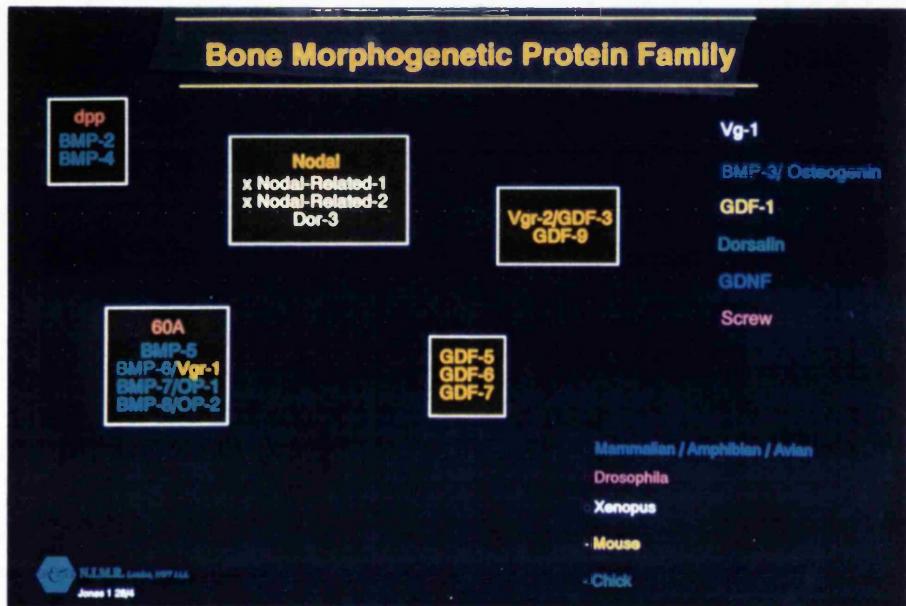


Figure 1.15: Subfamilies within the TGF- β superfamily.

(From C.M. Jones, personal communication)

XeB5	$\text{TGF}\beta_3$	$\text{TGF}\beta_2$	BNP5	BNP7	BNP6	BNP8	Dr60A	BNP2	BNP4	Drdpp	Ckdsl-1	XeVg1	muGOF3	GOF1	BNP3	INHBA	INHBB	nodal	m15	INHac	muGOF4
82	81	71	40	40	41	38	42	39	38	39	36	38	41	44	40	42	38	34	28	32	27
	72	74	44	44	45	38	39	40	39	42	36	46	39	40	41	45	41	34	27	32	27
	82	43	42	44	42	43	42	41	41	38	41	38	43	40	40	41	37	29	31	28	$\text{TGF}\beta_3$
		43	44	45	40	49	43	42	41	37	45	37	39	43	42	38	38	30	30	29	$\text{TGF}\beta_2$
			92	91	71	68	67	66	63	51	65	51	50	55	50	48	43	34	33	34	BNP5
				88	70	64	68	67	63	51	65	51	52	54	50	47	42	33	31	33	BNP7
					72	64	67	67	64	52	66	54	50	54	50	49	44	33	33	34	BNP2
						68	54	55	50	52	54	63	56	47	45	45	49	31	30	33	BNP8
							58	54	52	52	53	55	51	44	40	41	44	33	29	29	Dr60A
								95	81	53	68	55	51	58	53	52	47	35	30	35	BNP2
									82	55	66	55	52	57	51	52	48	36	30	35	BNP4
										56	59	51	48	55	46	48	44	33	31	31	Drdpp
											50	51	47	46	35	37	45	28	28	33	Ckdsl-1
												62	57	58	52	47	41	29	33	XeVg1	
													59	44	47	48	50	33	31	37	muGOF3
														43	45	42	45	38	29	36	GOF1
															43	47	44	35	33	31	BNP3
																69	42	34	32	36	INHBA
																	40	35	31	34	INHBB
																		28	28	34	nodal
																		22	28	31	m15
																			28	INHac	

Figure 1.16: Comparison of sequence identities within the TGF- β superfamily.

This phylogeny is based on human genes where possible. The numbers represent the percentage amino acid identities between each pair calculated from the first invariant cysteine residue to the C-terminus excluding gaps. (From Burt & Paton, 1992)

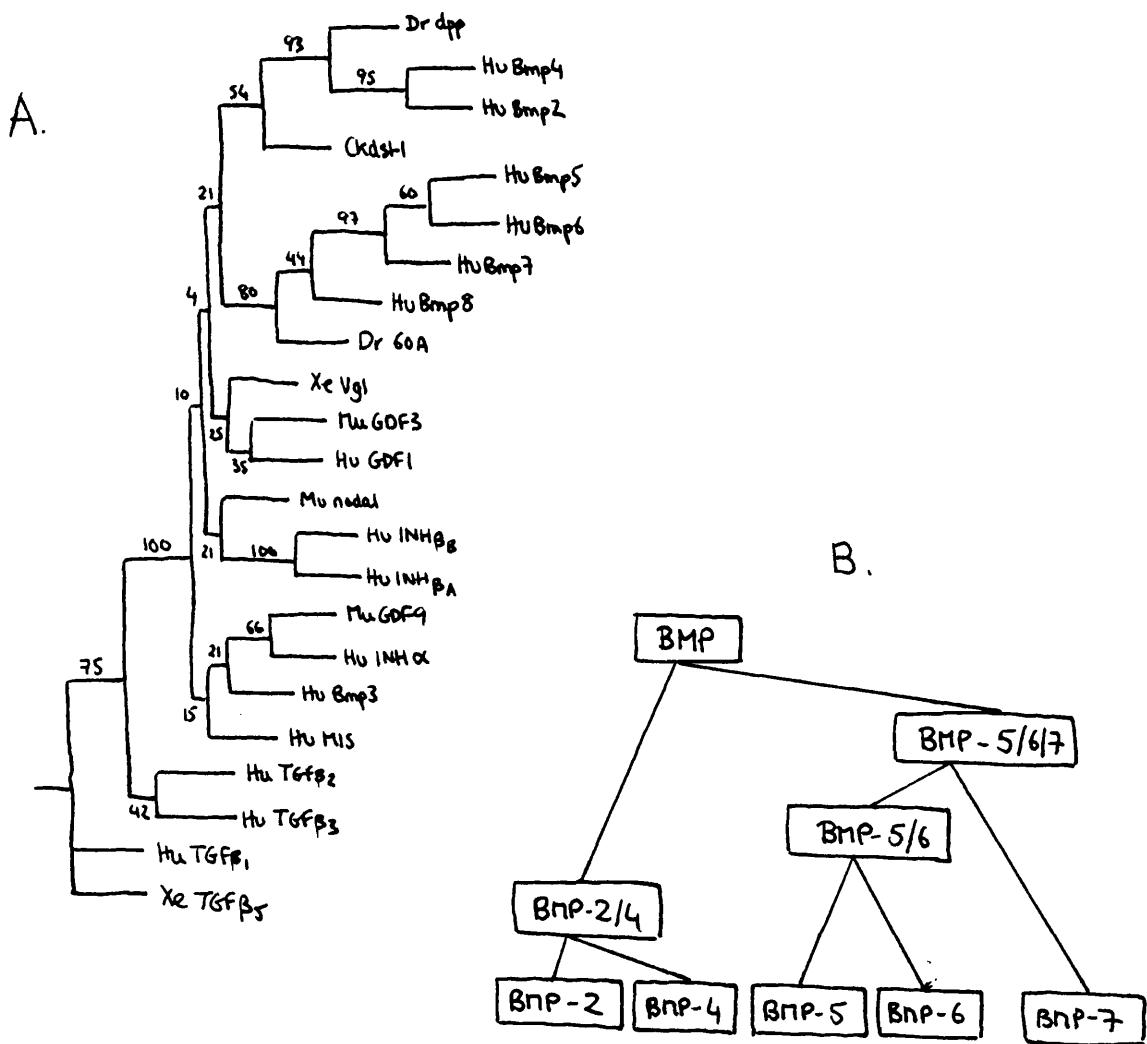


Figure 1.17: Phylogenetic trees of members of the TGF- β superfamily.

- a) Phylogenetic tree of the TGF- β superfamily, rooted to *Xenopus laevis* TGF- β 5 gene. Numbers at each fork indicate percentage likelihood of the grouping to the left of the fork occurring in a bootstrapping analysis (From Burt & Law, 1992)
- b) Phylogenetic tree of the BMPs. (From Lyons *et al*, 1991)

i) *Bmp-2* and *4* and *Decapentaplegic*: The *dpp* subfamily:

The *Bmp-2* and *4* genes are structurally more closely related to each other than to the other *Bmp* genes in vertebrates, and are also 75% identical at the amino acid sequence level to the product of the *decapentaplegic* (*dpp*) gene in *Drosophila*. Remarkably, human *Bmp-2* and *4* sequences can rescue the dorsoventral axis defects caused by *dpp* mutations in *Drosophila* (Padgett *et al*, 1993), and conversely, purified preparations of the DPP protein can induce bone and cartilage in mammals (Sampath *et al*, 1993).

dpp plays a fundamental role in dorsoventral body patterning, and null mutations lead to complete ventralisation of embryonic ectoderm (Gelbart *et al*, 1985; Irish and Gelbart, 1987; Padgett *et al*, 1987; St. Johnston *et al*, 1990; Ferguson and Anderson, 1992, Spencer *et al*, 1982; Gelbart, 1989) (Figure 1.18). *dpp* is normally expressed in the dorsal 40% of the *Drosophila* embryo (St. Johnston *et al*, 1987; St. Johnston and Gelbart, 1987). At later stages, *dpp* is also required to pattern the embryonic midgut and the imaginal discs, and for distal outgrowth of the adult appendages (reviewed by Hoffman, 1991; Gelbart, 1989; Raftery *et al*, 1991; Diaz-Benjumea *et al*, 1994). For example, normal wing blade development is solely dependent on *dpp* function in those anterior compartment cells that border the A/P compartment boundary of the wing imaginal disk: if any cells are lacking *dpp* in this area, no wing forms (Raftery *et al*, 1991; Posakony *et al*, 1991). *dpp* has also been demonstrated to act as a morphogen in *Drosophila* eye and limb development, with a gradient of *dpp* organising cell fates within the ectoderm (Ferguson and Anderson, 1992). The close sequence homology suggests that BMP-2 and 4 may perform analogous roles in vertebrate development.

Decapentaplegic in the fly is also of interest because it is a downstream target of *hedgehog*, the *Drosophila* homologue of *Sonic hedgehog*, in the developing eye and leg (Heberlein *et al*, 1993; Diaz-Benjumea *et al*, 1994). In early limb buds, transcripts of *Shh* and *Bmp-2* are found in nearly identical domains along the posterior limb bud margin (see below). Thus it seems likely that *Shh* may control the expression of *Bmp-2* in the vertebrate limb.

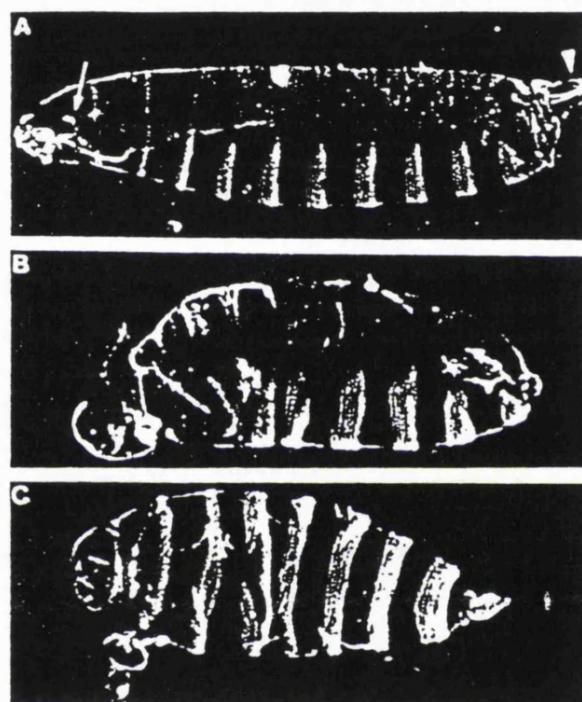


Figure 1.18: *Dpp* mutants.

- a) Lateral view of wild-type *Drosophila* embryo, with anterior to the left and dorsal up.
- b) Partial ventralisation due to *Tolloid* mutation.
- c) Complete ventralisation due to *Dpp* mutation.

ii) BMP-5-8 and 60A: The 60A subfamily:

The *Bmp-5*, *6*, *7* and *8* genes are on average 70% identical to the *Drosophila* 60A gene (Lyons *et al*, 1989; Özkaynak *et al*, 1990; Özkaynak *et al*, 1992), whereas 60A has only 53% amino acid sequence identity with *dpp*, and this homology is limited to the C-terminal mature region (Doctor *et al*, 1992). This gene was named 60A after its chromosomal map location and its normal function is not known as yet, but its expression pattern suggests that it is involved in multiple stages of *Drosophila* development, in particular in the formation of the embryonic gut (Wharton *et al*, 1991; Doctor *et al*, 1992; reviewed in Hoffmann, 1992). Ectopic expression of 60A has no effect on embryonic development but either causes defects in adult structures or the embryos die during metamorphosis (Staehling-Hampton *et al*, 1994b). Both *60A* and *dpp* can induce ectopic bone formation in the mammalian system, but have distinct effects on *Drosophila* development (Staehling-Hampton *et al*, 1994b). For example, ectopic expression of *60A* transgenes cannot respecify dorsal-ventral patterning in *dpp* mutant *Drosophila* embryos, and the spatial patterns of expression of *dpp* and 60A differ.

A mutation in the *Bmp-5* gene has been isolated: the mouse mutant "short ear", which displays localised cartilage defects (Kingsley *et al*, 1992; reviewed in Kingsley, 1994). The size, shape and number of many skeletal elements are reduced, and bone fracture healing is impaired (Green, 1968). Since only a subset of elements are affected, it has been suggested that the different BMPs may be responsible for skeletal formation in different body regions (Kingsley, 1994). This model predicts that mutations in other BMPs would disrupt other features in the skeleton, and that mutations could be combined to generate more severe defects. However, *short ear* animals also have an increased frequency of soft tissue abnormalities, such as lung cysts, liver granulomas and hydrotic kidneys, which may reflect a requirement for BMP-5 in normal soft tissue development as well as bone formation.

Bmp-6 (OP-1) is expressed in the developing skin and nervous system (Jones *et al*, 1992), and is also abundant in isolated calvaria, suggesting high levels of expression in osteogenic cells (Lyons *et al*, 1989). *Bmp-7* (previously known as OP-2) is expressed in highest levels in the kidney, but it induces bone formation *in vivo* (Özkaynak *et al*, 1991) and it has been suggested that it is synthesised in the kidney and then transported

to its physiological site of action, skeletal bone. It is also expressed in other organs such as the brain, heart and liver.

iii) The GDF group:

A new group within the DVR grouping has recently been found, by screening for homology to other family members. This is the subfamily of Growth Differentiation Factors (GDFs) (Lee, 1990; McPherron and Lee, 1993). GDF-1 is expressed primarily in the nervous system (Lee, 1990, 1991; Waterston *et al*, 1992; Boyer *et al*, 1993). It is only 52% homologous to its closest gene, *Xenopus Vg-1* (Lee, 1990).

GDF-3, or Vg-related gene 2 (McPherron and Lee, 1993; Jones *et al*, 1992b) is one of the only molecules in the TGF- β superfamily to lack one of the conserved 7 cysteines normally found in the mature region. It is expressed in ossifying skeletal tissue during embryonic development (Jones *et al*, 1992b), and in thymus, spleen, bone marrow, and adipose tissue in adults (McPherron and Lee, 1993). GDF-9 has also been identified (McPherron and Lee, 1993). It is only expressed in ovaries of mature animals (McPherron and Lee, 1993; Incerti *et al*, 1994). The functions of GDF-3 and 9 are at present unknown.

Storm *et al* (1994) isolated GDF-5, 6 and 7. A mutation in the GDF-5 gene results in the *brachypodism* defect, where the length and number of the limb bones is altered, but not the axial skeleton (Storm *et al*, 1994, reviewed by Tickle, 1994). GDF-5 is normally expressed in the limbs in the distal precartilaginous mesenchymal condensations and in the perichondrium of more proximal skeletal structures (Storm *et al*, 1994).

v) Activins:

There are two forms of inhibin/activin, A and B, consisting of a common α subunit and similar but distinguishable β subunits (Mason *et al*, 1985). α - β heterodimers, called inhibin, suppress the secretion of follicle-stimulating hormone (FSH) (reviewed by Ying, 1989). Two β subunits, β A- β A, β A- β B or β B- β B, form a new molecule, called

activin, that enhances FSH secretion (Ling *et al*, 1986; Vale *et al*, 1986; Mason *et al*, 1989).

Activin is also a candidate signalling molecules thought to induce mesoderm formation (Smith *et al*, 1990; van Eijnden-van Raaij *et al*, 1990; reviewed by Slack, 1989 and 1993; reviewed by Klein and Melton, 1994). There are two main classes of mesoderm inducing factors: the TGF- β s and the FGFs (Kimelman *et al*, 1988; Smith *et al*, 1989). Studies in amphibians, such as dominant negative receptor knockouts, suggest that FGFs are necessary for mesoderm formation (Amaya *et al*, 1991), and application of at least four members of the family can induce *Xenopus* animal caps to form mesoderm (Kimelman and Kirschner, 1987; Kimelman *et al*, 1988; Slack *et al*, 1987, 1988; Paterno *et al*, 1989). Injection of truncated dominant negative activin receptors also blocks mesoderm formation (Schulte-Merker *et al*, 1994). However, this could actually be an effect on *Vg-1* which also binds this receptor, since over-expression of follistatin, which binds activin but not *Vg1*, has no effect on development (Schulte-Merker *et al*, 1994). *Vg1* is another member of the TGF- β superfamily, which is expressed in the presumptive endoderm of *Xenopus* oocytes before gastrulation (Weeks and Melton, 1987; Thomsen and Melton, 1993). Injections of *Vg1* have no effect on development, but this may be due to problems in post-translational modification producing the mature functional protein (Thomsen and Melton, 1993; Klein and Melton, 1994). *Bmp-4* mRNA can also induce the formation of ventral mesoderm when injected into the animal hemisphere of fertilised *Xenopus* embryos (Köster *et al*, 1991; Dale *et al.*, 1992; Jones *et al.*, 1992). Activin can also induce the formation of axial structures in the chick (Mitrani *et al*, 1990).

It seems likely that members of both these families of molecules may be involved, in a combinatorial system also involving other modulating factors such as *Wnt-8* (Kimelman and Kirschner, 1987; Thomsen *et al*, 1990; Mitrani *et al*, 1990; Smith *et al*, 1989; Slack, 1993; Dale *et al.*, 1992; Jones *et al.*, 1992; Thomsen and Melton, 1993).

Activin expression has also recently been localised by *in situ* hybridisation to sites in the developing skeleton in rat limb buds (Roberts *et al*, 1991). Expression appears later than *Bmp-2* and *4* mRNAs, suggesting a role in later skeleton formation.

vi) Müllerian inhibiting substance (MIS):

MIS, a more distant relative of the BMPs, is an important morphogenetic signal in the developing reproductive system in mammalian embryos. MIS causes regression of the Müllerian ducts, the anlagen of the female reproductive structures, during male embryogenesis (Lee and Donahoe, 1993). It has also been proposed to control germ cell maturation, gonadal morphogenesis and suppress maturation (Lee and Donahoe, 1993).

vii) Homologies to TGF- β s:

Finally the BMPs are 30-40% homologous to the TGF- β s. Most cell types have been shown to possess TGF- β receptors, and the growth factor appears to affect an ever increasing number of regulatory functions in adult organisms (Ignatz and Massagué, 1986; Montesano and Orci, 1988; Massagué, 1990; Carrington and Reddi, 1990; Sporn and Roberts, 1992). For example, TGF- β 1 can be growth inhibitory as well as promoting growth, and can also lead to differentiation in culture (reviewed by Roberts *et al*, 1988). It is also thought to play an important role in wound healing. Many of the actions of TGF- β 1 can be explained in terms of effects on the extra-cellular matrix (Gelbart, 1989).

There are three isoforms of TGF- β in man, 1, 2 and 3 (Deryck *et al*, 1987a; Sporn and Roberts, 1992). TGF- β s are expressed in many tissues in the adult and embryos, but bone is the most abundant source of TGF- β in the body (Thorp *et al*, 1992). In chickens, TGF- β 1, 2 and 3 are all found in the chondrocytes of the growth plate, but not in resting or proliferating cells, only in hypertrophic and transitional cells. It is also expressed in osteoblasts, osteoclasts and in the bone marrow (Ellingsworth *et al*, 1986; Heine *et al*, 1987; Thompson *et al*, 1987; Sandberg *et al*, 1988; Pelton *et al*, 1989; Pelton *et al*, 1990; Thorp *et al*, 1992). *In vivo*, bone formation is stimulated but not induced by TGF- β (Noda *et al*, 1989; Joyce *et al*, 1990; Thorp *et al*, 1992). However, application of TGF- β 1 beads to developing chick limbs causes specific skeletal elements to be drastically shortened or missing completely (Hayamizu *et al*, 1991). The timing of this effect is consistent with an effect on early chondrogenesis, just prior to formation of the condensations.

iii) Other members of the TGF- β superfamily:

Other TGF- β -related molecules include the *nodal* gene, which is expressed at the tip of the primitive streak in mice, in an area equivalent to Hensen's node in the chick (Zhou *et al*, 1993). *Nodal* is essential for mesoderm formation and subsequent organisation of axial structures in early development. The BMP-3 or osteogenin gene also forms a group on its own (Wozney *et al*, 1988; Luyten *et al*, 1989). *Vgr-2*, in mice, is most closely related to *Vg-1* in *Xenopus* (Jones *et al*, 1992). It is expressed in developing bone in mid-gestation, when the bones are beginning to calcify (Jones *et al*, 1992).

Dorsalin, a gene expressed preferentially in the dorsal side of the developing neural tube in the chick (Basler *et al*, 1993) is also part of the TGF- β group. It may play a role in promoting outgrowth of neural crest cells from the neural tube, and in inhibiting formation of motor neuron cells on the dorsal side of the neural tube. However, like the BMPs, it has also been shown to stimulate the production of alkaline phosphatase, a marker of bone and cartilage formation, in bone marrow cell lines in culture. BMP-9 has also been described, and has more homology with *dorsalin* than with the other BMPs. It is expressed in the liver (Wozney *et al*, personal communication).

c) Receptor Molecules:

Receptors for the TGF- β s, activins, MIS and BMPs have recently been identified, and they belong to a novel family of transmembrane receptors that contain a serine/threonine kinase domain on their cytoplasmic surface (Gillespie *et al*, 1989; Georgi *et al*, 1990; Massagué, 1992; Mathews *et al*, 1992; Lin and Lodish, 1993; He *et al*, 1993; Baarends *et al*, 1994; Wrana *et al*, 1994b) (Figure 1.19). There are 2 types of receptor, Type I and Type II. It is not known what secondary messenger system is stimulated by these receptors: they do not stimulate any of the known systems such as cAMP or G-proteins.

The Type I receptors only show ligand-binding activity when co-expressed with Type II receptors (Attisano *et al*, 1993; Ebner *et al*, 1993b), and the binding specificity of Type I receptors may depend on the particular type II receptor with which they are

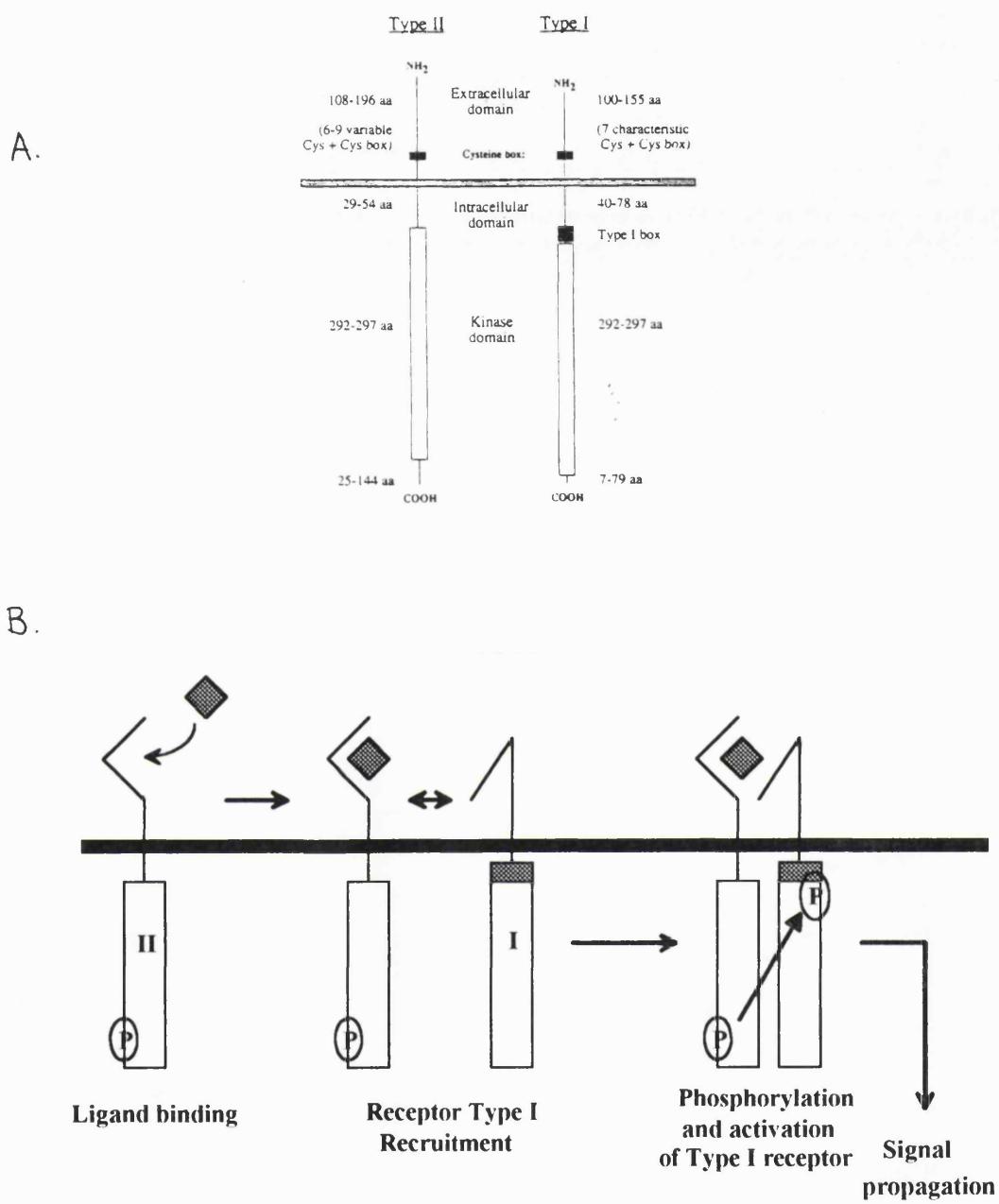


Figure 1.19: TGF- β superfamily receptors: structure and mechanism of action.

- a) Structure of Type I and Type II receptors. (From Kingsley, 1994a)
- b) General model for mechanism of action. (From Massagué, 1990)

expressed (Attisano *et al*, 1993; Wrana *et al*, 1994a). The ligand binds directly to the Type II receptor, which is a constitutively active kinase. This bound form of the ligand is then recognised by receptor Type I, which is recruited into the complex and becomes phosphorylated by the Type II receptor. This then allows the Type I receptor to propagate the signal to downstream substrates (Wrana *et al*, 1994a). With a large number of receptor subunits and receptor isoforms, it might be possible for the same factor to evoke different responses in different tissues and at different times.

TGF- β s also bind to some extracellular matrix proteins, such as betaglycan, decorin, and endoglin and it has been shown that these may act as storage low-affinity receptors (Yamaguchi *et al*, 1990; Massagué, 1991; Cheifetz *et al*, 1992; Moustakas *et al*, 1993). In this way they may either increase binding, or sequester ligand (Yamaguchi *et al*, 1990; reviewed by Massagué, 1994). Betaglycan has also been shown to present ligand to the Type II receptor (López-Casillas *et al*, 1993).

Various receptors have been isolated that bind BMPs *in vitro* and in transfected Cos cells (ten Dijke *et al*, 1994; Yamaji *et al*, 1994), such as murine BRK-1, a Type I receptor which binds BMP-2 and BMP-4, and can form complexes with the *C. elegans* *daf-4* gene product (Koenig *et al*, 1994). *daf-4* is a Type II receptor that can bind mammalian BMP-2 and 4, and not activin or TGF- β (Estevez *et al*, 1993). The worm ligand for this receptor has not yet been identified. A *Xenopus* BMP receptor has also been cloned: it expressed maternally, in the appropriate location to be involved in mesoderm induction (Graff *et al*, 1994). It binds both BMP-2 and 4 with high affinity, and a truncated form blocks BMP-4 signalling and converts ventral mesoderm to dorsal mesoderm (Graff *et al*, 1994).

Three specific receptors for *dpp* have also been isolated in *Drosophila*: *punt*, a Type II receptor (Childs *et al*, 1993; Ruberte *et al*, 1995; Letsou *et al*, 1995), *thick veins* (*tkv*) (Penton *et al*, 1994; Brummel *et al*, 1994) and *saxophone* (*sax*) (Penton *et al*, 1994; Brummel *et al*, 1994; Xie *et al*, 1994), which are both Type I receptors. Mutations in these genes lead to the same phenotype as weak *dpp* null mutants (Affolter *et al*, 1994; Xie *et al*, 1994). All three receptors are required for proper *dpp* signalling but it has been suggested that *tkv* and *punt* are needed everywhere and *sax* only acts at peak levels of *dpp* expression: in this way, distinct cell fates could be specified in response

to different concentrations of *dpp* achieved combinatorially by the three receptors (Nellen *et al*, 1994; Ruberte *et al*, 1995). *punt* is expressed ubiquitously throughout the embryo, and both *thick veins* and *saxophone* are expressed in complex spatial and temporal patterns in the areas where *dpp* is thought to act, such as the eye and gut (Penton *et al*, 1994; Brummel *et al*, 1994). Therefore both ligand and receptor are spatially expressed and regulated: this may allow a range of different responses to the same concentration of ligand.

d) BMP-1:

BMP-1 is structurally unrelated to the other BMP genes (Maéno *et al*, 1993). BMP-1 is related to metalloendoproteases found in sea urchins, *Strongylocentrus purpuratus*, called SpAN and BP10, which are expressed at very early stages in development and spatially restricted (Reynolds *et al*, 1992; Lepage *et al*, 1992), and astacin, a metalloendoprotease with novel specificity purified from the crayfish, *Astacus fluviatilis* (Titani *et al*, 1987; Stöcker *et al*, 1993). All the members of the Astacin family appear to be secreted peptides, and BMP-1 and tolloid are probably synthesised as pre-proteins (Wozney *et al*, 1988; Shimell *et al*, 1991). One of the domains of BMP-1 is homologous to the human C1s and C1r complement proteins, involved in the immune response (Leytus *et al*, 1986; Journet and Tosi, 1986; Arlaud *et al*, 1987; Mackinnon *et al*, 1987; Tosi *et al*, 1987).

BMP-1 is the human homologue of the *Drosophila tolloid* gene, which is required for proper dorso-ventral patterning (Shimell *et al*, 1991). It achieves this by interacting with *decapentaplegic*, the *Drosophila* homologue of BMP-2 and 4, so it is thought that BMP-1 may co-ordinate BMP-2 and 4 function in the same way in vertebrates. It may achieve this by proteolytically activating latent complexes of the other BMPs. Expression of *tolloid* and *dpp* overlap at early stages, but then diverge later.

1.8 Possible Roles for BMPs in Limb Development:

a) Expression patterns of BMP-2 and 4 in the limb:

As well as their role in inducing bone formation, it has also been found that, in mice, the *Bmp* genes, especially *Bmp-2* and *4*, are expressed in the developing limb, long before bone formation.

Work in Brigid Hogan's laboratory (Lyons *et al.*, 1990; Jones *et al.*, 1991) had previously shown that, in the mouse, BMP-2 and 4 are expressed in the AER and mesoderm in early limb development. The chick cDNAs of BMP-2 and 4 were cloned in our laboratory and their mRNA expression has been localised to several different areas of the developing chick limb by *in situ* hybridisation (Francis *et al.*, 1994) (Figure 1.20). *Bmp-2* transcripts are not detectable in the wing area until stage 16/17, and then, between stages 17 and 26, *Bmp-2* expression is restricted to the AER and a posterior region of the mesoderm, which co-localises with the polarising region. Later, at stages 25 and 26, there is also an additional domain of expression in the anterior proximal part of the limb bud; and then at later stages, it is expressed in the interdigital necrotic zone and around the cartilage elements.

The pattern of *Bmp-4* expression is very similar to that of *Bmp-2* in the AER, but transcripts are present throughout the wing mesenchyme at stages 16 to 17, and then expression becomes progressively restricted between stages 19 and 24, so that transcripts are found in two regions at the anterior and posterior margins of the limb bud, and in the Progress Zone. *Bmp-4* also is expressed interdigitally and around the cartilage elements at later stages.

BMP-4 expression overlaps that of BMP-2 in some areas so there may be some co-operation between BMP-2 and 4, possibly involving the formation of heterodimers.

The distribution of *Bmp-2* and *Bmp-4* transcripts in the developing limb suggests that BMP-2 and BMP-4 could play important roles in limb development. Both genes are expressed in the AER, and could be involved in maintaining the cells in the Progress Zone as undifferentiated cells (Lyons *et al.*, 1990; Jones *et al.*, 1991; Niswander and

Martin, 1993). Equally, both genes are expressed in chondrogenic regions in the later bud, suggesting a role in cartilage differentiation and morphogenesis (Lyons *et al*, 1989). This raises the possibility that, like the related *dpp* gene in *Drosophila*, which has been shown to act at several different stages in development (Ferguson and Anderson, 1992; Heberlein *et al*, 1993), the BMPs may be necessary for both initial patterning and for later skeletal morphogenesis in the limbs.

b) Interactions of BMPs with Retinoic Acid and *Hox* Gene Expression:

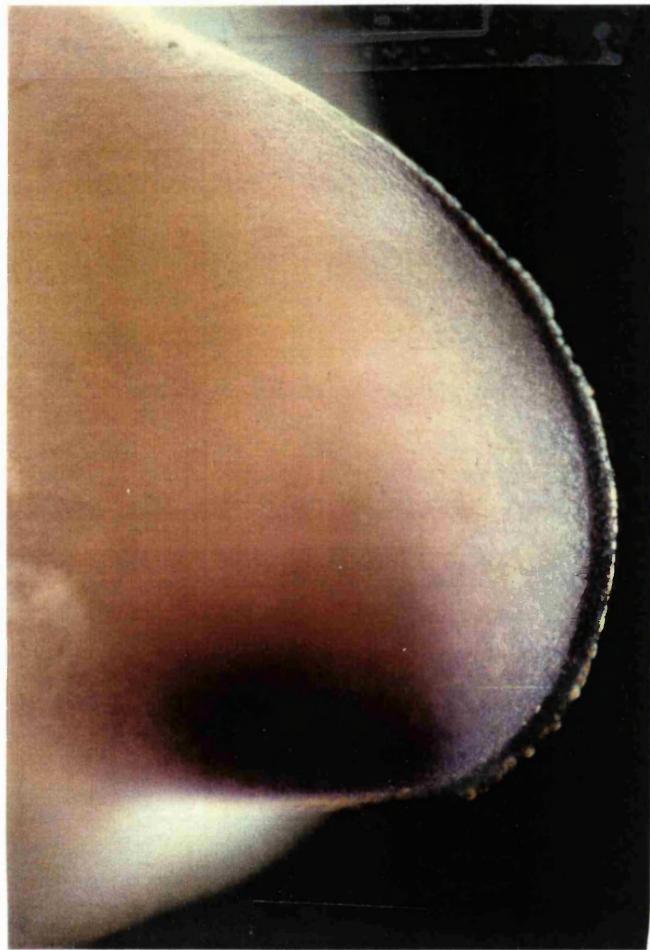
The co-localisation of BMP-2 expression with the ZPA also raises the possibility that BMP-2 may be one of the molecules involved in the specification of the antero-posterior axis, in the polarising region signalling pathway.

For example, if a RA-soaked bead is implanted into the limb such that it duplicates the ZPA activity and causes digit duplication, a mirror-image ectopic domain of BMP-2 expression forms as well (Francis *et al*, 1994) (Figure 1.21). The *Bmp-2* gene is therefore probably part of the response component of the polarising region signalling pathway, but BMP-2 is not itself a polarising molecule, as beads soaked in recombinant human BMP-2 protein, at concentrations of 0.2 to 2 mg ml⁻¹, do not cause the formation of additional digits (Francis *et al*, 1994).

RA treatment also causes the formation of an ectopic region of *Hox-d13* expression at approximately the same time and place as BMP-2 (See section 1.6 above; Figure 1.21). The normal domain of posterior expression of BMP-2 also overlaps considerably with that of *Hox-d13* (Izpisúa-Belmonte *et al*, 1991). This overlap is maintained throughout limb development and ectopic expression of *Bmp-2* can induce *Hoxd-11* and *d-13* expression in the developing chick limb (Duprez *et al*, in press).

There is evidence that other members of the BMP family can activate homeobox genes too: DPP, synthesised by mesoderm, activates expression of the homeotic gene, *labial*, in neighbouring ectoderm in *Drosophila* (Panganiban *et al*, 1990a), and, in developing teeth, BMP-2 and 4 can induce *Msx-1* expression, a homeobox-containing gene,

A



B

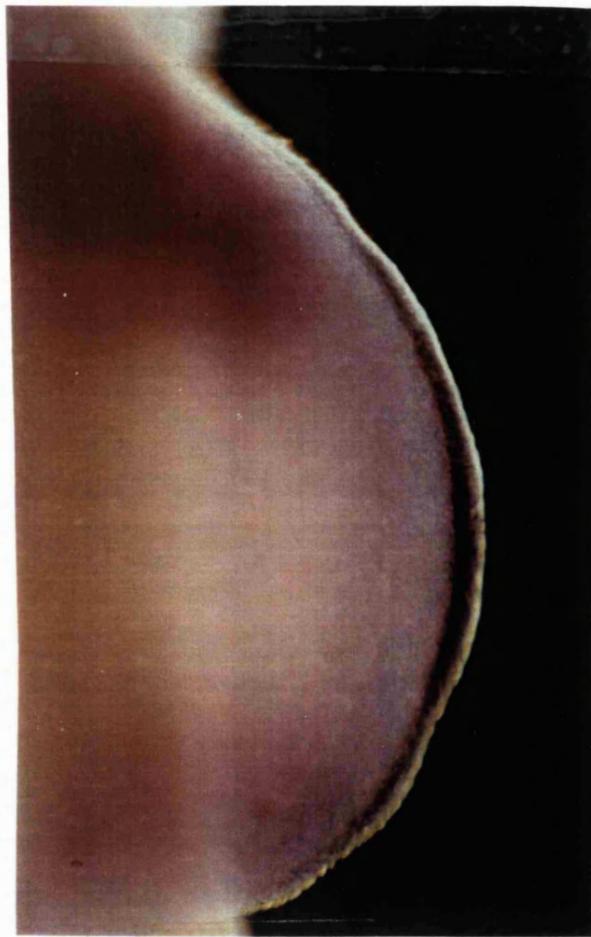


Figure 1.20: Expression of *Bmp-2* and *Bmp-4* in the chick limb. A. *Bmp-2* at stage 22; B. *Bmp-4* at stage 20. (Anterior = up)

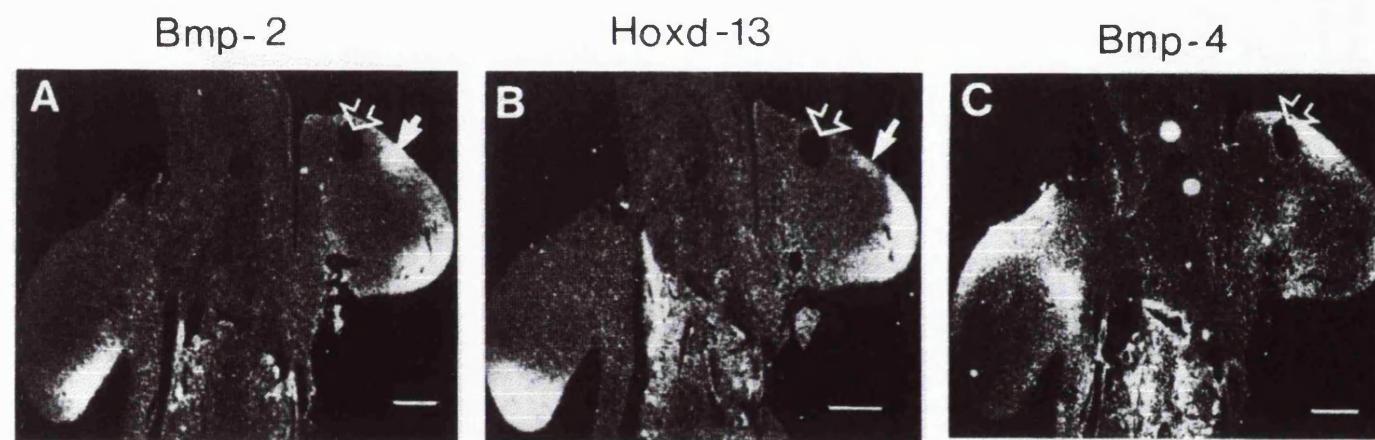


Figure 1.21: Ectopic activation of *Bmp-2* and *Hoxd-13* genes following RA treatment.

Ectopic expression of A. *Bmp-2*, B. *Hoxd-13*, and C. *Bmp-4* is shown by the arrow in each panel.

The normal expression pattern is shown on the contralateral limb.

homologue of the *msh* (muscle segment homeobox) gene of *Drosophila* (Vainio *et al.*, 1993). In limb buds stripped of the AER and cultured (Niswander and Martin, 1993a and 1993b), BMP-2 and FGF-4 co-ordinately regulate the expression of *Evx-1*, the murine homologue of the *Drosophila even-skipped* homeotic gene, which is normally expressed in the posterior Progress Region mesenchyme shortly after formation of the AER (Bastian and Gruss, 1990). BMP-2 on its own will not stimulate ectopic anterior expression of *Evx-1*, but 10 ng/ml of BMP-2 + 100ng/ml FGF-4 will switch on ectopic *Evx-1*, whereas in the presence of 100 ng/ml BMP-2 + 10 ng/ml FGF-4, no expression is seen. In this culture system, BMP-2 has also been shown to affect outgrowth of the limb, in concert with FGF: FGF stimulates outgrowth of the limb, and BMP-2 inhibits outgrowth (Niswander and Martin, 1993b). At 50 ng/ml of applied BMP-2, *Evx-1* expression is activated, but outgrowth of the limb bud is inhibited.

BMP-2 could also function in the polarising region signalling pathway in concert with BMP-4 or 7. The *Bmp-2* domain overlaps with that of *Bmp-4* and *Bmp-7* in the posterior mesenchyme of normal limb buds, and in the RA-treated limbs, the ectopic domain of *Bmp-2* also overlaps with the anterior expression domain of *Bmp-4* (Francis-West *et al.*, 1994). Members of the TGF- β family, including BMPs, have been shown to form both homodimers and heterodimers (Sampath *et al.*, 1990). BMP-2 and 4 and 7 might therefore act as homo- and/or heterodimers in the developing limb. This introduces further complexity because homodimers and heterodimers can have opposing effects, as in the case of the inhibins (Hsueh *et al.*, 1987). However, the distribution of *Bmp-2* and 4 transcripts may not accurately reflect the distribution of active protein, since there is the possibility of further control at the levels of translation, secretion and proteolytic activation after secretion, and no specific antibodies against individual BMPs have been made (reviewed by Wozney, 1989).

RA has also been shown to affect *Bmp* gene expression in cell lines. In F9 cells induced to differentiate with RA, *Vgr-2* and *Bmp-4* expression levels decrease, and *Bmp-2* and *Bmp-6* levels increase (Jones *et al.*, 1992). In embryonic stem cells and carcinoma cells induced to differentiate with RA, endogenous BMP-2 levels increase 11-fold and BMP-4 decrease 12-fold (Rogers *et al.*, 1992). TGF- β 1 and β 2 expression also increases in these cells, but β 1 receptor levels decrease dramatically (Weima *et al.*, 1989; Mummary *et al.*, 1990). In the C3H-10T1/2 mouse cell line, treatment with RA increases the

incidence of osteoblast differentiation. These cells normally express *Bmp-2* and *Bmp-4*, and low levels of *Bmp-6*: upon RA treatment, *Bmp-2* and *4* expression decreases transiently (4 days) and *Bmp-6* expression increases (Gazit *et al*, 1993). Recombinant BMP-2 can also stimulate osteoblastic maturation and inhibit myogenic differentiation in rat osteoblast cell lines (Yamaguchi *et al*, 1991).

c) Similarities with Insect Models of Development:

Insights into the possible roles of *Bmp-2* and *4* in vertebrates may also be gained by looking at the roles of their *Drosophila* homolog, *dpp*.

dpp has been shown to act as a morphogen in at least two systems in development in *Drosophila*: the eye, and the developing gut, as well as in specifying dorsal ectodermal cell fates (Heberlein *et al*, 1993).

In the retina in *Drosophila*, a wave of differentiation passes across the eye disc over a 2 day period. *hh* has been shown to induce *dpp* expression, which appears to be a primary mediator of movement of the furrow, marking differentiation (Heberlein *et al*, 1993). Both of these genes act as diffusible signals in this process: differentiating cells posterior to the furrow produce HH protein which diffuses anteriorly and mediates induction of *dpp* expression in the furrow (Heberlein *et al*, 1993).

dpp is also expressed in parasegments 4 and 7 of the embryonic visceral mesoderm (Hursh *et al*, 1993). Using an antibody specific to DPP (Panganiban *et al*, 1990b), *dpp* was found to be secreted and to influence the expression of different homeobox genes involved in the interaction between the outer mesodermal and inner endodermal layers of the midgut. DPP and wingless act in adjacent parasegments as the link between the mesoderm and the endoderm layers (Immerglück *et al*, 1990; Panganiban *et al*, 1990a; Hursh *et al*, 1993; Staehling-Hampton *et al*, 1994a). It is thought that *Ultrabithorax* expression in the mesoderm of parasegment 7, directly or indirectly switches on *dpp* expression (Reuter *et al*, 1990), DPP protein then moves locally to the endodermal layer where it elicits expression of the homeobox *labial*, in collaboration with *wingless*. Therefore *dpp* functions apparently both upstream and downstream of homeobox

genes. In addition, *Ubx* and *abd-A* homeotic genes and *dpp* are needed to form the second midgut constriction (Capovilla *et al*, 1994). Capovilla *et al* (1994) isolated an enhancer element in *dpp* controlling this expression, which contains binding sites for UBX and ABD-A. UBX directly switches on DPP activity, and ABD-A represses it (Capovilla *et al*, 1994).

1.9 Aims of the work described in this thesis:

The aims of the work described in this thesis all centered around attempting to elucidate the role of the chicken *Bmp-2* gene in the developing chick limb.

There were three main aims:

1. To isolate and characterise a chicken genomic *Bmp-2* clone. This is described in Chapter 3.
2. To design and optimise a system for transfecting chick primary mesoderm cells isolated from embryonic limb buds, and to use this to study the expression and regulation of promoter constructs of the chicken *Bmp-2* gene in different areas of the limb bud, and under various conditions. This work is described in Chapter 4.
3. To analyse the function of *Bmp-2* in the developing chick limb bud, by examining the effects of inhibiting *Bmp-2* gene function using antisense RNA. This work is described in Chapter 5.

Addendum and Corrections to Materials and Methods

2.4 b) iii) Culture of transfected chick mesenchyme cells:

Each batch of 2×10^7 mesenchyme cells was plated out into 4 wells in Micromass medium (see 2.4 b ii)). The "n" numbers shown on the graphs indicate the number of wells analysed, and not the number of transfections done. The medium was replaced with fresh medium after 24 hours and 72 hours. Any dead cells were removed along with the medium since they detach from the wells when they die.

2.4 b) iv) Normalisation of transfection experiments:

For each transfection experiment, cells from all four limbs were pooled. The number of cells were counted before transfection and the same number of cells plated out each time. For each transfection experiment, transfections were done with pGL2 Control and pGL2 Basic, and if any anomalous expression values occurred for these cells, all the readings from that batch of cells were excluded from the averages calculated. All cell samples were also examined before being lysed, and the number of cells per well estimated by eye, assessing the percentage coverage of the bottom of the well. This eliminated potential confusion of low luciferase activities with false-negative values resulting from loss of cells, since the dead cells detach and are removed with the medium.

2.4 i) Grafting of infected chick embryonic fibroblast cells:

When the cells were ascertained to be 100% infected with the retrovirus by GAG staining, the cells were trypsinised, resuspended in DMEM medium, transferred to an eppendorf and centrifuged at 6,500 rpm in a microfuge to pellet the cells. The pellet was then removed from the eppendorf tube using a sterile bent wire, and placed in Petri dish containing DMEM + foetal calf serum at 37°C in a CO₂ incubator for at least 1 hour to allow the pellet to solidify. The pellet was then chopped up into 1mm³ pieces with a tungsten needle and left again at 37°C for another hour. These pieces were then grafted into the posterior region of the forelimb of stage 17-21 chick embryos, by placing them either into slits, or into holes excavated in the mesenchyme by removing a similar volume of mesenchyme tissue using a tungsten needle. The infected cells were guided into place using a scoop specially made of shaped flattened metal and a tungsten needle or watchmakers forceps. The egg was then sealed with sellotape and replaced in the incubator. After 24 and 48 hours, the embryos were checked under the microscope to ensure that the grafts were still in place.

MATERIALS AND METHODS

2.1 Materials and Methods:

All materials were from standard laboratory suppliers, unless otherwise stated. The enzymes were supplied either by Gibco/BRL or Sigma, Poole, Dorset. The chicken Collagen Type X promoter construct was a gift from Professor M. Pacifici, School of Dental Medicine, Department of Anatomy and Histology, University of Pennsylvania, 4001 Spruce Street, Philadelphia, PA 19104-6003. The chicken RIHB promoter construct was a gift from Dr. M. Vigny, Unité INSERM 118, Associée CNRS, 29 rue Wilhelm, 75016 Paris, France. The retroviral vector, RCAS(BP)A, and the adaptor plasmid, Cla12 Nco, were gifts from Dr. S. Hughes: Bionetics, Research Inc., Basic Research Program, National Cancer Institute, Frederick Cancer Research Facility, P.O. Box B, Frederick, Maryland 21701-1013, U.S.A.

All the following techniques were carried out as described in Sambrook, Fritsch and Maniatis (1989), unless otherwise stated. All the following methods were carried out using aseptic techniques where required. The solutions used were either filter sterilised or autoclaved. All the water used was double de-ionised.

2.2 DNA Techniques:

a) DNA Isolation: Mini-preparations:

15 ml of L Broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, pH 7.2) containing 0.1 mg/ml ampicillin was inoculated, either from a bacterial colony or a glycerol stock, and incubated overnight at 37°C in a shaking incubator.

The culture was centrifuged at 3,000 r.p.m. for 20 minutes at 4°C in a Beckman J-6B centrifuge. The resulting pellet was resuspended in 200 µl of Tris-glucose (50 mM glucose, 25 mM Tris.Cl, pH 8.0, 10 mM EDTA, pH 8.0), transferred to an Eppendorf tube and incubated on ice for 5 minutes. 400 µl of freshly-made 1% SDS, 0.2 M NaOH

were added, the resulting solution mixed and left on ice for a further 10 minutes, to lyse the bacterial cells. Then 200 µl of ice-cold Solution III (60% (v/v) 5 M KAc, 11.5% glacial acetic acid) was added, the solution mixed well by inversion, and left on ice for 15 minutes. The solution was then microfuged at 13,000 r.p.m. for 15 minutes. The aqueous phase was removed, leaving behind the pellet of bacterial cell debris. The aqueous supernatant was then re-microfuged for a further 10 minutes. The supernatant was again taken, taking care not to take up any of the genomic DNA at the liquid surface. 600 µl of isopropanol was added to precipitate the DNA, the solution mixed well and microfuged for another 15 minutes. The pellet was washed with 70% ethanol and freeze-dried, and then resuspended in 200 µl of water. The DNA was then RNase treated with 1 µl of 10 mg/ml RNase A at 37°C for 1 hour.

The DNA was then cleaned by phenol/chloroform extraction. 1/10th of the volume of 3M NaAc was added and mixed well. An equal volume of 50/49/1 (v/v) phenol/chloroform/isoamyl alcohol was added and the solution vortexed. The solution was then microfuged for 5 minutes at 13,000 r.p.m. and the top aqueous layer removed. This was repeated until no debris was seen at the liquid interphase, and then an equal volume of water-equilibrated chloroform + 1% isoamyl alcohol was added and the solution vortexed. The solution was then microfuged for a further 30 seconds, and the top aqueous layer removed. 2.5 times the volume of absolute ethanol were added to precipitate the DNA, and the solution microfuged for 15 minutes at 4°C in the cold room. The resulting pellet was washed with 70% ethanol and freeze-dried before being resuspended in 50 µl of T.E. (10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

b) DNA Isolation: Large scale preparations: Lithium Chloride Method:

A conical flask containing 500 ml of L Broth was prepared and autoclaved, 500 µl of 0.1 mg/ml ampicillin was added, and the flask inoculated with bacterial glycerol stocks containing the DNA required. This was incubated in an orbital incubator at 37°C overnight. The next day, the culture was centrifuged at 3,000 r.p.m. in a Beckman J-6B centrifuge for 30 minutes at 4°C and the bacterial pellet was resuspended in 18 ml of Solution I (50 mM glucose, 25 mM Tris-Cl, pH 8.0, 10 mM EDTA). Then 40 ml of freshly-made up Solution II (0.2 M NaOH, 1% (v/v) SDS) was added and the solution

mixed thoroughly by inversion, and left at room temperature for 10 minutes. Then 20 ml of ice-cold Solution III (60% (v/v) 5 M KAc, 11.5% (v/v) glacial acetic acid). The mixture was then mixed thoroughly and left on ice for 15 minutes, to allow a precipitate to form. This was then centrifuged at 4,000 r.p.m. in a Beckman J-6B centrifuge at 4°C, with the brake switched off, for 15 minutes. The supernatant was taken off, leaving the bacterial cell debris behind, and filtered through four layers of cheesecloth into a fresh 250 ml Beckman centrifuge bucket. 0.6 volumes of isopropanol were added, and the solution mixed well, and left at room temperature for 15 minutes. This was then centrifuged for a further 15 minutes at 5,000 r.p.m. in a Sorval GS3 rotor, in a Sorval RC 5B centrifuge, at room temperature. The resulting pellet was carefully washed with 70% (v/v) ethanol, free-dried and resuspended in 3 ml of TE.

The DNA was then purified by PEG precipitation. The DNA in solution was transferred to a 15 ml centrifuge tube and 3 ml of ice-cold 5M LiCl was added to precipitate the high molecular weight RNA. The solution was mixed well and centrifuged at 10,000 r.p.m. in a Sorval SS34 rotor, in a Sorval RC 5B centrifuge for 10 minutes at 4°C. The pellet was discarded, and an equal volume of isopropanol was added to the supernatant, mixed well and then the DNA was recovered by centrifuging at 10,000 r.p.m. for 10 minutes at room temperature. The supernatant was then carefully poured away and discarded and the pellet was washed in 70% ethanol and freeze-dried, before being resuspended in 500 µl of TE. DNase-free pancreatic RNase A was added to a final concentration of 10 µg/ml and the solution was transferred to an Eppendorf and incubated at room temperature for 30 minutes. Then 500 µl of 1.6 M NaCl containing 13% (w/v) polyethylene glycol (PEG 8000) was added, the resulting solution mixed well, and the DNA recovered by microfuging at 13,000 r.p.m. for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 400 µl of TE (pH 8.0), after making sure that all the PEG-containing supernatant was fully removed by aspiration. The solution was then cleaned by phenol and phenol/chloroform extraction (see section 2.2a above), and the DNA was ethanol precipitated using 100 µl of 10 M NH₄Ac and 2 volumes of ethanol. The solution was left at 4°C for 15 minutes and then microfuged for 10 minutes at 12,000 r.p.m.. The pellet was washed carefully with 70% ethanol, and re-microfuged for 5 minutes. The ethanol was then removed by aspiration, and the pellet freeze-dried and resuspended in 500 µl of TE.

The concentration and purity of DNA recovered was ascertained by measuring the optical density (O.D.) of the DNA solution at 260 and 280 nm. (1 O.D. of DNA at 260 nm = 50 µg/ml; the O.D._{260/280} ratio for DNA = 1.8). The concentration of DNA was also estimated by gel electrophoresis.

c) **Restriction digestion:**

i) Full digestion:

For full restriction digestion of DNA samples, the DNA was mixed in water to the appropriate concentration to provide clear bands on a gel, 1 µl of the restriction enzyme and 1/10th of the final volume of the appropriate restriction enzyme buffer was added. The enzyme was kept on ice at all times. This mixture was then incubated at 37°C for at least 30 minutes.

ii) Double digestion (changing buffers):

When double digestions were performed using enzymes which required incompatible buffers, the digestion were carried out one at a time. The first digestion was carried out as above for full digestion, and then the mixture was heated to 95°C for 15 minutes and then put immediately on ice until it reached room temperature, to inactivate the enzyme, to prevent any inappropriate activity in the second buffer. The salt concentrations of the mixture were then adjusted to give the appropriate conditions for the second enzyme, and the second restriction digestion performed.

This technique proved to be more effective than phenol/chloroform extraction and ethanol precipitation of the digested DNA, prior to the second digestion, because of the low yield of digested DNA recovered after the extraction procedures.

iii) Partial digestion:

Various methods were essayed to produce partial digestions of cosmid DNA.

Initial experiments were carried out using the procedures described in Sambrook, Fritsch and Maniatis (1989), and recommended by other colleagues who have done such experiments before, such as using dilutions of the restriction enzyme (10%, 1% and 0.1% in glycerol buffer), or restricting the time of digestion at 37°C to 10 minutes or less. All these conditions resulted in either full digests of the cosmid clone or no digestion at all. It was found that even a digestion for 5 minutes at room temperature was sufficient to fully digest the cosmid clone. We discovered that it was necessary to perform the digestion on ice at 4°C for 10-30 minutes, followed by heat inactivation of the enzyme as described above, to generate partial digests.

d) Electrophoretic separation of DNA fragments:

Gels for electrophoresis were cast with 1 x TAE buffer (50 x stock: 24.2 % (w/v) Tris-Cl, 57.1 % (v/v) glacial acetic acid, 10 % (v/v) 0.5 M EDTA, pH 8.0), and 1% agarose, or 0.7% low-melting point agarose. The resulting solution was heated to melt the agarose, in a microwave oven, and then 10 µg/ml of ethidium bromide was added when the solution had cooled to 70°C. The gel was then poured into the cast and left to set. Electrophoretic separation of digested cosmid DNA had to be carried out in 20 x 20 cm gels, to provide sufficient distance for clear separation and resolution of the large cosmid bands. Once set, gels were run in 1 x TAE buffer at 100 mV for 30 minutes for small gels, and 5-6 hours for large gels with cosmid DNA. Loading buffer (6 x stock: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll type 400 in water) was added to the DNA samples to a final concentration of 1 x. Gels were photographed with a ruler, to enable estimation of the band sizes of hybridised bands in Southern blots, on a U.V. short wave transilluminator, using Polaroid type 667 film.

To estimate the sizes of the restriction fragments, including the very large ones whose sizes could not be estimated by direct comparison with the markers, standard log curves

of the marker sizes against distances travelled along the gel were plotted for each gel, and the sizes of the bands worked out from the standard graph.

e) **Southern blotting:**

After electrophoresis, small gels were washed in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 15 minutes, followed by 15 minutes in neutralising solution (0.5 M Tris-Cl, 3 M NaCl, pH 7.0). Large 20 x 20 cm gels were washed in denaturing solution twice for 30 minutes, and twice in neutralising solution for 30 minutes. The blot was assembled in the usual way described in Sambrook *et al* (1989), in 10 x SSC, using either Gene Screen Plus (DuPont, Dreieich, Germany) or Hybond N+ (Amersham International plc, Aylesbury, Bucks, U.K.) for the transfer of the DNA. The blot was left overnight. Before removing the membrane, the position of the wells were marked.

Initially a pre-wash of 10 minutes in 0.2 M HCl was used for the cosmid digestions, to acid-cut the very large DNA fragments, for more efficient transfer, but this proved to inhibit efficient hybridisation to the radioactive probes in some way, and so was discontinued.

i) Duplicate lifts:

For some cosmid digestions, duplicate lifts were taken of the same gel for hybridisation with a number of different probes. In this case, the first membrane was left on the gel for 25 minutes, the second one for 7 minutes, the third and fourth for 3 minutes, and the final one left overnight. A large amount of fresh dry tissues were used and replaced between each lift to ensure effective transfer of the DNA to the filters.

f) Hybridisation of Southern Blots:

i) Labelling probes:

Probes were normally isolated by excising the appropriate DNA fragment from a low-melting point agarose gel containing digested DNA, or by using DNA produced by PCR amplification. The required bands were physically cut out of the gel during visualisation on a short wave U.V. transilluminator. The gel containing the DNA was exposed to the U.V. light for as short a time as possible to prevent degradation of the DNA. The gel slices were trimmed to remove any excess agarose, weighed and then placed in 3 ml of double-distilled water per g of gel slice. This was then boiled for 5 minutes to melt the agarose before each use of the DNA.

ii) End-labelling of DNA probes:

Approximately 250 ng of DNA was used per probe. 10 µl of oligolabelling buffer was added to a final volume of 30 µl, and then 1 µl of T4 DNA polymerase (1-2 units) and 50 µCi of [³²P]αdCTP (New England Nuclear Inc., Boston, U.S.A.) was added. This solution was then incubated for two hours or overnight at 37°C, and then the probe purified by centrifuging it through a G-10 Sephadex column for 10 minutes at 2,000 r.p.m.. 1 µl of the probe was then TCA precipitated to remove any unincorporated radioactive label, and the radioactivity of the probe measured in a scintillation counter. To TCA precipitate the probe, it was placed on the side wall of an eppendorf containing 5 µl of 10% bovine serum albumen. Then 1 ml of 10% tris-carboxylic acid (TCA) was added and the resulting solution mixed and left on ice for 10 minutes to precipitate the probe. The precipitate was then vacuum-filtered onto a glass disc filter and rinsed first with 10% TCA, and then with methanol to dry the filter, and placed in Ecoscint scintillant (National Diagnostics, Atlanta U.S.A.). The specific activity of the probe was calculated, taking into account the amount of DNA used in the reaction. Probes were only used if the specific activity was above 5×10^7 counts per minute per µg. Probes were boiled for 5 minutes to denature the DNA before adding it to the hybridisation mixture, and could be stored at -20°C for up to 2 weeks before use.

Oligo-labelling buffer consists of 10:25:15 parts of solutions A, B and C. Solution A contains 1 ml of solution O (1.25 M Tris-Cl, pH 8.0, 0.125 M MgCl₂), 18 µl of β-mercaptoethanol, 5 µl 0.1 M dATP, 5 µl 0.1 M dTTP and 5 µl 0.1 M dGTP. Solution B consists of 2 M HEPES, pH 6.6, and Solution C consists of random hexamer at 90 O.D. units/ml in TE.

iii) Random primer labelling of DNA probes:

The protocol for random primer labelling of DNA probes was the same as described above for end-labelling, except that 2 units of Klenow polymerase was used instead of T4 DNA polymerase.

iv) Labelling primers:

A different protocol was used to label oligonucleotide primers to ensure a high activity. 50 picomoles of primer was used for each probe. 5 µl of 10 x polynucleotide kinase (PNK) buffer was added to a final volume of 50 µl. 2 units of T4 polynucleotide kinase enzyme and 50 µCi of [³²P] γATP (New England Nuclear Inc., Boston, U.S.A.) were used. The solution was incubated at 37°C for 1 hour, and then the probe was purified by centrifuging it through a G-25 Sephadex column at 2,000 r.p.m. for 10 minutes. 1 µl of the probe was TCA precipitated and the activity checked using a scintillation counter. PNK-labelled probes were not boiled before use, and could be stored at -20°C for up to 2 weeks before use.

10 x PNK buffer consists of 0.5 M Tris-Cl (pH 7.6), 0.1 M MgCl₂, 50 mM DTT, 1 mM spermidine and 1 mM EDTA.

v) Hybridisation of filters:

i.i. Hybridisation conditions and washing conditions for cDNA-based probes:

The prehybridisation and hybridisation solution used consisted of 6 x SSC. 5 x Denhardts solution, 1% SDS, and 0.1 mg/ml freshly boiled herring sperm DNA. 5 x Denhardts solution contains 0.5 g Ficoll 400, 0.5 g polyvinyl pyrrolidone and 0.5 g bovine serum albumen. Filters were air-dried for 30 minutes after Southern blotting, and then cross-linked in a U.V. stratalinker. They were then prehybridised in 10-20 ml of prehybridisation solution at 65°C for 1 hour to block non-specific binding. A Hybaid rotary hybridisation oven system was used. The probe was then denatured by boiling for 5 minutes and then added to fresh prehybridisation solution, and the filter hybridised for 4 hours or overnight at 65°C.

The filters were then washed in 2 x SSC + 0.5% SDS for 15 minutes at 50°C twice, and in 2 x SSC + 0.1% SDS for 15 minutes at 50°C twice, and then blotted dry, wrapped in Saran wrap and exposed in a cassette at -70°C with Kodak fast film (Kodak X-omat AR fast film, Kodak Ltd., Hemel Hempstead, Herts. U.K.) for the required amount of time.

i.ii. Hybridisation conditions and washing conditions for oligonucleotide primers used as probes:

The prehybridisation and hybridisation solution used for oligonucleotide probes consisted of 0.9 M NaCl, 1% SDS and 40 µg/ml tRNA. Filters were dried and U.V. cross-linked as described above for cDNA probes. Filters were then prehybridised for 30 minutes-1 hour at the melting temperature (T_M) of the primer, and then the probe was added and the filters hybridised for 1-4 hours at the primer T_M .

Filters were then washed for 10 minutes in 6 x SSC at the primer T_M , followed by 10 minutes in 4 x SSC at the same temperature, and then blotted dry and exposed as described above for cDNA-based probes.

i.iii. Re-hybridisation of filters:

Filters could be re-hybridised with a different probe after being stripped of the previous hybridising probe, provided that they had not dried, resulting in irreversible binding of the probe. Filters were stripped either by washing in 200 ml of 0.4 N NaOH for 30 minutes at 42°C, followed by 30 minutes in 200 ml of 0.1 x SSC, 0.1% SDS, 0.2 M Tris.Cl (pH 7.5) at 42°C, repeated until the filters were no longer radioactive, or by boiling in 1% SDS for 10-15 minutes. The protocol used depended on the strength of radioactivity of the filter and the type of membrane used. Stripped filters were checked by exposing them to film overnight to check that all the signal had been removed.

g) Library Screening:

i) λ gt11 and λ ZAP libraries:

Libraries were plated out and screened using the protocols described by the manufacturing company for each library. The appropriate bacterial strain of plating cells was inoculated with the phage containing the library and incubated at 37°C for 15 minutes. Libraries then were plated out at the required plaque-forming unit (PFU) density in L-broth + 10% agarose, 5% NaCl and 2% maltose, on 21 x 21 cm L-Broth agar (15%) plates including the appropriate antibiotic. These plates were grown for 8 hours or overnight and then duplicate lifts were taken off each plate. The plates were stored at 4°C for at least 1 hour before lifts were taken to prevent the top agarose from peeling off. Genescreen Plus or Hybond N+ membranes were used and labelled with a needle and India ink for later orientation of the hybridised filters against the original library plates. The first lift was left for 1 minute and the second for 5 minutes. Hybond N+ filters were then washed for 5 minutes in 10% SDS, to prevent the signals from the plaques from spreading too far, 1 minute in denaturing solution (0.5 M NaOH, 1.5 M NaCl), 5 minutes in neutralising solution (0.5 M Tris.Cl, 3 M NaCl, pH 7.0), and then rinsed in 2-3 x SSC. Genescreen Plus filters were washed in 0.5 M NaOH twice for 2 minutes, and then 1 M Tris.Cl (pH 7.5) twice for 2 minutes. The filters were then blotted dry, left to air-dry for at least 30 minutes and then U.V. cross-linked in a U.V.

Stratalinker from Stratagene. The filters were then hybridised and washed following the conditions described above for Southern blots.

After the films were developed, the autoradiographs of the duplicate filter lifts were aligned using the orientation marks from the master plate. Duplicate signals on both films were marked, the area on the plate identified and the plaque of interest picked off the plate and placed in 100 µl of SM buffer. The plaques were left in buffer for a few hours, shaking occasionally to wash out the phage, and this stock was then replated for secondary screening and tertiary screening, following the above protocol, but using smaller round plates. This was carried out until single pure hybridising plaques were obtained.

ii) PCR amplification of DNA inserts from λgt11 libraries:

PCR reactions were set up to isolate the inserts from hybridising plaques in λgt11 libraries. SM buffer alone was used as a negative control. The PCR reaction was set up using 5 µl of 10 x PCR buffer, 5 µl of nucleotide stock (1.25 mM each of dATP, dCTP, dGTP and dTTP), 1-5 µl of phage stock and 1 µl of each primer. The final volume was 50 µl. The smallest possible volume of phage stock to give a good signal was used because it contained Mg²⁺, which inhibits the PCR reaction. 1 unit of *Taq* polymerase was added and the reaction mixture was topped with 50 µl of paraffin wax to prevent evaporation. The PCR program used consisted of a melting step of 94°C for 2 minutes, an annealing step of 47-55°C for 2 minutes, and then a polymerisation step of 72°C for 3 minutes. The annealing step was worked out depending on the T_M of each primer, based on its G-C content: T_M = 4 x (G+C) + 2 x (A+T). The program was run for 20-25 cycles, and then the sample checked by electrophoresing an aliquot on an agarose gel to check the size and purity of the DNA product. 50 µl of chloroform was then added to the PCR reaction mix, after removing most of the paraffin layer, and the solution microfuged for 5 minutes at 13,000 r.p.m. The top aqueous layer containing the PCR product was then removed and purified using the Geneclean system, and then cloned into a TA vector (described below).

iii) Excision of Phagemids *in vivo* from λZAP vectors:

The plaque of interest was isolated as described above for λgt11 libraries. 200 µl of XL-1-Blue bacterial cells, at the stage of growth where the O.D._{600 nm} = 1, were combined with 100 µl of phage stock and 1 µl of ExAssist helper phage (titer > 1 x 10⁶ PFU/ml) in a 50 ml Falcon tube, and incubated for 15 minutes at 37°C. Then 3 ml of L Broth was added and incubated at 37°C for a further 2-2.5 hours in an orbital shaker. The tube was then heated to 70°C for 2 minutes to kill the bacteria and centrifuged for 15 minutes at 4,000 r.p.m. The supernatant containing the phagemids was decanted, incubated with 200 µl of SOLR bacteria at the growth stage where O.D._{600 nm} = 1.0, in an eppendorf tube at 37°C for 15 minutes. Then 100 µl of this mixture was plated onto L Broth agar + ampicillin plates at left to grow up overnight at 37°C. The resulting colonies contained the λZAP DNA in the form of stable phagemid plasmids, which could then be grown up and the DNA isolated as described above for plasmid mini-preparations or large scale preparations.

iv) Screening of cosmid libraries:

Aliquots of the cosmid library were diluted in L Broth plus ampicillin and spread evenly onto Hybond N+ filters covering large 21 x 21 cm L-Broth agar + ampicillin plates. The plates were incubated at 37°C overnight. The next day, the membrane was removed from the master plate and 2 further Hybond N+ filters were placed in turn on top of the colonies on the master membrane and then transferred, colony side up, to further fresh L-Broth agar + ampicillin plates. The filters were placed on the fresh plates to pre-wet them before placing them on the master plate to transfer the bacterial colonies. The secondary filters and the master filters were identically marked with needles dipped in India ink to provide orientation marks. The master membrane was replaced on its original plate and all three plates were re-incubated for 1-2 hours at 37°C to allow the bacterial colonies to re-establish themselves. The membrane were then removed from the secondary duplicate plates and floated in denaturing solution (0.5 M NaOH, 1.5 M NaCl), colony side up, for 7 minutes, and then neutralising solution (0.5 M Tris.Cl, pH 7.0, 3 M NaCl) twice for 3 minutes, and finally rinsed in 2 x SSC, whilst being shaken to remove the colonies. Any bacterial debris left was

carefully removed with a tissue and the filters were air-dried for 30 minutes and then U.V. cross-linked.

The filters were then hybridised as described above for λ phage libraries. When the films were developed, they were aligned using the orientation marks. Any duplicate signals were mapped to the correct area on the master membrane and the area carefully scraped to remove the bacterial colonies, which were then resuspended in 200 μ l of L Broth + ampicillin, and glycerol. These bacterial stocks were then used as stocks for further screening, using smaller plates, until individual pure hybridising colonies could be isolated. These pure colonies were then grown up and DNA isolated from them following the method described for large scale preparation of DNA described above. Only large-scale methods could be used, because the yield of cosmid DNA was low, because of its large size. Also, care had to be taken during the DNA isolation to prevent shearing of the DNA: for examples the solutions were never vortexed, only ever mixed by inversion.

h) Cloning:

i) Purification of inserts using GeneClean:

The GeneClean system from Bio 101 (1070 Joshua Way, Vista, California 92083, U.S.A.) was used to purify DNA isolated from low melting point gel fragments and produced by PCR, prior to cloning. 3 volumes of NaI were added to the DNA, or 3 μ l of NaI per mg of gel slice. This mixture was then incubated at 55°C for 5 minutes. Then 5 μ l of Glassmilk suspension was added, plus an additional 1 μ l per 0.5 μ g of DNA above 5 μ l. This suspension was transferred to an eppendorf or spin filter and left on ice for 5 minutes to allow the DNA to bind to the Glassmilk, vortexing every minute. The solution was then microfuged for 15 seconds at 13,000 r.p.m. The pellet was washed twice with ice-cold NEW Wash solution, and vortexed and centrifuged between each wash. The pellet was then dried with 80% ethanol and re-spun and the ethanol removed by aspiration. 1-2 times the volume of the glassmilk suspension of water was then added, and the solution vortexed to mix it, and then incubated at 55°C

to elute the DNA. The suspension was then finally re-centrifuged for 30 seconds and the supernatant, containing the DNA, used in cloning.

ii) TA vector preparation:

TA vectors were used to improve the efficiency of ligation of PCR fragments. The vector, usually Bluescript (Stratagene), is cut with a blunt-ended restriction enzyme, and then an A and T residue are added to the blunt ends to hybridise with the A and T residue left at the ends of PCR products by the *Taq* polymerase enzyme.

The Bluescript vector (Stratagene) was digested with *EcoRV* and then 10 µl was further incubated with 2 µl of 10 x *Taq* buffer, 2 µl of 20 mM dNTPP, 5 µl of water, and 1 unit of *Taq* polymerase per µg of DNA, at 70°C for 2 hours. The DNA was then phenol-chloroform and chloroform extracted and ethanol precipitated. This produced 4 µl of TA vector.

iii) Ligations:

Ligations were carried out with 100 ng of digested vector and 100 ng of insert. The final volume was made up to 10 µl with water, and warmed to 45°C to melt any cohesive termini that had re-annealed, and then placed on ice. For ligation of cohesive termini, 1 µl of 10 x bacteriophage T4 DNA ligase buffer, 1 µl of 5mM ATP and 1 unit of T4 DNA ligase was added. For ligation of blunt-ended termini, a 2: 1 ratio of insert to vector was used. These reactions were incubated at 16°C overnight, and then used to transform competent bacteria.

iv) CaCl₂ method for making competent cells:

DH5α bacterial cells were grown up in 10 ml of L Broth at 37°C in an orbital incubator overnight, and then 50 µl of this culture were used to inoculate 50 ml of L Broth. These cells were then grown at 37°C in a shaking incubator until they reached an

O.D._{600 nm} of 0.3-0.4. The cells were then transferred to a 50 ml Falcon tube and incubated on ice for at least 15 minutes, before being centrifuged at 2,000 r.p.m. at 4°C. The supernatant was then decanted and replaced with 5 ml of ice-cold 5 mM CaCl₂. Care was taken that the cells remain at 4°C during every step of the procedure, because this improved the competence of the cells. The cells were incubated on ice for a further 30 minutes and then centrifuged again for a further 15 minutes at 2,000 r.p.m. at 4°C. The cell pellet was resuspended in 1.5 ml of ice cold 5 mM CaCl₂, and then left on ice for 1 hours to overnight. The longer the incubation the better the competence of the cells produced. The competence of the cells was checked by transforming an aliquot of cells with known quantities of the Bluescript vector (Stratagene): levels of competence of 10⁷⁻⁸ transformed cells per mg of DNA were routinely achieved using this method.

v) Transformation of competent cells:

200 µl of competent cells were added to each ligation mixture and left on ice for 1-2 hours. The cells were then heat-shocked for 5 minutes at 42-45°C and then placed back on ice. Then 1 ml of L Broth was added to each mixture of cells and they were incubated at 37°C for 30 minutes, before being microfuged for 2.5 minutes at low speed, 6,500 r.p.m.. The cell pellet was then resuspended in 20 µl of 20 mg/ml X-Gal and plated out on to L Broth plus ampicillin agar plates. These were then left to air-dry and then incubated at 37°C overnight.

vi) Characterisation of positive clones:

Positive clones with the required inserts were identified either by blue-white selection using X-Gal: bacterial colonies containing only the Bluescript vector turn blue whereas colonies containing an insert in the vector remain white.

Alternatively, Grunstein-Hogness blots were carried out. Colonies were plated out with a yellow tip onto Genescreen Plus filters placed on small circular L Broth agar plus ampicillin plates. A positive and a negative control was also plated out on each plate. The plates were left to grow at 37°C overnight. The next day, the filters were removed

from the plates and washed for 5 minutes in 10% SDS, then 7 minutes in denaturing solution, 7 minutes in neutralising solution, and finally rinsed in 2 x SSC for 5 minutes. The filters were then blotted dry and hybridised following the procedures described above for Southern blots, using the appropriate probe for the required insert. Colonies containing inserts showed as positive signals on the autoradiograph.

i) **DNA Sequencing:**

DNA sequencing was generally carried out using the Sequenase 2.0 kit from United States Biochemical Corporation (P.O. Box 22400, Cleveland, Ohio 44122, U.S.A.). 2-3 µg of DNA was used in a final volume of 20 µl of water. 2 µl of 2 M NaOH was added and the solution incubated at 65°C for 10 minutes to denature the template. The solution was then neutralised with 0.4 volumes of 5 M NH₄Ac (pH 7.5) and then 4 volumes of ethanol added and the solution left at -70°C for 5 minutes to precipitate the DNA. The solution was then microfuged at 12,000 r.p.m. for 15 minutes at 4°C and the pellet freeze-dried and resuspended in 7 µl of water and 2 µl of Sequenase buffer, and 5 pmoles of sequencing primer were added. This solution was then heated to 65°C for 2 minutes to anneal, and then left to cool slowly at room temperature over 5-10 minutes. When the mixture had cooled, 2 µl of labelling mixture, diluted 1:5 with water, 1 µl of 0.1 M DTT, 0.5 µl of [³⁵S] dATP and 2 µl of Sequenase enzyme diluted 1: 7 in ice-cold glycerol dilution buffer were added. This reaction mix was mixed thoroughly and incubated at room temperature for 5 minutes. Then 3.5 µl of this labelling reaction was added to each of four tubes containing 2.5 µl of each termination mix, G, A, T and C, and these tubes incubated at 37°C for a further 5 minutes. The reactions were then terminated by adding 4 µl of Stop solution, mixed and stored at -20°C until used.

This basic protocol was altered in various ways to optimise it for sequencing of the genomic *Bmp-2* clone, and reduce compressions caused by secondary structures. Compressions occur when secondary structures in the DNA sequence being read cause the sequencing enzyme to skip bases and this gives rise to ambiguous signals in the gel where one or more bands appear at the same position. Secondary structure may also cause premature termination of sequencing and signals in the gel with all four bands at the same position. This secondary structure often occurs in regions of DNA with a high

G-C content, which can sometimes be recognised by clustering of sites for restriction enzymes with a high G-C ratio in their recognition sequence, such as *NotI*. Such an area occurs in the 5' region of the *Bmp-2* gene.

The optimal procedure was found to involve doing the termination reactions at 42°C, to reduce compressions on the sequencing gel caused by secondary structure. Sequencing using 5'-deaza- or dITP-nucleotides for the termination reactions did not resolve the problems which resulted in the formation of the compressions. The basic protocol also involved denaturing the DNA template at room temperature, and it was found that carrying out this step at 65°C for 10 minutes improved the resolution of the bands. For generating sequence information close to the primer, 1 µl of the Mn²⁺ buffer provided in the Sequenase kit was added to the labelling mixture. The addition of the Mn²⁺ ions reduces the average length of the DNA synthesised but intensifies the bands close to the primer.

The sequencing reaction were run out on denaturing sequencing gels made using the Sequagel components. The gels were poured between two glass plates taped together, and run for 1-5 hours at 1200 V, at a constant wattage. The gel was then denatured in 10% methanol + 10% acetic acid for 15 minutes and then vacuum-dried for a further 15 minutes before putting it down to expose overnight in a cassette with Kodak fast film at room temperature. To improve the resolution of bands, particularly when sequencing the 5' region of the *Bmp-2* genomic clone, 40% formamide was added to the sequencing gel. This further reduced the occurrence of secondary structure and compressions. These gels were run at the same wattage but a 60% higher voltage. This meant that they ran slower by a factor of about 50%.

Sequencing using the PCR Promega fmol Sequencing kit was also attempted to try to get better resolution of the bands. This was carried out following the protocol in the kit. This involved using primers labelled with ³²P, using the PNK labelling method described above, for sequencing and then using the reaction buffer and *Taq* polymerase enzyme provided in the fmol sequencing kit instead of the Sequenase enzyme and buffer. A master mix was made with 4-40 fmoles of template DNA, 1.5 µl of labelled primer, 4.25 µl of 5 x sequencing buffer and 1 unit of enzyme, in a final volume of 16 µl. 4 µl of this master mix was added to each of 4 termination tubes containing 1 µl of

d/ddNTP in a 1: 1 dilution with water. Finally 20 µl of mineral oil was added to each tube and the tubes placed in a thermal cycler machine, preheated to 95°C to prevent any non-specifically annealed primers from being extended and stabilised. Then a PCR program was used with an initial melting step of 95°C for 2 minutes, followed by 30 cycles of 95°C for 30 seconds, 42°C for 30 seconds and 70°C for 1 minute. Finally 3 µl of stop solution was added to each tube and the resulting mixture run on a normal or 60% formamide sequencing gel. Care was taken when running these gels and disposing of the buffer because the primers were labelled with ^{32}P .

j) Primer extension:

Primer extension experiments were carried out using the Promega AMV Reverse Transcriptase Primer Extension kit. Primers used for primer extension were labelled using the PNK procedure described above. The reaction was carried out by adding 1 µl of 20 x PCR buffer, 2 µl of labelled primer, and 7 µl of water to 25-50 µg of total RNA. An overlay of mineral oil was added, the reaction heated to 65°C for 10 minutes and then placed at the hybridisation temperature, based on the melting temperature of the primer, for 3 hours. The final reaction mixture was made up using 5 µl of 10 mM Tris.Cl, pH 8.3, 2.75 µl of 1 M KCl, 0.5 µl 1 M of DTT, 500 µM of dNTP mix (dATP, dGTP, dCTP and dTTP), 6 µl of 25 mM MgCl₂ and 12.5 µl of water. This was heated to 42°C for 1 minute and then 1 µl of AMV reverse transcriptase and 1 unit of RNase inhibitor was added, and this solution was added to the hybridisation mixture and incubated at 42°C for 1 hour. This solution was then purified by centrifuging it through a G-50 Sephadex column to remove any unbound label and a small amount run on a denaturing sequencing gel, alongside a sequencing reaction mix of some known sequence, usually the Bluescript vector sequenced with T3 or T7 primers, to provide size markers. Care was taken when running this gel and disposing of the buffer because the primers were labelled with ^{32}P .

2.3 RNA Techniques:

All the following techniques were carried out in an RNase-free environment, using RNase-free solutions, tubes and eppendorfs.

a) RNA extraction from cells:

Total RNA was extracted from tissue culture cells using the Acid Guanidinium Thiocyanate-Phenol-Chloroform extraction method, described by Chomczynski and Sacchi (1987).

The cells were harvested in 1-2 ml of solution D per 80cm² tissue culture flask (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (w/v) sarcosyl and 0.1 M 2-mercaptoethanol). To the resulting solution, 0.1 ml of 0.2 M sodium acetate, pH 4.0, 1 ml of phenol, and 0.2 ml of chloroform-isoamyl alcohol mixture (49:1 v/v) were added per ml of Solution D. After addition of each solution, the tube was inverted several times to ensure thorough mixing and left on ice for 15 minutes, then centrifuged at 10,000 r.p.m. in a Sorval SS34 rotor, in a Sorval RC 5B centrifuge for 20 minutes at 4°C. The aqueous phase containing the RNA was removed, leaving the DNA and protein in the interface and phenol phase. An equal volume of isopropanol was then added to the aqueous phase in a clean Sarstedt tube. The tube was placed at -20°C for a minimum of 1 hour to precipitate the RNA. The tube was then spun at 10,000 r.p.m. in a Sorval SS34 rotor for 20 minutes, at 4°C, and the resulting RNA pellet was dissolved in 0.3 ml of solution D. The RNA was then transferred to a 1.5 ml eppendorf tube. An equal volume of isopropanol was added and the solution placed at -20°C for a further hour to precipitate the RNA. The eppendorf was then microfuged for 10 minutes at 4°C, the pellet washed with 70% ethanol and respun for a further five minutes, and the pellet resuspended in 50 µl of RNase-free double-deionised water. The RNA was then cleaned by phenol-chloroform and chloroform extraction, ethanol precipitated and finally resuspended in 50 µl of RNase-free water. The O.D. at 260 and 280 nm was measured. (1 O.D. at 260 nm of RNA = 40 µg/ml; ratio of O.D.₂₆₀₋₂₈₀ nm for RNA = 2.0). The RNA was stored at -20°C, with 1/10th of the volume of 3M NaAc, and 2.5 times the volume of ethanol.

b) Extraction of poly(A)[±] RNA:

The PolyATtract mRNA isolation system from Promega was used to isolate poly(A)⁺ mRNA, according to the manufacturer's instructions. 100 µg of total RNA was made up to a final volume of 500 µl with double-deionised water in an RNase-free eppendorf tube. This was then placed in a 65°C water bath for 10 minutes, 3 µl of biotinylated-oligo(dT) probe and 13 µl of 20 x SSC (3 M NaCl, 0.3 M tri-sodium citrate) were then added, mixed well, and then left at room temperature until it had completely cooled. Whilst this was cooling, the streptavidin-linked paramagnetic particles (SA-PMPs) were washed by resuspending them in 0.3 ml of 0.5 x SSC by gently flicking the tube, then capturing the particles by placing the tube in the magnetic stand until the SA-PMPs had collected at the side of the tube, and then removing the supernatant. The SA-PMPs were washed three times. Next the particles were resuspended in 0.1 ml of 0.5 x SSC, the entire contents of the RNA annealing reaction were added, and incubated at room temperature for 10 minutes.

The magnetic stand was then used to collect the SA-PMPs with the bound poly[A⁺] RNA, and the supernatant removed. The beads were washed 4 times with 0.1 x SSC. After the final wash, the particles were resuspended in 0.1 ml of water to elute the RNA. The elution step was repeated using 0.15 ml of double-deionised water. The final pooled volume of water contains the extracted poly(A)⁺ RNA. The RNA was stored as described above for total RNA.

c) Northern Blots:

RNA samples were electrophoresed on formaldehyde denaturing agarose gels to allow separation of the RNA fragments. The gels contained 1% agarose in 1 x MEA (20 x MEA stock: 400 mM morpholinopropanesulphonic acid, 100 mM NaAc, 200 mM EDTA, (pH 7.2)), 5.4 % formaldehyde, 0.5 µg/ml ethidium bromide. 10-15 µg of total RNA or 1-2 µg of poly(A)⁺ RNA was run on each gel. The RNA (total and poly(A)⁺) was precipitated and the pellet resuspended in 20 µl of loading buffer (75% (v/v) de-ionised formamide, 9% (v/v) formaldehyde, 1.5 x MEA) and heated for 10 minutes at 65°C before loading into the gel. The gels were run at 5V/cm, until the loading

buffer had migrated three-quarters of the way down the gel. The gel was then photographed, with a ruler to enable the transcript sizes to be calculated later, on a U.V. short wave transilluminator, using Polaroid type 667 film. Chicken total RNA contains two transcripts of ribosomal RNA which can be used as size markers, 28S (approximately 5 kb) and 18S (approximately 2 kb).

After running the gels, they were rinsed twice for 20 minutes in 500 ml of 10 x SSC to remove the formaldehyde from the gel. The blot was assembled in the usual way as described in Sambrook *et al* (1989), in 10 x SSC, using either GeneScreen Plus (DuPont, Dreieich, Germany) or Hybond N+ (Amersham International plc, Aylesbury, Bucks, U.K.), for the transfer of the RNA. The blot was left overnight.

Before removing the membrane, the positions of the wells were marked. The filters were then removed, fixed by U.V. cross linking on a Stratalinker and then baked for 2 hours at 80°C. The filters were then hybridised.

d) Making riboprobes:

The template of the riboprobe was linearised with an appropriate enzyme, then cleaned by phenol-chloroform extraction and ethanol precipitated, as described above, and resuspended in water at 1 µg/µl. The transcription reaction was set up, consisting of 1 x transcription buffer (T3/T7 or SP6) buffer, 20 mM DTT, 0.5 mM GTP, 0.5 mM ATP, µM UTP and 2 µM CTP. Ribonucleotides were obtained from Pharmacia. 2-3 µg of linearised DNA was made up to 25 µl with water, and, to this, µCi of α [³²P] CTP (New England Nuclear Inc., Boston, U.S.A.), 50 units of RNase inhibitor (Boehringer Mannheim, Lewes, Sussex) and 10 units of transcription enzyme (T3/T7 or SP6 RNA polymerase) were added. This mixture was then incubated for 40 minutes at 37°C, after which time a further 10 units of fresh enzyme were added and the mixture re-incubated for a further hour at 37°C. The transcription reaction was then DNase treated to remove the template DNA, by adding 50 units of RNase inhibitor, 10 µg *E.coli* tRNA (Sigma, Poole, Dorset) and 10 units of BCL or BRL RNase-free DNase I, and incubating for 10 minutes at 37°C. After this incubation, 95 µl of water and 10 µl of

5 M LiCl were added, the solution was mixed and the RNA was ethanol precipitated in 2.5 volumes of ethanol at -70°C for 15 minutes or -20°C for 2 hours. The solution was then microfuged for 15 minutes and the pellet washed with 70% ethanol, air-dried and then resuspended in 50 µl of water. 1 µl of this was taken and counted in 8 ml of EcoScint (National Diagnostics, Atlanta, U.S.A.).

e) Hybridisation:

Northern blots were hybridised with RNA probes in Hybaid bottles at 65°C in a Hybaid rotary hybridisation oven. The prehybridisation solution consisted of 5 x SSC, 60% (v/v) de-ionised formamide, 20 mM NaPO₄ (pH 6.0), 5 x Denhardts solution (see above), 1% (w/v) SDS, 100 mg/ml herring sperm DNA, 100 µg/ml yeast total RNA and 10 µg/ml poly(A)⁺ RNA (Sigma, Poole, Dorset). The last three components were freshly denatured by boiling prior to adding them to the hybridisation solution. The filters were prehybridised for 2-4 hours at 65°C, and then the riboprobe added and fresh hybridisation solution with 7% Dextran sulphate added, and the filters hybridised overnight at 65°C.

The filters were washed for 30 minutes each in 3 x SSC + 0.5% (w/v) SDS at 65°C, at 65°C in 2 x SSC + 0.5% SDS, then at 70°C in 1 x SSC + 0.5% SDS, at 75°C in 0.1 x SSC + 0.5% SDS, and finally in 0.1 x SSC + 0.1% SDS at 80°C. After the final wash, the filters were blotted dry and wrapped in Saran Wrap and placed in a cassette to expose with Kodak fast film at -70°C for the required amount of time.

f) Whole mount *in situ* hybridisation:

This procedure is as described by Wilkinson (1992), as modified by J.-C. Izpisúa-Belmonte.

The chick embryos were removed from the egg, deviscerated and washed in PBS (phosphate buffered saline: 135 mM NaCl, 27 mM KAc, 10 mM NaHPO₄, 15 mM potassium dihydrogen phosphate, pH 7.5) and fixed overnight in 4% (w/v)

paraformaldehyde. The paraformaldehyde was made up in PBS, with a few drops of 10 M NaOH added and the mixture then heated to 65°C until the paraformaldehyde had dissolved. The embryos were then washed twice in PBT (PBS + 0.1% Tween-20) and hydrated through a series of 5 minute methanol washes - (v/v) 25%, 50%, and 75% methanol in PBT, then twice in 100% methanol. At this stage they can be stored at -20°C. In all the washes at room temperature, the embryos were gently rocked on a rocking platform (Luckman). Next the embryos were washed twice with PBT for 5 minutes. The embryos were then incubated in 6% (v/v) hydrogen peroxide in PBT for 1 hour to bleach them. This was then followed by three 5 minute washes in PBT. They were then treated with 10 µg/ml of proteinase K in PBT for 15 minutes. This was then removed and replaced with 2 mg/ml glycine in PBT for 5 minutes, followed by two 5 minute washes with PBT. The embryos were then placed in 0.2% gluteraldehyde/4% paraformaldehyde (v/v), rocking gently for 20 minutes, again this was followed by two 5 minute washes with PBT. The PBT was then removed and replaced with 1 ml of prehybridisation mix (50% (v/v) deionised formamide, 5 x SSC, pH 4.5, 50 µg/ml phenol/chloroform extracted yeast total RNA, 1% (w/v) SDS, and 50 µg/ml heparin). The embryos were transferred to a fresh vial and the prehybridisation mix removed and replaced with fresh solution, and incubated at 70°C for a further hour. The prehybridisation mix was then replaced with hybridisation mix (prehybridisaion mix containing 1 µg/ml digoxigenin-labelled RNA probe), and re-incubated at 70°C overnight. The prehybridisation and hybridisation mixes were preheated to 70°C before being added to the embryos.

Riboprobes labelled with digoxigenin (DIG) were prepared in the same way as radioactive riboprobes, but 0.7 µl of 10 mM DIG UTP (from Boehringer Mannheim GmbH, Germany), 1.3 µl of 10 mM rUTP, 2 µl of 10 mM rATP, rCTP and rGTP were added.

The next day the embryos were washed twice with solution I (50% (v/v) deionised formamide, 5 x SSC, pH 4.5, 1% (w/v) SDS) at 70°C for 30 minutes, followed by a 10 minute wash in an equal volume of solution 1: solution 2 (0.5 M NaCl, 10 mM Tris-Cl, pH 7.5, 0.1% (v/v) Tween-20), at 70°C. Three 5 minute washes were carried out in solution 2 at room temperature. Next they were washed twice with 100 µg/ml RNase A in solution 2 for 30 minutes at 37°C; this was followed by a 5 minute wash in

solution 2, and a 5 minute wash in solution 3 (50% (v/v) formamide, 2 x SSC, pH 4.5), both at room temperature. Two washes were then carried out in solution 3 for 30 minutes at 65°C, followed by three 5 minute washes in TBST (stock solution of 10 x TBS: 1.4 M NaCl, 27 mM KCl, 0.25 M Tris.Cl, pH 7.5, 0.1% Tween-20. This was diluted to 1 x TBST on the day of use and 100 µl of 2 mM levamisole was added.) The embryos were then preblocked for 60-90 minutes with 10% (v/v) sheep serum in TBST. The sheep serum was heat treated before use for 30 minutes at 70°C. The antibody was prepared by weighing out 3 mg of chick embryo powder, adding 0.5 ml of TBST and 5 µl of sheep serum and heat treating at 70°C for 30 minutes. This was cooled on ice and then 1 µl of anti-digoxigenin antibody was added and shaken gently at 4°C for 1 hour to reabsorb the antibody, and then microfuged for 10 minutes. This was then diluted to 2 ml with 1% (v/v) heat-treated sheep serum in TBST. The blocking serum was removed from the embryos, replaced with the antibody solution and the embryos rocked overnight at 4°C.

Chick embryo powder was prepared by homogenising 10 day old chick embryos in a minimum volume of PBS (pH 7.5), to which 4 times the volume of ice-cold acetone was added. This was mixed well, and then placed on ice for 30 minutes. This was centrifuged at 10,000 r.p.m. in a Sorval SS34 rotor for 10 minutes and the supernatant discarded. The pellet was washed with ice-cold acetone and re-centrifuged. The pellet was then placed on a piece of filter paper and allowed to air-dry before being ground into a fine powder with a mortar and pestle. The powder was then stored in an air-tight tube at 4°C.

After the overnight antibody incubation, the antibody was removed from the embryo and the embryo was washed 3 times in TBST for 5 minutes at room temperature. This was followed by five 1 hour washes in TBST. The embryos were then washed three times for 10 minutes with NTMT (100 mM NaCl, 100 mM Tris.Cl., pH 9.5, 50 mM MgCl₂, 0.1% Tween-20 and 2 mM levamisole). They were then incubated in NTMT including 4.5 µl NBT (nitroblue tetrazolium salt - 75 mg/ml in 70% (v/v) dimethylformamide) and 3.5 µl BCIP (5-bromo-4-chloro-3-indolyl phosphate - 50 mg/ml in dimethylformamide) per ml and this was rocked for the first 20 minutes. The embryos were kept in the dark after the addition of the colour reaction and left in this

solution until the colour had developed to the required extent. They were then washed twice for 5 minutes in PBT and stored in PBT at 4°C.

2.4 Embryology and Tissue Culture Techniques:

All tissue culture techniques were carried out under sterile conditions. The solutions were either autoclaved, filter-sterilised or purchased as sterile products ready for use from Gibco/BRL (Paisley, Scotland). The plasticware used was also sterile and purchased from Nunc.

a) Isolation of chick limb bud mesenchyme cells:

Chick limb bud mesenchyme cells were isolated from stage 18-21 chick embryos, which had been incubated at 37°C. The eggs came from Poydon Farm, Waltham Cross, Herts. 3 dozen embryos were dissected to provide sufficient cells for transfection.

The blunt end of the egg was opened using a pair of blunt forceps and scissors, and the embryo was removed into a sterile petri dish containing PBS, pH 7.5. All four limb buds were removed using tungsten needles and watchmakers' forceps, and all the limb buds pooled. The ectoderms were then removed by incubating the buds with 2% (v/v) trypsin in CMF (Gibco) for approximately 10-12 minutes on ice. As soon as the ectoderm could be seen separating from the mesenchyme, the trypsin was inactivated by the addition of foetal calf serum. The ectoderms were then teased away from the mesenchyme cells and discarded and the mesenchyme cells were triturated, counted using a hemocytometer and washed three times to remove the trypsin. The cells were then transfected.

b) Transfection of chick embryonic limb bud mesenchyme cells:

i) Transfection using Transfectam:

Initially mesenchyme cells were transfected using the Transfectam system provided by Promega. After isolation, the limb bud mesenchyme cells were resuspended in serum-free Dulbecco's minimal essential medium (DMEM) prior to transfection, because serum decreases the efficiency of the Transfectam. 5×10^5 cells were plated out in 1.5 cm dishes and 5 µg of plasmid DNA and 1-25 µl of Transfectam were added, and the cells incubated at 37°C for 1-4 hours. The medium containing the Transfectam was then removed and the cells resuspended in DMEM.

ii) Transfection by electroporation:

After isolation, 2×10^7 mesenchyme cells were placed in eukaryotic electroporation cuvettes with 50 µg of DNA. The cells were left on ice for 30 minutes: this is supposed to improve transfection efficiency by halting all the cells at the same phase of the growth cycle. The cells were then electroporated, using a capacitance of 960 µF and a voltage of 0.22 V. These are the conditions usually used in our laboratory for transfecting eukaryotic cells. After electroporation, the cells were spun down at 6,500 r.p.m. and resuspended in Micromass medium ((v/v) 45% DMEM, 45% F12 medium, 10% foetal calf serum, with antibiotic (1% of final volume) and L-glutamine (200 mM, 100x; 2% of final volume) before replating either as monolayers or in micromass culture conditions.

iii) Culture of transfected chick mesenchyme cells:

For monolayer culture, the transfected cells were plated out in 15 mm dishes by flooding the dishes with the cell suspension and then adding the medium straight away. For micromass culture, the cells were plated out in a 10 µl drop in the middle of the well, and then incubated for 1 hour at 37°C, 5% CO₂ before flooding the well with 350 µl of medium.

If all-*trans* RA was added, it was added at a concentration of 50 nM per well, in ethanol, and the RA was added in the dark, and the cells then cultured and lysed in the dark, to prevent light-mediated conversion of the all-*trans* RA to other forms of RA.

The cell were lysed after 24, 48, 72 or 120 hours using 250 µl of water containing 25 µl of Promega cell lysis solution, per well. The cell density was estimated by eye to see whether the cells were healthy or not, and how confluent they were, and after lysis, the wells were examined under the microscope to check that all the cells had been lysed. The cell lysates were then tested for luciferase activity in two batches by adding 80 µl of Promega luciferase reagent to 125 µl of the cell lysate, and using a luminometer. Positive and negative controls were carried out for each batch of cells isolated and transfected, by transfecting the cells with pGL2 Control and pGL2 Basic vectors, obtained from Promega.

The cell lysates were stored at -20°C or kept on ice immediately after harvesting, because the samples rapidly degraded. However, the optimum temperature for the luciferase reaction is room temperature, so the samples were warmed to room temperature before testing for luciferase activity.

c) Culture and passaging of chick embryonic fibroblast cells:

Chick embryonic fibroblast cells were plated out in 80 cm² tissue culture flasks at a density of 6 x 10⁵ cells per ml. 20 ml of medium was used (90% DMEM: Dulbecco's Modified Eagle's Medium, 10% Foetal calf serum, heat inactivated, virus screened, from Gibco/BRL, Paisley, Scotland), prewarmed to 37°C, and the cells were incubated at 37°C, 5% CO₂. When the cells became confluent, they were passaged. This was carried out by removing the medium from the cells and washing them with serum-free DMEM, to remove any serum that might otherwise inhibit the action of the trypsin. The cells were then trypsinised by adding 2 ml of TV (1:9 v/v trypsin/versene) and replacing the flask in the incubator for 1-2 minutes to allow the cells to dissociate from the bottom of the flask. This reaction was then terminated by the addition of 5 ml of medium containing 10% serum. The cells were counted using a hemocytometer and replated at a density of 5 x 10⁶ cells per ml, in 20 ml of medium in a 80 cm² flask.

d) GAG antibody staining:

CEF cells were infected with the retroviral vector constructs by adding the viruses to the culture medium. To assay cells for viral spread, they were stained with a monoclonal antibody, 3C2. The transfected cells were plated out into 15 mm plates and left overnight at 37°C, 5% CO₂. The medium was then removed from the cells and they were washed with 0.5 ml PBS (pH 7.5) per plate for 5 minutes. The cells were then fixed for 20 minutes with 2% (w/v) paraformaldehyde and the Vectastain ABC elite kit (Vector Labs, 16 Wulfric Square, Bretton, Peterborough, U.K.) was used to carry out the immunocytochemistry. All the solutions were provided in the kit except the primary antibody, the diaminobenzidine tetrahydrochloride (DAB) (Sigma, Poole, Dorset), the PBS and the Tris.Cl, pH 7.5.

After fixing, the cells were washed for 5 minutes in PBS, then 0.5 ml of 0.3 % (v/v) hydrogen peroxide (from a 30% stock solution) in methanol was added, to quench any endogenous peroxidase activity. This was left on the cells for 30 minutes, followed by a wash with 0.5 ml PBS for 20 minutes. Next 0.5 ml of blocking buffer was added for 20 minutes. The blocking buffer was prepared from the same species that the secondary antibody was derived from: in this case, 150 µl of horse serum was added to 10 ml PBS. The cells were then washed with 0.5 ml PBS for 5 minutes. 0.5 ml of a 1/10 (v/v) dilution in PBS of the primary antibody, isolated from 3C2 cells, was added. The cells were then washed 3 times for 5 minutes in PBS. Next, the cells were incubated with the secondary antibody, 50 µl of biotinylated antibody, and 150 µl of horse serum in 10 ml of PBS, for 30 minutes. Again the cells were washed three times with 0.5 ml PBS for 5 minutes, and then they were incubated with 0.5 ml of elite ABC reagent for a further 30 minutes. This consisted of 100 µl of reagent A and 100 µl of reagent B added to 5 ml of PBS and immediately mixed and left to stand for 30 minutes before adding it to the cells. The cells were then washed three times with 0.5 ml PBS for 5 minutes and then incubated with the colour reaction, DAB, for 2-7 minutes or until the colour was sufficiently intense. The DAB was made up of an equal volume of 0.02% (v/v) hydrogen peroxide (from a 30% stock) made up in double de-ionised water and 0.1% (w/v) DAB made up in 0.1 M Tris.Cl, pH 7.2. Once the colour was fully developed, the DAB was removed and the cells washed twice for 5 minutes in water. The cells were then stored in 50/50 (v/v) PBS glycerol.

e) Alcian blue staining of micromasses:

Cells in micromass culture were stained for cartilage with Alcian blue. The cells were fixed overnight in 4% paraformaldehyde at 4°C. The paraformaldehyde was then removed and replaced with filter sterilised 1% alcian blue for 3 hours at room temperature, then the cells were rinsed with water for 1 minute. The water was removed and 50% (v/v) ethanol added for 5 minutes, followed by 70% ethanol for 5 minutes, 90% ethanol for 5 minutes and finally absolute ethanol for 5 minutes. The last ethanol wash was then removed and the micromasses stored under 100% glycerol.

f) Cartilage staining of embryos:

10 day embryos were cleared and stained for cartilage using Alcian green. The embryos, when harvested, were removed from the egg into PBS (pH 7.5), where the head was removed and also the viscera. They were then fixed in 5% (w/v) TCA (trichloroacetic acid) overnight. The next day the TCA was removed and the embryos were rinsed in water, then stained with 0.1% (w/v) alcian green in acid alcohol ((v/v) 70% ethanol, 1% HCl) for 6 hours or overnight. The embryos were again rinsed in water, then washed with acid alcohol for 4 hours, then washed for a minimum of 2 hours in absolute ethanol. Finally the embryos were cleared and stored in methyl salicylate.

g) X-Gal staining for pCH1101 transfected cells:

The transfected limb mesenchyme cells were fixed for 15 minutes in 0.5 % glutaraldehyde in PBS, plus 2 mM MgCl₂ and 1.25 mM EGTA, and then washed several times in PBS. They were then incubated for 30 minutes to overnight in X-gal solution (1 mg/ml X-Gal, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆.3H₂O, 2 mM MgCl₂, 0.01% Na desoxycholate, 0.02% NP40 in PBS), then washed briefly in water, and stored under glycerol.

h) Photography:

Photographs of manipulated embryos were taken on either a Stemi-SV-6 Zeiss (4, Bennett Court, Bennett Road, Reading, Berks., U.K.) microscope, with a Yashica 108 multi program camera or on a Zeiss 4750 52-9901 dissecting microscope with an Olympus camera, using 64-T Ektachrome colour slide film. Cells were photographed on a Zeiss Axiovert 405M microscope with an in-built camera, using 64-T Ektachrome colour slide film.

CHAPTER 3

Isolation and Characterisation of a Chicken *Bmp-2* Genomic Clone

3.1 Introduction:

One of the aims of the work described in this thesis was to examine how expression of the *Bmp-2* gene is controlled and regulated. To do this I isolated a chicken *Bmp-2* genomic clone. Mapping and sequencing of this clone would then allow analysis of the promoter region of the gene. Studying the promoter region could permit identification of the regulatory regions responsible for the spatial and temporal regulation of the *Bmp-2* gene.

In this chapter I shall describe the isolation and characterisation of #16F, a chicken *Bmp-2* genomic clone.

3.2 Results:

a) Analysis of the Number of Copies of the *Bmp-2* Gene in the Chick Genome:

A Southern blot of chicken genomic DNA, digested with a range of restriction enzymes, was hybridised with a chicken *Bmp-2* cDNA probe, previously isolated in our laboratory (Francis *et al*, 1994). The probe used was a 929 bp *Eco*RI fragment of the cDNA clone p5.1. This probe corresponds to part of the coding region of the chicken *Bmp-2* cDNA. (Figure 3.1.)

The Southern blot (Figure 3.2a and b) was hybridised at 65°C and washed at 50°C twice in (2 x SSC + 0.5% SDS) for 30 minutes, followed by another two 15 minute washes in (2 x SSC + 0.1% SDS).

As shown in Figure 3.2b, the chicken *Bmp-2* cDNA probe hybridised to genomic fragments of the following approximate sizes:

<i>Hind</i> III	9.4 kb
<i>Eco</i> RI	1.0 kb
<i>Bam</i> HI	12.1 kb
<i>Pst</i> I	4.5 kb and 2.0 kb
<i>Pvu</i> II	2.5 kb

Since most of these digests contain only a single hybridising fragment, these data indicate that there is a single *Bmp-2* gene in the chicken genome.

b) Isolation of a Chicken *Bmp-2* Genomic Clone:

To isolate a genomic clone containing the chicken *Bmp-2* gene, a chicken genomic library was screened with the same chicken *Bmp-2* cDNA probe, using the same hybridisation and washing conditions as for the genomic DNA Southern blot described above.

The library, supplied by Stratagene, contained fragments of genomic DNA isolated from an eight month Cornish White Rock cockerel. This DNA had been partially digested with *Mbo*I and cloned into the *Bam*HI site of the pWE15 cosmid vector (Figure 3.3).

The library was titered and 300 000 colonies were plated out on to each of two 20 x 20 cm plates and screened. Given that the size of the haploid chicken genome is approximately 1.2×10^9 bp (1.1-1.4 pg of DNA), a single copy gene should be represented once, at 95% probability, in every 1×10^5 colonies of this library, using the Clarke and Carbon equation (Brown, 1990). This number of colonies should therefore contain representatives of the whole genome on each plate (Olofsson and Bernardi, 1983).

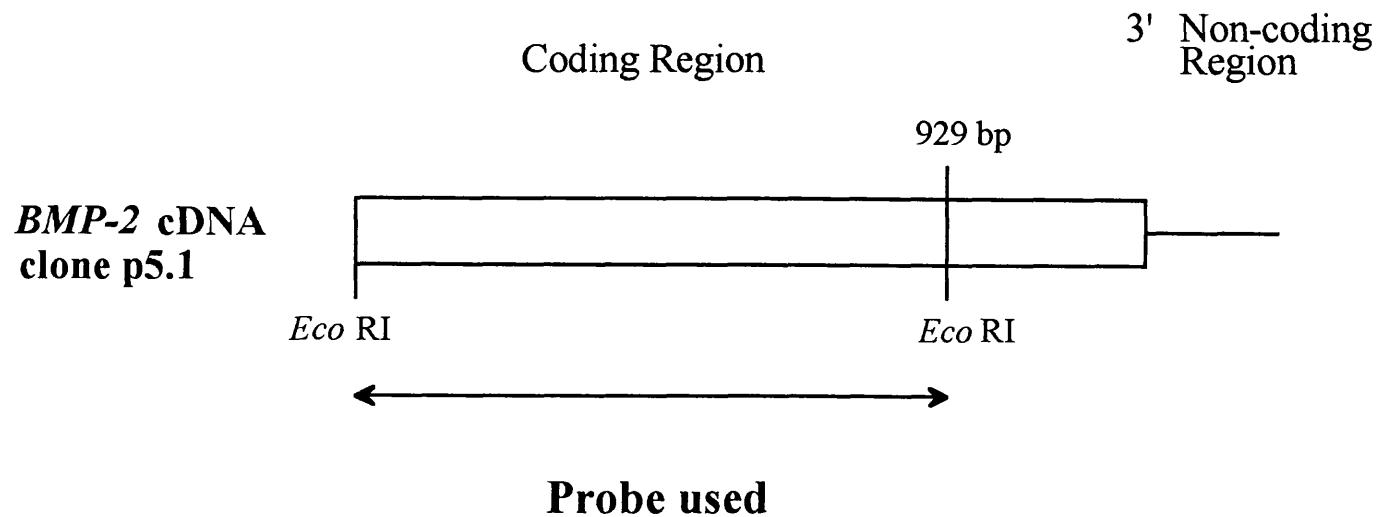


Figure 3.1: *Bmp-2* cDNA probe, used for Southern Blot hybridisation and Library Screening.

The cDNA clone p5.1 lacks the 5' non-coding region and the 5' end of the coding region of the chicken *Bmp-2* mRNA (Francis *et al*, 1994).

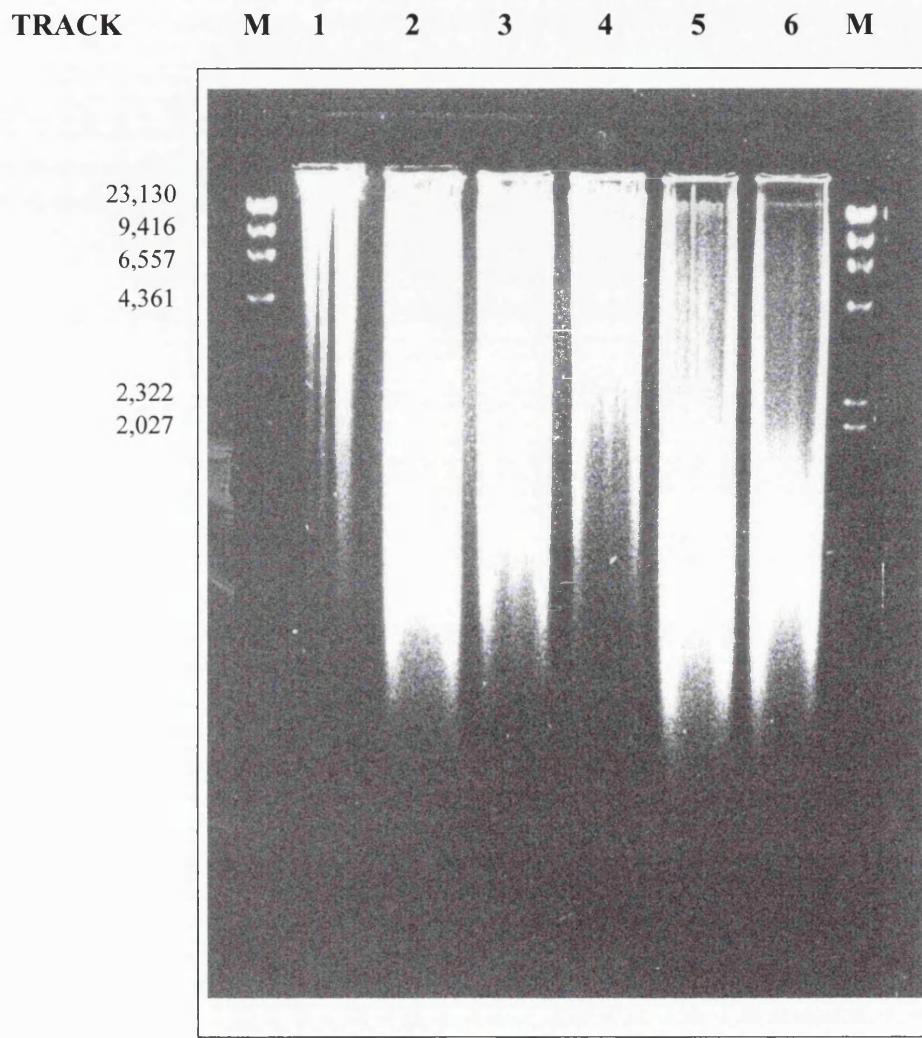


Figure 3.2: Southern Hybridisation Analysis of Chicken Genomic DNA.

a) Gel containing chicken genomic DNA. Track (1) contains undigested DNA. Other tracks contain DNA digested with: (2) *HindIII*, (3) *EcoRI*, (4) *BamHI*, (5) *PstI* and (6) *Pvull*. Track (M) contains *HindIII*-cut Lambda DNA markers (sizes indicated in basepairs). This gel was blotted on to nylon membrane and hybridised with the chicken *Bmp-2* cDNA probe shown in Figure 3.1. The blot was a gift from Dr. David Darling in our laboratory.

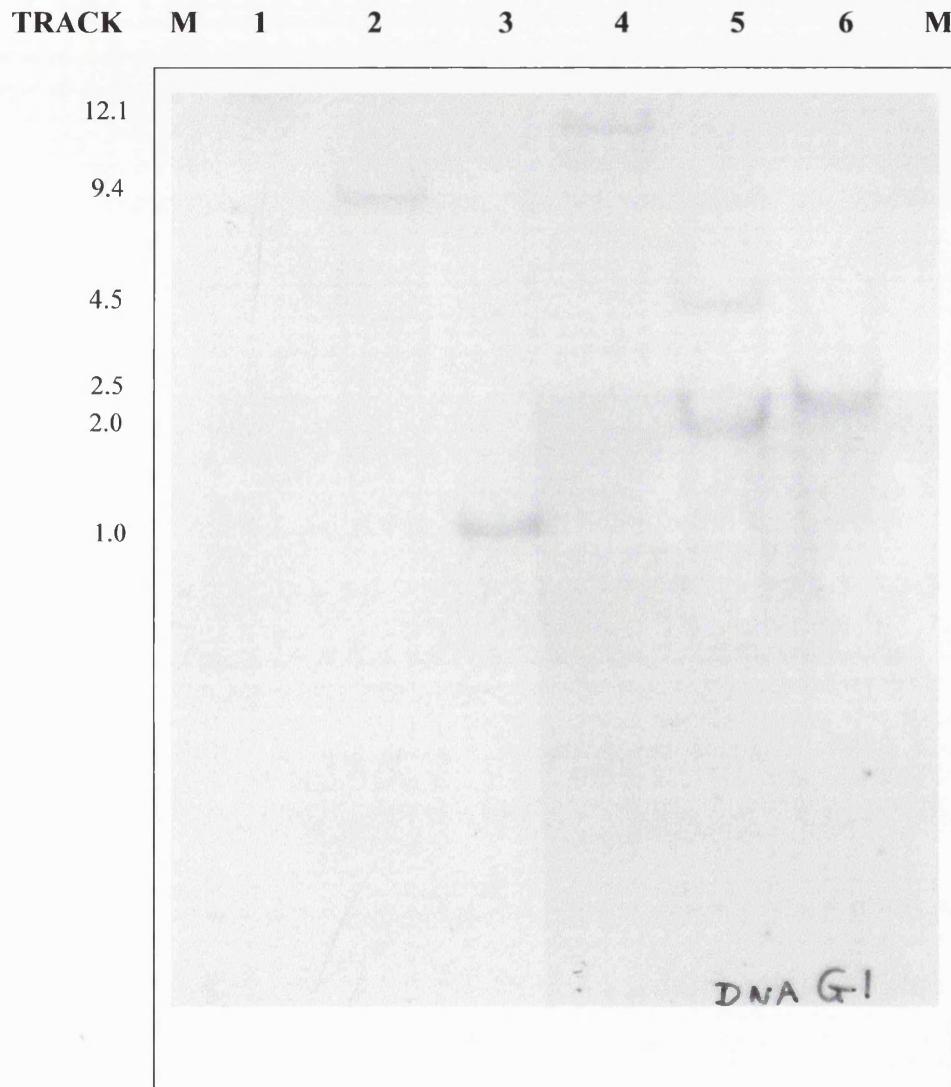


Figure 3.2: Southern Hybridisation Analysis of Chicken Genomic DNA.

b) Autoradiograph of a Southern blot of the gel shown in a) hybridised with the chicken *Bmp-2* cDNA probe. Approximate sizes of hybridising fragments are indicated in kilobases. Track (1) contains undigested DNA. Other tracks contain DNA digested with: (2) *Hind*III, (3) *Eco*RI, (4) *Bam*HI, (5) *Pst*I and (6) *Pvu*II. Track (M) contains *Hind*III-cut Lambda DNA markers (sizes indicated in basepairs).

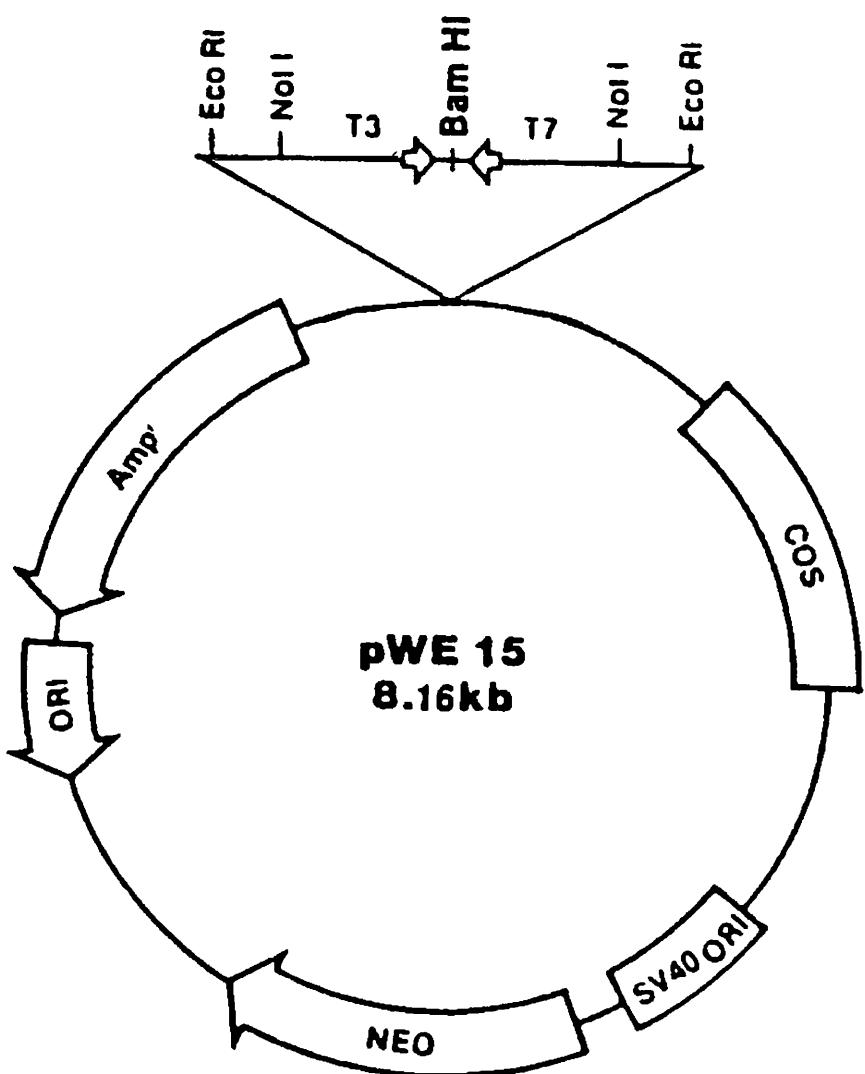


Figure 3.3: Map of pWE 15 Cosmid Vector (Stratagene)

Thirty-two clones hybridised to the probe. This is more than would be expected of a single copy gene. This probably reflects the fact that certain genes are over-represented in this particular genomic library, rather than indicating that the probe was hybridising non-specifically, because the same effect was seen when the library was screened for various other genes in the laboratory, all of which were thought to be present as a single copy in the genome, such as the RXR γ gene (E. Seleiro, personal communication).

A number of strongly hybridising clones were picked but I concentrated on one clone, #16. The others were stored at -70°C. #16 was picked, grown up and put through two rounds of further screening, until all the colonies on the filters hybridised with the probe, indicating that all the colonies derived from a single positive colony. One of these colonies (#16F) was then picked, grown up, and DNA was isolated from it and restriction mapped. Examples of primary, secondary and tertiary screens are shown in Figure 3.4a, b and c.

c) **Confirmation that the Cosmid Clone #16F Contains the *Bmp-2* Gene:**

To confirm that the cosmid clone #16F contained the chicken *Bmp-2* gene, DNA from the cosmid clone was digested with the same enzymes used in the total genomic Southern blot shown in Figure 3.2. Hybridisation with the chicken *Bmp-2* cDNA probe showed that the same bands were present in the genomic clone as in the total genomic Southern blot. (Figure 3.5.) One exception to this was the presence of an additional *Eco*RI band, of 13.8 kb. However, since this band gives only a weak signal with the cDNA probe, it is possible that this signal was not detected on the total genomic DNA Southern blot because of the low intensity. There was also an additional band present in the *Bam*HI lane: this was later found to be a result of partial digestion of the cosmid DNA. This is therefore further proof that there is only a single copy of the *Bmp-2* gene present in the chicken genome, and that the genomic clone #16F contains this gene.

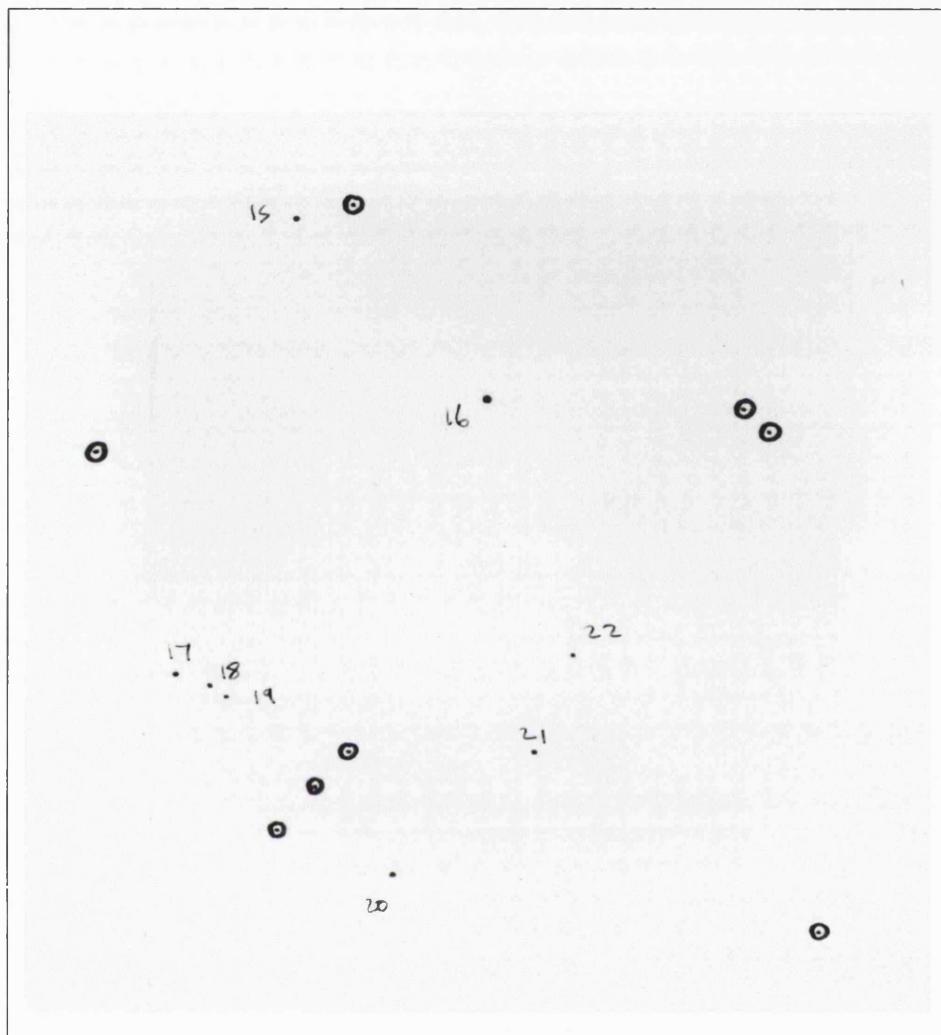
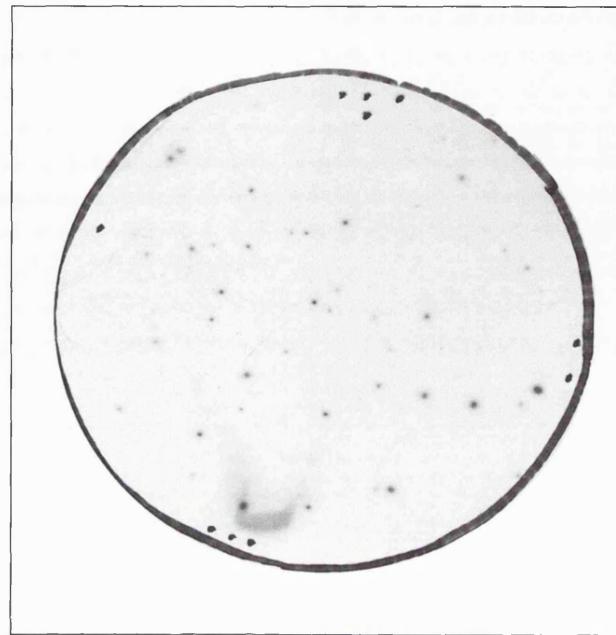


Figure 3.4: Results of Genomic Library Screening.

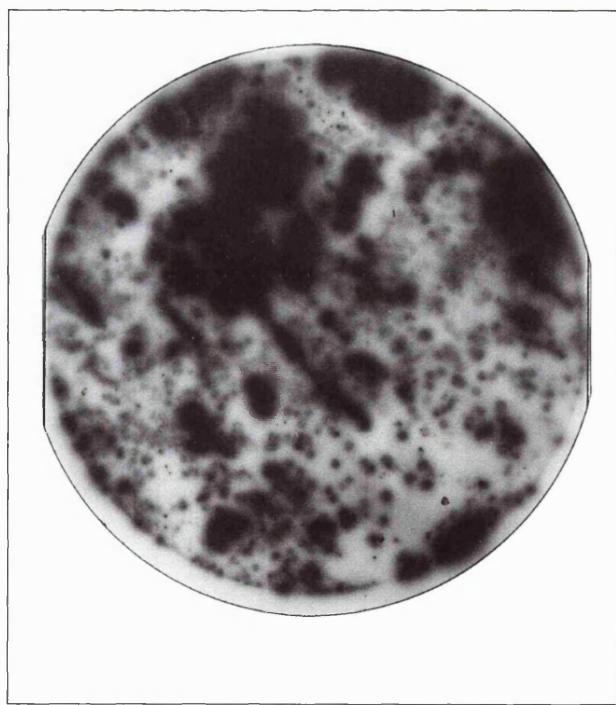
The autoradiographs show the signals from colonies that hybridised to the chicken *Bmp-2* cDNA probe shown in Figure 3.1.

- a) Primary Screen: A total of thirty-two duplicate signals were found on two plates. #16 was picked and screened further.
- b) Secondary Screen.
- c) Tertiary Screen: Clone #16F was picked and analysed.

b)



c)



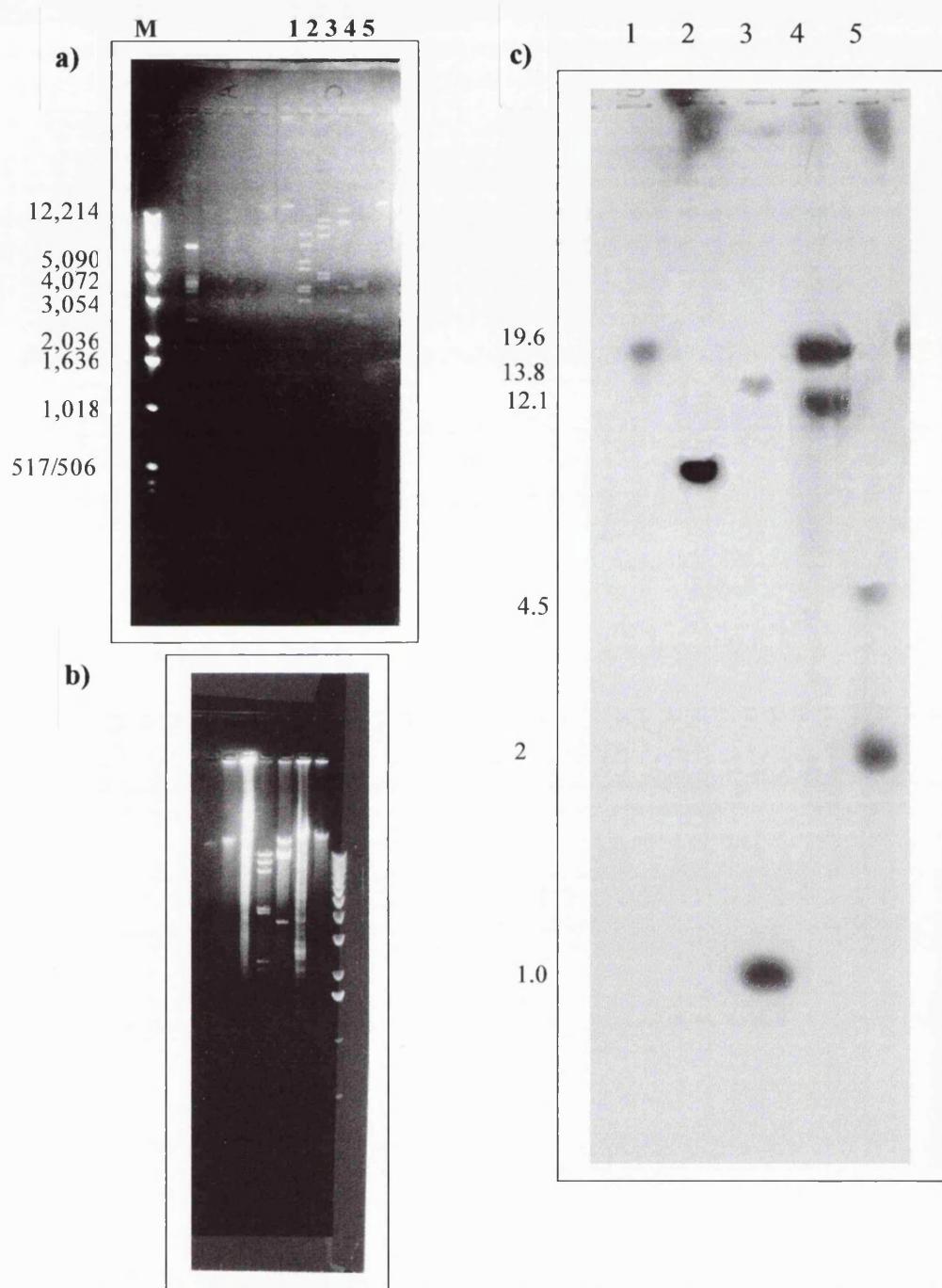


Figure 3.5: A Southern blot of cosmid #16F DNA, digested with the enzymes used in the analysis of chicken genomic DNA.

a) and b) Gel containing cosmid #16F DNA. Track (1) contains undigested DNA. Other tracks contain DNA digested with: (2) *Hind*III, (3) *Eco*RI, (4) *Bam*HI, and (5) *Pst*I. Track (M) contains 1 kb Ladder Markers (sizes indicated in kilobases).

c) Autoradiograph of a Southern blot of the gel shown in a) hybridised with the *Bmp-2* cDNA probe, (shown in Figure 3.1). Approximate sizes of hybridising fragments are indicated in kilobases. The majority of hybridising fragments from the cosmid #16F correspond in size to those in the Southern blot of chicken genomic DNA, shown in Figure 3.2 (b).

d) Mapping of Cosmid Clone #16F:

i) Results of Restriction digests and Southern hybridisation:

DNA from the cosmid clone #16F was digested with a range of restriction enzymes, both singly and in combination, and the digests were run out on 30 x 30 cm agarose gels. A photograph of a representative gel is shown in Figure 3.6a.

The table shown in Appendix A summarises the approximate fragment sizes, in base pairs, worked out for all the restriction enzymes used, and the averages for the total insert size for each digest.

From the averages shown in Appendix A, the approximate size of the insert of #16F was calculated as 41.5 kb.

In order to map the location of the *Bmp-2* gene within this cosmid insert, Southern blots were made of each gel and hybridised with various probes. One example of an autoradiograph of a Southern blot is shown in Figure 3.6b.

The probes used included the chicken *Bmp-2* cDNA (Figure 3.1), the full-length human *Bmp-2* cDNA, and various oligonucleotides that were designed both from the sequence of the chick cDNA and later from sequence of the cosmid insert itself. (Figure 3.7.)

In addition, the cosmid vector pWE15 has the bacteriophage T3 and T7 primers flanking the cloning site (Figure 3.3). By hybridising blots of #16F with the T3 and T7 primers, it was possible to map the fragments at the ends of the insert.

The hybridisation and washing conditions used for the large probes, such as the cDNA probe, were the same as described for the genomic Southern blot described in Section 3.2.a, but substantial optimisation of hybridisation and washing conditions was required before successful hybridisation with the short oligonucleotide probes was achieved.

a)

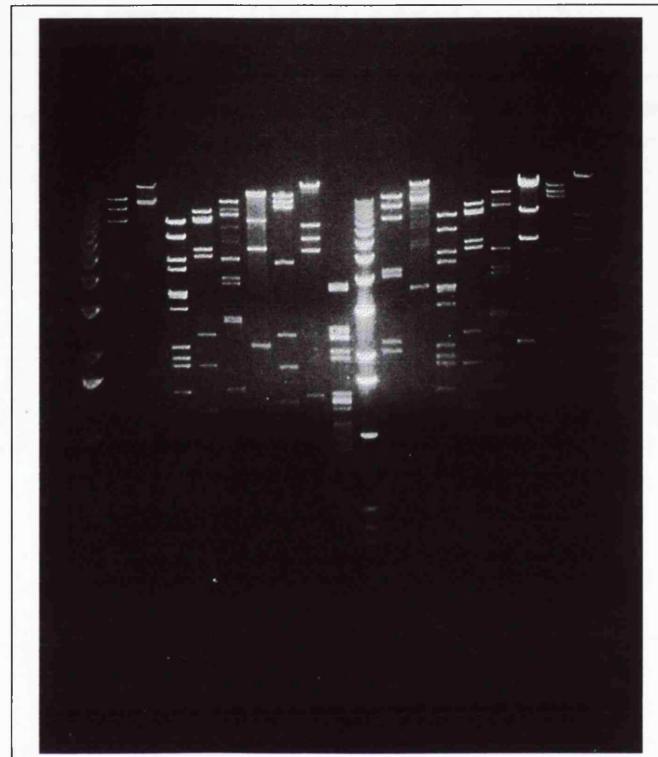


Figure 3.6 : Examples of restriction digests and Southern blots of cosmid clone, #16F

- a) Photograph of gel with DNA from cosmid #16F, digested with various restriction enzymes.
- b) Autoradiograph of Southern blot of the gel shown in a), hybridised with the *Bmp-2* cDNA probe.

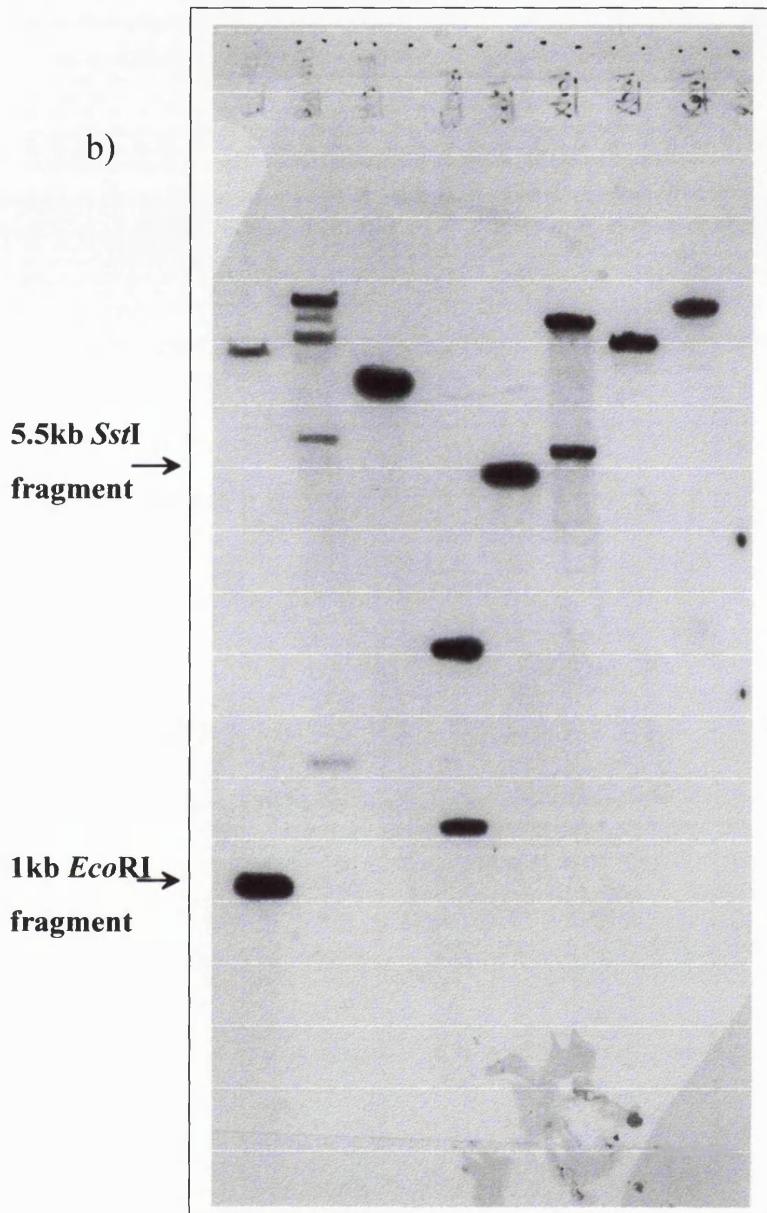
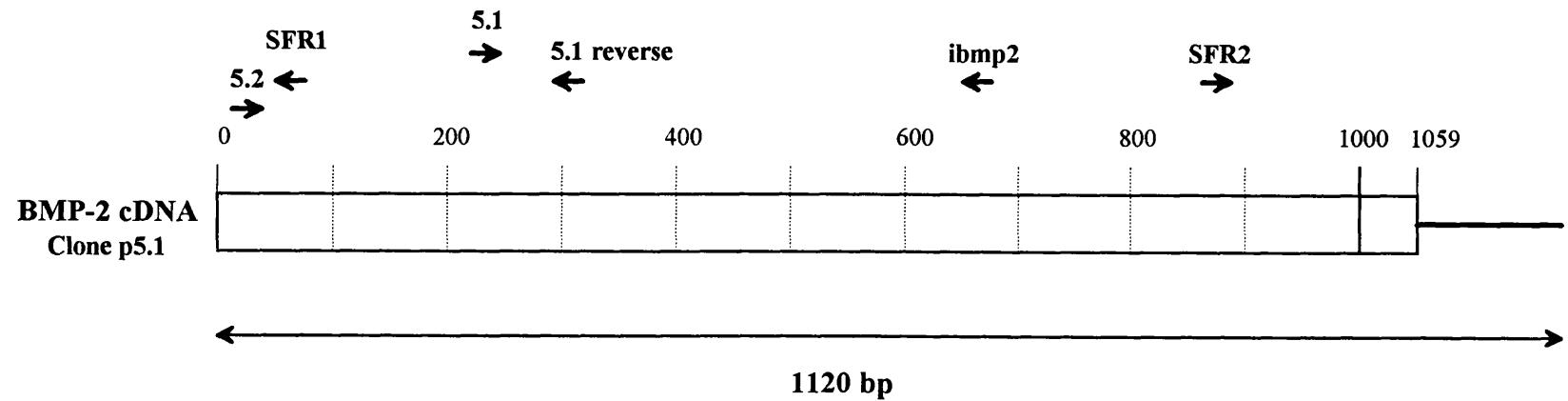


Figure 3.6 : Examples of restriction digests and Southern blots of cosmid clone, #16F

- Photograph of gel with DNA from cosmid #16F, digested with various restriction enzymes.
- Autoradiograph of Southern blot of the gel shown in a), hybridised with the *Bmp-2* cDNA probe.



5.2:	5' AGGACCTCCTGGGC 3'	20-33bp	5.1 reverse:	5' CTGAGGTGACAGACTC 3'	310-295 bp
SFR1:	5' TCAGCCCGAACATGTGG 3'	70-54bp	ibmp2:	5' CAACGGATGCCTTTGCAAC 3'	774-755 bp
5.1:	5' CTGCCAGAAACAAGTG 3'	229-244 bp	SFR2:	5' TTCCGCTGGCAGATCACC 3'	863-880 bp

Figure 3.7: Sites and sequences of oligonucleotides used as hybridisation and/or sequencing primers.

The results of the Southern hybridisations performed with these probes are also summarised in Appendix A. Analysis of these restriction digestion and Southern hybridisation data, along with nucleotide sequencing of selected regions of the insert (see below), gave rise to the map shown in Figure 3.8. The following sections illustrate how this map was derived from the data.

ii) Mapping the T7 end of the insert:

Hybridisation with the T7 primer permitted the mapping of fragments at the T7 end of the insert.

In cases where the cosmid vector does not contain sites for the restriction enzyme used, it was possible to identify which fragment corresponded to the uncut vector and flanking insert DNA. For example, the T7 primer hybridises to a *Bam*HI fragment of 12.1 kb. Given the size of this fragment and the fact that there are no *Bam*HI sites in the vector, this fragment must therefore contain the entire vector as well as some flanking DNA from the insert on both sides. However, it was not possible to determine how long the flanking DNA from the insert was on either side, without doing further digestions, such as double digests.

In other cases, it was possible to determine the length of insert DNA flanking the vector ends, particularly at the T7 primer end. For example, the T7 primer hybridises to:

- an *Eco*RI fragment of 4.5 kb:

there is an *Eco*RI site 32 bp from the T7 end of the vector,
therefore there must be an *Eco*RI site in the insert approx. 4.5 kb
from the T7 end of the vector.

- an *Hind*III fragment of 2.3 kb:

there is an *Hind*III site 64 bp from the T7 end of the vector,
therefore there must be an *Hind*III site in the insert approx. 2.3 kb
from the T7 end of the vector.

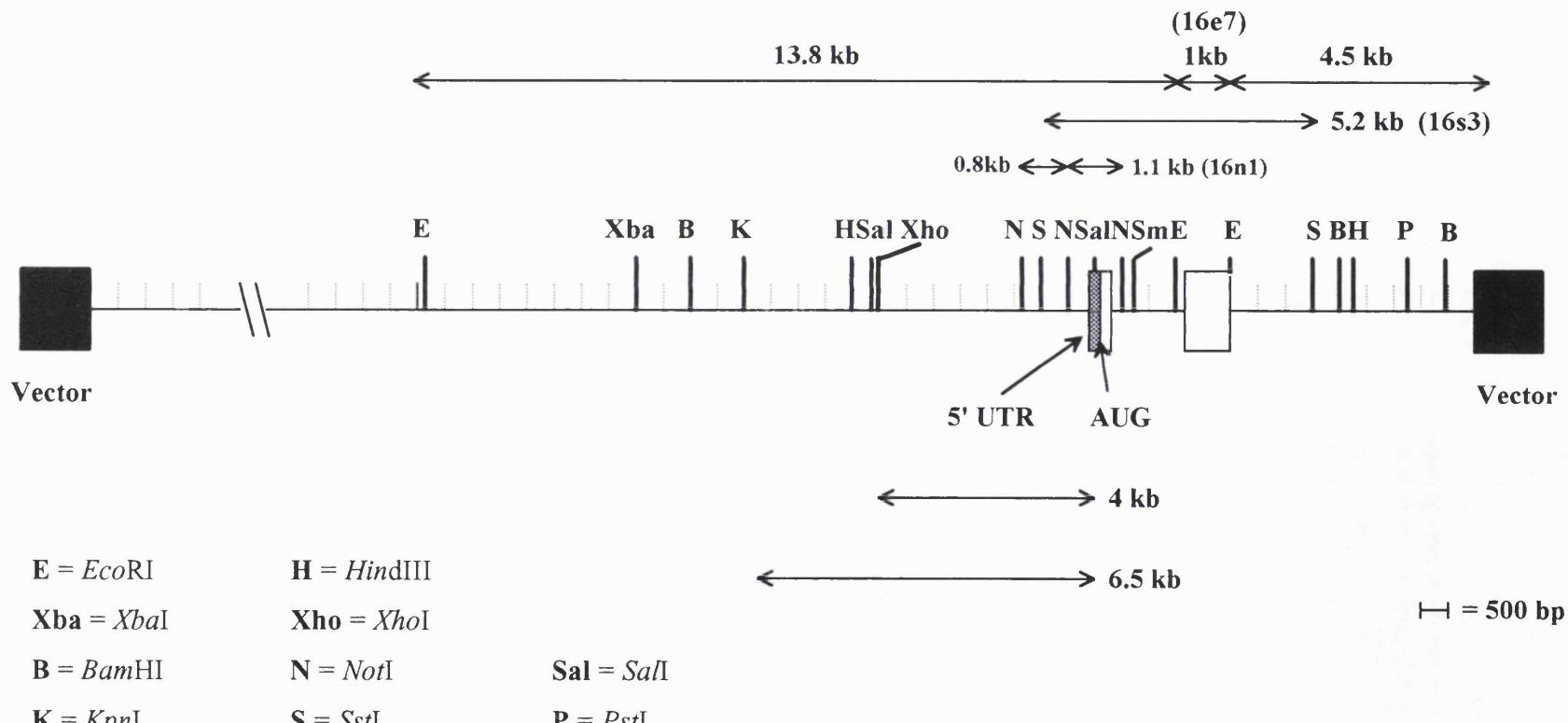


Figure 3.8: Map of cosmid #16F.

(The 5' UTR, represented by the grey box, is shown larger than its actual relative size, for clarity. The exons are represented by the white boxes.)

- a *Pst*I fragment of 4.6 kb:

there is a *Pst*I site 3.3 kb from the T7 end of the vector,
therefore there must be a *Pst*I site in the insert approx. 1.3 kb
from the T7 end of the vector.

- a *Sma*I fragment of 11.3 kb:

there is a *Sma*I site 4.9 kb from the T7 end of the vector,
therefore there must be a *Sma*I site in the insert approx. 6.4 kb
from the T7 end of the vector.

- a *Not*I fragment of 7.4 kb:

there is a *Not*I site 25 bp from the T7 end of the vector,
therefore there must be a *Not*I site in the insert approx. 7.4 kb
from the T7 end of the vector.

- a *Sal*I fragment of 13.5 kb:

there is a *Sal*I site 2.4 kb from the T7 end of the vector,
therefore there must be a *Sal*I site in the insert approx. 11.1 kb
from the T7 end of the vector.

From this it was possible to construct a map of this end of the cosmid insert.

(Figure 3.9)

iii) Mapping the insert using partial digestion:

The pWE 15 vector has been constructed in such a way to facilitate mapping of the insert. It has *Not*I sites flanking the polylinker so that most genomic inserts, though not, as it turns out, this one, can be excised as a single large restriction fragment. Once the insert has been excised with *Not*I, partial and complete digests can be done with other enzymes and then hybridised to labelled T3 and T7 primers. This allows faster mapping of the insert than can be done with single and partial digests alone. (Figure 3.10)

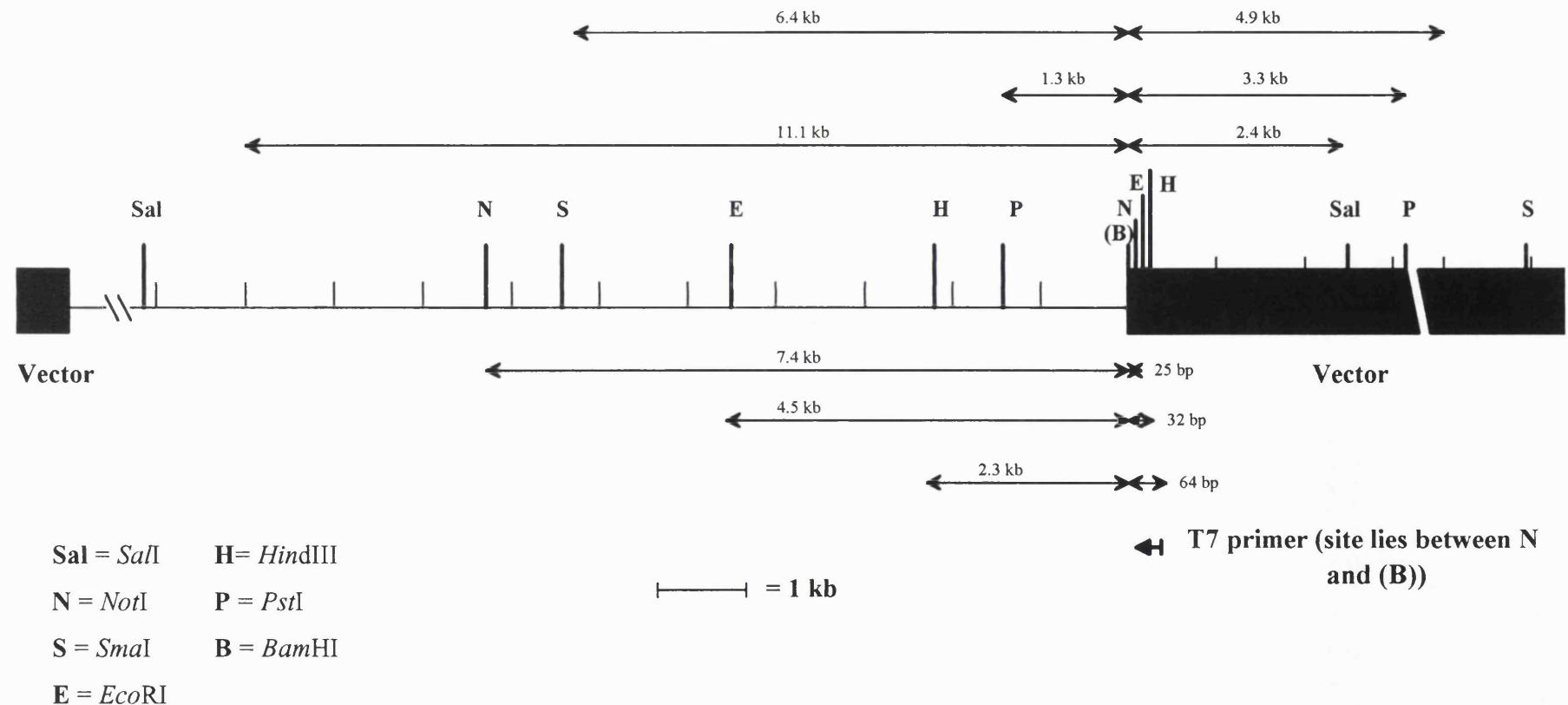
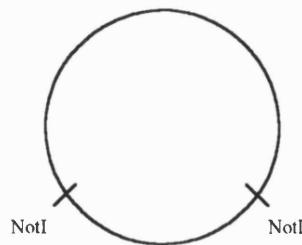


Figure 3.9: Map of T7 end of cosmid #16F.



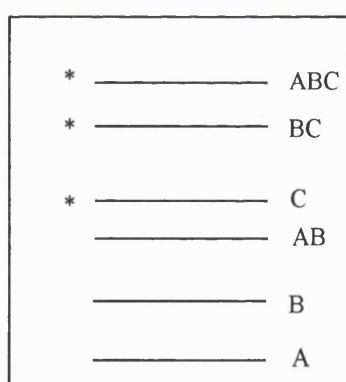
Cosmid is digested with NotI to excise the insert.



Cosmid is then digested with other enzymes, under conditions giving partial digestion.



partial digestion



Southern blots of this gel are then hybridised with the T3 & T7 primers, and compared to the hybridisation results given by fully digested DNA.

e.g. fragment C, BC & ABC will hybridise to the T7 primer (*), and fragments A, AB & ABC will hybridise to the T3 primer.

Figure 3.10: Strategy for mapping the cosmid insert using *NotI* digestion followed by partial digestion and hybridisation with T3 or T7 primer.

The digestion conditions for generating partial digests of the cosmid clone were optimised and it was found that it was necessary to perform digestions on ice at 4°C for 10-30 minutes.

Initial mapping of the cosmid with *NotI* revealed that there were 4 *NotI* sites in the insert. This meant that the strategy for mapping the insert was more complicated than anticipated, but partial digests of the cosmid clone with *NotI* enabled me to construct a *NotI* map of the whole of the cosmid insert (Figure 3.11). 3 of the 4 *NotI* sites mapped to within 2 kb of one another.

iv) Mapping of *Bmp-2* cDNA sequences:

Using the *Bmp-2* cDNA probe and the oligonucleotide primers designed from the sequence of the chick cDNA (Figure 3.7), I mapped the position of the coding region of the *Bmp-2* gene in the cosmid insert.

In *EcoRI* digests, all the primers from the chick *Bmp-2* cDNA sequence hybridised to a single 1 kb *EcoRI* fragment (Appendix A and Figure 3.6). The cDNA probe hybridised strongly to both this fragment and a 13.8 kb *EcoRI* fragment. In *SstI* digests, all the probes and primers hybridised to a 5.2 kb *SstI* fragment. The cluster of *NotI* fragments also mapped within this region, and the cDNA probe hybridised to a 1.1 kb *NotI* fragment.

These three fragments were subcloned into the Bluescript vector (Stratagene). Clone 16s3 contains the 5.2 kb *SstI* fragment, clone 16e7 contains the 1 kb *EcoRI* fragment, and clone 16n1 contains the 1.1 kb *NotI* fragment.

By carrying out restriction digests of these clones, and hybridising Southern blots of the electrophoresed fragments, using cloned fragments of the cosmid as probes, it was ascertained that the 5.2 kb *SstI* fragment contained both the 1 kb *EcoRI* fragment and the 1.1 kb *NotI* fragment. The clone, 16s3, was also used to further subclone a smaller *NotI-EcoRI* fragment of 1.2 kb, situated between 16n1 and 16e7, called 16en1.

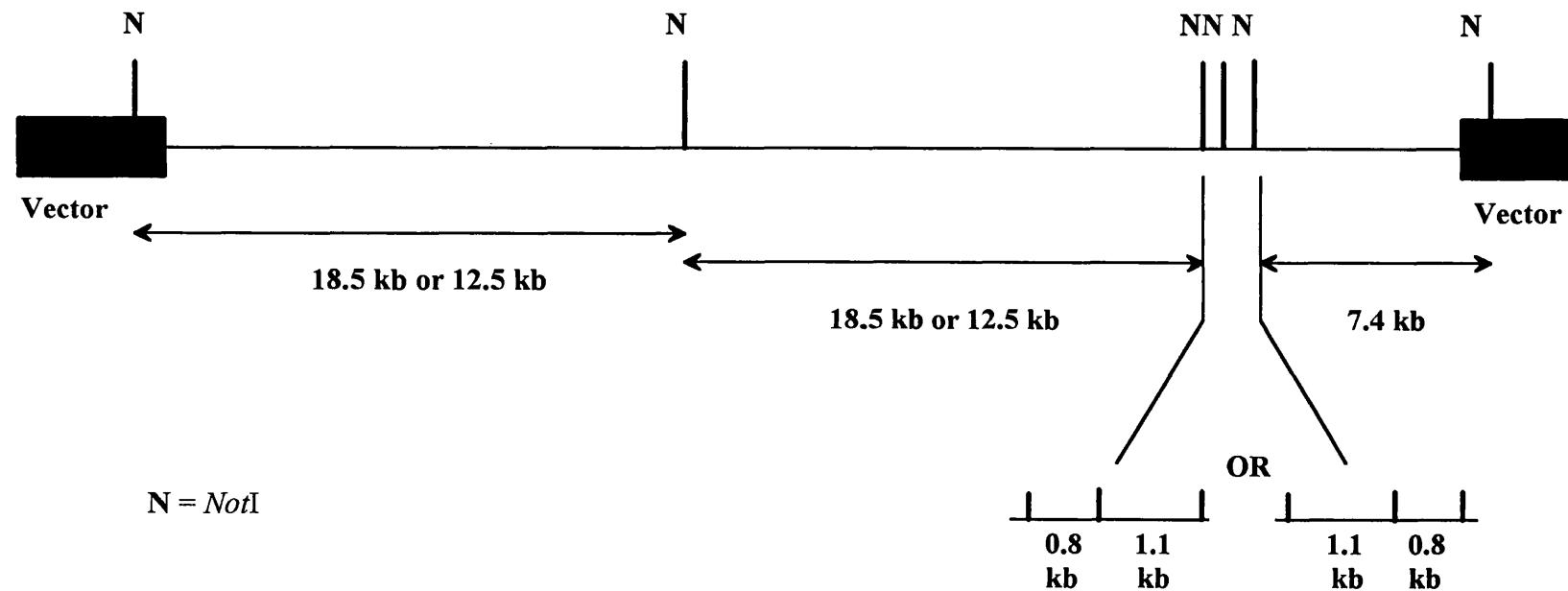


Figure 3.11: Map of cosmid #16F generated using *NotI* digest data.

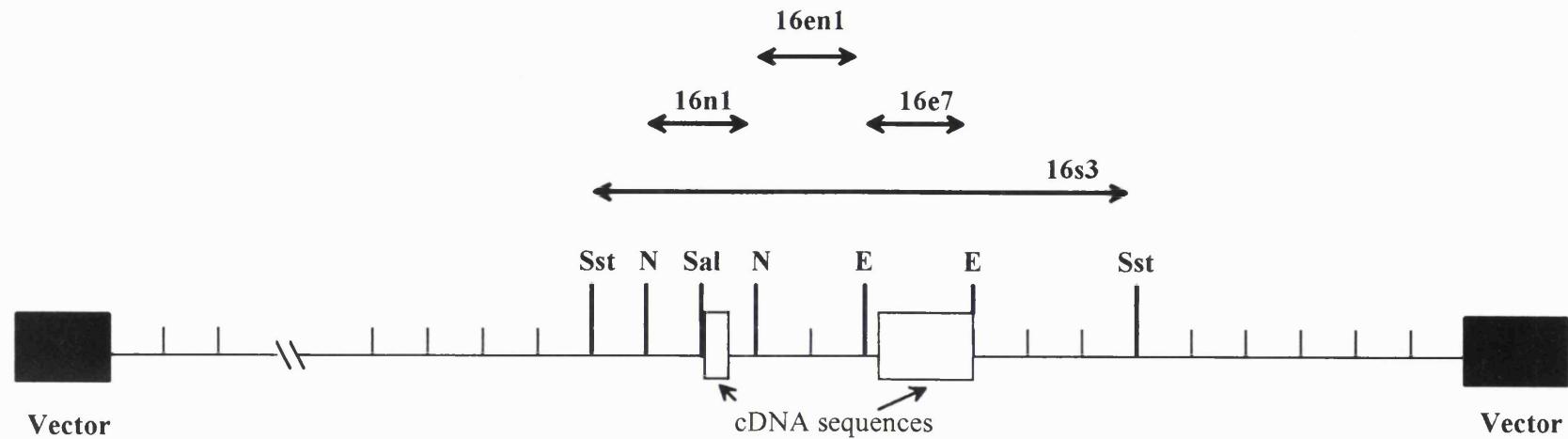
These 4 fragments were restriction mapped and/or sequenced to determine their positions relative to one another, and a map of the structure of the *Bmp-2* cDNA sequences present in this cosmid clone was constructed (Figure 3.12).

Further flanking enzyme sites were added to this map of the *Bmp-2* exons using data from hybridisation of double digests and partial digests with the *Bmp-2* cDNA probe. (Figure 3.13; see Appendix A for data.)

For example, in the *Eco*RI + *Kpn*I double digest, the fragments that hybridise to the cDNA probe are 1 kb and 9 kb. The 1 kb fragment corresponds to the 1 kb *Eco*RI fragment that was already subcloned as 16e7. Hybridisation of blots of single digests with these two enzymes gave a 13.8 kb fragment and a 1 kb fragment with *Eco*RI, and a 19.5 kb fragment with *Kpn*I, when probed with the cDNA probe. The 13.8 kb *Eco*RI fragment must lie upstream of the 1 kb *Eco*RI fragment, because the *Not*I restriction data does not provide enough room for the 13.8 kb *Eco*RI fragment to be downstream of the 1 kb fragment, given the position of the vector (Figure 3.11). From the double digest information, there must therefore be a *Kpn*I site approximately 9 kb upstream of the 1 kb *Eco*RI fragment.

Similarly, in the *Eco*RI + *Bam*HI double digest, the fragments that hybridise to the cDNA probe are a 1 kb fragment, which corresponds to the 1 kb fragment that was subcloned as 16e7, a 1.6 kb fragment, a 9.5 kb fragment, and a 5 kb partial digest band that was not visible on the gel. From this information, there must be two *Bam*HI sites upstream of the 1 kb *Eco*RI fragment, one giving the 9.5 kb fragment, and the other the 1.7 kb upstream fragment, although it was not clear which site was closer to the *Eco*RI fragment. From the partial restriction band, there must be another *Bam*HI site 4 kb downstream of this 1 kb fragment, since no combination of the upstream sites could give rise to a partial fragment of this size.

Similar analysis of other double digests resulted in the map shown in Figure 3.13. The locations and sizes of the fragments on this map were also checked by doing various other double digests, such as combinations of these enzymes with *Sst*I, or *SaII*. In particular, it was possible to use double digests with *SaII* and to hybridise to the 16n1



Sst = *SstI*

N = *NotI*

Sal = *SalI*

E = *EcoRI*

— = 500 bp

Figure 3.12: Map of cosmid clone, #16F, showing positions of 16e7, 16n1, 16s3 and 16en1.

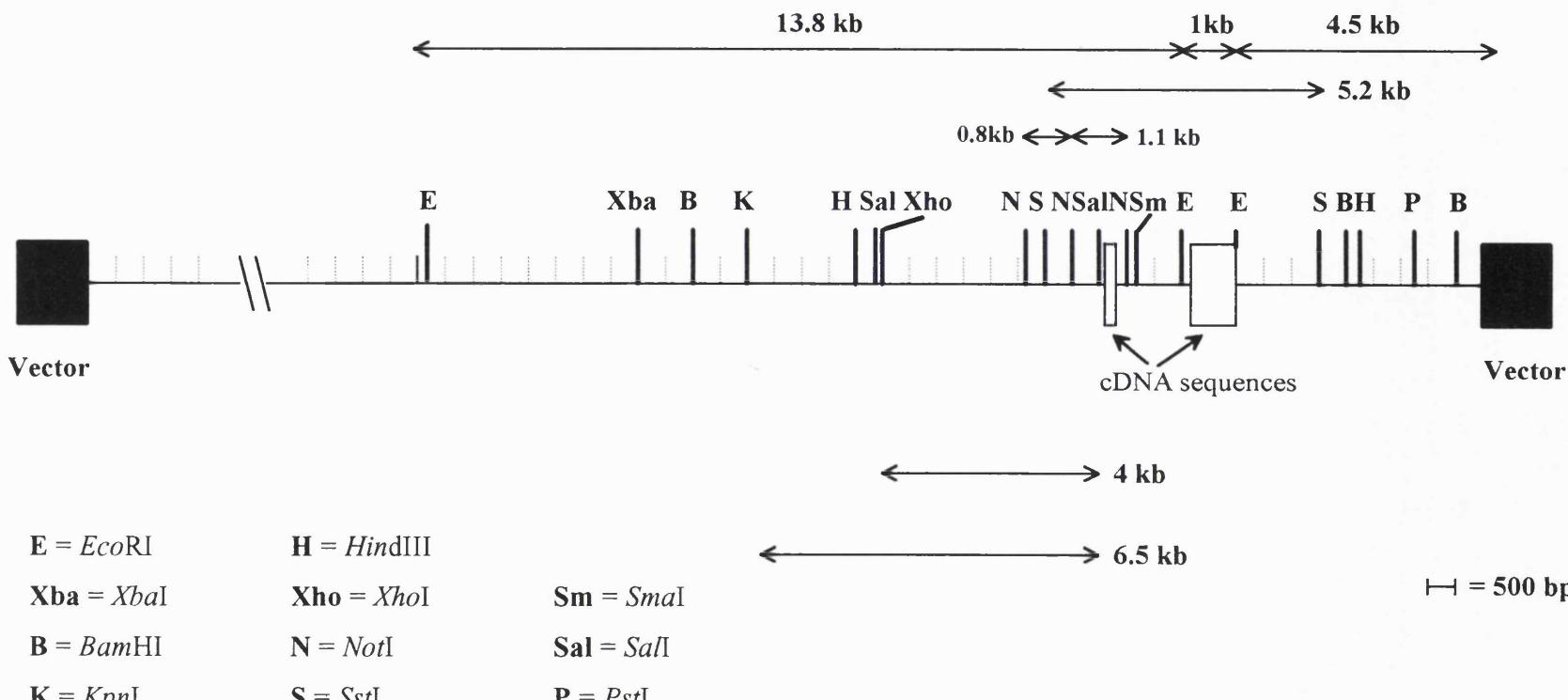


Figure 3.13: Restriction map of cosmid #16F.

insert probe, the 1.1 kb *NotI* fragment, to get more accurate sizes for these fragments, and these figures are used on the map shown in Figure 3.13.

This localisation of the region of the cosmid insert containing the *Bmp-2* gene relative to the ends of the cosmid vector was determined using the *NotI* and *EcoRI* full and partial digest data. In this way, the map of the T7 end of the vector (Figure 3.9) could be incorporated with the map of the *Bmp-2* encoding region to give the full map shown in Figure 3.13. From these data, the 1 kb *EcoRI* fragment containing the majority of the coding region of the *Bmp-2* gene is 4.5 kb away from the T7 end of the cosmid vector. These figures were verified using the data from *SstI* digests of the cosmid, both alone and in combination with *NotI* and *EcoRI*.

e) Sequence Analysis of the *Bmp-2* gene:

As noted above, the 1 kb *EcoRI* fragment (16e7) and the 1.1 kb *NotI* fragment (16n1) were partially sequenced, using the T3 and T7 primers in the flanking arms of the Bluescript vector, and the primers designed for sequencing the chick *Bmp-2* cDNA: 5.1, 5.2 and ibmp2 (Figure 3.7). Further primers, SFR1 and SFR2, were designed and synthesised during the course of the sequencing using sequence data from these clones and the *Bmp-2* cDNA clone, and the positions and sequences of these primers are also shown on Figure 3.7.

Sequencing of the 5' untranslated region, the 5' coding region and the introns proved difficult and optimisation of sequencing reaction conditions and the gel composition was required to generate clear sequence data and eliminate compressions. A number of approaches were tried, and the optimum protocol for sequencing DNA from this cosmid clone was found to involve using the routine Sequenase kit and method, but carrying out the termination reactions at 42°C and running the reactions out on sequencing gels containing 40% formamide.

The regions sequenced are shown in Figure 3.14 and the sequence is shown in Figure 3.15. As described below, these data show that the *Bmp-2* coding region lies in two exons separated by an intron of approximately 1.65 kb.

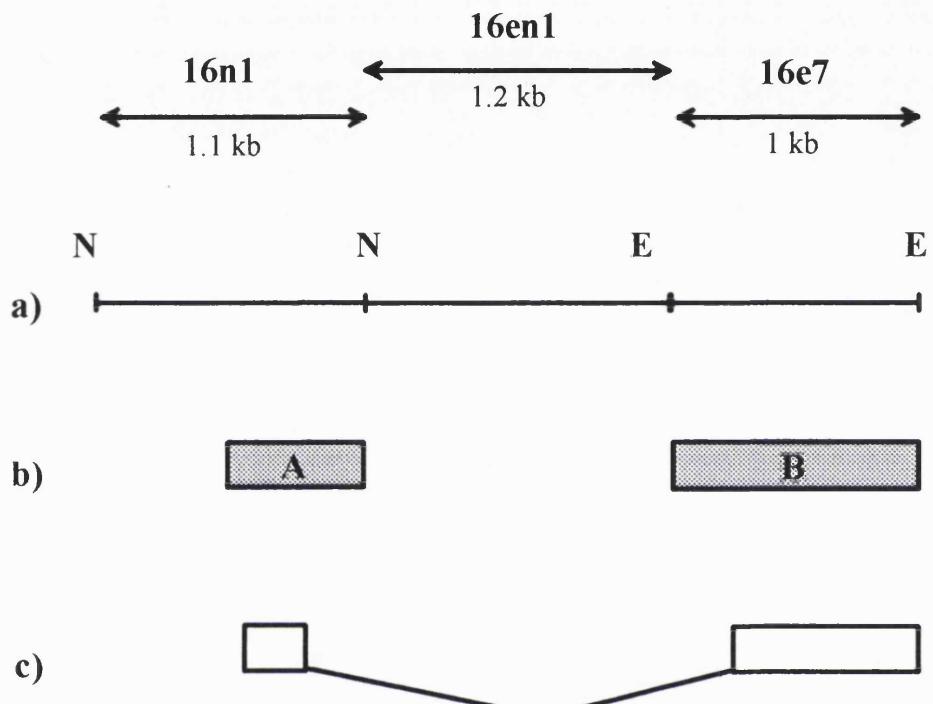


Figure 3.14: Sequence analysis of chicken *Bmp-2* genomic clone, #16F

- a) Portion of the genomic clone containing the cDNA sequences, from map in Figure 3.13.
- b) Regions sequenced, shown as boxes A and B. The sequences are shown in Figure 3.15.
- c) Intron/exon structure of this region of the chicken *Bmp-2* gene, as determined from sequence comparison of the genomic clone and the cDNA.

A)	TCCGTGC <u>A</u> GTCGACC <u>AT</u> GGTTGCNGNNACCGCTCCCTCCTGGNGNTGC TGCTCTGCCGGGTGCTGCTGGCGGNNGGTGGCCTCATGCCGGAGGTG GGACGGCGGNGCTTCAGCGNACCGGGNNNNNCTCGGCCNGCAGCGN NNNGAGGACCTCCTGGCGAGTTGAGCTGCCTGCTCCACATGTTCGG GCTGAAGCGGCAGGCCAGCCCCGGCAAGGACGTCGTATCCCCCTACA TGTTGGACCTCTATCGCCTGCACGCCGGCAGCAGCTGGCTACCCGCTG GAGAGGGCCGCCTGCCGCGCCAACACCGTGTGCAGCTCCACCACGAAG <u>G</u> <u>T</u> AGGGGCCGAGGGACGNNGGAGGGANNNNAGGAGNNNNNGGAGNNN NNNGAGGNNNNNGGNNNNNNNNAGGGAGGGCNGGAGGAGGGCG GCGGGGCGGCTCCNGNNNCGGAGGGGGTNCCGG	50 100 150 200 250 300 350 400 450 484
B)	TTCCATCGTATGCTTCATGTTCTCGGCACTCGTAATGACTACCTGGAAT GCAGAGAGCACCGGTGTTCCATCAAAGTGCATGACAATGAACGAAGCGTT CTTAACTTGGTGCTATACATGCTTGTAAATAAACAGAAGGATGAGGAG GCTGTGCTATGCCTAAGTCTTCAGGTCTTGTGCGATGCCAGCAGC ACGTATCTGAGGNGTTGGTTGCTTGTCTCAGTCCTAACTTTTTCT CTCTCCCTCTGCTCCNTGCCCNNNCTCTGTTAATCAAATT <u>AGAA</u> GTTTTGNNAAGAACTGCNNNAACAAGTGGAAAACAGCACGACGTTCTT CTTTAATTAACTTCATCCCTAATGAGGAGCTGTCACCTCAGCTGAAC TCCAGATTTTCCGGGGAGCAGGTGCACGAAGCCTTGAGAGCAACAGC AGCTACCATCACCGTATTAAATTATGAAATTATGAAGCCAGGCCACAGC CNCCTCCAAGGACCCGTACGAGACTTTGGACNCCAGGTTGGTNCTA CTAATGAACGTAAAATGGGAAGTTTGATGTAANNCCAGCTGTTGNNG TGGCTTGCACACAGGCAACCAATCCATGGTTGTGGAGGTGGTCA CTTGGACAAAGAGAACATGGCTCCAAGAGGCACGGTAGGATTAGCAGGT CTTACATCAGGATGAAGATAGCTGGTCTCAGCTCAGGCCGTTGTTAGTG ACGTTGGCATGATGGCAAGGGACACCCGCTCCACAAAGAGAAAGCG TCAAGCGAAACACAAACAGCGTAAGCGTCACAAATACAGTGAAAGGAC TTCCGTTGTATGGACTTCAATGACGTGGGGTGGAAATGACTGGATTGTT NNCCCGCCGGGTACAGTGCCTTTACTGCCATGGGAATGTCCTTCC GCTGGCAGATCACCTAAACTCAACAAACCATGCCATTGTTAGCTTGG TCAATTGGTGA	50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000 1013

Figure 3.15: Sequence of #16F.

- a) Sequence of Box A, shown in Figure 3.14, from 16n1.
- b) Sequence of Box B, shown in Figure 3.14, from 16e7.

N = A, C, T or G, base not resolvable from sequence data.

Putative ATG codon and splice acceptors and donor site shown in red and underlined.

f) Comparison of DNA sequences of #16F and *Bmp-2* and *Bmp-4* cDNA clones:

The cDNA clone of *Bmp-2* isolated by Francis *et al* (1994), p5.1, contains the 3' untranslated region of the gene, and most of the coding region, but lacks the 5' untranslated region and the 5' end of the coding region. Figure 3.16 shows an alignment of the sequence of p5.1 with that of #16F.

Except for a few bases, the two sequences are identical. These few differences may be due to sequencing errors or ambiguities. The region corresponding to the last 200 bp of the cDNA was not analysed in the genomic clone. This is the region downstream of the 1 kb *EcoRI* fragment (16e7), and it contains the 3' end of the coding region and 3' untranslated region of the gene.

From the map and sequence data, it is clear that there is an intron, flanked by consensus splice acceptor and donor sites, shown in red in Figures 3.15 and 3.16, 214 bp downstream from the 5' end of the cDNA clone. As shown in Figure 3.14, this intron comprises the 1.2 kb *NotI-EcoRI* fragment (16en1), approximately 130 bp of the 3' end of the *NotI* fragment (16n1) and approximately 300 bp of the 5' end of the *EcoRI* fragment (16e7).

The genomic sequence also contains 135 bp upstream of the 5' end of the p5.1 cDNA sequence. Part of this sequence was identified as the remaining *Bmp-2* coding region by the homology of its predicted amino-acid sequence with the mouse and human BMP-2 sequences (Figures 3.17 and 3.18). Part of this new sequence also overlapped the 5' end of the cDNA, p5.1, which was not homologous to the mouse and human *Bmp-2* sequences. This cDNA sequence is therefore likely to have arisen from a fault in the original cloning of the cDNA, incorporating sequence from elsewhere, and the genomic sequence represents the true chicken *Bmp-2* sequence.

This new 5' coding sequence compares well with the mouse and human *Bmp-2* sequences at the amino acid level over the residues that could be compared (Figure 3.18): it has 66 % sequence identity with the human sequence (12 differences out of 35 amino acids), and 60 % sequence identity with the mouse sequence (14 differences out of 35 amino acids) over the codons that could be compared. The mouse and human

16F-A	TCCGTGCAGGTCGACCATGGTTGCNGNNACCGCTCCCTCCTGGNGNTGCTGCTCTGCCGGTGC	119
cDNA	GGTCGCTGAAGCGCCCCGAGGACCTCTGGCGAGTTGAGCTGCGCCTGCTCACATGTTGGCTGAAGCGGCCGAGCCCCGGCAAGGACGTCGT	104
16F-A	NACCGGGNNNNNNNCTCGGCCNGCAGCGNNN-----	239
cDNA	CCCCCCCTACATGTTGGACCTCTATGCCTGCACGCCGGCAGCAGCTGGCTACCCGCTGGAGAGGGCGCCTGCCGCCAACACCGTGTGCAGCTCCACCA	214
16F-A	GAGGGGGCGAAG	359
16F-A	AGGGACGNNGGAGGGANNNNAGGAGNNNNNGGAGNNNNNNNGGAGNNNNNNNNNNNNNNNNNNAGGGAGGGCNGGAGGAGGGCGGGGGCGGCTCCGNGNNNCGGAGGGGT	479
16F-A	NCCGG	484
16F-B	TTCCATCGTGTGACTGTTCTCGGACTCGTAATGACTACCTGGAATGCAGAGAGCACCGGTGTTCCATCAAAGTGCA	120
16F-B	TGACATGACAAGCGACGAGGCTGTGCTATGCCAAAGTCTTCAGGTCTTGTGCATGCCAGCACGTATCTGAGGNGTTGGTTGCTTCAGTTCTAAC	240
cDNA	AAGTTTGGAAAGAACTGCCAGAACAAAGTGGAAAACAGCACGACGTTCTTAAATT	276
16F-B	TTTTTTCTCTCCCTCTGCTCCNTGCCCNNNCTCTGTTAATCAAATTAG-----NN-----NNNN-----N-----N-----	360
cDNA	ACTTCCATCCCTAATGAGGAGTCTGTCACCTCAGCTGA	396
16F-B	ACTCAGACTCCAGATTTCCGGGGAGCAGGTGCACGAAGCCTTGAGAGAACAGCAGCTACCATCACCGTATTAATATTTATGAA-----T-----	480
cDNA	ATTATGAAGCCAGCCACAGCCACCTCCAAGGACCTGTACGAGACTTTGGACACCAGGTTGGTCATCATAATGCAAGTAATGGAAAGTTCTGATGTAACGCCAGCTGTTGAGG	516
16F-B	-----N-----N-----N-C-AC-----A-C-----AT-----G-----T-----NN-----N-----N-----	600
cDNA	TGGATTGCACACGGACAACCTAACATGGTTGTGGAGGTGGTCACTGGACAAAGAGAACAGTGCCTCCAAGAGGCACGTTAGGATTAGCAGGTCTTACATCAGGATGAAGAT	636
16F-B	---C-----A-G-----A-T-----TG-----G-----	720
cDNA	AGCTGGTCTCAGCTCAGGCCGTTAGTGACGTTGGCATGATGGCAAGGGACACCCGCTCCACAAAAGAGAAAAGCGTCAAGCGAACACAAACAGCGTAAGGCCACAAATACAGT	756
16F-B	-----T-----	840
cDNA	TGCAAAAGGCATCCGTTATGTGGACTTCATGACGTGGGTGAATGACTGGATTGTTGCCCGCCGGGTACAGTGCCATTGCGCTGGCAGAT	876
16F-B	-----G-A-T-----NN-----	960
cDNA	CACCTAAACTCAACAAACCATGCCATTGTCAGACTTGGTCAATTGGTGAATTCCAAATCCCAAGGCTTGCTGTGCCGACAGAACTGAGTGCTATCTCAATGCTCACCTGAT	996
16F-B	-----	1013
cDNA	GAGAACGAAAAGGTCGTACTAAAGAACTATCAAGATATGGTTGGAGGGCTGCCGCTGACACCGCGAGTGTATCGGGACAAATGCAAAAGAAAAAGCTGACACTTAATATT	1116
cDNA	CCCG	1129

Figure 3.16: DNA sequence comparison of chicken *Bmp-2* genomic clone, #16F, with chicken *Bmp-2* cDNA, p5.1.

- - = base identical between sequences. 16F-A and 16F-B correspond to the sequences shown in Figure 3.15, and are numbered as in that figure.

TCCGTGCAGGTCGACCATGGTTGCNGNNACCGCTCCCTCCTGGNG 46
 M V A D/E/G T R S L L E/G
 V/A V/A

 NTGCTGCTCTGCCGGGTGCTGCTGGCGGNGNGTCGGCCTCATG 92
 L/M L L C R V L L G G E/G V G L M
 V V/A

 CGGGAGGTGGGACGGCGGNNGCTTCAGCGNACCGGGNNNNNCCT 138
 P E V G R R S/G F S E/G P G - - A
 C/R V/A

 CGGCCGNGCAGC [GCCCGAGGACCTCCTGGCGAGTCGAGCTGC 184
 S A S/G Q [R P E D L L G E F E L
 C/R

 GCCTGCTCACATGTTGGGCTGAAGCGGCGGCCGAGCCCCGGCA 229
 R L L H M F G L K R R P S P G

Figure 3.17: Translation of the 5' end of the chicken genomic clone of *Bmp-2*.

- = residues that cannot be accurately determined from the sequence data.

Where multiple residues are shown, these represent all possible translations of the bases sequenced.

Sequence in black after the brackets is present in the chicken cDNA clone, p5.1 (Francis *et al* 1994). The first four amino-acid residues of the cDNA sequence have been omitted because they do not align with the genomic sequence.

Chicken	MVA-TRSLL--LLCRVLLGG-VGLMPEVGRR-FS-
	* ** * * * *
Human	MVAGTRCLLALLLPQVLLGGAAGLVPELGRKFAA
	* * * *
Mouse	MVAGTRCLLVLLLQVLLGGAAGLIPELGRKKFAA
Chicken	PG--ASA-Q T RPEDLLGEFELRLLHMFGKRR

Human	ASSGRPSSQ PSDEVLFELRLLSMFGKQR
	* * *
Mouse	ASS-RPLSR PSEDVLFELRLLSMFGKQR

Figure 3.18: Comparison of the predicted amino acid sequences of the amino-termini of mouse (Dickinson *et al*, 1990), human (Wozney *et al*, 1988) pre-pro BMP-2. Sequence in black after the brackets is present in the chicken cDNA clone, p5.1 (Francis *et al*, 1994).

-- = residues that cannot be accurately determined from sequence data.

* = residues that differ between the genes

blue = residues that differ between the genes

sequences have 86 % sequence identity with one another over this region (6 differences out of 44 amino acids). 3 of the amino acids that differ between the chicken sequence and the other two sequences are residues that differ between the mouse and human sequences too. The other amino acid differences between the mouse and human sequences are at sites where the chicken genetic sequence could not be read sufficiently clearly to specify an amino-acid residue. Of these residues, however, from the sequence data available, only one could definitely be said to be different (amino acid 32 = C, R, S or G, not K), whereas the others could be the same as the residues in the mouse and human sequences..

It is to be expected that the chicken sequence should diverge more from its human and mouse counterparts, since the species are not as closely related, and, in general, homology between chick and mammalian sequences is less than between mammalian sequences. Also this region represents the pre-pro-peptide, whose sequence is less rigorously conserved than the mature region sequence. This divergence is also clearly shown in comparisons of the chicken *Bmp-2* sequence generated from the genomic clone with that of the chicken *Bmp-4* sequence (Figure 3.19).

The genomic sequence derived from #16F also contains 16 bp upstream of the putative translation initiation codon (Figure 3.16 and 3.17). There is a consensus splice acceptor site 7 bp upstream of the translation initiation codon, shown in red in Figures 3.16 and 3.17. This suggests that there is an intron here, 7 bp upstream of the ATG codon.

g) Comparison of #16F with Murine *Bmp-2* and *Bmp-4* Genomic Clones:

During the course of this work, a genomic clone of murine *Bmp-4* was described by Kurihara *et al* (1993), and a genomic clone of murine *Bmp-2* was described by Feng *et al* (1994). Özkaynak *et al* (1992) also reported the isolation of a human *Bmp-4* clone, but no sequence of this clone was published.

Like the chicken *Bmp-2* gene, all three of these genes have a single intron in the coding region of the gene, and the murine *Bmp-2* and *Bmp-4* genes also have an intron 7 bp upstream of the translation initiation codon. Moreover, the intron in the chicken

Chicken <i>Bmp-2</i>	MVA-TRSLL--LLCRVLLGG-VGLMPEVGRR-FS-
	** * * * * ***** * *
Chicken <i>Bmp-4</i>	MIPGNRMLMVLLCQVLLGGTNHASLIPETGRKKV
	* ** *
Human <i>Bmp-4</i>	MIPGNRMLMVLLCQVLLGGASHASLIPETGKKV
Chicken <i>Bmp-2</i>	PG- -ASA-Q RPEDLLGEFELRLLLHMFGLKRR
	** ** *
Chicken <i>Bmp-4</i>	AELQGQAGS GRRSAQSHELLRGFETTLLQMF
	* * *
Human <i>Bmp-4</i>	AEIQGHAG- GRRSGQSHELLRDFEATLLQMF

Figure 3.19: Comparison of the predicted amino acid sequences of amino-termini of pre-pro chicken BMP-2 and pre-pro BMP-4 from chicken (Francis *et al.*, 1994) and human (Wozney *et al.*, 1988). Sequence in black after the brackets is present in the cDNA clone, p5.1 (Francis *et al.*, 1994).

-- = residues that cannot be accurately determined from sequence data.

* = residues that differ between the genes

blue = residues that differ between the genes

Bmp-2 coding region is in the same position as that in the murine *Bmp-2* clone, taking into account the differences between the mouse and chick amino-acid sequences (Figure 3.20). The intron in the mouse *Bmp-4* gene is also located in the same place, taking into account the greater differences between the BMP-2 and BMP-4 amino-acid sequences. Therefore, the positions and sizes of the coding exons are similar for all 4 clones.

The sizes of the introns, however, are very different. The intron in the coding region was reported as being 6 kb in the mouse *Bmp-2* gene and only 1 kb in the mouse and human *Bmp-4* genes. The intron directly upstream of the translation initiation codon was 12 kb in the mouse *Bmp-2* gene and 1 kb in the mouse *Bmp-4* gene. The intron sizes in the human *Bmp-4* gene were reported to be 0.37 and 0.86 kb, respectively (Özkaynak *et al*, 1992). I was unable to ascertain how long the intron upstream of the transcription initiation codon was in the chicken *Bmp-2* clone, but the size of the intron in the coding region was approximately 1.65 kb. This is closer to the size of the intron in the human and mouse *Bmp-4* clones than that of the supposedly more homologous mouse *Bmp-2* clone.

The DNA sequences flanking the splice acceptor and donor sites are also very similar between the three genes. In fact, there is only a single base difference between the sequences around the splice acceptor site immediately upstream of the coding region, in the chicken and mouse *Bmp-2* genes (Figure 3.21).

Whilst I have not determined directly whether the coding region downstream of the *EcoRI* site at position 892 in p5.1 lies in the same exon as the coding sequence upstream of this site, the similarity of the chicken *Bmp-2* gene with the mouse *Bmp-2* and *Bmp-4* genes in other respects suggests that it will be.

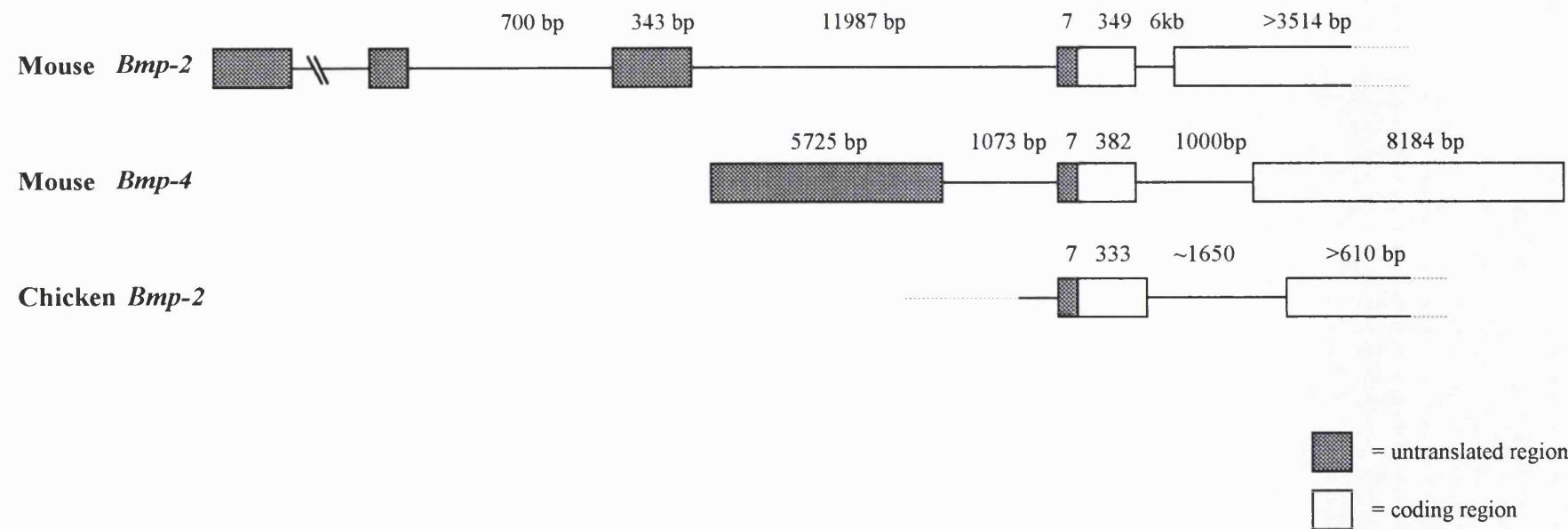


Figure 3.20: Comparison of the structures of *Bmp-2* and *Bmp-4* genomic clones
 (Feng *et al.*, 1994; Kurihara *et al.*, 1993)

Chicken <i>Bmp-2</i>	TCCTGC AG	/	GTCGACC ATG
	*		
Mouse <i>Bmp-2</i>	CCCTGTCC-GC AG	/	GTCGACC ATG
	*** **		****
Mouse <i>Bmp-4</i>	CCCCTCCC-- CAG	/	AGACACC ATG

Figure 3.21: Sequence comparison between splice acceptor sites upstream of the initiation codon in the *Bmp-2* & *4* genomic clones (Feng *et al*, 1994; Kurihara *et al*, 1993). Gaps have been introduced in the sequences for alignment purposes.

* = divergent bases; / = intron/exon boundary;

ATG = translation initiation codon

h) Mapping the 5' End of the Gene:

Analysis of the structure of the genomic clone of *Bmp-2* showed that there must be at least one exon upstream of the one containing the translation initiation codon. Since I was interested in isolating and analysing the promoter region of this gene, I tried various methods of mapping the 5' end of the gene.

i) Screening cDNA Libraries for chicken *Bmp-2* cDNA clones containing the 5' end:

At the time that the original chicken *Bmp-2* cDNA clone p5.1 was isolated (Francis *et al*, 1994), I isolated a number of other clones from the same library, using the mouse *Bmp-2* cDNA probe provided by Dr. Brigid Hogan. I analysed 8 of these clones using restriction enzyme digestion (Figure 3.22). The patterns of fragments generated by these enzymes were compared with those given by p5.1, and all of these other clones were found to be smaller than the BMP-2 cDNA clone, and wholly contained within it. (Figure 3.23.)

I also used p5.1 as a probe to screen several other cDNA libraries, such as a stage 10 chicken embryonic liver cDNA library in λ ZAP, and a stage 12-15 chicken embryonic cDNA library in λ ZAP. I isolated 14 cDNA clones, but none extended further upstream than p5.1, as shown by restriction digestions.

ii) Primer Extension:

Since none of the cDNA libraries screened contained clones incorporating the 5' end of the chick *Bmp-2* gene, I used the SFR1 primer in primer extension experiments, to determine if it was possible to generate cDNA corresponding to sequences at the 5' end of the chicken *Bmp-2* mRNA, which could then be isolated by PCR. The primer SFR1 is located 173 bp downstream of the translation initiation site in the chicken *Bmp-2* genomic clone #16F (Figures 3.7 and 3.24).

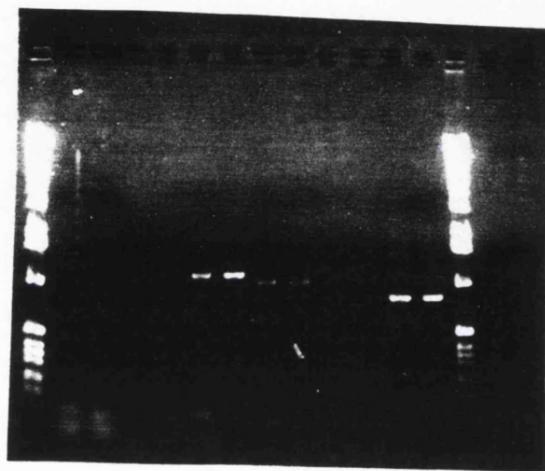


Figure 3.22: Restriction digest analysis of cDNA clones of *Bmp-2*.

Gel showing restriction patterns of further smaller cDNA clones isolated.

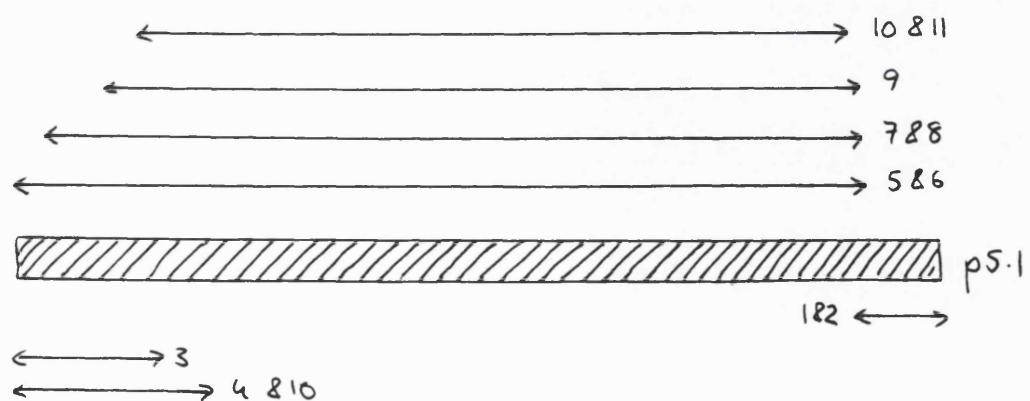
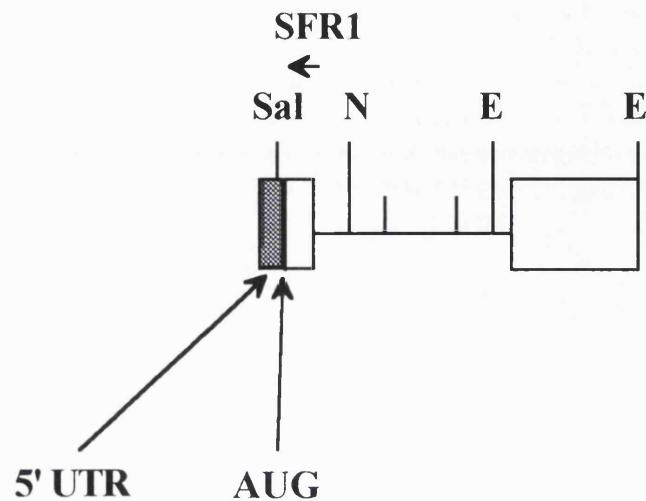


Figure 3.23: Alignment of p5.1 and further smaller cDNA clones.



70-54bp from the 5' end of the cDNA clone, p5.

182 bp downstream of the splice acceptor site in the genomic clone,
 173 bp downstream of the translation initiation codon.

Figure 3.24: The location of SFR2 relative to the cDNA and genomic clone.

RNA was extracted from stage 24 chick limb buds, when *Bmp-2* transcripts should be abundant. Yeast total RNA was used as a negative control, and the control RNA and primer supplied with the Promega kit were used as a positive control.

The positive control experiments generated fragments of the length expected, indicating that the technique was working successfully (Figure 3.25). With limb bud RNA, the SFR1 primer gave primer extension products of 58 bp and 74 bp, which were not seen with yeast RNA (Figure 3.25). The 5' ends of these products map to a position within the predicted *Bmp-2* coding region, downstream of the ATG codon, and close to the 5' end of cDNA clone p5.1 (Figure 3.26).

It therefore seems likely that there is some secondary structure in this region that causes the reverse transcriptase enzyme used in the primer extension reaction to fall off the template at this point. Since the same enzyme is used to construct the inserts in cDNA libraries, this could also explain why all the cDNA clones isolated of *Bmp-2* have 5' ends at this region and do not contain any sequence upstream of this region.

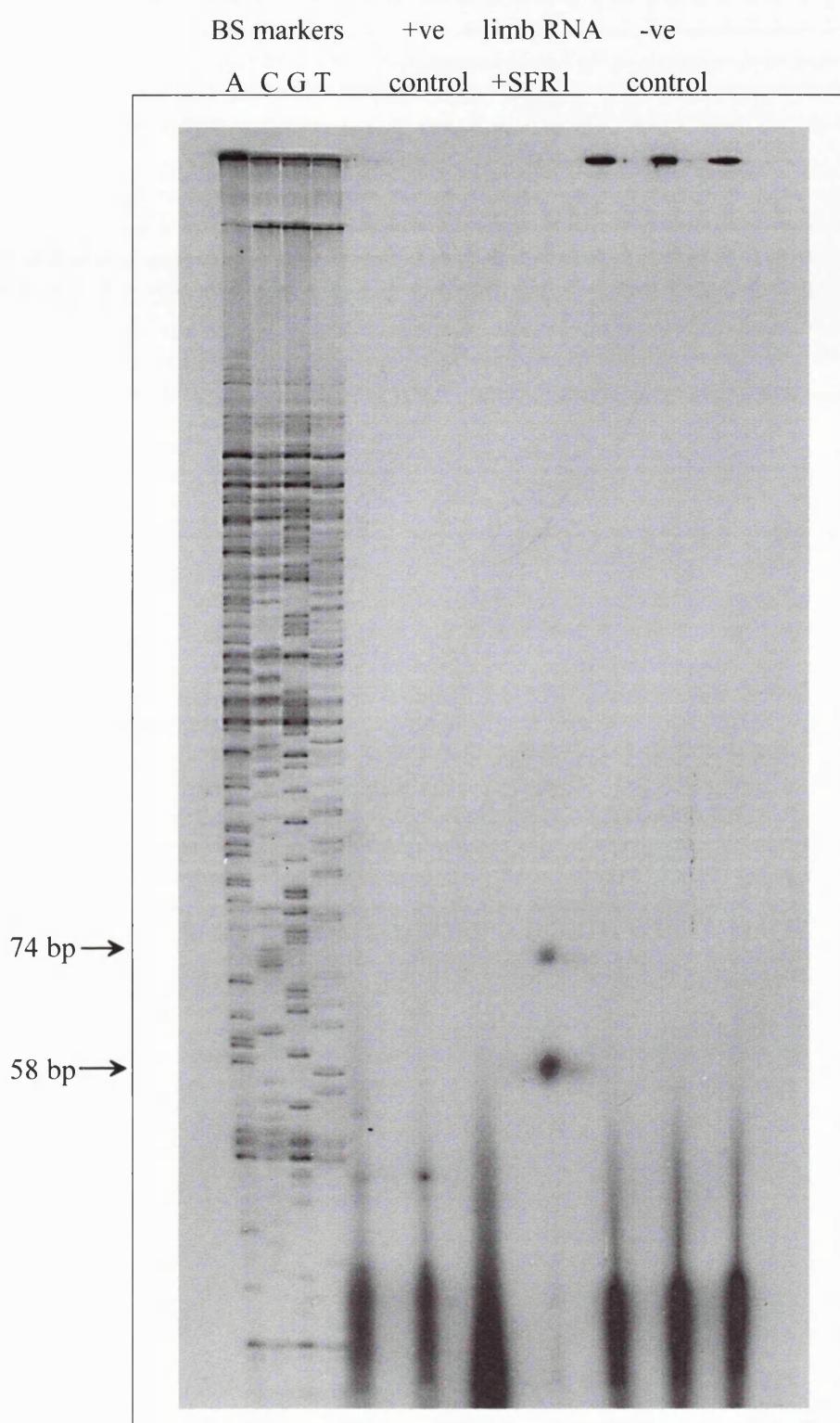


Figure 3.25: Result of primer extension experiments.

Autoradiograph of results of primer extension experiments, with controls.

16F TCCGTGCAGGTCGACCATGGTTGCNGNNACCCGCTCCC
 TCCTGGNGNTGCTGCTCTGCCGGGTGCTGCTGGCGGN
 GNGGTGGCCTCATGCCGGAGGTGGGACGGCGGNGCTT
 CAGCGNACCGGGNNNNNNNNCOTCGGCCGNGCAGCGNNN
 5' end of cDNA, p5.1
 NGAGGACCTCCTGGCGAGTTCGAGCTGCGCCTGCTC
 ACATGTTCGGGCTGAAGCGGCGGCCGAGCCCCGGCAAAG
 SFR1
 GACGTCGTCATCCCC

Figure 3.26: Positions of the primer extension products relative to the genomic clone and the cDNA, p5.1.

The translation initiation codon and splice site in the 5' UTR are shown in red and underlined. The sites of the primer extension products are boxed and shown in blue.

3.3 Discussion:

a) The chicken *Bmp-2* gene:

As described in this chapter, I have successfully isolated a genomic clone of the chicken *Bmp-2* gene. I have also been able to identify the 5' coding sequence that was missing from the original *Bmp-2* cDNA clone. The sequence of the *Bmp-2* gene is highly evolutionarily conserved between species, and the chicken sequence is very similar to the mouse and human *Bmp-2* sequences (60-66% over the amino acids that could be compared). I also examined the intron-exon structure of this gene and compared it with that of *Bmp-2* and *Bmp-4* genes from other species. The location of intron-exon boundaries examined was also almost identical in the *Bmp-4* and *Bmp-2* genes in humans, mice and chickens.

The high evolutionary conservation of sequence in the 5' untranslated region of the chicken and mouse *Bmp-2* genes indicates that these regions of the gene may be important for the function of the gene. In this case it is probably involved in ensuring correct splicing, since there is an intron splice site in this region, immediately upstream of the AUG codon. This conservation may be necessary to control alternative splicing.

The possibility of alternative splice variants of *Bmp* genes was demonstrated in the mouse *Bmp-4* clone by Kurihara *et al* (1993), and suggested by the results of Francis *et al* (1994) which showed that there are two different sized *Bmp-2* transcripts present in the chick limb bud. Since from the results described here, there appears to be only one copy of this gene in the chicken genome, the same as in humans and mice, these different transcripts are therefore likely to result from alternative splicing, or possibly the use of multiple promoter regions, rather than the presence of two closely related genes. Splice variants have also been reported for *Bmp-7* (Özkaynak *et al*, 1990), *Bmp-3* and *Bmp-8* (Özkaynak *et al*, 1992), MIS (Guerrier *et al*, 1990; Lee *et al*, 1992; Lee and Donahoe, 1993), TGF- β 1 (Kim *et al*, 1991; Geiser *et al*, 1991), TGF- β 2 (Fujii *et al*, 1986; Madisen *et al*, 1988; Webb *et al*, 1988; Noma *et al*, 1991; Burt and Paton, 1991; O'Reilly *et al*, 1992) and TGF- β 3 (Arrick *et al*, 1994). In MIS, the size difference between the different transcripts are due to differences in the amount of 3' polyadenylation, whereas in TGF- β 2, the differences are due to differences in the

length of the 5' UTR, as well as differential polyadenylation and alternative splicing of the final exon. In the case of TGF- β 3, the shorter of the two transcripts is a truncated version of the full-length transcript, lacking 870 bp from the 5' non-coding region, rather than an alternatively spliced variant.

Kurihara *et al* (1993) showed that there are also two alternative transcriptional start sites for the mouse *Bmp-4* gene, using RNase protection assays. They also showed that there are at least three exons upstream of the first coding exon. However, Feng *et al* (1994) showed that the mouse *Bmp-2* gene has only one exon upstream of the first coding exon (Figure 3.20).

I was unable to identify further exons upstream of the first coding exon in the chicken *Bmp-2* gene, since I was hindered by the absence of full-length cDNA clones containing the 5' coding region in screened cDNA libraries, and the failure of primer extension products to go beyond the position where the cDNA clones started.

b) Comparison with other members of the TGF- β gene superfamily:

Genomic clones have also been isolated of various other members of the TGF- β superfamily.

In *Drosophila*, the coding regions of the *dpp* gene are contained in 2 exons, but there are also 5' non-coding exons, similar to those found in the *Bmp-2* and *Bmp-4* genes (Padgett *et al*, 1993). The position of the intron between the coding exons is exactly the same for all three genes, and the splice sites are at identical positions (Padgett *et al*, 1993). The coding region of the *dpp* gene spans only 6 kb (Gelbart *et al*, 1989), but there is an elaborate *cis*-regulatory apparatus that encompasses more than 55 kb (St. Johnston *et al*, 1990). These *cis*-regulatory regions control the timing, location and quantity of *dpp* transcription (Gelbart, 1989; St. Johnston *et al*, 1990; Blackman *et al*, 1991). There are 5 different overlapping transcripts of *dpp*, each with 3 exons, and which differ only in the first 5' untranslated exon (Gelbart, 1989). Each appears to be initiated from a different promoter, which range in size from 150 bp to 2 kb, but they all use the same splice acceptor site in the second exon. Each transcript has its own

temporal and presumably spatial pattern of expression (Gelbart *et al*, 1985; St. Johnston *et al*, 1990).

The 60A gene in *Drosophila* is thought to have no introns, but the genomic clone lacks the 5' end of the gene, so there could be a small one there (Wharton *et al*, 1991).

A mouse genomic clone of *Bmp-6* was reported by Lyons *et al*, 1991: *Bmp-6* has 7 coding exons spanning over 70-80 kb. Human genomic clones of *Bmp-7* (OP-1) and *Bmp-8* (OP-2) have also been isolated (Özkaynak *et al*, 1990; 1992). They both also have 7 exons, whose sizes vary between 78 bp and 418 bp. *Bmp-8* spans 27 kb. The locations of the introns and splice junctions are identical between *Bmp-7* and -8, but are not the same as in the *Bmp-4* gene (Özkaynak *et al*, 1992).

A murine genomic clone of *Vgr-2* has also been isolated (Jones *et al*, 1992). It has 2 coding exons and a similar intron location to *Bmp-2* and *Bmp-4* (Jones *et al*, 1992). The mouse *Gdf-9* gene also has 2 exons, separated by a 2-9 kb intron (Incerti *et al*, 1994). There are multiple putative transcription start sites between 31 and 57 bp upstream of the start site of translation. Hötten *et al* (1994) isolated a human genomic clone of *Gdf-5*: it also has two coding exons, and it has no introns in the untranslated region of the longest cDNA clone obtained. The single intron is approximately 2 kb long.

The inhibin α , β_A and β_B subunit genes equally all have two exons (Stewart *et al*, 1986; Feng *et al*, 1989; Mason *et al*, 1989; Albiston *et al*, 1990; Pei *et al*, 1991; Rodgers *et al*, 1991; Tanimoto *et al*, 1991; Su *et al*, 1992; Thompson *et al*, 1994). The intron is approximately 1.5-2.1 kb long in α , 9-10 kb in β_A and 2.6-13.4 kb in β_B . Primer extension and S1 nuclease mapping showed 3 adjacent sites for transcription of α , approximately 70-80 bp upstream of the translation initiation codon, and several sites for β_B , over 150 bp upstream of the ATG codon (Feng *et al*, 1989; Pei *et al*, 1991). Neither α nor β_B has an obvious TATA or CCAAT box, and β_B is very G-C-rich. β_A , on the other hand, has a CCAAT box and a TATA box (Tanimoto *et al*, 1991; Su *et al* 1992; Thompson *et al*, 1994).

MIS has 5 exons (Cate *et al*, 1986; Haqq *et al*, 1992; Lee and Donahoe, 1993; Münsterberg and Lovell-Badge, 1991). The 5' UTR is only 8-10 bp long (Cate *et al*,

1986; Lee and Donahoe, 1993), and the major site of transcription initiation (88% of transcripts) is 10 bp upstream of the ATG (Guerrier *et al*, 1990). The murine gene has a putative TATA box, but neither the human nor bovine clones have TATA or CAAT boxes, but they do have a TTAA motif which may represent a degenerated TATA box (Guerrier *et al*, 1990; Münsterberg and Lovell-Badge, 1991).

The TGF- β s all have 7 exons, and the splice sites are very highly conserved between TGF- β 1, 2 and 3 (Derynck *et al*, 1987a, 1987b; Burt and Paton, 1991; Burt *et al*, 1995). The TGF- β 1 gene does not have a TATA or CAAT box motif in the promoter in mice or humans but the sequence is very G-C rich, with eleven CCGCCC repeats (Geiser *et al*, 1991; Kim *et al*, 1989). S1 mapping showed 2 transcriptional start sites, separated by 290 bp, containing 866 or 576 nucleotides of 5' UTR. Kim *et al* (1992) showed that the 5' UTR of TGF- β 1 can inhibit gene expression in reporter constructs by as much as 22-fold, in a cell-type-specific manner. The 5' UTR of TGF- β 1 is 840 nucleotides long and highly G-C-rich. The region between +11 and +147 of the 5' UTR forms a stable secondary stem-loop structure spanning, and may possibly be involved in post-transcriptional regulation of TGF- β 1 gene expression. A region like this needs to be very close to the start site of the RNA to have any effect (Kim *et al*, 1992).

The upstream region of TGF- β 2 shows no homology to the promoter of TGF- β 1 or 3 (Malipiero *et al*, 1990; Burt and Paton, 1991). It does not have any G-C rich areas around the transcription start site in the mouse (Miller *et al*, 1984), and has a TATA consensus sequence 30 bp from the transcriptional start site, but no consensus CAAT box (Noma *et al*, 1991). The 5' UTR is 1 kb long (Burt and Paton, 1991). S1 mapping showed a single transcription start site, 1357 nucleotides upstream of the coding sequence in humans (Noma *et al*, 1991).

The TGF- β 3 gene also has a TATA box, 21 bp upstream from the transcription start site in humans (Lafyatis *et al*, 1990). It is also very G-C rich, but these bases occur in the 5' UTR instead of the transcription start site as in TGF- β 1. Burt *et al* (1995) found that the chicken TGF- β 3 gene has 2 sites of transcriptional initiation, one with ATF/IRE TBP/TATA sequence motifs, the other with no known sequence motifs. The 5' UTR is 1.1 kb long in the human gene and has 11 ORFs, which inhibit translation of RNAs containing this region due to formation of non-functional transcriptional

complexes and secondary structures (Arrick *et al*, 1991). There might therefore be 2 separate promoters present in this gene, one giving rise to a long mRNA with a G-C-rich 5' UTR and extensive secondary structure, and the other lacking these sequences, and therefore being translated at a higher rate.

In summary, there seems to be some degree of conservation of intron/exon structure amongst the members of the TGF- β superfamily, with a number of members having only two exons of coding sequence, similar to the postulated structure of the chicken *Bmp-2* gene described in this chapter. However, various members of the gene family are organised differently, although members of sub-groups within the family appear to maintain the same structure and number of exons. Lyons *et al* (1991) suggested that the members of the TGF- β superfamily arose by gene duplication, and that the most recent duplication gave rise to the *Bmp-2* and *Bmp-4* genes, because they are the most closely related on the basis on intron/exon structure and sequence similarity, and the *Bmp-6*, 7 and 8 genes are more divergent, having 7 coding exons, rather than two. This is supported by the data described in this chapter.

However, the mouse *Bmp-4* gene and the *Drosophila dpp* genes were until now the only members of the TGF- β superfamily to contain multiple exons for the 5' untranslated region; the other genes may have multiple transcriptional start sites, but do not have introns in the 5' UTR. The chicken *Bmp-2* gene now joins the former group. The extensive sequence homology and similarities in genomic organisation between these genes lends further support to the theory that *dpp* and *Bmp-2* and 4 are derived from a common ancestral gene.

c) HpaII Tiny Fragment (HTF) Islands:

Although I was not able to locate the promoter region in the chicken *Bmp-2* gene, there is preliminary evidence in favour of the idea that the promoter region of the *Bmp-2* gene is located not far upstream of the first coding exon, because of the clustering of three *NotI* sites, within 2 kb of one another, around the AUG codon. This is highly unlikely to occur by chance: the probability of there being three such restriction sites consisting of 8 bp within 2 kb of one another is 1 in 63,536. However, this clustering of

restriction sites for rare-cutting enzymes with G-C-rich sites is a documented phenomenon and the resulting region is called a *Hpa*II tiny fragment island (HTF island), so named after one of the enzymes diagnostic of this (Bird, 1986). *Not*I is another diagnostic enzyme, since it is a rare-cutting enzyme with only G and C residues in its restriction site (Allikmets *et al*, 1994).

An HTF island (or CpG island) is a region of DNA in which the frequency of non-methylated CpG dinucleotides is substantially higher than elsewhere in the genomic DNA (Shiraishi *et al*, 1995; reviewed by Bird, 1987). This gives rise to an apparent G-C-richness of the sequence. HTF islands are usually approximately 1.5 kb in length (Bird *et al*, 1987). Approximately 56 % of human genes are associated with CpG islands: almost all house-keeping genes and many tissue-specific genes have an HTF island at the 5' end (Gardiner-Garden and Frommer, 1987; Shiraishi *et al*, 1995). HTF islands in the 5' region of genes usually extend upstream and downstream of the site of transcriptional initiation (Gardiner-Garden and Frommer, 1987), and most 5' CpGs start before the ATG codon, but the exact location varies between 100 bp and 2 kb upstream (Gardiner-Garden and Frommer, 1987). Almost all islands studied cover at least part of one exon and usually the promoter (Larsen *et al*, 1992).

Examples of genes containing HTF islands include TGF- β 1, and TGF- β 3, and Müllerian Inhibiting Substance (Larsen *et al*, 1992; Burt *et al*, 1995). CpG islands are not necessarily conserved between species: for example, they are depleted in mice, rats and *Xenopus*, but strongly conserved in bovine, chickens and humans (Gardiner-Garden and Frommer, 1987).

It has been suggested that the demethylation of the HTF island correlates with a change in chromatin structure which allows access to the promoter region for proteins involved in transcription (Tazi and Bird, 1990), and it has been shown that methylation of an HTF can act as a silencer and inhibit transcription (Renzo *et al*, 1989; Brandeis *et al*, 1993).

From the location of the HTF island spanning the first coding exon of the chicken *Bmp-2* genomic clone, it seems likely that the promoter is not far upstream from the ATG codon.

However, HTF islands may also be found in the second exon of genes as well as the first one and I was unable to map the size of the intron preceding the translation start site (Gardiner-Garden and Frommer, 1987).

d) Further studies:

Further mapping, subcloning and sequencing of the genomic *Bmp-2* clone #16F would enable me to identify the 3' region of the chicken *Bmp-2* gene fairly readily. This would also show whether the intron-exon structure of the chicken gene maintained the family trend or whether there is another downstream intron interrupting the coding region.

However, the main priority would be to identify the 5' end of the *Bmp-2* gene.

In the work described here, primer extension failed to progress past points 116 and 132 bp downstream of the translation initiation codon, probably due to some secondary structure here. Primer extension using a primer situated upstream of this point might generate further sequence of the 5' end of the *Bmp-2* gene. Alternatively, the problem might be resolved by using the SFR1 primer at higher reaction temperatures.

Özkaynak *et al* (1990) had the same problem isolating a cDNA of *Bmp-7* containing the 5' region: all the clones they initially isolated ended at various points in the 5' portion of the second to last exon. This turned out to be due to secondary structure, and they resolved this by using cDNA libraries that were simultaneously primed by random primers and oligo(dT) or that were reverse transcribed under reaction conditions designed to eliminate RNA secondary structure, such as those sold by Stratagene and Clontech, to obtain full length clones (Özkaynak *et al*, 1990). The same problem was also encountered isolating full-length cDNAs of human inhibin β_B , which lacked the 5' end because it is very G-C-rich (Mason *et al*, 1989). Burt *et al* (1995) were unable to carry out S1 mapping, RNase protection and primer extension on the chicken TGF- β 3 gene because of the high G-C ratio of the 5' region, which forms stable RNA/DNA secondary structures. They used Reverse Transcriptase-PCR followed by DNA hybridisation to detect low levels of product (Burt *et al*, 1995). Burt and Paton (1991)

used 7-deaza-2'-deoxyguanosine instead of dGTP in extension reactions to weaken secondary structures in G-C-rich mRNA in the 5' region of the chicken TGF- β 2 gene.

Further mapping of this region in the genomic clone #16F, including subcloning and sequencing, might also resolve these questions. Identification of the transcription start site using RNase protection assays would not be possible without a template containing the 5' region, which would have to be subcloned from the genomic clone. It might also be possible to isolate a longer cDNA clone from libraries using cloned fragments of the 5' end of the genomic clone, such as 16n1 or 16s3, and to use this to map the upstream region of the gene.

Once the 5' end of the gene had been identified and cloned, sequencing would allow us to study the promoter in more depth and ascertain what response elements are present in the promoter, such as RAREs, which might be expected to be present from the response of the gene to RA *in vivo* and *in vitro* (Francis *et al*, 1994; Rogers *et al*, 1992).

One further method of studying the 5' promoter region of this gene would be to create reporter constructs of large regions of the genomic clone upstream of the transcription initiation start site, cloned into a vector such that they drive the expression of a reporter gene. In this way, fragments of the putative promoter region could be rapidly screened for promoter activity, by examining which fragments could drive expression of the reporter gene.

An ideal restriction site for such experiments would be the *Sall* site, located a few bases upstream of the AUG codon and just downstream of the splice acceptor site. The map of the cosmid would provide upstream sites. Any regions that showed promoter activity could then be subjected to deletion analysis to map in further detail the regions needed to regulate the patterning, timing and amount of expression of the *Bmp-2* gene.

To do this, I needed a cell system that would mimic as closely as possible the conditions of the developing limb, where the *Bmp-2* gene is normally expressed. I therefore designed a novel system involving transfecting primary chick mesenchyme cells for this purpose, and this will be described in the next chapter.

Addendum to Chapter 4 Discussion

There was a significant amount of variability between readings for the various luciferase experiments, between experiments. In some instances, this was of the order of ten-fold (compare Figure 4.6 and Figure 4.11). This could have been due to variability in the transfection efficiency of cells from chick embryos of different stages. In order to obtain sufficient cells, limbs from chicks of various stages between 17 and 21 were pooled. This was necessary because it is not possible to get all the embryos to exactly the same stage at any given time, due to small differences in the rate of development of different embryos.

However, the ratios between the expression levels of the various constructs remained the same between experiments and could be compared.

The levels of expression of pGL2 Control were much lower in Figure 4.9 than in the other figures. This is because, in these experiments where untransfected cells were mixed with transfected cells, fewer cells were initially transfected than the normal 2×10^7 , because the number of cells which could be isolated for any one experiment was limiting, and we had to reserve some of these cells to use as untransfected cells in the final mixed culture. In this way, the final number of cells plated out was the same as in the other experiments, but not the number of cells transfected. The lower number of transfected cells could therefore account for the lower expression levels.

CHAPTER 4

A Transfection System for Chick Mesenchyme Cells

4.1 Introduction:

In order to study the promoter region of a gene using reporter constructs, it is necessary to have an appropriate cell system in which to express these constructs. The cell system used must provide an appropriate environment in which to look at expression of the gene of interest. In the case of the gene that I was interested in, chicken *Bmp-2*, a cell line or primary cell system was needed which normally expresses high levels of the *Bmp-2* gene, and preferably one of chicken origin, because the elements in the chick promoter region may require specific signals which may not be present in cells from other species.

All the cell lines previously used in research on the *Bmp-2* gene were of mouse origin, such as the 10T1/2 cell line, derived from early mouse mesoderm, used by Katagin *et al* (1990). In addition, it has not so far been possible to immortalise chick cells to establish cell lines.

However, as described in Chapter 1, *Bmp-2* is strongly expressed in the posterior region of the chick limb bud at stage 17-21, but is not expressed in the anterior region (Francis *et al*, 1994). These cells would thus represent a convenient system in which to examine the control of expression of reporter constructs carrying fragments of the putative *Bmp-2* promoter.

I therefore used a cell culture system based on primary chick mesoderm cells from limb buds, and explored various methods of transfecting these cells.

4.2 Advantages of a transfected chick cell system:

These chick mesenchyme cells are important as a model because they are closer to the *in vivo* state than cell lines. They also express high levels of the *Bmp-2* gene at the stage at which they are harvested, i.e. stage 17-21 (see Chapter 1), which might be important since it has been suggested that *Bmp-2* can autocatalyse its own transcription via a positive feedback loop, in a similar way to TGF- β 1, and *dpp* in *Drosophila* (Van Obberghen-Schilling *et al*, 1988; Hursh *et al*, 1993).

These cells can be maintained in culture for up to 7 days, under various conditions. They can be cultured either as a single layer, or in a dense pellet as a micromass. The micromass culture system gives rise to conditions similar to those of cells in developing limbs *in vivo*, because the cells are plated at very high densities, and so experience close cell-to-cell contact. After 5-7 days, the cells differentiate into cartilage cells, a cell type not found in monolayer culture but characteristic of the developing limb. The mesenchyme cells can also be pelleted and placed into an ectodermal jacket and grafted back on to a host wing bud, where they will develop into ectopic limbs, which appear distorted but have recognisable digits (Zwilling, 1961; Hardy *et al*, 1995).

When cultured as a single layer, cells isolated from either the anterior or posterior halves of the limb will maintain their polarising or non-polarising characteristics for some time in culture, in particular when cultured in the presence of FGF (Honig, 1983; Vogel and Tickle, 1993).

This cell system would therefore prove useful for analysing the promoter regions of genes that are expressed in the developing limb in a spatially restricted way. The *Bmp-2* gene is one example of such a gene, since it is expressed in posterior but not anterior cells in the limb *in vivo*. The system could also be used to identify regulatory sequences that respond to polarising signals, such as might be present in genes downstream of RA or *Sonic hedgehog*, by carrying out deletion mapping on the promoter construct of interest and looking for regions of the promoter that preferentially direct expression in cells isolated from the polarising region of the limb.

These cells can also be treated in culture with various substances, such as growth factors or RA. This would make it possible to map and isolate regions of promoters which respond to these factors. For example, it would be possible to determine which regions of the *Bmp-2* promoter are responsible for its reported RA inducibility in anterior limb bud cells *in vivo* (Francis *et al*, 1994).

Additionally, it may be possible to estimate whether induction by a given growth factor is direct or not, by studying the time required for induction by that factor, and the kinetics of the induction.

In collaboration with Adrian Hardy, of the Anatomy Department, I therefore designed a system to isolate these chick mesenchyme cells and transfect them with luciferase reporter constructs. To our knowledge, we are the first people to have successfully transfected these cells.

4.3 Results:

a) Isolation of Cells:

In order to isolate mesenchyme cells, limb buds were freshly dissected from stage 17-21 chick embryos. Ectoderm was removed by trypsinisation and the mesenchymal cells were triturated until only single cells were present, by pipetting them up and down using a Gilson pipette.

These cells were adherent and could be cultured for up to 7 days, either as a monolayer, or under micromass culture conditions (Figure 4.1).

a)



b)

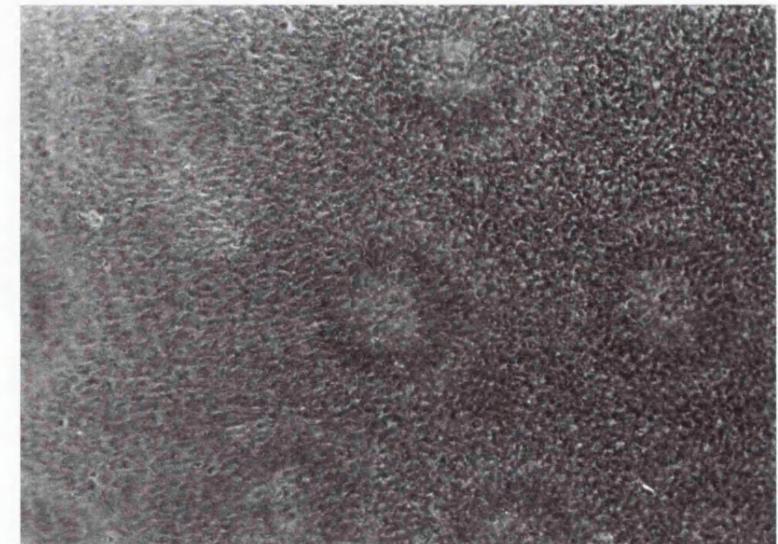


Figure 4.1: Primary Chick Mesenchyme Cells in Culture.

- a) Micromass culture: high power view of mesenchyme cells.
- b) Micromass culture: lower power view showing whorls of cartilage cells forming after 5 days.

b) Transfection using "Transfectam":

Previous work in our laboratory had shown that it is not possible to transfet primary chick mesenchyme cells efficiently using the standard calcium phosphate precipitation method.

Initially, I decided to use Transfectam, a lipoprotein product from Promega, which is designed to facilitate transfection of primary cells and cells which are difficult to transfet by other means. Transfectam binds to the DNA, coating it with a lipid layer, which then associates with the cell membrane and allows the DNA to pass into the cell. It is designed to give high transfection efficiencies without the high cell death rate often reported for electroporation, because it disrupts the cells less, and it is particularly recommended for primary cells.

The transfection was carried out on attached cells, in serum-free medium, and at the end of the incubation period the cells were washed and cultured in "Micromass Medium".

To test the feasibility of this method of transfection, cells were transfected with pCH1101, a construct in which the SV40 promoter drives expression of the *E.coli lacZ* gene, which encodes β -galactosidase. Cells can then be stained for β -galactosidase activity, providing a visual assay of transfection efficiency.

The cells were successfully transfected and expressed the β -galactosidase marker, but the efficiency of transfection was very low (approximately 4 stained cells per 100,000) and most of the cells appeared to have lysed, leaving cell debris over the bottom of the well. (Figure 4.2.)

I also performed transfection experiments using luciferase as a marker instead of β -galactosidase. The assay for luciferase is more sensitive than that for β -galactosidase, or CAT assays, and there is no endogenous luciferase activity in vertebrate cells. Initially, a Beta Radiation Counter was used to measure the luminescence in transfected cell extracts. This method is not as sensitive at measuring this type of luminescence as a luminometer, but was sufficient to give an indication of the extent of transfection.

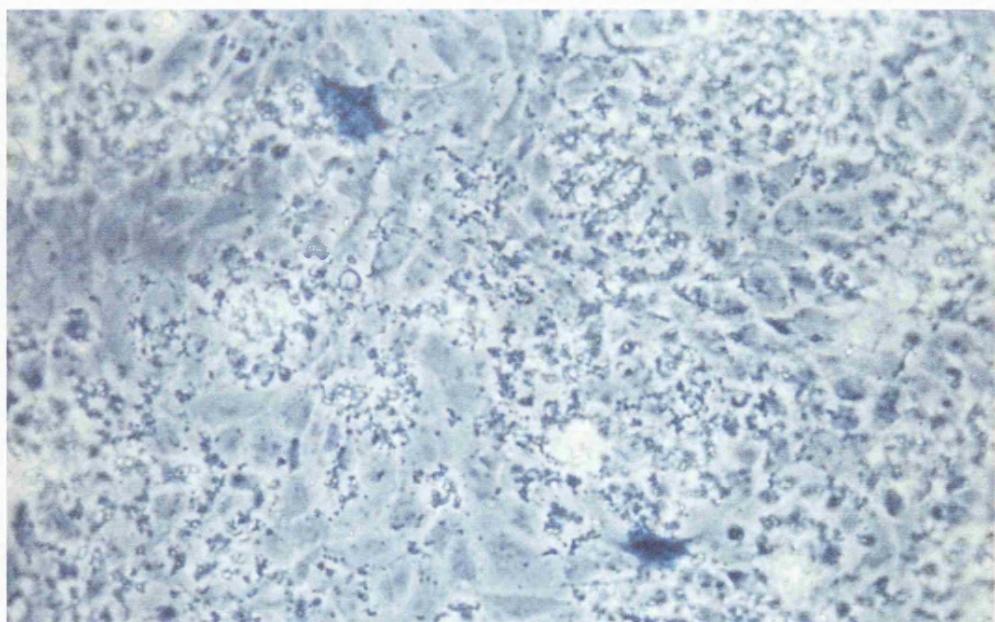
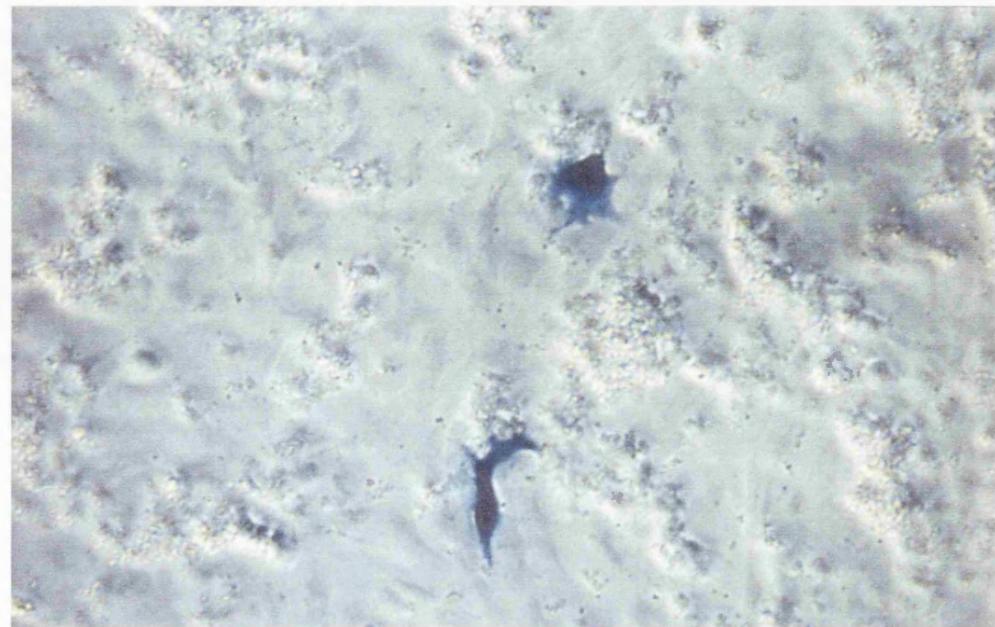


Figure 4.2: Expression of β -galactosidase directed by the construct pCH1101 (supplied by Stratagene) in transfected chick mesenchyme cells. Only a very small number of cells expressed the marker, and the majority of cells appeared to have lysed, leaving clearly visible cell debris.

In the initial studies, cells were transfected with the pGL2 Control and Basic luciferase constructs, supplied by Promega (Figure 4.3). pGL2 Basic contains only the luciferase gene with no upstream promoter regions to drive expression, and it should give an indication of the background luciferase activity generated by this cell system. pGL2 Control contains the luciferase gene linked to the SV40 promoter. This should drive strong expression of the reporter gene in any vertebrate cell.

After optimising conditions, I found that using the minimum recommended amount of Transfectam, and incubating the cells with the Transfectam for the minimum recommended time (1 hour) at 37°C, gave high luciferase activity (Figure 4.4), but that 90-95% of the cells lysed during the period of incubation. All the other conditions tried resulted in the lysis of all the cells.

Experiments in which untransfected cells were incubated in serum-free medium showed that the cells can survive the incubation period without serum when no Transfectam is present, demonstrating that it is the Transfectam that is responsible for the high death rate.

This high cell death rate was a problem because I wanted to make micromasses, and the transfected cells recovered and plated out in micromass culture were not experiencing the conditions of high cell density and close cell-cell contact that are needed for chondrogenesis, because of the low levels of live cells. It is not practicable to compensate for cell death by increasing the number of cells used, as it is difficult to isolate large numbers of primary cells from chick embryos, and the cells have to be used within hours of their isolation and therefore cannot be accumulated over a period of time, because the condition of the cells deteriorates rapidly when they are kept in PBS buffer.

Given that the transfected cells that do survive express the luciferase construct at high levels, I decided to try transfecting these cells by electroporation, in the hope that the levels of cell death might be lower.

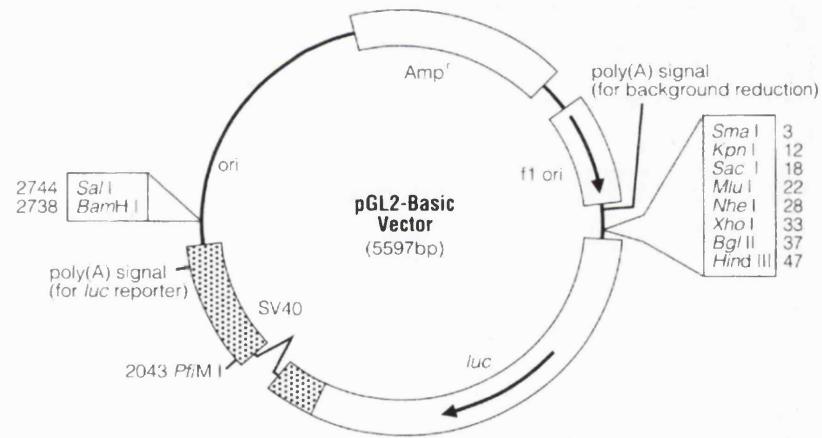
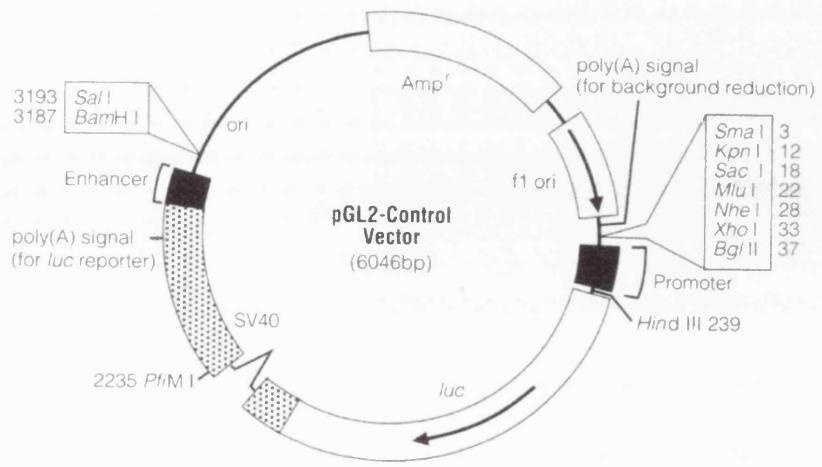


Figure 4.3: Maps of the luciferase constructs, pGL2 Control and pGL2 Basic, supplied by Promega.

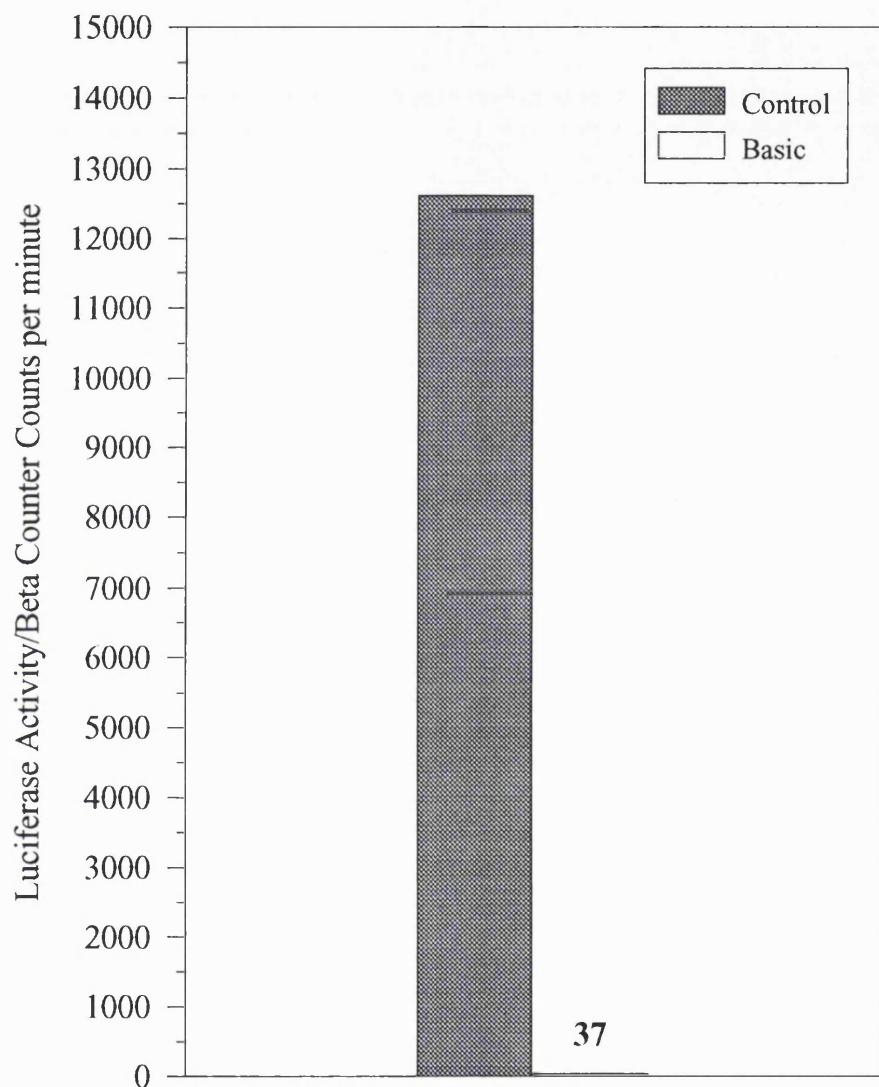


Figure 4.4: Luciferase Expression Directed by pGL2 Control and pGL2 Basic in Chick Mesenchyme Cells Transfected using Transfectam.

Readings of luciferase activity were made 24 hours after transfection, using a scintillation beta-counter. (n = 1)

c) Transfection by Electroporation:

i) Setting up the system: positive and negative controls:

I isolated chick mesoderm cells as above and transfected them by electroporation using a capacitance of 960 μ F and a voltage of 0.22 V, in eukaryotic transfection cuvettes. These conditions are those usually used in our laboratory for transfecting eukaryotic cell lines.

Under these conditions, cell death is much reduced compared to the cells transfected with Transfectam and sufficient cells survive to plate out in high density micromass culture.

Cells were transfected with the positive and negative controls, pGL2 Control and pGL2 Basic. 50 μ g of DNA was used per 2×10^7 cells. Transfected cells were assayed for luciferase activity after 24, 48, 72 or 120 hours.

In all of these experiments, luciferase activity was measured using a luminometer machine and the counts shown on the graphs are in arbitrary luminometer units. The heights of the bars in the histograms represent the arithmetic averages of all counts measured for each batch of transfections and the error bars on the graphs show the standard deviation from the mean estimated for the population, based on the sample size, n, for each batch, shown in the legend of each histogram. t tests were carried out, comparing the average luciferase activities for each construct or culture condition, to assess whether there were significant differences between constructs or culture conditions and the results are shown on the histograms and explained in the legends. Where more than four comparisons were to be made using t tests, one-way analysis of variance tests were also carried out to ensure that the differences found were actually significant, and not a function of the large number of t tests carried out.

Cells transfected with pGL2 Control DNA showed high levels of luciferase expression, after 24 hours, much higher than the values for the negative control, pGL2 Basic, and the background level of luminescence. (Figure 4.5.)

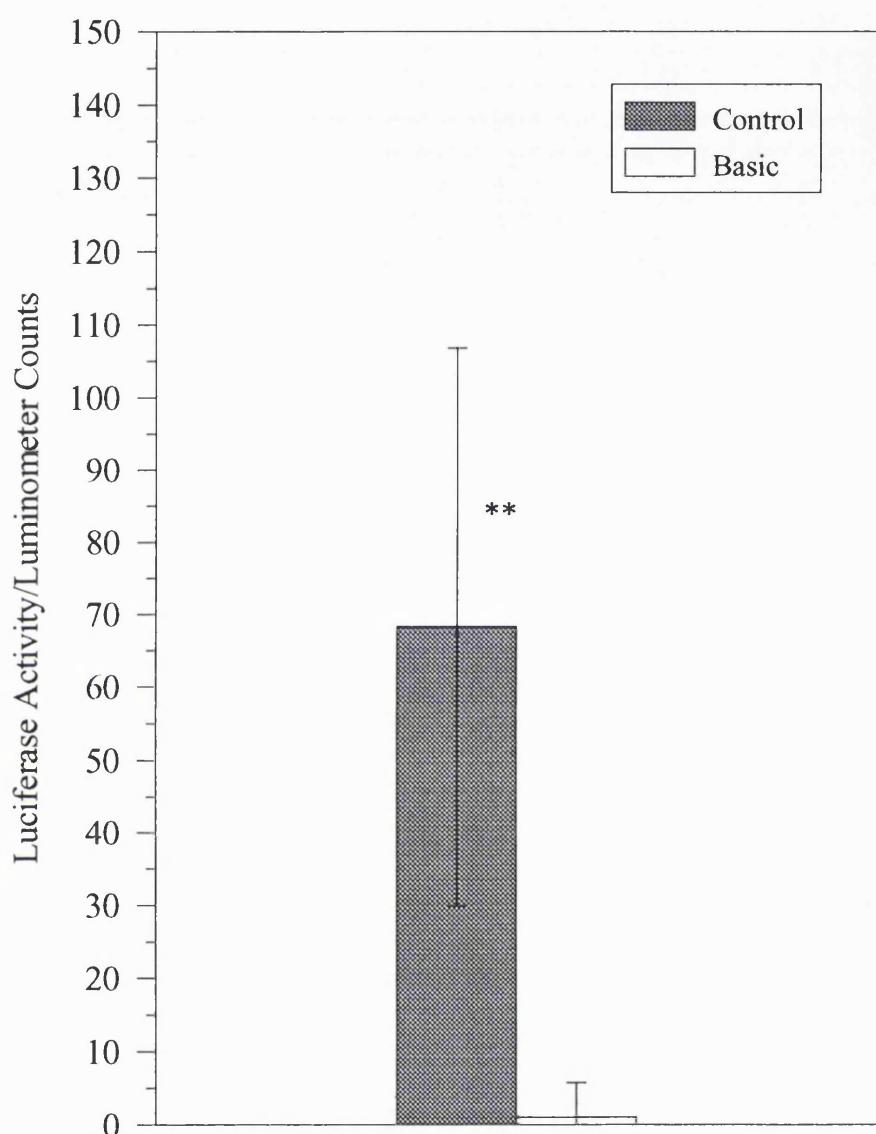


Figure 4.5: Luciferase Expression Directed by pGL2 Control and pGL2 Basic in Chick Mesenchyme Cells Transfected by Electroporation.

Readings of luciferase activity were made 24 hours after transfection using a luminometer. (n = 10)

(** = significant difference between Control & Basic at the 99% confidence limit)

The luciferase expression drops off very quickly though, and is indistinguishable from background levels after 72 hours (Figure 4.6). Experiments by other people in the laboratory in which other cell lines were transfected with this vector indicate that this rapid drop-off is unusual, since, in their experiments, expression in transfected cell lines can last 5 days or more. However, the levels of expression directed by the positive control construct, pGL2 Control, are sufficiently high to be easily distinguishable from background levels and from the levels of expression directed by the negative control construct in this cell line, after 24 and 48 hours.

ii) Micromass culture of transfected cells:

To form micromasses, the cells are initially plated out in a single drop on the culture dish, where they then adhere, and then the dish is flooded with culture medium. In this way the cells develop in a multilayer, high density clump. (Figure 4.7.) If sufficient cells are plated out, cartilage nodules and whorls form after 5-7 days (Figure 4.8a). However, if too few cells are present, no cartilage will form, since the cells are not in sufficiently close contact with one another.

I was able to generate micromass cultures using transfected limb bud mesenchyme cells, which formed cartilage nodules. (Figure 4.8b and c) The cartilage took longer to form than with untransfected cells, and less was formed, as judged by staining with Alcian blue, despite the fact that I plated out more cells than are usually used to form a micromass, since I was expecting large numbers of the transfected cells to die. However, it proved difficult to generate successful micromass cultures reproducibly.

This poor reproducibility could be due to the high levels of cell death resulting in cell densities that were too low to give chondrogenesis. To overcome this potential problem, I mixed transfected cells with untransfected cells in a 1:1 and a 10:1 ratio and then plated the resulting mixtures out in micromass culture. However, although this dilution of transfected cells did not substantially affect the levels of luciferase signal (Figure 4.9), and there was no significant difference between the luciferase activity in mixed and unmixed cultures, the micromass cultures still did not reproducibly survive past 5 days or produce cartilage.

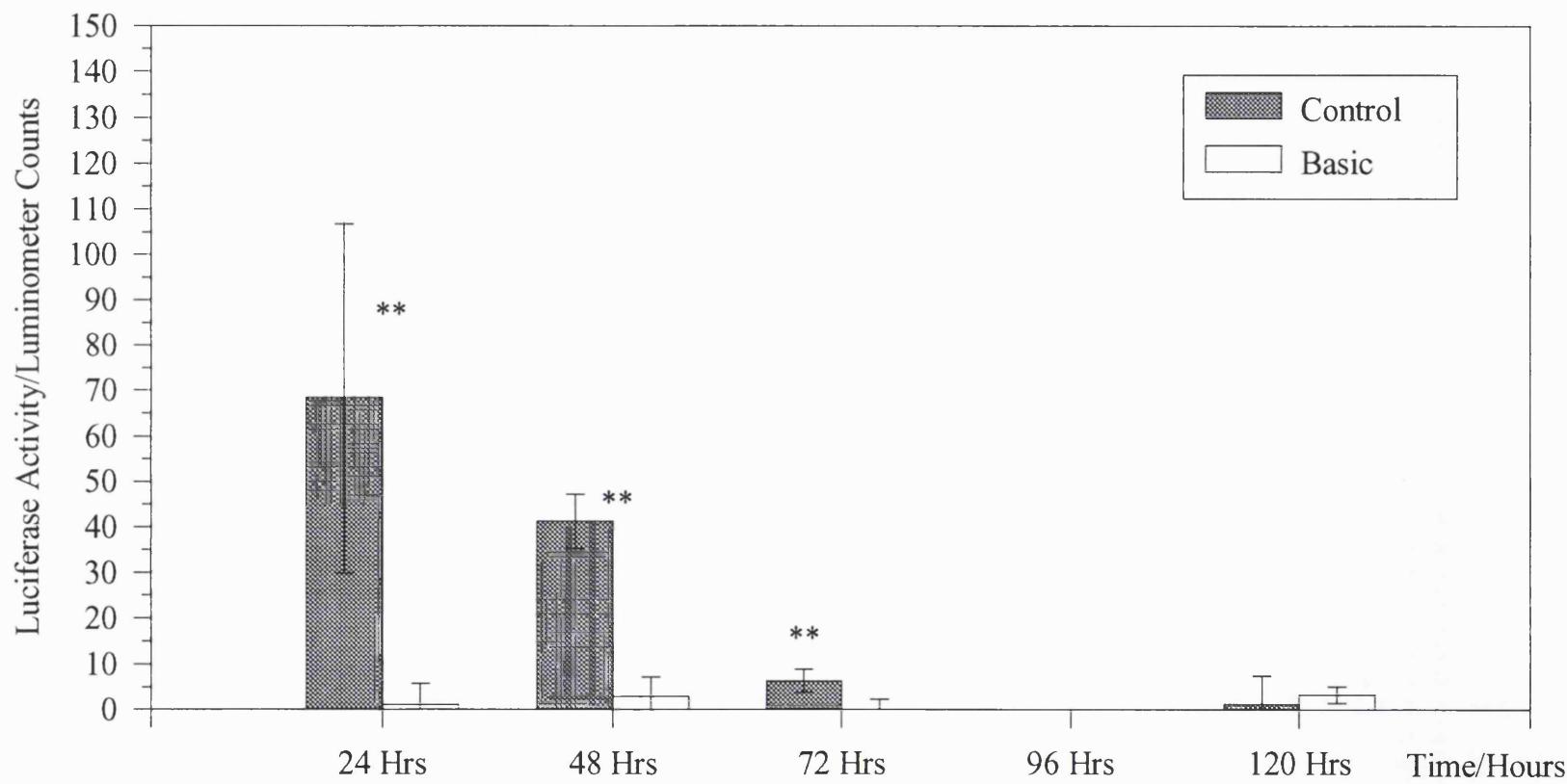


Figure 4.6: Time Course of Luciferase Expression Directed by pGL2 Control and pGL2 Basic in Chick Mesenchyme Cells.

(** = significant difference between Control and Basic at the 99% confidence limit) (24, 48 & 120 hrs: n = 10; 72 hrs: n = 8)

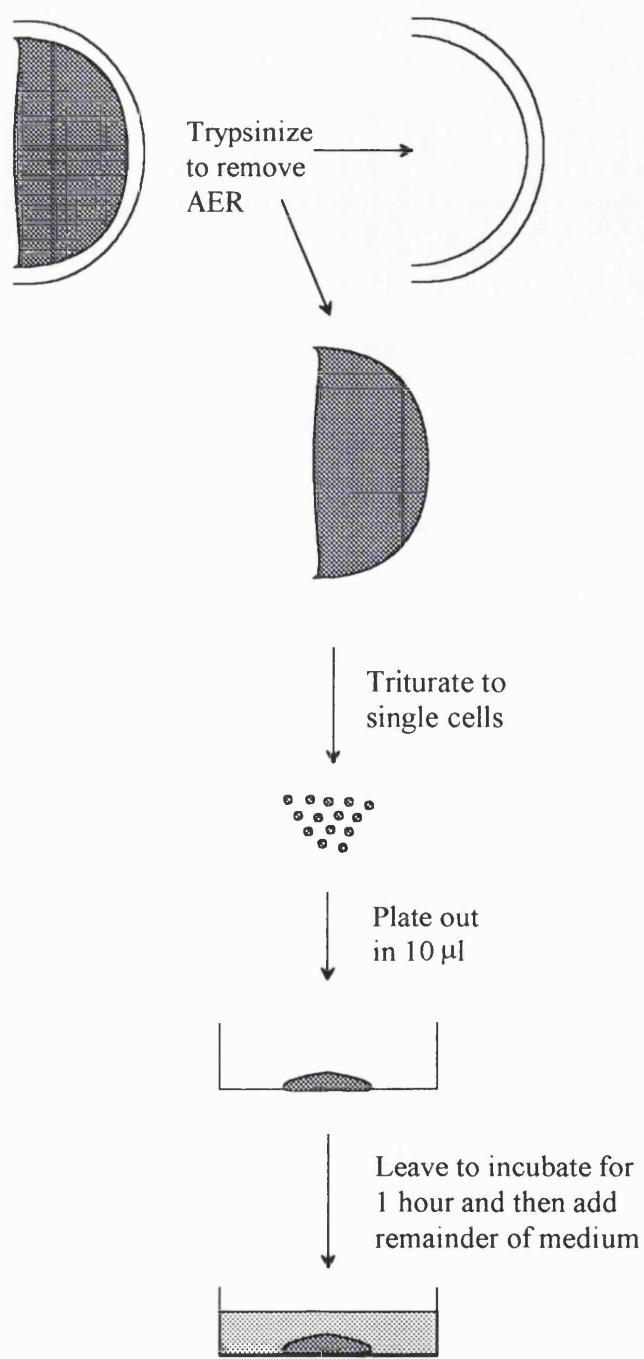


Figure 4.7: Technique for micromass culture.

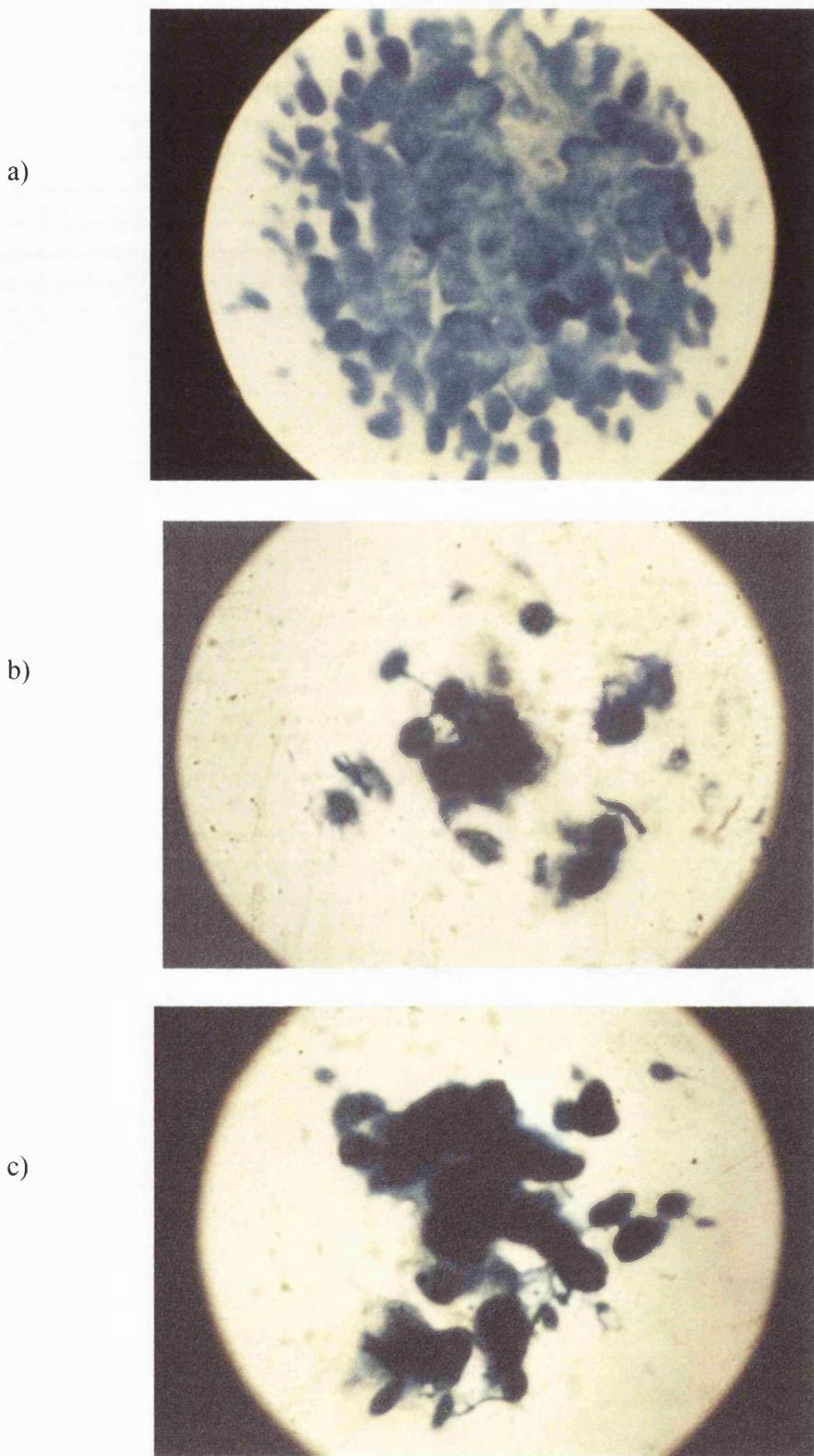


Figure 4.8: Chick limb bud mesenchyme cells in micromass culture, stained for cartilage with Alcian blue after 7 days in culture.
a) Untransfected chick limb bud mesenchyme cells.
b) and c) Transfected chick limb bud mesenchyme cells.

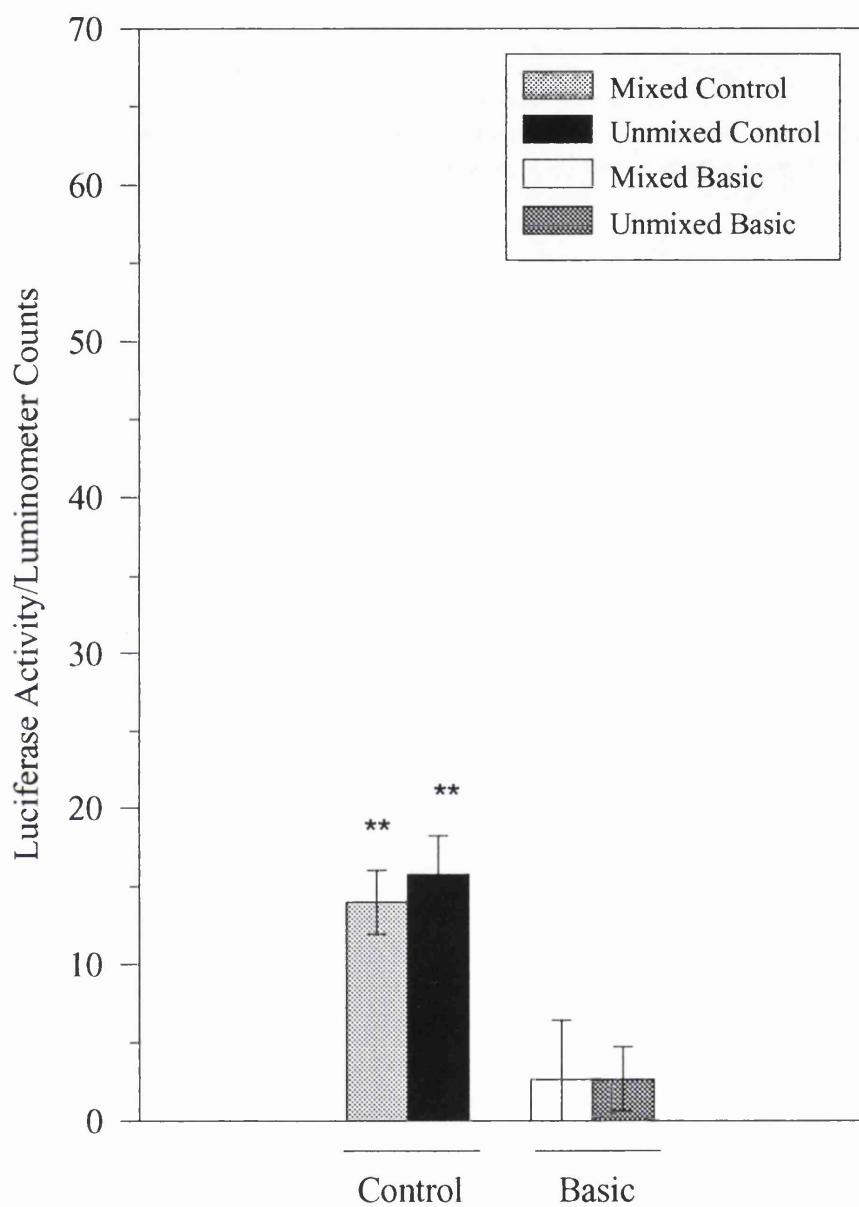


Figure 4.9: Comparison of Luciferase Expression in Micromass Cultures Containing a Mixture of Transfected and Untransfected Cells and in Micromass Cultures Containing Only Transfected Cells.

Luciferase expression was measured 24 hours after transfection.

(n = 4)

(** = significant difference between Control & Basic at the 99% confidence limit)

In many cases, clumps of dead cells were observed within the micromass cultures. These could affect the viability of the micromass culture either by disrupting the close cell-to-cell contact required for cartilage formation, or by actually causing the other cells to die.

It is therefore possible that successful micromass cultures would result reproducibly if dead cells were removed from the transfected cell suspension before plating out, for example by using Ficoll gradients. The yield from such separation techniques would be low however, and the transfected cells would probably have to be mixed with untransfected cells.

In all subsequent experiments, the cells were plated out as monolayers.

d) Transfection of Cells with Promoter Constructs for Specific Markers of Chick Limb Development:

Given the success of transfection with pGL2 Control and pGL2 Basic, I transfected chick limb bud cells with two reporter gene constructs containing promoters from genes that should be expressed at specific times during chick limb bud development.

i) Type X Collagen:

Chicken type X collagen luciferase promoter constructs were a gift from Prof. M. Pacifici, of the University of Pennsylvania (LuValle *et al*, 1993) (Figure 4.10). Type X collagen is only expressed in hypertrophic cartilage, and is not expressed in undifferentiated mesenchyme cells, or in the cartilage formed in micromasses (LuValle *et al*, 1993). It should therefore not be expressed in these transfected cells, either plated in micromass culture or cultured in single layers. It therefore provides a negative control, as a gene which is normally expressed in the developing limb, but not at the stages and conditions used in these experiments.

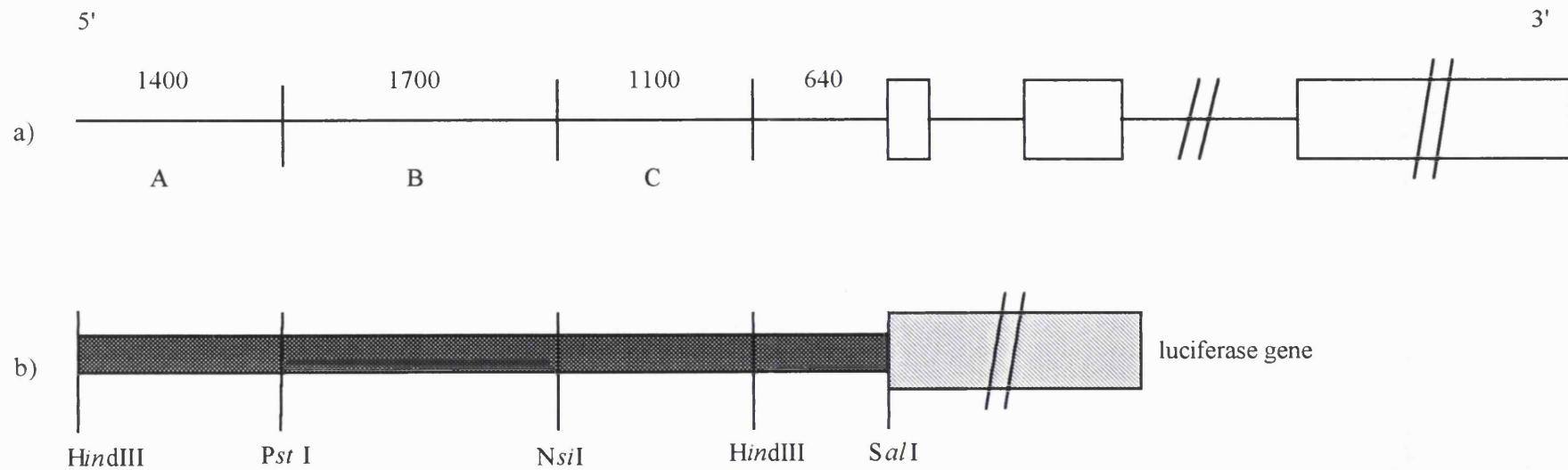


Figure 4.10: Map of the chicken Type X Collagen promoter construct. This luciferase construct was a gift from Professor M. Pacifici, of the University of Pennsylvania (LuValle *et al*, 1993).

- a) Map of promoter region of genomic clone of chicken Type X collagen. The white boxes represent the exons.
- b) Map of chicken Type X collagen luciferase promoter construct. This construct contains all of the promoter region required for correct expression.

Chick limb bud cells from whole limbs were transfected with this construct, and luciferase activity was measured after 24 hours, 48 hours, 72 hours and 5 days (Figure 4.11).

From these graphs it can be seen that, as expected, the type X collagen constructs are not expressed by these cells, even after 5 days in culture.

ii) Retinoic-Acid-Induced-Heparin-Binding Protein:

The Retinoic-Acid-Induced-Heparin-Binding Protein (RIHB) promoter construct was a gift from Dr. D. Duprez and Dr. M. Vigny, of INSERM, Paris (Duprez and Vigny, 1994) (Figure 4.12).

RIHB is a gene that is expressed very strongly and ubiquitously in the developing limb bud between stages 17 and 21. Expression then declines until it is no longer detectable by stage 26. It is later expressed again in developing cartilage. Expression of this gene can also be induced by RA in cell lines (Duprez and Vigny, 1994). This gene therefore provides a convenient positive marker to test the suitability of our transfection and culture system for genes expressed in the developing limb, and their induction by factors introduced to the culture medium, such as RA.

I transfected whole chick limb bud cells with this construct, cultured the cells, both in the presence and absence of RA, and measured luciferase activity after 24 hours, 48 hours, or 72 hours. (Figures 4.13 and 4.14.)

The RIHB gene constructs were expressed in these cells, but only very weakly (Figure 4.13). Expression was close to background levels after 24 hours, but was higher after 48 and 72 hours. The kinetics of the expression directed by this promoter construct were therefore different to that directed by pGL2 Control, where maximal expression occurs after 48 hours, and then declines at 72 hours. Although statistically significantly different than the background levels, this maximum value is very low (approximately 10% of pGL2 Control). This is, however, comparable to the levels of luciferase expression, in relation to SV40 control expression levels, found by Dr. Duprez and Dr.

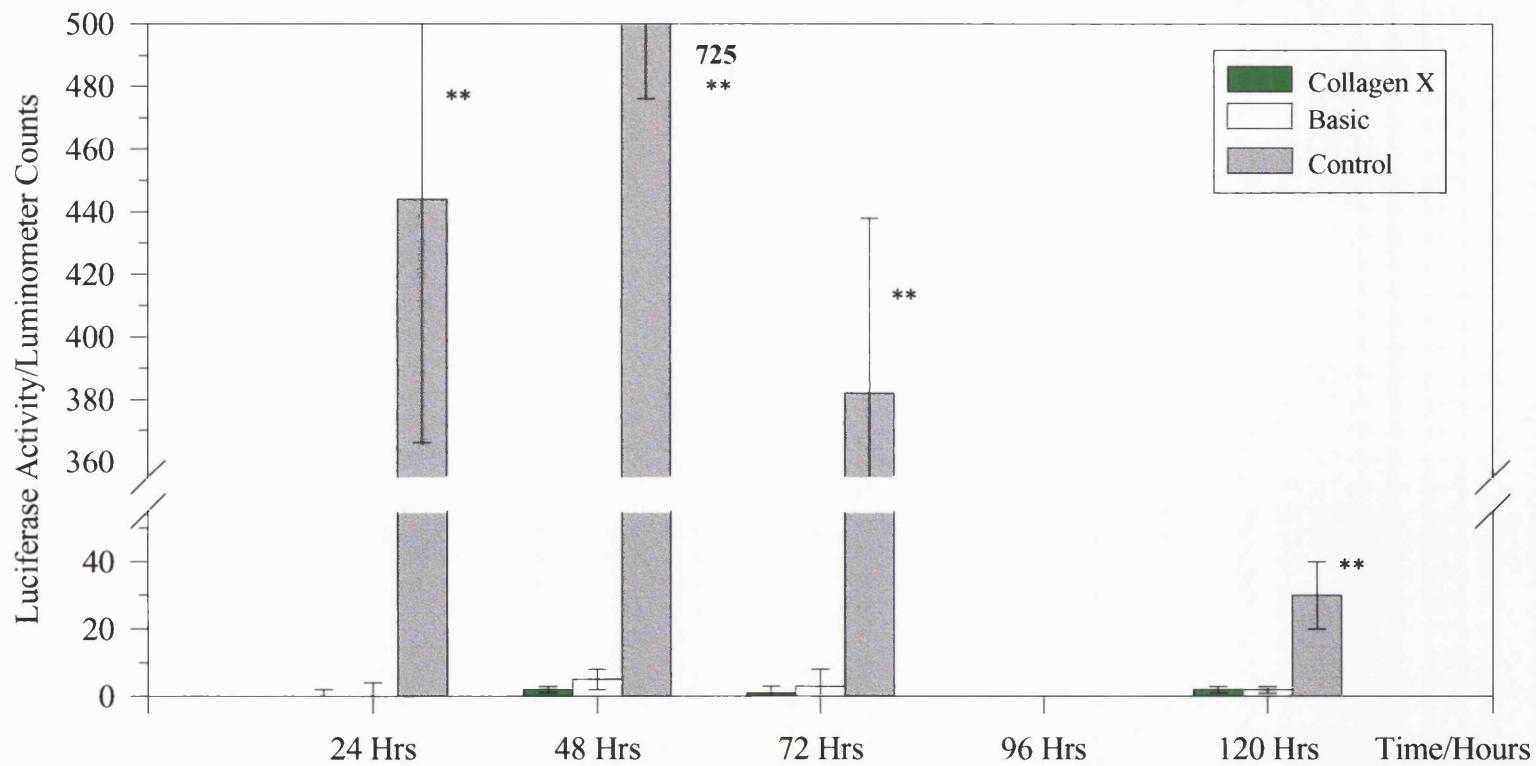


Figure 4.11: Expression of Type X Collagen Reporter Constructs.

(** = significant difference between Control & Basic at the 99% confidence limit) ($n = 4$) Note: In this figure, Control luciferase activity is higher than in previous figures. This variation was a function of the system. For this reason, transfections with pGL2 Control and pGL2 Basic were included in each transfection experiment.

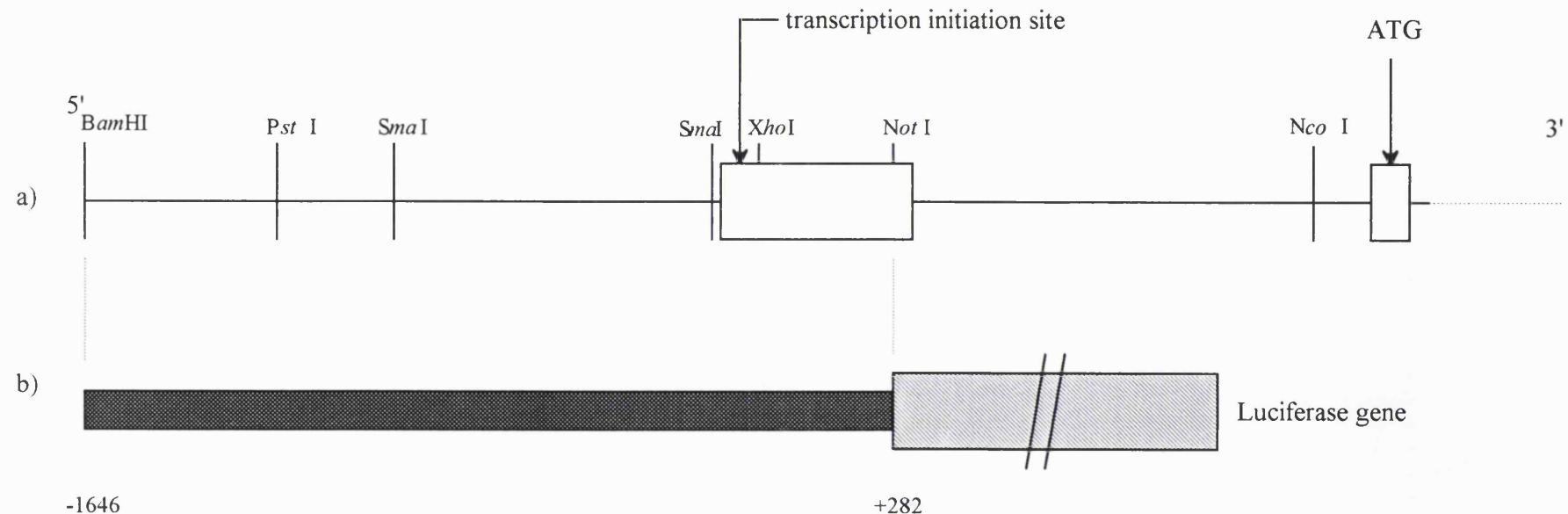


Figure 4.12: Map of the chicken RIHB promoter luciferase construct. This construct was a gift from Dr. D. Duprez and Dr. M. Vigny, from INSERM, Paris (Duprez & Vigny, 1994).

- a) Map of promoter region of genomic clone of chicken RIHB. The white boxes represent the exons.
- b) Map of chicken RIHB luciferase promoter construct, D129. This construct contains all the promoter region required for correct expression.

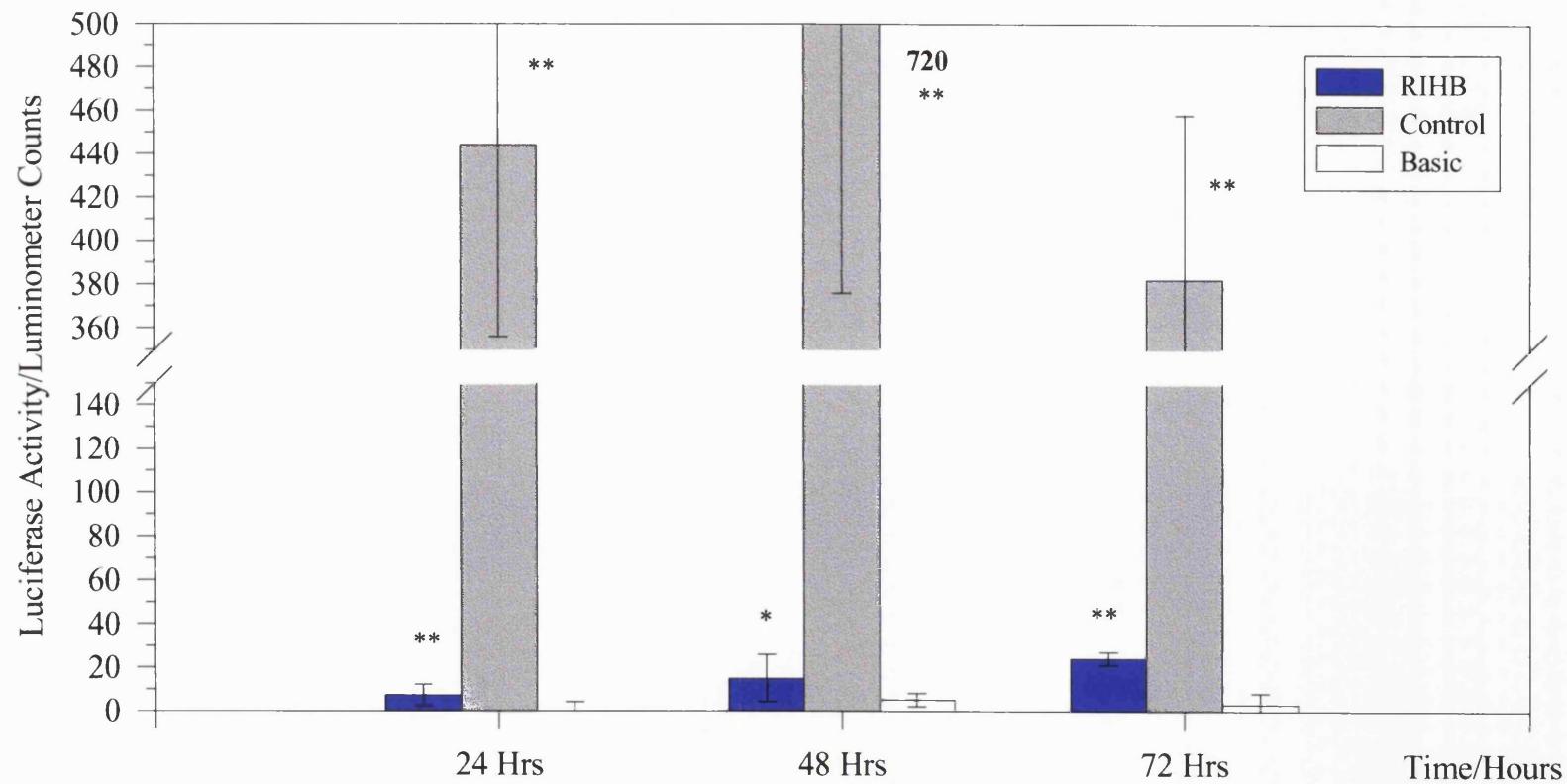


Figure 4.13: Expression of RIHB Promoter Construct.

(** = significantly different from Basic at the 99% confidence limit; * = significantly different from Basic at the 95% confidence limit) (n = 8)

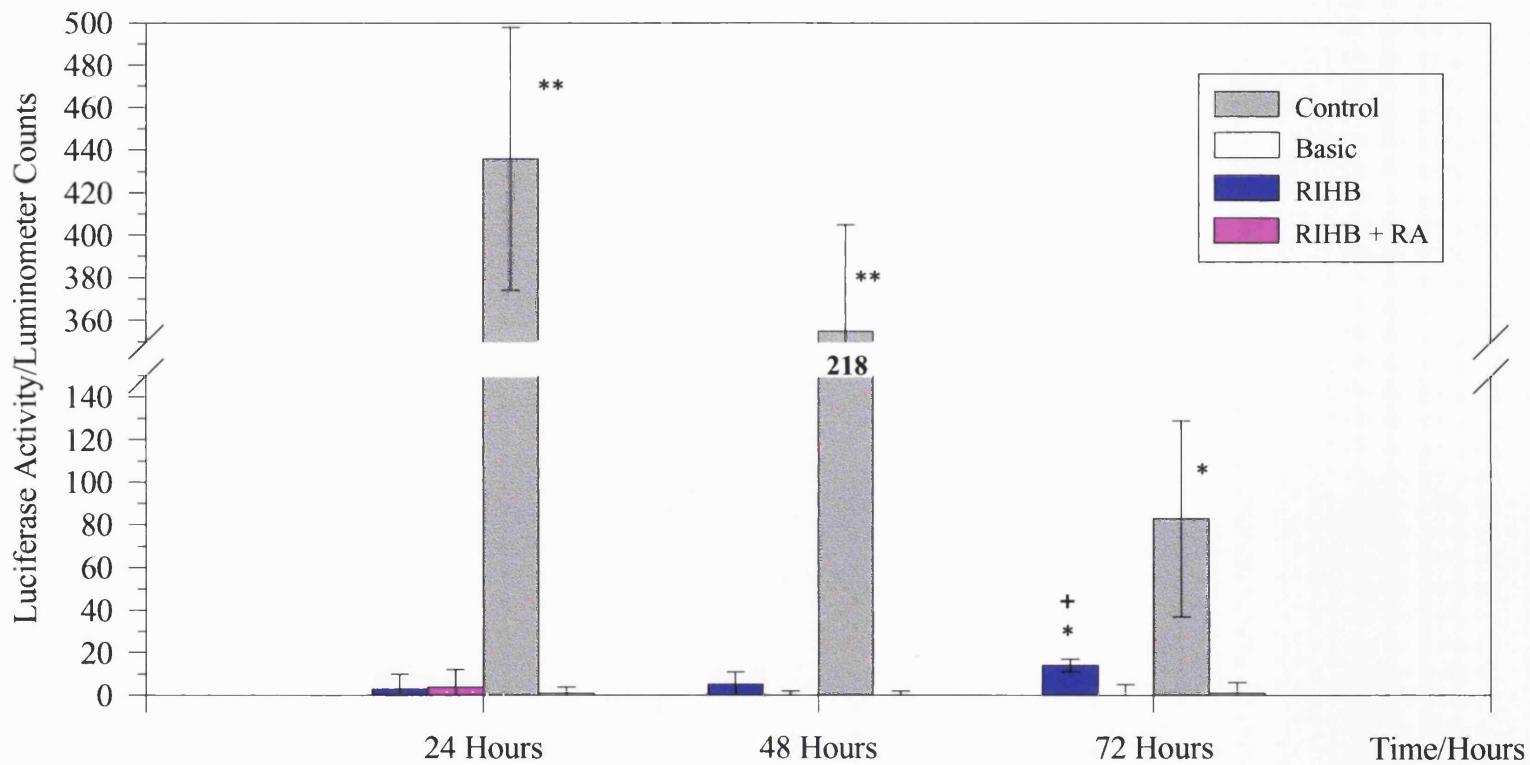


Figure 4.14: Effects of RA on RIHB Promoter Construct Expression.

(** = significantly different from Basic ($p=99\%$); * = significantly different from Basic ($p=95\%$);

+ = significantly different from RIHB + RA ($p=95\%$)) ($n = 3$)

Vigny in their cell line transfection experiments (Duprez, personal communication). In our cell system, since the pGL2 Control levels are relatively weak themselves compared to pGL2 Basic, RIHB expression levels are barely above background, despite the sensitivity of the luciferase assay system.

Cells transfected with the RIHB promoter construct were treated with RA to see if it was possible to stimulate RIHB promoter activity, as described by Cockshutt *et al* (1993) and Raulais *et al* (1991). At first RA was added to cells isolated from the whole limb bud. In these initial experiments, the addition of RA did not stimulate luciferase activity, if anything it appeared to repress activity (Figure 4.14).

However, other experiments in which I added RA to cells isolated from either the anterior or the posterior halves of limb buds, (see below, Section e) iii.)), showed that application of RA to cells, at the doses used in these experiments (50 nM), caused the posterior cells to die, whilst anterior cells survived. This could account for the drop in luciferase activity seen in the RA-treated cells transfected with the RIHB promoter construct (Figure 4.14).

I therefore transfected cells isolated from anterior and posterior halves of the limb bud with the RIHB promoter construct and then treated the anterior cells with RA.

Again, there was minimal luciferase activity in anterior or posterior cells after 24 or 48 hours in the presence or absence of RA (Figure 4.15.). At 72 hours, luciferase expression is significantly above background levels in both posterior and anterior cells. There was no significant difference between luciferase activity in posterior and anterior cells not treated with RA (Figure 4.15). This agrees with the data of Duprez and Vigny (Duprez, personal communication) showing that RIHB is expressed uniformly across the limb and shows no differences in anterior-posterior expression.

RA induces a small but significant increase in luciferase activity in anterior cells (Figure 4.15). So, RIHB expression is inducible by RA in these cells, as well as in the cell lines tested by Duprez and Vigny, Cockshutt *et al* (1993) and Raulais *et al* (1991).

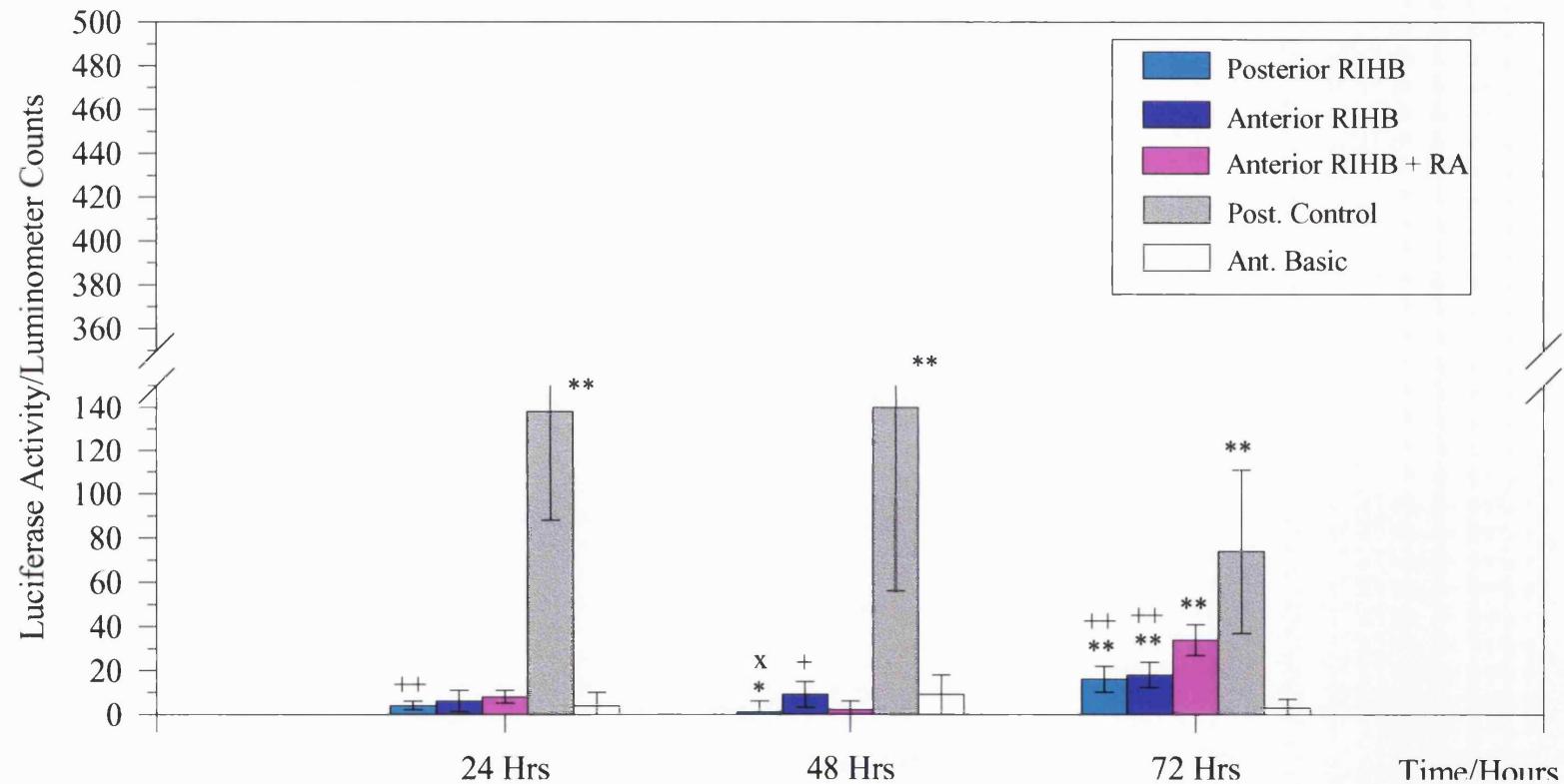


Figure 4.15: Effects of RA on RIHB Promoter Construct Expression in Cells Isolated from Anterior and Posterior Halves of Limb Buds. (** = significantly different from Basic ($p=99\%$); * = significantly different from Basic ($p=95\%$); ++ = significantly different from RIHB + RA ($p=99\%$); + = significantly different from RIHB + RA ($p=95\%$); x = significantly different from Anterior RIHB ($p=95\%$)) ($n = 8$)

Thus, we have demonstrated that a promoter construct of RIHB, a gene normally expressed ubiquitously in the developing limb, is expressed in this transfected cell system, with the correct spatial pattern, and expression is inducible by exogenous factors added to the cell medium, in a similar way to that reported in cell lines. In summary, therefore, these cells seem to maintain their natural expression characteristics after isolation and electroporation, and this system appears to provide a good model to study the regulation of expression of genes that are normally expressed in the developing limb bud.

e) Transfection of Mesenchyme Cells with a Neuronal Reporter Construct:

Another set of chick reporter constructs being studied in our laboratory was based on the promoter of the neuronal nicotinic acetylcholine receptor $\alpha 2$ subunit gene (Bessis *et al*, 1993). Although this gene is normally only expressed in a specific region of the chick brain, in a small group of cells in the diencephalon, the reporter constructs do not contain the tissue-specific regions of the promoter. They contain the SV40 promoter, preceded by varying numbers of tandem repeats of an *Oct*-like motif, which has been shown to act as either a silencer or enhancer, depending on the number of repeats present, and the type of cell in which it is expressed (Figure 4.16) (Bessis *et al*, 1993). These constructs have been shown to be expressed in non-neuronal cell lines as well as in chick fibroblast cells, so it was decided to test expression of these constructs in our primary chick mesenchyme cells, as a further control.

The constructs used in initial transfection experiments were the two longest promoter constructs: $\alpha 2.6$, containing all six *Oct*-like motifs, and $\alpha 2.4$, which lacks two of the *Oct*-like motifs (Figure 4.16d and e).

The $\alpha 2.6$ construct acted as a silencer in all the transfection experiments done by Bessis *et al* (1993), whereas the $\alpha 2.4$ construct acted as a strong enhancer of the SV40 promoter driving luciferase expression in all the cell types tested. Further deletion of this promoter region decreases the enhancer effect, until all the *Oct*-like motifs have been deleted, when the promoter region has no effect on reporter promoter activity (Figure 4.16d and e).

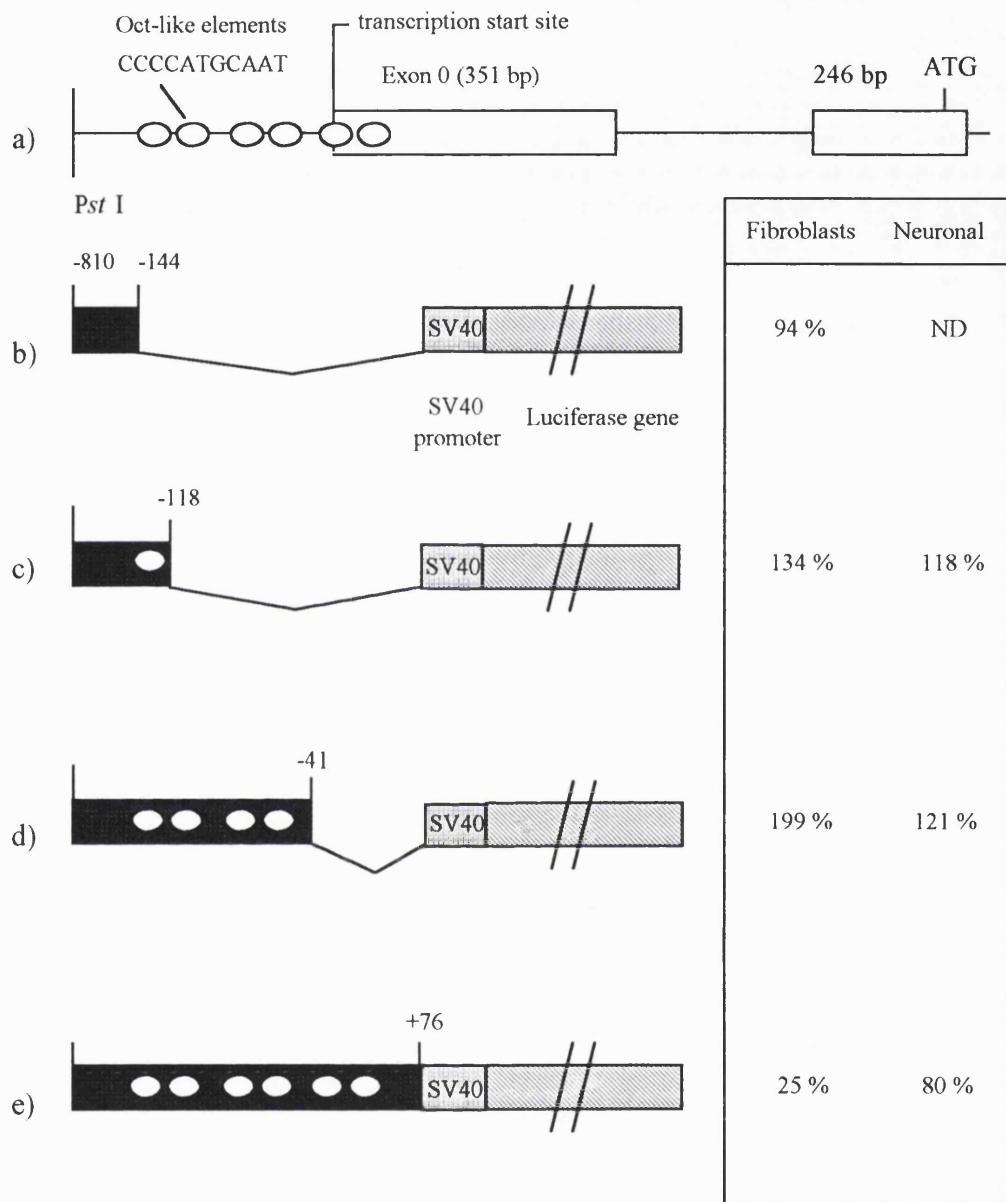


Figure 4.16: Maps of Neuronal Nicotinic Acetylcholine Receptor $\alpha 2$ Subunit Gene

Promoter Constructs, and their percentage luciferase activities in fibroblasts and cells of the SVLT neuronal cell line, compared to activity driven by the SV40 promoter alone.

a) Map of $\alpha 2$ subunit gene. b) Map of $\alpha 2.0$.

c) Map of $\alpha 2.1$. d) Map of $\alpha 2.4$.

e) Map of $\alpha 2.6$.

i) Transfection results with the α 2.6 promoter construct:

Cells isolated from whole limb buds were transfected with the α 2.6 construct and then harvested after 24, 48 or 72 hours, and assayed for luciferase expression.

Surprisingly, the α 2.6 construct directed significantly higher levels of luciferase expression in the transfected limb bud cells than the SV40 promoter construct, pGL2 Control, even though the α 2.6 construct contains the same SV40 promoter preceded by six elements which supposedly act as silencers (Figure 4.17) (Bessis *et al*, 1993).

Luciferase activity in cells transfected with α 2.6 was 10-100 times stronger than in those transfected with the SV40 promoter positive Control. Activity also lasted longer in cells transfected with this construct than with Control, with luciferase levels still significantly above background ($p=99\%$) after 72 hours (Figure 4.18).

ii) Expression of α 2.6 in Cells Isolated from Anterior and Posterior Halves of Limb Buds:

To further investigate the properties of the α 2.6 construct in the chick limb bud cells, cells were isolated from either the anterior or the posterior halves of chick limb buds and transfected with this construct. These cells were harvested and assayed for luciferase expression 24, 48 or 72 hours after transfection.

There was a marked difference in the levels of expression of these constructs between cells isolated from the anterior and posterior halves of the limb bud, at stages 17-21. Expression was almost twice as high in posterior cells as it was in cells isolated from the anterior half of the limb bud, and this difference was maintained throughout culture, for 48 hours (Figures 4.19 and 4.20).

To control for differences in transfection efficiency, experiments were also done comparing levels of expression of pGL2 Control and Basic in anterior and posterior cells, and these constructs showed no significant difference in expression between anterior half or posterior half cells (Figure 4.21). This was supported by experiments in which dot blots of lysed transfected cells were hybridised to a probe complementary to the luciferase gene (Abken and Reifenrath, 1992). The levels of hybridisation signal

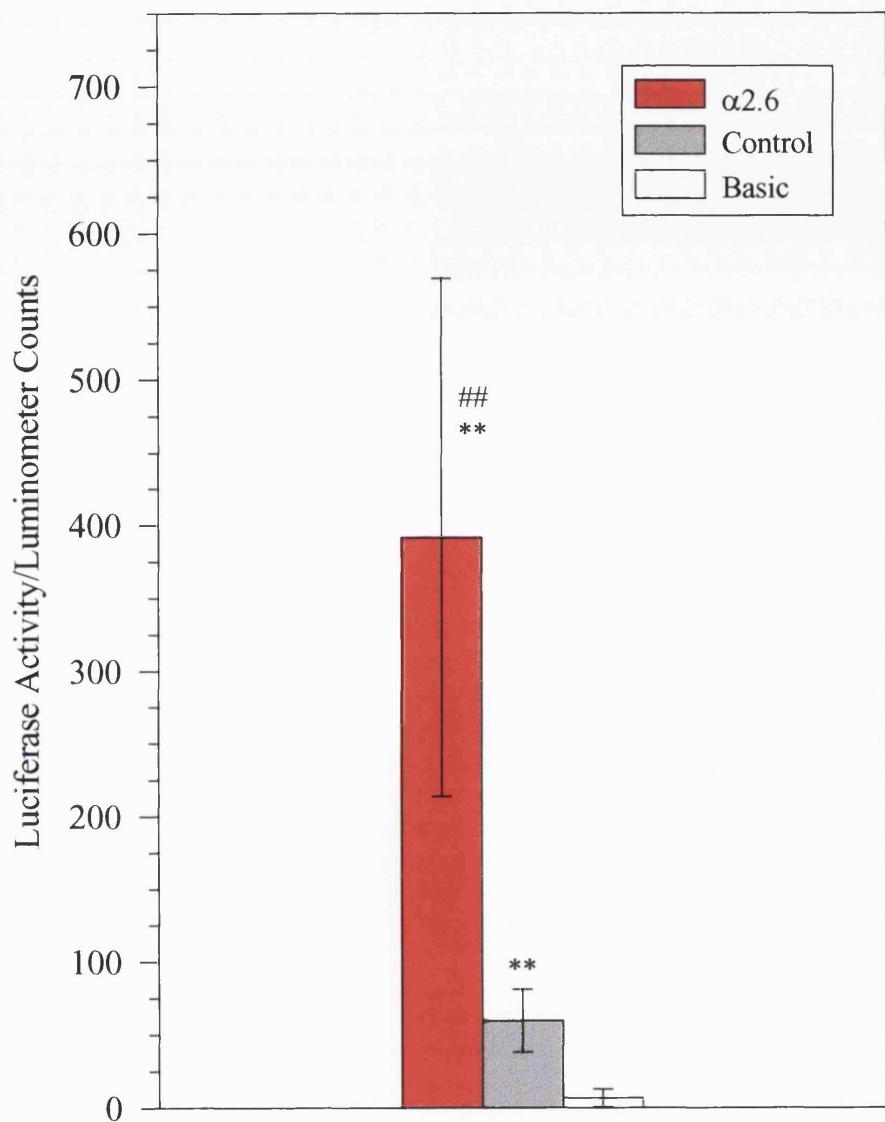


Figure 4.17: Expression of α 2.6 in Chick Mesenchyme Cells.

Luciferase expression was measured 24 hours after transfection.
(n = 8)

(** = significantly different from Basic (p=99%);

= significantly different from Control (p=99%))

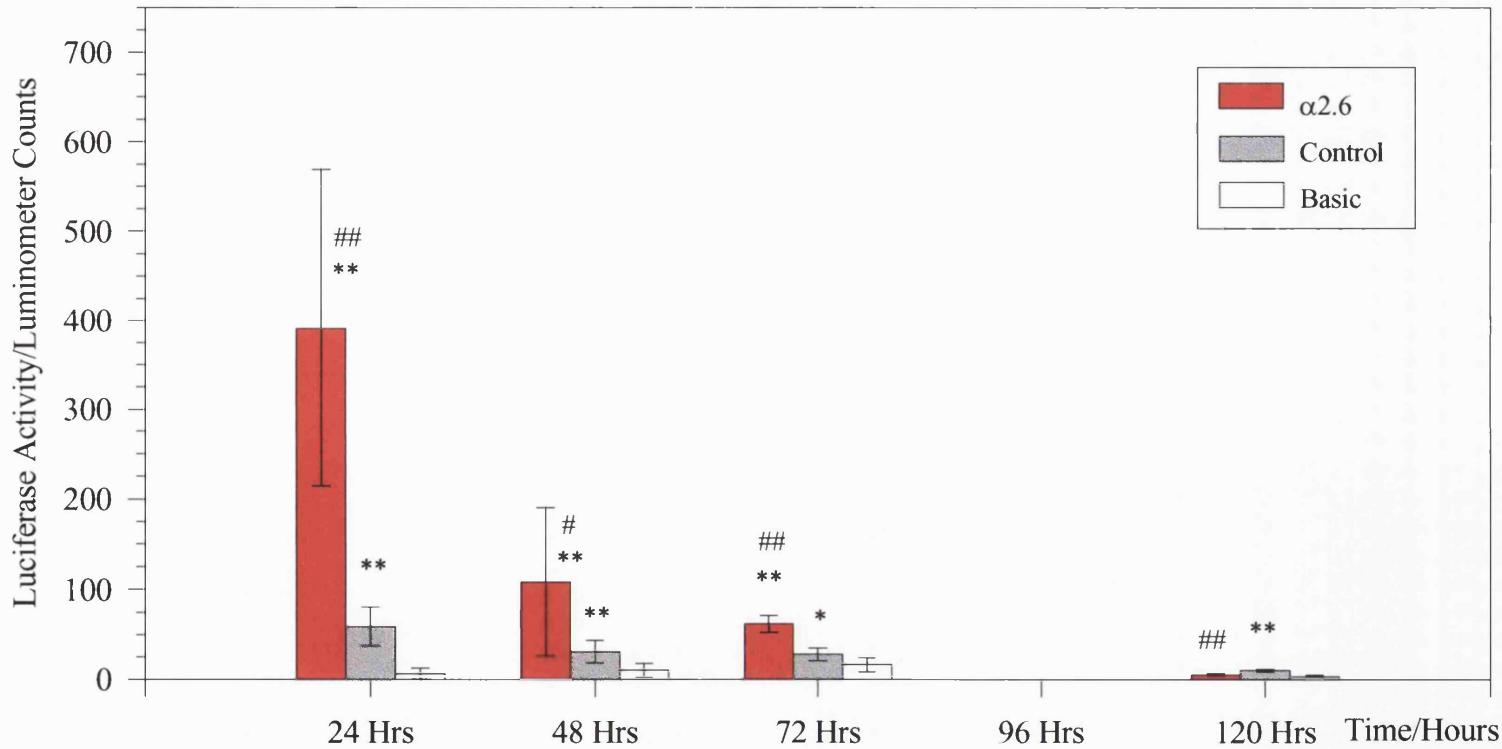


Figure 4.18: Time Course of Expression of α 2.6 in Chick Mesenchyme Cells.

(** = significantly different from Basic ($p=99\%$); * = significantly different from Basic ($p=95\%$); ## = significantly different from Control ($p=99\%$); # = significantly different from Control ($p=95\%$)) (24 Hrs, 48 Hrs: $n = 8$; 72 Hrs: $n = 6$; 120 Hrs: $n = 4$)

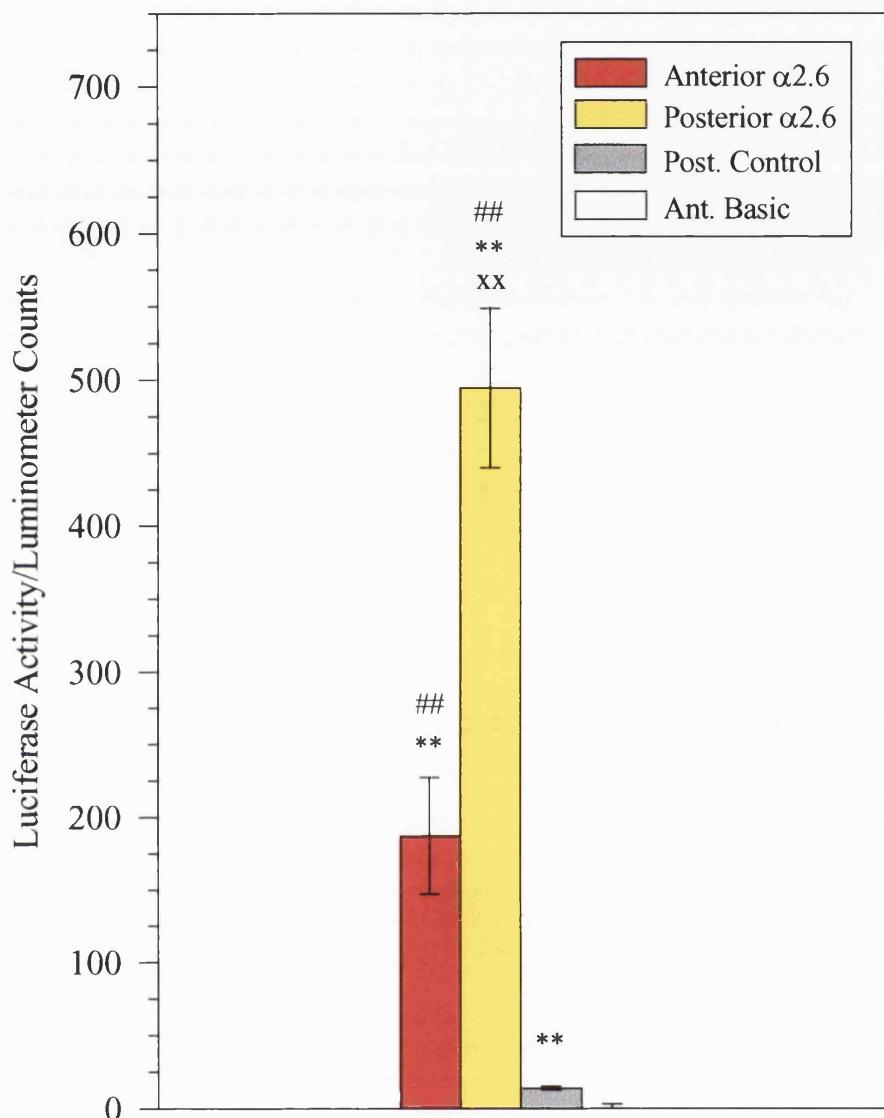


Figure 4.19: Expression of α 2.6 in Cells Isolated from Anterior and Posterior Halves of Limb Buds.

Luciferase expression was measured 24 hours after transfection.

(** = significantly different from Basic ($p=99\%$); xx = significantly different from Anterior α 2.6 ($p=99\%$); ## = significantly different from Control ($p=99\%$)) ($n = 10$)

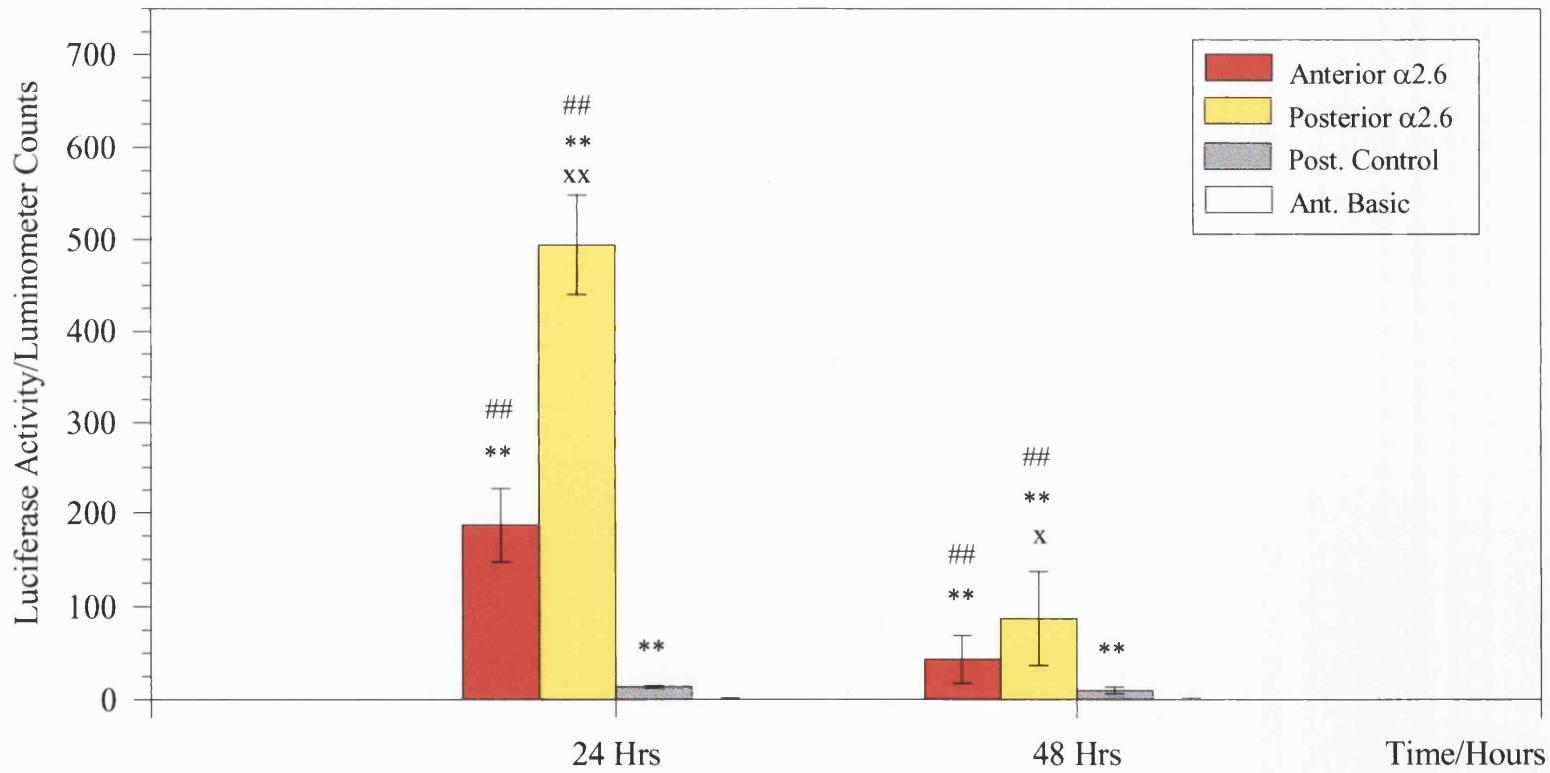


Figure 4.20: Time Course of Expression of α 2.6 in Cells Isolated from Anterior and Posterior Halves of Limb Buds.

(** = significantly different from Basic ($p=99\%$); xx = significantly different from Anterior α 2.6 ($p=99\%$);

x = significantly different from Anterior α 2.6 ($p=95\%$); ## = significantly different from Control ($p=99\%$)) ($n = 10$)

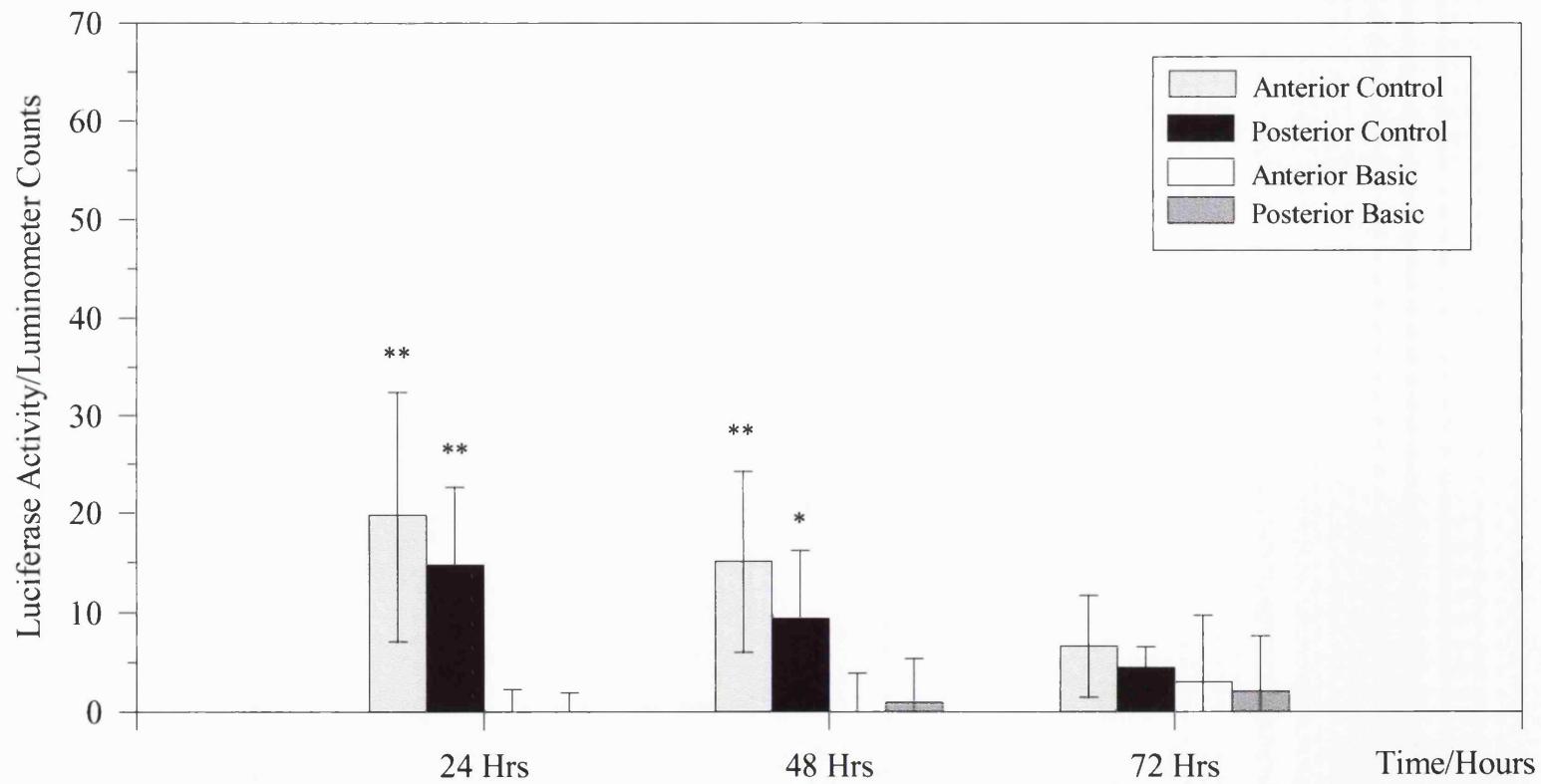


Figure 4.21: Expression of pGL2 Control and pGL2 Basic in Cells Isolated from Anterior and Posterior Halves of Limb Buds.

(** = significantly different from Basic ($p=99\%$); * = significantly different from Anterior Basic ($p=95\%$))

(24 Hrs: n = 6; 48 Hrs: n = 4; 72 Hrs: n = 3)

showed that approximately equal amounts of the different plasmid constructs were present and therefore that similar amounts of the different constructs are taken up by the cells (Figure 4.22).

Expression is not entirely absent in anterior cells. This could be the true pattern of expression directed by this promoter in these cells. Alternatively it could be due to contamination by posterior cells, or could result from the anterior cells gaining posterior characteristics in culture, as described by Wanek *et al* (1994). One way to check this would be to grow the cells in the presence of FGF, which should maintain the cells as they were in either the posterior or anterior state (Vogel and Tickle, 1993; see Section 4.4). If the low levels of expression in anterior cells were the result of anterior cells gaining posterior characteristics during the culture period, treatment with FGF should abolish luciferase activity in anterior cells transfected with these constructs.

Alternatively, the regulatory elements in these promoters may be responding to something other than a simple anterior-posterior difference, such as a possible morphogen gradient from the polarising region. If this were the case, expression would be expected to be lower, but not completely abolished in the anterior half of limb buds.

iii) RA Treatment of Cells Transfected with α 2.6:

To further investigate the anterior/posterior spatial signals that the α 2.6 construct was responding to in the chick mesenchyme cells, transfected cells were treated with all-*trans*-RA during culture (See Chapter 1; Thaller *et al*, 1993). Hayamizu and Bryant (1992) have shown that, under micromass culture conditions, anterior cells acquire posterior properties after culture in the presence of RA, and that digit duplications result if these RA-treated cells are grafted into the anterior region of host chick limb buds. All their RA-treated grafted cells were washed for 60 minutes before grafting to eliminate the possibility of carry-over of RA. The best results (>70% polarising activity) resulted after culture in the presence of 50 nM RA for 20 hours. This concentration is similar to that used to get full mirror-image digit duplications and ectopic expression of putative patterning genes when exogenously applied to the limb

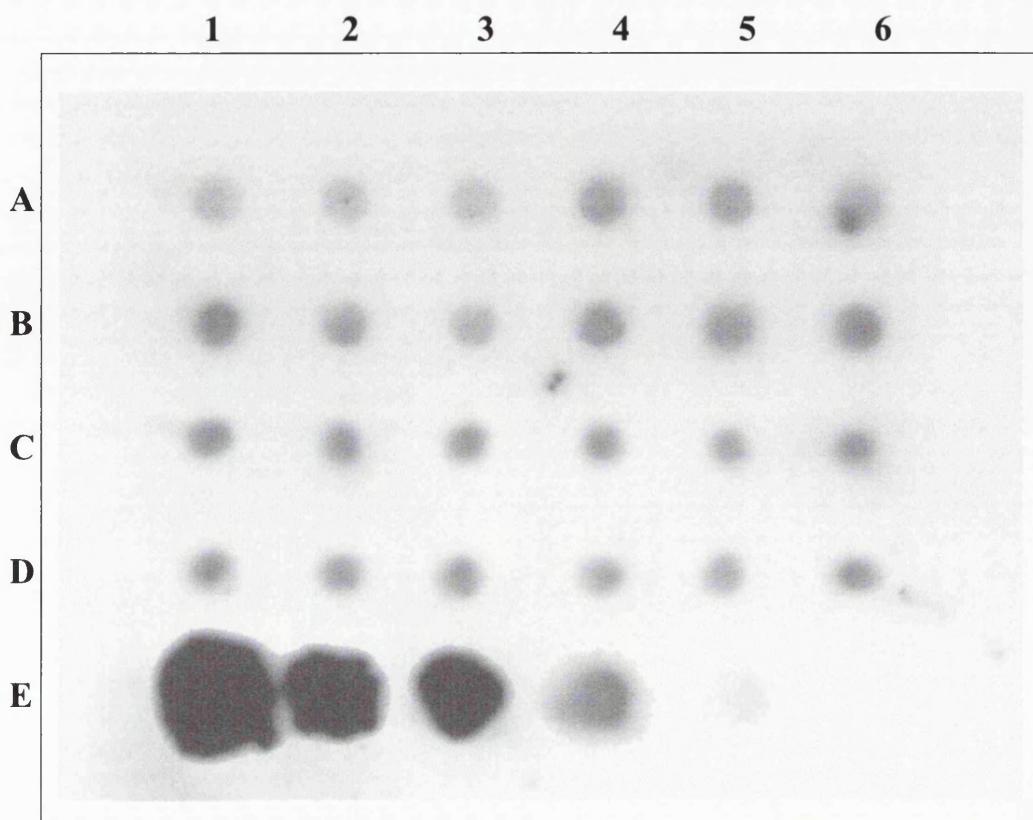


Figure 4.22: Dot Blot of lysed transfected chick limb bud mesenchyme cells showing levels of expression of different constructs. The cells were lysed 24 hours after transfection. Similar results were seen after 48 hours (data not shown). This blot was hybridised to a probe complementary to the luciferase gene.

A 1 - 3: Anterior cells transfected with pGL2 Basic

A 4 - 6: Posterior cells transfected with pGL2 Basic

B 1 - 3: Anterior cells transfected with pGL2 Control

B 4 - 6: Posterior cells transfected with pGL2 Control

C 1 - 3: Anterior cells transfected with α 2.6

C 4 - 6: Posterior cells transfected with α 2.6

D 1 - 3: Anterior cells transfected with α 2.4

D 4 - 6: Posterior cells transfected with α 2.4

E 1 - 4: Positive Control: Luciferase gene DNA at 1 μ g, 100 ng, 10 ng, and 1 ng

E 5: Negative control: Untransfected mesenchyme cells

bud (25 nM) (Tickle and Eichele, 1985), and to the concentration found to be present endogenously in chick limb bud (approx. 25 nM: 50 nM posteriorly and 20 nM anteriorly) (Thaller and Eichele, 1985, 1987).

I applied all-*trans*-RA by diluting it in the culture medium, to a final concentration of 50 nM. The cells were then cultured in the dark, to prevent the isomerisation of the all-*trans* RA to other forms.

Treating anterior cells, transfected with α 2.6, with all-*trans*-RA at 50 nM induced luciferase activity in anterior cells to the same level as found in posterior transfected cells, within 48 hours of treatment (Figures 4.23 and 4.24).

Experiments were also done treating anterior and posterior cells transfected with pGL2 Control with RA to check whether the RA-induction effect was specific to the nicotinic acetylcholine receptor constructs or whether it affected transcription in the cells in a more general way. RA treatment of pGL2 Control-transfected anterior cells had no effect on luciferase activity, whereas treatment of posterior cells transfected with pGL2 Control actually decreased luciferase activity (Figure 4.25). This effect was also seen to some extent in posterior cells transfected with α 2.6 and treated with RA. Examination of the RA-treated posterior cells showed that there were far fewer cells present in the culture after 24 hours than were initially plated out, or than were present in the other wells. The RA therefore appears to have a toxic effect on cells isolated from the posterior halves of limb buds, but not on anterior cells. There is some evidence for this in experiments done with high concentrations of RA applied to limb buds causing loss of digits in chicks, mice and amphibians (Tickle and Eichele, 1985; Summerbell, 1983; Lee and Tickle, 1985; Maden, 1985).

iv) Kinetics of Induction of α 2.6 by RA:

Time courses were also carried out to inquire into the kinetics of the induction effect of RA on the promoter construct α 2.6. (Figure 4.26.) Cells were transfected and cultured as described above, but samples were lysed and luciferase activity measured at time points of 1 hour, 2 hours, 4 hours, 6 hours or 24 hours after transfection.

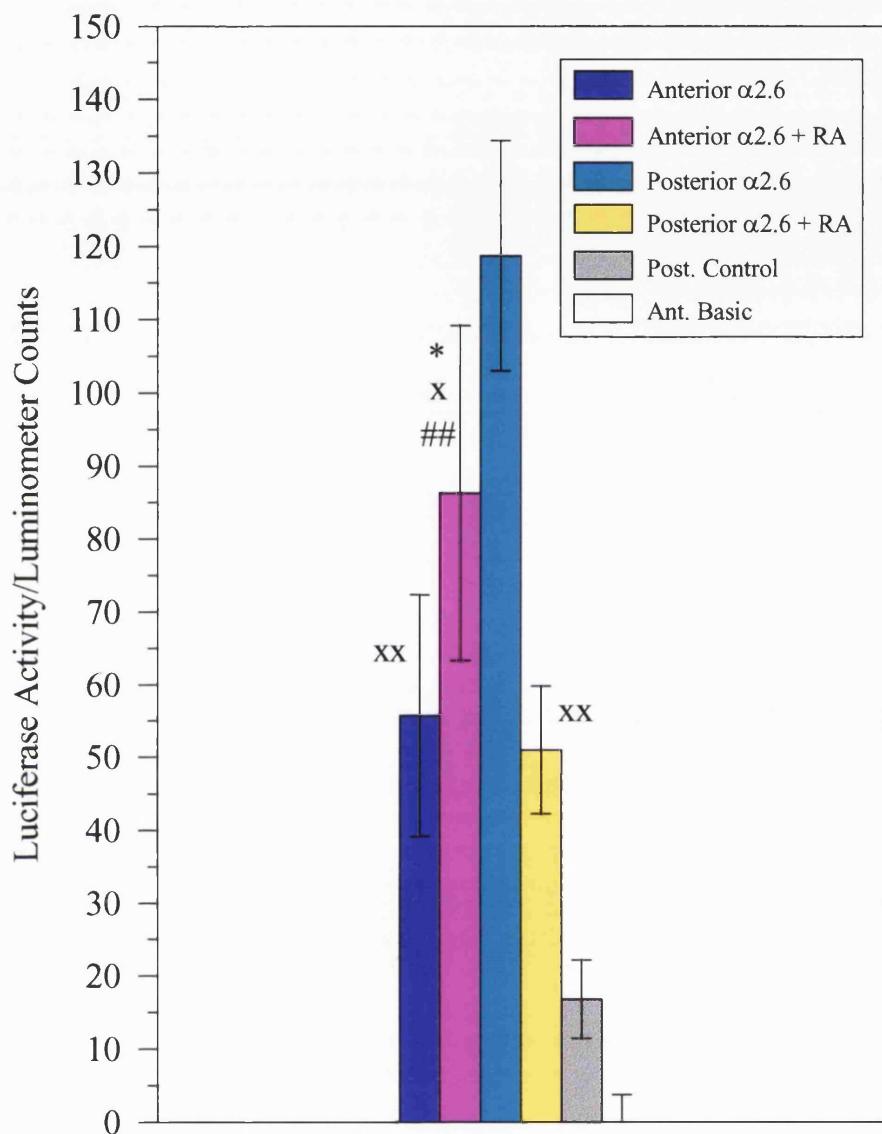


Figure 4.23: Effects of RA on Expression of α 2.6.

Luciferase expression was measured 24 hours after transfection.

(* = significantly different from Anterior α 2.6 ($p=95\%$); xx = significantly different from Posterior α 2.6 ($p=99\%$); x = significantly different from Posterior α 2.6 ($p=95\%$); ## = significantly different from Posterior α 2.6 + RA ($p=99\%$)) ($n = 6$).

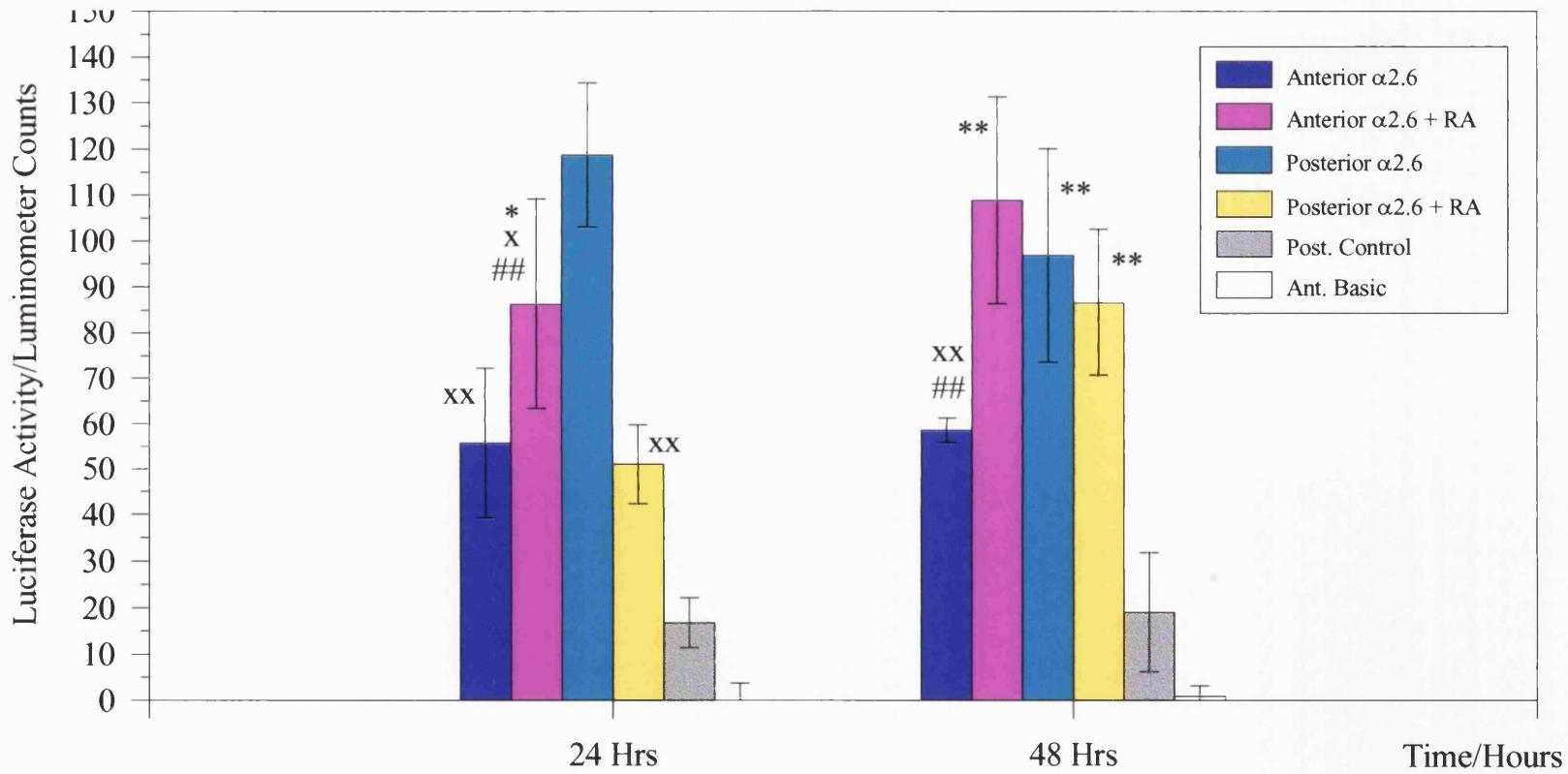


Figure 4.24: Effects of RA on Expression of α 2.6.

(* = significantly different from Anterior α 2.6 ($p=95\%$); ** = significantly different from Anterior α 2.6 ($p=99\%$); xx = significantly different from Posterior α 2.6 ($p=99\%$);
 x = significantly different from Posterior α 2.6 ($p=95\%$); ## = significantly different from Posterior α 2.6 + RA ($p=99\%$) ($n = 6$).

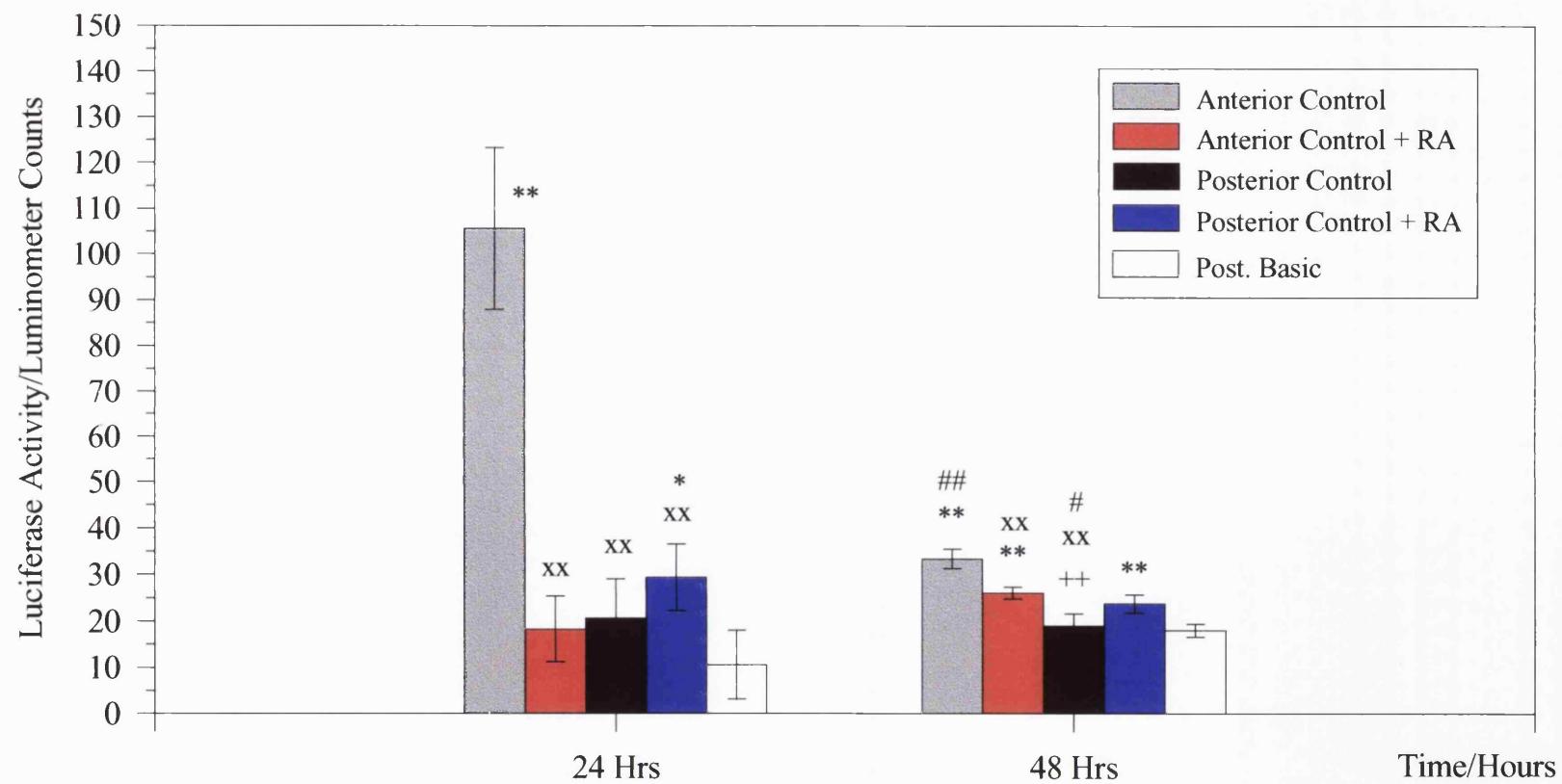


Figure 4.25: Effects of RA on Expression of pGL2 Control.

(** = significantly different from Basic ($p=99\%$); * = significantly different from Basic ($p=95\%$); xx = significantly different from Ant. Control ($p=99\%$); ## = significantly different from Post. Control+RA ($p=99\%$); ++ = significantly different from Ant. Control+RA ($p=99\%$); # = significantly different from Post. Control+RA ($p=95\%$) (n = 4)

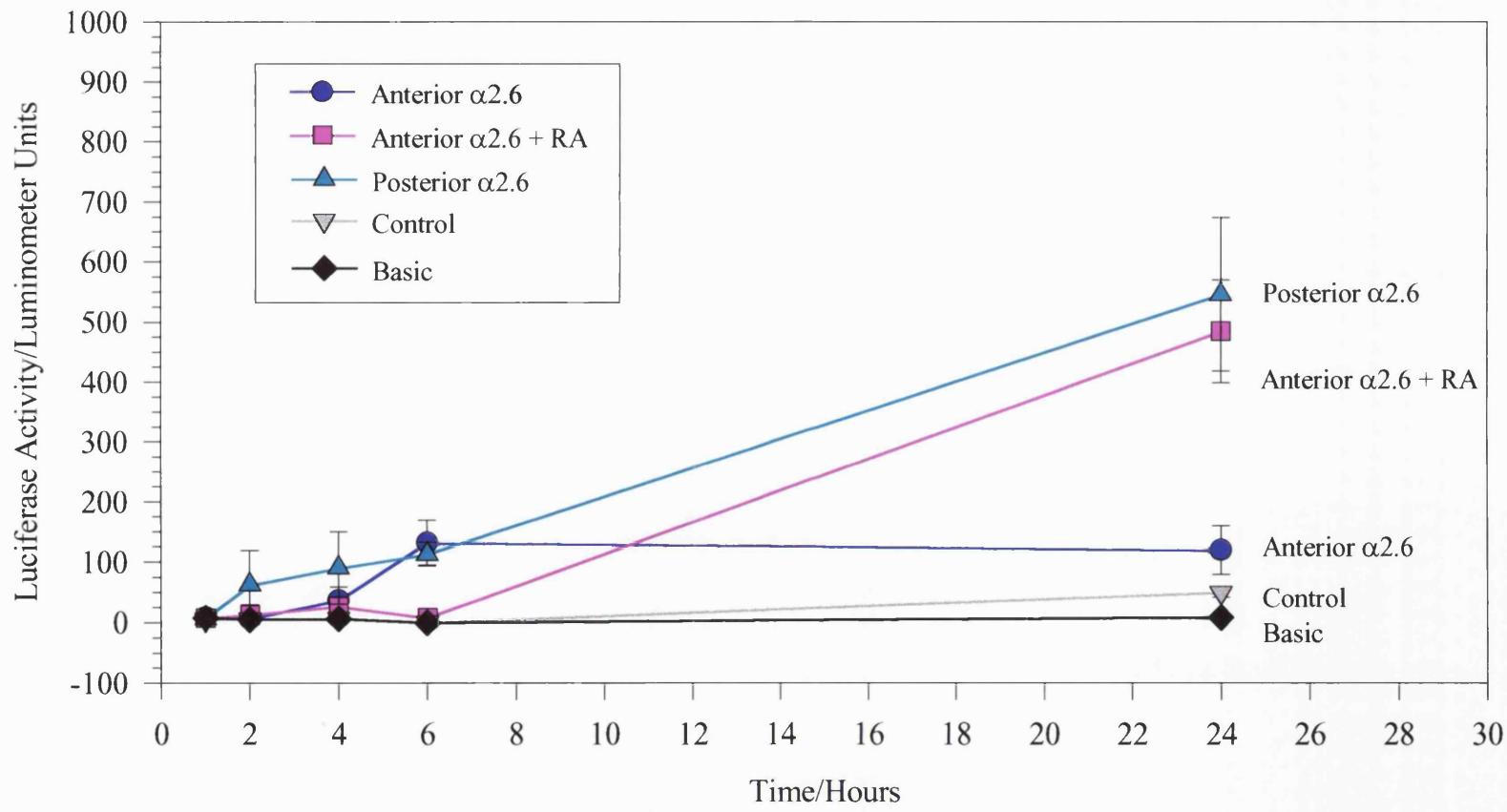


Figure 4.26: Time Course of the Effects of RA on Expression of α 2.6.

For figure legend, see next page.

Legend for Figure 4.26: After 2 hours, there are significant differences between Posterior α 2.6 and Basic levels ($p = 99\%$), and Anterior α 2.6 + RA and Basic ($p = 95\%$); Posterior α 2.6 is also significantly different from Anterior α 2.6 + RA ($p=95\%$).

After 4 hours, Posterior α 2.6 is significantly above Basic ($p = 99\%$), Anterior α 2.6 ($p = 99\%$) and Anterior α 2.6 + RA levels ($p = 99\%$); and Anterior α 2.6 and Anterior α 2.6 + RA are significantly above Basic ($p = 99\%$).

After 6 hours, Posterior α 2.6 and Anterior α 2.6 are both significantly above Basic ($p = 99\%$) and Anterior α 2.6 + RA ($p = 99\%$). There is no significant difference between Basic and Anterior α 2.6 + RA at this time.

After 24 hours, Posterior α 2.6 and Anterior α 2.6 + RA are both significantly above Basic ($p = 99\%$) and Anterior α 2.6 ($p = 99\%$); and Anterior α 2.6 is significantly above Basic ($p = 99\%$). There is no difference between Posterior α 2.6 and Anterior α 2.6 + RA. Control is significantly above Basic levels for the first time ($p = 99\%$).

It was found that more than 6 hours are needed for RA to induce luciferase activity in anterior cells transfected with these constructs. This means that RA is relatively unlikely to be acting directly on these promoters. However, the kinetics of the induction are not straightforward: the RA has no effect for the first 4 hours, with luciferase activity in anterior RA-treated cells being indistinguishable from background, despite the fact that luciferase activity in anterior and posterior cells transfected with α 2.6 is above background after 4 hours. In fact luciferase activity in posterior cells transfected with α 2.6 is above background levels after only 2 hours, long before pGL2 Control shows activity. Between 4 hours and 6 hours, however, RA treatment seems to inhibit luciferase expression in anterior cells to levels below those expressed in untreated anterior cells. Subsequently, at 24 hours, the levels rise to far exceed those of untreated anterior cells, and are close to the levels expressed by untreated posterior cells.

v) Transfection results with the α 2.4 promoter construct:

Similar experiments to those described above were also carried out with the α 2.4 construct, which lacks two of the *Oct*-like motifs found in α 2.6. In the experiments done by Bessis *et al* (1993), α 2.4 acts as a moderately strong enhancer of expression, in all the cell lines tested.

This shorter construct, α 2.4, was also expressed more strongly than the SV40 positive Control in limb bud mesenchyme cells, but less strongly than the α 2.6 construct, with luciferase counts being approximately 2.5 times those of the positive control, pGL2 Control (Figure 4.27).

The kinetics of the time course of expression of α 2.4 over the course of 72 hours are also similar to those of α 2.6 (Figure 4.28).

In all other respects, the expression of the α 2.4 construct is very similar to that of the α 2.6 construct.

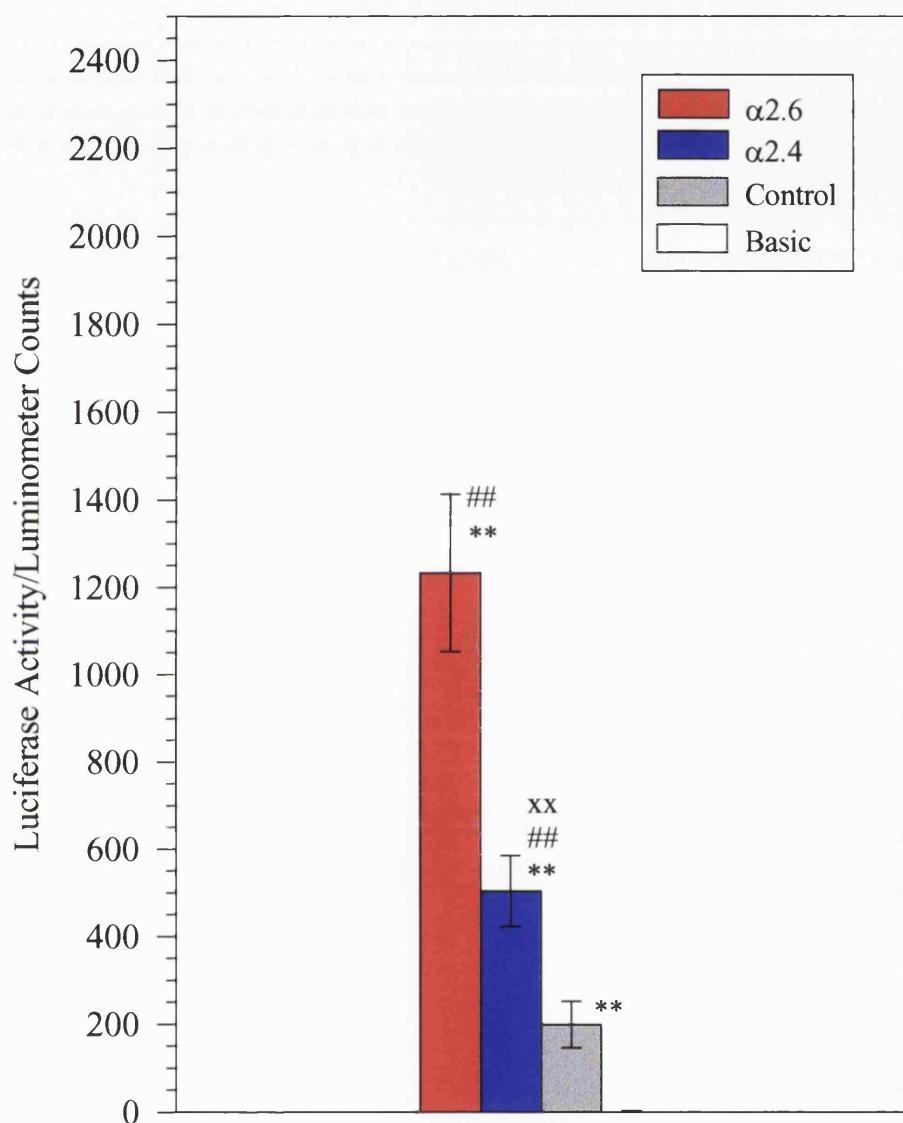


Figure 4.27: Expression of α 2.6 and α 2.4.

Luciferase expression was measured 24 hours after transfection.

(**) = significantly different from Basic ($p=99\%$);

(##) = significantly different from Control ($p=99\%$);

xx = significantly different from α 2.6 ($p=99\%$) ($n = 6$)

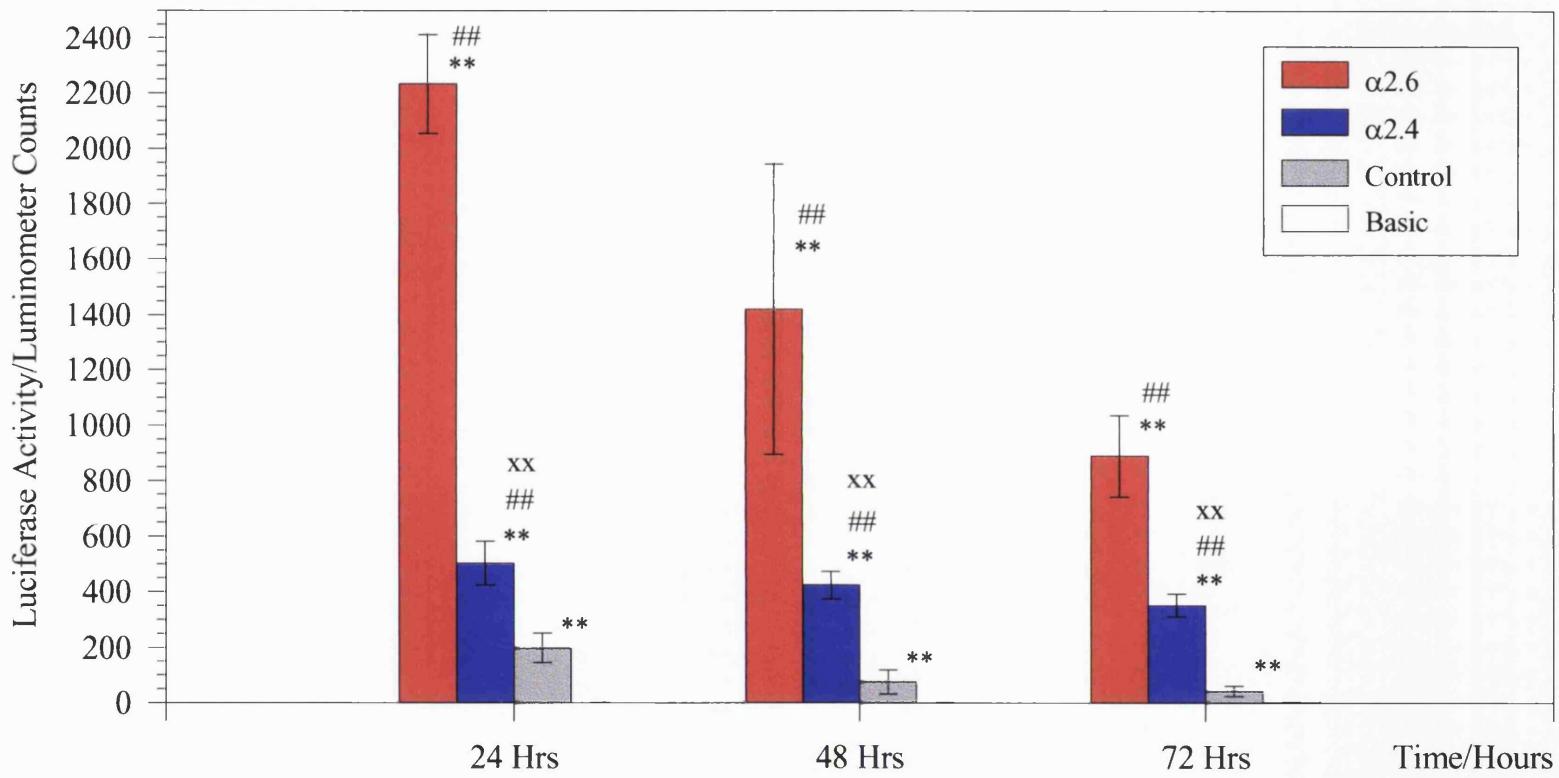


Figure 4.28: Time Course of Expression of α 2.6 and 2.4.

(** = significantly different from Basic ($p=99\%$); ## = significantly different from Control ($p=99\%$);

xx = significantly different from α 2.6 ($p=99\%$) ($n = 6$)

It is more strongly expressed in posterior cells than anterior ones (Figures 4.29 and 4.30): α 2.6 is still expressed at higher levels than α 2.4, although the ratio between anterior cells and posterior transfected cells is similar between the two constructs, with the expression levels in posterior cells being roughly double those in anterior ones.

Expression of α 2.4 is also inducible by RA in anterior cells, raising expression levels to approximately the same levels as those found in posterior cells (Figures 4.31 and 4.32).

vi) Transfection Results with other Deletion Constructs:

Anterior and posterior cells were also transfected with shorter α 2 constructs (α 2.0 and α 2.1) (Figure 4.16b and c), and then harvested and assayed for luciferase activity after 24 or 48 hours. Construct α 2.0 contains none of the enhancer elements, only the promoter region upstream of this region, and had no effect on the expression of the SV40 promoter in the experiments done by Bessis *et al* (1993). Construct α 2.1 contains the promoter region plus one enhancer element, and was found by Bessis *et al* (1993) to be a weak enhancer of luciferase activity.

In transfection experiments with chick mesenchyme cells, α 2.0 directed no luciferase activity, in either anterior or posterior cells, despite the presence of the SV40 promoter in this construct. (Figure 4.33)

α 2.1 was found to have very weak activity, approximately 10-50% of that of the SV40 promoter alone. The α 2.1 construct also showed no apparent anterior-posterior differences in expression. (Figure 4.33)

In addition, application of RA did not induce the expression of either of these two constructs, and, in fact, appeared to inhibit expression. (Figure 4.34)

The apparent lack of anterior-posterior differences and lack of RA inducibility of these two constructs could occur because the region conferring RA inducibility has been deleted in these shorter constructs (Figure 4.35).

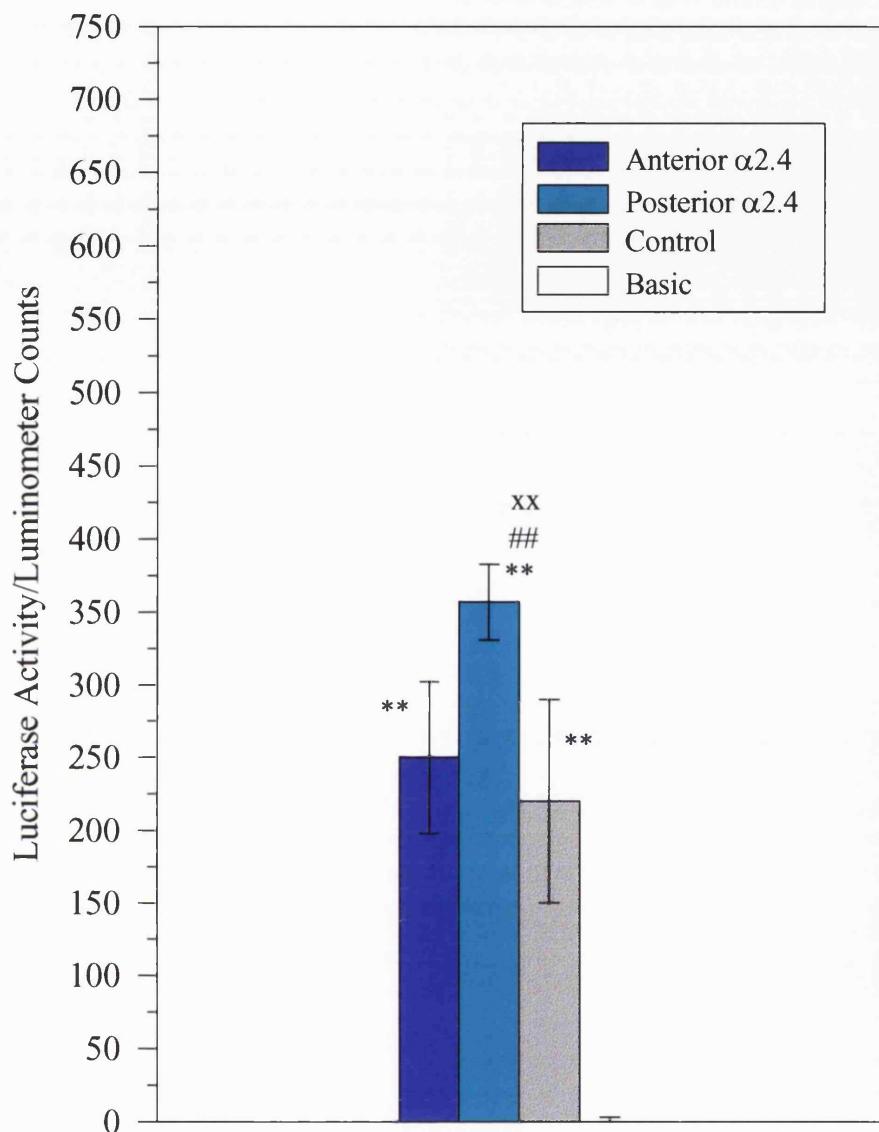


Figure 4.29: Expression of $\alpha 2.4$ in Cells Isolated from Anterior & Posterior Halves of Limb Buds.

Luciferase expression was measured 24 hours after transfection.

(** = significantly different from Basic ($p=99\%$);

= significantly different from Control ($p=99\%$);

xx = significantly different from Ant. $\alpha 2.4$ ($p=99\%$) ($n = 8$)

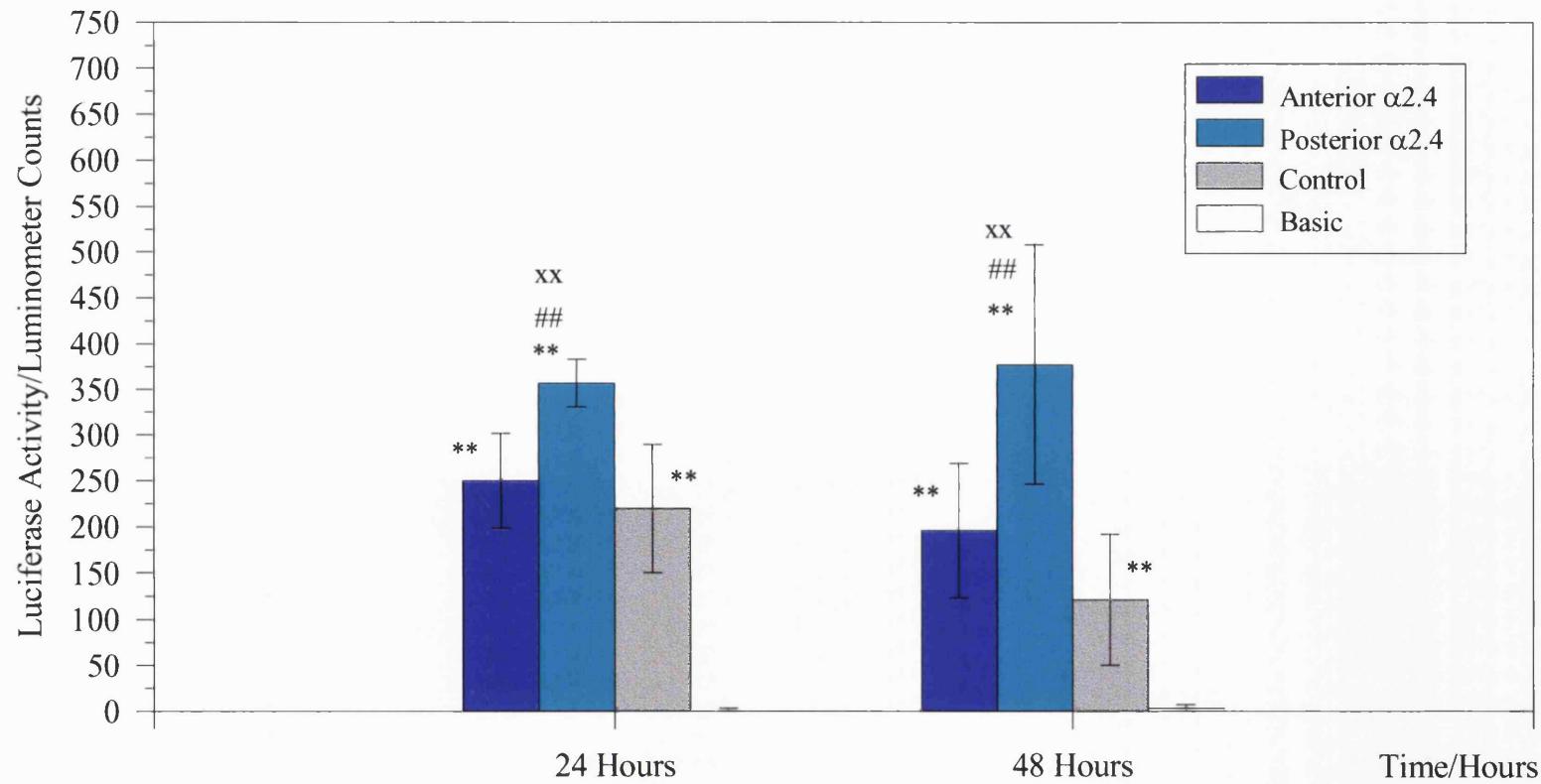


Figure 4.30: Expression of α 2.4 in Cells Isolated from Anterior and Posterior Halves of Limb Buds.

(** = significantly different from Basic ($p=99\%$); ## = significantly different from Control ($p=99\%$);

xx = significantly different from Ant. α 2.4 ($p=99\%$) ($n = 8$)

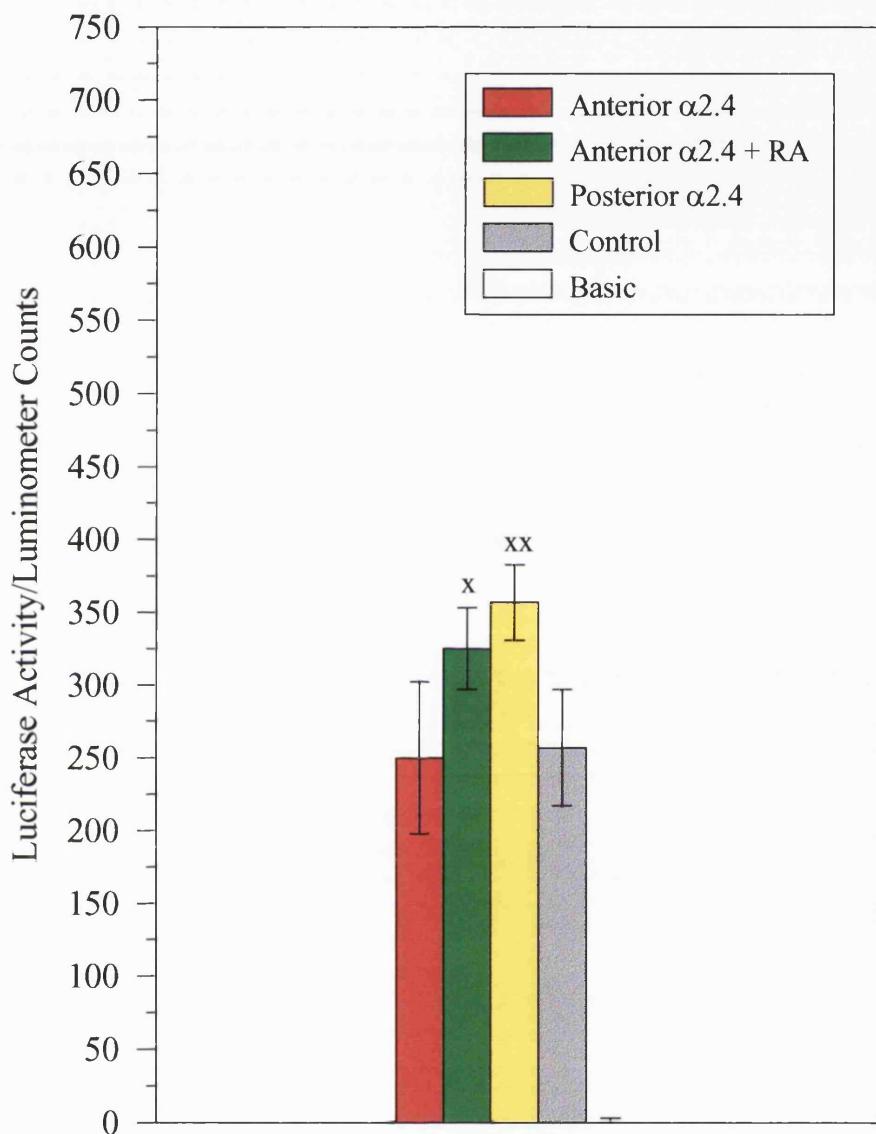


Figure 4.31: Effects of RA on Expression of α 2.4.

Luciferase expression was measured 24 hours after transfection.

(xx = significantly different from Ant. α 2.4 (p=99%);

x = significantly different from Ant. α 2.4 (p=95%) (n = 6)

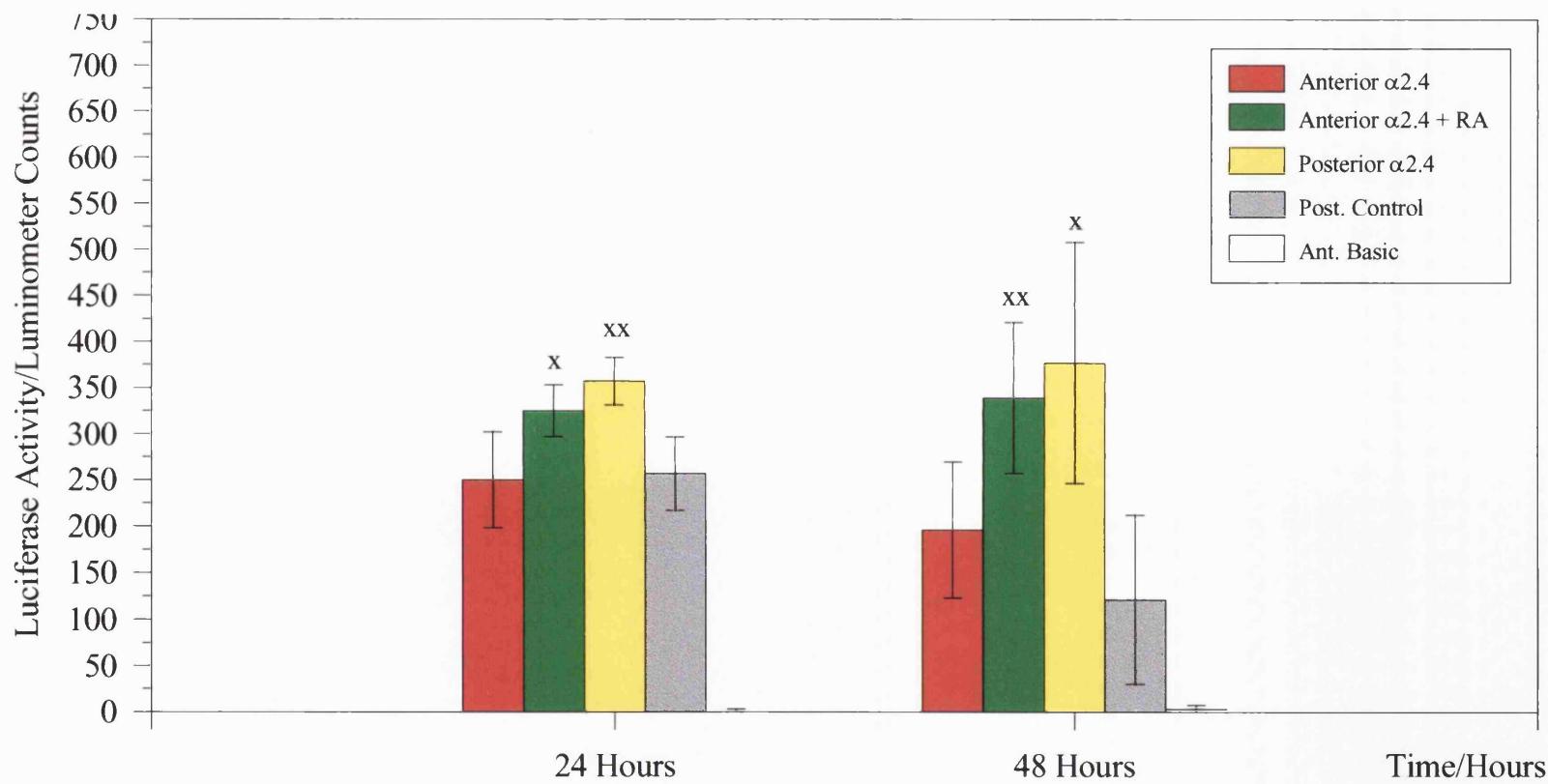


Figure 4.32: Effects of RA on Expression of $\alpha 2.4$.

(xx = significantly different from Ant. $\alpha 2.4$ ($p=99\%$); x = significantly different from Ant. $\alpha 2.4$ ($p=95\%$)) ($n = 6$)

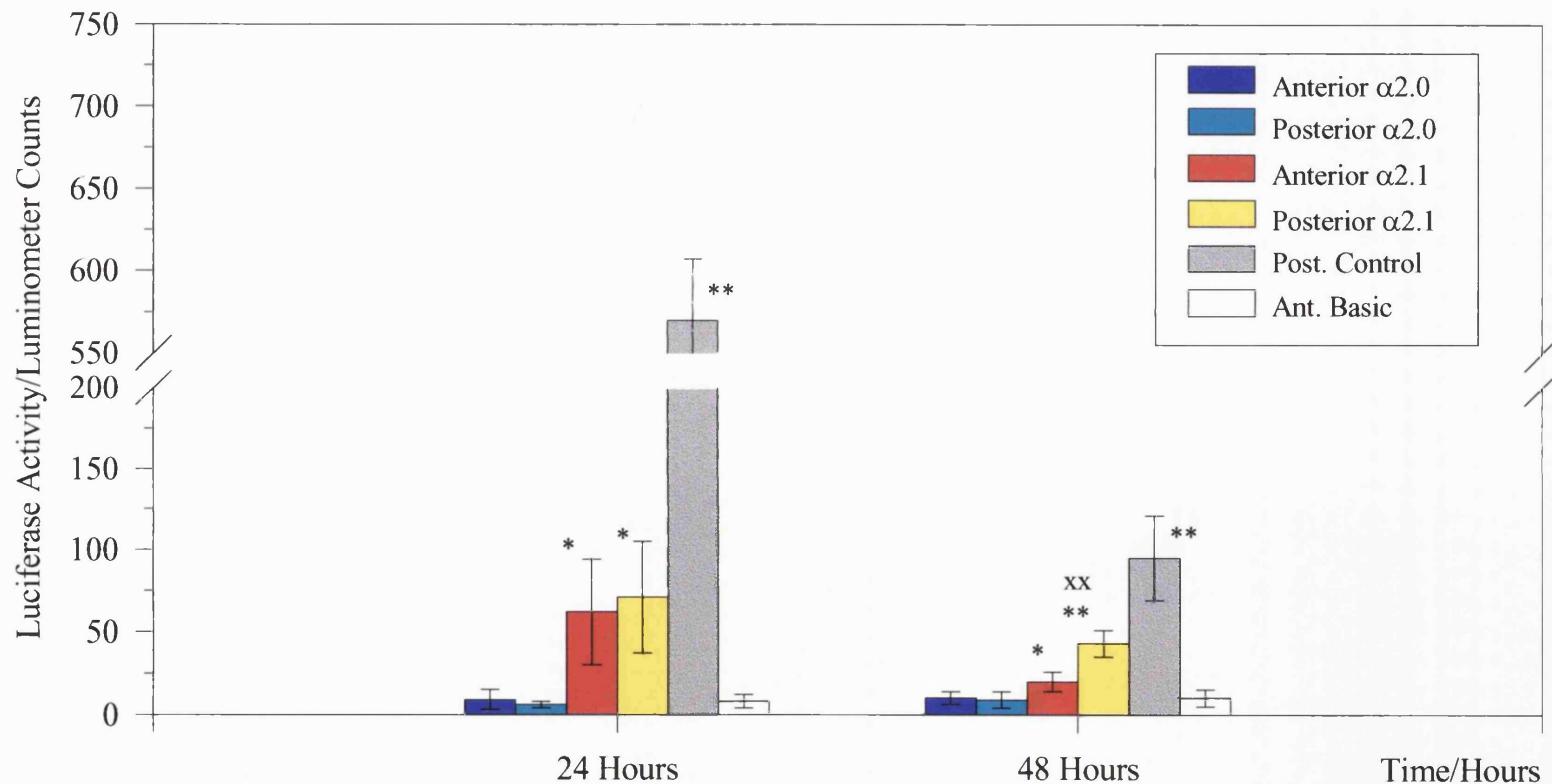


Figure 4.33: Expression of α 2.0 and α 2.1 in Cells Isolated from Anterior and Posterior Halves of Limb Buds.

(** = significantly different from Basic ($p=99\%$); * = significantly different from Basic ($p=95\%$); xx = significantly different from Ant. α 2.1 ($p=99\%$)) ($n = 4$)

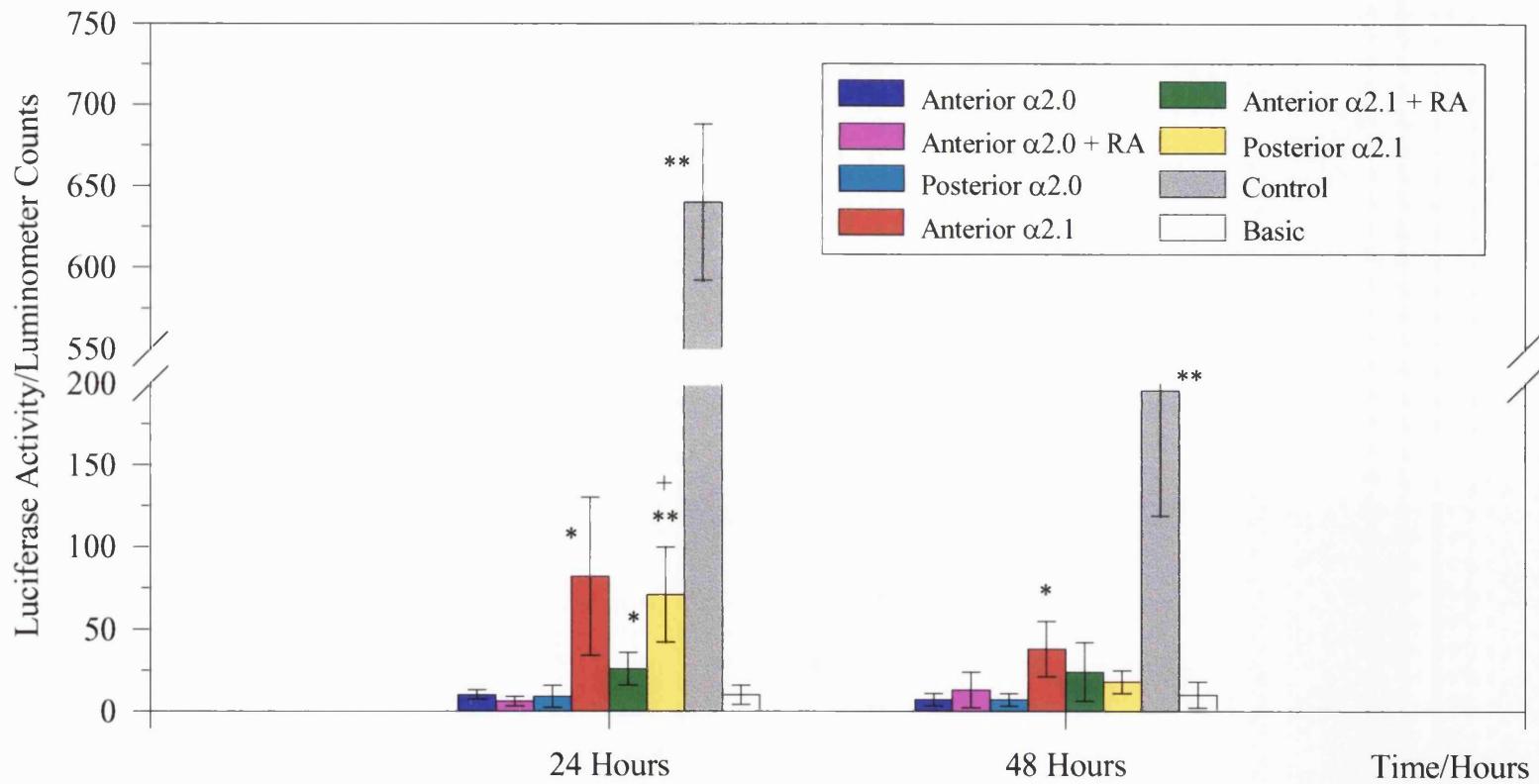


Figure 4.34: Effects of RA on Expression of α 2.0 and 2.1.

(** = significantly different from Basic ($p=99\%$); * = significantly different from Basic ($p=95\%$); + = significantly different from Ant. α 2.1+RA ($p=95\%$)) ($n = 4$)

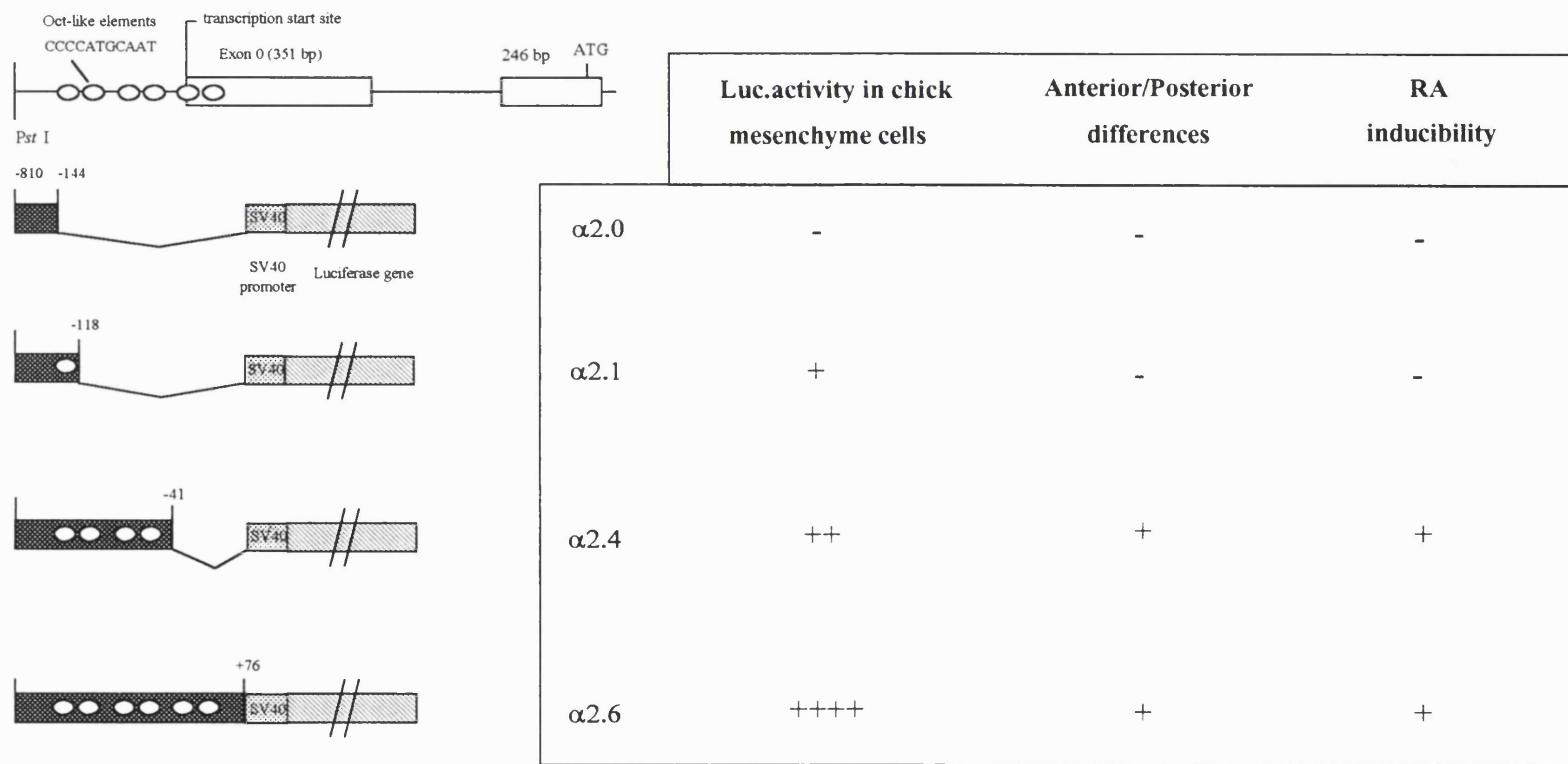


Figure 4.35: Properties of Neuronal Nicotinic Acetylcholine Receptor $\alpha 2$ Subunit Gene Promoter Constructs in transfected chick mesenchyme cells. The SV40 promoter alone directing luciferase activity (pGL2 Control) shows luciferase activity intermediate between $\alpha 2.1$ and $\alpha 2.4$, no differences in activity in anterior and posterior cells, and no RA inducibility. (- = no activity; + = activity (number of + signs indicates relative strength of activity))

-815 ctgcagaatg gggagagaag tacattctgg tgaccc ccaa
 -735 gcaaactgtt ctccgggtcc cc~~d~~**agggd**tg gaaattgcag tcct~~ggc~~**a**t cggtcgcaagg aaggttgtgc agtacatttc
 -655 ctagcaggaa ggaaggaggg agagtggtct **gggtc**agggc t~~g~~a~~g~~c~~t~~**tgc** ccaggagggaa ggcaagaagg atgagctcag
 -575 gtttccc~~at~~ cctcttgcag atggagagcc accccttgc~~c~~ aaagctccgt ccccatccct cg~~ttt~~gc~~a~~ cat cctatggaca
 -495 cctcagtctg cctgttaggtg caaggaaaaa tggtcg~~tgc~~ gggtagggaa gatgcaggat gcttccaa ttgtgtgacg
 -415 ttctccttgg tatcatttt~~a~~ tggag~~tgc~~ cc~~at~~**tg**gcag gttgg~~tcc~~ aaaccagctc **aggc**agg~~gg~~ ggt~~ga~~**at**ggg
 -335 caaaccctaa gatggtgggg **ggc**a~~g~~ctgtc ccattcctgt gcagcagtgc atgggaacag cccatac~~a~~c cct~~t~~gcaaca
 -255 ctgtcg~~a~~tg **ccct**ctgtac cacc~~t~~**gaa** a~~t~~acttcatg ca~~ttc~~act ataacac~~c~~tc cacgtgatgc ccctcataa~~c~~ac
 -175 cagagagcac gtaccc~~cc~~gtt **ca**~~t~~**goccta** ttcaata~~ccc~~ ccatg~~caat~~ c~~cc~~catg~~ca~~ at~~ttt~~ccct gcaataacccc
 -95 atacaat~~tcc~~ **c**catg~~caat~~a **tgc**cctttgc aaag~~cccc~~ t~~g~~caat~~cc~~ cttgc~~at~~ctac cctacg~~cggt~~ **gccc**ccacag
 -15 ca~~cccc~~catgc a~~a~~atccctt gcaata~~cccc~~ atg~~caat~~**g**cc cattgc~~at~~gta tcccacgc~~ag~~ **tgccc**aacac gtggccccat
 +66 gcgtgctgca **cccc**ad~~tgc~~ cc~~c~~cgctgcac atccctacat cccacccg~~ga~~ cg~~c~~aggag~~gg~~ caagaagg~~gg~~ cagg~~gg~~**ggc**a
 +146 cagaaagcct tcctccctc tcctcctcct cctgcagcgc tctggg~~tca~~ **cc**ctcct~~cc~~ t~~c~~c~~t~~ccctt tcg~~t~~**cacc**gc
 +226 tctgaggaca gcagaacatc ctccactgga gcccattgca cgccggagag cctcctcggc acagcatccc cacc~~cc~~g~~tgc~~
 +306 **cct**gggctca g~~cg~~aagg~~gg~~aa cggagcag~~ga~~ cctctctcca gaaaaaaaaa agg~~g~~agg~~aa~~ tct~~tgg~~tca gaggc~~ct~~gga
 +386 cacactatcc agctctgtcc atgcgtcctc tggagcttgc tgctgg~~tgg~~ gacacacagg acacc~~c~~t~~g~~ct ggagatgcct
 +466 cgtgatggct tttcctactg agcccgtcgc tgtgcctacg tctgg~~gtca~~ cactgc~~ag~~ga agcatg

Figure 4.36: Sequence of the upstream region of chicken $\alpha 2$ subunit gene, showing lack of consensus RAREs.

Boxed sequences in red and blue show putative RARE sequences: there are no consensus direct repeat RARE sequences (consensus sequence = $(^G_A G^T / _G TCA) X_{1-5} (^G_A G^T / _G TCA)$). Ringed sequences in black are the enhancer/silencer elements. The bold black lines show where the various constructs end.

Alternatively, the levels of luciferase activity directed by these constructs could be so low that the induction effect is obscured by the variability that occurs at the threshold of detection of the luminometer, such that a doubling in expression levels would not appear to be statistically significant. This could be resolved by generating and testing further constructs with the upstream silencer region found in α 2.0 deleted.

If α 2.1 is truly not RA inducible, the RA-responsive region must lie between nucleotides -118 and +76 of the chicken α 2 subunit gene (Figure 4.36).

vii) Absence of RA Response Elements in the Promoter Region of α 2.6 and 2.4:

In the light of the fact that α 2.6 and 2.4 are RA-inducible in this system, but that no RA response elements (RAREs) were reported when Bessis *et al* (1993) isolated and described this region of the promoter of the neuronal nicotinic acetylcholine α 2 subunit gene, I carried out a sequence search on the promoter. Consensus RARE sequences were taken from Rowe and Brickell (1993), and Blomhoff (1994).

From the sequence analysis, there do not appear to be any RAREs in the promoter of the neuronal nicotinic acetylcholine α 2 subunit gene (Figure 4.36). In the only regions where there are two individual RARE sequences, they are either not direct repeats, or are separated by too many bases, or, in the case of the possible RARE at +193 bp, they are actually located within the transcribed region of the gene, rather than in the promoter region.

In particular, there are no direct repeats of RARE consensus sequences in the region of the promoter that is included in α 2.4 and α 2.6, but excluded from α 2.1, between nucleotides -118 and +76 (Figure 4.36). This region is thought to contain the RA responsive element, because α 2.4 and α 2.6 are both RA-responsive, whereas α 2.1 appears not to be.

f) Promoter Constructs with Regions of the Putative *Bmp-2* Promoter Region:

The aim of designing this reporter system was to test regions of the putative chicken *Bmp-2* promoter from the *Bmp-2* cosmid clone #16F, described in Chapter 3. To further examine the putative promoter region of the *Bmp-2* gene, I attempted to generate reporter constructs, linking various fragments from the upstream region from the cosmid #16F in front of a luciferase gene, so that they could drive luciferase expression.

My aim was to generate a set of constructs of varying lengths, to examine how much upstream sequence was required to drive expression of the luciferase reporter gene. Once a construct was generated that showed promoter activity, further smaller constructs could be made, by creating nested deletions of the promoter fragment used, to further map the regions of the promoter needed to drive correct spatial and temporal expression of the *Bmp-2* gene.

A variation of the pGL2 Basic vector was used, created by Dr. Panos Kefalas and Timothy Brown in our laboratory, incorporating the Bluescript polylinker into the polylinker of pGL2 Basic, because it provides more restriction sites into which to clone. It is called pGL-BSK⁺ (Figure 4.37).

From the sequence obtained of the 5' coding region and 5' untranslated region (Figure 3.15 and 3.16), it was decided to use the *SaII* site which starts 7 bases immediately upstream from the translation initiation codon as the 3' end for all the fragments cloned into the luciferase reporter vector. The only other suitable site was an *NcoI* site, overlapping the initiation codon, but there were no suitable sites in the vector into which to ligate this restriction site. The overhanging end of the *SaII* site anneals to the overhang of the *XhoI* site in the luciferase vector, and the sites are then lost during the ligation.

Two fragments in particular were targeted: the 6.5 kb *KpnI-SaII* fragment and the 4 kb *XhoI-SaII* fragment. These fragments were chosen because they are large enough to span a large amount of the putative promoter region, and of a reasonable size to subclone, and the restriction sites are easy to ligate into the vector in the correct

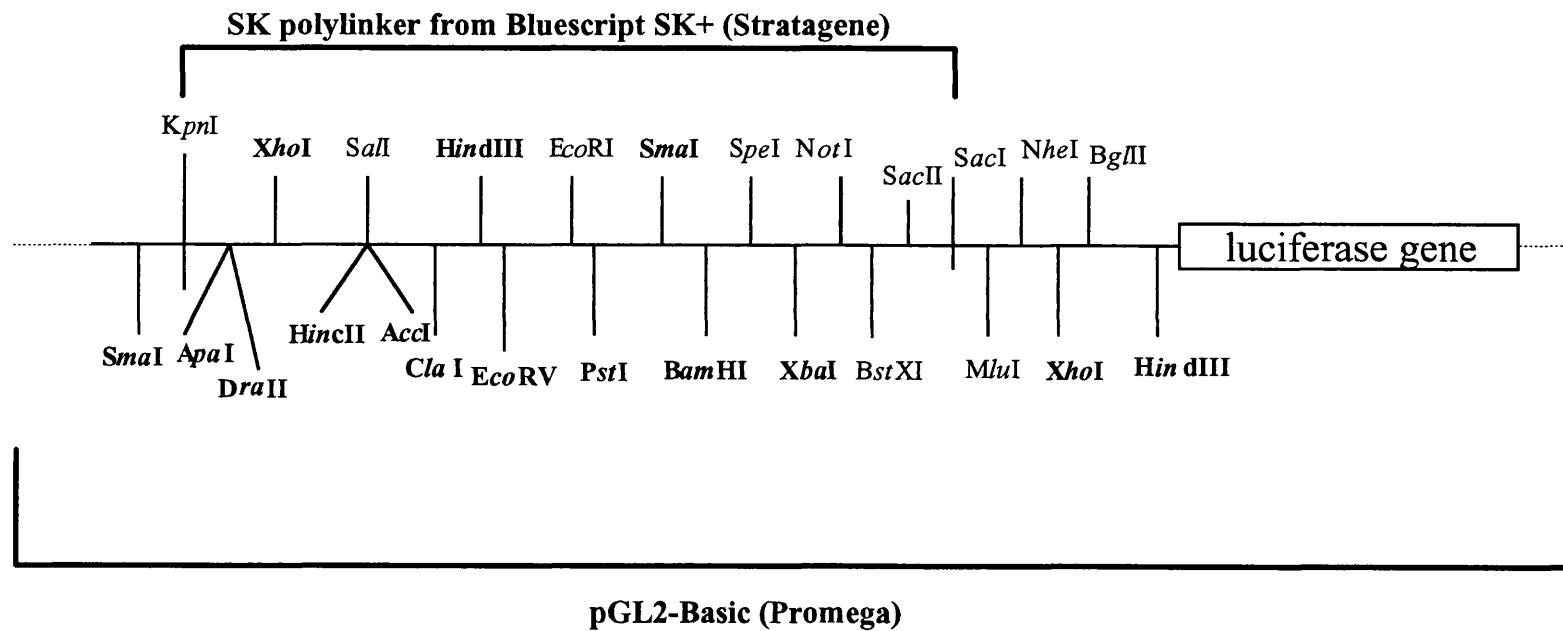


Figure 4.37: Map of the Vector, pGL-BSK⁺.

orientation. The *Xba*I-*Sal*II fragment may ligate into the vector in either orientation, but this could be resolved by sequencing, and restriction mapping using *Sst*I and *Not*I.

However, despite numerous attempts, both by myself and by other members of the laboratory, we were unable to generate promoter constructs incorporating these fragments of the putative promoter region of the *Bmp-2* gene. This may be because this region of the *Bmp-2* contains some unstable intervening region, which causes it to recombine out of the constructs, or to fail to ligate. This is supported by the fact that the expected constructs would be very large, and may have been too big for the bacteria to carry. Attempts to clone these fragments into Bluescript also failed repeatedly.

This area might be expected to be prone to recombination because it is very G-C rich, potentially forming Z-DNA, a negatively supercoiled form of DNA structure which is unstable (Reich *et al*, 1993; van Holde and Zlatanova, 1994; reviewed by Hames and Glover, 1990).

This problem might be circumvented either by using a strain of bacteria for cloning which can support unstable forms of DNA and large constructs, or possibly by using the new pGL3 vector introduced by Promega, which is more stable.

4.4 Discussion:

a) The chicken limb bud mesenchyme cell transfection system:

I have shown that this newly designed system for transfecting and culturing primary chick mesenchyme cells is a good model for gene expression in the developing limb. Experiments with Type X Collagen and RIHB promoter constructs show that the cells appear to maintain at least some of their characteristics, in terms of stage and positional identity, in culture.

This system should prove very useful, since a great many genes have been studied that are expressed in the developing limb, and the limb is a good model for development. This system provides a fast, efficient way of analysing regions of the promoters of these genes and the sequences required for correct positional, temporal and quantitative expression of these genes.

However care should be taken to ensure that these cells are actually maintaining their positional identity in culture. Previous experiments have shown that, in culture, the main characteristic of positional identity in these cells, polarising activity, is completely lost after 96 hours in micromass culture (Vogel and Tickle, 1993). This was assayed by grafting cells from the posterior region of the limb into the anterior region of a host limb, after varying times in culture, and examining the degree of digit duplication induced by these cells. After 24 hours, the cells exhibit 20% of the activity of a graft of freshly isolated, uncultured cells. However, other experiments have shown that these posterior cells can still induce full duplications after 30 hours in culture, and that activity only begins to tail off after 48 hours (Hayamizu and Bryant, 1992; Anderson *et al*, 1993), and cells from the quail limb bud can maintain polarising activity in culture for 48 hours (Stocker and Carlson, 1988).

The loss of polarising activity can be surmounted by varying the culture conditions. Honig (1983) showed that although polarising activity was rapidly lost from posterior cells after only 30 hours in culture at 38°C, cultured ZPA cells could maintain polarising activity for many days in culture at 24°C (60-75% activity after 72 hours). Cells can also be induced to maintain polarising activity for longer in culture by adding

ectodermal jackets to the culture or by culturing fragments of the posterior limb with the AER still attached. Addition of approximately 80 ng/ml of FGF-4 can also maintain polarising activity in culture for some time, substituting for some signal from the AER, but still all activity is lost after 96 hours (Vogel and Tickle, 1993). The FGF needs to be added at the start of the culture, or it has no effect, and an equal concentration of heparin sulphate needs to be added as well, to promote the stability of the FGF.

The experiments carried out with anterior and posterior limb mesenchyme cells transfected with the α 2 neuronal promoter constructs indicate that these cells are maintaining some anterior-posterior differences after 48 hours in culture in the absence of FGF, but these results need to be treated with caution since there is no evidence that this reflects normal expression of this gene, and we do not know precisely what anterior-posterior signals these constructs are responding to. Therefore, these cultured transfected cells should be assayed for maintenance of polarising activity by grafting transfected cells into the anterior region of a host limb bud after various times in culture, and examining the degree of digit duplication induced by these cells.

This cell culture system provides a faster and more convenient technique than creating transgenic mice, and it overcomes the problem of the lack of stable chicken cell lines in which to study the expression of chicken genes which may require species-specific factors for correct expression. These cells can also be cultured in a variety of different ways: as micromasses, monolayers, or grafted back into host limbs either as pellets or as reaggregates in ectodermal jackets. In this way it would be possible to study gene expression in a variety of different environments.

These cells are also very amenable to culture manipulations, such as studying the effect on gene expression of exogenously added growth factors or RA. They present a model system that is closer to the *in vivo* state than stable cell lines for this kind of study. One possible application for this cell system is to investigate the genes and sequences involved in the polarising pathway, downstream, and possibly upstream of *Sonic hedgehog*.

Any reporter gene may be used in this system, so it is not limited to the quantitative approach that has been described here, lysing the cells and measuring luciferase activity

in a luminometer. Direct visualisation of the localisation of promoter gene activity may be achieved using the Green Fluorescent Protein system (Chalfie *et al*, 1994; Wang and Hazelrigg, 1994), or antibodies to the luciferase gene recently available from Promega. This would be particularly useful if the transfected cells were used in reaggregates or grafted back into host limb buds.

The system could also be used for expression of genes, in addition to promoter reporter studies, and it may prove particularly useful for this, since there are no stable chicken cell lines.

In addition, it would be possible to perform double transfections putting two or more constructs into the same cells, to examine the effects of co-expression of two genes in the same cell.

b) The α 2 Neuronal Promoter Constructs:

Surprisingly, some of the α 2 neuronal promoter constructs were found to drive luciferase activity in the chick limb bud mesenchyme cells used in this system at very high levels, higher than those of the SV40 promoter alone, despite the fact that the α 2 constructs contain putative silencer elements upstream of the SV40 promoter (Bessis *et al*, 1993). In addition, these promoter constructs appeared to show anterior-posterior differences in expression and to be RA-inducible. There is no evidence that this reflects normal expression of this gene.

The results obtained from transfection of these enhancer and silencer constructs into chick limb bud mesenchyme cells showed different effects on luciferase transcription than expected from the transfection experiments done by Bessis *et al* (1993) (Figures 4.16 and 4.35), and this probably reflects differences in the cell systems used. The limb bud mesenchyme cells would be expected to contain different transcription factors than those found in neuronal cells and fibroblast cells.

This is supported by the fact that other transfection experiments done in our laboratory, such as those by Dr. N. Milton using neuronal cells (Milton *et al*, 1995), and P.

Georgiadis, using rat H9 myoblast cells, using these constructs, have shown that the constructs can have very different properties from those described by Bessis *et al* (1993) in their cell systems.

The experiments performed by P. Georgiadis also showed that, in that rat H9 myoblast cells, the α 2.6 construct shows RA responsivity (P. Georgiadis, personal communication).

However, the sequence search has shown that there are no consensus RAREs in the construct. Therefore these promoter constructs cannot be responding directly to induction by RA. They must either be induced indirectly, by a later component of a cascade induced by RA, or be responding to some other anterior/posterior signal in the limb bud, which is itself induced or mimicked by RA.

The complex kinetics of the time course of activation of α 2.6 by RA, over the first 24 hours of induction also suggest that RA does not act directly on the neuronal promoter construct. One possible explanation, supported by the kinetics of activation, is that RA has multiple effects on the expression of these constructs, using both an inhibitory and an inducing loop or cascade. Alternatively, these constructs may be responding to some other signal, which is itself induced in a complex manner by RA, or which is induced normally between 6 and 24 hours after RA application, and which then overcomes a direct inhibition of the α 2.6 construct by RA. One possible candidate for this is the *Sonic hedgehog* gene.

In summary, therefore, further analysis of these neuronal constructs could be done in this cell system, using further deletion mapping, to investigate the nature of the anterior-posterior signals that they are responding to, and the effects of RA on expression of these constructs. This might shed further light on the signalling involved in normal patterning of the limb, and the putative role of RA as an endogenous signal in limb development.

CHAPTER 5

Inhibition of *Bmp-2* Gene Expression by Antisense RNA

5.1 Why Inhibit *Bmp-2* Gene Expression ?

As described in the Introduction, the *Bmp-2* gene has been posited to play important roles in the development of the chick limb. It has been suggested that *Bmp-2* acts during early patterning of the limb, possibly being involved in the polarising pathway specifying the anterior-posterior axis of the limb, and potentially regulating outgrowth of the limb in the proximo-distal axis through its expression in the AER. *Bmp-2* is also likely to play a key role in later limb development, in the morphogenesis of the skeletal elements.

One way to test these putative roles for the *Bmp-2* gene is to disrupt its expression. If *Bmp-2* plays an important role in limb development, inhibiting normal gene expression would be expected to perturb the normal development of the limb. For example, repressing expression of *Bmp-2* in the AER might be expected to alter outgrowth (Niswander and Martin, 1993a), whilst blocking its function in the polarising pathway might alter anterior-posterior pattern. Inhibition of the effects of *Bmp-2* later in development might be expected to affect the size and shape of the cartilage elements. Since recombinant BMP-2 protein induces extra cartilage and bone formation, knocking out *Bmp-2* gene expression might cause the limb bones to be thinner.

Possible clues to the phenotypic effects of reducing *Bmp-2* levels also come from looking at mutations in the *Bmp-2* gene. No specific *Bmp-2* mutations have been described, possibly because the role of the *Bmp-2* gene is sufficiently important in development that any embryos that lack expression die early in development. In support of this, a *Bmp-2* loss-of-function mouse produced by homologous recombination dies during gastrulation (C.M. Jones, personal communication). However, several mutations have been tentatively suggested to be linked to the *Bmp-2* gene. For example, the *tight-skin* (*Tsk*) mutation in mice is found near the chromosomal location of the *Bmp-2* gene on chromosome 2 (Dickinson *et al*, 1990).

Mice heterozygous for the *Tsk* mutation, a dominant allele, have tight skins, which are thicker and tightly bound to subcutaneous and deep muscular tissues, due to excessive accumulation of collagen in the dermis and subdermal tissue. They also show increased growth of cartilage and bone, and small tendons with hyperplasia of the tendon sheath (Green *et al*, 1976; Jimenez *et al*, 1986; Jimenez *et al*, 1988) The overall size of the skeleton is increased and all the bones are larger in width and length, but normal in shape. The skin tightness is not recognisable until 7 days after birth. Homozygous mice die *in utero* at 7-8 days.

In humans, the *Bmp-2* gene has been localised to chromosome 20, in the region of the locus of the Fibrodysplasia Ossificans Progressiva (FOP) disease (Tabas *et al*, 1991, Kaplan *et al*, 1990). FOP is a rare autosomal dominant disorder characterised by progressive accumulation of heterotopic bone, ossification of the soft connective tissues and several malformations of the foot and hand (Schroeder and Zasloff, 1980).

Heterotopic bone deposition is generally asymmetrical, whereas the feet and hand abnormalities are bilaterally symmetrical. Abnormalities include fusion of the digits and reductions in the lengths of the middle phalanges. Kaplan *et al* (1990) described gradients of effect in the foot and hand malformations of FOP, with most malformations occurring in the big toe or thumb, and digit V, and markedly fewer defects of the middle digits (Figure 5.1). The proximal phalanx of the first toe is abnormally shaped, usually broader and more square than normal and generally fused with the terminal first phalanx. Typically also the big toe may be absent from both feet.

Both of these diseases were postulated to result from gain-of-function mutations in the *Bmp-2* gene (Kaplan *et al*, 1990; Dickinson *et al*, 1990).

Mutations in other TGF- β superfamily members may also give clues to potential effects of *Bmp-2* inhibition. As described in Chapter 1, a mutation in *Bmp-5*, the *short ear* mutation, in mice leads to reductions in the size, shape and number of many skeletal elements, including some bones of the wrist (Kingsley *et al* 1992; Kingsley, 1994).

Mice carrying deletions in the *Gdf-5* gene (*brachypodism*) have alterations in the length of limb bones but not the axial skeleton (Storm *et al*, 1994). It has been suggested that different members of the BMP family control the formation of different morphological features in the mammalian skeleton, with the skeleton being a mosaic structure built of

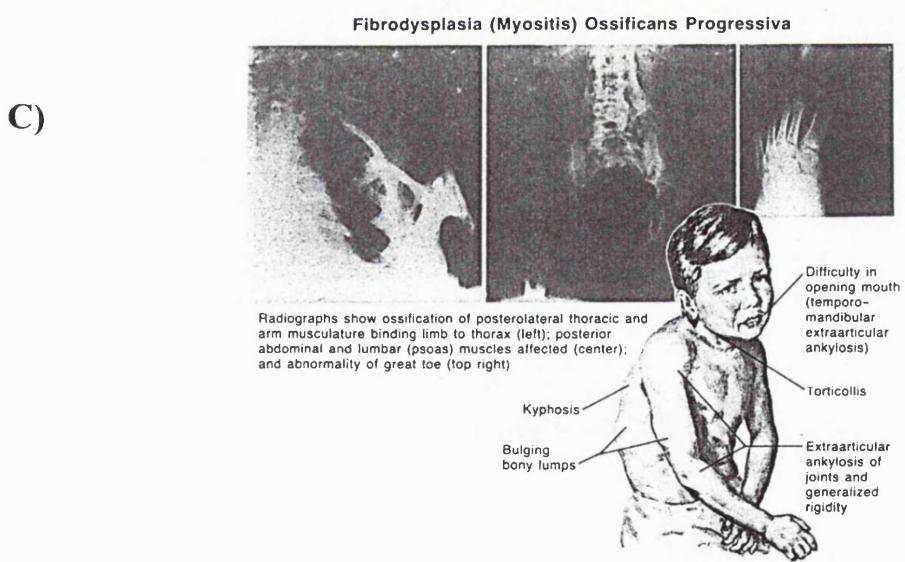
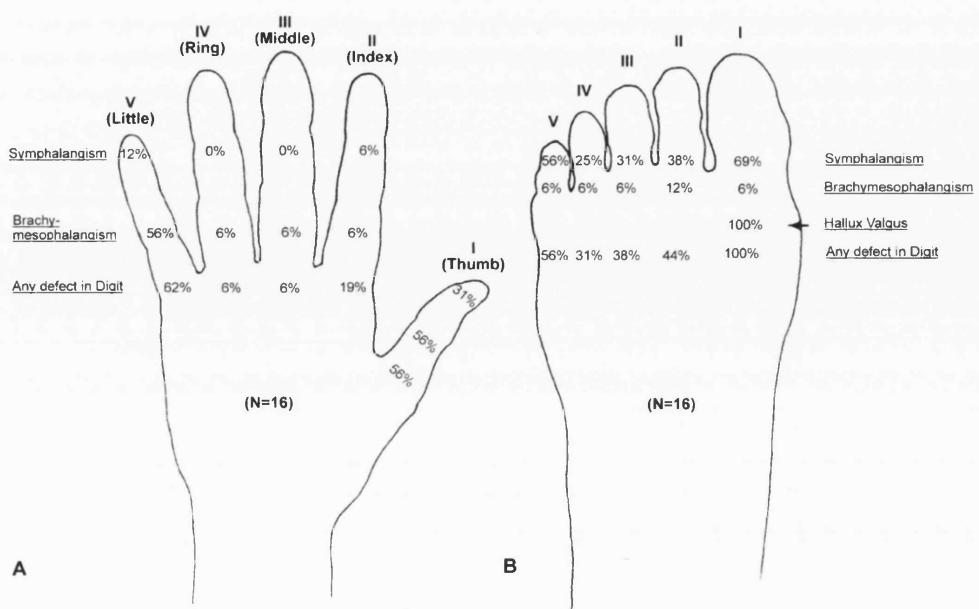


Figure 5.1: Symptoms of Fibrodysplasia Ossificans Progressiva (FOP).

- Patterns of digit deformities in FOP, as manifested a) in the hands, and
- b) in the feet. (Adapted from Kaplan *et al*, 1990).
- Defects characteristically seen in FOP. (Adapted from Kaplan *et al*, 1990).

composite patterns of activity of BMP-like proteins (Kingsley *et al*, 1994; Storm *et al*, 1994). Inhibition of *Bmp-2* expression might therefore be expected to cause malformations in specific skeletal elements of the limb.

One way to inhibit *Bmp-2* expression in the limb is to use antisense nucleic acids. Antisense strategies have been used to inhibit expression of TGF- β 3 and TGF- β 1 (Potts *et al*, 1991; Khanna *et al*, 1994). Application of modified synthetic oligodeoxyribonucleotides complementary to the area around the initiation codon, which is not conserved between the different TGF- β s, inhibited the transforming effect of TGF- β 3 on embryonic cardiac endothelial cells. A 15 bp phosphorothioate-modified antisense oligomer to the transcription start site of TGF- β 1 has been shown to reduce TGF- β 1 secretion and prevent TGF- β 1-dependent inhibition of DNA synthesis in A-549 human adenocarcinoma cells (Khanna *et al*, 1994).

5.2 Antisense Nucleic Acids:

a) Overview of antisense techniques:

Antisense nucleic acids are single-stranded RNA or DNA molecules that are complementary to the sequence of their target, usually RNA, and that bind to and inhibit the function of this target.

In 1981, it was discovered that prokaryotes such as *E.coli* use natural antisense RNA to regulate various processes such as plasmid replication and membrane structure (reviewed by Inouye, 1988; Simons, 1988; Thomas, 1992). Various naturally-occurring antisense genes have also been found in eukaryotes, but, in most cases, it is not known if they actually functionally inhibit expression of their complementary genes (Hildebrandt and Nellen, 1992; Lee *et al*, 1993; Wightman *et al*, 1993). For example, there is an antisense transcript of *Fgf-2* expressed in the *Xenopus* oocyte, which is known to interact with the sense *Fgf-2* mRNA, although it is not known precisely what effect this has (Kimelman and Kirschner, 1989; Volk *et al*, 1989). This discovery led to the development of artificial RNA regulatory systems, both in prokaryotes and eukaryotes, to inhibit the expression of numerous exogenous

endogenous genes (Coleman *et al*, 1984; Izant and Weintraub, 1984, 1985; Kim and Wold, 1985; Pepin *et al*, 1992; reviewed in Murray, 1992; and Eguchi *et al*, 1991).

Antisense techniques can be used to completely or partially inhibit the function of a given gene in a cell line or organism. This strategy can in principle be used for any gene for which sequence information is available and in any system into which antisense nucleic acids can be introduced, by external application, such as by microinjection, or by nuclear expression from a construct containing an artificial antisense gene (reviewed in Colman, 1990).

Antisense regulation has been shown to be successful in a large number of systems, including plants, *Dictyostelium*, *Drosophila*, mice and *Xenopus* oocytes, as well as in various cell lines (reviewed in Murray, 1992; van der Krol, 1988c), and the antisense approach is clearly particularly attractive for those eukaryotic organisms that do not have well-defined or amenable genetic systems, or which are difficult to make transgenic *in vivo*, such as chickens (Salter *et al*, 1987; Bosselman *et al*, 1989; Perry, 1988; Mitrani and Eyal-Giladi, 1982; Petitte and Eches, 1988; Love *et al*, 1994).

Because of the dramatic developmental changes that occur, embryos are especially suited for antisense RNA studies. Often the inhibition of an important gene expressed during embryogenesis leads to significant morphological and sometimes lethal defects. Antisense injections have been tried successfully on the following genes in *Drosophila* embryos: *Krüppel* (Rosenberg *et al*, 1985), *snail* (Boulay *et al*, 1987), *wingless* (Cabrera *et al*, 1987), *knirps* (Nauber *et al*, 1988) and *pecanex* (Labonne *et al*, 1988).

Affecting a single gene can also down-regulate whole complexes of proteins. In plants, both nuclear and chloroplast proteins are required in the chloroplast for the production of RUBISCO complexes used in photosynthesis: if the nuclear component is inhibited by antisense transcripts, levels of the entire protein complex are reduced (Rodermel *et al*, 1988). A similar effect has also been observed in antisense transgenic mice (Katsuki *et al*, 1988; Kimura *et al*, 1989): inhibition of the expression levels of a single myelin basic protein mRNA by introducing antisense myelin genes into wildtype mice induces the shiverer phenotype, characteristic of mice completely lacking myelin surrounding their nerves.

Antisense inhibition can be precisely targeted to a single gene because of the sequence specificity of the complementary nucleic acids. For example, McGarry and Lindquist (1986) showed that antisense genes targeted to *hsp26* will cause a reduction only in HSP26 protein, and none of the other HSP proteins, despite their sequence similarities (77% over 310 bp area). Galileo *et al* (1992) showed that a chicken antisense gene transfected into mouse cells stably transfected with the chicken target gene, will cause a 60% reduction in expression of the chicken gene, but without affecting the mouse sequences. Chang *et al* (1991) showed that a single point mutation in the sequence of an antisense 8- or 11-mer deoxyribonucleotide will completely abolish its inhibitory effects. Khanna *et al* (1994) showed that a 15 bp antisense DNA oligomer complementary to the nucleotides flanking the transcription start site of the human TGF- β 1 gene only inhibited TGF- β 1 expression, and had no effect on the other TGF- β s.

In general terms, the antisense sequence is thought to hybridise to its target and then block expression by one or more of several means. Inhibition can occur at the level of transcription, post-transcriptional modification, transport or translation of the mRNA, and is thought to occur by the formation of duplex hybrids within the cell. Figure 5.2 illustrates the numerous points at which antisense inhibition may act in the stages from the production of the mRNA, to its translation into functionally active protein. These may, however, be simplified into two basic alternative explanations that can account for antisense effects: either the antisense:sense hybrid formation directly blocks mRNA function, or interaction with the complementary antisense sequence destroys or inactivates the target mRNA.

However, despite many successes, various aspects of antisense inhibition remain to be clarified. For example, the precise mechanism by which the antisense molecules inhibit gene function in any given system is not known. Equally, some systems, such as *Xenopus* embryos and cultured mammalian cells, appear to have an enzyme that selectively unwinds RNA duplexes, potentially negating inhibition (Bass and Weintraub, 1987; Rebagliati and Melton, 1987; Wagner and Nishikura, 1988), although the discovery that unwinding is associated with irreversible base modification of the RNA complicates this interpretation.

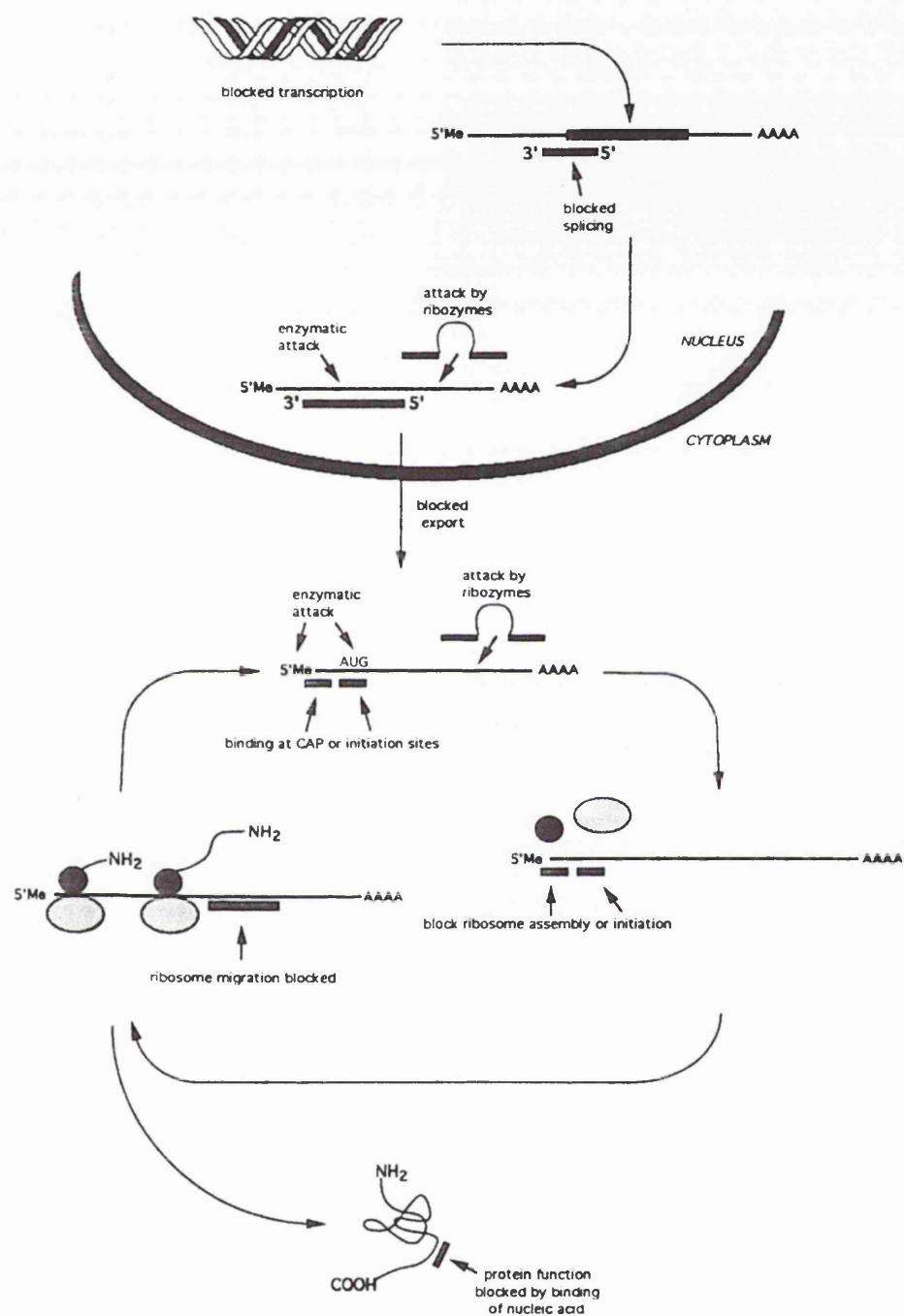


Figure 5.2: Possible sites of action for inhibition by antisense molecules.

(Adapted from Murray, 1992)

The diversity of results described in the literature indicates that there may be differences in the cellular responses to the presence of antisense RNAs. These differences may depend on the biological system and on the specific target gene.

b) Factors to consider in designing an antisense strategy:

In practice, therefore, the application of antisense techniques has been complex, partly due to the wide range of different strategies that can be used and the many different stages at which the RNA can be affected. In addition, the lack of information on the precise method of action of antisense constructs makes it difficult to rationally design reagents and strategies (Colman, 1990; Wagner, 1994).

To optimise inhibition, a number of factors must be considered:

i) Type of antisense molecule to use:

There are two main types of antisense molecule: antisense RNA and synthetic antisense DNA oligomers.

Antisense RNA is the more commonly used type of antisense molecule. It can be transcribed *in vitro* and then subsequently micro-injected into cells. This allows a large amount of RNA to be applied, but the effect is only temporary. Alternatively, antisense RNA can be obtained by nuclear expression of an engineered antisense construct. Antisense constructs contain all or part of the normal gene sequence in an inverted, "wrong" orientation downstream of a promoter, so that the noncoding strand, complementary to the target sequence, is transcribed. Driving continuous production of antisense RNA using a promoter allows the production of a large amount of antisense RNA. Specific promoters can also be used to drive expression of the antisense RNA, giving more control over the level, timing, inducibility, or tissue specificity of the antisense inhibition (Amini *et al*, 1986). Such antisense expression vectors can easily be constructed using standard procedures and introduced into cells by any of the normal means of transferring DNA, such as transfection, electroporation, microinjection, or, in the case of viral vectors, by infection.

Antisense DNA is generally used in the form of short synthetic oligomers (reviewed by Toulmé and Hélène, 1988; Wagner, 1994). The length of such synthetic constructs is usually limited to 10-30 bp. They must be applied to the cells externally, or micro-injected, and have only a transient effect because they have no means of replicating and do not integrate into the host cell DNA. Various modifications can be made to the DNA to improve cell stability or cellular uptake, or even to allow it to cleave the target RNA and degrade it. Examples include backbone modifications such as methylphosphonates and phosphorothioate, or terminal modifications, such as the addition of acridine or cholesterol (reviewed in Colman, 1990; Wagner, 1994). However, care must be taken that these substances are not toxic to the cells.

Some antisense DNA molecules may be able to form triple-helices with chromosomal DNA, but this approach has been limited so far by effects of physiological pH values on target specificity, although this approach has been shown to be effective in inhibiting expression of the human TGF- β 1 gene (Cooney *et al*, 1988; Khanna *et al*, 1994). Antisense oligonucleotides have also been made that bind to proteins (Blackwell and Weintraub, 1990; Blackwell *et al*, 1990; Tuerk and Gold, 1990), or that have ribozyme activity and cleave the target molecule (Kim and Cech, 1987 Green *et al*, 1990; reviewed by Perriman and Gerlach, 1990).

ii) Region of gene to use:

Many regions of mRNA have been targeted for hybrid formation with diverse results. Antisense RNA directed at 5' untranslated regions appears to give the greatest levels of inhibition, although targeting 3' untranslated regions also reduces expression of certain genes (Strickland *et al*, 1988; Xiao *et al*, 1988; Ch'ng *et al*, 1989; reviewed by van der Krol *et al*, 1988b), and both successes and failures have been noted for antisense RNA complementary to the 5' or 3' coding regions and intron-exon boundaries in unspliced RNA (Kim and Wold, 1985; Knecht and Loomis, 1987; Melton, 1985; Wormington, 1986; Sandler *et al*, 1988; Rothstein *et al*, 1987). Oligodeoxynucleotides targeted to splice sites have been shown to efficiently inhibit expression, but intronless genes have been effectively inhibited too (van der Krol, 1988b).

It is important to pick a region of the gene that will produce an mRNA without secondary structure which might limit binding to the target, or reduce stability of the antisense construct, but it often difficult to know which regions of the mRNA might form secondary structures (Inouye, 1988, van der Krol, 1988b).

iii) Size of fragment to use:

Antisense inhibition has been successfully carried out with synthetic oligonucleotides as small as 10-12 bp (Chang *et al*, 1991; Khanna *et al*, 1994) or RNAs as large as the whole coding region (Cornelissen and Vandewiele, 1989; Sheehy *et al*, 1988).

In one study in bacteria, there was a strong correlation between the degree of inhibition and the length of complementary sequence in the antisense molecule (Ellison *et al*, 1985). This study found that the length of the antisense molecule was more important even than the position of the antisense sequence compared to the target gene, as long as the molecule was long enough to form stable duplexes. No such experiments have been done in eukaryotes to date, but it is expected that antisense molecules corresponding to fragments of the cDNA sequence of the target gene would inhibit gene function more effectively than the whole cDNA sequence, because antisense molecules based on the whole cDNA sequence would be shorter than the unspliced target RNA and the duplex formed would be less stable, because of the hairpin loops that would need to form around the unspliced introns. Additionally, antisense inhibition may occur at the level of RNA splicing, in which case it would be important to include the intron/exon boundaries in the antisense molecule.

iv) Level of antisense transcripts compared to sense:

In some systems, inhibition requires the antisense RNA to be in vast excess (50-fold or higher) over the endogenous RNA (Izant and Weintraub, 1984, 1985; Kim and Wold, 1985; Rosenberg *et al*, 1985) while in others a 1-10-fold excess is sufficient (Crowley *et al*, 1985; Izant and Weintraub, 1985; McGarry and Lindquist, 1986; Strickland *et al*, 1988). In one case in which natural antisense RNA is known to be produced, in the

Dictyostelium EB4 gene, the non-coding antisense RNA is very unstable, and this might explain why an excess is needed for inhibition (Hildebrandt and Nellen, 1992). Production of such high levels of antisense transcripts requires either expression of an antisense construct *in situ* driven by a strong promoter, or the efficient application of very large amounts of RNA or DNA. Another alternative is to select for high expression in stably transfected cell lines (Kim and Wold, 1985; Amini *et al*, 1986).

v) Method of applying antisense molecules:

Antisense nucleic acids can be introduced by external application, by microinjection, or by nuclear expression of an antisense construct in transgenic organisms or cells. The choice of method of application depends on the type of antisense molecule being used, how long inhibition is required for, and the amount of antisense construct that needs to be applied, and the cell system or organism being used.

Micro-injection can be used for antisense RNA or DNA. Inhibition is only transient, but large amounts of antisense material can be injected. For this technique, however, the target cells must be accessible to micromanipulation: this limits the targets to certain cell lines, *Xenopus* oocytes (Bass, 1992; Colman, 1992), the preimplantation mouse embryo (Levy *et al*, 1992), where the cells are large, and early *Drosophila* embryos in the syncytial stage, before cellularisation occurs (Patel and Jacobs-Lorena, 1992).

External application of antisense nucleic acids can be used for antisense RNA or DNA. Uptake by this method is usually poor. Synthetic antisense oligonucleotides can be chemically modified to enhance uptake or improve stability once it has been taken up, but these modifications are expensive and complex to do. Small oligomers are taken up more efficiently than larger ones.

Nuclear expression, introducing antisense constructs into transgenic cells or animals, as described above, is a simple, effective and flexible way of producing large amounts of antisense RNA. By choosing an appropriate promoter and transfection system, antisense constructs can be produced stably or transiently, ubiquitously or restricted to a particular place, time or tissue.

c) Strategy used for antisense inhibition of the chicken *Bmp-2* gene:

The aim of the experiments described in this chapter was to completely or partially inhibit expression of the *Bmp-2* gene in the chick limb during embryonic development using antisense molecules.

In these experiments, I chose to use antisense RNA rather than synthetic DNA oligonucleotides, because it is simpler and cheaper to produce. I decided to base the antisense constructs on fragments of the chicken cDNA clone p5.1 (Francis *et al*, 1994), which would be expected to form more stable duplexes with unspliced *Bmp-2* RNA than either the whole cDNA or short oligomers.

No one specific region of the gene was targeted, since different regions have been shown to be effective in different systems. Instead, the cDNA clone p5.1, and a shorter cDNA, p4, which lacked the first 80 bp of p5.1, were digested with *Pvu*II and *Eco*RI, and the resultant fragments were subcloned into an expression vector (Figure 5.3).

The antisense RNA had to be targeted specifically to the limb, because inhibition of *Bmp-2* gene function in other regions of the developing embryo might be expected to seriously perturb normal development. This is demonstrated by the fact that transgenic knockout mice which lack *Bmp-2* gene expression die during gastrulation, before the formation of the limbs (C.M. Jones, personal communication). For this reason, we used a replication-competent retroviral vector to express the antisense RNA. This virus can be used to target antisense RNA expression to the developing limb by injection of concentrated virus or by grafting virus-infected cells into the developing limb bud.

Using this retroviral vector also gives a high predicted level of expression of the antisense RNA, since expression is driven by the viral enhancer and promoter, which is a strong promoter, and the virus is present at high copy numbers. This should ensure that the antisense RNA molecules are expressed at a higher level than the endogenous sense RNA molecules, giving a sufficiently high ratio for effective duplex formation and inhibition.

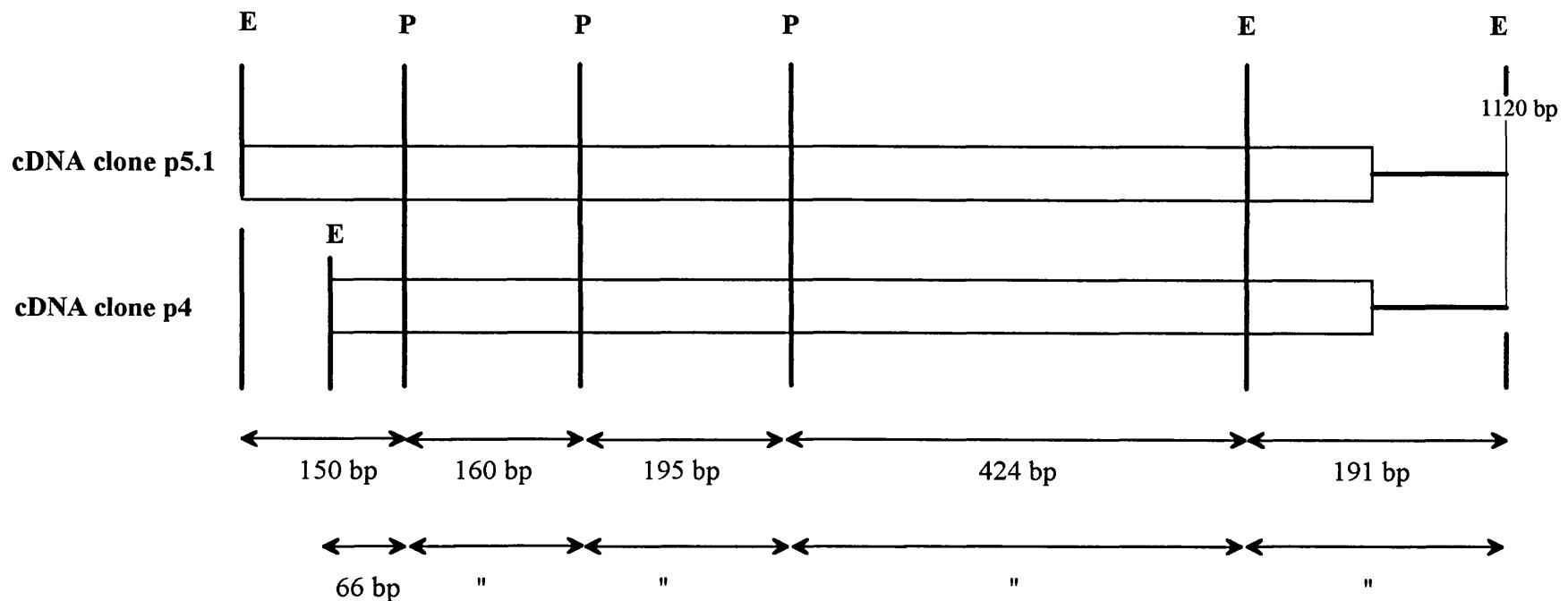


Figure 5.3: Comparison of chicken *Bmp-2* cDNA clones p4 and p5.1, showing *Eco*RI (E) & *Pvu*II (P) sites.

The open box represents the coding region of the mRNA, the line the 3' untranslated region

d) Retroviral vectors:

Retroviruses are powerful tools in the study of the function of genes during development. Vectors based on infectious viruses can be used to introduce non-viral genes into mitotic cells *in vivo* or *in vitro*: the gene will be stably introduced into the host chromosome, efficiently expressed and passed on to infected cells' progeny.

Retroviruses are characterised by a unique pathway of replication (Figure 5.4) (Stoker, 1993). Infection by retroviruses generally does not lead to cytopathic effects in host cells, but instead establishes the chronic production of progeny viruses that then bud from the cell surface. The retrovirus contains 3 genes: *gag*, *pol* and *env*, that are flanked by the long terminal repeats (LTR) which contain *cis*-acting sequences that regulate proviral transcription. All three genes have specific functions that allow the replication and production of new virus particles.

There are two types of retroviral vector: replication defective and replication competent. Replication defective viruses infect the initial target cells, integrate into the host genome and are then passed on to the progeny of these cells as proviruses during cellular DNA replication. They cannot produce infectious virus particles because they lack essential parts of their coding sequences. Replication competent viruses, on the other hand, spread actively from cell to cell by directing the assembly of infectious virus particles.

For many years, replication defective retroviral vectors have been used to investigate the lineage of various cells during development, for example neural crest cells (Frank and Sanes, 1991) and cells of the nervous system (Price *et al*, 1987). Targeted cell lines can be infected with a replication defective virus encoding β -galactosidase and the lineage of the cells can be followed by staining for β -galactosidase activity (Sanes *et al*, 1986). More recently, these replication defective vectors have been used as vectors to express specific genes ectopically during development, such as ectopic expression of *Fgf-2* in the limb bud (Riley *et al*, 1993) (See Chapter 1). de la Pompa and Zeller (1993) showed that by grafting aggregates of retrovirus-producing cells roughly the same size as RA-soaked beads into chick wing buds, either apically or anteriorly, at

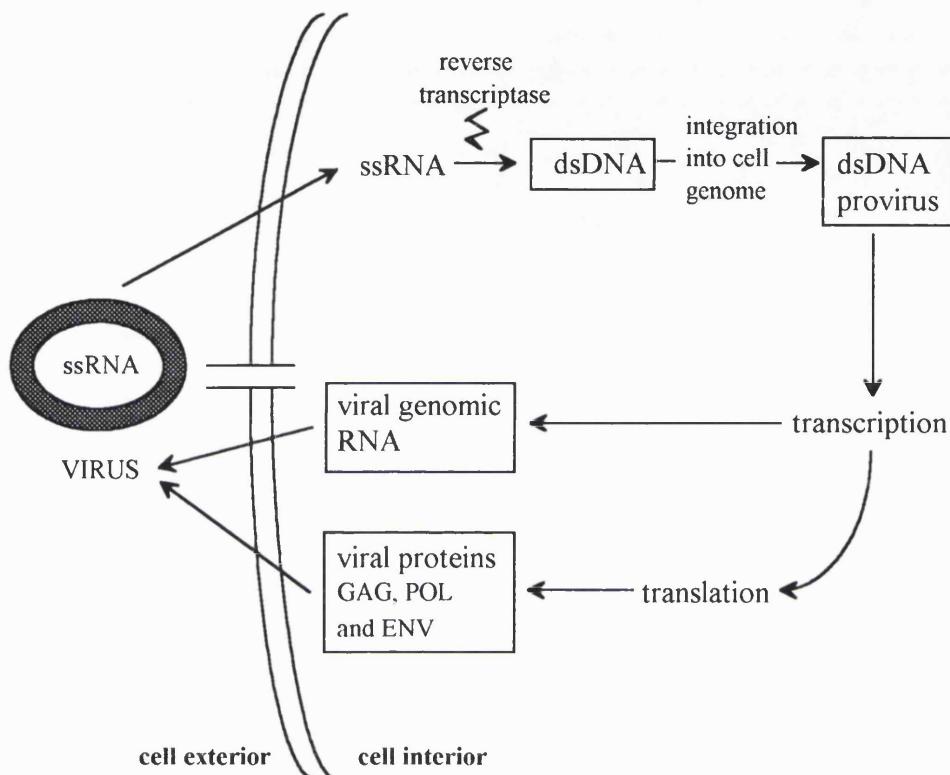


Figure 5.4: The life cycle of a replication competent retrovirus.

(Adapted from Stoker, 1993)

Retroviral particles consist of an encapsidated ribonucleoprotein core ensheathed by a membrane envelope coat. They contain two copies of a plus-strand RNA genome and replicate through a reverse transcription step in which the single-stranded genomic RNA is converted into double-stranded viral cDNA. The virus enters the cell via receptors on the cell surface to which the viral *env* protein binds. The provirus cDNA then integrates covalently into the chromosomes of their host cells. Only a single proviral copy is inserted at a given site. The integrated viral DNA is then transcribed and translated by host mechanisms, leading to the synthesis of the structural proteins required for viral maturation, and the production of more viral particles.

stage 17-18, widespread expression of a reporter gene, β -galactosidase, could be achieved without affecting morphogenesis.

Replication defective viruses have also been constructed to allow the efficient expression of two genes from the vector (Ghattas *et al*, 1991). This means that genes that are thought to act as heterodimers could be ectopically expressed in parallel, or a marker gene could be expressed in tandem with the bioactive gene of interest, to check the spread of the virus.

Replication competent retroviral vectors, however, are better suited for expressing genes ectopically in the limb. They are more stable than replication defective viral vectors because they are helper-virus-independent, eliminating the possibility of rearrangements through recombination with homologous sequences in the helper viruses. They also normally occur at a higher titre and do not need to carry selectable markers because the sequences are passed along passively during replication. Spread of the virus in the embryo is also more efficient because the viruses can actively spread through infection, rather than being limited to propagating with division of the cells originally infected. This makes them more flexible with respect to the areas that can be affected, since effective spread of the virus is not limited to areas of high cell proliferation. For example, cells in the limb bud can be infected at a later stage and in areas other than the limited areas of rapid cell division, and still achieve expression across a large area of the limb bud, as required in these antisense *Bmp-2* experiments (Riley *et al*, 1993; Riddle *et al*, 1993).

These replication competent retroviral vectors have also been used successfully in the chick embryo, targeting not only the limb (Morgan *et al*, 1992; Riddle *et al*, 1993; see Chapter 1), but also other structures, for example the nervous system (Fekete and Cepko, 1993a and 1993b). Specific structures can be targeted by injecting virus in specific areas or by grafting virus-infected tissue or cells: the viral vectors will then spread into the surrounding structure. Morgan *et al* (1992) showed that by injecting virus into the primordia of the limb at stage 10-12, complete infection can be achieved by stage 21, and because of the kinetics of viral spread, infection and ectopic expression are largely limited to the limb bud at this stage, although the infection will continue to spread as development proceeds.

If infection is only required in a very limited area, then grafts can be made into a strain of chicken which is not susceptible to infection by the particular strain of viral vector being used (Riddle *et al*, 1993; Fekete and Cepko, 1993a), this allows viral spread to be limited over the course of several days, without the need for a tissue-specific promoter. This is useful if the gene being expressed has multiple distinct functions during development, because it simplifies interpretation of the results and reduces the likelihood of development in other areas of the embryo being disrupted.

One disadvantage of replication competent vectors, however, is that only approximately 2 kb of exogenous coding sequences can be inserted, because the vector must contain all the genes required for transcription, *gag*, *pol* and *env*, and the virus particle will only accommodate viral genomes of limited size (Hughes *et al*, 1987).

Retroviral vectors are therefore well-suited to performing targeted gene inhibition in chick embryos using antisense sequences. The retroviral vectors can be injected or infected cells grafted into the embryo at a specific chosen developmental stage, targeting a particular tissue or area, and the embryos can then be re-incubated and harvested at a later stage (reviewed by Tickle, 1992). Retrovectors containing antisense constructs were first used to inhibit virus replication in infected cells. This was first successfully done by von Ruden and Gilboa in 1989, and then again in 1990, by Rhodes and James. Galileo *et al* (1992) used a retroviral vector encoding antisense integrin RNA, targeting injections to the optic tectum and found that neuroblast migration was inhibited, due to inactivation of the endogenous integrin message.

5.3 Aims of the work described in this chapter:

The RCAS retroviral system was used to attempt to inhibit *Bmp-2* gene expression in the developing limb of chick embryo, using the retroviral vector, RCAS(BP)A, directing synthesis of antisense chicken *Bmp-2* RNA (Hughes *et al*, 1993). (Figure 5.5a).

RCAS (RC: replication competent; A: Avian Leukosis Virus Long Terminal Repeat; S: with a splice acceptor) is derived from the Schmidt-Ruppin strain of Rous Sarcoma Virus (RSV), which was discovered in 1911 by Peyton Rous (Rous, 1911). The *src* gene has been excised from the virus and been replaced with a synthetic DNA linker containing the recognition site for the *ClaI* enzyme (Hughes and Kosik, 1984). RCAS has envelope subgroup A specificity. RCAS(BP) is an unpublished version of RCAS (Hughes *et al*, 1987) that contains *pol* sequences derived from the Bryan high-titer strain of RSV (BH RSV) which allow more efficient replication of the virus in host cells (Sudol *et al*, 1986; Petropoulos and Hughes, 1991). (Figure 5.5)

5.4 Results:

a) Construction of antisense viruses:

In order to generate antisense constructs, fragments of two chicken *Bmp-2* cDNA clones were cloned into the retroviral expression vector, RCAS(BP)A, in the sense or antisense orientation . The initial cloning was carried out by Dr. Philippa Francis-West.

The cDNA, p5.1 (Francis *et al*, 1994), and a shorter cDNA clone, p4, which lacked the first 80 bp of p5.1 (Figure 5.3), were digested with *Pvu*II and *Eco*RI, and the resultant mixture of fragments was ligated to an adaptor plasmid, Cla12 Nco (Hughes *et al*, 1987), cut with *Sma*I, or *Sma*I and *Eco*RI (Figure 5.6). This plasmid was used to facilitate cloning, since the retroviral vector RCAS(BP)A only has a single cloning site, a *Cla*I site, whereas the adaptor plasmid contains many restriction sites, flanked by two *Cla*I sites. In this way the cDNA sequence of interest can be cloned into the adaptor plasmid using the sites in the polylinker, and then excised as a *Cla*I fragment and

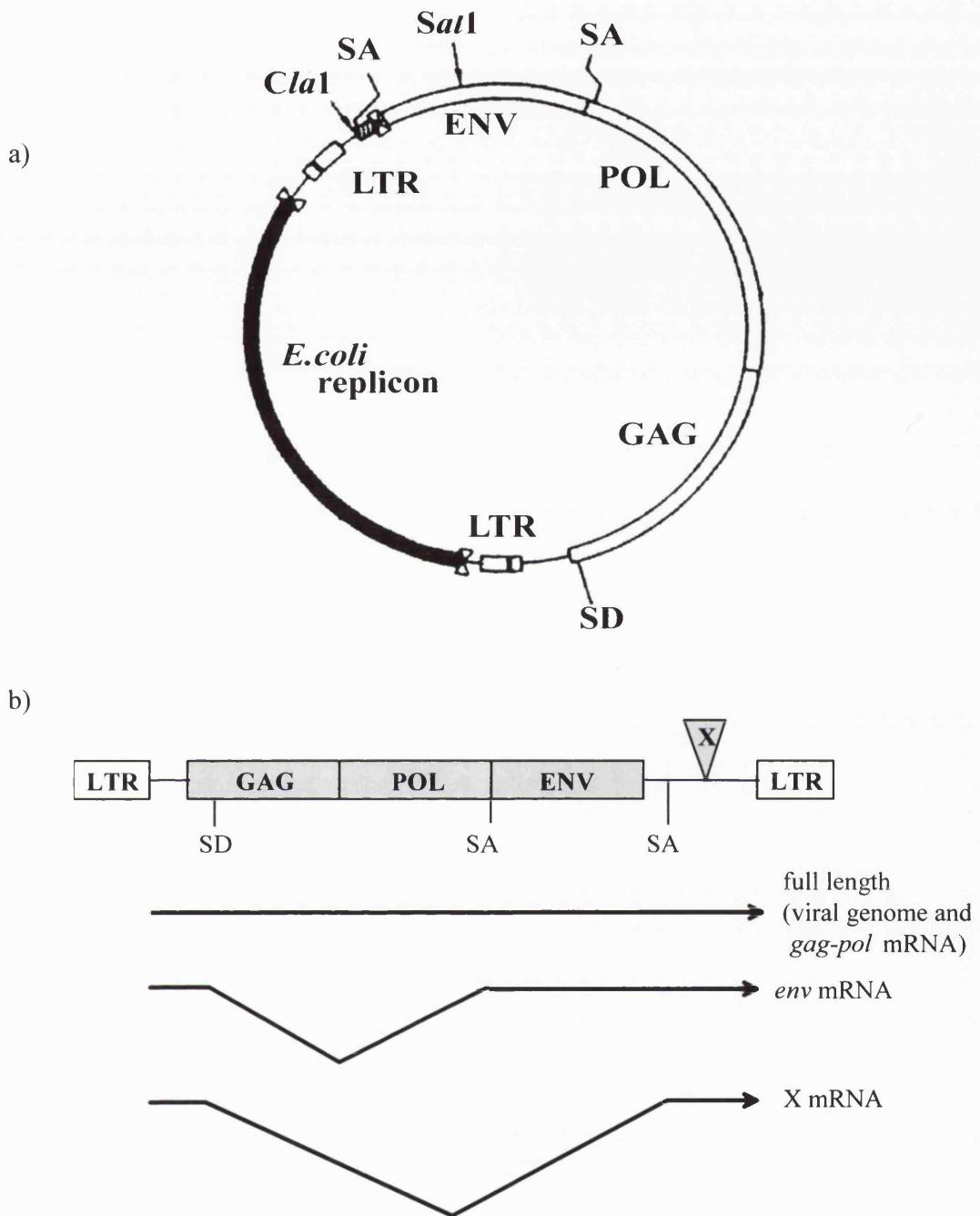


Figure 5.5: Structure of RCAS(BP)A.

- a) Map of the proviral form of RCAS(BP)A (Adapted from Hughes *et al*, 1987)
- b) Splicing products produced from the primary transcript of RCAS(BP)A.

SA = splice acceptor site; SD = splice donor site; LTR = long terminal repeat

X = gene inserted into vector

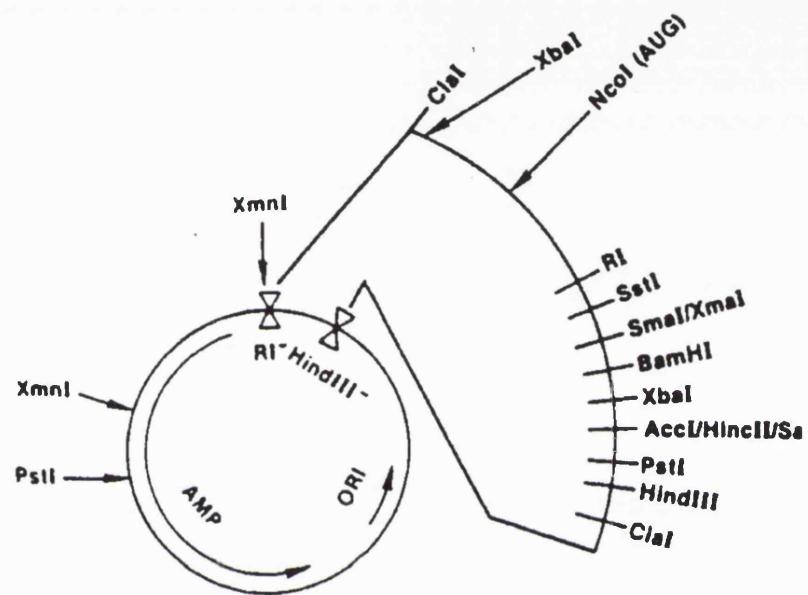


Figure 5.6: Map of the adaptor plasmid Cla 12 Nco

(Adapted from Hughes *et al*, 1987)

inserted into the *Clal* site of the RCAS(BP)A vector. (See Figure 5.7 for Cloning Strategy).

Clones containing the adaptor plasmid with a *Bmp-2* insert were identified by radioactive screening, using the *Bmp-2* cDNA clone p5.1 as a probe. Screening was performed in this way, because the adaptor plasmid has no provision for blue-white selection. Clones containing inserts were grown up and the inserts were removed by *Clal* digestion and ligated to *Clal*-cut RCAS(BP)A.

Nine clones containing inserts were identified and isolated as described above. Seven of these were further characterised: they were called 55/2 and 55/58, 65 and 88, 30/63 and 30/45, and 10 + 1. The other two clones contained inserts already represented in the other seven clones, as determined by restriction mapping.

I determined the size of the insert in each clone by *Clal* digestion (Figure 5.8). From these digests, the insert in 10 + 1 was approximately 300 bp long, those in 65, 88, 30/63 and 30/45 were approximately 1 kb, and the insert in 55/2 and 55/58 was slightly less than 1 kb. *SaII* digests were also performed to determine the orientation of the insert in each clone (Figure 5.9). There is one *SaII* site in the RCAS(BP)A vector and one site in the polylinker of the adaptor plasmid, flanking the sites used to ligate the insert. Therefore, if identical size inserts are present in different orientations, *SaII* digestion should yield fragments of two different sizes, depending on whether the insert separates the two *SaII* sites or not (Figure 5.9b). From this digest data, it can be seen that 55/2 and 55/58 represent a pair of complementary sense and anti-sense constructs, as do 65 and 88, and 30/63 and 30/45.

To determine the precise orientation and the position of each antisense insert compared to the *Bmp-2* cDNA, each clone was sequenced using primers corresponding to sequences in the RCAS(BP)A flanking regions and the polylinker of the adaptor plasmid. The positions and orientations of all the clones are summarised in Figure 5.10. The insert in 10 + 1 is 310 bp long, that in 55/2 and 55/58 is 619 bp long, that in 30/63 and 30/45 is 779 bp long, and the one in 65 and 88 is 970 bp long.

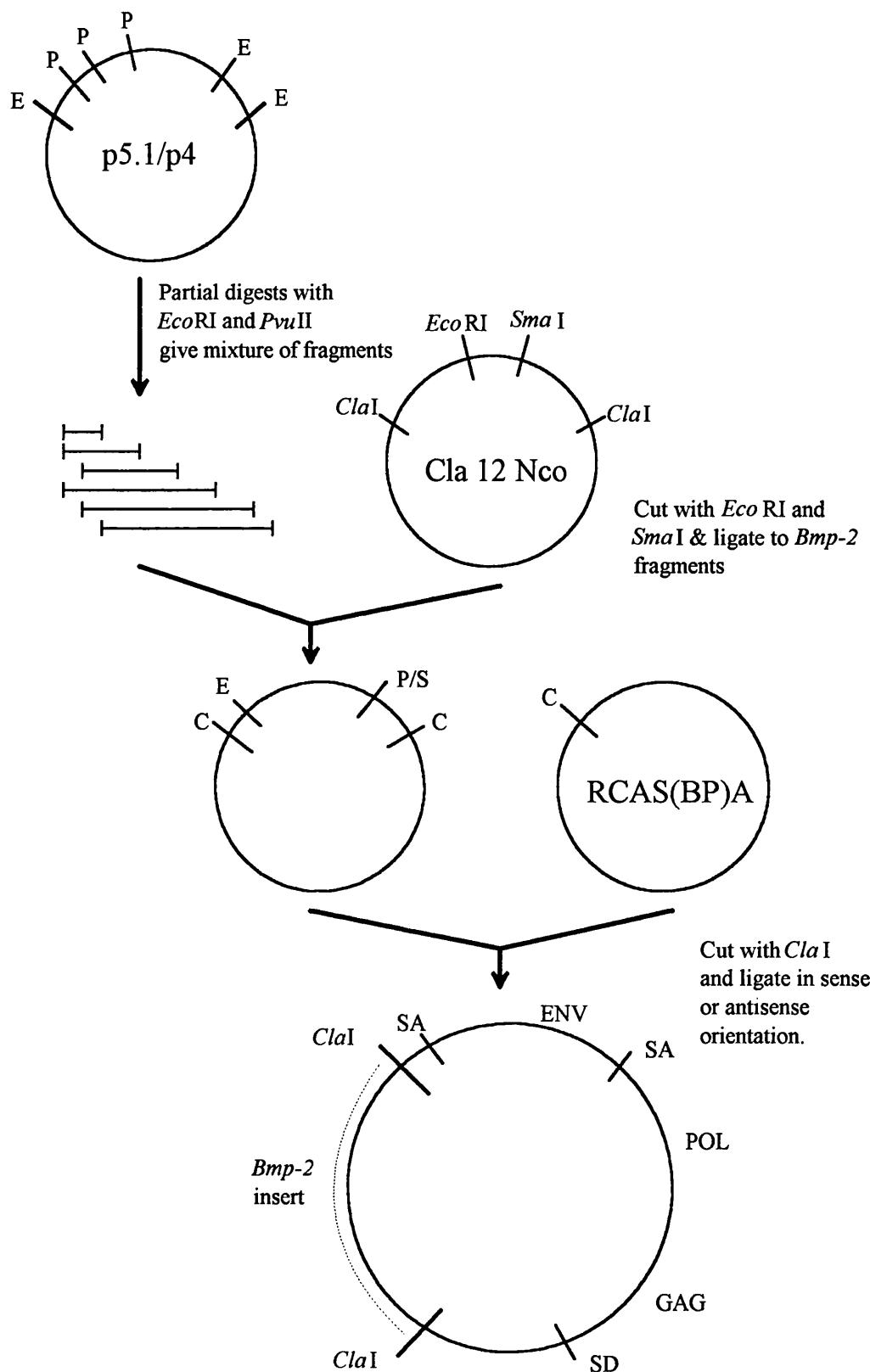


Figure 5.7: Cloning strategy for cloning chicken *Bmp-2* fragments into the retroviral vector RCAS(BP)A.

SA = splice acceptor site; SD = splice donor site; E = *Eco*RI; P = *Pvu*II; C = *Cla*I; P/S = *Pvu*II/*Sma*I

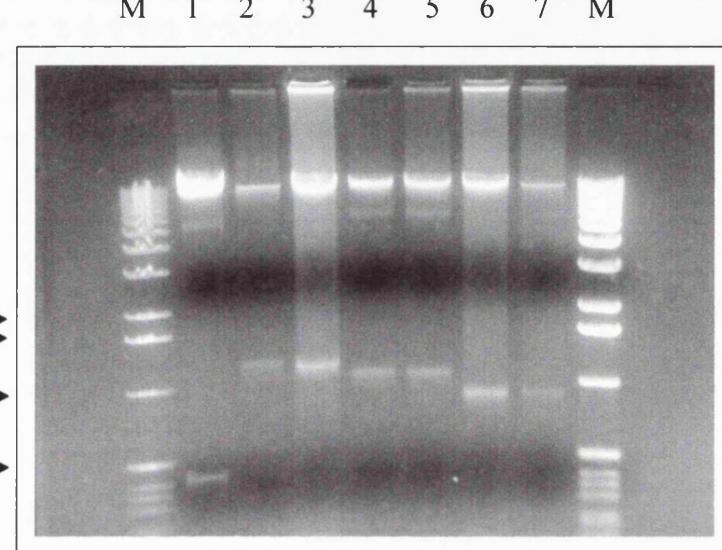


Figure 5.8: Determination of the sizes of the inserts in the antisense retroviral clones, using *Clal* digestion.

M: 1kb ladder markers;	1: 10+1;	2: 65;	3: 88;
4: 30/63;	5: 30/45;	6: 55/2;	7: 55.58

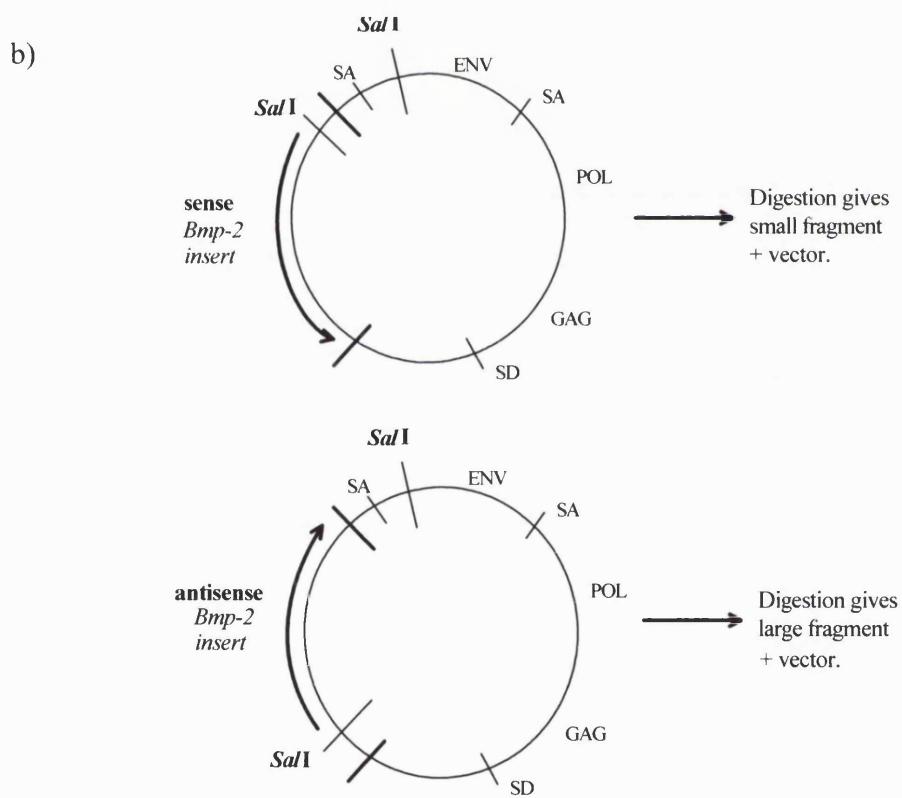
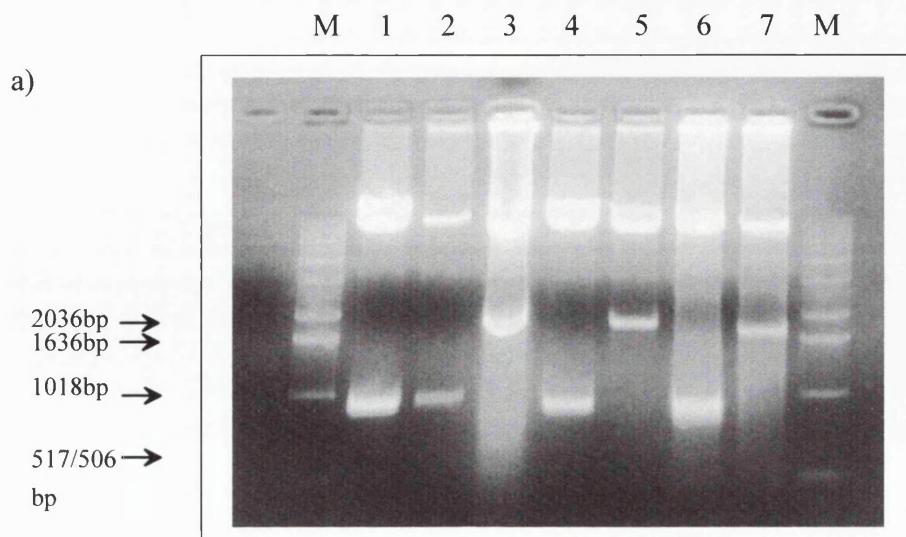


Figure 5.9: Determination of the orientation of the inserts of antisense retroviral clones, using *SalI* digestion.

- Photograph of gel showing *SalI* digests. (M: 1kb ladder markers; 1: 10+1; 2: 65; 3: 88; 4: 30/63; 5: 30/45; 6: 55/2; 7: 55.58)
- Maps of the retroviral constructs, showing the locations of the *SalI* sites in sense and antisense constructs..

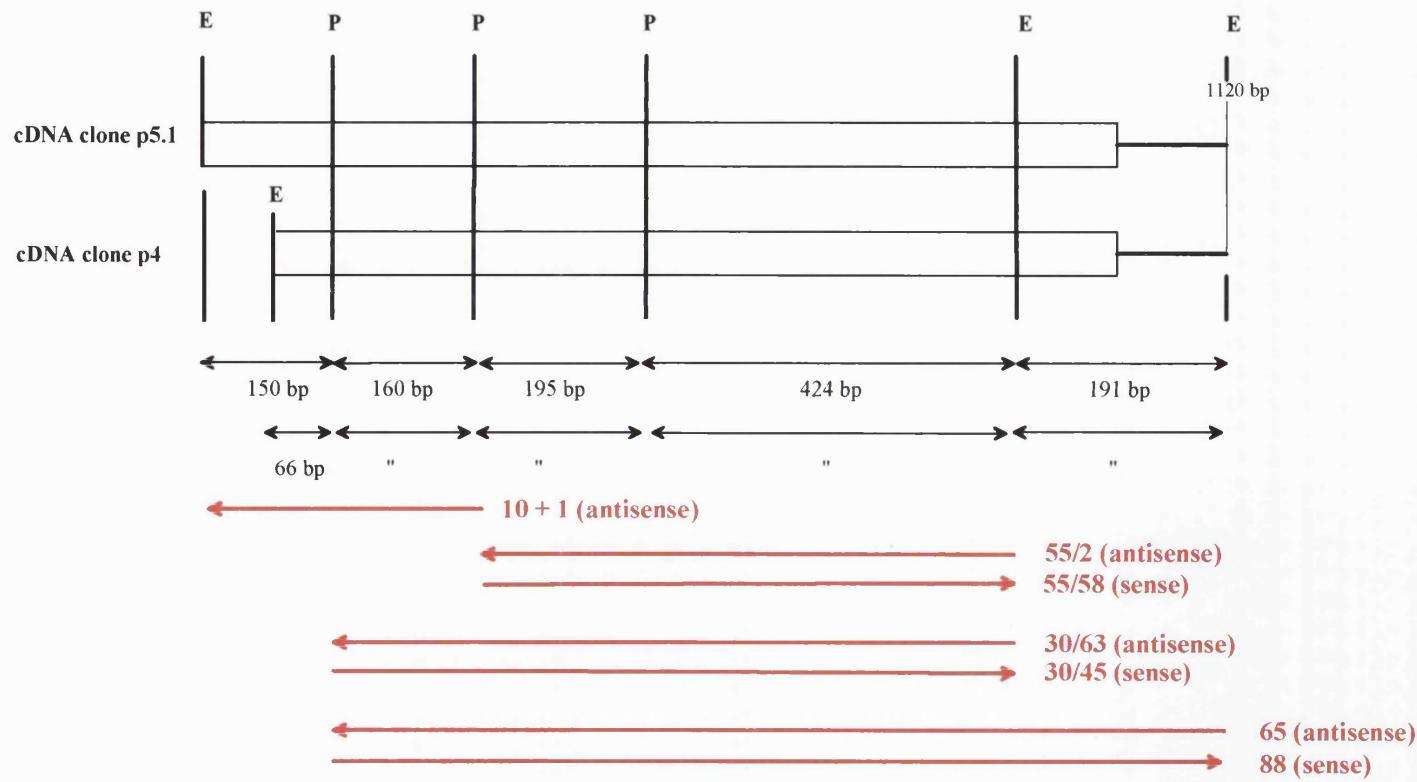


Figure 5.10: Positions and orientations (with respect to the upstream retroviral promoter) of the constructs obtained by subcloning of fragments from p5.1 & p4, compared to the cDNA clones.

b) Infection of chick fibroblast cells and tissue culture:

In order to infect chick embryo limb buds, pellets of cells infected with the viral constructs were grafted into limb buds, to provide a source of infectious virus to infect the surrounding limb bud tissue. The non-manipulated contralateral limb provides a convenient internal control for the effects of grafts on development (Summerbell and Wolpert, 1973).

The cells used for grafting were primary chick embryo fibroblasts (CEFs) isolated from ten day old 0 line chick embryos (Astrin *et al*, 1979). These cells were used because they contain no endogenous retroviruses, unlike cells derived from normal outbred chickens, such as White Leghorn chickens (Weiss *et al*, 1984). There is therefore no risk of recombination of the transfected constructs with endogenous sequences. Line 0 cells were used instead of any other virus-free strain of chicken cell because they are susceptible to infection by the RCAS(BP)A retroviral vector.

The disadvantage of using CEFs was that these cells do not keep growing continuously and become senescent after approximately six weeks (about 18 passages). For this reason, supernatant from these cells, which contains viral particles, was frozen down at -70°C, and used to re-infect fresh CEF cells at various intervals.

The conditions used for culturing these cells are described in the Materials and Methods Chapter. The viral constructs were transfected into the chick embryonic fibroblasts using the standard calcium phosphate method.

An advantage of using replication competent retroviruses is that, even if the initial transfection efficiency of the 0 line cells is low, the virus will spread to all the cells within a few days of transfection. However, it was important to ensure that all the cells were infected before grafting to ensure efficient infection of the limb bud after grafting. For this reason, the spread of the virus was monitored in the transfected 0 line cells using a monoclonal antibody (3C2) which recognises the viral protein gag (Potts *et al*, 1987). This protein is found in the protein core of the viral particle.

Using this staining method, transfected cells were only used for grafting after they had been confirmed to be 100 % positive for viral *gag* proteins, ensuring that all the cells contained the retroviral antisense constructs (Figure 5.11a, b and c).

c) Analysis of expression of *Bmp-2* constructs in CEF cells by Northern hybridisation:

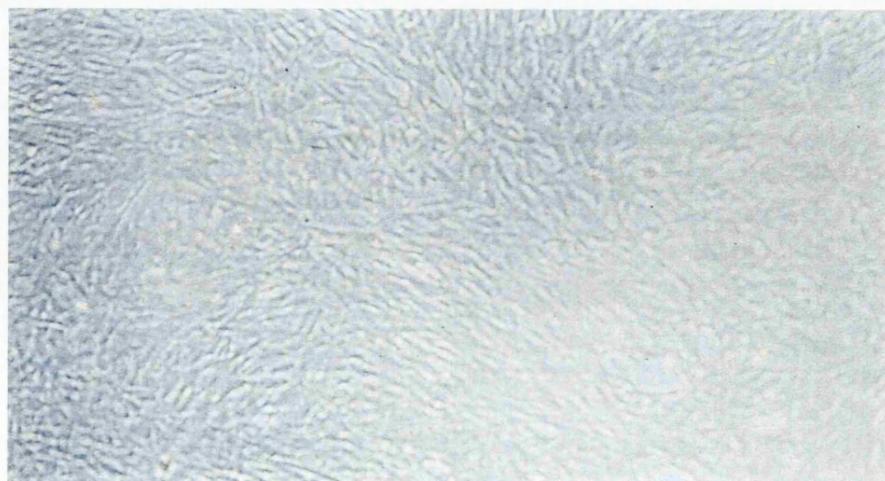
Poly(A)⁺ RNA was extracted from transfected CEF cells and analysed by Northern hybridisation to determine which viral transcripts were present. ³²P radioactive ribonucleotide probes complementary to the antisense *Bmp-2* sequences were used for hybridisation. RNA isolated from untransfected CEF cells was used as a negative control (Figure 5.12).

The antisense constructs appear to be expressed and spliced correctly in these transfected cells, giving the three spliced transcripts expected from the RCAS(BP)A-*Bmp-2* constructs. The relative abundances of all three splice variants vary between the constructs: 10 + 1 has the highest levels of *Bmp-2* insert compared to the other splice variants. and 55/2 the least.

All three transcripts could have an antisense effect, since they all contain the antisense *Bmp-2* sequences. However, the smallest spliced transcript, containing only the antisense *Bmp-2* message might be expected to be the most efficient at forming duplexes with the endogenous sense transcripts and effecting inhibition, because it does not contain any sequences that are not complementary to the sense transcripts which might reduce the efficiency of hybridisation or the stability of RNA:RNA duplexes (Murray, 1992).

d) Grafting of virally infected cells and extent of viral spread:

Once O line cells were ascertained to be 100 % infected with the retrovirus, pellets of these cells were grafted into the wing buds of stage 16-21 chick embryos, when patterning is occurring. The grafts were placed in the posterior region of the

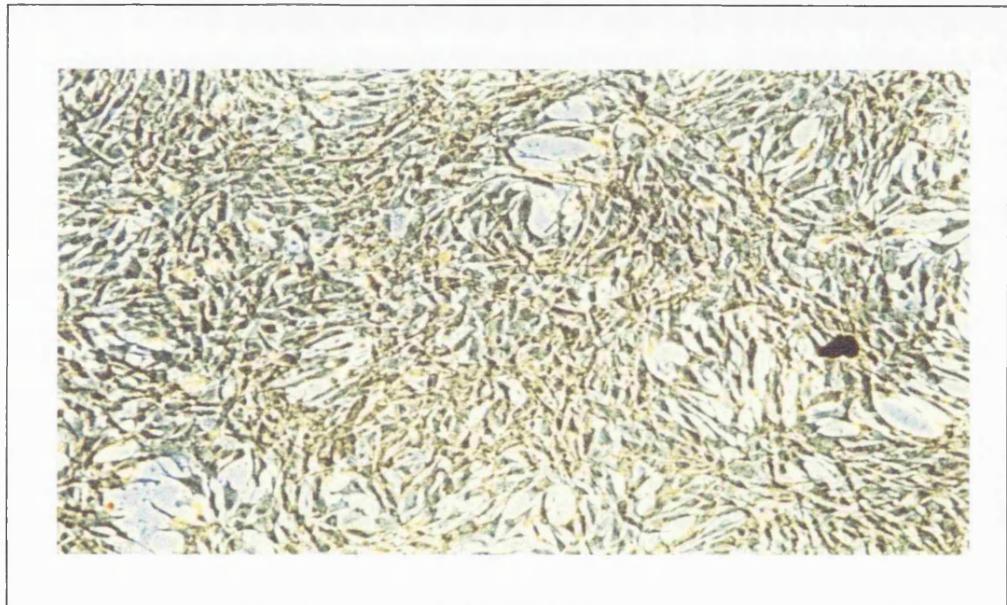


a) Negative control: untransfected CEF cells.



b) CEF cells transfected with antisense construct, 10+1, one week after transfection.

Figure 5.11: *gag* staining of 0 line cells transfected with the antisense retroviral constructs, to show efficiency of virus uptake and transfer.



c) High power view of CEF cells transfected with antisense construct, 10+1, viewed under phase contrast.

Figure 5.11 (cont.): *gag* staining of 0 line cells transfected with the antisense retroviral constructs, to show efficiency of virus uptake and transfer.

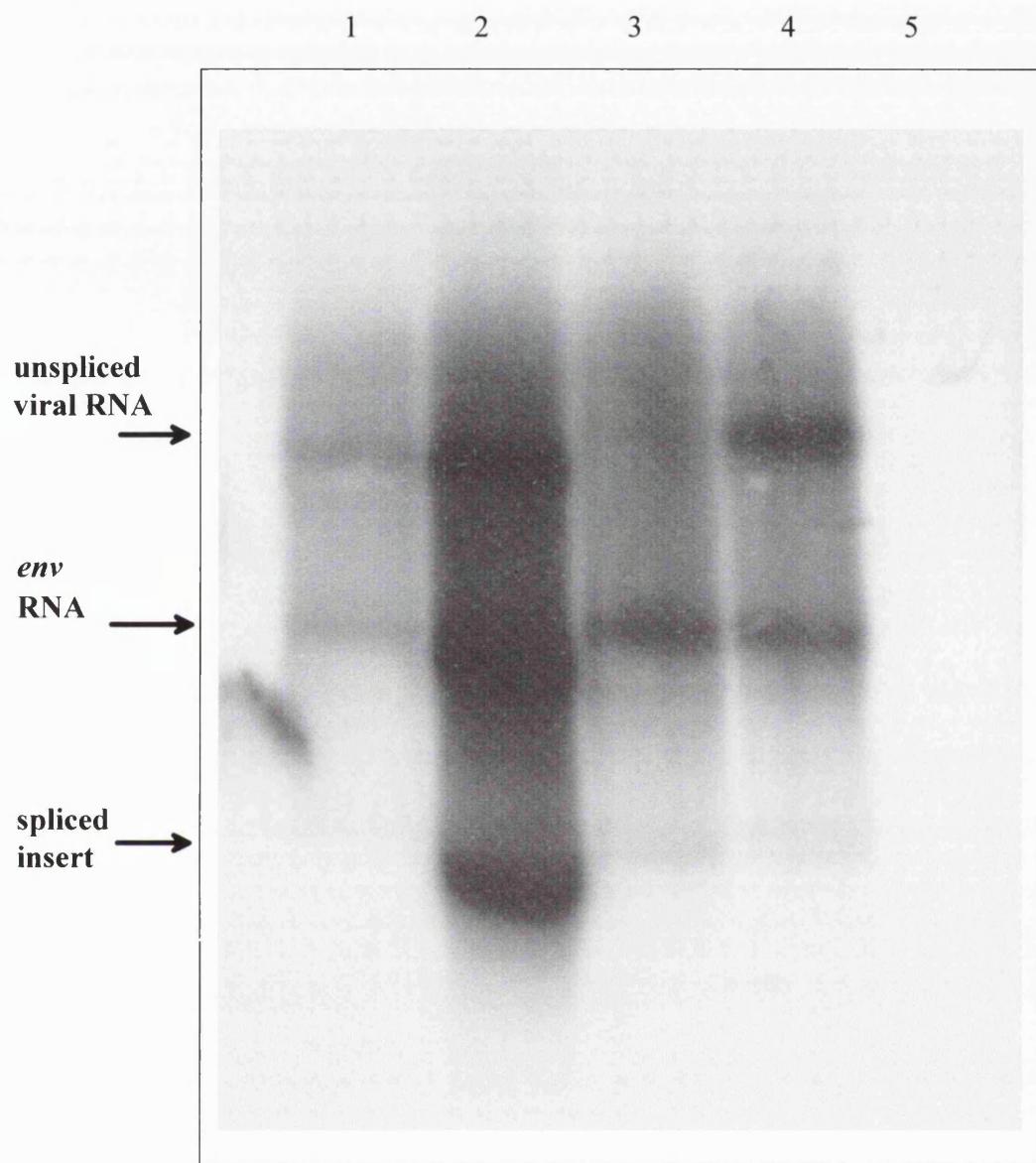


Figure 5.12: Northern blot of Poly(A)⁺ RNA isolated from CEF cells transfected with antisense *Bmp-2* constructs, hybridised with a radioactive probe complementary to the antisense *Bmp-2* sequence.

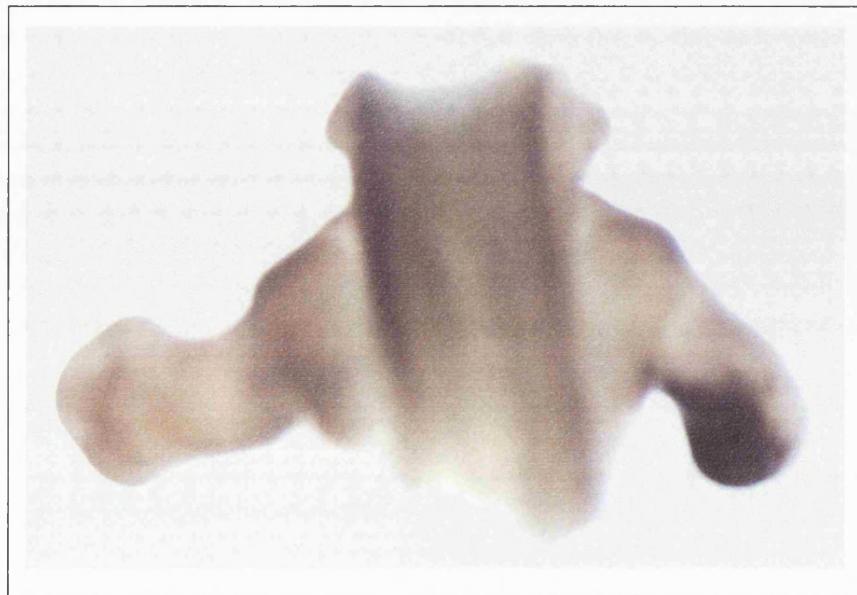
1: construct 55/2; 2: construct 10+1; 3: construct 65;
 4: construct 30/63; 5: untransfected cells.

mesenchyme, because this is where the highest levels of endogenous *Bmp-2* gene expression are found at this stage and where the polarising region is localised (Francis *et al.*, 1994; see Chapter 1). The virus will, however, continue to be expressed at later stages of development.

To determine the extent of viral spread, embryos were harvested 2–5 days after grafting. The embryos were fixed in 4 % paraformaldehyde and whole mount *in situ* hybridisation was performed using digoxigenin-labelled RNA probes specific for antisense chicken *Bmp-2* RNA.

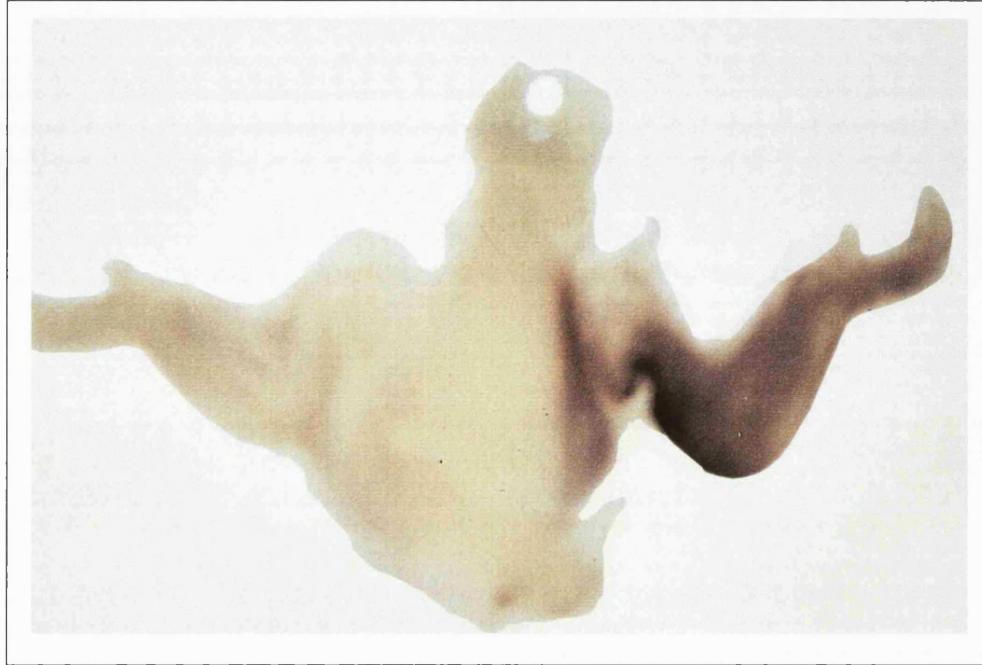
Figure 5.13 shows the presence and extent of spread of the antisense retroviral constructs in the early developing chick wings, 3 days after grafting. These hybridisation data show that the antisense transcripts are readily detectable in the limb bud cells. The spread of the viral constructs was variable and the constructs did not infect the entire wing bud within the incubation time, but the spread was sufficient to cover the normal domain of expression of endogenous sense *Bmp-2* expression, and therefore should allow duplex formation and inhibition of gene function. 12 embryos were analysed in this way by whole mount *in situ* hybridisation for spread of the *Bmp-2* constructs: the spread was variable, but the example in Figure 5.13 is representative of the typical extent of spread.

Figure 5.14 shows the presence and extent of spread of the antisense retroviral constructs in the developing chick wings at a later stage, at embryonic day 10. The antisense *Bmp-2* transcripts are still present at high levels at this stage. Again, the spread of the viral constructs was variable but the spread was amply sufficient to cover the normal domain of expression of endogenous sense *Bmp-2* expression, in some cases encompassing the entire wing, and therefore to allow duplex formation and inhibition of gene function. 8 embryos of this stage were analysed by whole mount *in situ* hybridisation, and the example shown in Figure 5.14 is a typical representative of the extent of viral spread.



Chick embryos harvested 3 days after grafting.

Figure 5.13: Presence and spread of antisense retroviral constructs in two developing right chick limb buds. These embryos have been hybridised with sense chicken *Bmp-2* riboprobes labelled with digoxigenin. The purple colour shows where the probe has hybridised to antisense *Bmp-2* RNA. Note the absence of hybridisation to the uninfected contralateral limb in the upper panel.



Embryonic day 10 chick embryo

Figure 5.14: Presence and spread of antisense retroviral constructs in developing limb bud of chick embryo at embryonic day 10. This embryo has been hybridised with sense chicken *Bmp-2* riboprobes labelled with digoxigenin. The purple colour shows where the probe has hybridised to antisense *Bmp-2* RNA. Note the lack of hybridisation to the uninfected contralateral limb.

e) Phenotype of antisense-grafted limbs:

A number of embryos were harvested at embryonic day 10 to assess the effect of the antisense constructs on development of the manipulated limbs. The embryonic limbs were stained for cartilage using Alcian Green to examine the limb phenotype in more detail. The limbs were then examined for defects and the lengths of the skeletal elements of the limb, relative to the control contralateral limb, were estimated.

Tables 5.1 and 5.2 summarise the effects seen, and Figures 5.15 to 5.18 show some specific examples.

In 27% of the cases (4/15), there was a fusion of the metacarpals in digits 3 and 4 (Figure 5.15, 5.16, 5.17 and 5.18a). This effect was seen in 25 % of the limbs infected with 10 + 1 (1/4), in 14% of those infected with 65 (1/7), and in both of the limbs infected with 30/63 (100%). This effect was not seen in any of the limbs infected with the sense constructs, and may therefore constitute a true effect of the antisense constructs, and inhibition of *Bmp-2* expression, on developing limbs. However, caution must be exercised in interpreting these results because of the low sample sizes ($n = 15$) (See Table 5.1).

In 67% of cases (10/15) there was a shortening and thickening of the skeletal elements, in particular of the radius and distal elements (Figure 5.18b). However, this effect was also seen in 88% (15/17) of the control limbs infected with sense constructs. This effect, therefore, was probably caused by grafting cells into the posterior zone of the developing limb, and hence disrupting formation of the skeletal elements, rather than an effect of the antisense viruses.

Table 5.1: Effects of antisense & sense constructs on chick limb morphology.

Key: n = normal length & shape.

S = element shorter than control contralateral element (the number following gives an approximate indication of severity of effect, from 1-5, with 1 representing least effect).

T = element thicker than control contralateral element (the number following gives an approximate indication of severity of effect, from 1-5, with 1 representing least effect).

tc = terminal claw phalanx missing.

Fusion = Fusion of metacarpals of digits 3 & 4.

Construct	Specimen	Effects						Notes
		Hum.	Radius	Ulna	D2	D3	D4	
10 + 1: Antisense	V1	S2/T2	S1	S2	n	S2	S2	Fusion.
	V3	S2/T1	S1	S3	S2	S2tc	S2tc	
	V4	n	n	n	n	n	n	D3/4 separated.
	V7	n	n	n	n	n	n	
55/2: Antisense	V14	n	n	S2	n	S2	S4	
	V17	n	S1	S1	S3	S3	S3	
55/58: Sense	V18	n	n	S2	n	S2	S2	
	V22	S1	S2	S3/T2	S1	S2	S4	
65: Antisense	V24	n	S1	S2	n	S1	S1	
	V25	n	S1	S3/T2	S1	S2	S2tc	
	V27	n	n	n	n	n	n	
	V30	n	n	n	n	n	n	
	V34	n	S1	S1	S1	S3	S3	Fusion.
	V37	S2/T1	S1	S2	S1tc	n	n	
	V38	n	n	n	n	n	n	

Table 5.1 (cont.): Effects of antisense & sense constructs on chick limb morphology.

Construct	Specimen	Effects						Notes
		Hum.	Radius	Ulna	D2	D3	D4	
88: Sense	V23	n	S3/T2	S3/T2	S4	S3tc	S3tc	
	V26	n	S3	S3	S2	S3	S4	
	V28	S2/T2	S2	S3/T2	S2	S3	S4	
	V29	n	S1	S3	n	S2	S2tc	
	V32	S1	S2	S3/T1	S2	S2	S2	
	V33	n	S1	S3/T3	S1	S2	S2tc	D4 metacarpal thin.
	V35	n	S1	S3/T3	n	n	S1/T1	
	V39	n	S1	S2/T2	n	n	n	
30/63: Antisense	V44	n	S1	S1/2	n	S2	S2	Fusion.
	V45	S2	S1	S2/T2	S1	S2	S2	Fusion.
30/45: Sense	V41	n	n	n	n	n	n	Defect in H/U joint.
	V43	S2/T2	S2	S3/T2	S2	S3	S3/T4	
	V48	S1	S1	S2/T2	S1	S1	S1	
	V49	n	S1	S4	n	n	n	
	V50	n	n	n	n	n	n	
	V51	n	n	n	S4	S4	n	
	V52	n	S1	S2	n	S2	S2	

Table 5.2: Summary of Effects of Antisense & Sense Constructs on Limb Development

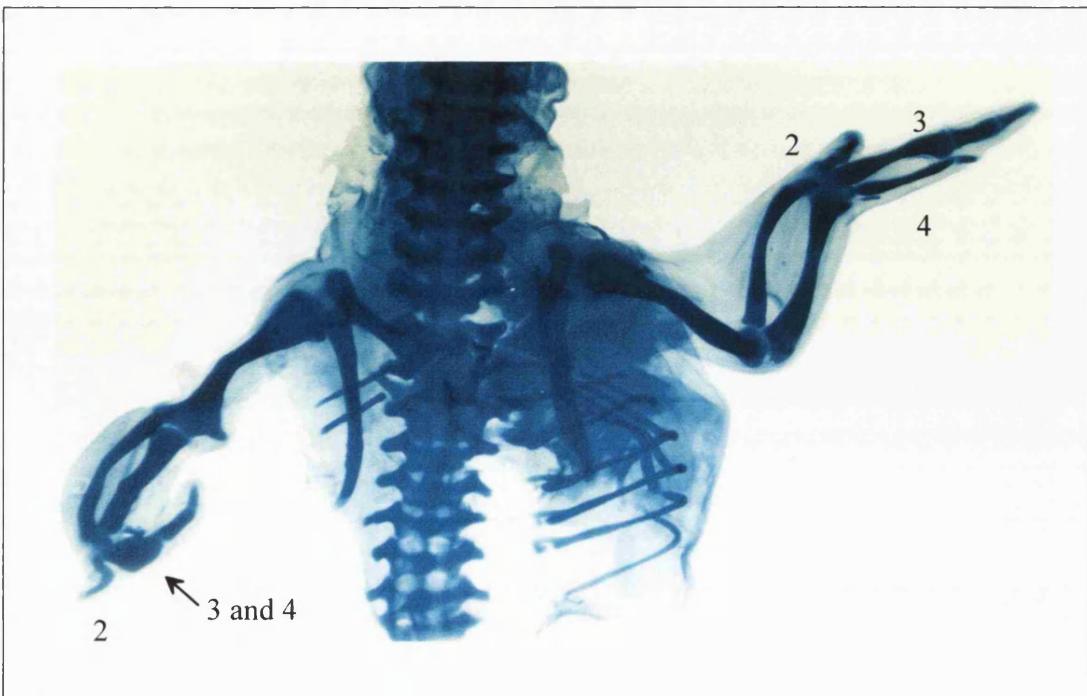
Construct	Change in Length & Shape							Fusions
	Hum.	Radius	Ulna	D2	D3	D4	Total	
10+1 Antisense	50% (2/4)	50% (2/4)	50% (2/4)	25% (1/4)	50% (2/4)	50% (2/4)	50% (2/4)	25% (1/4)
55/2 Antisense	- (0/2)	50% (1/2)	100% (2/2)	50% (1/2)	100% (2/2)	100% (2/2)	100% (2/2)	- (0/2)
55/58 Sense	50% (1/2)	50% (1/2)	100% (2/2)	50% (1/2)	100% (2/2)	100% (2/2)	100% (2/2)	- (0/2)
65 Antisense	14% (1/7)	57% (4/7)	57% (4/7)	43% (3/7)	43% (3/7)	43% (3/7)	57% (4/7)	14% (1/7)
88 Sense	25% (2/8)	100% (8/8)	100% (8/8)	62% (5/8)	75% (6/8)	88% (7/8)	100% (8/8)	- (0/8)
30/63 Antisense	50% (1/2)	100% (2/2)	100% (2/2)	50% (2/2)	100% (2/2)	100% (2/2)	100% (2/2)	100% (2/2)
30/45 Sense	29% (2/7)	57% (4/7)	57% (4/7)	43% (3/7)	57% (4/7)	43% (3/7)	71% (5/7)	- (0/7)

Total Antisense	27% (4/15)	60% (9/15)	67% (10/15)	47% (7/15)	60% (9/15)	60% (9/15)	67% (10/15)	27% (4/15)
Total Sense	29% (5/17)	76% (13/17)	82% (14/17)	53% (9/17)	71% (12/17)	71% (12/17)	88% (15/17)	- (0/17)

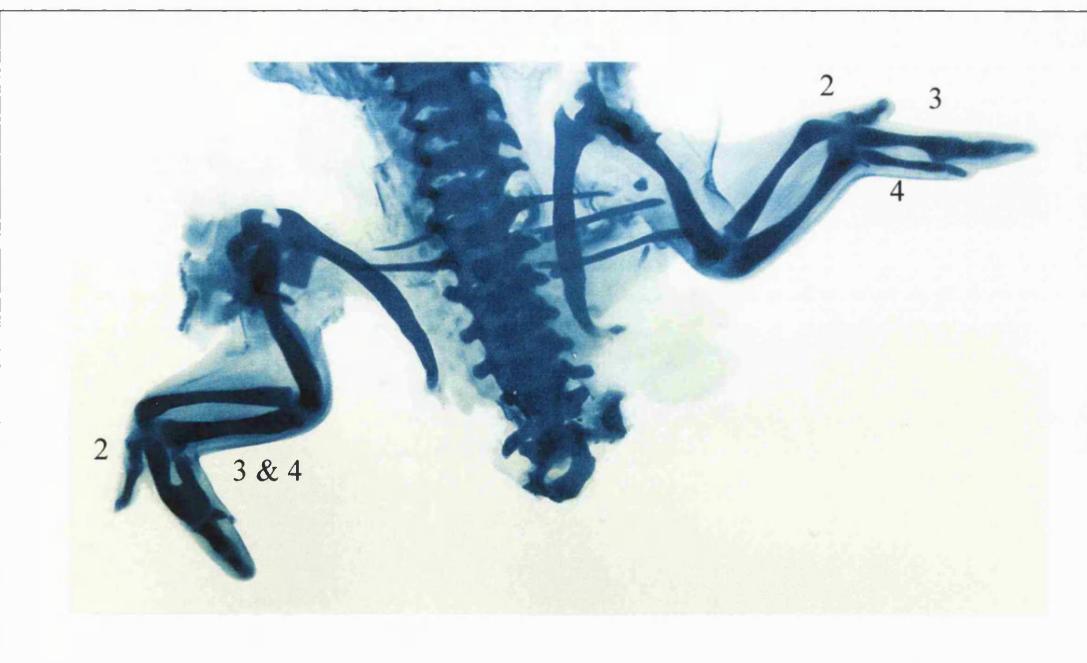
Key: Total Changes in Length & Shape = total number of limbs showing any effect with that construct.

Total: Antisense = sum total of affected cases for all antisense constructs.

Total: Sense = sum total of affected cases for all sense constructs.

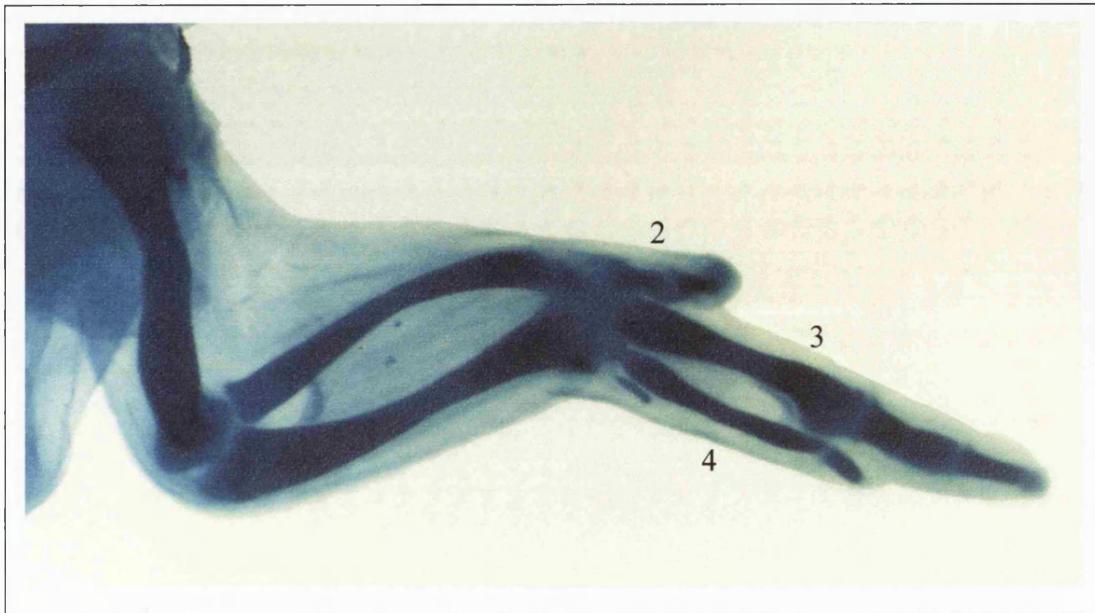


a) Fusion of metacarpals of digits 3 and 4 seen in limb infected with antisense *Bmp-2* construct 30/63 (numbers = digit identities) (specimen number V44).

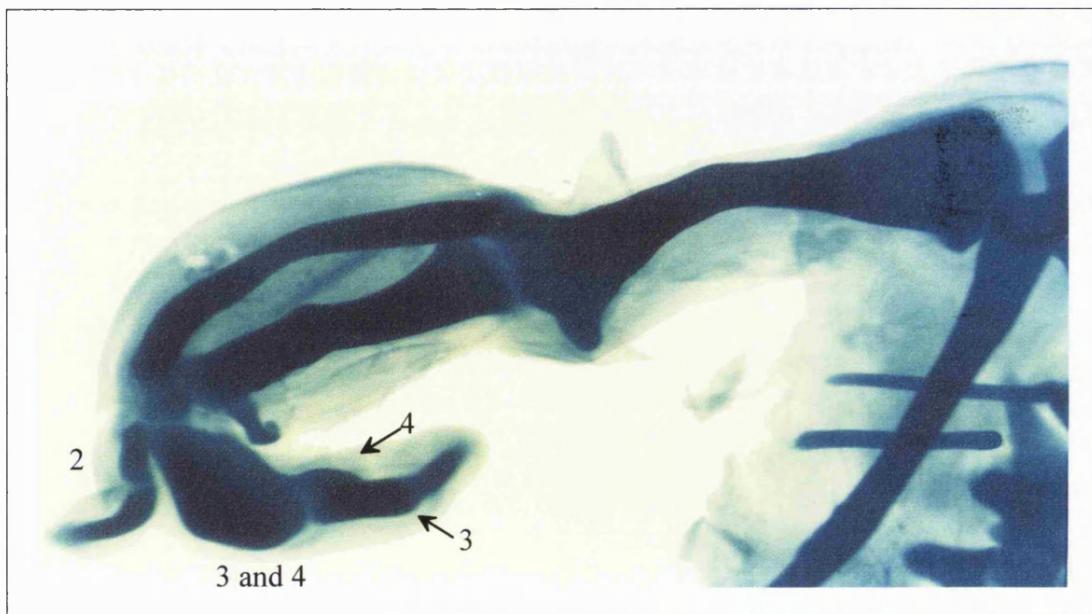


b) Fusion of metacarpals of digits 3 and 4 seen in limb infected with antisense *Bmp-2* construct 30/63 (numbers = digit identities) (specimen number V45).

Figure 5.15: Cartilage staining of embryos to show effects of *Bmp-2* retrovirus grafts.
(See Figures 5.16 and 5.17 for high power views).



a) Control contralateral limb of embryo shown in Figure 5.15 (Panel a).

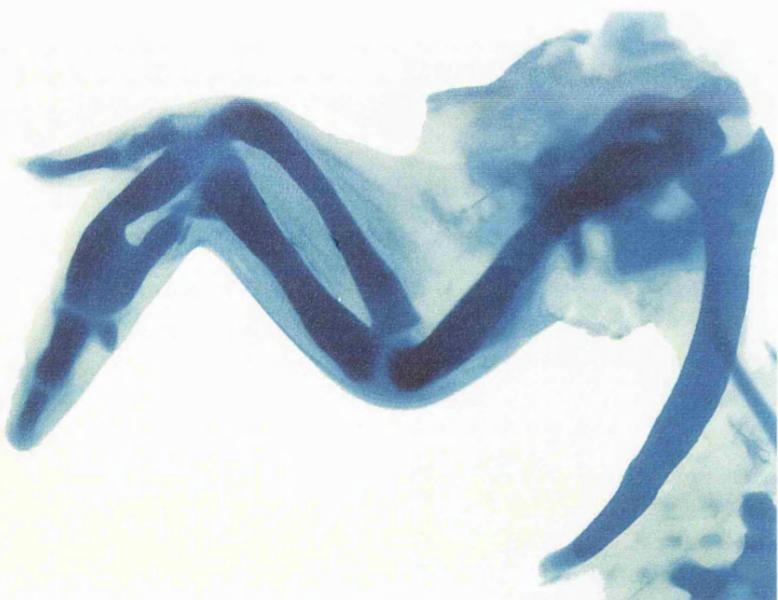


b) Limb infected with antisense *Bmp-2* construct 30/63 shown in Figure 5.15 (Panel a).

Figure 5.16 : Cartilage staining of embryos to show effects of *Bmp-2* grafts.

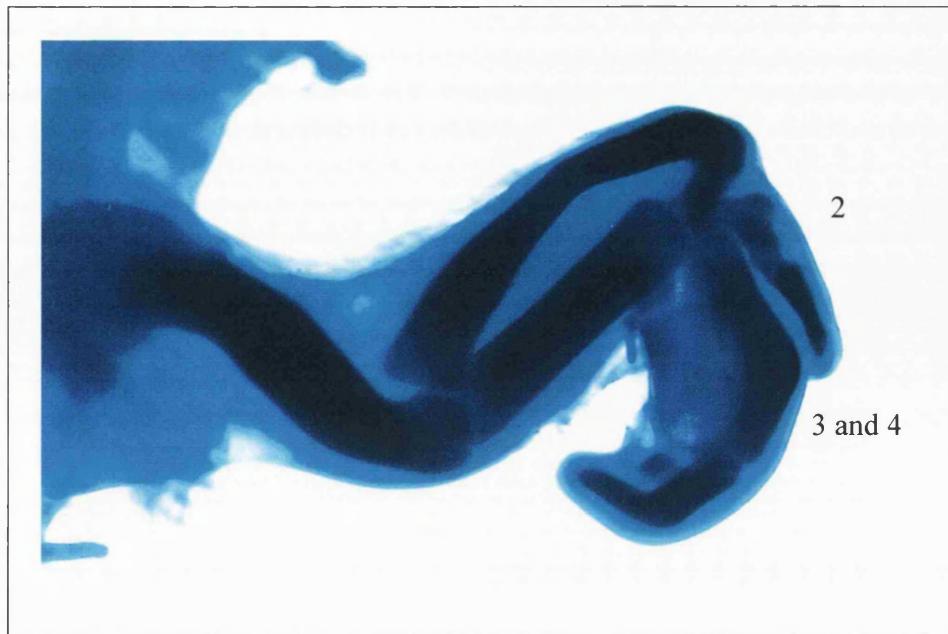


a) Control, contralateral limb of embryo shown in Figure 5.15 (Panel b).

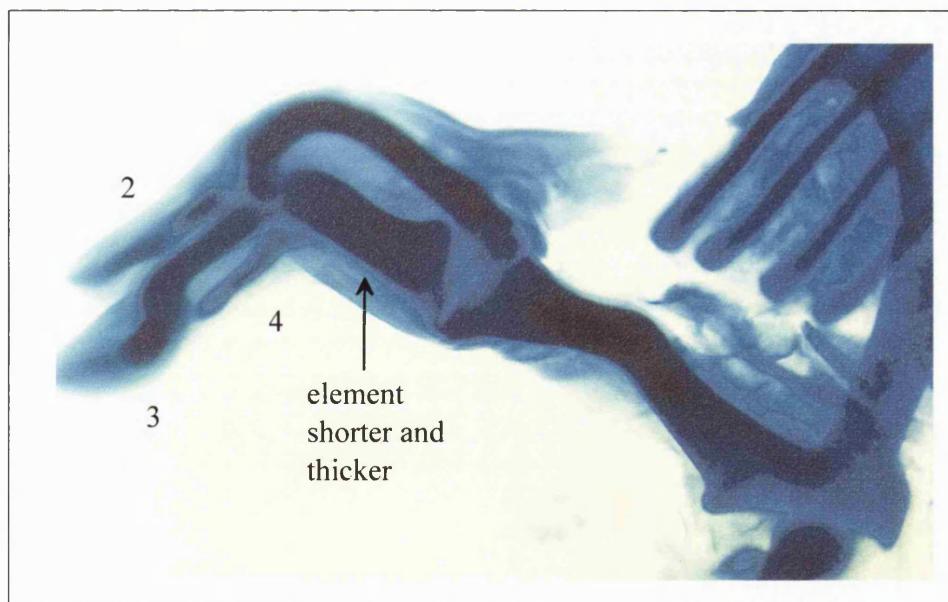


b) Limb infected with antisense *Bmp-2* construct 30/63 shown in Figure 5.15 (Panel b).

Figure 5.17 : Cartilage staining of embryos to show effects of *Bmp-2* grafts.



a) Limb infected with antisense *Bmp-2* construct 10+1, showing severe fusion of metacarpals 3 and 4. (Numbers indicate digit identities) (Specimen number V1)



b) Limb infected with sense *Bmp-2* construct 88, showing shortening and thickening of cartilage elements. (Numbers indicate digit identities) (Specimen number V29)

Figure 5.18: Cartilage staining of embryos to show effects of *Bmp-2* grafts.

f) Analysis of levels of endogenous sense *Bmp-2* transcripts:

Embryos were analysed by wholmount *in situ* hybridisation, using digoxigenin-labelled RNA probes specific for endogenous sense chicken *Bmp-2* RNA, to compare the levels of endogenous sense *Bmp-2* transcripts present in the control and manipulated limbs.

In some cases, there appeared to be a slight decrease in the levels of endogenous sense *Bmp-2* transcripts in the experimentally manipulated limbs, compared to the control limbs (Figure 5.19a). In other cases, however, there did not appear to be any difference in the intensity of staining between the control contralateral limbs and the experimentally manipulated limbs (Figure 5.19b).

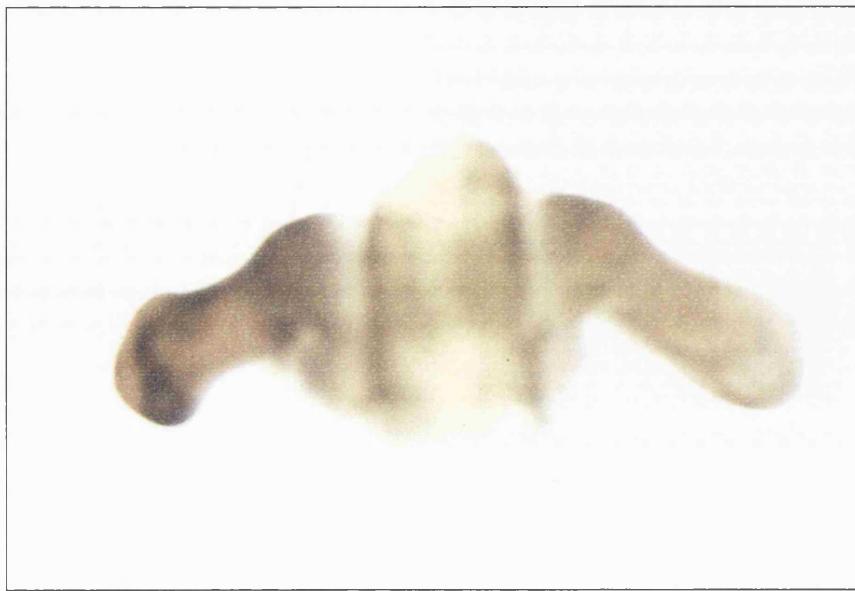
g) Analysis of antisense and sense transcripts by Northern Hybridisation:

Northern hybridisation was also used to determine the levels of antisense and sense *Bmp-2* RNA in transfected limbs. This is a more quantitative method than whole mount *in situ* hybridisation.

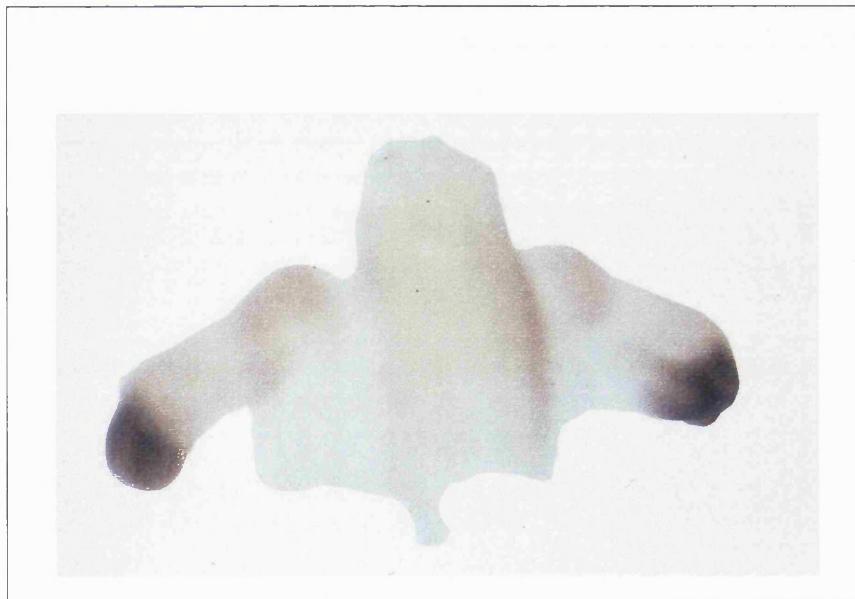
Total RNA was extracted from single transfected limb buds, 2-5 days after grafting, and analysed by Northern hybridisation to check for expression levels and splicing of the retroviral constructs. ^{32}P radioactive ribonucleotide probes complementary to the antisense *Bmp-2* sequences were used for hybridisation.

The total RNA isolated from individual transfected limbs was also used to check for any possible reduction in the levels of sense endogenous *Bmp-2* RNA. In this case, radioactive ribonucleotide probes complementary to the sense *Bmp-2* sequences were used, and a positive control of total RNA isolated from each contralateral control limb was used.

In all these Northern hybridisations, it was impossible to see expression of either the antisense or endogenous sense *Bmp-2* transcripts, because of the background levels of hybridisation of the ribonucleotide probes to the ribosomal RNAs present in the total



- a) Example of embryo with decreased levels endogenous of sense *Bmp-2* transcripts in the experimentally modified (right) limb compared to the contralateral (left) control.



- b) Example of embryo with similar levels of endogenous sense *Bmp-2* transcripts in control (left) and experimentally modified (right) limbs.

Figure 5.19: Levels of endogenous *Bmp-2* sense transcripts in experimentally modified developing chick limb buds, at embryonic day 10. These embryos have been hybridised to antisense chicken *Bmp-2* riboprobes labelled with digoxigenin. The purple colour shows where the probe has hybridised to the antisense constructs.

RNA. An example is shown in Figure 5.20. Extraction of poly(A)⁺ RNA was not carried out, because only very small amounts of RNA could be isolated from these individual limb buds, and any further extraction procedure would have resulted in yet more losses.

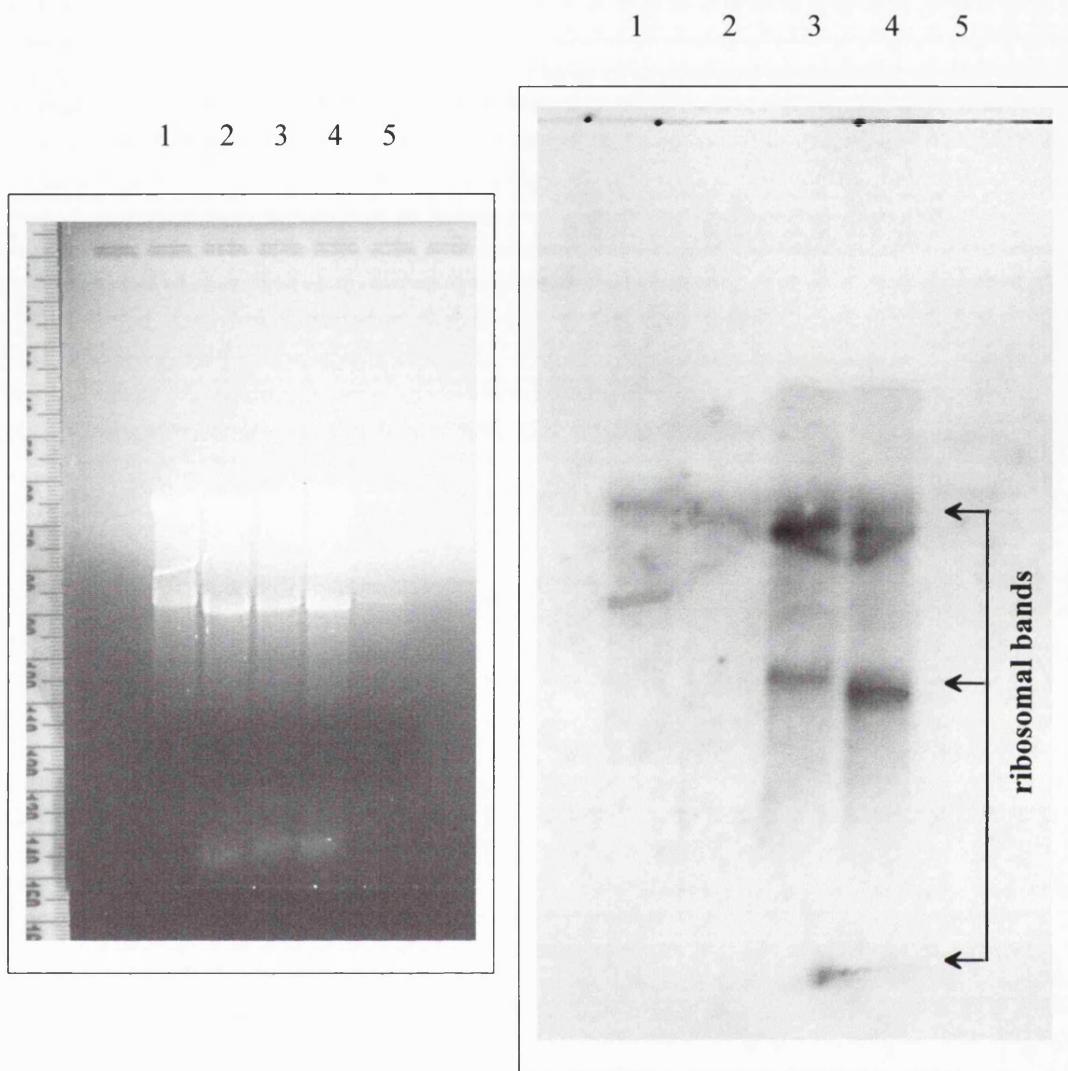


Figure 5.20: Northern blot of total RNA isolated from cells of individual limb buds transfected with *Bmp-2* constructs. This blot was hybridised with a radioactive probe complementary to the antisense *Bmp-2* sequence to show expression levels and splicing patterns of the viral antisense *Bmp-2* RNA in the infected limb bud. However, only non-specific binding to ribosomal RNA bands present in total RNA can be seen. This was seen in all Northern hybridisations attempted with total RNA isolated from infected limbs.

- a) Photograph of gel containing total RNA isolated from individual limb buds.
- b) Autoradiograph of Northern blot of gel shown in a), hybridised with a probe complementary to the antisense *Bmp-2* sequence.

1: Antisense construct 55/2; 2: Antisense construct 10+1;

3: Antisense construct 65; 4: Antisense construct 30/63;

5: Untransfected cells.

5.5 Discussion:

Constructs have been generated containing fragments of the chicken *Bmp-2* gene in both orientations in a retroviral expression vector, RCAS(BP)A. These constructs direct expression of sense or antisense *Bmp-2* RNA. Chick limb bud cells were infected with these retroviral constructs by grafting virus-producing cells into limb buds of stages 16 to 20, and the effects of antisense inhibition of the *Bmp-2* gene observed.

a) Effects of expression of antisense *Bmp-2* constructs in the developing chick limb:

Expression of these antisense constructs appeared to result in a decrease in the levels of endogenous sense *Bmp-2* transcripts in the developing chick limb in some specimens. The experimentally manipulated limbs appear to develop normally up to embryonic day 10, except for a fusion of the metacarpals in digits 3 and 4 in 27% of cases. This effect only occurred in limbs grafted with antisense constructs and not with the control sense constructs, but the number of observed cases was very small, and so it cannot be said with certainty that this effect was caused by antisense inhibition.

If this were indeed an effect of the inhibition of the *Bmp-2* gene, it could arise either as a function of a disruption of patterning of the skeletal elements, or due to the production of excess ectopic cartilage.

As described in Section 5.1, there are two mutations thought to be linked to *Bmp-2*: *Tight-skin* in mice and FOP in humans (*Tsk*: Dickinson *et al*, 1990; Green *et al*, 1976; Jimenez *et al*, 1986, 1988; FOP: Kaplan *et al*, 1990; Schroeder and Zasloff, 1980; Tabas *et al*, 1991). Both of these result in the production of excess bone and cartilage, and were therefore thought to result from a dominant gain of function, rather than a deletion in the *Bmp-2* gene (Kaplan *et al*, 1990; Dickinson *et al*, 1990). However, the results of expression of antisense *Bmp-2* constructs might suggest that increases in bone and cartilage formation could occur as a result of inhibition, as well as over-expression, of *Bmp-2* (Wozney *et al*, 1988). FOP also results in the fusion of the metacarpal

phalanges, lending support to the suggestion that *Tsk* and FOP represent mutations in the *Bmp-2* gene.

If the fusions between metacarpals of digits III and IV are specific effects of *Bmp-2* inhibition, this might indicate that *Bmp-2* is responsible for the formation of these specific cartilage elements, either in terms of the anterior-posterior axis (i.e., digits III and IV) or the proximo-distal axis (i.e., metacarpal level). This is supported by the fact that most malformations in FOP occur in the digits, and that the big toe and digit V are affected much more frequently than the middle digits (Figure 5.1). This would also lend credence to the theory that the skeleton is a mosaic structure built of composite patterns of activity of BMP-like proteins, with different members of the BMP family controlling the formation of different elements and morphological features of the skeleton, as suggested by Kingsley *et al* (1994) and Storm *et al* (1994) on the basis of mutations in *Bmp-5* and *Gdf-5*.

However, the abnormalities in the number of bones in the *Gdf-5* mutations have also been compared with the defects found in *Hoxd* gene knockout mice (Tickle, 1994; Dollé *et al*, 1993). This link seems plausible because, as discussed in the Introduction, the *Drosophila* homologue of *Bmp-2*, *decapentaplegic* has been shown to interact with various homeobox genes during development in the fly. In addition, ectopic expression of *Bmp-2* can induce *Hoxd-11* and *d-13* expression in the developing chick limb (Duprez *et al*, in press).

As described in the Introduction, *Hoxd-13* is the last gene of the *Hox-d* cluster, and the most distally restricted gene (Dollé *et al*, 1989b). Early in limb development, it is normally expressed posteriorly in the major body axis and, in the limbs, its expression is restricted to distal and posterior cells, in the area around the ZPA (Dollé *et al*, 1989b; Izpisúa-Belmonte *et al*, 1991; Hayamizu *et al*, 1994). Expression in the mesenchyme is graded along the A-P axis, with expression maximal at the posterior margin and decreasing progressively anteriorly. There is no expression in the AER.

Hoxd-13 loss-of-function mutant mice have skeletal alterations along all the body axes (Dollé *et al*, 1993). In the limbs, there is a reduction in the length of some of the bony elements, loss of phalanges, bone fusions and an extra rudimentary posterior digit is

sometimes seen in the fore and hind-limb. The shortening effect is most pronounced in digits I, II and V. There are also fusions between the articulations, particularly the metacarpal-phalangeal joint, and between the metacarpals (Figure 5.21).

There is no obvious change in development in the mutant mice until day 13.25 *post coitum*, when the individual cartilage condensations become apparent. In the mutants, the separation of the metacarpal condensations from the phalanges is visible but ill-defined, and the condensations appear smaller. Ossification is also much delayed and in some areas does not even occur. Dollé *et al* (1993) suggest that these effects are best explained by a retardation in limb morphogenesis. They also suggest that the extra element forms because the extra space in the limb due to the small size of the other elements leads to spontaneous condensation, rather than due to respecification of the anterior-posterior axis, such as occurs with ZPA grafts or RA.

The fusions of the metacarpals seen in *Hoxd-13* mutant mice resemble those seen in our experiments inhibiting *Bmp-2* expression in the developing chick limb. This therefore might indicate that, in normal development, *Bmp-2* regulates expression of *Hoxd-13*.

Francis *et al* (1994) showed that, in normal chick limb buds, the expression domain of *Hoxd-13* in the mesoderm of the chick limb is initially contained within the domain of *Bmp-2* expression, and then spreads to form a domain that overlaps with and spreads beyond the *Bmp-2* domain. Limited diffusion of BMP-2, such as occurs for *Drosophila* DPP (Panganiban *et al*, 1990), could account for the *Hoxd-13* domain becoming slightly larger than the *Bmp-2* domain (Francis *et al*, 1994). When *Bmp-2* and *Hoxd-13* are ectopically induced anteriorly by RA, the same relation occurs between the ectopic domains of expression (Francis *et al*, 1994). Duprez *et al* (in press) have shown that BMP-2 on its own can activate expression of *Hoxd-11* and *Hoxd-13* in anterior mesenchyme.

It has also been shown that a signal from the AER is required to maintain expression of *Hoxd-13* (Izpisúa-Belmonte *et al*, 1992b; Hayamizu *et al*, 1994), and this might either be fulfilled by expression of *Bmp-2* in the AER, or by signalling between *Bmp-2* in the mesenchyme and an ectodermal signal, possibly one of the FGFs (Duprez *et al*, in press). This would be similar to the signalling by DPP found in the development of

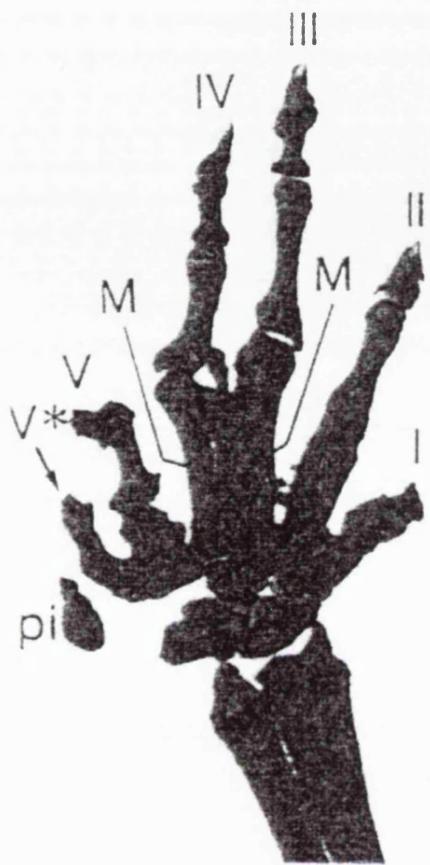


Figure 5.21: Defects seen in limbs of homozygote null *Hoxd-13* mutant mice (adapted from Dollé *et al.*, 1993).

This figure shows a dorsal view of a *Hoxd-13*^{-/-} forelimb autopod skeleton. The fusions between the skeletal elements are clearly shown, especially between metacarpals (M) III and IV. The arrow points to an extra postaxial digit (V*), which in this animal was a single bone, fused to the base of the fifth metacarpal.

the *Drosophila* gut, where DPP is synthesised in the mesoderm, and activates expression of the homeotic gene *labial* in neighbouring endoderm (Panganiban *et al*, 1990).

Therefore, inhibition of *Bmp-2* expression by antisense sequences would result in lower levels of *Hoxd-13* expression. However, antisense techniques usually only result in a decrease in the production of the target product, rather than complete inhibition, and this might account for the fact that the phenotype of the *Hoxd-13* knock-out mice is more severe than the results described in this chapter.

However, the phenotype observed upon expression of antisense *Bmp-2* constructs was not what might have been predicted from the proposed role of *Bmp-2* in the chick limb. A much more severe effect on development was anticipated. The fact that this did not occur can be explained in several different ways.

For example, the role of the *Bmp-2* gene in development might not be as important as was thought. Its early expression pattern might not correlate with a role in initial development of the limb pattern, but instead be a by-product of expression needed for a function later on in development, for example, in correct bone formation. Alternatively, this may be an example of functional redundancy, with an alternate pathway providing a back-up system so that the entire process of limb development does not fail because of disruption in a single gene. This could be another gene which could take over the function of the *Bmp-2* gene in the event of a decrease in expression of *Bmp-2*. This role may be played by another gene in the BMP family, such as *Bmp-4* or *Bmp-7*, both of which are present in the developing chick limb (Francis *et al*, 1994; Francis-West *et al*, 1995).

However, the fact that an abnormal phenotype was observed in limb buds infected with antisense *Bmp-2* constructs, the fusion of the metacarpals in digits 3 and 4, indicates that not all the functions of *Bmp-2* can be compensated for by other genes. The similarity of this observed phenotype of inhibition of *Bmp-2* to the effect of inhibiting *Hoxd-13* expression might indicate that regulation of the *Hoxd-13* gene is specific to *Bmp-2*, and cannot be compensated for by *Bmp-4* or *Bmp-7*. Alternatively, this may be the only role that requires high levels of BMP-2, possibly in the form of homodimers,

and that the other roles can be carried out by either by lower levels of *Bmp-2* expression, or by heterodimers formed with other TGF- β family members, requiring lower levels of production of BMP-2 protein.

b) Possible optimisation of the antisense system and further experiments:

However, the most plausible explanation for this lack of effect on limb development is that the low and variable levels of reduction of the endogenous sense *Bmp-2* genes achieved in the limbs infected with antisense constructs were not sufficient to affect development significantly. There are several ways in which this antisense system could be optimised to give more efficient inhibition of *Bmp-2* expression.

The low levels of reduction may occur because insufficient levels of antisense transcripts are being expressed. Despite the fact that the whole mount *in situ* hybridisation data seem to indicate that relatively high levels of retrovirus are being produced, and that the viruses are spreading throughout the limb sufficiently to cover the normal expression domain of *Bmp-2*, effective antisense inhibition may require a vast excess of antisense transcripts (50-fold or higher) over the endogenous sense transcripts, as described in Section 5.2b. A more accurate assessment of the levels of antisense and endogenous sense RNA in the infected limbs could be achieved by hybridising radioactive probes to sections of the limb and counting the silver grains in the developed emulsion, or by performing RNase protection on the isolated RNA from individual limbs. Hybridisation of probes to sections of the limb would also show whether the antisense transcripts were present throughout the limb tissue.

Whole mount *in situ* hybridisation followed by staining for cartilage with Alcian Green could also be used to correlate viral spread and antisense and sense RNA levels with the severity of phenotypic effect on cartilage. In this way, the severity of the malformations in the skeletal elements could then be directly observed, and correlated with whether the same embryo has high levels of antisense transcripts, or a large reduction in endogenous sense *Bmp-2* levels.

Analysis of the levels of antisense and endogenous sense transcripts in individual limbs by Northern hybridisation did not yield results in these experiments, because of the high levels of non-specific hybridisation of the *Bmp-2* ribonucleotide probes to the ribosomal bands present in total RNA isolated from limb bud cells. It was not considered practical to extract poly(A)⁺ RNA from these limbs because of the low levels of total RNA isolated from individual limb buds. This problem might, however, be circumvented by using a cDNA probe, which would recognise both sense and antisense RNA transcripts, or by pooling total RNA from a number of limb buds and then extracting poly(A)⁺ RNA.

The stability of the antisense constructs in the limb mesenchyme cells may also be influencing transcript levels. Previous work has also shown that not all the constructs are correctly spliced out of the retroviral vectors and not all are stable (E. Bell, personal communication). Although correct splicing of the antisense constructs to isolate them from the viral transcripts may not be necessary for duplex formation and inhibition, it would be expected that the smaller transcripts, not associated with the viral RNA, would be more efficient at forming duplexes and blocking *Bmp-2* activity. Correct splicing and stable expression of antisense *Bmp-2* constructs were shown in infected CEF cells, but I was unable to show correct splicing in limb bud mesenchyme cells due to problems with Northern hybridisation to RNA isolated from single limb buds, as described above.

In some cases in the literature, however, it was not possible to detect any antisense transcripts on Northern gels (van der Krol, 1988b; Colman, 1990). This may be because the antisense RNAs bind to the sense transcripts in the nucleus and trigger nuclease degradation by a dsRNA unwindase/modifying enzyme (Bass, 1992). This enzyme was originally found in *Xenopus* oocytes, but has since been found in most eukaryotic systems tested. This enzyme specifically attacks RNA duplexes, and changes adenosine bases to inosine, which causes the duplex to unwind, as these bases hybridise less stably. The resulting complexes are undetectable by hybridisation and untranslatable (Cornelissen, 1989). Efficient transcription of the antisense gene, however, can be demonstrated by nuclear run-on experiments (Crowley *et al.*, 1985).

A reduction in steady-state sense mRNA levels also usually occurs in the presence of antisense transcripts, perhaps because of increased turnover or degradation of duplexes (Crowley *et al.*, 1985; Khokha *et al.*, 1989). However, in some cases, 70-90% inhibition in enzyme activity has been described with no concomitant reduction in sense mRNA transcript levels (Strickland *et al.*, 1988), and in such case nuclear run-off experiments demonstrated that there was equally no effect on transcription (Sheehy *et al.*, 1988).

Another factor influencing inhibition is the timing of the antisense expression compared to the endogenous sense transcripts. Melton (1985) showed that by injecting both antisense and sense globin RNA into the cytoplasm of *Xenopus* oocytes, complete abolition of sense gene initiation was achieved if the antisense genes are injected first, but if the sense genes are injected first, only downregulation of expression was possible. In another case, however, Harland and Weintraub (1985) showed that, in *Xenopus* oocytes injected with antisense and sense TK and CAT constructs, even if the antisense transcripts are injected first, there may still not be complete inhibition: 5-10% of enzyme activity was detected even with vast excesses of antisense RNA.

There are several ways in which antisense expression could be optimised in this system. The retroviral promoter is a strong one which has been shown to direct insert expression efficiently in these cells, but the levels and spread of the retroviral constructs could be increased by applying the viruses earlier, possibly before the endogenous *Bmp-2* gene is expressed. Grafting of transfected cells into the limb cannot be carried out any earlier than stage 15-16, because the limb primordia are difficult to identify and target properly. However, microinjection of viruses has been successfully carried out in the flank in the area of the presumptive limbs as early as stage 7 in our laboratory, and this might provide a way of optimising viral spread and levels.

In more general terms, it may not be possible to achieve complete antisense inhibition in this chick expression system. The reported reduction in endogenous sense RNA levels varies between 6% to 90% (Kim and Wold, 1985; Rothstein *et al.*, 1987; Smith *et al.*, 1988; Sheehy *et al.*, 1988; Kasid *et al.*, 1989), but complete inhibition is generally not achieved. This may be because a vast excess of antisense to sense transcripts is required, because the non-coding antisense transcripts are unstable, or because the translation machinery can denature the RNA:RNA duplexes given sufficient time

(Harland and Weintraub, 1985). Antisense duplex formation may be inhibited by lack of availability of target sequences due to hybridisation with proteins or nucleoprotein complexes. A given cell system may also possess enzymes to degrade antisense:sense duplexes.

For the great majority of genes studied, complete inhibition was not required for an effect. In some systems, however, even low levels of expression of a gene are sufficient for normal gene action. For example, expression of antisense mRNA to an oncogene, *H-ras*, in *H-ras*-transformed cells caused no phenotypic reversion, despite the fact that the two genes were simultaneously expressed, driven by a strong promoter, the SV40 early promoter, and a 10-20 fold excess of antisense over sense was found (Salmons *et al*, 1986). They suggested that only complete suppression of *H-ras* would result in an observable effect.

There may also be variation in the effects of antisense *Bmp-2* constructs depending on where the virus integrates into the host genome. Sequences in the genome bordering the site of integration may repress or stimulate expression of the viral constructs. Such positional effects have previously been shown to be important in plants, when an antisense construct to the flower pigment gene, chalcone synthase, was transformed into petunias (van der Krol *et al*, 1988a). When the totipotent transformed cells were grown up into full plants, the resulting flowers had varying spatial expression and amounts of expression of the antisense sequence, depending on the genomic environment of the insert. This potential attenuation of the effects of antisense *Bmp-2* constructs could account for the mild effects of *Bmp-2* inhibition seen in this study, and further grafting experiments could be done to examine this.

However, the most direct way of studying the effects of antisense inhibition is to examine the levels of target protein produced, and this was used in the chalcone synthase experiments to directly show variations in the levels of protein production, as shown by the levels of pigmentation in the flowers (van der Krol *et al*, 1988a). It is generally assumed that, in the presence of antisense RNA, a substantial reduction in detectable levels of the target protein will be observed, and this has generally been observed (Pecorino, 1988; Khokha *et al*, 1989). In the case of *Bmp-2*, however, specific antibodies have to date not been available to study the levels of actual protein

produced in limbs. Until, specific antibodies are available, examination of variation in antisense construct expression is limited to examining RNA levels by *in situ* hybridisation.

Further variation in expression of the antisense constructs may arise from restrictions on the expression of the viral vector. Different patterns of expression of different *env* subgroup viruses have been shown in newly hatched chicks (Fekete and Cepko, 1993a; Brown and Robinson, 1988). Subgroup A can efficiently infect a wide range of connective, endocrine and reproductive tissues, including bone marrow, osteocytes, the adrenal cortex, thyroid, bursa, gonads, skeletal muscle and neural tissues, but is less effective at infecting haematopoietic tissues. Subgroup B viruses are better able than A to infect haematopoietic tissue, and subgroup E effectively infects the bursa, thymus, haematopoietic tissue and neural tissue. Subgroups A and E also efficiently infect cultured chicken embryonic fibroblasts, with better infection shown by A than E (Brown and Robinson, 1988). These experiments have not been carried out in embryos, and various other experiments have shown that retroviruses can be used to efficiently infect the developing chick limb (de la Pompa and Zeller, 1993; Riley *et al*, 1993; Riddle *et al*, 1993; E. Bell, personal communication), but it might be possible to make constructs expressing antisense *Bmp-2* sequences in other retroviral vectors to examine efficiency of *Bmp-2* inhibition. Synthetic antisense oligonucleotides to *Bmp-2* could also be used to try to inhibit *Bmp-2* gene function.

Finally, the non-specific phenotypic effects shown in these experiments, the shortening and thickening of the skeletal elements could be eliminated, to give a clearer picture of the specific effect of inhibiting *Bmp-2* expression. These non-specific effects are probably due to grafting cells into the posterior region of the limb, mechanically disrupting development of the limb structures. Since Tickle *et al* (1978) showed that fibroblast cells implanted into the embryonic chick wing do not affect morphogenesis, this is unlikely to be an effect of the cultured CEF cells, and previous experiments have shown that grafts of retrovirally-infected cells apically or anteriorly do not affect morphogenesis (Riley *et al*, 1993; E. Bell, personal communication). However, the antisense *Bmp-2* transcripts must be targeted to the posterior region of the developing limb in order to interact with the endogenous *Bmp-2* sense transcripts. One way to overcome this is to isolate the polarising region, infect it with an antisense *Bmp-2* virus

and then to graft it anteriorly into a host limb bud, or to graft a pellet containing a mixture of uninfected polarising cells and CEF cells infected with antisense virus anteriorly. This would be expected to show the effects of *Bmp-2* inhibition on the *Bmp-2* expression induced ectopically by the ZPA graft (Francis *et al*, 1994).

In summary, constructs containing antisense *Bmp-2* sequences in retroviral expression vector have been successfully produced, and used to infect embryonic chick limb buds. This provides a useful method for inhibiting gene expression in the developing limb bud, which has previously been resistant to gene inhibition techniques, but nonetheless provides an important system in which to study development because of its amenability to embryonic manipulation experiments.

Further grafting experiments are needed to fully determine the results of antisense inhibition of *Bmp-2* in the chick limb and to optimise the system we have designed.

CONCLUSIONS

The aims of the work described in this thesis were to determine the structure of the chicken *bone morphogenetic protein-2* gene, and to analyse its role in the development of the embryonic chick limb.

I have isolated and characterised a chicken genomic clone of *bone morphogenetic protein-2* (*Bmp-2*), #16F, and analysed the 5' coding region and intron-exon structure. Analysis of the sequence of this clone enabled me to identify a portion of the 5' coding region that was absent from the partial *Bmp-2* cDNA, p5.1, that was previously isolated in our laboratory (Francis *et al*, 1994). The sequence of the *Bmp-2* gene is highly evolutionarily conserved between species, and in this region the chicken sequence is very similar to the mouse and human *Bmp-2* sequences (60-66% over the amino acids that could be compared). I also examined the intron-exon structure of this gene by restriction mapping and sequencing, and compared it with that of *Bmp-2* and *Bmp-4* genes from other species. The location of intron-exon boundaries examined was also almost identical in the *Bmp-4* and *Bmp-2* genes in humans, mice and chickens. Preliminary mapping of the 5' non-coding of the gene showed that there is an intron 7 bp upstream of the ATG codon. An intron is found in the same position in the mouse *Bmp-2* and *Bmp-4* genes, and the *Drosophila decapentaplegic* gene. No other member of the TGF- β superfamily has multiple 5' non-coding exons. This supports the hypothesis that *Bmp-2*, *Bmp-4* and *dpp* are very closely related.

All attempts to identify additional sequence from the 5' non-coding region upstream of this intron, both by cDNA cloning and primer extensions, failed to generate any sequence longer than the original cDNA clone, p5.1. There appears to be some region of secondary structure at this position in the gene, which might be causing the polymerase enzymes to terminate prematurely. It might be possible to circumvent this problem by carrying out primer extension or cDNA library screening under conditions designed to

eliminate secondary structure, such as higher temperatures. Once the 5' end of the gene has been identified and cloned, sequencing would allow further study of the promoter region, and identification and analysis of any response elements that might be present, for example, by band shift experiments.

In order to analyse the regulation of the *Bmp-2* gene and locate the regions required for correct spatial and temporal expression of the gene in the limb, I designed and optimised a system for transfecting primary chick mesoderm cells, isolated from embryonic limb buds. Initially, I attempted to transfet these primary cells using Transfectam, supplied by Promega, but this caused cell lysis and extensive cell death. However, transfection of these cells by electroporation resulted in high levels of expression of the luciferase reporter constructs. In addition, these cells appeared to maintain some of their normal *in vivo* characteristics in culture after transfection, as judged by the expression of reporter constructs incorporating the promoters of the chick RIHB gene (Vigny and Duprez, 1994) and the chicken Type X collagen gene (LuValle *et al*, 1993), a positive and negative control for expression, respectively. The RIHB also proved to be inducible by RA in these cells, as anticipated. However, another set of constructs were also used as a control: promoter constructs of the neuronal nicotinic acetylcholine receptor $\alpha 2$ subunit gene (Bessis *et al*, 1993). These constructs consisted of the SV40 promoter driving luciferase expression, preceded by 6 *Oct*-like motifs, which act as either a silencer or enhancer depending on the number of repeats present. In this cell system, the $\alpha 2.6$ construct, containing 6 *Oct*-like motifs, although described by Bessis *et al* (1993) as a repressor, acted as a strong enhancer of luciferase expression, giving a 10-100-fold enhancement of the expression driven by the SV40 promoter alone. This construct also showed differences in expression depending on which area of the limb the transfected cells originated from: expression was stronger in cells isolated from posterior halves of limb buds, where the ZPA is located. Expression of this construct was also inducible by RA in anterior cells, to levels similar to those normally found in posterior cells. Similar expression characteristics were also found in a slightly shorter construct, $\alpha 2.4$, which contained only 4 of the *Oct* repeats, but the A/P

differences in expression and RA inducibility were lost in constructs α 2.0 and α 2.1, which contained only one or no enhancer elements. In fact, in these constructs, the remaining neuronal α 2 subunit promoter region acted as a silencer of expression. Analysis of the region between these constructs, that must be responsible for the A/P differences and RA inducibility, did not reveal any consensus RARE sequences. This pattern of expression does not in any way represent the normal expression of this gene, which is usually restricted to a small part of the chick brain. There must therefore be a response element present in this sequence which is responding to some signal which is present in a spatially defined way in anterior and posterior limb cells, and which probably acts downstream of RA. Further analysis of this promoter region would be needed to ascertain what this response element is, and what signal it is responding to in the chick limb.

Despite numerous attempts to generate reporter constructs containing fragments of the putative chick *Bmp-2* promoter region from clone #16F, I was unable to clone these fragments. This could be because there is some region of instability in this region, possibly around the intron in the 5' non-coding region.

Instead, I attempted to investigate the role of the chick *Bmp-2* gene in the developing limb by studying the effects on development of inhibiting *Bmp-2* gene function using antisense RNA. Constructs were generated using different antisense fragments of the chick *Bmp-2* gene in a retroviral expression vector, RCAS(BP)A. These constructs were targeted to the limb buds at stages 16-21 by grafting pellets of fibroblast cells infected with the retroviral constructs. *In situ* hybridisation showed that these antisense constructs were expressed at high levels in the developing limb buds and had spread during the course of infection such that expression of the antisense transcripts encompassed the entire expression domain of the endogenous sense transcripts. A decrease in the levels of the endogenous *Bmp-2* sense transcripts was also seen in the infected limbs of some specimens, again using *in situ* hybridisation. One potential phenotype that resulted from this inhibition was a fusion of the metatarsals of digits III

and IV in infected limbs. Further experiments using these antisense constructs need to be carried out to further elucidate the role of *Bmp-2* in the developing chick limb.

In summary, therefore, I have reported the structure of the chicken *Bmp-2* gene, designed a novel system for transfecting primary chick limb bud mesoderm cells, which should prove useful for testing the basis of regulation of genes such as *Bmp-2*, and inhibited chick *Bmp-2* gene function using antisense RNA.

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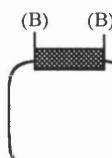
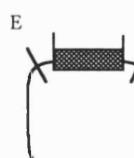
APPENDIX A

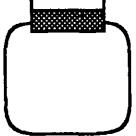
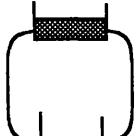
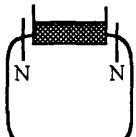
Summary of Restriction Patterns & Hybridisation Data for Cosmid #16F

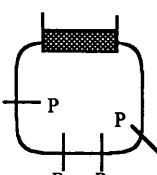
Bands shown in parentheses were thought to result from partially digested DNA, on the basis of lower intensity of fluorescence. Where a dash is shown for standard deviation, it was not possible to work out the standard deviation because only one reading had been taken of that band size. The notation [f.] represents bands that only showed faint signal intensity, and those marked [v.f.] were very faint. In cases where two adjacent bands are marked with ticks followed by question marks, it was not possible to allocate the signal band on the autoradiograph to a single band on the gel, where the bands were close together: it could represent either or both of the two bands.

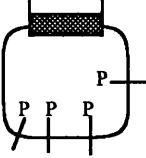
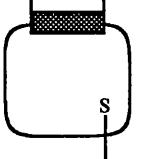
The average insert sizes were calculated by summing all the fragment sizes for each restriction digest, and subtracting the size of the vector, 8,164 bp. The total insert sizes vary considerably: underestimates of the insert may arise from the omission of small bands which have either run off the end of the gel or are not visible on the gel given the distribution of the ethidium bromide; or be due to omission of doublet or multiple bands of the same size counted as single bands; overestimates may be due to the inclusion of partially digested bands as fully digested ones. In-built inaccuracies also arise from the method of estimating band size from the standard graph, including the problems of extrapolating the standard line beyond the values given by the standard markers: these inaccuracies may lead to either over- or underestimates of band size.

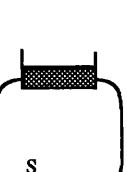
In some cases separate digestions with a single enzyme resulted in some fragment sizes of slightly differing sizes. It was necessary to resolve whether these fragments were in fact the same fragment or whether the bands were new partial digest fragments or new fragments that were not visible on previous gels. It was usually possible to resolve these ambiguities by careful observation of the gel photographs, but standard deviations were also worked out as percentages of the average value: if the inclusion of the new band size raised the standard deviation to more than 25% of the average, a new band was established. In most cases, standard deviations were less than 15%, and high values tended to occur at either end of the standard marker graphs, where sizes had to be estimated by extrapolating the line on the standard graph beyond the actual values for the markers.

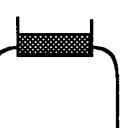
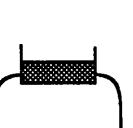
Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	5.1	5.2	ibnp2	SFR1	SFR2	T3	T7	hBMP2	cloned	Notes
<i>Bam</i> HII	No sites: 1 destroyed.  Uncut V = 8,164 bp	19.6 13.5 12.1 (3.7) 3.75	4.8 0.8 1.4 - 0.4	40.8 +/- 7.4	✓ ✓ ✓ -	✓ - - -	✓ - - -	v.f v.f v.f -	f. ✓ -	- -	✓ - -	✓ v.f f. -	.. at least 4 sites in insert		
<i>Eco</i> RI	2 sites: 0 & 65  V = 8,100 bp V arm = 32 bp V arm = 32 bp	13.8 11.3 9.7 (9.2) 4.5 4.3 2.4 2.15 1 0.99 0.72 0.63 0.615	1.3 1.5 1.45 - 0.4 0.47 0.17 0.17 0.07 0 0 0 0	44.0 +/- 5.5	✓ - - - - - - - - - - -	- - - - - - - - - - - -	- - - - - - - - - - - -	✓ v.f v.f -	✓ -	- -	- -	- -	3rd band = 8,100 bp cut V * .. at least 10 sites in insert		

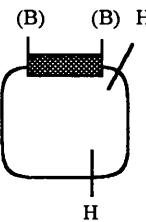
Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	5.1	5.2	ibmp2	SFR1	SFR2	T3	T7	hBMP2	cloned	Notes
<i>Kpn</i> I	No sites.  Uncut V = 8,164 bp	38 19.5 11 9 7.1 5.9 1.5	- 1.5 0.2 0.5 0.3 0.8 0	46 +/- 3.3	✓ - - - - - -	✓ - - - - - -	✓ - - - - - -	✓ v.f. v.f. v.f.	- - - -	- - - -	- - - -	- - - -	1st band = uncut cosmid, ∴ not incl. in total ∴ at least 6 sites in insert		
<i>Nco</i> I	2 sites: 3491 & 4516  V = 1,025 bp V ^L = 3,680 bp V ^R = 3,459 bp	12.8 7.1 5 2.9 1.4 1.1 0.27 0.19	1 0 0.2 0.1 0.01 0 - -	22.6 +/- 1.3	- - - - - - -	- - - - - - -	- - - - - - -	- - - - - - -	- - - - - - -	- - - - - - -	✓ ✓ -	6th band = 1,025 bp cut vector ∴ at least 6 sites in insert			
<i>Not</i> I	2 sites: 7 & 57  V = 8,114 bp V arms = 25 bp	38.9 18.5 12.5 9.3 7.4 1.1 0.86	- 3 0.86 0.68 0.5 0.1 0.03	41.5 +/- 5.17	- - - - - ✓ -	- - - - - -	- - - - ✓ -	- - - - -	- - - - -	- - - - -	*	1st band = uncut cosmid, ∴ not incl. in total 4th band = 8,114 bp cut vector ∴ at least 4 sites in insert			

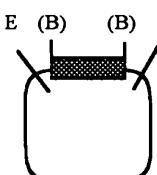
Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	5.1	5.2	ibmp2	SFR1	SFR2	T3	T7	hBMP2	cloned	Notes	
<i>Pst</i> I	4 sites: 3249, 4133, 5056 & 7413	38.9 4.5 4.2 2.8 2.7 2.55 2.2 2.1 1.5 1.4 1.35 1.28 1.15 1.1 1 0.99 0.94 0.89 0.815 0.69 0.64 0.56 0.545 0.525	- 0.3 0.3 0.03 0.05 0.04 0.03 0.1 0 0 0 0 - - - - - - - - - - - - - - -													1st band = uncut cosmid, ∴ not incl. in total.
	 <p>V¹ = 884 bp V² = 923 bp V³ = 2,357 bp V^L arm = 783 bp V^R arm = 3,217 bp</p>															∴ at least 19 sites in insert

Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	5.1	5.2	ibmp2	SFR1	SFR2	T3	T7	hBMP2	cloned	Notes
<i>Pvu</i> II	4 sites: 648, 3828, 4188 & 4950  $V^L = 3180\text{bp}; V^2 = 360\text{bp};$ $V^3 = 762 \text{ bp}$ $V^L \text{ arm} = 3,246 \text{ bp}$ $V^R \text{ arm} = 616 \text{ bp}$	31.6 19.1 14.5 5.4 3.8 3.4 3 2.6 2.3 2.25 1.19	- - - - - - - - - - -	49.4 $+/- (-)$								✓ - - - - - - - - - -		1st band = uncut cosmid - not incl in total V^2 & V^3 seem to have run off the end of the gel \therefore at least 6 sites in insert	
<i>Sal</i> I	1 site: 2457  $V^L \text{ arm} = 6,216 \text{ bp}$ $V^R \text{ arm} = 2,425 \text{ bp}$	40.7 23.5 13.5	- - 3.4	28.8 $+/- 3.4$	- - ✓							- - ✓		1st band = uncut cosmid - not incl. in total Either of these bands could contain either of the V arms. \therefore at least 1 site in insert	

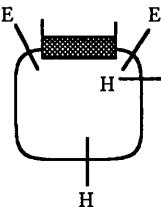
Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	5.1	5.2	ibmp2	SFR1	SFR2	T3	T7	hBMP2	cloned	
<i>Sma</i> I	1 site: 4921  $V^L\text{arm} = 3,275 \text{ bp}$ $V^R\text{arm} = 4,889 \text{ bp}$	11.3	0.6	46.5 +/- 3.15	-	-	-	-	-	-	-	✓	-	-	∴ at least 9 sites in insert
		10.2	0.7		-	-	✓	-	-	-	-	-	-	-	
		9.5	0.5		-	-	-	-	-	-	-	-	-	-	
		6.2	0.3		-	-	-	-	-	-	-	-	-	-	
		5.65	0.3		-	-	-	-	-	-	-	-	-	-	
		4	-		-	-	-	-	-	-	-	-	-	-	
		3.1	0.3		✓	✓	✓	✓	✓	✓	-	-	-	-	
		2.3	0.3		-	-	-	-	-	-	-	-	-	-	
		1.3	0.1		✓	-	-	-	f.	✓	-	-	-	v.f.	
		1.1	0.05		-	-	-	-	-	-	-	-	-	-	

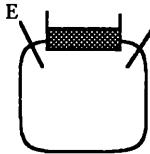
Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	5.1	5.2	ibmp2	SFR1	SFR2	T3	T7	hBMP2	cloned	Notes
XbaI	No sites. 	38.9 23.4 16.1 14 11.6 (7.1) 5.9 (4.2) 2.7 1.9 1.4	- - 1.5 1.6 1.9 - 0.04 - 0.02 0.01 0	45.4 +/- 5.1	- - ✓? ✓? ✓? - - - - - - -	- - ✓? ✓? ✓? - - - - - - -	- - ✓? ✓? ✓? - - - - - - -	- - ✓ - - - - - - - - -	- - ✓? ✓? ✓? - ✓ - - - - -	- - ✓? ✓? ✓? - - - - - - -	- - ✓ - - - - - - - - -	- - ✓? ✓? ✓? - - - - - - -	- - ✓? ✓? ✓? - - - - - - -		
XhoI	No sites. 	38.9 16.5 12.5 9.55 6.45 2.3 0.7 0.6	- 2.7 0.7 0 0.6 0.1 0 0	40.4 +/- 4.1	- ✓ - ✓ - - - -	- ✓ - ✓ - - - -	- ✓ - ✓ - - - -	✓ - - - - - - -	- ✓ - ✓ - - - -	- - ✓ - - - - -	- - ✓ - - - - -	- - ✓ - - - - -			

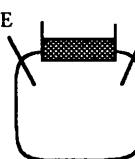
Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	5.1	5.2	ibmp2	SFR1	SFR2	T3	T7	hBMP2	cloned	Notes
<i>Bam</i> HI + <i>Hind</i> III	2 sites: 96 & 3600  V = 3,504 bp V ^L arm = 4,596 bp V ^R arm = 64 bp	5.8 5.4 4.75 4 3.7 3.2 2.8 2.5 2.2 2 1.85 1.7 1.5 0.96	0.7 0.4 0.4 0.1 0.2 0.2 0.25 0.15 0.03 0.03 0.05 - - - -	34.2 +/- 2.51											5th band = 3,504 bp cut V? Any of these bands could contain V ^R arm. ∴ at least 12 sites in insert (expect at least 4 <i>Bam</i> HI sites & 10 <i>Hind</i> III sites)

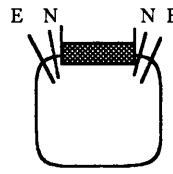
Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	5.1	5.2	ibmp2	SFR1	SFR2	T3	T7	hBMP2	cloned	Notes
<i>EcoRI + BamHI</i>	2 sites: 0 & 65.  V = 8,100 bp V ^L arm = 32 bp V ^R arm = 32 bp	10.7 9.4 7.4 4.3 4.1 3.75 2.1 1.9 1.6 1.45 (1.3) 1 0.76 0.7 0.6 (0.5)	1.2 1.2 0.7 0.23 0.2 0.3 0.1 0.25 0.1 - - 0.04 - - - -	41.6 +/- 4.3	- ✓ - - - - - - - - ✓ - - - - - - -									∴ at least 12 sites in insert (expect at least 4 <i>BamHI</i> & 10 <i>EcoRI</i> sites)	

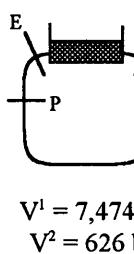
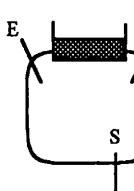
Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	5.1	5.2	ibmp2	SFR1	SFR2	T3	T7	hBMP2	cloned	Notes
EcoRI + HaeIII	<p>37 sites: 0 & 65; 9, 59, 240, 363, 456, 713, 973, 2206, 2330, 2338, 2402, 2636, 2725, 2746, 2797, 2854, 3067, 3524, 3530, 3539, 3582, 3991, 4165, 4556, 4583, 5079, 6282, 6293, 6304, 6322, 6756, 7214, 7294, 7561, 8148.</p> <p>V¹= 9bp; V²= 6bp; V³=181bp; V⁴= 123bp; V⁵= 93bp; V⁶= 257bp; V⁷= 260bp; V⁸= 1233bp; V⁹= 124bp; V¹⁰= 8bp; V¹¹= 64bp; V¹²= 234bp; V¹³= 89bp; V¹⁴= 21bp; V¹⁵= 51bp; V¹⁶= 57bp; V¹⁷= 213bp; V¹⁸= 457bp; V¹⁹= 6bp; V²⁰= 9bp; V²¹= 43bp; V²²= 409bp; V²³= 174bp; V²⁴= 391bp; V²⁵= 27bp; V²⁶= 496bp; V²⁷= 1203bp; V²⁸= 11bp; V²⁹= 11bp; V³⁰= 18bp; V³¹= 434bp; V³²= 458bp; V³³=80bp; V³⁴= 267 bp; V³⁵= 587bp; V³⁶= 16bp</p> <p>V^Larm = 23 bp V^R arm = 27 bp</p>	<p>1.7 1.4 1.3 1.15 (1.1) 1 0.82 (0.8) 0.73 0.69 0.62 0.59 0.54 0.44 0.38 0.32 0.31 0.26 smear</p>	<p>0 0.01 0.06 0.02 - 0.01 0 - 0.01 - - - - - - - - - -</p>	<p>4.1 +/- 0.11</p>	<p>✓ - f. f. - - ✓ - - - - - - - - - - -</p>										The average total insert is very low for this digest. It is probably an underestimate, from the fact that the 4 bp recognition site for the HaeIII enzyme will occur very frequently in the insert, giving rise to very small bands that will run off the end of the gel.

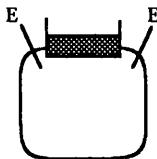
Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	5.1	5.2	ibmp2	SFR1	SFR2	T3	T7	hBMP2	cloned	Notes
<i>Eco</i> RI + <i>Hind</i> III	4 sites: 0, 65, 96 & 3600.  V ¹ = 31 bp V ² = 3,504 bp V ³ = 4,564 bp V ^L arm = 32 bp V ^R arm = 32 bp	11.2 (10.55) 9.75 8.6 (8.15) (6.9) (5.9) (5) 4.7 4.55 4.3 4 (3.7) 3.6 3.1 2.25 2.1 2 1.75 1.6 1.5 1.3 1.1 0.99 0.72 0.64	- - - - - 0.3 0.25 0.05 0.01 0.01 - 0.1 - 0.1 0.05 0.06 0 0.04 0.1 - 0.08 0.02 - - - - - - -	61.6 +/- 1.21	- ✓ - ✓ - f. f. f. - - - f. - - - - - - - - - - - - - -										∴ at least 16 sites in insert (expect at least 10 <i>Eco</i> RI sites & 10 <i>Hind</i> III sites)

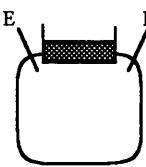
Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	5.1	5.2	ibmp2	SFR1	SFR2	T3	T7	hBMP2	cloned	Notes
<i>EcoRI</i> + <i>KpnI</i>	2 sites: 0 & 65  V = 8,100 bp V^L arm = 32 bp V^R arm = 32 bp	9 8.6 8.15 4.3 2.9 2.2 2.1 1.9 (1.35) 1.25 1.1 1 0.72 0.63 0.44 0.32	- - - - - - - - - - - - - - -	36.4 +/- (-)	✓ - - - - - - - - - - - - - -	- - - - - - - - - - - - - - -	3rd band = 8,100 bp cut V ? Any of these bands could contain either of the V arms. ∴ at least 13 sites in insert (expect at least 10 <i>EcoRI</i> sites & 6 <i>KpnI</i> sites)								

Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	5.1	5.2	ibmp2	SFR1	SFR2	T3	T7	InBMP2	cloned	Notes
<i>Eco</i> RI + <i>Nde</i> I	2 sites: 0 & 65  V = 8,100 bp V ^L arm = 32 bp V ^R arm = 32 bp	(16.8) 11.5 8.9 7.7 6.3 4.1 4 2.2 2 1.2 1 0.65 0.59	- - - - - - - - - - - - -	42 +/- (-)	f. ✓ - - ✓ - - - - - - - -									3rd or 4th bands = 8,100 bp cut V? Any of these bands could contain either of the V arms. ∴ at least 10 sites in insert (expect at least 10 <i>Eco</i> RI sites; no single digest data for <i>Nde</i> I)	

Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	5.1	5.2	ibmp2	SFR1	SFR2	T3	T7	hBMP2	cloned	Notes
<i>EcoRI + NotI</i>	4 sites: 0, 7, 57 & 65  V ¹ = 8 bp V ² = 8,099 bp V ³ = 7 bp V ^L arm = 25 bp V ^R arm = 25 bp	17 14.8 13.4 11.6 9.7 8.3 7.7 6.5 5.45 4.5 4.2 3.8 3 2.7 2.25 2 1.65 1.1 0.96 0.83 0.65 0.59 0.43	- - 0.56 0.15 - 0.2 0.02 0.38 - - 0.07 0.18 - 0.07 0.06 0.02 0.1 0.08 0.06 0.02 - - - - -	69.7 +/- 1.97	- - - - - - ✓ - - f. - - ✓ - - - - - - - - - -									6th band = 8,100 bp cut V ? Any of these bands could contain either of the V arms. V ¹ and V ² have probably run off the end of the gel. ∴ at least 15 sites in insert (expect at least 10 EcoRI sites & 4 NotI sites)	

Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	5.1	5.2	ibmp2	SFR1	SFR2	T3	T7	hBMP2	cloned	Notes
<i>EcoRI</i> + <i>PvuI</i>	3 sites: 0, 65 & 7539  $V^1 = 7,474 \text{ bp}$ $V^2 = 626 \text{ bp}$ $V \text{ arms} = 32 \text{ bp}$	11.5 9.16 7.12 4.1 4 2.2 2 1 0.65 0.59	- - - - - - - - - -	34.16 +/- (-)	✓ - - - - - - - - -										
<i>EcoRI</i> + <i>SalI</i>	3 sites: 0, 65 & 2457  $V^1 = 2,392 \text{ bp}$ $V^2 = 5,708 \text{ bp}$ $V \text{ arms} = 32 \text{ bp}$	11.5 7.7 5.7 4.1 4 2.4 2.2 2 1 0.65 0.59	- - - - - - - - - -	33.7 +/- (-)	✓ - - - - ✓ - - ✓ -									∴ at least 8 sites in insert (expect at least 10 <i>EcoRI</i> sites & 1 <i>SalI</i> site)	

Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	s.1	s.2	ibmp2	SFR1	SFR2	T3	T7	hBMP2	cloned	Notes
<i>Eco</i> RI + <i>Xba</i> I	2 sites: 0 & 65  V = 8,100 bp V arms = 32 bp	11.4 10.2 8.6 4.5 4.3 (3.7) (3.4) 2.35 2.25 2.15 (2.1) (2) (1.5) 1.4 (1.35) 1 0.72 0.66 0.64	- - - - - - - - - - - - - - - - -	42 +/- (-)	- ✓ - - - - - - - - - - - - - - - -									∴ at least 11 sites in insert (expect at least 10 <i>Eco</i> RI sites, and 7 <i>Xba</i> I sites)	

Enzyme	Sites in Vector	Ave. Size /kb	+ / -	Total Insert Size	cDNA	5.1	5.2	ibmp2	SFR1	SFR2	T3	T7	hBMP2	cloned	Notes
<i>Eco</i> RI + <i>Xho</i> I	2 sites: 0 & 65  V = 8,100 bp V arms = 32 bp	9.55 8.8 5.55 4.25 3.9 2.55 2.3 2 1.7 1.5 1.4 1.36 1 0.72 0.64 0.54 0.47	- - - - - - - - - - - - - - - -	40.1 +/- (-)	- ✓ - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - -	2nd band = 8,100 bp cut V ? Any of these bands could contain either of the V arms. ∴ at least 15 sites in insert (expect at least 10 <i>Eco</i> RI sites & 7 <i>Xho</i> I sites)								