Regulation of Insulin-like Growth Factor-I

Gene Expression in Bone Cells

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for the degree of Doctor of Philosophy by

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Abstract

The rat insulin-like growth factor-I (IGF-I) gene is characterised by the synthesis of multiple mRNA transcripts differing in the 5' and 3'-untranslated regions (UTRs). Transcription is initiated from either of the leader exons, exons 1 or 2, with further heterogeneity in the 5'-UTR conferred by multiple transcription start sites and differential splicing within exon 1. Differences in the nature of transcript expression are likely to reflect cell- and hormone-response-specific regulation. IGF-I is important in skeletal growth and development, therefore, the initial aim of this study was to determine the pattern of transcript expression in osteoblast-enriched primary cultures from rat long bones. Subsequently, a possible role for RNA-protein interactions in the regulation of translation of IGF-I was examined. The presence of all IGF-I transcripts was demonstrated by RT-PCR, while Northern analysis and RNase protection assays revealed that transcript expression in osteoblasts was heterogeneous and differed from that in liver. The relative proportion of transcripts varying in the 5'-UTR was quantitated and revealed a lower expression of exon 2-derived transcripts in bone cells compared to liver and that individual start site usage in exon 1 differed greatly. Transcripts synthesised by osteoblasts and whole bone were predominantly initiated from start site 3. In contrast, the majority of the transcripts in liver were the alternatively spliced mRNAs. Interestingly, the transcripts predominating in bone cells are the less translatable mRNA variants. Such heterogeneity is often indicative of post-transcriptional regulation, which is also likely to involve regulatory proteins. Indeed, crosslinking experiments revealed proteins with apparent molecular masses of 48, 63 and 69 kDa binding with different affinities to the different 5'-UTRs. In summary, since the mRNA variants exhibit different properties, including half-life and translatability, such cell-specific variation in their relative expression and the transcript-specific interactions with cytosolic proteins may reflect differential regulation of IGF-I synthesis in these tissues.
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Chapter 1

General Introduction
Part 1 - Insulin-like Growth Factor-I

History

Insulin-like growth factor-I (IGF-I) is a potent mitogen, mediating growth and anabolic responses in many tissues (Daughaday and Rotwein, 1989; Werner et al., 1994; Jones and Clemmons, 1995). Initially, the presence of IGFs was inferred by the inability to obtain the same responses to growth hormone (GH) in vitro as could be obtained in vivo. One of the main examples of this variance involved the accumulation of sulphate in cartilage (Salmon and Daughaday, 1957). Hypophysectomised rats no longer have the ability to synthesise GH and have a defect in cartilage glycosaminoglycan synthesis due to incorrect sulphation of chondroitin in the formation of chondroitin sulphate, a component of cartilage. This fault was characterised by the lack of incorporation of radiolabelled sulphur into chondroitin sulphate, and impaired synthesis could be rectified upon administration of GH in vivo. Conversely, the defect persisted when sections of cartilage from hypophysectomised rats were cultured in vitro, even upon addition of GH to the culture medium. However, when the medium was supplemented with normal rat serum, the defect was corrected, whereas serum from hypophysectomised rats had no effect. Therefore it appeared that the actions of GH, in terms of incorporation of sulphate into cartilage were transduced through an intermediate factor, thus named sulphation factor. Subsequently, since the actions of this factor were also found to affect DNA (Daughaday and Reeder, 1966), protein and proteoglycan synthesis (Salmon and DuVall, 1970; Hall and Uthne, 1971; Daughaday et al., 1975), the name was altered to somatomedin (mediator of somatotropin), of which
there were known to be two forms, somatomedins A and C. Furthermore, it was proposed that all the effects of GH were mediated through circulating somatomedins produced by the liver, which was termed the somatomedin hypothesis (Daughaday and Rotwein, 1989).

Simultaneously to the discovery of somatomedins, factors with insulin-like activity were studied. These were observed to mediate insulin-like action even in the presence of anti-insulin antibodies. For this reason they could be assayed and were termed non-suppressible insulin-like activity (NSILA) (Froesch et al., 1966). However, it was not until 1978 that the proteins of NSILA were sequenced and found to comprise two peptides, one basic and one acidic, both of which had a molecular weight of approximately 7,500Da (Rinderknecht and Humbel, 1978a and 1978b). These peptides had 70% homology in amino acid sequence to each other and approximately 50% similarity to human proinsulin, which led Rinderknecht and Humbel (1978b) to call these insulin-like growth factors (IGF) -I and -II respectively.

Furthermore it was also observed that the NSILA, now termed IGF-I and -II, could stimulate sulphate deposition into cartilage, and conversely, the somatomedins could mediate the incorporation of glucose into fat (Froesch et al., 1966). This observation and the successful sequencing of the somatomedin peptides demonstrated that somatomedin C was identical to IGF-I (Klapper et al., 1983) while IGF-II was proven to be the same as somatomedin A (Enberg et al., 1984). In 1987, in order to avoid confusion, the nomenclature of these factors was standardised to IGF-I and IGF-II (Daughaday et al., 1987).
Roles of IGF-I

IGF-I is a highly important growth factor, regulating the growth and differentiation of many cell-types (reviewed by Werner et al., 1994). As has already been described, the somatomedin hypothesis suggested that many of the effects of GH are mediated through hepatic production of IGF-I. This view is supported by the observation that GH-deficient rats have reduced body growth and serum IGF-I levels, both of which increase upon GH-replacement (Butler et al., 1994). A further example of GH-effects mediated through IGF-I is given by syndromes such as Laron-type dwarfism, which is due to a defect in the GH receptor, thereby making the patients GH-resistant (Werner et al., 1994). However, a significant increase in growth rate may be obtained upon stimulation with IGF-I, to some extent reversing the phenotype.

It appears, however, that the somatomedin hypothesis as originally stated was an oversimplification. For instance, GH-deficient rats which were given GH-replacement grew in a similar manner to normal rats. However, rats given IGF-I instead had diminished linear growth with respect to normal controls (Skottner et al., 1987; Guler et al., 1988; Skottner et al., 1989). Furthermore, IGF-I infusion resulted in the disproportionate growth of the spleen, kidney and thymus in comparison to bone (Schoenle et al., 1982; Skottner et al., 1987 and 1989; Pell and Bates, 1992). Therefore, these data suggest that not all GH actions are mediated through hepatic IGF-I production. Indeed, previous to this, IGF-I immunoreactivity was demonstrated to be up-regulated in extra-hepatic tissue after GH-stimulation (D’Ercole et al., 1984). For instance, the IGFs have
been shown to be locally synthesised partly under GH-stimulation in tissues such as the ovary (Giudice, 1992) and kidney (Chin et al., 1992). Similarly, it has been demonstrated that GH increases IGF-I synthesis in cultured rat adipocytes (Vikman et al., 1991).

In bone, it has also been shown that both GH and IGF-I individually infused into the arterial supply of the hindlimb of a hypophysectomised rat could stimulate growth of the epiphyseal plate. In contrast the non-infused limb remained unaltered by this stimulation. Co-infusion of anti-IGF-I antibodies with GH abolished this growth, demonstrating that localised GH-stimulated IGF-I production was responsible for the growth of the epiphyseal plate (Schlechter et al., 1986). It would therefore appear that while IGF-I alone may stimulate cell replication and may mirror the gross effects of GH, GH must also stimulate local production of IGF-I. In turn, IGF-I may then function in a paracrine or autocrine manner.

IGF-I though, does not only mediate the actions of GH, but also mediates the local actions of many other hormones and growth factors. For example, it has been shown that oestrogen is a potent stimulator of IGF-I production in the uterus, but not in the liver, of rats and this has been postulated to mediate a potent mitogenic response locally (Murphy and Friesen, 1988). Also inflammatory mediators, such as advanced glycosylation end products (reviewed by Chappey et al., 1997) may stimulate IGF-I synthesis from monocytes, while tumour necrosis factor (TNF)-α and interleukin (IL)-1 promotes IGF-I production by macrophages, suggesting a role for IGF-I in cellular proliferation and tissue repair at sites of damage. Indeed, at sites of injury, IGF-I is amongst the factors released promoting repair of connective tissue by platelets and
macrophages. Furthermore, since IGF-I can promote fibroblast- and capillary endothelial cell-proliferation, along with development of connective tissue, it seems probable that IGF-I is involved with wound healing and neovascularisation (Arkins et al., 1995). Interestingly, unregulated production of IGF-I by alveolar macrophages after stimulation by non-physiological factors such as asbestos, may lead to such serious diseases as idiopathic pulmonary fibrosis and asbestosis (Rom et al., 1988; Kovacs and DiPietro, 1994). IGF-I also plays an important anabolic role in bone formation, for instance, increasing collagen synthesis and deposition (McCarthy et al., 1989; Schmid et al., 1989) and it appears to mediate some of the anabolic actions of parathyroid hormone (PTH) which will be discussed further later.

**IGF-I Protein Structure**

IGF-I is a 70 amino acid basic peptide with a high degree of structural similarity to the A and B chains of proinsulin (Figure 1.1). For instance, the A domain of IGF-I has 52% amino acid homology to the A-chain of insulin, while the B-chains of both molecules are 48% homologous (Jansen et al., 1983). The mature IGF-I peptide also contains a C-domain linking the A- and B-domains. However, this is slightly smaller than that found in proinsulin and is not removed during the post-translational processing, unlike insulin in which the C-peptide is specifically cleaved during the conversion of proinsulin to insulin. Another structure which is absent from proinsulin, the D-domain, is linked to the A-domain.

The N- and C-terminal ends of the pro-hormone are variable in rat. For instance, a C-terminal E-peptide is present in the pro-hormone, which is cleaved during processing,
Figure 1.1 - A schematic representation of the IGF-I gene and mature protein. The boxes represent the exons and the protein. The mature IGF-I protein contains B, C, A and D domains. The mature protein is encoded by the solid boxes. The sequence coding the unprocessed hormone is denoted by the grey boxes and the untranslated sequence is represented by the open boxes. The transcripts are shown in Figures 1.2 and 3.1. The diagram is not shown to scale.
prior to release (Werner et al., 1994). In rats, two types of E-peptides may be produced by alternate splicing of the mRNA transcripts, and although their synthesis appears to be regulated by GH and in differentiating chondrocytes, their function is, as yet unknown (Werner et al., 1994). Additionally, at least three leader sequences have been characterised of 48-, 32- and 22-amino acids in length in the rat (Yang et al., 1995). While the rat is the best characterised system, previous studies suggest that leader sequences of 48-amino acids are present in humans, chicken and Xenopus (Rotwein et al., 1987; Kajimoto and Rotwein, 1989; Kajimoto and Rotwein, 1990) although other signal peptides (of 32- and 22-amino acids in length) are postulated in humans (Rotwein et al., 1986; Tobin et al., 1990; Jansen et al., 1991). Furthermore rat prepro-IGF-I containing these signal sequences have been co-translationally processed to smaller forms by canine pancreatic microsomes suggesting that these signal sequences have a role in post-translational processing of IGF-I (Yang et al., 1995).

**IGF-I Gene Structure**

The human and rat genes consist of six exons spanning over 80 kb (Figure 1.2), from which multiple heterogeneous mRNA transcripts may be generated. These arise by a combination of the use of many transcription initiation sites, alternate splicing and differential polyadenylation (Adamo et al., 1991a; Kim et al., 1991; Jansen et al., 1992; Shemer et al., 1992; West et al., 1996).

In rat, transcription is initiated from either of the alternate leader exons, 1 and 2, which also encode the 5'-untranslated regions (UTRs) and some sequence coding for one of the signal peptides, depending on the translation start site used (Roberts et al., 1987a;
The rat IGF-I gene

Figure 1.2 - The rat IGF-I gene and the transcript variations. The rat IGF-I gene comprises 6 exons. Exons 1 and 2 code for alternate leader sequences. Exons 3 and 4 code for the mature protein. Exon 5 may be alternately spliced and exon 6 codes for the 3'-UTR.
Shimatsu and Rotwein, 1987a and 1987b; Bucci et al., 1989). The heterogeneity of transcription start site usage is described in more detail in Chapter 3. Exons 1 or 2 are spliced to exons 3 and 4, which encode the mature 70 amino acid basic peptide while exon 4 also codes for the proximal portion of the E-peptide. Exon 5 may be alternately spliced to exon 6, thereby altering the translation reading frame and introducing an alternate translation stop codon within exon 6, resulting in the synthesis of different E-peptides (Casella et al., 1987; Roberts et al., 1987b; Shimatsu and Rotwein, 1987b; Hoyt et al., 1992). Excision of exon 5 results in the production of the Eₐ-peptide whereas when it is present, the Eₐ'-peptide is produced. Exon 6 itself, is greater than 6 kb in length, the majority of which codes for the 3' -UTR and contains multiple putative polyadenylation signals (Hoyt et al., 1992).

Although the gene structure above describes that found in the rat, the human gene is very similar and there is a great deal of sequence homology (Jansen et al., 1983; Daughaday and Rotwein, 1989). Transcripts are transcribed from multiple transcription start sites within two alternative leader exons in a similar manner to that in the rat, and the protein is encoded in exons 3 and 4 (Jansen et al., 1991; Kim et al., 1991; Hoyt et al., 1992). In contrast to rat though, exons 5 or 6 are alternatively spliced to exon 4, and encode alternate E-peptides and 3'-UTRs, whereas in rat, exon 6 is found in all IGF-I mRNAs (Rotwein, 1986; Daughaday and Rotwein, 1989; Hoyt et al., 1992).

The proteins encoded are also highly similar. The IGF-I peptide in the rat is identical to the human in 67 of the 70 amino acid residues (Shimatsu and Rotwein, 1987b). Furthermore, the Eₐ peptide in human, which is coded for by alternate splicing of exon 4 to 6, is homologous to the rat peptide in 17 of the 19 amino acids. In contrast, the Eₐ,
peptide in human (encoded by transcripts with the splice variant exon 4 to 5) differs greatly to that found in rat (Daughaday and Rotwein, 1989).

IGF-I Nomenclature

It is worth noting that there has been a great deal of confusion over the nomenclature of the various transcripts which may be obtained, especially since three papers describing the sequence of the IGF-I exons and transcripts were produced simultaneously by two different groups. Initially it was believed that the IGF-I gene comprised only 5 exons only one of which contained the 5'-UTR (Shimatsu and Rotwein, 1987b) and to an extent, this was borne out by the sequencing of cDNA clones (Roberts et al., 1987a; Shimatsu et al., 1987a). Therefore, the exon 1-derived transcripts obtained were termed class C transcripts (after finding homology with clone C - Roberts et al., 1987a), or IGF-IB (due to sequence similarity with mouse IGF-IB transcripts - Bell et al., 1986). These are now termed exon 1-derived transcripts.

It was not until 1989 that the second leader exon, with homology to class A and B transcripts as defined by Roberts et al. (1987a), was cloned and sequenced, and was called exon 1A (Bucci et al., 1989). However, Jansen et al. (1991) termed this sequence exon 1B, due to the homology to class B clones. The transcripts containing leader exons 1/1C or 1A/1B were also termed class 1 and class 2 transcripts, respectively. However, to simplify nomenclature, exons 1/1C, 1A/1B, 2, 3, 4 and 5 were designated exons 1 to 6 respectively, with exon 1A/1B becoming exon 2 (Adamo et al., 1991a).
A further complexity was added by the apparent alternate splicing of sequences within exon 2. However, data obtained from RNase protection assays by Adamo et al. (1991b) demonstrated that the initial clone was artefactual. However, the class A transcripts which had been observed by Lowe et al. (1987) and Adamo et al. (1989), in fact corresponded to exon 2-derived transcripts, initiated from both an upstream and downstream start site.

**IGF-I and Development**

From work performed using IGF-I and IGF-I receptor knock-out mice and studies of rat transcript expression, it became apparent that IGF-I was not solely a post-natal growth factor, but played an important role in pre-natal development (Lund et al., 1986; Adamo et al., 1989; Baker et al., 1993). For instance, while there was no difference in the embryonic development of the IGF-I knock-out mice in comparison to the wild-types during the first 13 days of embryogenesis, from day 13 onwards growth was significantly reduced. These IGF-I knock out mice also continued to exhibit retarded growth for the first two weeks following birth.

This is in contrast to the observations in mice deficient in GH. Studies were undertaken using mice which have mutations in the receptor for the GH-releasing hormone (lit mice) thereby reducing GH secretion to 10% of the normal amount. These mice showed identical embryonic development to the wild-type mice, and were indistinguishable for the first two weeks post-natally. Further post-natal growth was much diminished, with mice reaching approximately 50% of the normal size (Cheng et al., 1983). This would
therefore suggest that the growth-promoting actions of IGF-I are not stimulated by GH until two weeks post-natally and that before this time, IGF-I functions independently.

One of the main phenotypes of the IGF-I knock-out mice is the reduced size. Since cell division is not arrested in the absence of IGF-I, this retarded growth rate has been postulated to involve an increase in the length of the cell cycle. Therefore the growth retardation of the IGF-I knockout mice compared to their wild-type litter mates would be due to the cells having undergone fewer cell divisions during the time given for embryonic development than for the wild-type mice (Baker et al., 1993). The G1-phase of the cell cycle has two control points, V (requiring essential amino acids before progression) and R (the point after which the cells are committed to the S-phase, irrespective of environmental conditions). Importantly, it has been found that the only requirement in terms of growth factor for cells to pass into S-phase, is the presence of IGF-I prior to the R-stage (Pardee, 1989; Baker et al., 1993; Baserga and Rubin, 1993).

**Growth Hormone-Dependent Regulation of IGF-I**

As has been demonstrated using hypophysectomised and GH-deficient rats, the regulation of IGF-I synthesis and especially circulating concentrations of IGF-I, is largely GH-dependent (Daughaday and Rotwein, 1989; Lund, 1994). For instance, the GH-deficient rat (chw), whose reduced GH levels are due to a defect in the GH-releasing factor signal transduction pathway, has greatly reduced IGF-I levels in serum (Charlton et al., 1988; Butler et al., 1994). Furthermore, perfusion of rat livers with GH demonstrated that the rate of hepatic secretion of IGF-I was sufficient to account for all
the circulating IGF-I (Schalch et al., 1979; Schwander et al., 1983; Lund, 1994). As a corollary to this, Mathews et al. (1988) studied IGF-I expression in transgenic mice over-expressing GH, and noticed a marked elevation in hepatic IGF-I levels.

The effect of GH on IGF-I synthesis was further confirmed at the level of transcription by Roberts et al. (1986). This group demonstrated that there was a reduction in IGF-I transcript expression in livers of hypophysectomised rats compared to normal rats. Furthermore this response could be reversed upon GH-replacement. Tannenbaum et al., (1979) observed that food deprivation in rats also resulted in a significant decrease in the peak value of IGF-I attained during GH-pulses. Simultaneously, there was a decrease in the number of high affinity GH receptors in liver, while the low affinity receptors were unaltered (Baxter et al., 1981). In this manner, liver was less sensitive to GH and this led to a large decrease in serum IGF-I, which could be reversed upon re-feeding (Maes et al., 1983).

However, the mechanism of regulation of these processes was found to be more complex after it was observed that the specific transcription start site used and thus the 5' -UTR and leader peptide of the transcripts was also regulated by GH (Foyt et al., 1992). Also, Adamo et al. (1991b) demonstrated that all IGF-I mRNA transcripts were down-regulated during food-deprivation, and that there was a co-ordinate decrease in start site usage, rather than the specific fall observed upon hypophysectomy. This could indicate that the decrease in mRNA during calorific fasting may not solely be due to a lack of GH-stimulation, but that another factor may be acting synergistically.
It is interesting to note that the preferential production of transcripts with a particular 5'-UTR indicates that these transcripts may be post-transcriptionally regulated. Regulation of this kind commonly involves an alteration in either the stability or the efficiency of translation of the transcripts. The manner whereby this could be achieved is discussed in more detail in Section 3 of Chapter 1. However, it is interesting to note that the 5'-UTR present in the IGF-I transcripts as been found to have an effect upon translatability, while the length and sequence of the 3'-UTR alters the half-life of the transcripts as is discussed in Chapter 3 (Hepler et al., 1990; Yang et al., 1995).

Growth Hormone-Independent Regulation of IGF-I

IGF-I synthesis is not regulated solely by GH, but is also controlled by factors such as oestrogen (Murphy et al., 1988), dexamethasone (Luo and Murphy, 1989), interferon-γ (Arkins et al., 1995) and prostaglandin E₂ (Bichell et al., 1993).

Regulation in macrophages - Inflammatory macrophages synthesise IGF-I and other cytokines during the wound-repair process as has been demonstrated by Noble et al. (1993). When tissue is damaged, macrophages are recruited to the area, where they secrete numerous proteases which digest the extracellular matrix. Hyaluronic acid, which is thought to be released following this digestion, appears to interact with the cell surface adhesion molecule CD44 and stimulates the production of tumour necrosis factor-α (TNF-α) and IL-1β. TNF-α in turn, functions in an autocrine/paracrine manner inducing IGF-I mRNA and protein expression which stimulates cellular proliferation and repair.
In contrast to the proposed model for IGF-I synthesis by macrophages as described above, interferon-γ (IFN-γ) represses IGF-I production. If IGF-I is secreted in an uncontrolled manner during wound repair, this is likely to cause the production of fibrous overgrowth, due to the over-proliferation of mesenchymal cells and the concomitant increase in extracellular matrix deposition (Arkins et al., 1995). Therefore the manner in which IGF-I is synthesised is carefully regulated. In turn, this control of IGF-I could explain the observed functions of IFN-γ, which has been characterised as mediating a potent anti-fibrogenic and anti-angiogenic response both directly by inhibiting cell replication and matrix synthesis, and by regulating the production of fibrogenic and angiogenic cytokines by the inflammatory macrophages (Hosang et al., 1988; Kovacs et al., 1994; Sunderkotter et al., 1994).

**Glucocorticoids** - Glucocorticoids, such as dexamethasone, are known to inhibit skeletal growth by reducing the responsiveness to serum IGF-I. The mechanism by which this occurs has been characterised and shown to involve IGF-binding proteins. However, dexamethasone also acts directly to decrease IGF-I mRNA synthesis in liver, kidney, tibia and lung in both normal and GH-treated hypophysectomised rats (Luo et al., 1989). Similarly, Adamo et al. (1988) found that stimulation of glial and neuronal cells with dexamethasone, *in vitro*, decreased IGF-I mRNA.

**Effects of fasting/protein deprivation** - Fasting and protein-deprivation have also been observed to reduce IGF-I production. While the effects of fasting itself are likely to be mediated through a reduction in the number and affinity of GH receptors in liver
(Tannenbaum et al., 1979; Baxter et al., 1981), protein-deprivation alone appears not to significantly effect liver GH receptor/GH binding protein mRNA. Therefore the decrease in IGF-I in this circumstance is unlikely to be a GH-mediated response and must involve some other mechanism. However, as was observed by Straus and Takemoto (1990), VandeHaar et al. (1991) and Thissen et al. (1994), levels of IGF-I mRNA were diminished with a protein-restricted diet. Furthermore, it was also shown that particular transcripts were preferentially transcribed or degraded in hepatocytes. Therefore, this evidence suggests that amino acids can directly regulate hepatic IGF-I production.

Sex steroids - Oestrogen in rats is known to induce an increase in uterine size in control and ovariectomised rats, an effect accompanied by an increase in IGF-I (Sahlin et al., 1994). Surprisingly, simultaneous GH administration into the uterus of rats attenuated the oestrogen-induced increase in IGF-I synthesis at the level of gene expression (Murphy et al., 1987; Murphy and Friesen, 1988). Uterine IGF-I synthesis also increases during pregnancy in the mouse, although progesterone and oestrogen appear to mediate their responses through discrete populations of cells. For example, oestrogen up-regulates IGF-I produced by the epithelial cells, whereas progesterone increases synthesis from the stromal cells (Kapur et al., 1992; reviewed by Werner et al., 1994).

In the testes, the Sertoli and the Leydig cells synthesise IGF-I. The Leydig cells are responsible for the production of testosterone, whereas the Sertoli cells are involved with both spermatogenesis and the regulation of the Leydig cells. IGF-I stimulates testosterone production and it is known that IL-1 reduces IGF-I expression by the Leydig cells in parallel to inhibiting the gonadotropin-mediated responses of these cells.
Therefore, IGF-I is important in the regulation of steroidogenesis in the male reproductive system (reviewed by Werner et al., 1994).
Part 2 - Osteoblasts and Bone

Introduction

Bone is a highly specialised form of connective tissue within the body, involved in both support and protection. This connective tissue is a composite of organised collagen fibres and mineral deposits, forming a rigid structure while maintaining a certain degree of elasticity inherent to collagen. Of the organic matrix, approximately 95% is in the form of type I collagen, the remaining 5% comprising proteoglycans, glycoproteins, such as osteonectin, fibronectin and bone sialoproteins, and many non-collagenous proteins. The mineral deposits are predominantly in the form of hydroxyapatite crystals, and these salts are the body’s major store of calcium and phosphate. Therefore, in spite of its apparent rigidity, bone is constantly in a state of flux, both undergoing repair/remodelling and being degraded/replaced during calcium and phosphate homeostasis.

Bone is not homogeneous, however, and morphologically there are two types. Cortical or compact bone consists of tightly packed collagen fibrils, deposited in concentric lamellae, with the fibrils in each adjacent layer perpendicular to the previous one. In this manner great strength is conferred to cortical bone, whose function is primarily mechanical and protective. In comparison to this dense structure, the collagen fibrils of cancellous or spongy bone are loosely organised and form a porous matrix. One of the main roles of cancellous bone appears to be metabolic.
The Major Cell Types Involved with Bone Homeostasis

There are four main types of cell involved with the upkeep of bone: i) osteoblasts; ii) osteocytes; iii) bone-lining cells; iv) osteoclasts.

i) Osteoblasts

Osteoblasts are fully differentiated cells responsible for forming bone (Figure 1.3). Briefly, osteoblasts line the surface of bone, upon which they secrete fresh bone matrix which is subsequently mineralised. Osteoblasts are highly active cells, producing a great deal of protein, and as such have a large rough endoplasmic reticulum and Golgi body. The Golgi is generally polarised towards the bone surface being actively formed, from which type I collagen and other proteins are secreted.

The mineralisation process of the deposited protein may also involve osteoblasts, although this depends on the type of bone. This is exemplified by the differences in mineralisation of woven and lamellar bone. Woven bone is bone which is rapidly formed, often during histogenesis or fracture healing, and in which there is no preferential organisation of the collagen fibrils. The nucleation of mineralisation of the type of bone appears to be similar to that observed in cartilage mineralisation, insofar as it may be initiated within vesicles which have budded from the plasma membrane of osteoblasts, called matrix vesicles. In contrast, matrix vesicles may not be involved with the mineralisation of lamellar bone which appears to be initiated in the space between adjacent collagen fibrils by collagen itself or another of the matrix proteins (Landis et al., 1993).
Figure 1.3(a) - A diagrammatic representation of osteoblast cells and bone. The bipolar nature of the osteoblasts (1) is demonstrated, with the nucleus (2) furthest away from the surface of the bone. Collagen is synthesised in the rough endoplasmic reticulum (3), positioned near the bone surface, and secreted, forming the osteoid layer (5), which gradually mineralises to form new (6). As time progresses, the osteoid layer may engulf the osteoblasts, which then become osteocytes (7), which are linked to each other as well as the osteoblasts via the canaliculi (8). Bone-lining cells (4), which may be preosteoblasts, are often found covering the bone and the active osteoblasts.

Figure 1.3(b) - A schematic diagram of the structure of a growing long bone taken in cross section. The different types of bone formed during bone growth are indicated, as are the growth plates, both fused and still in the process of growth. (Adapted from Baron, 1996)
ii) Osteocytes

While the secretion of bone matrix by osteoblasts is usually polarised towards the bone surface, it may become generalised and in this manner, surround the cell. The cell may become engulfed in the matrix which then mineralises, and thereby become an osteocyte (Figure 1.3). Indeed, approximately 20% of osteoblasts become embedded in the bone matrix. These cells, only occupying a small cavity or lacunae, no longer divide but may deposit and resorb bone matrix to a limited extent. Indeed, depending on their role, osteocytes may contain lysosomal vacuoles if osteolytic, while if synthesising matrix they may have a more osteoblastic morphology with an extensive rough endoplasmic reticulum and Golgi body. In this manner, osteocytes may be responsible for the maintenance of mineralised bone. Osteocytes are not isolated, however, and fine filopodial processes permeate through bone, along canaliculi which radiate from each lacunae, connecting with neighbouring osteocytes, blood vessels and the endosteal and periosteal surfaces.

iii) Bone Lining Cells

These cells are thought to be precursors to osteoblasts and are found on bones which are undergoing neither resorption nor formation. Characteristically they are flat, elongated and are inactive with few organelles, although relatively little is known about them.

iv) Osteoclasts

During the processes of growth and development, as well as calcium homeostasis, bone must be resorbed, an event which is performed by osteoclasts. Unlike the three types of cell described above, which all originate from mesenchymal osteoprogenitor cells,
osteoclasts arise from the fusion of mononuclear precursors originating from some haematopoietic tissues. The cells, therefore, are large and multinucleate. Osteoclasts are highly polarised cells and when resorbing bone, the plasma membrane may be defined as two separate areas. The ruffled border is a highly involuted area of plasma membrane which is in close apposition to the bone surface, and is the site at which resorption occurs. The osteoclasts are attached to the bone around the ruffled border, forming a tight seal between the bone and an area of the plasma membrane called the clear zone, which is free of organelles but is rich in microfilaments. The cells contain an extensive Golgi body which buds to form lysosomes which exocytose their contents across the ruffled border. Osteoclasts also have many mitochondria and the nuclei themselves are found polarised to the opposite end of the cell to the ruffled border, interconnected by cytoskeletal proteins.

The Osteoblast Lineage

Osteoblasts are derived from multipotential mesenchymal stem cells from which adipocytes, smooth muscle cells and chondrocytes are also derived (Figure 1.4) (Grigoriadis et al., 1988). Numerous hormones and factors induce the cells to differentiate into one of these pathways but the system is still poorly understood. For instance, it has been observed that incubation of marrow stromal fibroblastic cells with dexamethasone and basic fibroblast growth factor (bFGF) stimulates adipogenesis (Grigoriadis et al., 1988; Locklin et al., 1995), while others have demonstrated that these factors stimulate mineralised bone nodule formation from these cells and also from foetal calvarial cells (Bellows et al., 1986a; Noff et al., 1989). Similarly transforming growth factor-β (TGF-β) has been shown to mediate different responses
Figure 1.4 - A diagrammatic representation of the mesenchymal lineage. The figure depicts the four possible ways in which the multipotential mesenchymal cells may differentiate.
in a number of osteoprogenitor systems, stimulating proliferation and inhibiting differentiation although the results from studies conflict (reviewed by Triffitt, 1996).

Many mesenchymal stem cells, which are by nature immature and undifferentiated and which usually give rise to tissue other than bone, can be induced into the osteoblast lineage by exposure to a variety of hormones and growth factors (reviewed by Triffitt, 1996). The cells which are responsive in this manner are known as inducible osteoprogenitor cells and may be more primitive than the determined osteoprogenitor cells which have the inherent ability for osteogenesis such as the osteoblast stem cells lining the bone and in the marrow. Furthermore, Urist and Mikulski (1995) demonstrated that the induction of osteoprogenitors in this manner appears to be self-perpetuating once initiated, in non-skeletal sites. Indeed, this may also result in the creation of a functional bone haematopoietic microenvironment.

However, it is likely that osteoprogenitors do not form a discrete population of cells, especially from the observations that bone may be formed via two routes and that separate embryonic lineages are involved in the synthesis of particular parts of the skeleton. As will be discussed later, bone is predominantly formed in an endochondral manner. This involves the creation of a ‘template’ of the bones in cartilage by chondroblasts, which is then replaced by bone. The majority of the skeleton is made in this manner, including the long bones and the vertebrae. In contrast, the creation of the flat bones of the skull and the addition of bone on the periosteum is mediated by intramembranous ossification. Here, the mesenchymal stem cells instead of differentiating into chondroblasts, differentiate directly into osteoblasts. Furthermore, embryological studies have demonstrated that craniofacial skeletal tissues arise from the
neural crest while the mesoderm is involved with forming the postcranial limb, rib and appendicular skeletons.

The study of the progression of cells through the osteoblast lineage has been complicated by the differences observed in the behaviour of cells in different systems. For instance, cells lines such as ROS 17/2.8 and UMR-106, have aberrant regulation of proliferation/differentiation and have been found to be regulated by hormones in a pleiotropic manner. While these differences may reflect the heterogeneous nature of the cells in bone, and the stages of differentiation and maturation of osteoblastic cells in vivo, these alterations may also be due to artefacts caused by the immortalisation process. In contrast, primary cultures of osteoblasts, predominantly obtained foetal rat calvaria, synthesise type I collagen, alkaline phosphatase and other non-collagenous proteins and are responsive to hormones and cytokines. A common criticism of these cultures is that they contain a heterogeneous population of cells, both in terms of different lineages and stages of differentiation/maturation. However, this heterogeneity in the cell population may have a great similarity to the cross-section of cells found in bone in vivo and this type of culture system could thereby be more biological. Therefore, the majority of the characterisation of the osteoblast lineage has been performed using cells obtained from primary culture. Indeed, formation of bone and the action of osteoblasts in vivo can be mirrored in vitro. For instance, initially in bone formation in vivo the osteoprogenitor cells proliferate while simultaneously laying down bone matrix. These differentiate into osteoblasts and embedded osteocytes (which become quiescent) and further proliferation of the osteoprogenitors occurs as required, for bone remodelling or fracture-repair. In vitro, this is seen as exponential growth of the osteoprogenitors which eventually reach confluency. This is accompanied by matrix
production, which continues even through post-confluent proliferation as bone-like tissue is established. Following this, mineralisation occurs in association with limited apoptosis and compensatory proliferation and up-regulated collagenase activity.

Historically, osteoblast development has been taken to be a linear sequence of events (Figure 1.5) from mesenchymal stem cells to osteoprogenitors, to preosteoblasts and finally to osteoblasts, osteocytes or bone lining cells (Aubin et al., 1995). Typically, osteoblasts have been defined by their ability to lay down collagen type I and mineralise it, synthesise alkaline phosphatase (Beresford et al., 1986; McCarthy et al., 1988; Whyte, 1994; Puzas, 1996), osteocalcin, osteopontin and bone sialoprotein (Stein and Lian, 1993; Aubin et al., 1995) and respond to PTH (Wong and Cohn, 1974; Wong and Cohn, 1975; Luben et al., 1976; Rao et al., 1977; Nijweide et al., 1986). However, the differentiation of osteoblasts is complex, and may be broadly subdivided into three defined stages: i) the proliferation period; ii) the extracellular development and maturation stage; iii) the mineralisation stage. These stages have been characterised by the cells' production of particular proteins which coincide with observable alterations to phenotype (Stein and Lian, 1993; Aubin et al., 1995).

i) The Proliferative Period

This period is characterised by an increase in cell number with a concomitant rise in type I collagen and histone synthesis. Histones are produced in parallel with DNA replication, providing the necessary basic chromosomal proteins for packaging the newly packaged DNA into chromatin. At the peak of collagen/histone production, there is a gradual rise in alkaline phosphatase production. The role of alkaline phosphatase in collagen maturation/bone mineralisation is not fully understood and is discussed below
Figure 1.5 - A schematic of the osteoblast developmental pathway. This figure demonstrates the postulated manner in which mesenchymal stem cells differentiate to form cells of the osteoblast lineage.
(Whyte, 1994). Simultaneously there is a fall in c-fos and c-jun expression: members of a large family of transcription factors (the bZip family) and major constituents of the AP-1 transcription factor complex. They contain a DNA binding domain which is highly basic and a leucine zipper region which allows the formation of heterodimers with other Fos and Jun family members and other nuclear proteins. The complexes thus formed may bind the AP-1 consensus sequence and thereby act as transcription factors and so regulate gene expression. c-fos is known to play an important role in bone development, since over-expression leads to the development of bone tumours (Rüther et al., 1987 and 1989). These tumours tend to be chondroblastic osteosarcomas, which comprise a foci of cartilage around which neoplastic bone has been deposited. In contrast, c-fos knock-out mice suffered from osteopetrosis, characterised by net increases in bone mass due to impaired osteoclast function. It would therefore appear that c-fos plays an important role in regulating the differentiation and function of osteoblasts, chondroblasts and osteoclasts (Grigoriadis et al., 1993; Wang et al., 1994).

**ii) The Maturation Stage**

At this time, the cells no longer divide and c-fos and c-jun production falls to almost undetectable levels and synthesis of histone and collagen decreases to less than 50% of their maximal production. Simultaneously, alkaline phosphatase production peaks and this is followed by a sharp rise in the levels of osteopontin and osteocalcin whilst calcium deposition commences.

*Alkaline Phosphatase* - As already stated, the role of alkaline phosphatase in not clear. Alkaline phosphatase was first characterised by Robison and Soames in 1924 and was found to have an alkaline pH optimum (pH 9.2-10.5) when hydrolysing phosphate ester,
thereby increasing the local free inorganic phosphate concentration. However, further experiments demonstrated that while alkaline phosphatase could aid in mineralisation, it was also found in other non-mineralising tissues such as liver and intestine (reviewed by Whyte, 1994). Subsequently, alkaline phosphatase was found in abundant amounts in the small membrane-bound matrix vesicles. These were first observed as buds of chondrocyte plasma membrane but have also been seen to arise from osteoblasts at fracture sites and in membranous and cortical bone (Anderson et al., 1969). It is postulated that hydroxyapatite crystals form before rupturing and allowing extravesicular crystal growth to continue (Ornay et al., 1985). The critical role of alkaline phosphatase in initiating mineralisation was demonstrated by Bellows et al. (1991), when lavamisole, an inhibitor of alkaline phosphatase activity, prevented mineralisation in vitro. However, once initiated, mineralisation could continue in the absence of alkaline phosphatase or high concentrations of inorganic phosphate. The manner in which alkaline phosphatase aids mineralisation is little understood (Whyte, 1994), and a plethora of putative substrates and functions have been proposed. For example, one hypothesis is that alkaline phosphatase acts to hydrolyse an inhibitor of mineralisation, such as PPi, inorganic pyrophosphate which has been shown to inhibit hydroxyapatite crystal formation when present in high concentrations (Caswell, 1991). Other possible actions of alkaline phosphatase include a role as transmembrane phosphate transporter, a calcium/magnesium ATPase and an extracellular calcium binding protein stimulating calcium deposition, amongst others (Whyte, 1994).

**Osteopontin** - Osteopontin is an acidic phosphorylated glycoprotein found in many tissues. In bone, it is synthesised by preosteoblasts, osteoblasts and osteocytes and secreted into the bone matrix where it is found in especially high concentrations at the
mineralisation front. Interestingly, the amino acid sequence contains an Arg-Gly-Asp (RGD) sequence which is a well characterised binding motif for cell surface integrins. Indeed it was observed that cellular attachment of ROS 17/2.8 to culture plastic was aided if the surface was coated with osteopontin, and inhibited by RGD peptides in the medium (Oldberg et al., 1986). However, while this indicated a possible role for osteopontin in promoting osteogenesis by aiding osteoblast cell attachment to the extracellular matrix, recently it has been suggested that this may also be involved in osteoclast attachment and resorption (Reinholt et al., 1990; Ross et al., 1993). Furthermore the interaction of osteopontin with osteoclasts via integrins is likely to be important in signal transduction. For instance, both transient increases in intracellular calcium in rat osteoclasts and secretion of calcium via an ATPase in a calcium-dependent manner by chicken osteoclasts are induced by such interactions (Miyauchi et al., 1991; Zimolo et al., 1994). Another possible role for osteopontin, in addition to cell attachment to matrix, may be to allow migration of cells, but this has yet to be demonstrated for osteoclasts or osteoblasts, in bone formation/remodelling.

Osteopontin also inhibits both hydroxyapatite nucleation and crystal growth, the repression of which could be relieved by the removal of the phosphate groups from osteopontin (Hunter and Goldberg, 1993). These studies suggest that osteopontin may interact with the crystal faces and thereby regulate their size and shape.

Several other proposals have been put forward for the function of osteopontin. These include integrin-mediated interactions allowing the cell to accurately place itself with respect to its surroundings and possibly thereby act as a mechanosensor for bone strain (Aarden et al., 1994). Also, in a similar manner to that observed in macrophage-induced
production of osteopontin during tissue repair, osteopontin could be deposited on
damaged mineralised tissue and thereby act as a substructure for osteoclast cell
attachment (Murry et al., 1994; Senger et al., 1995).

**Osteocalcin** - Osteocalcin is one of a large family of gla proteins, so called because
some glutamic acid residues within the protein have been γ-carboxylated. This, in turn,
confers high affinities for hydroxyapatite crystals and calcium to these proteins.
Although osteocalcin comprises 1% of the organic content of bone, its role is not well
defined but it is thought that it could be involved with the regulation of mineralisation
and resorption. Three possible roles have been postulated: (a) *In vitro* experiments
demonstrated that osteocalcin inhibited both the conversion of brushite \([\text{Ca}_4(\text{HPO}_4)_2\text{6H}_2]\) to hydroxyapatite \([\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]\) and the growth of hydroxyapatite crystals
themselves, suggesting that osteocalcin may limit mineralisation (Romberg et al., 1986;
Van de Loo et al., 1987; Ducy et al., 1996); (b) as already stated, for the
osteocalcin protein to be functional, specific glutamic acid residues within the protein
must be γ-carboxylated. This is performed by a vitamin K-dependent γ-carboxylase
which is inhibited by the anti-coagulant warfarin and rats treated with warfarin
produce bone deficient in osteocalcin. When these bones were implanted into normal
rats, the bones were shown to be resistant to resorption and had a reduced ability to
recruit and differentiate osteoclast progenitors, suggesting a role for osteocalcin in
recruitment of osteoclasts and thereby resorption (Lian et al., 1984; Defranco et al.,
1992); (c) osteocalcin was observed to associate with osteopontin and promote
adhesion of osteoclast-like cells *in vitro*. Therefore, osteoclasts could be recruited to
sites to be resorbed by osteocalcin and attach via an osteopontin-integrin interaction
(Chenu et al., 1994).
iii) The Mineralisation Stage

Calcium deposits rise quickly, whilst osteocalcin and osteopontin production peaks then begins to fall. Alkaline phosphatase production falls to almost basal levels and the cells gradually become quiescent and thereby progress into bone lining cell-morphology.

The Process of Bone Formation and Remodelling

The manner in which bone is initially formed depends upon the type of bone. For instance, flat bones are created by intramembranous ossification, whereas long bones are formed by a more lengthy process called endochondral ossification. These two mechanisms are described below.

i) Intramembranous Ossification

In this process, the osteoprogenitor stem cells within a highly vascularised area of connective tissue differentiate directly into preosteoblasts and finally into osteoblasts. These cells synthesise collagen and secrete it in a non-regular manner, with no particular orientation to the bundles. As bone formation continues a great many large osteocytes are embedded in the bone matrix. The matrix itself is not calcified in an orderly manner but mineralises in irregularly distributed patches. The blood vessels infiltrating the woven bone form haematopoietic bone marrow. Meanwhile, the bone is progressively replaced with lamellar bone, as described below for endochondral ossification.
ii) Endochondral Ossification

The mesenchymal stem cells initially differentiate into prechondroblasts and chondroblasts which synthesise and secrete a collagenous matrix and thereby create the cartilage 'template'. In a similar manner to osteoblasts, the chondroblasts can become embedded in the matrix, but due to the greater elasticity of the matrix, the chondrocytes thus formed continue to proliferate. Furthermore, they continue to secrete collagenous matrix between the chondrocytes and thereby undergo interstitial growth. Simultaneously appositional growth occurs, whereby mesenchymal stem cells lining the cartilage continue to proliferate and differentiate. In the growth plates, the chondroblasts form regular columns called isogenous groups which enlarge with age, and become hypertrophic. Initially the hypertrophic chondrocytes were thought to senesce by apoptosis or programmed cell death, but it is now considered that at least some cells survive. Indeed, following vascularisation of the area surrounding the chondrocytes, some of these cells have been identified as differentiating into osteoblasts (Galotto et al., 1994; Roach et al., 1995). Further away from the growth plates and the hypertrophic zone, the cartilage progressively mineralises forming the zone of provisional calcification.

Simultaneously, preosteoblasts and osteoblasts are recruited to the midshaft and lay down a ring of woven bone, under the perichondrium, which thus becomes the periosteum. Mononuclear cells with a distinct morphology (Lee et al., 1995) then invade allowing vascularisation of the bone to occur, thereby admitting the blood supply which will become the haematopoietic bone marrow. The osteoclasts partially resorb the calcified cartilage towards the growth plate, permitting further vascularisation.
Following resorption and vascular invasion, osteoblasts form a layer of woven bone on the remnants of the cartilaginous longitudinal septa. In this manner, cartilage is replaced by trabecular bone, called the primary spongiosum. Further down, this bone in turn, is progressively replaced with lamellar bone in a second remodelling process.

**iii) Remodelling**

During adult life, bone is only replaced at sites where resorption has previously occurred and since the resorptive process is not random, this is a carefully controlled mechanism. The sequence of events is essentially the same for both trabecular or cortical remodelling and may be divided into discrete sections (Figure 1.6).

Initially osteoclasts are activated and resorption is initiated. In more detail, quiescent osteoclasts prior to resorption may lie in amongst the cells lining bone which are probably of the osteoblast lineage. However, when resorption is stimulated, the bone lining cells retract and osteoclasts invade the space left behind. The osteoclasts then form a tight seal on the bone around the ruffled border at which resorption occurs. Once the bone has been resorbed to a sufficient depth, the osteoclast either progresses along the bone or becomes detached. This is known as the reversal phase.

Following this, macrophage-like mononuclear cells attach to the freshly resorbed bone and form a cement line, marking the limit of resorption and creating a layer onto which the new bone can attach. The preosteoblasts then attach and differentiate into osteoblasts while secreting bone matrix, marking the formation phase. At this stage, osteoblasts regulate at least two of the three main processes involved with bone formation, namely, the synthesis and deposition of collagen and the aggregation of
Figure 1.6 - A schematic of bone remodelling. Osteoclasts resorb bone (frame 1) after which uncharacterised mononuclear cells migrate to the site of remodelling marking the reversal phase (frame 2). Osteoblasts lay down new collagen (frame 3 - red) forming the osteoid layer which matures and gradually mineralises (black), while the osteoid layer continues to grow (frame 4). Eventually, the rate of collagen deposition decreases, while mineralisation rate remains constant, resulting in a decrease in osteoid thickness (frame 5).
collagen molecules. However, the exact role of osteoblasts in the process of maturation of the collagen and the nucleation/growth of hydroxyapatite crystals within the collagen matrix has not been fully determined. The processes themselves are described below.

**a) The Synthesis and Deposition of Collagen**

Collagen polypeptides are translated by ribosomes on the rough endoplasmic reticulum as pro-α-chains in which selected proline and lysine residues may be hydroxylated. Collagen molecules comprise three α-chains which are wrapped round each other in a helical manner. Many different types of collagen exist, but the characteristic type found in bone consists of two different collagen polypeptides, α1 and α2, in the ratio of 2:1 (Puzas, 1996). The collagen polypeptides aggregate to form procollagen molecules, after which they are secreted at the bone forming surface.

**b) The Aggregation of Collagen Molecules**

The propeptides of the procollagen molecules are removed enzymatically which allows the aggregation of collagen molecules, through covalent bonds, to form collagen fibrils. These are then usually arranged along the major axis of stress and, in mature bone, are often found lying parallel to each other in sheets, with the sheets arranged almost at right angles to each other. This unmineralised collagen is known as the osteoid seam.

**c) The Maturation and Nucleation**

This process is still relatively poorly understood. In brief, the process involves the nucleation of hydroxyapatite crystals with magnesium and carbonate within the fibrils. The crystals remain small and form long, thin plates or tubes enwrapping the collagen microfibrils, while water occupies ~70% of the fibril volume. *In vivo*, a lag time is seen
between the deposition and mineralisation of collagen. It is thought that maybe alkaline phosphatase plays an important role in nucleation (Whyte, 1994) but in a manner similar to many bone proteins, including osteocalcin and osteopontin (Stein and Lian, 1993), the exact role it plays is not well defined. Eventually, the new bone fills the pit and the osteoblasts become quiescent, which is referred to as the resting phase.

**The Regulation of Bone Remodelling and Hormonal Effects on Bone**

As previously mentioned, bone remodelling, both for structural repair and calcium/phosphate homeostasis, is a complex process, the regulation of which is critical for maintaining bone mass. To this end, many growth factors, hormones and steroids are known to regulate both osteoblast and osteoclast function (Figure 1.7). Their actions may regulate the proliferation of the precursor cells, the recruitment of these cells to sites of bone remodelling and the end-point differentiation into their active phenotypes. The factors may also mediate their actions in an endocrine manner, for example PTH, dihydroxyvitamin D₃ and calcitonin, or a paracrine (effective on cells of a different class) or autocrine (effective on cells of the same class) way, as seen for fibroblast growth factor (FGF)-1 and -2, platelet-derived growth factor (PDGF) and IGF-I and -II. These may act in a number of ways, either directly stimulating or repressing the individual cells or indirectly, for instance by altering receptor number or affinity, the synthesis of binding proteins or through the production of another factor, all of which may affect bone resorption or formation. Since faulty regulation has been linked to many diseases, a great deal of work has been performed in order to characterise these factors and the actions of some of these are described below. The
Figure 1.7 - The effects of hormones and growth factors on osteoblasts (right) and osteoclasts (left). The various responses of the bone cells to the factors are denoted by '+' for stimulatory and '-' for repressive. The effects of hormones which have not been clearly elucidated are prefixed by a '?'.
first part is devoted to systemic regulators of bone remodelling while the second part concentrates on the effects of some local factors.

**i) Systemic Factors**

*Parathyroid Hormone (PTH)* - The overall role of this hormone is to raise calcium levels in the blood, predominantly by stimulating bone resorption and regulating calcium uptake from the kidneys and, to a lesser extent the gastrointestinal tract. Interestingly, PTH does not appear to increase the numbers of osteoclast progenitors in the short-term (up to 4 days), although the data is contradictory (Lorenzo *et al.*, 1983). However, PTH does seem able to stimulate the fusion of the committed progenitors into multi-nucleated osteoclasts, and also activates resorption by preformed osteoclasts, thereby releasing calcium ions locked in mineralised bone (reviewed by Nijweide *et al.*, 1986; Martin and Ng, 1994).

The manner in which PTH mediates its response in bone is confused, but it has been postulated that PTH does not act directly on osteoclasts but rather that the response is mediated through osteoblasts releasing an, as yet, uncharacterised second messenger (McSheehy and Chambers, 1986). This could involve IL-11 which is released into the medium of co-cultures of osteoblasts and marrow cells (from which osteoclasts may be derived) when stimulated with PTH (Girasole *et al.*, 1994) and indeed, neutralising antibodies to IL-11 inhibited PTH-induced osteoclastogenesis. However, a direct effect of PTH on osteoclasts is indicated since it has been shown to bind to osteoclasts (Teti *et al.*, 1991) and has been demonstrated to activate bone resorption by osteoclasts directly (Miller and Kenny, 1985; Murrills *et al.*, 1990). It is therefore possible that both
these mechanisms are employed to stimulate osteoclastogenesis and that they work synergistically.

The apparent conflicting data of the effects of PTH upon osteoblasts are likely to be due in part, to observations that the differentiation stage of the osteoblast may also affect the response to PTH. For example, in pre-confluent cultures, PTH stimulates the synthesis of alkaline phosphatase and calcitonin, whereas in post-confluent cultures, both components were down-regulated. Therefore it would appear that PTH could stimulate differentiation of preosteoblasts, as are present in the sub-confluent cultures, while inhibiting the mature phenotype in confluent cultures (Bellows et al., 1990). This could be indicative of the role of PTH in vivo, whereby PTH stimulates both bone resorption while simultaneously enhancing bone remodelling in order to maintain bone mass (Isogai et al., 1996). PTH may also aid stimulation of resorption by regulating calpain- or E-cadherin-dependent (Babich and Foti, 1994) osteoblast cell attachment to bone (reviewed by Fitzpatrick and Bilezikian, 1996) since removal of osteoblasts allows the subsequent attachment of osteoclasts and thereby resorption (Murray et al., 1995).

Many experiments have been performed, studying the effects of PTH upon osteoblast activity in vitro. In culture, responsiveness to PTH, as measured by increased intracellular cAMP, was initially used as a marker for osteoblasts (Luben et al., 1976). PTH was found to interact with a specific receptor on the osteoblast surface whereupon one of the first functions described was to mediate amino acid transport (Rosenbusch and Nichols, 1967). It has also been shown that continuous incubation of osteoblasts with PTH inhibited collagen and matrix synthesis at the transcriptional level and inhibited nodule formation in vitro, supporting the initially catabolic function of PTH. In
contrast, intermittent treatment with PTH stimulated bone formation and collagen synthesis (Kream et al., 1986; Lomri and Marie, 1988). This response to PTH is probably mediated through the secretion of anabolic factors such as IGF-I and TGF-β into the extracellular milieu along with regulation of TGF-β receptor expression on osteoblasts (Centrella et al., 1988). As a corollary to this hypothesis, it is known that one of the primary responses of osteoblasts to PTH is a rise in levels of cyclic adenosine monophosphate (cAMP), which increases the synthesis of skeletal IGF-I (Donahue et al., 1988). Furthermore, other inducers of cAMP production in osteoblasts have a similar effect upon IGF-I production.

The dual anabolic/catabolic roles of PTH are compatible with the coupled resorption/formation model for remodelling, whereby the hormone may transiently increase serum calcium by resorption, but also accelerate repair using such factors as IGF-I. It is also interesting to note that PTH appears to act on osteoblasts and osteoclasts on particular types of bone preferentially. For instance, patients with elevated levels of PTH in hyperparathyroidism, suffer from osteopenia in the cortical bones predominantly and to a much lesser extent in trabecular bone.

Calcitonin - Calcitonin is a 32 amino acid polypeptide hormone which inhibits osteoclastic bone resorption. Since responsiveness to calcitonin is an osteoclastic phenotype, it was one of the first factors used to distinguish osteoclasts in culture and therefore the ability of cells to respond was used as a biochemical marker of osteoclasts (Luben et al., 1976; Mundy, 1996). Morphologically, calcitonin appears to cause cytoplasmic contraction of the osteoclast plasma membrane, which has been linked with a decrease in its resorptive ability (Chambers and Magnus, 1982).
Furthermore, calcitonin has the ability to inhibit osteoclast progenitor cell proliferation and differentiation whilst also causing the dissolution of mature osteoclasts into precursor mononuclear cells (reviewed by Mundy, 1996).

1,25 dihydroxyvitamin D₃ - Vitamin D is a pleiotropic hormone predominantly synthesised by the kidneys under the tight control of PTH and phosphorous. In turn, vitamin D regulates PTH synthesis by both interacting directly with the chief cells in the parathyroid gland and by raising serum calcium. It acts on the gastrointestinal tract primarily, but also regulates osteoblast and osteoclast formation (DeLuca, 1980). The main function of vitamin D has been well characterised, that is stimulating uptake of calcium from the gut, whereas its action on bone is still not completely elucidated. Overall, it appears to increase osteoclastic bone resorption by either directly stimulating differentiation and fusion of osteoclast precursors (Roodman et al., 1985), or by the indirect stimulation of other cells to produce various resorptive factors. For instance, vitamin D is a potent immunoregulatory factor and may stimulate certain cells with monocyte characteristics to produce IL-1 (Tsoukas et al., 1984) which is an enhancer of osteoclastic resorption.

The action of vitamin D on osteoblasts is more complex. It is known to be necessary for normal bone formation and mineralisation and stimulates osteoblastic production of osteocalcin (Fritsch et al., 1985; Lian et al., 1985; Harrison and Clark, 1986). Importantly, vitamin D may also stimulate the synthesis of certain IGF binding proteins (Delany et al., 1994) which may potentiate or inhibit the action of IGF-I by prolonging the half-life of IGF-I and regulating its rate of clearance, aiding its localisation to a
particular type of cell or tissue or modulate the affinity of IGF-I for its receptor (reviewed by Jones and Clemmons, 1995).

*Growth Hormone* - GH is required, *in vivo*, for the maintenance of bone mass by stimulating bone formation, a hypothesis corroborated by the observations that GH-deficient patients have decreased bone mass which may be reversed upon GH-replacement. However, it is difficult to gauge the effects of GH directly on bone, since GH causes a large rise in hepatic, and thereby systemic, IGF-I production. *In vitro* studies, though, have demonstrated that while the effects of GH directly on bone are not large, it does stimulate an increase in IGF-I mRNA abundance in the rat rib growth plate of hypophysectomised rats mediated by osteoblasts (Isgaard *et al*., 1988; McCarthy *et al*., 1989). GH also increases serum calcium predominantly through an increase in vitamin D production resulting in enhanced uptake from the gastrointestinal tract. Interestingly, GH has been reported to have a pronounced stimulatory effect on bone resorption (Spencer *et al*., 1991; Eriksen *et al*., 1996).

*Insulin* - Like GH, insulin stimulates bone and cartilage matrix synthesis and is also necessary for normal mineralisation and bone growth. In a manner very similar to GH, insulin also promotes hepatic IGF-I production, to which some of its *in vivo* responses may be attributed. However, unlike IGF-I, insulin does not stimulate preosteoblast replication but acts by promoting matrix production by the differentiated osteoblasts.

*Glucocorticoids* - The actions of glucocorticoids are many and varied. Systemically, *in vivo*, they repress calcium absorption from the gut which is thought to mediate a large increase in PTH secretion which in turn, leads to bone resorption (Delany *et al*., 1994).
However, stimulation of bone tissue with glucocorticoids \textit{in vitro}, appears to inhibit osteoclastic resorption and induce differentiation of cells of the osteoblast lineage (Raisz \textit{et al.}, 1972). Longer-term stimulation, though, decreases the replication of both osteoblastic and osteoclastic cells, and inhibits their respective actions of bone formation and resorption (Canalis, 1983). Glucocorticoids mediate many other actions on osteoblasts consistent with decreasing bone collagen and matrix, including down-regulating type I collagen synthesis while increasing production of matrix metalloproteinase (MMP)-13 (a protease important in the breakdown of collagen), both of which are regulated post-transcriptionally (Delany \textit{et al.}, 1995). Therefore, these two actions are consistent with glucocorticoids acting to decrease bone collagen.

Glucocorticoids are also found to inhibit the action of TGF-\(\beta\) by making it bind to non-signal transducing receptors on the cell surface of osteoblasts, thereby preventing TGF-\(\beta\) mediated stimulation of proliferation of osteoblastic progenitors and collagen synthesis (Centrella \textit{et al.}, 1991).

Glucocorticoids reduce transcription of IGF-I in osteoblasts, the number of IGF-II receptors and alter the expression of IGFBPs, so that the expression of IGFBP-1 and -6 is increased while synthesis of IGFBP-2 to -5 is inhibited (McCarthy \textit{et al.}, 1990; Delany and Canalis, 1995). The actions of IGF-I itself, in bone are described below. While these are only examples of the manner in which glucocorticoids affect the responses of bone cells to various factors, it is clear that they decrease bone turnover if not mediate a net loss of bone mass.

\textit{Sex Steroids} - Oestrogens and androgens appear to be predominantly involved with the inhibition of resorption, and post-menopausal bone resorption and osteoporosis is
linked to a decrease in oestrogen levels. It has been suggested that oestrogen mediates this response indirectly through the down-regulation of production of cytokines IL-1 and -6, which are thought to increase osteoclast activity and bone resorption (Pacifici et al., 1989; Jilka et al., 1992; Girasole et al., 1992; Horowitz, 1993), although osteoclasts themselves may also be a target for oestrogen (Hughes et al., 1996). In contrast to these actions on osteoclasts, it would appear that osteoblasts are weakly stimulated by oestrogen, if at all, especially since the bone mass of patients on oestrogen replacement tends not increase, but, on the whole, remains static. However, oestrogen therapy has been observed to lead to an increase in bone mass occasionally. Indeed, supplementation of oestradiol (E2) in vitro has been observed to increase the steady state levels of IGF-I and collagen mRNA in rat calvarial cell lines and primary cultures (Ernst et al., 1989).

Thyroid Hormones - These hormones have an anabolic role in cartilage development and growth. This is in contrast to their effect on bone, where at high concentrations thyroid hormones have been shown to stimulate osteoclast bone resorption but have no effect on bone matrix deposition or osteoblast cell proliferation (Mundy et al., 1976). However, their precise mode of action is not well characterised, for at low concentrations they stimulate IGF-I synthesis and bone formation (Lakatos et al., 1993).

ii) Regulation of Remodelling in the Bone Microenvironment

Many factors are produced locally, both by the bone cells themselves and by stromal cells or by macrophages/monocytes and other cells of the immune and haematological system as they pass through bone, thereby releasing cytokines into the bone
microenvironment. Seeing that bone resorption occurs in discrete areas of the skeleton, it would be imperative to regulate both the extent of the resorption, and the course of the remodelling, locally. A great many factors have been found to play a role, and a few are described below.

*Transforming Growth Factor (TGF)-β Family* - This family comprises many factors including the TGF-βs and the bone morphological proteins (BMPs) which have great amino acid sequence homology. TGF-β has wide-ranging effects upon the stimulation of bone formation. For instance, these factors stimulate proliferation of osteoblast progenitors and stimulate type I collagen synthesis (Centrella *et al.*, 1987). Further roles include the suppression of resorption, which may be achieved through osteoclast apoptosis. Interestingly though, TGF-β is released as an inactive precursor attached to a binding protein. This inactive form is activated during bone resorption as stimulated by PTH for example, thereby increasing the local concentration of TGF-β which inhibits osteoclastic resorption of bone and stimulates new bone synthesis. The BMPs are osteoinductive factors, working through their own receptors, and while they have similar activities to TGF-β, they can induce differentiation of progenitors of osteoblasts (Wozney *et al.*, 1988).

The regulation of TGF-β is quite complex since three forms are synthesised, TGF-β1, -β2 and -β3 (Canalis *et al.*, 1993) each with their own regulatory elements but with similar biological activities. Furthermore, there are three possible receptors with which TGF-β may interact. TGF-β receptor-I and -II transduce the TGF-β signal, whereas TGF-β receptor-III or betaglycan is a non-signal transducing type of receptor.
Therefore, alterations in the relative amounts of the TGF-β receptor-III may regulate the responsiveness of a particular cell to TGF-β.

**Fibroblast Growth Factors (FGFs)** - Generally these are considered to be potent angiogenic factors, but they also have pleiotropic effects upon bone regulation. Two types of FGF exist, basic and acidic FGF, both of which are capable of stimulating bone cell replication although basic FGF is more potent. FGFs are synthesised by osteoblasts when stimulated by FGF itself or TGF-β* in vitro* (Canalis *et al.*, 1988; Hurley *et al.*, 1994). Systemic administration of FGFs increases the numbers of preosteoblasts and bone formation is stimulated (Nakamura *et al.*, 1995) although FGFs inhibit collagen synthesis in mature osteoblasts (Hurley *et al.*, 1993). Fitting in with the role of FGFs in remodelling/repair, it has been observed that MMP-13 production may be stimulated in osteoblasts by FGF. However, FGFs do not stimulate osteoclastic resorption.

The activity of FGFs can also be modulated by binding to proteoglycans in the extracellular matrix. FGFs have four types of receptor, FGFR-1 to -4 (Wang *et al.*, 1994) and while these have not been well elucidated in bone, their importance is clear from studies into achondroplasia. This is due to a mutation in FGFR-3, leading to severe skeletal abnormalities including dwarfism and early closure of the cranial sutures (Shiang *et al.*, 1994). It is likely that different bone types vary in the FGF receptors expressed, and thereby mediate the actions of FGF in a site-specific manner.

**Platelet-derived Growth Factor (PDGF)** - PDGF is predominantly a systemic factor important in the early stages of wound repair (Heldin and Watermark, 1987). This factor has two main roles in bone cell regulation: (1) to stimulate proliferation of
osteoblast precursors, thereby stimulating collagen synthesis in a manner similar to FGF insofar as PDGF does not stimulate differentiation to a mature phenotype (Hock and Canalis, 1994) and (2) stimulation of resorption, both by osteoclasts and by the up-regulation of MMP-13 in osteoblasts. However, the action of PDGF depends on the isoform mediating the response. PDGF comprises of a dimer of PDGF-A and/or PDGF-B which may form homodimers (PDGF-AA or -BB) or heterodimers (PDGF-AB), all of which are synthesised by osteoblasts (Rydziel et al., 1994). The proliferative response to PDGF appears to be ubiquitous to all PDGF isoforms, while the resorptive response has been shown with PDGF-BB (Canalis, 1996).

TGF-β can stimulate the synthesis of both PDGF-A and -B, but PDGF (A or B) itself may stimulate production of PDGF-A. In a similar manner to FGF, PDGF-B can also interact with one of the matrix proteins, osteonectin, thereby decreasing its affinity for the PDGF receptors (Raines et al., 1992). Furthermore, there are two forms of PDGF receptor, -α and -β, whose expression can be regulated thereby controlling the binding affinities of the receptors for the PDGF isoforms (Centrella et al., 1992).

_Interleukin (IL)-1 and -6 and other Cytokines_ - While the two forms of IL-1, IL-1α and β, are predominantly synthesised by activated monocytes, they are also made by other cells including osteoblasts (Mundy, 1996). They are both highly resorptive agents with similar actions and work through the same receptor, stimulating osteoclast progenitor proliferation and maturation, and also stimulating osteoclast activity indirectly through another cell (Thomson et al., 1986; Pfeilschifter et al., 1988). Infusion of IL-1 _in vivo_ stimulates resorption resulting in a large increase in serum calcium concentration, and
increased levels of IL-1 have been implicated in certain cases of osteoporosis which may be reduced with oestrogen administration (Sabatini et al., 1988; Boyce et al., 1989; Pacifici et al., 1989; Horowitz, 1993). Similarly, IL-6 stimulates osteoclast formation but it is less potent than IL-1. It is predominantly produced by osteoclasts in response to factors such as PTH, vitamin D and IL-1 (Feyen et al., 1989). Since it is stimulated by IL-1, it is not surprising that it has been implicated in post-menopausal osteoporosis and that oestrogen may also diminish the production of IL-6 (Manolagas and Jilka, 1995).

TNFα and lymphotoxin are structurally similar to IL-1 and mediate almost identical responses, and may be synergistic with IL-1, but act though different receptors than those used by IL-1 (Mundy, 1996). The molecules are released from macrophages and T-lymphocytes respectively and are potent stimulators of osteoclast progenitor proliferation, maturation, fusion and resorption, mediating their actions through osteoblasts (Bertolini et al., 1986; Thomson et al., 1987; Johnson et al., 1989).

Other factors have also been demonstrated to be important in the regulation of bone remodelling such as macrophage and granulocyte/macrophage colony-stimulating factors (GM-CSF) and γ-interferon. The critical role for GM-CSF-1 was ascertained from a knockout mouse which was observed to suffer from osteopetrosis due to decreased osteoclast formation, but which could be cured upon CSF-1 replacement (Felix et al., 1990). In contrast γ-interferon, which is also an immune cell product, inhibits osteoclastic bone resorption, mainly by preventing the differentiation of the precursor cells and, to a lesser degree, decreasing the rate of precursor proliferation (Gowen et al., 1986; Takahashi et al., 1987).
IGF-I and Bone

As already described, IGF-I is an important and abundant growth factor mediating responses both systemically and locally. In bone, it is one of the most abundant growth factors and is produced by cells of the osteoblast lineage, acting locally as a growth and differentiation factor, thereby effecting anabolic responses (Isgaard et al., 1988; Ernst et al., 1989; Bichell et al., 1993; Delany et al., 1994). These involve the stimulation of osteoblast proliferation, the up-regulation of type I collagen synthesis and secretion, which is partly dependent upon DNA synthesis, and a decrease in the production of MMP-13 (Hock et al., 1988; Canalis et al., 1995). This leads to an overall increase in the rate of bone remodelling, as has been observed with short-term infusions of IGF-I in vivo in humans (Ebeling et al., 1993). Furthermore, the stimulation of hormonally intact rats with IGF-I resulted in an increased body mass accompanied by an increase in tibial epiphyseal width and tibial protein synthesis (Hizuka et al., 1986; Martinez et al., 1994), while an increase in bone mass was seen in infused sheep (Cottam et al., 1992). IGF-I also appears to play an important role in fracture healing in rats. It was found that in both normal and hypophysectomised rats, IGF-I was produced at the fracture callus, initially by chondroblasts and chondrocytes, but also by cells surrounding the fracture site, such as muscle cells, satellite cells and osteoblasts, reaching its zenith at 8 days. However indomethacin, an anti-inflammatory drug which is an inhibitor of prostaglandin synthesis, was observed to reduce fracture healing (Edwall et al., 1992). This impaired fracture healing was accompanied by a 50% decrease in maximal IGF-I
expression, which was delayed, suggesting that local production of IGF-I may act as an important tropic factor in bone regeneration (Edwall et al., 1992).

The importance of IGF-I as an anabolic agent is evident from the number of agents which regulate its synthesis and availability, not only systemically but also locally including tissues such as bone. For instance, thyroid hormones appear to mediate their anabolic effects through IGF-I synthesis in bone as does progesterone (Lakatos et al., 1993; Barengolts et al., 1996). GH also enhances IGF-I production by osteoblasts, in a limited manner, thereby mediating its anabolic action on bone. Interestingly, acromegaly, the overproduction of GH, results in tall stature and slightly increased or normal bone mineral density. In contrast, in rats perfused with IGF-I, only the anabolic effects of bone formation are observed. This is due to GH having both anabolic and resorptive effects on bone formation, while the actions of IGF-I are purely anabolic and do not appear to be coupled to resorption (Spencer et al., 1991).

Factors which increase the intracellular messenger cAMP, such as PTH and PGE₂, mediate large increases in IGF-I synthesis (McCarthy et al., 1990). Interestingly, the IGF-I-enhanced synthesis of type I collagen is due to the growth factor affecting the differentiated function of the osteoblast rather than simply due to an increase in cell numbers, as has been described for FGF and PDGF. Furthermore, FGF and PDGF appear to prevent the differentiation of cells of the osteoblast lineage, an effect which may be due in part to an inhibition of the osteoblast IGF-I production. Other inhibitors include glucocorticoids, TGF-β and oestrogen (Barengolts et al., 1996).
It must be remembered that while many factors may affect the production of IGF-I by osteoblasts, they may also regulate the synthesis of IGF-I binding proteins, which can affect the half-life of IGF-I or enhance/inhibit its binding ability to the receptors. However the manner in which the binding proteins are synthesised is likely to be tightly regulated. For instance, in addition to increasing IGF-I production, PTH and PGE$_2$, tend to stimulate the synthesis of IGFBP-3, -4 and -5. Interestingly, especially since these factors have both anabolic and catabolic effects upon bone remodelling, IGFBP-4 has inhibitory activity and IGFBP-3 and -5 enhance the effect of IGF-I (Delany et al., 1994). Indeed, it has been observed that PGE$_2$, GH and the IGFs all regulate these three binding proteins, with individual patterns of expression (McCarthy et al., 1994). This regulation of binding proteins would therefore appear to correlate with the overall action of the factors. The manner in which the action of particular factors is mirrored by their regulation of IGFBPs is further exemplified by glucocorticoids and mitogenic growth factors (Chen et al., 1991). These have been shown to mediate part of their action, not only through the down-regulation of IGF-I synthesis, but through the co-ordinated decrease in IGFBP-5 expression (Canalis et al., 1995).

IGF-I is often found associated with IGFBP-5 within the bone matrix itself (Bautista et al., 1990). This may form a further regulatory mechanism for maintaining bone mass and it has been postulated that the anabolic effects of intermittent PTH treatment may be, at least in part, due to an increase in the concentration of matrix IGF-I (Pfeilschifter et al., 1995). Also, the concentrations of matrix IGFs are known to be elevated in diseases linked with increased bone density, such as osteoarthritis, and depressed with the age-related decrease in bone mass.
Many of the factors described in this section exert their influence upon other hormones and growth factors, in particular IGF-I, at the level of gene expression. The manner whereby this regulation may be mediated is described in the next section.
Part 3 - Regulation of Protein Synthesis

The process of protein synthesis is complicated and regulated at several points. Briefly, the gene is transcribed in the nucleus, producing a heterogeneous nuclear RNA (hnRNA) transcript. Following transcription, a methylguanine cap ($m^7G$) is added to the 5'-end of the transcript, and a poly(A) tail added to the 3'-end, after which the introns are spliced from the molecule. The mature messenger RNA (mRNA) is then transported into the cytoplasm, where it may be translated, degraded or stored in an untranslatable form, usually bound to proteins forming messenger ribonucleoprotein particles (mRNP).

If the mRNA is to be translated into protein, a complex of many proteins including the 40S ribosomal subunit binds to the 5'-cap. This complex scans the mRNA in a 5' to 3' direction until an AUG initiation codon is reached at which point the 60S ribosomal subunit associates, forming the active ribosome. Importantly, while this ribosome is synthesising and elongating the polypeptide chain, other ribosomes may initiate translation from the same transcript, thereby forming polyribosomes or polysomes, so that many peptide chains may be synthesised from a single transcript simultaneously.

Proteins to be exported, such as growth factors, or for embedding in the plasma membrane, for example receptors, have an amino-terminal signal sequence of variable length. This interacts with the endoplasmic reticulum, directing the transport of the nascent protein through the membrane. Subsequently, the signal sequence is cleaved from the protein which may be followed by post-translational modifications, such as glycosylation or sulphation.
It is apparent from this brief description that protein synthesis may be regulated both at the level of transcription, RNA processing, translation and post-translational events. However, under the remit of this project, only transcriptional and translational regulation was studied. Therefore, the mechanisms and implications of regulation at these two points will be considered in the following section.

Section A - Transcriptional Regulation

Introduction

The manner in which genes are transcribed may be either constitutive (basal) or modulated. The type of transcription largely depends upon the presence of a number of defined regions upstream of the start site. For instance, TATA and CCAAT boxes tend to be associated with constitutive transcript expression, whereas regulated transcription requires hormone response elements, enhancers, silencers etc. Both these groups are described in more detail below.

Elements Involved with Constitutive Gene Expression.

Many housekeeping genes have one or more of the upstream elements, the TATA box, CCAAT box or GC box within their promoter region. The TATA box is often found approximately 25 bases upstream of the start site and is a well conserved 8 bp consensus sequence which tends to consist entirely of A-T base pairs. Analysis of mutations of the TATA box have shown that while it has little effect on the rate of
transcription, it plays an important role in determining the precise start point. The CCAAT box is also well-conserved, and often located at position -80 with respect to the transcription start site. However, unlike the TATA box, it appears to act as a modulator of the frequency of initiation and only determines the general region from which the transcripts will be initiated. Similarly, the GC box (GGGCGG), otherwise known as the binding site for the transcription factor Sp1, also modulates the efficiency of the promoter and its general location. Interestingly, the GC box may be present in multiple copies and, in a similar manner to the CCAAT box, its position and orientation is not critical to its function. Indeed, from these observations, it is possible that the CCAAT and GC boxes serve to bring the RNA polymerase into the general vicinity of the transcription start site, whereupon a TATA-binding protein (a component of the transcription initiation factor TFIID) interacts with the TATA motif. Subsequently, this factor may interact with other transcription factors and align RNA polymerase II so that transcription is initiated from the correct start site.

Other Regulatory Elements

Several other elements, called enhancers and silencers, may also regulate the rate of gene transcription. These are often gene-specific and may be positioned both up- or downstream of the transcription start site, and can also be situated at great distance from it. In addition, they appear to function independently of their orientation, but unlike the promoters, they may not initiate transcription and can only modulate the rates of transcription. Importantly, the action of enhancer elements is often dependent upon the cell type and therefore cell-specific regulation of transcription can be achieved. In this manner, unlike the interactions with the promoter elements, tissue-specific proteins
may interact with the enhancer elements and modulate gene expression. Many other regulatory elements are found in genes including cAMP response elements and steroid hormone response elements.

The manner in which proteins interacting with enhancer elements, which are sometimes several kilobases away from the transcription start site, regulate the rate of transcription is still unclear. However, three models have been proposed (Dynan and Tjian, 1985): (1) the protein interacts with its enhancer site and migrates along the DNA until it is in close proximity to the start site, whereupon it mediates its action on the transcription complex; (2) in binding, the protein stimulates a cascade of proteins binding along the gene; (3) the protein binds, causing the DNA to ‘loop out’ thereby bringing the enhancer protein into the region of the transcription complex.

The Use of Alternate Promoters

The transcriptional regulation of several genes, including IGF-I, which have multiple transcription start sites may be even more tightly controlled. The use of particular start sites may be tissue-specific, driven by a particular stimulus, differentially expressed from several promoters or a combination of these. In addition, the heterogeneous transcripts produced may be processed or translated at different rates or they may encode different proteins.

An example of different proteins being encoded by transcripts initiated from alternate promoters is provided by the α2(I) collagen gene. Both cartilage and bone cells were observed to transcribe the gene, but of these tissues, only bone expressed collagen. This
was found to be due to tissue-specific usage of different promoters (Bennet et al., 1989). Subsequently, it was found that the transcription start site used in cartilage was preceded by the sequences CCAATT and TGTAAA, which taking into account their position with respect to the start site and their close homology to the consensus promoter sequence, would suggest that these elements are likely to have basal promoter activity (Bennet and Adams, 1990). An interesting aspect to the regulation of this gene is that the use of the alternate promoters may change with development. For instance, α2(I) collagen is synthesised by prechondrogenic mesenchymal cells, but this ceases when these cells differentiate into chondroblasts. Furthermore, this alteration in the mRNA transcribed results in the production of a low-molecular weight non-collagenous protein whose function has not been identified as yet.

Murine parathyroid hormone-related peptide is another example of multiple promoter use but in this case results in the synthesis of the same protein. Two TATA-less promoters are present, the downstream one containing several GC boxes and which is found to be active in all tissues studied. The upstream promoter region, in contrast, contains a CCAAT box which appears to be tissue-specific since these transcripts have been only found strongly expressed in the kidneys and weakly expressed in the liver (McCuaig et al., 1995). The resulting variations in the length and sequence of the 5′-UTR thus produced, may mediate changes in the translatability of the transcripts and thereby alter the expression of the protein.

The rat mitochondrial aspartate aminotransferase gene is also initiated from multiple transcription start sites, but the manner of expression may be regulated in response to testosterone in a tissue-specific manner. In more detail, the 200 bases upstream of the
start sites are highly GC-rich and contain three putative Sp1 binding sites but no TATA box (Juang et al., 1995). In total, transcription may be initiated from seven sites and all are used in liver and the prostate. However, testosterone only induces enzyme synthesis from the prostate epithelium, simultaneously altering the pattern of start site usage within the tissue. Therefore it would appear that while certain promoters mediate constitutive expression, others may contain androgen-response elements, thereby allowing tissue-specific hormonal regulation of expression.

The Regulation of the Rate of Transcription

Thus far, the alterations in start site usage, as mediated through regulation of promoter usage has been described. However, modulation of the abundance of particular transcripts, often in a tissue-specific manner, is frequently controlled though other regulatory elements, such as enhancers and hormone response elements. The enhancers may, therefore, dictate the extent to which a particular promoter is used sometimes even to the extent of preventing expression.

Such regulation has been implicated in the expression of growth hormone receptors. The number of receptors in foetal tissues is low, rising rapidly postnatally (Mathews et al., 1988) and there is evidence to suggest that this increase in expression is mediated through an enhancer element in the gene. Indeed, a sequence over 3 kb upstream of the start site has been implicated in enhancing expression of the GH receptor gene in adult hepatocytes, while having little effect on foetal tissue (Menon et al., 1995). Furthermore, a protein which is present only in adult hepatocytes, has been found to interact with the putative enhancer region. This would indicate that interaction of this
protein with the enhancer may play an important role in regulating the expression of the receptor during development. However the exact mode of action including how the binding profile changes with development has yet to be elucidated. In contrast to this developmentally-specific regulation, pro-\(\alpha_1(I)\) collagen has been demonstrated to be regulated in a cell-specific manner. An enhancer region was shown to interact with an osteoblast-specific protein, which may thereby confer cell-specific expression of this collagen variant to osteoblasts (Rossert et al., 1996).

In contrast to the enhancing effects of the elements so far described upon gene expression, silencer elements may decrease transcription. An example of a gene regulated in such a manner is provided by the parathyroid hormone gene. The silencer element, termed the calcium-response element, is positioned over 3 kb upstream of the initiation site, and appears to interact with nuclear proteins in response to increased extracellular calcium concentrations. This, in turn, results in a down-regulation of PTH gene expression (Okazaki et al., 1991).

Section B - Post-transcriptional Regulation of Protein Synthesis

Introduction

The variability in the 3'- and 5'-UTRs of many transcripts has been shown to be dependent upon some tissue-specific aspect or the physiological situation in which the cell expressing the transcripts finds itself. These changes may have subsequent effects upon the manner in which translation will occur. Furthermore there are many cases of changes in the levels of protein synthesis not correlating with an alteration in the rates
of transcription or the amount of mRNA. This post-transcriptional regulation allows cells to alter protein synthesis in response to acute changes in stimuli, without the need to initiate gene transcription, thereby permitting rapid changes.

There are predominantly three points at which translation of RNA transcripts may be regulated, which may be summarised thus: changes in (1) the rate of processing of nuclear RNA prior to export to the cytoplasm; (2) the stability of the mRNA transcripts; (3) the efficiency of translation.

There are many examples of alterations to nuclear processing rate affecting the rate of translation, including alkaline phosphatase (Kiledjian and Kadesch, 1991), prolactin (Billis et al., 1992) and adenosine A1 receptors (Ren and Stiles, 1994). However, while this may be an important regulatory step, whereby alternate splicing of exons can be controlled, and thereby mediate changes in the sequence of the mRNA ultimately exported, this project has focused on the study of cytoplasmic mRNA expression. One control mechanism in the cytoplasm is changes in RNA stability which occurs during, for example, synthesis of GH (Paek and Axel, 1987; Murphy et al., 1992), follicle-stimulating hormone (Attardi and Winters, 1993) and α1(I) collagen (Delany et al., 1995). Additionally, regulation of translational efficiency will occur in the cytoplasm,

The Effects of the 3'-UTR on Stability

Alterations in the rate of transcript turnover is known to be an important mechanism of post-transcriptional regulation. This would allow the rapid degradation, or prolonged presence, of transcripts within the cytoplasm, thereby altering the period of time for
which the transcripts may be translated. Importantly, the half-lives of transcripts may not only be regulated in response to exogenous factors, but may be cell-specific. For instance, a greater number of insulin receptors were found to be present in HepG2 liver cells than in fibroblasts (Reddy et al., 1988; Hatada et al., 1989), which was, at least in part, attributed to variations in the half-lives of the transcripts (Tewari et al., 1991).

The 3'-UTR has been heavily implicated in mediating regulation by affecting the stability of transcripts. For instance, proteins may bind and thus hinder RNases from degrading the RNA, thereby increasing the half-life of the mRNA on polysomes. There are three main features which are indicative of regulation by the 3'-UTR, which include (i) variability in the length of the 3'-UTR and poly(A) tail; (ii) the presence of AU-rich cis-acting elements which denote transcript instability and (iii) sequences to which trans-acting factors bind.

i) The half-life of some transcripts has been found to be dependent upon the particular polyadenylation signal used and the length of the poly(A) tail. For example, IGF-I alters its transcript-stability by varying the polyadenylation signal used and thus the length of the 3'-UTR (Lund, 1994). Variations in the length of the poly(A) tail mediate changes in the half-life of transcripts, and this has been demonstrated in the regulation of several genes by exogenous factors. For example, the half-life of rat thyrotropin β-subunit mRNA appears to decrease concomitantly with the thyroid hormone-induced shortening of the poly(A) tail (Krane et al., 1991). Conversely, the poly(A) tract of GH increases in length under stimulation by glucocorticoids and thyroid hormone, resulting in more stable transcripts (Paek and Axel, 1987; Murphy et al., 1992). This type of mechanism is thought to involve cis- or trans-acting factors interacting with the 3'-UTR, thereby
protecting RNase-sensitive sites or altering the conformation of the mRNA. One example of these is the poly(A)-binding protein (PABP), which will be discussed later.

ii) A sequence commonly linked with labile mRNAs is the AUUUA sequence, as seen in many cytokine mRNAs (Shaw and Kamen, 1986). Two hypotheses exist to explain their action; (a) these sites are preferentially targeted by proteins for rapid degradation (Malter, 1989; Brewer, 1991); (b) these sequences form secondary structures with the poly(A) tail which prevents the binding of PABPs and thereby decrease stability (Wilson and Treisman, 1988).

iii) Two good examples of transcript translatability/stability regulated by trans-acting factors binding the 3'-UTR are TSHβ and transferrin receptor transcripts. Regulation of TSHβ mRNA stability is known to involve a 12 base sequence within the 3'-UTR which interacts with an 80 - 85 kDa protein. The protein binding is in turn regulated by levels of thyroid hormone, T3 (Leedman et al., 1995). Similarly, transferrin receptor transcripts have been found to contain iron response elements (IRE) within the 3'-UTR. These are thought to mediate their action through recruitment of iron response factors (IRF), thereby preventing RNases from gaining access to the 3'-UTR, by steric hindrance (Casey et al., 1988). Angiotensinogen is another example of a transcript whose translation is regulated by protection of mRNA from enzymatic digestion. Angiotensin II is known to regulate angiotensinogen production by positive feedback post-transcriptionally. The regulatory system is dependent upon the presence of the 3'-UTR, which may interact with a 12 kDa protein (Klett et al., 1995). It appears that de novo protein synthesis is not required for regulatory mechanism, but rather that the 12 kDa protein is a subunit of a 45 kDa storage complex, which may become
phosphorylated in response to angiotensin II (Klett et al., 1995), thereby stabilising the transcripts within 30 minutes.

The Mechanism of Translation

In order to fully comprehend the mechanisms involved in translational regulation, the process of translation must be fully considered. Translation may be split into several discrete events as shown diagrammatically in Figure 1.8. Broadly these may be described as initiation, elongation and termination.

i) Initiation

All eukaryotic mRNA is modified during transcription to add a 5'-terminal G in reverse orientation, which may then act a substrate for methylations. This acts as the first feature to be recognised by some of the initiation factors required for the 40S ribosomal subunit to bind to the mRNA. One function of these proteins is to unwind the 5'-end of the mRNA and remove any secondary structure, so that the ribosome binding sites are in the single-stranded state for ease of access. The process of initiation involves at least nine proteins called eukaryotic initiation factors (eIFs). In more detail the process of initiation may be described thus: (1) GTP binds to eIF-2; (2) eIF-2 now has increased affinity for Met-tRNA; to which it binds; (3) the free 40S subunits associate with two elongation factors, eIF-3 and eIF-4C forming a 43S ribosomal complex. The ternary complex of Met-tRNA, eIF-2 and GTP binds directly to the 43S ribosomal complex which is now in a state capable of binding to the mRNA (4). However, firstly the mRNA must have any secondary structure melted at the 5'-end, to which the ribosome will bind. Thus (5) the eIF-4F multimeric factor, comprising eIF-4A, eIF-4E and p220,
Figure 1.8 - A summary of the eukaryotic translational mechanism. The numbers refer to specific stages in the translational mechanism and are described in detail in the text.
must bind to the 5' cap, along with eIF-4B, at which point (6) it may catalyse the unwinding of the first 15 bases of the mRNA, obtaining the energy to do so from the hydrolysis of ATP. The eIF-4F multimer is a complex which contains both the cap binding protein (CBP), which specifically recognises the 5' cap and the RNA helicase, eIF-4A, which assist in unwinding the mRNA and thus remove secondary structure. Further unwinding (7) is required prior to the 40S - Met-tRNA\textsubscript{i} - eIF-2 - GTP complex binding and involves the interaction of eIF-4A and eIF-4B. The 40S ternary complex may then bind the unwound mRNA (8), through association with eIF-3, along with eIF-4A and B, spanning up to 60 bases in length.

The scanning model of translation predicts that the 40S ribosomal subunit then migrates down the mRNA molecule in the 5'- to 3'-direction in search of an AUG initiation codon in an ATP-dependent manner (9). While migrating, the 40S subunit must melt any secondary structure in order to travel in a linear manner. It has been found that the 40S subunit may melt any structures of less than -30kcal, but will be impeded by any structures of greater stability. eIF-6 is found associated with the large 60S subunit and maintains the ribosome in its dissociated state. Upon association of the 60S subunit with the initiation complex, the eIF-6 factor is released. Simultaneously eIF-5 acts to release eIF-2, eIF-3 and eIF-4C from the initiation complex, after which the 40S and 60S subunits may join, for which eIF-4C is necessary. It seems probable that after the 80S ribosome formation, all the remaining factors are released. Once both subunits come together on the mRNA, the area covered contracts to 30-40 bases.
ii) Elongation

The complete ribosome contains two major compartments, the A site, or entry site, at which the codon coding for the next amino acid is exposed, prior to the entry of the aminoacyl-tRNA with the correct anticodon. Upstream to the A site lies the P site in which the nascent protein is attached to the tRNA complementary to the upstream codon, in the form of peptidyl-tRNA. The peptide is elongated by the addition of the peptide chain on the peptidyl-tRNA in the P site onto the aminoacyl-tRNA at the A site, a process catalysed by peptidyltransferase, an integral part of the large subunit. The new peptidyl-tRNA now present at the A site is translocated to the P site, while the ribosome moves one codon downstream.

The elongation of the peptide chain involves at least three complexes, called elongation factors (eEFs). The recruitment of aminoacyl-tRNAs to the A site of ribosomes is dependent on both the presence of a peptidyl-tRNA in the P site of the ribosome and the hydrolysis of GTP. A binary complex of eEF-1α - GTP binds to an aminoacyl-tRNA, which may then enter the A site. The GTP is hydrolysed to GDP at which point the eEF-1α - GDP complex is released from the ribosome. This inactive complex is recycled using the aid of eEF-1βγ, exchanging the GDP for GTP. The process involved with the translocation of the ribosome involved the protein eEF-2 which acts as a translocase, dependent upon GTP hydrolysis.

iii) Termination

There are no tRNAs complementary to the stop codons. Instead a release factor, eRF, recognise the termination codons and binds to the ribosome in a GTP-dependent manner. The process of termination involves the hydrolysis of the peptide from the last
tRNA, release of the tRNA and the dissociation of the ribosomal subunits from the mRNA. At some point, it is known that the GTP on the eRF is hydrolysed to GDP, but the exact function of eRF is not clear as yet. However, it seems likely that eRF is implicated in diverting the usual transfer of the peptide from the peptidyl-tRNA to aminoacyl-tRNA, and may also alter the conformation of the ribosome and thus aid dissociation from the mRNA. It is possible, though, that other proteins are involved.

Both initiation and elongation have been demonstrated to act as regulatory points in some systems. For example, the translation of the transferrin transcripts has been well characterised and is known to be regulated at initiation (Melefors and Hentze, 1993). Another example involves global control of protein synthesis by insulin (see below) in which ribosomal association to transcripts in adipocytes is affected by insulin-stimulation (Pause et al., 1994). Similarly, mRNA-ribosomal interactions are stimulated in germ cells during co-ordinate activation of dormant mRNA (Bachvarova, 1992). Meanwhile, an increase in the rate of elongation is implicated in the increased translational yield of insulin (Welsh et al., 1986), ornithine aminotransferase (Muekler et al., 1983), β-glucuronidase (Bracey and Paigen, 1987) and gonadotropin-releasing hormone receptors (Tsutsumi et al., 1995).

**Global Regulation of Translation**

On the whole, alterations to the rate of total protein synthesis are mediated through modifications of general transcription factors. For example, eIF-2α is phosphorylated in response to heat shock (Hershey, 1989) and following serum starvation the phosphorylation of eIF-4E is reduced (Kaspar et al., 1990). As a corollary to this, over-
expression of eIF-4E or F, which may allow translation of transcripts which are
normally poorly expressed, produced a malignant phenotype (Zimmer et al., 1994).

Other control mechanisms have also been implicated in insulin-dependent translation in
adipocytes, which appear to involve alternate phosphorylation states of a regulatory
protein. This protein was found bound to eIF-4E, in its unphosphorylated state, thereby
inhibiting initiation. However, when stimulated by insulin, the protein was
phosphorylated and dissociated from the initiation factor, thereby allowing translation
to occur (Pause et al., 1994).

Another example of global control of translation has been observed in oocytes. The
transcriptional apparatus is inactive in these cells, and the maternal mRNAs are dormant
and are untranslatable (Wickens, 1990; Richter, 1991). It is thought that this is achieved
through the binding of ‘masking proteins’ to the 3'-UTR which may cause a
conformational change in the mRNA, so that the 5'-cap is no longer accessible to the
translational machinery, thereby inhibiting initiation of translation. However, once these
proteins dissociate, translation may proceed normally. Examples of these proteins have
been found in both Xenopus oocytes (Y-box proteins, Wolffe, 1994) and in mammalian
germ cells associated with protamine-2 mRNA (Kwon and Hecht, 1991).

**Gene-specific Regulation of Translation**

Sequences within the 5'-UTR, and to a lesser extent the 3'-UTR, of transcripts of many
genes are known to affect the efficiency of translation, or translatability. In contrast, the
stability of the transcripts has been well characterised as being dependent upon the 3'-
UTR. It is, therefore, interesting to note that IGF-I transcripts are known to be
heterogeneous and differ in both their 3'- and 5'-UTRs. However, a large number of
genes are now characterised as being regulated through their UTRs, often involving
RNA-protein interactions.

i) The 5'-untranslated Region

The role of the 5'-UTR in regulating translation has been characterised in many genes
and has been found to differ greatly. For instance, initiation of translation may be
regulated, as has been characterised for ferritin mRNA (Rouault et al., 1988). Briefly,
regulatory proteins bind to the 5'-UTR and thereby inhibit translation initiation. In
contrast, translational control of insulin synthesis involves the modulation of the rates of
initiation and elongation (Welsh et al., 1986). The glucose-induced increase in insulin
synthesis was found to be due to both the dissociation of a translationally-inhibitory
protein, and through increased rates of initiation and elongation, as seen by the
formation of polysomes. Similarly, increased translational efficiency, in terms of
enhanced initiation and elongation, has been implicated in the stimulation of ornithine
aminotransferase synthesis in liver (Muekler et al., 1983).

Translation is thought to be initiated according to the ‘scanning model’ as proposed by
Kozak (1991). As already described, this hypothesis proposes that ribosomes scan
along the mRNA until an AUG initiation codon is reached. However, the start codon is
often not in the optimal context for the ribosome to recognise ("^GCCA/GCCAUGG/A^4"). The RNA, though, may adopt a secondary structure such as
a stem loop, downstream of the start codon thereby slowing the scanning, since the
ribosome will need to ‘melt’ the structure in order to progress further. Furthermore,
proteins may bind to this secondary structure and thereby increase its stability. This
allows a longer temporal interaction with the suboptimal start codon and increases the probability of initiation from this site. This may be particularly important in genes which may be translated from several different start sites.

Conversely, instead of aiding initiation, the presence of stem-loop or hairpin-loop structures (which may be stabilised by proteins) upstream of the initiation codons may prevent the ribosome from scanning along the mRNA and thereby decrease the translatability of the transcripts. It is this latter mechanism which has been implicated in the iron-dependent regulation of translation of ferritin, and this is described in detail in Chapter 4. Interestingly, thymidylate synthase production is also regulated by a protein binding within the 5'-UTR, but this protein is the enzyme itself. This is therefore an example of autoregulation of gene expression mediated through the translation product inhibiting further protein synthesis (Johnson, 1994).

One could also envisage that the nature of the 5'-UTR may play a critical role especially in the regulation of genes which encode different transcripts varying in their 5'-UTR. For example in multiple transcripts, translation could be diverted from a poorly translated transcript to a more translatable one, without the need for de novo transcription. To this end, it is interesting to note that different IGF-I mRNA transcripts have been observed to be differentially regulated (Yang et al., 1995).

\textit{ii) Autoregulation}

PABPs are an interesting example of transcript-stability regulation at the 3'-end and translational auto-regulation at the 5'-end. PABP bind to poly(A) tails and it is known that translation \textit{in vivo} is inhibited when there is an over abundance of binding proteins,
in comparison to poly(A) tails. As expected, therefore, during periods of growth the protein is produced in large amounts, whereas translation is repressed during, for example, the terminal stages of erythropoiesis (Standart and Jackson, 1994). PABP mRNA transcripts, however, contain an A-rich motif within the 5'-UTR, and it is thought that translation is controlled through auto-regulation. In this manner, PABPs would usually bind poly(A) tails with high affinity, but during over-production, they would bind to the 5'-UTR of their message. While this binding may be of lower affinity than for the poly(A) tails, it would still be great enough to prevent the 48S ribosomal subunit from dislodging it, even though it is over 70 bases from the cap (Standart and Jackson, 1994). This is especially interesting since it appears to disagree with the displacement theory put forward to explain the IRE/IRF-interaction (see Chapter 4). It may, therefore, be that inhibition may still occur if the interaction between the binding protein and motif are strong enough.

iii) The Effect of the 3'-UTR on Post-transcriptional Regulation

The 3'-UTR may also influence translational efficiency, as seen with the amyloid precursor protein. The gene codes for two types of transcripts, varying only in the length of the 3'-UTR, with the longer one having enhanced translatability (de Sauvage et al., 1992). In contrast, the human β-interferon mRNA has sequences within the 3'-UTR which reduce translational efficiency. Surprisingly the regulatory sequences, UUAUUUUAU, are those more usually associated with determining stability, however the variations in translatability were not due to changes in stability (Kruys et al., 1987).

Tumour necrosis factor (TNF) mRNA and other cytokines also appear to contain similar motifs. TNF is known to be translated by macrophages in response to
lipopolysaccharide (LPS), but is usually repressed. Removal of UA-rich motifs from the 3′-UTR abolished this inhibition, which was thought to be mediated through proteins interacting with these motifs. A 38 kDa protein which binds specifically to the UA-rich sequences in TNF mRNA has been identified in macrophages and furthermore, it has been shown that LPS reduces binding of the protein to the motif, which suggests that this protein is a translational repressor (Kruys and Huez, 1994).

PTH synthesis also appears to be post-transcriptionally regulated through the 3′-UTR (Hawa et al., 1993). For instance, low calcium induces an increase in PTH production at the translational level, while apparently altering the half-life of the mRNA associated with ribosomes. It therefore seems possible that following association of the mRNA with polysomes, thereby increasing translatability, the half-life of this mRNA alters. The exact method by which this is achieved is unclear, but seems to be mediated by two proteins which interact with the 3′-UTR (Vadher et al., 1996).

The examples described above only alter the rate of translation in a subtle way. In contrast, the regulation of rabbit erythroid 15-lipoxygenase involves a more noticeable effect, similar to the ‘dormancy’ observed with oocyte transcripts. This mRNA is produced in red blood cells during their development and maturation in the marrow but the transcripts are maintained in an inactive form until the cells reach the peripheral circulation, at which point translation occurs. The translational block has been found to involve a 48 kDa protein binding to a pyrimidine-rich 19 nucleotide repeat sequence in the 3′-UTR (Ostareck-Lederer et al., 1994). The manner in which the translational block is relieved is unclear but may involve either the degradation or phosphorylation/dephosphorylation of the repressor protein.
However in general, the mechanisms by which RNA-protein interactions within the 3'-UTR affect the translation at the 5'-end are poorly understood. Several hypotheses have been proposed including that proteins may bind to the 3'-UTR and alter the secondary structure so that either the ribosomes are hindered in their access to the cap, thereby decreasing the efficiency of translation, or conversely place the termination codon in juxtaposition to the cap, and thus increase the rate of re-initiation.
Part 4 - Aims of the Project

In this chapter, it has become clear that IGF-I is important in bone growth and development and mediates many of its actions through synthesis in osteoblasts. However, the mechanism of action of the osteoblasts, in terms of bone growth and responsiveness to hormones and growth factors, is site- and age-specific. Many of the hormonal responses are mediated through variations in IGF-I expression, in particular regulating its autocrine and paracrine synthesis by osteoblasts. IGF-I expression, though, may be regulated at both the transcriptional and the post-transcriptional levels.

The aim of this project was to study the transcript expression of IGF-I in osteoblasts * from the femurs of 28-day old female Sprague Dawley rats, with respect to heterogeneous start site and alternate leader exon usage. Subsequently I wished to determine whether any cytosolic proteins interacted with different 5′-UTRs and therefore may be regulatory. Therefore in the first instance (as described in Chapter 2), the manner in which primary osteoblast cultures was optimised and the cultures themselves validated for the presence of cells with osteoblastic characteristics. In Chapter 3, I have described the various methods by which transcript expression was studied, comparing the pattern of expression to liver (a well characterised control) and whole bone. Finally, in Chapter 4, protein-RNA interactions were examined in order to determine whether any variations in binding patterns were obtained using transcripts with different 5′-UTRs.

* - the term "osteoblast" is used to cover all cells of the osteoblast lineage.
Chapter 2

Preparation of Primary Osteoblast Cultures
Introduction

Typically, osteoblasts are regarded as non-replicating, fully-differentiated cells which are highly metabolically active. As previously described, they contain a large rough endoplasmic reticulum which is polarised towards the bone surface (Figure 1.3a in Chapter 1) and they secrete large amounts of type I collagen. They also synthesise alkaline phosphatase, whose function is still unclear, and produce other secreted proteins including osteocalcin, osteopontin and bone sialoprotein (Stein and Lian, 1993; Aubin et al., 1995).

The Use of Osteoblasts in Culture

In order to study the direct effect of hormones on bone, functionally as well as morphologically in vitro, bone organ cultures have been used since at least 1965 (Raisz, 1965; Stracke, 1984). However, in order to examine the manner in which the hormonal response is modulated at the cellular level, cultures of bone cells, especially osteoblasts, are required. To this end, primary cultures of osteoblasts have been used in a wide variety of experiments, mostly involving the study of hormonal effects, including androgens and dihydroxyvitamin D₃ (Gray et al., 1992), growth hormone (Kassem et al., 1993), transforming growth factor β (TGF-β) and epidermal growth factor (EGF) (Antosz et al., 1987; Antosz et al., 1989), glucocorticoids (Bellows et al., 1987), prostaglandin E₂ (Pash et al., 1995) and calcitonin (Luben et al., 1976).
However, a common difficulty with primary cultures was identifying both the cells present, and their maturity since collagenase digestion of bone is likely to result in the release of many different cell types. Therefore, a great many studies have also been undertaken to characterise the cells released from collagenase digests. Early studies in the 1970s relied on the knowledge that bone cells were responsive to parathyroid hormone (PTH), which was indicated by an increase in cAMP, for the characterisation of osteoblasts in culture (Wong and Cohn, 1974; Luben et al., 1976). However, this response was seen to diminish with time, although Rao et al. (1976) succeeded in obtaining a culture which still responded to PTH after repeated subculture and storage at -80°C. Despite this, researchers were unable to form mineralised bone nodules in vitro without devising techniques, such as the inoculation of cells intramuscularly (Groot et al., 1983; Moskalenski et al., 1983) which made controlled investigations of the process of bone formation very difficult.

In 1986, Bellows et al. (1986a) succeeded in inducing bone nodules in culture from foetal rat calvaria, with the addition of ascorbic acid, and these nodules mineralised upon addition of organic phosphate in the form of β-glycerophosphate (Bellows et al., 1987; Bhargava et al., 1988). They also reported a significant increase in the number of nodules formed upon stimulation with dexamethasone, and in 1988 ultrastructural analysis of the bone nodules by Bhargava et al., confirmed that they resembled true bone. While this now allowed the study of the process of mineralisation, several papers were published which suggested that the cells isolated by collagenase digests were not only of the osteoblast phenotype, but that there were also several other populations of cells (Bellows et al., 1986b) derived from mesenchymal origin (Grigoriadis, 1988). While the heterogeneity of the collagenase-obtained cultures had been widely observed
(Peck et al., 1964; Wong and Cohn, 1974; Luben et al., 1976; Aubin et al., 1982), both of these studies were performed on clonally-derived cell populations isolated from foetal calvaria using limited dilution cloning. This involves the seeding of between 1-5 cells in a multiwell plate and culturing them for 10 days, after which those wells containing a single colony are harvested and grown as distinct clonal cultures, all having originated from only one cell. This should therefore result in a culture of cells with the same phenotype. However, long-term cell culture of clones (Bellows et al., 1986b) which produced almost exclusively type I collagen (indicative of osteoblasts - Marie et al., 1989), resulted in the down-regulation of this phenotype with a concomitant increase in type III collagen synthesis, normally produced by fibroblasts. Grigoriadis et al. (1988) performed experiments using a PTH- and 1,25-dihydroxyvitamin D$_3$-responsive clone which also had other osteoblast-associated characteristics, such as a polygonal morphology. Upon culturing in the presence of ascorbate, β-glycerophosphate and dexamethasone however, four distinct cell types developed, all of mesenchymal origin, including osteoblasts, adipocytes, chondrocytes and myotubes. While the osteoblast and myotube phenotypes could be generated spontaneously in the absence of dexamethasone, both the chondrocytes and adipocytes were dependent upon the presence of the glucocorticoid. This, and other similar observations, have demonstrated the need for a greater understanding of osteoblast development, and within the last 10 years a great many papers have focused upon this area.
Culturing Osteoblasts

Over the years, three main procedures have been consistently used to culture osteoblasts *in vitro*, including: i) immortalised cell lines; ii) explants from bones; iii) sequential trypsin and collagenase digests.

*i) Immortalised Cell Lines*

Many osteoblast cell lines exist and are commonly used. Examples of these include the rat lines ROS 17/2.8 (Stein and Lian, 1993), UMR-106 (Lakatos *et al.*, 1993), RTC (Ernst *et al.*, 1989) and the human osteosarcoma cell lines SaOS-2 and MG-63 (Ström *et al.*, 1994). The cell lines were produced either from the transformation of normal osteoblasts or from culture of tumour cells. Since all the cells are identical, the consistency of the culture is maintained, but there are several drawbacks. For instance, often the characteristics of the cells of a cell line differ from those found *in vivo*. Such a divergence in behaviour from that expected by osteosarcoma cells, is exemplified by the expression of osteocalcin and osteopontin by ROS 17/2.8. These proteins are expressed at high levels by osteoblasts at the onset of extracellular matrix mineralisation, whereas ROS 17/2.8 cells express these proteins irrespective of the extracellular matrix (Stein and Lian, 1993). In this manner, drawing comparisons with *in vivo* studies is made more complicated.

*ii) Explants From Bone*

While Wong and Cohn performed an explant in 1974, the practice had been prevalent since at least the 1960s. The principle behind this procedure is that osteoblastic cells are known to migrate from bone explants in tissue culture (Ecarot-Charrier *et al.*, 1983;
Lomri et al., 1988). The cultures obtained are highly enriched for osteoblasts and exhibit many osteoblastic characteristics, such as responsiveness to PTH and 1,25 dihydroxyvitamin D₃. They have also been demonstrated to synthesise and mineralise type I collagen in vitro (Ecarot-Charrier et al., 1983; Lomri et al., 1988). This procedure is still widely used (Marie et al., 1989; Wong et al., 1990; Ström et al., 1994) and is useful when only small amounts of bone are available. However, the length of time needed to obtain cells, and the low number of cells eventually released are both major drawbacks.

**iii) Sequential Collagenase Digests**

This was first developed as a method of culturing bone cells by Peck et al. in 1964, digesting rat foetal calvarial bone fragments for varying lengths of time with collagenase. While the cells released appeared to stain strongly for the presence of alkaline phosphatase shortly after isolation, indicating the presence of cells of the osteoblast lineage, prolonged culturing and subculturing resulted in overgrowth by fibroblasts. Wong and Cohn in 1974 successfully alleviated the problem of fibroblasts out-growing the other cells present. Briefly, this involved digesting fragmented mouse calvaria for 20 minutes in a trypsin/collagenase solution sequentially to yield 3 populations of cells (that is, those cells released after 20, 40 and 60 minutes of digestion) which were then grown in culture along with the remaining bone fragments. The first population of cells were fast-growing, spindle-shaped cells and resembled fibroblasts, whereas fraction 3 and those grown out from the bone fragments were relatively large cells with highly convoluted dendritic borders. The second population contained cells with both these morphologies, but the spindle-shaped cells out-grew the larger cells. Wong and Cohn also demonstrated that both PTH and calcitonin could
elicit a larger increase in cytosolic cyclic AMP (cAMP) from cells of population 3 than
the other fractions. Furthermore, this increase was additive.

In 1975, the methodology was modified to increase the number of sequential digests
and the concentration of collagenase used (Wong and Cohn, 1975). Populations of
parathyroid- and calcitonin-sensitive cells could be differentiated in the primary cultures
obtained, indicating that the additive increase observed previously was mediated by
different cell types. Luben et al. (1976) identified those cells initially released, which
were responsive to calcitonin, as osteoclasts, since they also displayed several
biochemical markers indicative of osteoclastic cells, such as acid phosphatase activity.
Those cells released after further digestion, which responded to PTH but not calcitonin,
had both alkaline phosphatase activity and the ability to synthesise collagen, from which
this fraction was inferred to contain cells of the osteoblast lineage. The methodology
was further refined by Rao et al. in 1977, with the addition of DNase, amongst other
substances, to the collagenase digest mix.

By far the majority of osteoblast-enriched cultures used have been obtained by
sequential collagenase digestion, particularly from foetal rat calvaria. For while the
extraction procedure is more complex than that of explants, a great many cells of the
osteoblast lineage may be obtained rapidly and in a reproducible manner. Examples of
the use of these cultures include studies on androgen- and 1,25 dihydroxyvitamin D₃-
activity (Gray et al., 1992), biochemical characterisation of collagen synthesis
(McCarthy et al., 1988), prostaglandin E₂-mediated transcriptional regulation of IGF-I
(Pash et al., 1995) and growth hormone-stimulation (Kassem et al., 1993).
The general aim of the project was to investigate the synthesis of IGF-I transcripts by skeletal tissue, and in particular, osteoblasts from long bone. Calvarial bone has been extensively studied, but it is a very specific type of bone, membranous bone. In contrast to membranous bone, long bones such as the femurs undergo a large amount of longitudinal growth. Furthermore, it is known that trabecular bone, which is a major constituent of long bone, is the type primarily affected during postmenopausal osteoporosis. Additionally, data from Ernst et al. (1989) suggests that oestrogen suppresses PTH-stimulated adenylate cyclase activity to a greater extent using primary osteoblasts from trabecular bone than from calvaria.

We chose to obtain primary cultures of osteoblasts from 28-day old rats, when more of the receptor-effector systems are expressed than in the foetus. For instance, the levels of IGF-I protein and IGF-I receptor mRNA in rat bone are known to decrease markedly during foetal development and rise during postnatal growth (Bickle et al., 1994). Furthermore, growth hormone has been demonstrated not to mediate an increase in IGF-I during foetal growth, whereas postnataally, growth hormone up-regulates IGF-I synthesis (Baker et al., 1993). Therefore, the aim of the work in this chapter was to optimise the extraction procedure to obtain primary rat osteoblast cultures from the long bones of 28-day old rats and confirm the synthesis of IGF-I in vitro.
Methods and Results

In order to study primary osteoblasts from 28-day old rats in vitro, the cells were extracted from femurs by collagenase digestion. However, the majority of experiments undertaken to date, studying osteoblasts, were performed using cells obtained from foetal calvaria and therefore the extraction procedure required optimising. Many of the general procedures and solutions used are described in Appendices I and II, but the steps requiring optimisation are described in detail in this chapter.

Optimisation of the Extraction Protocol

i) Basic Protocol

The original protocol was obtained from T. Arnett (Pers. Comm.). Briefly, 40 femurs were obtained from 28-day old female Sprague Dawley rats in a sterile manner and transported in buffered medium (for details refer to the Final Protocol, page 108). The bones were cleaned, cut finely and washed twice in calcium- and magnesium-free phosphate-buffered saline (PBS). Bone chips were transferred into 2 culture flasks (75cm² growth area, 250cm³ total volume) and were incubated in 25ml trypsin for 30 minutes at 37°C and shaken vigorously. The supernatant was discarded and the bone chips were digested sequentially in 30ml crude collagenase (Type IA, Sigma) in PBS for 30 minutes at 37°C. The cells released were collected as separate fractions and either frozen in aliquots (Appendix I) or grown in nystatin-containing medium for 2 days before trypsinising the cells and culturing them in standard medium (refer to Final Protocol).
Bone cells were extracted by sequential collagenase digestion of long bone from 28-day old female Sprague Dawley rats. Using this basic protocol, cell counting directly after digestion was not possible due to the large amount of debris present. A very viscous layer was also obtained, which was hard to disaggregate without the addition of DNase.

In the first instance, each fraction was cultured individually and the following a day it was readily apparent that few viable adherent cells had been released. Therefore, in order to ascertain the number of viable cells released by collagenase digestion, each fraction was grown separately in identical conditions, trypsinised after two days and counted. From the figures obtained it was apparent that digestions 3 and 4 yielded many more cells than were isolated from the first two digestions (Table 2.1). This raised the possibility that more cells might be released using optimal digestion conditions.

<table>
<thead>
<tr>
<th>Collagenase Digest</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Number of Cells/Flask</td>
<td>750</td>
<td>9,300</td>
<td>25,000</td>
<td>22,000</td>
</tr>
</tbody>
</table>

*Table 2.1 - An example of cell counts/flask after 2 days growth using cells extracted following the initial protocol. An aliquot of each digestion fraction was seeded in an identical manner and counted after two days in culture.*

Additionally, when the cells were grown in culture, they were observed to differ greatly in morphology irrespective of the fraction used. Cell types present included small spindle- and polygonal-shaped cells, although the majority were large and dendritic in
shape (Plates 2.1 - 2.11). This indicated that the primary cell cultures obtained comprised a heterogeneous population of cells.

**ii) Optimisation of Digestion Conditions**

In order to increase the yield of osteoblast-like cells, various components of the methodology were optimised including the use of nystatin, the addition of calcium and magnesium to the collagenase digestion mix, the collagenase type used and the ratio of bone : collagenase.

**a) Nystatin** - This was added to the transport medium and in the initial stages of growth. However, the length of time it was present in the medium was minimised and the manner of its removal optimised. This was found to be 2% nystatin for two days, after which the precipitated nystatin was removed by swirling PBS rapidly over the cells.

**b) PBS** - The PBS used during the collagenase digestion was changed from calcium- and magnesium-free to PBS containing calcium and magnesium since collagenase requires chelating ions for optimal function (Appendix II for details). This alteration had a large visible effect, including an increased total number of cells released in each fraction after digestion. However, when the cells were stained with trypan blue in order to determine the viability of the cells, many of the cells were stained, indicating that cell lysis was occurring during digestion. This was borne out by the observations that the number of viable cells after several days in culture was unaffected. These results are summarised qualitatively in the table below (Table 2.2).
### Table 2.2 - A qualitative comparison of the effects on cell number and debris produced, using PBS with or without calcium and magnesium during the collagenase digest. The viable cells were those seen to be adherent after 24 hours.

<table>
<thead>
<tr>
<th>Type of PBS</th>
<th>Cells Released</th>
<th>Debris Visible</th>
<th>Viable Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS without Ca(^{2+}) and Mg(^{2+})</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>PBS with Ca(^{2+}) and Mg(^{2+})</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

Therefore, the alterations in PBS used appeared to enhance the digestion of the bone chips, but did not increase the number of viable cells due to cell damage and lysis during digestion.

c) **Collagenase** - The crude collagenase type was changed from type IA to type II (Sigma) under recommendation from T. Arnett (pers. comm.), since different collagenase types have been observed to work optimally on different tissues. However, this alteration had a very limited effect on the number of viable cells released and the fraction from which they were obtained remained unchanged.

d) **Digestion Duration and Shaking** - The number of cells released from the bones using the basic protocol appeared to be greatest from fractions 3 and 4, as described previously. Therefore, since this may indicate that more cells could be released in prolonged digestion, the incubation time in collagenase was increased to three sequential digestions of an hour each. Simultaneously, the vigour with which the bone
chips were shaken throughout the incubation was much decreased to a gentle rocking (~10 shakes/second). Both of these alterations were made in conjunction with the use of PBS with calcium and magnesium and collagenase type II.

The cumulative effects of both these changes were very significant. The alterations resulted in a marked decrease in the amount of particulate matter and viscous material, which was thought to be DNA released from lysed cells. The decrease in the amount of viscous material in the fractions made the addition of DNase to release cells within the aggregate unnecessary. However, the total number of cells released appeared to be undiminished but of these, a much greater proportion were viable. Therefore, a long gentle incubation released many more viable cells, indicating that the cell lysis may have been due to the rapidity with which the chips were shaken. The results are summarised qualitatively in Table 2.3.

<table>
<thead>
<tr>
<th>Digestion Time / Shaking</th>
<th>Cells Released</th>
<th>Debris Visible</th>
<th>Viable Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS without Ca(^{2+}) and Mg(^{2+})</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Short/Vigorous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS with Ca(^{2+}) and Mg(^{2+})</td>
<td>++++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Short/Vigorous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS with Ca(^{2+}) and Mg(^{2+})</td>
<td>++++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Long/Gentle</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 2.3 - A qualitative comparison between short digestion/vigorous shaking (using PBS with or without calcium/magnesium) and long digestion/gentle shaking.*
e) The Ratio of Bone : Collagenase - Since the digestion conditions were much more effective at releasing viable cells, the number of femurs was reduced in order to ascertain whether the bone chips were present in excess for the amount of collagenase used. Furthermore, the incubation was performed in one flask as opposed to two. Overall, the digestion of 20 femurs in one 75cm² flask in 60ml of 0.2% collagenase resulted in the same yield of cells as were released from 40 femurs in two flasks using 30ml of collagenase in each. The final concentration of collagenase was approximately 0.2g bone/ml collagenase mix (0.2%).

iii) Final Protocol

Following the optimisation of various parameters, the following protocol was used to obtain primary osteoblast cultures from 28-day old rat femurs. 20 excised femurs were transported at 4°C in Waymouth medium containing HEPES (20mM), sodium bicarbonate (0.06%), penicillin/streptomycin (100U/ml), amphotericin (0.25µg/ml) and nystatin (100U/ml). The bones were cleaned, cut up through the marrow to help release bone marrow cells, finely chopped and shaken vigorously in calcium- and magnesium-free phosphate buffered saline (PBS). The supernatant was discarded and the bones were washed again to reduce contamination from bone marrow cells. The bone chips were transferred into one culture dish (75cm² growth area, 250cm³ total volume) and were incubated in 50ml trypsin/EDTA in calcium- and magnesium-free PBS at 37°C for 30 minutes and shaken gently. The supernatant was discarded and the tissue incubated in crude collagenase Type II (0.2% w/v - Sigma) in PBS containing calcium and magnesium for 30 minutes at 37°C with continual gentle shaking*. The supernatant was discarded and a further 3 collagenase digests were performed for 1 hour each, keeping the fractions separate. The fractions were centrifuged and the pellets

* - refer to Addendum
were washed in calcium- and magnesium-free PBS. Each fraction was split into 5, 4 of which were resuspended in storage buffer (Appendix II) and frozen. The remaining aliquot was grown in phenol red-free Dulbecco’s Modified Eagle Medium (DMEM) containing sodium pyruvate and glucose (1000mg/ml), heat-inactivated foetal calf serum (10%), penicillin/streptomycin (100U/ml), amphotericin (0.25μg/ml) and nystatin (100U/ml) for 24 hours at 37°C with 5% CO₂. The medium was replaced with fresh nystatin-containing medium and the cells were allowed to grow for a further 24 hours after which the cells were washed in PBS for 20 minutes. This was discarded and replaced with medium comprising FCS (10%), penicillin / streptomycin (100U/ml) and amphotericin (0.25μg/ml) in DMEM. The cells were grown to confluence and tested for alkaline phosphatase activity. The cells were trypsinised (see above) and counted (Appendix I), after which the cells were pooled and grown in nystatin-free medium at a seeding density of ~2400 cells/cm².
Identification of Cells of the Osteoblast Lineage

The cells obtained from collagenase digestion of bone chips had many different morphologies, suggesting that the primary cultures were comprised of a heterogeneous population of cells. Therefore, the presence of cells of the osteoblast lineage amongst those released by collagenase digestion needed to be validated.

i) Alkaline Phosphatase Staining

Alkaline phosphatase is an easily recognisable marker for cells of the osteoblast lineage, as described previously. The cells were obtained as described in the final protocol and grown to confluence, after which they were stained for alkaline phosphatase (Appendix I). This protocol resulted in the production of an insoluble blue dye in the presence of alkaline phosphatase, and the staining displayed the presence of a heterogeneous population of cells (Plates 2.1 - 2.4). As can be seen clearly, the intensity with which the cells are stained varies. Approximately 40% of the cells present stained positively for alkaline phosphatase, although some cells stained a darker blue than others as clearly seen in Plates 2.2 and 2.3. Interestingly, in both these photographs, the morphology of the cells staining for alkaline phosphatase can be seen to vary from a fibroblastic spindle-shape to a larger, less stellate, morphology. Furthermore, in Plate 2.3 some multi-layering of cells is visible, which is an indication of osteoblastic phenotype and is known to occur during bone nodule formation.

In addition to these short-term cultures, cells were grown for longer periods in order to permit the formation of mineralising bone nodules. In this case the medium contained ascorbic acid (50μg/ml) and β-glycerophosphate (10mM). Dexamethasone (10⁻⁹M) was
Plate 2.1 - A photograph of a population of cells obtained by collagen digestion of rat long bone stained for alkaline phosphatase. The synthesis of alkaline phosphatase is a good marker for osteoblastic phenotype, and may be clearly seen as an insoluble blue colouration in those cells in which it is present. This photograph shows the heterogeneity of the cell-types obtained by collagenase digestion of bone.

Plate 2.2 - A primary culture enriched for osteoblasts stained for alkaline phosphatase. This photograph was taken under higher magnification than Plate 2.1. It is more evident that the morphology of the cells staining for alkaline phosphatase varies from slender and spindle-shaped to large and less stellate. Again, it is clear from this view that the culture comprises a heterogeneous population of cells.
Plate 2.3 - A photograph of alkaline phosphatase-stained cells enriched for osteoblasts viewed under higher magnification. The cells clearly have varying morphologies and stain for alkaline phosphatase with different intensity. Some multi-layering of cells is also apparent.

Plate 2.4 - Mineralising cultures enriched for osteoblasts stained for alkaline phosphatase. Under the microscopy conditions used, the alkaline phosphatase stain appears to have a more purple hue. The areas which appear to be black are sites of mineral deposition, thus preventing light from penetrating.
Plate 2.5 - Kidney fibroblasts stained for the presence of alkaline phosphatase. These cells were grown and stained in parallel to the primary cultures of osteoblasts. In this manner they were used as a negative control for alkaline phosphatase activity and no staining was apparent.
also added to some cultures in order to determine its effects upon bone nodule formation. Plate 2.4 shows a typical example of such a culture stained for alkaline phosphatase and demonstrates that the nodules and surrounding cells stain intensely. Indeed, due to mineral deposition, the mineralised nodules themselves appear to be almost black in colour.

In contrast to the primary osteoblast cultures, skin fibroblast cells, which were used as negative controls, were unstained when tested for alkaline phosphatase (Plate 2.5). This demonstrated that the stain was specific to a population of cells and did not stain all cells ubiquitously. Therefore, overall, a large proportion of those cells obtained by collagenase digestion of 28-day old rat femurs were of the osteoblastic lineage.

**ii) Tartrate-Resistant Acid Phosphatase (TRAP) Staining**

Tartrate-resistant acid phosphatase (TRAP) is an enzyme found only in osteoclasts and may thus be used as a specific marker. TRAP staining was performed in order to verify the absence of any osteoclasts in the primary cultures enriched for cells of the osteoblast lineage. The TRAP staining protocol (Appendix I), results in the production of a vivid pink, insoluble compound within the osteoclasts, while those cells lacking tartrate-resistant acid phosphatase remain unstained. A micrograph of a typical long-term primary cell culture stained for TRAP is shown in Plate 2.6. When compared to a positive control of osteoclast culture (Plate 2.7 - kindly donated by M. Horton), it may be easily determined that no osteoclasts are contaminating the primary osteoblast culture.
Plate 2.6 - A primary osteoblast culture stained for tartrate-resistant acid phosphatase (TRAP). TRAP-staining is used as an indicator of osteoclasts in culture, and are normally clearly visible by a red/pink pigment deposit. No such colouration was visible in any primary culture enriched for osteoblasts.

Plate 2.7 - Rabbit osteoclasts stained for the presence of TRAP. This photograph gives a good example of the intensity of stain colouration expected with osteoclasts.
iii) von Kossa Staining

The von Kossa staining protocol is used to detect areas of calcium deposition, and is therefore used to visualise nodules at which mineralisation is occurring. The stain produces a dense black colouration at these sites, as may be seen in Plates 2.8 and 2.9. Cells were grown in long-term culture in the presence of dexamethasone and after approximately 30 days bone nodules could easily be distinguished by eye. Under low-power magnification (Plate 2.8), the mineralising bone nodules are clearly visualised. It is interesting to note that when studied under higher magnification (Plate 2.9), the individual cells between the nodules may be distinguished. However, those cells bordering the nodules are not as clearly defined. This is probably due to the multi-layering of the cells of the osteoblast lineage which occurs during bone-nodule formation. In contrast, when long-term culture was performed in the absence of dexamethasone, no bone nodules were apparent. Skin fibroblasts were grown in parallel as negative controls, both in the presence and absence of dexamethasone, in long-term culture. The cells became detached around the edges of the flasks and senesced from approximately day 20. Therefore, by day 30 - 40, when the primary osteoblast cultures were stained, almost no viable cells were present and no nodules could be seen. Therefore, the cultures of cells enriched for osteoblasts may form nodules and deposit calcium.

iv) Alizarin Red S Staining

Another characteristic marker for mineralised bone nodules is the presence of phosphate deposits. The alizarin red S protocol stains inorganic phosphate deposits crimson, allowing the easy identification of sites of mineralisation and therefore, bone nodules. Examples of long-term cultures grown in the presence of dexamethasone for 30 to 40
Plate 2.8 - A photograph of a mineralising osteoblast-enriched primary culture following von Kossa staining. The von Kossa stain is used to detect the presence of calcium deposits in culture and is visualised as a dense black colouration. Therefore, mineralising bone nodules, such as those seen here, are clearly visible.

Plate 2.9 - von Kossa-stained primary osteoblast cultures. This photograph was taken from the same field of view as Plate 2.8 under higher magnification, so that the cells may be distinguished. Between the bone nodules (stained black), the cells are attached to the culture plastic in a monolayer and their morphologies may be clearly defined. Surrounding the bone nodules, however, the individual cells are no longer distinguishable. This is likely to be due to the cells becoming multi-layered as has been well reported in ectopic bone nodule formation in culture.
days and stained are shown in Plates 2.10 and 2.11. Under low-power magnification, the sites of mineralisation may be visualised as light-impermeable areas (Plate 2.10). Surrounding these, a border of crimson is apparent, signifying the presence of inorganic phosphates. Under higher magnification, (Plate 2.11) the graduation of colour from orange to deep crimson is easily visible as one approaches the bone nodules.

The bone nodules themselves appear to be black which is probably due to both the amount of phosphate present resulting in an intense stain, and the extent of light-impermeable calcium deposition as ectopic bone is formed in culture. As noted when stained using the von Kossa protocol, the cells in unstained areas between the nodules may each be individually distinguished. However, at the sites of phosphate deposition, the cells are less well defined which is probably due to the cells becoming multi-layered. Once more, in the absence of dexamethasone, no bone nodules were apparent and as previously explained, the negative control skin fibroblasts did not survive in long-term culture long enough to stain.

In this section, I have demonstrated that the primary culture enriched for osteoblasts obtained from 28-day old rats contain cells of the osteoblast lineage. These cells stained for the osteoblast-specific marker, alkaline phosphatase, and could form ectopic bone nodules in the presence of dexamethasone and ascorbic acid. Furthermore, in the presence of β-glycerophosphate these nodules became mineralised as determined by the von Kossa and alizarin red S staining protocols. Additionally, it was demonstrated that no osteoclasts were detected in culture.
Plate 2.10 - Mineralising osteoblast-enriched primary cultures stained with alizarin red S. Alizarin red S is used to detect the presence of inorganic phosphate deposits, and may, therefore, be used to visualise mineralising bone nodules. These are clearly seen as areas staining red, surrounding the light-impermeable bone nodules.

Plate 2.11 - A detailed view of ectopic bone nodules stained with alizarin red S. The depth of the staining increases from orange to crimson as one approaches the bone nodules. The interior of the bone nodule appears black due to both the intensity of the staining and the amount of calcification which has occurred. Interestingly, some areas between the nodules have stained orange and from this magnification it appears that the cells are multi-layered, and may be early sites of mineralisation.
Seeding Densities and Growth Curves

The optimal seeding density needed to be determined in order to achieve maximal proliferation from the limited number of cells obtained from the collagenase digests.

i) Plating Density

Cells were grown to confluence, trypsinised and plated out at various seeding densities between ~2,400 and 40,000 cells/cm². After three days, the number of cells present was counted. A typical example of data obtained is represented graphically in Figure 2.1, showing the effect of seeding density upon the final number of cells obtained after three days.

These data illustrate that cell number increased 3-fold for a doubling in seeding density between ~2,400 to 5,000 cells/cm². However, although the cell number continued to increase at higher seeding densities, the relative change in final number was smaller. For instance, from ~5,000 to 25,000 cells/cm², a large increase in plating density was required for only a comparatively small increase in final cell number. Specifically, over a four-fold increase in plating density was needed to achieve a doubling in the final number of cells present after three days. While only one seeding density above ~25,000 cells/cm² was used, the final number of cells diminished to ~80% of the greatest number of cells obtained after three days.

In summary, the maximal rate of proliferation was achieved at a seeding density of ~5,000 cells/cm², while the greatest number of cells would be obtained from plating at ~2,500 cells/cm² but the cells would have to be left in culture for longer.
Figure 2.1 - Optimisation of the seeding density of cultures of primary osteoblast-enriched cells. Seeding densities were varied from 2,000 to 40,000 cells/cm², and the total number of cells (x10^5) obtained after 3 days growth in 7cm² culture dishes were counted. From these data it is apparent that the greatest proliferation occurs with the lower seeding densities, between 2,500-5000 cells/cm².
ii) Growth and Differentiation

In order to study cells at particular proliferation stages, growth curves were required. Also, as already mentioned, studies by Peck et al (1964) demonstrated that multiple passages of primary cultures enriched for osteoblasts had a deleterious effect upon the population of cells of the osteoblast lineage present in culture due to out-growth by fibroblastic cells. Therefore, alkaline phosphatase expression was determined * and compared after 1, 2 and 3 passages.

Cells were grown at the seeding density of 2,500 cells/cm² and counted on a daily basis. In parallel, an identical culture was stained for alkaline phosphatase activity. Typical data obtained is shown graphically in Figures 2.2 and 2.3 and an example of cells staining for alkaline phosphatase is shown in Plate 2.12.

Since cells were initially plated at 2,500 cells/cm² in a 9cm² culture dish, the cells were seeded at ~ 23,000 cells/dish on day 0 (Figure 2.2). After 24 hours, the number of adherent cells from passage 1 (black line) in culture dropped to 20%. However, from day 1 to day 6, the number of cells in culture increased logarithmically with a doubling time of approximately 24 hours. Following this log phase of growth, the rate of proliferation declined as the cells achieved confluence, reaching a maximum density of approximately 2.2 x 10⁵ cells/cm². From day 7 to day 8, at which the study was terminated, the cell number remained constant having reached a plateau.

Alkaline phosphatase staining (Figure 2.3) of cells from the first passage (black line) was barely visible for the first 3 or 4 days with only ~1 to 2% of the cells staining positively. From day 4 to 5, there was an approximate 5-fold increase in the proportion

* - refer to Addendum
Figure 2.2 - Sequential growth curves for osteoblast-enriched primary cultures. Cells were plated at 2,500/cell in 7cm² culture dishes and the total number of cells in the dishes were counted every 24 hours. Passage 1 refers to those cells grown directly after collagenase digestion of rat long bones. Passages 2 and 3 were obtained after trypsinisation of confluent cells from passage 1 and 2 respectively and reseeding into culture dishes.

Figure 2.3 - Sequential alkaline phosphatase staining of primary cultures of osteoblasts. Alkaline phosphatase staining was performed on cultures grown in parallel to those used in Figure 2.2. Cells staining were counted every 24 hours* and the mean percentage was plotted. Interestingly, multiple passages appeared to have a deleterious affect on the final proportion of alkaline phosphatase-positive cells.

* - refer to Addendum
<table>
<thead>
<tr>
<th>Day</th>
<th>Day</th>
<th>Day</th>
<th>Day</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Plate 2.12 - A photograph demonstrating the increase in proportion of osteoblast-enriched cells staining for alkaline phosphatase with time. The cells were grown in 7cm² culture dishes seeded at 2,500 cells/cm² and stained every 24 hours. This photograph clearly shows that alkaline phosphatase production rises rapidly from day 4 and reaches a maximum at approximately day 8, persisting until day 10.
of cells staining, a rate of increase which diminished to ~3-fold from day 5 to 6, so that 35% of the cells were alkaline phosphatase positive. As the cells approached confluence, the rate at which the proportion of cells staining for alkaline phosphatase increased then gradually declined and by day 7, had reached a plateau of 40%.

Passage two (blue line) followed a similar trend in terms of proliferation and alkaline phosphatase expression to that seen in the first passage. The growth curve from cells from passage three (red line) also followed a similar growth curve. However, the rate of proliferation was slightly greater with a doubling time of 20 hours, though the same maximal cell density was achieved after 7 days. In contrast, alkaline phosphatase expression was much reduced. The proportion of cells staining positively after five days in culture was comparable to that seen in passages 1 and 2 comprising approximately 13% of the cells in culture. From day 5 to 6, however, the increase the proportion of cells staining for alkaline phosphatase was negligible and reached a maximum proportion of 20% from day 6 to 8.

In summary, the rate of proliferation and the growth curves of cells from primary culture enriched for osteoblasts have been determined. It has also been demonstrated that the osteoblastic phenotype, as assessed by alkaline phosphatase expression, is expressed in the late stages of proliferation as the cells reach confluence. Furthermore, multiple passages appear to result in a decline in the proportion of cells staining for alkaline phosphatase. This maybe an indication that cells of the osteoblast lineage proliferate at a slower rate in comparison to other cells obtained from the collagenase digest and that therefore the number of passages should be kept to a minimum. Also,
since the initial number of cells obtained from collagenase digestion was limited, the use of the lower seeding density of 2,500 cells/cm² was vindicated.
Measurement of IGF-I in Medium

Radioimmunoassays (RIA) were performed to ascertain whether IGF-I was produced by the primary cultures of osteoblasts. Cells were seeded at 2,500 cells/cm² in a 28cm² tissue culture dish. The cells were grown for 4 days in 2ml of DMEM without phenol-red supplemented with 10% FCS, penicillin/streptomycin (100U/ml), amphotericin (0.25μg/ml) and ascorbic acid (50μg/ml). The culture medium was aspirated and the cells were rinsed with PBS (1.5ml). Serum-depleted culture medium (2ml), comprising DMEM without phenol red, supplemented with standard amounts of penicillin/streptomycin, amphotericin and ascorbate in the absence of FCS, was added. Following 24 hours in culture, the medium was removed and stored at -70°C. In order to remove IGF-binding proteins (IGF-BPs) which might interfere with the assay, the samples were extracted either by (i) acid-ethanol or (ii) C18 Sep Pak column extraction, after which they were assayed using the RIA kit (Nichols Institute Diagnostics). However, extraction conditions needed to be optimised before IGF-I could be detected.

i) Acid-Ethanol Extraction

Acid-ethanol extraction is a simple procedure and involves the precipitation of binding proteins in 87.5% ethanol and 12.5% 2N HCl. The supernatant containing the soluble IGF-I is neutralised with 0.855M Tris-base prior to performing the RIA. However, the IGF-I levels consistently fell below the levels of detection. This could have been due to either a lack of sensitivity of the assay procedure or due to low production of IGF-I in culture. However, the extraction by acid-ethanol, itself, has several drawbacks. The initial sample size to be measured is small (100μl from 2ml of culture medium) and this is diluted at least 15-fold prior to being assayed. Therefore only 1/300th of the whole
sample is being measured. Furthermore, this extraction method is not highly efficient at removing the binding proteins, which may interfere with the assay procedure and therefore the alternative method of column extraction was used.

**ii) C18 Sep-Pak Column Extraction**

This extraction protocol is based on the differential binding affinities of IGF-I and IGF-BPs to silicic acid under conditions of varying hydrophobicity. Briefly, the columns are prepared by sequential washes with isopropanol, methanol, and 4% acetic acid. The samples are acidified to dissociate the binding proteins from the IGF-I, and added to the prepared columns, to which both bind. The binding proteins are eluted with 4% acetic acid, following which the bound IGF-I is eluted in methanol. The IGF-I is recovered after drying the samples *in vacuo*. This protocol has several major advantages over acid-ethanol extraction. A much larger sample size may be used (for example 1.6ml of the 2ml of culture medium was commonly used) and no dilution was necessary.

Since the protocol was altered from that specified in the kit instructions to allow greater sensitivity with a larger sample size, the extraction procedure was optimised and validated.

The extraction method was performed using a known quantity of $^{125}\text{I}$-labelled IGF-I tracer in order to elucidate the percentage recovery of IGF-I. Furthermore, this procedure allowed verification that a consistent recovery was being obtained. Triplicate samples of $^{125}\text{I}$-IGF-I tracer (100μl) were acidified and extracted as per the protocol in Appendix I. Upon addition of the sample, the eluents were collected and counted on a gamma-counter. Similarly, the eluents were collected after the addition of acetic acid.
and methanol. The percentage recoveries were calculated and tabulated as shown below (Table 2.4).

<table>
<thead>
<tr>
<th></th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Addition</td>
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<td>15%</td>
<td>14%</td>
</tr>
<tr>
<td>Acetic Acid Wash</td>
<td>9%</td>
<td>9%</td>
<td>10%</td>
</tr>
<tr>
<td>Methanol Wash</td>
<td>27%</td>
<td>24%</td>
<td>28%</td>
</tr>
</tbody>
</table>

Table 2.4 - A comparison of the percentage recoveries of $^{125}$I-IGF-I from C18 Sep-Pak columns after initial addition and elution with acetic acid and methanol. The extraction was performed in triplicate in order to confirm the consistency of recovery.

Data from trial 1 demonstrates that 15% of the IGF-I does not bind to the columns and runs through. However, upon addition of acetic acid, the majority of the IGF-I remains bound and only 9% of the total IGF-I is eluted from the column. Of the 76% of IGF-I initially added, which is still bound to the column, only 27% is eluted in methanol which is used to wash the IGF-I from the column. While this represents a recovery of only one quarter of the IGF-I initially added, the ability to extract the larger volumes used represents a significant advantage over the acid-ethanol extraction procedure, although this low recovery will be addressed in the future. Furthermore, comparison of the recoveries from the three extractions demonstrates that the recovery is consistent with very little variation.
Measurement of IGF-I in primary osteoblast culture medium

In order to ascertain whether IGF-I was produced by osteoblasts in culture, cells were cultured in serum-depleted medium (2ml) containing 0% FCS for 24 hours *. The medium was frozen until used, when an aliquot (1.6ml) was extracted. Simultaneously, serum-depleted medium (1.6ml) which had been handled in an identical manner, but which had not been incubated with cells, was extracted as a blank control. The amount of IGF-I was measured in both sets of samples by RIA in duplicate, and also following serial dilution. The dilutions were performed to obtain readings from the centre of the standard curve and verify the consistency of the measurements acquired. Typical results are shown below (Table 2.5) and are displayed as the mean of the duplicates performed for every dilution. The standard curve is shown in Figure 2.4.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>1 : 4</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Blank</td>
<td>1 : 8</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Blank</td>
<td>1 : 16</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>1 : 4</td>
<td>1.6 ng/ml</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>1 : 8</td>
<td>0.72 ng/ml</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>1 : 16</td>
<td>0.29 ng/ml</td>
</tr>
</tbody>
</table>

Table 2.5 - Measurements of IGF-I in culture medium after 24 hours in culture with primary osteoblasts. The blank samples refer to medium which was not incubated with cells. Dilutions of 1:4, 1:8 and 1:16 were used in order to obtain readings from the centre of the standard curve and to confirm that the measurements obtained after dilution were linear.
Figure 2.4 - A standard curve obtained in the IGF-I radioimmunoassay.
The results shown clearly demonstrate that the amount of IGF-I present in the culture medium prior to incubation with the cells was below the level of detection. However, following 24 hours in culture, IGF-I was easily detectable in the culture medium, although a 16-fold dilution gave results at the limits of detection. The 8- and 4-fold dilutions both gave readings in the linear section of the standard curve and produced values which were consistent with little variation. From the figures, it was apparent that osteoblasts in primary culture produce IGF-I to a final concentration of approximately 4ng/ml over a 24 hour period.
Discussion

In this chapter, the extraction methods for obtaining primary cultures enriched for osteoblasts from 28-day old rat femurs have been described, along with the optimisations necessary. Furthermore, a high proportion of these cells have been demonstrated to belong to the osteoblast lineage and have been shown to be capable of forming mineralised bone nodules in vitro.

The cells were extracted by sequential collagenase digestion, as first described by Peck et al. (1964). As previously described, this allows a rapid preparation of primary cultures and results in the release of an adequate number of cells for most metabolic studies. Furthermore, osteoblastic cells obtained in this manner have been extensively characterised, both in terms of markers of the osteoblast phenotype, such as alkaline phosphatase (Luben et al., 1976; Bellows et al., 1991; Aubin et al., 1995), PTH- and 1,25 dihydroxyvitamin D₃-responsiveness (Wong and Cohn, 1974; Luben et al., 1976; Marie et al., 1989; Wong et al., 1990) and mineralised bone nodule formation (Bellows et al., 1986a; Bellows et al., 1987; Bhargava et al., 1988), as well as in terms of the effect of various hormones and other factors including, for example, thyroid hormones T₃ and T₄ (Lakatos et al., 1993), growth hormone (Kassem et al., 1993), prostaglandin E₂ (Bichell et al., 1993; Nagata et al., 1994), oestrogen (E₂) (Ernst et al., 1989) and androgens (Gray et al., 1992).

However, the vast majority of these cultures were obtained from foetal rat calvaria and these are likely to differ in regulation from those extracted at different periods. For
example, the embryonic role of IGF-I was unaffected by both the ablation of the pituitary gland and in mice with a mutation in the gene for the growth hormone releasing hormone receptor, resulting in only 10% of the normal circulatory levels of growth hormone (lit mice). This demonstrated that, prenatally, the regulation of IGF-I by growth hormone, as predicted by the somatomedin hypothesis, does not occur (Chard, 1989; Baker et al., 1993). Therefore, while IGF-I appears to mediate a prenatal response, as observed in IGF-I knock-out mice, it may be regulated postnatally to a greater extent (Shemer et al., 1992; Baker et al., 1993).

In contrast, it has been demonstrated, during postnatal life, that growth hormone enhances IGF-I transcription and increases IGF-I mRNA abundance (Daughaday and Rotwein, 1989). For instance, in lit mice, growth was almost indistinguishable from that of normal mice for the first two weeks. However, by weeks 3 to 5 the body weight was much reduced, a phenotype which could be reversed upon growth hormone-replacement, resulting in a corresponding increase in circulatory IGF-I. Therefore, by 3-weeks of age the growth hormone-IGF-I axis is active and the cells obtained from animals of this age are likely to be more responsive to postnatal growth factors. Thus, osteoblasts were extracted from 4-week old rats, even though cells from these rats may be capable of fewer divisions than foetal cells.

Another difference to previous studies is that cells were obtained from femurs rather than calvaria. Calvarial bone is comprised of a different type of bone, flat bone, than that found in long bones such as femurs, and it is possible that there are differences in the osteoblast population. As a corollary to this, it is known that flat bones are first created by intramembranous ossification, whereas long bones are synthesised via
endochondral ossification. Additionally, as has been observed in skeletal abnormalities, such as progressive diaphysial displasia and achondroplasia, osteoblasts present on different bones appear to be differentially regulated, as will be discussed later. Furthermore, long bones undergo great longitudinal growth under the effects of growth hormone and other factors, which are likely to be mediated through IGF-I. Long bones, which are predominantly supportive, may also have to be responsive to a greater variety of mechanical strain than flat bones whose function is primarily protective. Therefore, osteoblasts were obtained from femoral bones.

However, as previously stated, most primary cultures of osteoblasts have been obtained from foetal rat calvaria. Therefore, in order to obtain a consistent population of cells from 4-week old rat femurs, the digestion protocol had to be extensively optimised. To this end, the two main alterations affecting cell numbers released were the type of PBS used and the digestion duration/ferocity of shaking. Alterations to the PBS used to that containing calcium and magnesium may be easily explained, since collagenase requires divalent cations as chelating ions for maximal enzymatic function. However, while medium containing calcium and magnesium is sometimes used (Rao et al., 1976; Aubin et al., 1982; Bellows et al., 1987), other cultures have been prepared in the absence of magnesium and calcium (Luben et al., 1976; Isogai et al., 1996). However, in my hands, the presence of calcium and magnesium ions greatly increased the total number of cells released, although the number of viable cells remained unaltered.

The amplitude and rapidity of shaking first used, while releasing cells, also resulted in the production of a large amount of debris, and fractions containing a viscous substance. That this was DNA could be inferred by its disappearance upon addition of
DNase into the collagenase/trypsin digestion mix, as was also used by Rao et al (Rao et al., 1976). This signified that cells were being lysed during the digestion through either over-lengthy digestion, or excess impact within the digestion vessel, thereby damaging the cells. It was unlikely that this was due to the length of digestion since nearly all digestions involve the incubation of the bone fragment in a collagenase digestion mix for at least 30 minutes (Rao et al., 1976; Bellows et al., 1986a; Antosz et al., 1987; Antosz et al., 1989; Gray et al., 1992). Indeed, this assumption was vindicated by the decrease in cell debris after less vigorous shaking but a longer digestion period. In fact, the presence of DNase was no longer found to be necessary once these conditions had been optimised, and the procedure was so effective that the yield remained high while the number of femurs were decreased.

The cells obtained were morphologically heterogeneous as had been observed in the literature (Wong and Cohn, 1975; Rao et al., 1976; Luben et al., 1976). Furthermore, cells in culture tend to assume shapes grossly different to those normally observed in vivo, which makes morphological characterisation of the cells almost impossible, especially since cells in vivo are usually identified by both their position in the extracellular matrix and their morphology (Luben et al., 1976). Therefore, cells must be identified according to known biochemical and histochemical markers. A marker commonly used to validate enrichment for cells of the osteoblast lineage in culture is alkaline phosphatase (Bellows et al., 1991; Aubin et al., 1995). This protein is commonly associated with the bone cell phenotype and is known to rise markedly following down-regulation of proliferation and increased differentiation (Stein and Lian, 1993).
As observed in the results, the cells stained for alkaline phosphatase differentially, which may indicate cells at various stages of differentiation or the presence of cells of lineages other than osteoblastic. Either way, the proportion of stained cells was high. Furthermore, the cells in culture displayed the characteristic rise of alkaline phosphatase expression with increasing numbers of days in culture (Stein and Lian, 1993), continuing to rise as proliferation slows down (Aubin et al., 1995). It is also interesting to note that repeated sub-culture of the osteoblast-enriched culture resulted in a decrease in the expression of this marker. This could be reflecting the observations made by Peck et al. (1964) and to a lesser extent Wong and Cohn (1974), in which spindle-shaped cells, which were taken to be fibroblastic, proliferated faster than the other cells, thereby transforming the culture from predominantly osteoblast cells to mostly fibroblasts, resulting in a loss of responsiveness to PTH. However, the consistency of the staining in the first two passages indicated that while the cells should preferentially not be passaged more than twice, the proportion of cells of the osteoblast lineage was high for the first two passages and appeared to remain constant.

The ability of cells from primary culture of bone to form mineralised bone nodules in culture has been taken as an indicator of the presence of cells of the osteoblast lineage (Bellows et al., 1986a; Bhargava et al., 1988). These mineralised nodules are often formed when the culture medium is supplemented with ascorbic acid, β-glycerophosphate and dexamethasone, and may be easily visualised using various staining methods. The von Kossa stain allows the detection of calcium deposits which are seen as a dense black colouration, while alizarin red S may be used to visualise phosphate within the extracellular matrix, staining phosphate a rich crimson colour. It
has been shown in this chapter that mineralised bone nodules may be formed in vitro, confirming the presence of cells of the osteoblast lineage in the preparations of osteoblast-enriched primary cultures.

However, many groups have reported that nodule formation and their mineralisation was possible in the absence of dexamethasone, solely with the addition of ascorbic acid and β-glycerophosphate (Bellows et al., 1986a; Bellows et al., 1987; Bellows and Aubin, 1989; Nagata et al., 1994). This was not apparently possible using the preparations of cells obtained. There are two main reasons accounting for this variation, including differences in 1) the bone from which the cells originated and 2) the age of the animals at the time of the extraction. Most of the studies performed involved the use of cells originating from foetal calvaria. The differences between calvaria and long bones are such that there may be variance in the cell populations released. For instance, certain diseases causing skeletal abnormalities affect only certain types of bone. An example is achondroplasia which is caused by a mutation in the fibroblast growth factor receptor-3 gene, resulting in both dwarfism and early closure of cranial sutures (Shiang et al., 1994). Progressive diaphyseal displasia, known as Camurati-Engelmann’s disease, also demonstrates the differences in which particular bones develop, predominantly affecting the periosteal and endosteal surfaces of long bones (Hundley and Wilson, 1973). The age of the tissue from which the cells were extracted may also play an important role in determining the characteristics of the osteoprogenitors and osteoblasts present in culture. This has been demonstrated by the apparent absence of a growth hormone-mediated increase in IGF-I prenatally, whereas postnatally IGF-I transcripts rise in synergy with growth hormone, as already described. However, the variations in the responses of the cultures from 4-week old rat femurs compared to
those published reports from foetal calvaria demonstrate the importance of not restricting osteoblast studies to foetal calvaria.

As already stated, one of the aims of this project was to study the production of IGF-I transcripts in osteoblast-enriched cultures. Therefore, the synthesis of IGF-I in culture was verified and measured by RIA. The presence of IGF-I observed, was consistent with previous reports, both in the osteoblast cell line UMR-106 and from primary cultures from foetal limb bones (Lakatos et al., 1993). This, therefore, supports the premise that IGF-I is produced in cultures enriched for osteoblasts and lends credence to the further investigation of IGF-I synthesis.

In summary, in this chapter, the conditions for obtaining osteoblast-enriched cultures from 4-week old rat femurs have been optimised. The cells released have been characterised and it has been demonstrated that the primary cultures contain a high proportion of cells of the osteoblast lineage which are capable of forming mineralised bone nodules. Furthermore, IGF-I production by these cells has also been shown to occur in vitro. Therefore, we have obtained a well-characterised system to pursue our studies of IGF-I transcripts in primary osteoblast cultures.
Chapter 3

IGF-I Gene Expression
Introduction

As has already been described in chapter 1, the rat and human IGF-I genes contain 6 exons spanning over 80 kb (Adamo et al., 1991a; Shemer et al., 1992; Delany et al., 1994). Exons 1 and 2 code for alternate leader exons, while exons 3 and 4 code for the IGF-I protein. Exon 5, which may be alternately spliced, and exon 6 code for separate peptides, E-peptides, and exon 6 encodes the 3'-UTR.

Both the 5'- and 3'-end of IGF-I transcripts are diverse in both rat and human. The heterogeneity in the 5'-end stems from alternate leader exon usage, multiple transcription start sites and alternate splicing (Roberts et al., 1987a; Shimatsu and Rotwein, 1987a; Bucci et al., 1989; Jansen et al., 1991). This was determined by a combination of methods including primer extension and RNase protection assays in both rat (Adamo et al., 1991a; Adamo et al., 1991b) and human (Jansen et al., 1991; Kim et al., 1991). The 5'-UTR of the rat transcripts may thereby vary in length from between ~40 to ~900 bases.

Transcription of the IGF-I Gene

i) Transcription From Start Sites Derived From Exon 1

In detail, exon 1-derived transcripts may be initiated from 4 transcription start sites (denoted start sites 1, 2, 3 and 4) within the exon (Figure 3.1). Start sites 1 and 2 are in close apposition (containing 405 and 362 bases of exon 1 respectively). However, start sites 2, 3 and 4 are not closely clustered and therefore the length of the 5'-UTR present
Figure 3.1 - A diagrammatic representation of the transcription start sites which are situated in the first 3 exons of the IGF-I gene, also showing the 186 base alternatively spliced region within exon 1. The start sites within exon 1 are denoted start site 1 to 4, while those within exon 2 are represented by arrows. Exons 4-6 are not shown.
in the transcripts may vary greatly. Specifically, transcripts initiated from start sites 3 and 4 contain 260 and 28 bases of leader exon 1 respectively. The multiplicity of transcripts produced is further increased due to the alternate splicing of a 186-base region positioned downstream of start site 2. Therefore, those transcripts initiated from start site 1 and 2 might only contain 219 and 176 bases of exon 1.

\textit{ii) Transcription From Start Sites Derived From Exon 2}

Exon 2 is a large exon, spanning over 700 bases. There are at least 3 transcription start sites within the exon (Figure 3.1). The upstream start site, containing 772 bases of exon 2, is widely separated from the two downstream start sites, which, in contrast, are in close apposition. In rat liver, transcripts are initiated predominantly from the second start site, 70 bases upstream of the 3'-end of the exon. The remainder use the third transcription start site, 50 bases upstream (Shemer \textit{et al.}, 1992).

\textbf{Regulation of Gene Expression and the 5'-UTR}

Heterogeneity of this kind in transcription start site usage and varying 5'-UTRs has been observed in several other genes, such as those encoding rat growth hormone/growth hormone-binding proteins (Domené \textit{et al.}, 1995) and the rat glucocorticoid receptors (Gearing \textit{et al.}, 1993). Indeed, possible consequences of this heterogeneity are transcripts which are translated with different efficiencies, that is, exhibit different translatabilities. This has been shown for the transcripts encoding IGF-II (Nielsen \textit{et al.}, 1990; Teerink \textit{et al.}, 1994; de Moor \textit{et al.}, 1995), somatostatin (Danoff and Shields, 1988), inducible nitric oxide synthase (Chu \textit{et al.}, 1995), murine tissue inhibitor of metalloproteinases (Waterhouse \textit{et al.}, 1990) and retinoic acid receptor-\(\beta_2\)
(Zimmer et al., 1994). Another effect which may be mediated through alternate start site usage includes tissue-specific expression of the protein product, such as that observed for the α2(I) collagen gene (Beck et al., 1991). Furthermore, alternate splicing within the 5'-UTR of c-fos altered the extent to which the transcripts were destabilised by an AU-rich element within the 3'-UTR (Roy et al., 1992).

**Tissue-Specific and Hormonal Regulation of IGF-I Transcripts**

Transcription of the IGF-I gene has been shown to be hormonally regulated. For instance, from studies using hypophysectomised rats, it has become evident that GH mediates an up-regulation of hepatic IGF-I mRNA synthesis predominantly (Roberts et al., 1986). In addition, low doses of oestrogen (E2) act synergistically with GH in stimulating hepatic IGF-I mRNA, even though E2 alone had no affect on hepatic IGF-I transcription, although chronic E2 administration can attenuate a GH-stimulated rise in hepatic IGF-I mRNA (Murphy and Friesen, 1988; Murphy and Luo, 1989).

GH has also been observed to mediate an increase in the abundance of transcripts in the proximal tibia, lung and kidney (Luo and Murphy, 1989), cultured rat adipocytes *in vitro* and adipose tissue *in vivo* (Vikman et al., 1991). GH-induced up-regulation of transcription in these extrahepatic tissues may also be regulated. For instance, dexamethasone not only significantly reduced the GH-induced increase in IGF-I transcripts in liver, but also mediated a similar response in the tibia, the kidney and the lung.
Recent studies on the effects of GH on leader exon usage in hypophysectomised rats have demonstrated that the increase in IGF-I gene transcription is not uniform. For instance, hepatic IGF-I transcripts derived from exon 2 are preferentially increased in GH-stimulated rats. Furthermore, it has been shown that GH induces a specific increase in transcription from particular exon 1-derived start sites (Foyt et al., 1991). Profiles have also been obtained in normal rats during development (Adamo et al., 1989; Adamo et al., 1991b). In a similar manner to the GH-stimulated hypophysectomised rats, exon 2-derived transcripts were specifically increased during growth hormone-dependent development. However, the expression of hepatic IGF-I transcripts initiated from exon 1 start sites differed, since initiation of transcripts was co-ordinately increased from all start sites (Adamo et al., 1991b). Further differences in the manner in which GH appears to regulate start site usage have been characterised. For instance when GH-deficient, rather than hypophysectomised rats, were given growth hormone there was a uniform increase in both exon 1- and exon 2-derived transcript. Taken together, these data suggest that transcripts initiated from exon 2 may also be regulated by a pituitary-dependent factor other than GH (Butler et al., 1994).

Transcripts from other tissues are also developmentally regulated as demonstrated by Shemer et al. (1992). For instance, transcription start site usage within exon 1 in kidney differs from that observed in the liver, with particular initiation sites only being increased at the time of weaning, rather than a co-ordinate increase in transcription from all start sites. Furthermore, exon 2-derived transcripts were only detectable in kidney postnatally. This indicates that expression of particular transcripts may be both tissue-specific and developmentally-regulated. Studies on expression of transcripts in adults further vindicated the hypothesis that relative start site usage may be tissue-
specific. For example, while kidney and brain have a similar pattern of exon 1 start site expression to liver, other extrahepatic tissues including stomach, heart and muscle preferentially use different transcription initiation sites. In addition, transcripts derived from exon 2 were not observed at all in heart, brain or muscle (Shemer et al., 1992).

These examples of tissue- and developmentally-specific control of start site usage, could indicate that translation of IGF-I transcripts is post-transcriptionally regulated. This hypothesis is supported by the observations of Foyt et al. (1991), who demonstrated that transcripts arising from alternate start sites were differentially associated with polysomes. Since polysomal association of transcripts is indicative of greater translatability than those which are not found in the polysomal fraction, this difference is likely to mean that the efficiency of translation is regulated, at least in part, by the choice of transcription start site used.

IGF-I and Osteoblasts

As described in Chapter 1, IGF-I is present in both human and rat bone (Canalis et al., 1988; Martinez et al., 1994) and mediates many anabolic effects including stimulation of foetal metatarsal bone growth in vitro (Scheven and Hamilton, 1991) and formation of trabecular/cortical bone in vivo (Spencer et al., 1991; Mueller et al., 1994). In particular, IGF-I stimulates osteoblast proliferation, increases type I collagen synthesis and secretion (Hock et al., 1988; McCarthy et al., 1989; Schmid et al., 1989), and decreases the production of MP-13 (Hock et al., 1988; Canalis et al., 1995a). Furthermore, many hormones mediate their actions on bone through alterations in IGF-I mRNA expression and synthesis of the protein. These include T3/T4 (Lakatos et al.,
1993) GH (Isgaard et al., 1988; Nilsson et al., 1990), PTH (Pfeilschifter et al., 1995), PGE\(_2\) (Bichell et al., 1993; Pash et al., 1995) oestrogen (Ernst et al., 1989; Ernst and Rodan, 1991) and progesterone (Barengolts et al., 1996) which mediate some, if not most, of their local actions through an increase in IGF-I transcription and translation. Conversely, glucocorticoids (Luo and Murphy, 1989; McCarthy et al., 1990; Delany et al., 1994a), TGF-\(\beta\) (Okazaki et al., 1995), PDGF and bFGF effect their responses, in part, by decreasing IGF-I expression.

However, the majority of these studies involved the use of cell lines or whole body in vivo effects of the factors upon bone IGF-I. Furthermore, to date the regulation of IGF-I gene expression has mostly been assessed by measuring changes in total RNA. On the whole this has involved the quantitation of the intensity of the bands/dots obtained from Northern analysis or dot/slot blots respectively, although RT-PCR has also been employed to verify the presence of particular transcripts. However, there are major draw-backs to these analytical approaches when studying IGF-I, which are explained below.

\textbf{i) Northern Analysis and Dot Blots}

Both these techniques are relatively sensitive and easily quantifiable. Dot blots involve the addition of aliquots of RNA onto nitrocellulose. These are hybridised to radiolabelled probes, and exposed to film. The resulting dots on the autoradiographs are measured densitometrically, from which the amount of the particular RNA may be ascertained. Therefore overall changes in transcript abundance may be detected, but, the nature of the particular RNA in a heterogeneous population, as with IGF-I, cannot be ascertained. In contrast in northern analysis, RNA is separated in an agarose gel.
according to length prior to hybridisation to radiolabelled probes. Using this technique, both the total amount of RNA, as well as the size and proportion of particular RNA transcripts may be ascertained. While this methodology is therefore more suited to the examination of specific start site usage, if, as in the case of IGF-I, there are also variations in the 3'-UTR concomitant with alternate start site usage (and the potential presence of unprocessed RNA), the transcription initiation site may be masked.

**ii) RT-PCR**

This methodology allows the detection of RNA down to a single transcript and is a powerful method for determining the presence of specific mRNAs. For quantitative analysis, though, all the transcripts have to be amplified to the same extent in order to draw a comparison between the amounts of particular transcripts present. This may be possible when only one type of transcript is expressed and inter-tissue variations are being examined. However, in the case of IGF-I which may encode multiple transcripts, many different primer-pairs would be needed in order to differentiate between the amplified fragments. It would therefore be very difficult to optimise the RT-PCR conditions and primers in order to amplify all transcripts to the same degree. Furthermore, other techniques, such as RNase protection assays, might be employed which while they might not be as sensitive, are comparatively simple.

**iii) RNase Protection Assays**

This technique allows the detection of very low concentrations of RNA. Basically, it involves the hybridisation of a radiolabelled antisense riboprobe to total cellular RNA or mRNA. Single-stranded RNA is digested using RNase A and T₁, leaving the double-stranded RNA hybrid. These are separated on denaturing polyacrylamide gels and
visualised using autoradiography. Therefore, using suitable riboprobes, the expression of different start sites can be quantitated by comparing the intensities of the protected bands.

Aims

The general aim of this chapter was to elucidate whether transcripts were expressed in a tissue-specific manner in bone. This was achieved in a variety of ways and the results are described in two sections. In part 1, RT.PCR was used to determine the nature of the transcripts present in bone and osteoblasts compared to liver. In part 2, I elucidated and quantified the nature of the transcripts expressed in osteoblasts and whole bone in comparison to liver (control) using RNase protection assays.
Methods and Results

Part 1 - Determination of the Presence of IGF-I Transcripts in Bone Cells

Initially, studies were performed in order to verify the presence of IGF-I transcripts in primary cultures enriched for osteoblasts as well as in whole bone. This was undertaken using RT-PCR. Liver, in which an abundance of IGF-I transcripts has been detected, was used as a control tissue. The methods and solutions related to this section may be found in full in Appendices I and II respectively. Any modifications made to the basic protocol in order to optimise conditions are mentioned within the body of the text.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

i) Preparation of RNA

Femurs and livers were removed from 28- and 50 day-old female Sprague Dawley rats and transported in dry ice. The tissues were frozen in liquid nitrogen and stored at -70°C. RNA was extracted from frozen bone (~4g), liver (~2g) and the osteoblast-enriched culture described in the previous chapter (~1.5 x 10⁷ cells) using the single step isolation procedure introduced by Chomczynski et al (1987). Briefly, tissues were homogenised in a guanadinium thiocyanate-based solution followed by phenol-chloroform extraction and isopropanol precipitation.
**ii) Primer Optimisation**

RT-PCR was using the primers shown in Figure 3.2. In the first instance, primer 3, near the 5'-end of exon 1, was used in conjunction with primer 14 at the 3'-end of exon 3, in order to amplify fragments from liver corresponding to transcripts initiated from start sites 1 and 2. Two fragments were predicted, corresponding to both the full-length transcripts and those from which the 186 base region had been spliced out. The expected band sizes are shown below in Table 3.1.

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Size of full-length</th>
<th>Size with deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer set 3-14</td>
<td>473 base pairs</td>
<td>287 base pairs</td>
</tr>
</tbody>
</table>

*Table 3.1 - Expected fragment sizes after RT-PCR amplification using primer set 3-14.*

The two predicted fragments of 473 and 287 bp in length were amplified from liver RNA. However, a third band of intermediate size (~400 bp) was also consistently detected. This may have been due to non-specific amplification and therefore, in order to test this, the magnesium chloride concentration was varied.

An example of amplification products obtained from liver RNA using the primer combination 3-14 in various magnesium chloride concentrations (1-3 mM) is shown in Figure 3.3. No bands were visible in the control lanes representing samples amplified in the absence of template (RNA) or reverse transcriptase (RT), indicating that the intermediate-sized band was not due to either contamination of the solutions or the presence of DNA. Decreasing concentrations of magnesium chloride in the incubation also had little effect upon the amplification of the middle band.
Key:  → - Primer direction  
    ▲ - Transcription start sites

Figure 3.2 - A diagrammatic representation of RT-PCR primers and their orientation. The primers are denoted by numbers; their sequence and exact positions are shown in Appendix III.

Figure 3.3 - Optimising magnesium chloride concentrations for primer pair 3-14. Magnesium chloride concentrations were varied between 1.0-3.0mM. Neither control, without RNA or RT, contained any bands. However, altering the magnesium chloride concentration made no difference to the intensity of the middle band. The sizes of the fragments are shown to the right, and DNA size markers are displayed to the left.
In order to investigate the possibility that the amplification of the ~400 bp fragment may have been due to primer 3 having poor specificity, 2 primers complementary to the region 5' of primer 3 in exon 1 were constructed (primers 1 and 2 - Figure 3.2).

<table>
<thead>
<tr>
<th>Primers set</th>
<th>Size of full length</th>
<th>Size with deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer set 1-14</td>
<td>523</td>
<td>337</td>
</tr>
<tr>
<td>Primer set 2-14</td>
<td>498</td>
<td>312</td>
</tr>
</tbody>
</table>

*Table 3.2 - Expected fragment sizes after RT-PCR amplification using primer sets 1-14 and 2-14.*

Primer 2 is specific for a region 25 bases upstream of primer 3 within exon 1 and should therefore produce fragments 25 bp longer than those synthesised with primer 3 when used in conjunction with primer 14 (312 and 498 bp - Table 3.2). Similarly, primer 1 is complementary to a region 50 bases upstream of primer 3, and therefore the amplification products from primer pair 1-14 would be predicted to be 523 and 337 bp in length. Figure 3.4 shows an example of the fragments amplified using the three primer combinations. No bands were visualised in the control lanes in the absence of RNA and RT demonstrating that there was no contamination of the RT-PCR reaction. However, three fragments were amplified using primers 1-14 of approximately 520, 450 and 340 bp in length. The largest and smallest bands represented products amplified from full-length and alternatively-spliced transcripts, the middle band corresponding to the uncharacterised band. In a similar manner, RT-PCR with primers 2-14 and 3-14 resulted in three bands each. These represented the unspliced transcripts (approximately 500 and 470 bp respectively), the middle bands of ~430 and 400 bp and the transcripts
Figure 3.4 - The downstream primer 14 was used in conjunction with the upstream primers 1, 2 and 3. In all cases, three fragments were amplified from osteoblast RNA, corresponding to the full length transcripts, an uncharacterised band and alternatively spliced transcripts. The bands are labelled to the right as full-length and deletion, amplified from those transcripts both with and without the 186 base region respectively. The middle band refers to those uncharacterised fragments of intermediate size. DNA size markers are displayed to the left.
from which the 186 base region was excised (~310 and 280 bp). Therefore, while the two expected bands for each primer combination were amplified, an intermediate band was also consistently present.

The amplification of this transcript with all three primer combinations could be due to the existence of a transcript not previously described in the literature. In order to confirm that this is a novel species, RT-PCR was performed under stringent conditions. To this end, the magnesium chloride concentration was optimised for all primers, and other conditions including variations in the annealing temperatures and hot starts were also used. For instance as already demonstrated, three fragments were amplified using primer combination 1-14, of approximately 520, 450 and 330 bp in length representing the full-length, the uncharacterised band and alternatively-spliced transcripts. Increasing the magnesium chloride concentration in the incubation (1.6 and 3 mM) appeared to lead to a diminution of the band of 450 bp in length (Figure 3.5a). However, the fragment amplified from full-length transcripts (~520 bp) also decreased in intensity. Therefore the uncharacterised band was not specifically affected by the concentration of magnesium chloride present in the reaction.

In order to denature any secondary structure which may be interfering with reverse transcription of the IGF-I transcripts, the RNA was heated to 85°C or 95°C. Examples are shown in Figure 3.5b, from which it can clearly be seen that three bands were still obtained, irrespective of denaturation of the RNA at 85°C when compared to undenatured RNA. In addition, random hexamers and poly(T)₁₆ were used as primers in the RT-step, also in combination with denaturing the RNA, in order to determine whether the intermediate fragment was an artefact due to reverse transcription with
Figure 3.5a - Denaturing RNA and alterations to the magnesium chloride concentration in the RT-PCR of osteoblast RNA using primer pair 1-14. Changes to the magnesium chloride concentration between 1.0 and 3.0mM made no appreciable difference to the amplification of the middle band, and three bands were consistently produced. Indeed, any change in intensity was accompanied by a concomitant decrease in the amplification of the full-length fragment, as observed using 1.6 and 3.0mM. Denaturing the RNA prior to amplification also had no appreciable effect. The sizes of the fragments are indicated to the right, and DNA size markers are shown to the left.

Figure 3.5b - The effect of denaturing the RNA template on the production of the middle band, prior to RT-PCR using primers 1-14. Liver RNA was heated to 85°C and immediately reverse transcribed in the standard manner (Appendix I). Three bands were consistently amplified, irrespective of denaturing the RNA. The absence of bands in the control without any RNA demonstrated that the middle band was not due to contamination of the reaction mix.
Figure 3.5c - Denaturation of the RNA and the use of random hexamers in the reverse transcription step of RT-PCR. The use of random hexamers during reverse transcription following denaturation of the RNA resulted in the production of three well defined bands. Irrespective of the denaturation temperature, 85 or 95°C, the middle band was consistently amplified. No bands were apparent in the absence of any RNA. The molecular size marker is displayed to the left while the fragment sizes observed are indicated to the right.

Key:
- Primer direction
- Transcription start sites
primer 14 (Figure 3.5c). These parameters appeared to make little difference, however, and the three bands described previously were consistently obtained. Furthermore, increasing the annealing temperature from \( T_m - 5^\circ C \) to \( T_m \), thus decreasing any non-specific binding of the primers to the template had no noticeable effect on the production of the intermediate-sized fragment.

The production of this uncharacterised band using three different upstream primers could either reflect a deletion of ~80 bases within these transcripts by alternate splicing, or the formation of secondary structure within the RNA which has not been melted under the rigorous conditions utilised. If the former, the position within which the deletion occurs may be anywhere between primers 3-14 and in order to define it further, three upstream primers 4, 5, and 6 were used in conjunction with the downstream primer 14. These primers are complementary to regions downstream of primer 3 and start site 3, and are specific to the sequence within the 186 base alternatively spliced region (see Table 3.3 for expected fragment sizes). Therefore, one band would be expected for each primer when used in combination with primer 14 since only the full-length transcripts containing the 186 base region could be amplified.

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Expected fragment size</th>
</tr>
</thead>
<tbody>
<tr>
<td>primer set 4-14</td>
<td>389 base pairs</td>
</tr>
<tr>
<td>primer set 5-14</td>
<td>336 base pairs</td>
</tr>
<tr>
<td>primer set 6-14</td>
<td>254 base pairs</td>
</tr>
</tbody>
</table>

*Table 3.3 - Expected fragment sizes after RT-PCR amplification using upstream primers 4, 5 and 6 and downstream primer 14.*
Figure 3.6 - The downstream primer (primer 14) was used in conjunction with the upstream primers 4, 5 and 6 in RT-PCR of rat liver RNA. One fragment was produced per primer pair (shown below) corresponding to the full length transcripts (389, 336 and 254 bp respectively). The observed fragment sizes are indicated to the right, while the DNA marker sizes are shown to the left.

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Figure 3.6 - The downstream primer (primer 14) was used in conjunction with the upstream primers 4, 5 and 6 in RT-PCR of rat liver RNA. One fragment was produced per primer pair (shown below) corresponding to the full length transcripts (389, 336 and 254 bp respectively). The observed fragment sizes are indicated to the right, while the DNA marker sizes are shown to the left.

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Figure 3.6 - The downstream primer (primer 14) was used in conjunction with the upstream primers 4, 5 and 6 in RT-PCR of rat liver RNA. One fragment was produced per primer pair (shown below) corresponding to the full length transcripts (389, 336 and 254 bp respectively). The observed fragment sizes are indicated to the right, while the DNA marker sizes are shown to the left.
An example of fragments amplified from rat liver RNA, using these primers with appropriate controls, and separated on a 2% agarose gel is shown in Figure 3.6. Each primer combination yielded one fragment only of approximately 390, 340 and 250 bp for primers 4-, 5- and 6-14 respectively. Therefore, the bands corresponded to the amplification of transcripts initiated from start sites 1 and 2 (full-length) and 3 and, as predicted, transcripts with the 186 base region excised were not detected. Furthermore, no bands were detected corresponding to the amplification from transcripts with a novel 80 base alternatively-spliced region. Combining these results with those obtained from primers 1, 2 and 3, it would seem likely that the region containing a putative splice site/secondary structure may be situated downstream of primer 3 and upstream of primer 5. This is demonstrated diagrammatically in Figure 3.7 and the region is delineated by the grey box spanning start site 3. The position cannot be localised more exactly using these primers since the postulated deletion/secondary structure may span primer 4.

A putative splice acceptor splice site resides approximately 80 bases downstream of the donor site for the 186 base alternatively-spliced region (AG/AT). Therefore, in order to further characterise and sequence the intermediate band which may be derived from novel alternate splicing of transcripts initiated from start site 1 and 2, the fragment was ligated into a TA vector plasmid. This was performed using the complete RT-PCR mix after amplification, and therefore contained not only the uncharacterised fragment, but also the products amplified from full-length and alternatively-spliced transcripts. A typical PCR, testing for insert within TA-clones, is shown in Figure 3.8. Test-PCR was performed using primers flanking the multiple cloning site, adding 240 bp to the inserted-fragment size. The insert ligated into the TA vector was obtained using primers
Figure 3.7 - The area in which the putative splice site/secondary structure resides. The area lies between primer 3 and 5 and is delineated by the filled rectangle. The upstream primers used are indicated by broken arrows beneath the rectangle representing the exon. The transcription start sites are represented by the solid arrows above the exon.
Figure 3.8 - Test-PCR using primers flanking the insertion site of amplified fragments (a) 2-14 and (b) 3-14 ligated into TA vector. The use of these primers adds an extra 240 bases to the predicted size of the insert. Only two fragment sizes were amplified, corresponding to full length transcripts and those from which the 186 bases have been deleted for primer pair 2-14, while only the fragments corresponding to the amplification of alternatively spliced transcripts appear to have been ligated after RT-PCR using primer pair 3-14. Other test PCRs demonstrated that the full-length products were also present in another selection of clones. However, no intermediate-sized fragments were found to have been ligated. The observed fragment sizes are indicated to the right and the DNA size markers are shown to the left.
2- and 3-14. Therefore, the predicted fragment sizes would be 738, ~660 and 552 bp for primers 2-14 (Figure 3.8a) and 713, ~630 and 527 bp for primers 3-14 (Figure 3.8b). However, from these results it is apparent that although the fragments representing the products obtained from the full-length (lane 9) and alternatively spliced transcripts (lanes 1, 3, 4, 10, 12 and 17-20) were cloned, no clones contained the intermediate fragment. The remainder of the lanes were obtained from clones transfected with TA vector plasmid into which no DNA had been ligated (240 bp band).

Since the absence of the intermediate band could be due to a large excess of these two fragments being amplified in comparison to the middle band, the RT-PCR products were separated on a 2% agarose gel, from which the middle band was excised, purified and cloned into TA vector plasmid. Surprisingly, this also resulted in the production of fragments corresponding to those both with and without the 186 base deletion and the absence of the predicted band.

To confirm the presence, within the intermediate fragment, of the full-length fragments, and those from which the 186 base region had been excised, the RT-PCR was repeated. The fragments amplified were separated for a longer period of time on a 2% agarose gel to allow clear resolution of the bands. The middle band was excised and the isolated DNA taken through a second round of PCR. The products obtained were separated on a 2% agarose gel and are shown in Figure 3.9. Clearly, once again all three bands were amplified, irrespective of whether primer combination 1-, 2- or 3-14 was used. This procedure was repeated several times to confirm that there was no contamination of the middle band DNA with either the longer or shorter species, and the result was highly reproducible.
Figure 3.9 - Fragments re-amplified using primers 1-, 2- and 3-14 after isolating the middle bands on a 2% agarose gel. Three bands are again produced, corresponding to the full length transcripts, the uncharacterised fragment and the transcripts from which the 186 base region has been excised. The bands produced are labelled to the right of the photograph while the molecular size markers are shown to the left.

Figure 3.10 - A test-PCR of TA plasmids amplifying two clones containing insert of the size predicted for the middle band produced with primer pair 1-14. The fragment corresponding to the alternatively spliced transcripts amplified by RT-PCR were found ligated into TA clones. These were amplified using flanking primers and are visualised as fragments of 577 bp. The 240 bp fragment represents clones lacking the insert. Full length fragments were observed in other selections of clones as bands of 763 bp. However, two clones of ~700 bp were also apparent, which could represent the ligation of the DNA from the middle band into TA vector. The DNA size marker is indicated to the left and the observed fragment sizes are displayed to the right.
DNA from the isolated intermediate fragment was ligated into TA vector, from which two clones were obtained of the size predicted should the middle band from primer pair 1-14 have been successfully inserted, as shown in Figure 3.10. Once again, the fragments were produced by PCR of the vector using primers flanking the insertion site, adding an extra 240 bp onto the predicted size of the insert. The expected lengths were therefore 763, ~680 and 577 for the insertion of full-length, intermediate and alternatively-spliced fragments into TA vector plasmid. The figure demonstrates that four types of clone were obtained: those without any insert (240 bp), those containing full-length and alternatively-spliced fragments and two clones containing an insert of the appropriate size to correspond to ligation of the intermediate band into TA plasmid (lane 4 and 10).

The insertion of not only the intermediate-sized fragment, but also those corresponding to the full-length and alternatively-spliced transcripts, into the plasmid was surprising. However, this suggested that the middle band could be an artefact caused by heteroduplex formation. The sequence of the two clones, though, did not correspond to any known in the IGF-I gene or the vector. Primer mis-pairing was ruled out since sequence analysis revealed that the primer sequences were conserved. It is therefore possible that the DNA fragment which had been inserted into the vector had been an artefact of the RT-PCR reaction. Recently, though, having used the new Taq DNA polymerase, Taq Gold (ABI-Perkin Elmer), in conjunction with primer sets 1-14 and 2-14 the middle band was eliminated, suggesting that it was an artefact.
iii) RT-PCR of Osteoblast RNA

Having demonstrated the presence of both full-length and alternatively-spliced exon 1-derived transcripts in liver, RT-PCR was performed on RNA from primary osteoblast culture *. The results obtained using primers 1-6 in combination with primer 14 are shown in Figure 3.11 in lanes 1 to 6 respectively. The fragments of the predicted size were obtained with the addition of the intermediate fragments amplified using primers 1-, 2- and 3-14 as previously described. Therefore, the full-length and alternatively-spliced exon 1-derived transcripts are produced by osteoblasts in culture.

RT-PCR using a primer complementary to exon 2 (primer 11 - see Figure 3.2 for position) has been performed in conjunction with downstream primer 14 to amplify exon 2-derived transcripts (Figure 3.12). This primer is complementary to a region 120 bp (primer 11) upstream of the 3'-end of exon 2 and has been successful in amplifying exon 2-derived transcripts in liver. Amplifying this fragment from bone and osteoblast RNA was more difficult. This could have been due to a low expression of these transcripts by bone and osteoblasts, although no fragments were visualised upon increasing the number of amplification cycles. However, it has been previously shown by Shemer et al (1992), that over 90% of the exon 2-derived transcripts are initiated from the start site 70 bases upstream of the 3'-end of the exon, with nearly all the rest being derived from a start site 20 bases further upstream. A new upstream primer (primer 12) was designed to the 21 bases at the extreme 3'-end of exon 2, which when used in conjunction with primer 14 would result in the production of 161 bp fragment. Using this primer combination (Figure 3.13), exon 2-specific mRNA was amplified from osteoblast cells and bone, confirming its presence in this tissue.

* - refer to Addendum
Figure 3.11 - RT-PCR of RNA from primary osteoblast cultures using upstream primers 1 to 6 with downstream primer 6. This figure shows that IGF-I transcripts are produced by bone cells in vitro. The primers used are shown along the top and some of the expected fragment sizes are given to the left and right.

Key: → - Primer direction
     Γ - Transcription start sites
**Figure 3.12** - RT-PCR amplification of liver IGF-I RNA using upstream primer 11 and downstream primer 14. The fragments produced correspond to the exon 2-derived transcripts initiated from the upstream start site. The size of the fragment is given to the right, while the DNA size marker is displayed to the left.

**Figure 3.13** - RT-PCR amplification of osteoblast IGF-I RNA using the exon 2-specific primer pair 12-14. Since the majority of exon 2-derived transcripts are initiated from the second and third start sites, upstream primer 12 which is specific to the extreme 3'-end of the exon, was used in conjunction with the downstream primer 14. The fragment amplified is of the correct size to correspond to exon 2-derived transcripts, and its size is given to the right. The DNA marker used is displayed to the left.

**Key:**
- **→** Primer direction
- **Γ** Transcription start sites
In summary, in this section both cultured osteoblasts and liver have been demonstrated to produce exon 1- and exon 2-derived transcripts. Also it has been shown that alternate splicing occurs in both liver and osteoblasts and the presence of the full-length and alternately-spliced transcripts have been detected in this manner. However, it would not be possible to demonstrate the use of more downstream transcription start sites using RT-PCR since transcripts originating from more upstream start sites could be used as templates, along with the shorter transcripts.
Part 2 - Quantitative Comparison of IGF-I Transcripts

Northern Analysis

So far, I have demonstrated the presence of both exon 1- and exon 2-derived transcripts in osteoblasts, bone and liver using RT-PCR. The drawback of RT-PCR though, while it may allow the detection of minute concentrations of particular transcripts, is that quantitative analysis of the results is subjective. Therefore, in order to quantitate the particular transcripts present, with a view to studying transcription start site usage, different technologies were employed. In the first instance, Northern blots were performed in order to examine the heterogeneity of the IGF-I transcripts produced by osteoblasts, bone and liver.

Initially the integrity of the RNA was determined by visualisation of the 18S and 28S ribosomal bands after ethidium bromide staining of RNA gels (Figure 3.14) and shown to be intact. This RNA was transferred onto a nitrocellulose membrane (Appendix I) and hybridised to an IGF-I antisense riboprobe. The riboprobes (690 bases) were prepared from an insert cloned from IGF-I cDNA spanning the region from the Sau 3A restriction site upstream of exon 1 to a Nla IV site in exon 4 (Figure 3.15). The cDNA clone (500ng), was linearised with Eco RI and used as template for transcription, as described in Appendix I.

Northerns probed with the IGF-I riboprobes demonstrated the presence of 2 major species of transcript in 50-day old liver RNA (Figure 3.16). The transcripts were
Figure 3.14 - Ethidium bromide staining of RNA separated on a 1% agarose gel. This was performed in parallel to Northern analysis in order to verify the intact nature of the RNA. The two bands visualised represent the 18S and 28S ribosomal RNA bands and show little sign of degradation.

Figure 3.15 - The area spanned by the Sau 3A-Nla IV Riboprobe. Exon 1 transcription start sites (arrows) are shown in their appropriate positions. This construct was orientated within the vector so that anti-sense riboprobes could be synthesised from a T7 promoter. Refer to Appendix I for details of the methodology.
Figure 3.16 - Northern analysis performed on RNA from bone and liver RNA from 50-day old rats. The size of the transcripts visualised vary from ~7.4 kb to 1.6 kb. The pattern of expression is clearly different between bone and liver transcripts. For instance, bone produces transcripts of ~7.4 kb in length, which were not apparent in transcripts from liver. In contrast, liver tended to express shorter transcripts than those found in bone. For more details of the comparison of transcript expression, refer to the text.
represented by a discrete band of ~1.9 kb in length, and a broad band corresponding to longer transcripts between ~2.7 and 5.1 kb.

IGF-I RNA from bone, in contrast, was visualised as three bands. These included the ~1.9 and ~2.7-5.1 kb transcripts, the larger species appearing less intense than its counterpart from liver. However, a longer transcript of ~7 kb in length was detected in bone but not liver. Therefore, it appeared that while bone and liver were both producing heterogeneous transcripts, they were being synthesised in a tissue-specific manner.

The transcripts were analysed quantitatively, and the proportion of each species of transcript was found to differ between the two tissues. In liver, the ~1.9 kb transcripts, constituting ~15% of the total IGF-I transcripts, were ~7 times more prevalent than in bone from 50-day old rats (~2%) in which they are only a very minor type of transcript. The transcripts between ~2.7-5.1 kb comprised the majority in both tissues - ~85% in liver and ~75% in bone. However, the largest IGF-I transcripts of ~7 kb in length, were only found in bone from 50-day old rats where they constituted 20-25% of IGF-I transcripts.

In summary, the IGF-I transcripts detected varied in length from ~1.9 to ~7 kb. This heterogeneity in the transcript length may be due to alternate start site usage within exon 1 and 2, or the termination of transcription at different polyadenylation sites within exon 6. The length of the 5'-UTR due to different start site usage may vary between ~30 to ~900 bases. In contrast, exon 6, which spans over 6 kb, contains many putative polyadenylation signals. It is therefore evident that the smaller variations in the 5'-end of the transcripts are likely to be masked by the much larger differences in the 3'-UTR.
Thus, Northernss, which only allow the determination of the absolute length of the
transcript, do not permit the characterisation of start site usage.
RNase Protection Assays

i) Comparison of Start Site Usage by RNase Protection Assays

RNase protection assays were performed as a sensitive means of quantitatively elucidating different transcript production. As previously described, differences in transcripts may be detected by RNase protection assays. Specifically, the use of IGF-I antisense riboprobes, which span from Sau 3A, upstream of the start sites within exon 1, to Nla IV in exon 4 (Figure 3.15) allowed the elucidation and quantitation of all transcription start sites used within exon 1. The manner in which this is achieved is summarised in Figure 3.17. RNase protection assays are performed by allowing a radiolabelled riboprobe to hybridise to RNA. RNase is added which digests any single-stranded RNA, while leaving the duplex intact. In the case of IGF-I, the use of start sites 1 to 4 in the initiation of full-length transcripts (in other words, transcripts from which the 186 bases have not been excised) will result in the progressive truncation of the radiolabelled riboprobe. These are separated on a polyacrylamide gel and following autoradiography the presence of transcripts initiated from different start sites may be determined. Densitometry may also be performed on the autoradiographs from which the relative abundance of the transcripts may be calculated. Transcripts initiated from start sites 1 and 2 from which the 186 bases have been alternatively spliced, though, will only be visualised as one band. This is because the radiolabelled riboprobe will only hybridise to the transcripts from within the region coded by exon 4 as far as the 3’-splice junction transcribed from exon 1. In a similar manner, transcripts initiated from the different start sites within exon 2 will only be detectable as one band.
Figure 3.17 - A diagrammatic representation of the riboprobe used in the protection assay and the predicted fragments. RNase protection assays involve the annealing of an antisense radiolabelled riboprobe (the arrow) to transcripts. Addition of RNase results in the digestion of any single-stranded RNA. Multiple transcription start site usage, as depicted above will lead to the production of a multiplicity of truncated riboprobes, which may be separated by PAGE and visualised by autoradiography. The expected fragment sizes using the Sau 3A-Nla IV riboprobe are indicated to the right.
Figure 3.18 - Autoradiograph obtained from an RNase protection assay using liver RNA, using the method described by Lowe et al. (1987). The riboprobe was incubated in the absence of RNA, ethanol precipitated and loaded on the gel in the standard manner, as shown in the left-hand lane, marked probe. Riboprobe was also incubated with tRNA (marked tRNA) and RNased in the normal fashion, in order to confirm that the bands observed in the RNase protection assay of liver RNA (marked liver RNA) were not due to incomplete digestion of the riboprobe. Exon 1 transcription start sites 1 to 4 are marked on the left, along with those initiated from start sites 1 and 2 from which the 186 base deletion has been excised (deletion). Exon 2 has at least 3 start sites, but cannot be differentiated due to the probe used. A lengthy exposure was required in order to visualise transcription start site 1 within exon 1. The sizes of the fragments were ascertained from comparison with end-radiolabelled marker DNA and from the literature, and are given in bases to the right. The probe and tRNA lanes were exposed for 4 hours, while the liver RNA lane was exposed for 24 hours.
Protection assays on liver RNA extracted from 50-day old rats clearly show the bands produced (Figure 3.18). The top band at 530 bases represents transcripts initiated at start site 2 (ss2). The next two bands at 428 and 260 bases depict start site 3-initiated transcripts (ss3) and those from start sites 1 and 2 from which the 186 base region has been spliced (del), respectively. The band below these two, at 197 bases is produced by transcripts initiated from start site 4 (ss4), whereas the smallest band at 168 bases depicts exon 2-derived transcripts. Transcripts derived from start site 1 (ss1), at 573 bases in length, could only be visualised after much longer exposure.

Interestingly, no band was identified from RNase protection assays which would correspond to a transcript from which 60-80 bases had been deleted, as suggested from the RT-PCR amplifications of whole liver, bone and osteoblast RNA, confirming that this was an artefact (see section 1).

**ii) Confirmation of the Identity of RNase Protection Assay Fragments**

The identity of the protected bands was confirmed by performing a protection assay on six aliquots of liver RNA (40 μg each), and eluting the fragments from the gel individually. These were amplified by RT-PCR using the appropriate primers specific for each start site (see Appendix III). The RT-PCR products were ligated into a TA vector and sequenced using flanking primers (see Appendix I for methodology). Three examples of sequence obtained are given in Figures 3.19-3.21. Comparison of the sequence obtained with that previously published (Appendix III) revealed that the bands had been correctly identified.
Figure 3.19 - Sequence analysis to confirm the identity of the band corresponding to exon 1-derived transcripts initiated from start site 1.
Figure 3.20 - Confirmation of the identity of the RNase protection assay band representing transcripts initiated from start site 2 of exon 1.
Figure 3.21 - Characterisation of the band, obtained from RNase protection assays, representing exon 1-derived transcripts initiated from start site 3.
iii) RNase Protection Assay Optimisation

In my hands, the RNase protection assay procedure described by Lowe et al (1987) required surprisingly lengthy exposure before any bands could be visualised. For instance, IGF-I mRNA levels are known to be high in liver; however, bands could only be detected after exposure of between 24 to 48 hours for 20μg of RNA. Osteoblasts and bone were unlikely to contain a similar abundance of IGF-I mRNA and in order to minimise exposure-time, the conditions were optimised. This was undertaken as suggested by Lau et al (1992), using a range of RNase A:T1 concentrations between 40:2μg/ml to 10:0.5μg/ml respectively. Simultaneously, the incubation time was also varied from between 15 to 60 minutes.

The effects of these changes can be clearly seen in Figure 3.22. This figure shows an example of an autoradiograph obtained after 15, 45 and 60 minutes of digestion. The concentration of RNase A:T1 used in each panel was increased from 10:0.5μg/ml (used by Lau et al., 1992) to 40:2μg/ml as used by Lowe et al (1987).

The bands obtained after 15 minutes incubation, using the minimum amount of RNase, were most intense, and could be easily visualised after 24 hours exposure. However, using increasing concentrations of RNase led to a rapid reduction in signal. For example, when the highest concentration of RNase was used, the autoradiograph had to be exposed to film for at least four days to obtain a measurable signal, demonstrating that while the protected fragments were present, they were less abundant. In contrast, the duration of the digestion appeared to play a minor role. This resulted in a slight reduction of band intensity from that observed for each RNase concentration after 15 minutes digestion. For example, the autoradiograph obtained from samples digested...
Figure 3.22 - RNase protection assay optimisation. The RNase digestion protocol was optimised for time, from 15 to 60 minutes, and RNase concentration. The RNase concentrations used varied between RNase A: RNase T, 10 µg/ml:0.5 µg/ml to 40 µg/ml:2 µg/ml respectively. The optimisation assays were performed in parallel and the autoradiographs were exposed for the same length of time. It is clear that while the incubation time affects the intensity of the signal produced, the concentration of RNase used plays a more critical role. The conditions given by Lowe et al. (1987) involved the use of the highest concentration of RNase for 60 minutes, under which no bands are visible except after lengthy exposure. The start sites are denoted as ss l-4. Del refers to those transcripts from which the 186 base region has been alternatively spliced and exon 2-derived transcripts are denoted Ex 2. The protection assays shown in the three panels were exposed for 24 hours.
Figure 3.23 - Comparison of exon 2 band intensity with varying RNase concentrations for incubation periods of between 15 to 60 minutes. This graph compares the intensity of the band representing exon 2-derived transcripts after 15 minutes digestion with 10μg/ml RNase A:0.5μg/ml RNase T₁ (denoted maximal intensity) to all the digestion conditions used from autoradiographs exposed for identical periods of time. It may be clearly seen that increasing RNase concentrations results in a rapid diminution of exon 2 signal strength. Increasing digestion time also resulted in loss of signal intensity, but its effect was less marked. The RNase concentrations shown below the graph are given as μg/ml.
using the lowest concentration of RNase for 1 hour only required exposure to film for
one day to produce a signal of enough intensity to be measured densitometrically.
Simultaneously, experiments performed in the absence of RNA demonstrated that the
probe was completely digested under all the conditions used.

As may be inferred from above, the autoradiographs obtained were analysed
quantitatively. After an identical exposure time, the absolute intensity of the band
representing exon 2 was measured by densitometry. The maximum signal was obtained
after 15 minutes with 10µg/ml RNase A and 0.5µg/ml RNase T1. This was therefore
referred to as 100% signal strength. The intensity of the signals produced after
increased RNase concentration and incubation period was compared to this band and
plotted as the proportion of the maximum signal strength (Figure 3.23). It is clear from
the graph that increasing RNase in the digestion led to the almost complete ablation of
the signal. Similarly, increasing the incubation period diminished the intensity of the
band. However, even an incubation of one hour only reduced the signal by ~50% and
appeared to be less critical. Therefore 15 minutes digestion with 10µg/ml RNase A and
0.5µg/ml RNase T1 were chosen as sufficient to give complete digestion while
producing an intense signal, since increasing either the RNase concentration or
digestion period would necessitate longer exposure of the autoradiographs.

iv) Start Site Usage in Osteoblasts, Bone and Liver

Having optimised the technique, RNase protection assays were used as a means of
quantitatively elucidating different IGF-I transcript production from primary osteoblast
cultures, obtained from 28-day old female Sprague Dawley rats *. The pattern of start

* - refer to Addendum
site usage was compared to that found expressed in whole rat bone and liver (control) at 28 and 50 days of age (Figure 3.24).

In this example, qualitative analysis of protection assays performed on both bone (lanes 3 and 4) and osteoblast cultures (lanes 5 and 6) demonstrated the presence of transcripts initiated from start sites 2, 3 and 4 within exon 1 and exon 2-derived transcripts. However, in contrast to liver, these transcripts could only be visualised after long exposure which may indicate that they are expressed at lower levels. Even though the intensities of the bands is not directly proportional to the abundance of the transcripts, the pattern of start site use is clearly different between liver from 50-day old rats and whole bone/osteoblast RNA, while age has apparently little effect on transcript expression in liver (lanes 1 and 2). The most marked difference in this respect is the relative absence of exon 2-derived transcripts from bone and osteoblast culture in comparison to liver.

a) The proportion of exon 1-and exon 2-derived transcripts

Quantitative analysis of the protection assays was performed to elucidate the relative proportion of transcripts derived from either exon 1 or exon 2. Transcripts initiated at the start sites in exon 1 were therefore combined and compared to exon 2 usage, as represented in Figure 3.25.

At 28-days of age, liver tended to preferentially transcribe IGF-I mRNA from leader exon 1 (59% ± 5%). However, a large proportion of transcripts were initiated from start sites within exon 2 (41% ± 5%). In contrast, the majority of IGF-I transcripts in bone from 28-day old rats were derived from exon 1 (92% ± 2%) and only a small
Figure 3.24 - Examples of RNase protection assays performed using RNA from osteoblast-enriched cultures, bone and liver (control) from rats of 28- and 50-days of age. These protection assays demonstrate that all start sites are used in primary osteoblasts, whole bone and liver. Transcripts initiated from start site 1 could only be seen after lengthy exposure. However, while the pattern of expression appears to be similar for bone and osteoblast RNA, the intensities of the bands relative to each other appear to differ greatly between bone/osteoblast and liver RNA. For instance, the band representing start site 3-derived transcripts is the most predominant in bone and osteoblast RNA, while the band for exon 2 is the most intense from liver RNA. Therefore, the relative proportion with which each start site is used may be tissue-specific. This is discussed further in the text. The bands are labelled to the left as ss 2 - ss 4 referring to start sites 2-4, del as the alternatively spliced variant, and Ex 2 as exon 2-derived transcripts. The sizes of the bands are displayed to the right. The liver lanes were exposed for 24 hours, while the bone and osteoblast lanes were exposed for two weeks.
Figure 3.25 - A histogram showing the differences in the proportion of exon 1- and exon 2-derived transcripts in rat bone and liver at 28- and 50-days of age and osteoblast cells. In summary, this graph shows that the majority of the transcripts in bone and osteoblasts are derived from exon 1, while exon 2-derived transcripts comprise a much greater proportion of those expressed in liver. Also, age appeared to have little effect on leader exon usage. The data shown is the mean value ± S.D. (n≥5).
proportion of the transcripts were exon 2-derived (8% ± 2%). Transcripts originating from primary osteoblast cultures were also found to preferentially use leader exon 1 (92% ± 2%) in a similar manner to that seen in whole bone with correspondingly low exon 2-usage. In contrast to this inter-tissue variation, the effect of age on leader exon-usage in either bone or liver was minimal, and no significant difference was seen from rats at 28- and 50-days of age.

b) Start site usage within exon 1

Having ascertained that there was inter-tissue variation in the usage of leader exons 1 and 2, start site usage in bone from 28-day old rats was analysed and compared to liver (Figure 3.26). In order to study the differences in exon 1 start site use, the proportions with which each start site was used was calculated as a percentage of all exon 1-derived transcripts. Also, as already mentioned, transcripts initiated from start site 1 were often not of sufficient intensity to be measurable, even with long exposure. In liver (red bars), transcripts with the 186 base region deleted (deletion) predominated comprising about half the transcripts derived from exon 1 (47% ± 9%). Start site 4-derived transcripts were the next most prevalent, constituting 32% ± 7% of the transcripts using exon 1 as leader exon. In contrast, transcripts using start site 2 and 3 were both were poorly expressed and present in similar quantities (10% ± 3% and 11% ± 3% respectively).

There were several marked differences between the liver and bone transcription start site usage as epitomised by start site 3 usage and those transcripts with the 186 base region excised. Start site 3-derived transcripts in bone (blue bars) comprised half the transcripts initiated from within exon 1 (45% ± 11%), representing a significant increase
Figure 3.26 - Exon 1 transcription start site usage in liver and bone from 28-day old rats. The data presented are a comparison of the proportion of exon 1-derived transcripts initiated from each of the start sites within the exon, except start site 1 which could not be measured. In summary, the majority of transcripts in bone (blue) are initiated from start site 3, whilst liver (red) predominantly synthesises transcripts from which the 186 bases have been excised. The values plotted are the mean ± S.D. (n≥6)

Figure 3.27 - A comparison of exon 1 transcription start site usage in primary osteoblast culture and whole bone from 28-day old rats. The start site usage is plotted as a proportion of total exon 1-derived transcripts. There is a great deal of similarity in the pattern of expression between osteoblasts (yellow) and bone (blue). The values are plotted as the mean ± S.D. (n≥7)
from that found in liver. This was offset by a fall in the deletion IGF-I transcripts and those initiated from start site 4 which comprised only 24% ± 5% and 22% ± 9% respectively of exon 1-derived transcripts. The remaining start site, start site 2, was poorly expressed (<9%) and did not differ significantly from the proportion found in liver.

RNA derived from osteoblast cultures was compared to whole bone RNA from 28-day old rats so as to compare RNA from rats of similar age (Figure 3.27). Overall, exon 1 start site usage in osteoblasts (yellow bars) was very similar to that found in whole bone (blue bars). This was exemplified by the predominating use of start site 3 which did not differ significantly. In a similar manner to the expression of start sites in whole bone, the next most prevalent transcript type had the 186 base region excised (20% ± 3%). Although there was a slight increase in the proportion of start site 2-derived transcripts in bone cells (14% ± 5%) this did not differ greatly. Similarly, usage of start site 4 remained unchanged.

Although, there was no significant alteration in leader exon usage with age, transcripts could be differentially transcribed from particular start sites within the leader exons. Therefore transcription start site usage in liver from both 28- and 50-day rats was examined in order to ascertain whether any change occurred in the pattern of expression (Figure 3.28). Liver RNA from 50-day old rats (crimson bars) followed a broadly similar pattern of start site usage to that found at 28-days of age (red bars) and transcripts initiated from start sites 1 and 2 with the 186 base region excised still comprised the vast majority (47% ± 9%). However, the levels of start site 4-derived transcripts were significantly decreased in those from 50-day old rats (20% ± 12%)
Figure 3.28 - Comparison of exon 1 start site usage in liver RNA from 28- and 50-day old rats. The graph displays start site usage as a proportion of total exon 1-derived transcripts, having excluded exon 2-derived transcripts. Transcripts initiated from start site 1 were present in such low abundance as to be undetectable except after very lengthy exposure. There is a trend for rat liver to initially express transcripts from start site 4 and the alternatively spliced variants, the abundance of which decreases in favour of transcripts initiated from start site 2 and 3 later in life. 50-day old liver is shown in crimson while 28-day old liver is displayed in red. The data is presented as the mean value ± S.D. (n≥5)

Figure 3.29 - The affect of age upon exon 1 transcription start site usage in whole bone. The graph shown represents the amount with which the start sites are used as a proportion of exon 1-derived transcripts. Age appears to have little effect, and transcripts initiated from start site 3 predominate both at 28- (blue) and 50- (dark blue) days of age. mean ± S.D. (n≥6)
compared to 28-days of age. This was compensated by a corresponding increase in the amount of transcripts initiated from start site 3 (21% ± 8%). Similarly, the expression of start site 2 was slightly higher than in younger rats.

The effect of age on start site usage in liver was quite marked in that start site 4 usage decreased in older rats, while the proportion of start site 3-derived transcripts increased. Therefore the transcripts from bone at 28- and 50-days of age were similarly studied to ascertain whether those variations seen in liver were ubiquitous. The data is shown in Figure 3.29, and demonstrates the similarity in start site usage irrespective of age. Bone from 50-day old rats (dark blue bars) still predominantly expressed transcripts initiated from start site 3 (54% ± 3%) and while they appeared to be greater at 50-days of age compared to those at 28-days (blue bars), this was not significant. Transcripts with the 186 base region spliced out did not alter significantly with age and comprised 23% ± 3% making it the second most abundant type of transcript. However, the expression of start site 4-derived transcripts at 50-days of age (16% ± 3%) was significantly lower than that seen at 28-days, even though only a small decrease was involved. The remaining transcripts initiated from within exon 1 (start site 2), did not vary significantly with age and comprised less than 15% of exon 1-derived transcripts.

Overall, in liver, transcripts with the shorter 5'-UTRs (start sites 1 and 2 with the deletion and start site 4) are preferentially used rather than initiating full-length transcripts from start sites 1 and 2. In comparison, bone and osteoblast cultures tend to primarily express transcripts initiated from start site 3, which have long 5'-UTRs.
In summary, in this section it has been shown that a heterogeneous population of IGF-I transcripts is produced by bone, and in particular osteoblasts. Northern analysis has demonstrated that these transcripts may vary in length from ~1.9 kb to ~7 kb and that the pattern of expression may be tissue-specific. However, this technology did not allow the elucidation of start site usage, for which the more sensitive method of RNase protection assays was employed. This technique first needed to be optimised for both digestion duration and the concentration of RNase A and T1 used. Finally, it was demonstrated that all transcription start sites were used by osteoblasts/bone and liver, but that the proportion with which the particular sites themselves were used varied. Briefly, it appears that osteoblasts and bone tend to use transcription start sites which will result in long 5'-UTRs, whereas liver transcripts predominantly have short 5'-UTRs.
Discussion

In this chapter, it has been shown that both exon 1- and exon 2-derived transcripts are produced in bone and osteoblast cells from Sprague-Dawley rats. Furthermore, it has been demonstrated that these transcripts are expressed in a tissue-specific manner.

RT-PCR

RT-PCR was employed as a method of verifying the presence of IGF-I transcripts in bone and osteoblasts from which it was demonstrated that transcripts initiated from exon 1 start site 1, both with and without the 186 base region, were transcribed as were exon 2-derived transcripts. Unfortunately, the presence of transcripts initiated from exon 1 start sites, other than start site 1, could not be determined using this technique. For instance, start sites 2, 3 and 4 lie downstream of start site 1 and their sequence is therefore contained within transcripts initiated from start site 1. Therefore, when amplifying DNA using primers to a particular start site, such as start site 3, any fragment obtained could have been amplified from sequence internal to transcripts initiated from start sites 1 and 2, as well as those from start site 3 itself. A similar argument can be used in explaining the inability to distinguish between transcripts initiated from start sites 1 or 2 from which the 186 bases have been excised.

As mentioned above, the presence of exon 2-derived transcripts has been shown in osteoblasts and bone, using liver as a control, since the presence of exon 2-derived transcripts has been well characterised in this tissue (Adamo et al., 1991a and 1991b;
Figure 3.30 - A diagrammatic representation of the area containing the putative 60 base alternatively spliced site. The black bar denotes the area in which the 60-70 base deletion may be alternatively spliced. Interestingly, 67 bases from the 5'-splice donor site of the 186 base region, there is a putative splice acceptor site.
Foyt et al., 1991). However, while osteoblast and bone mRNA transcripts could be amplified using primers specific to the 5'-end of the exon, no fragments could be amplified using primers complementary to the most upstream start site. Transcripts initiated from this start site contain over 800 bases of exon 2, and will thereby have a long 5'-UTR. The inability to amplify fragments of the size corresponding to transcripts initiated from this site might indicate poor expression of these transcripts. Indeed, it has been shown that IGF-I mRNA initiated from this start site comprises less than 1% of total IGF-I transcripts (Adamo et al., 1991a).

Amplification using RT-PCR also appeared to produce a fragment which could correspond to a transcript from which 60-70 bases have been alternatively spliced near the 5'-end of the 186 base deletion in exon 1 (Figure 3.30). It was shown that this was not due to non-specific binding of the primers, since it was produced using three different upstream primers. Furthermore, there is a sequence (AG/AT - solid arrow) 60-70 bases downstream of the 5'-donor site within the alternatively spliced region which has a great deal of homology to the 3'-acceptor site (AG/AC). It is interesting to note that the 3'-acceptor site for the 186 base region does not conform to the consensus sequence (AG/GT). This divergence could be due to the alternately spliced nature of this region, since the consensus sequence may be more common in regions to be constitutively spliced.

However, the intermediate-sized fragment may be due to heteroduplex formation when the single-stranded amplified DNA re-anneals. In this manner a single strand of the full-length fragment may anneal to the complementary region on the fragment from which the 186 base region has been excised (Figure 3.31). This possibility may explain the
Figure 3.31 - The mechanism by which the middle fragment may be produced by heteroduplex formation. Initially, the same downstream primer is used to reverse transcribe all transcripts. This may then be amplified by PCR using an upstream primer. When using a primer combination flanking the alternatively spliced region, two products would be expected. However, it is possible that the 5'-3' strand of the long product could anneal to the 3'-5' alternatively spliced product. This would be separated on an agarose gel as a band of intermediate size. Furthermore, amplification of this heteroduplex would result in the production of the full-length and the alternatively spliced products along with the heteroduplex. The transcription start site is labelled as expected for start site 1 of IGF-1 mRNA and the primers are displayed as thick arrows both above and below the DNA strands (black lines). The region in the full-length product which would be alternatively spliced is shown in red.
ability to amplify the full length, the intermediate fragment, as well as the deleted length when taking the middle band DNA, eluted from the gel, through another round of PCR. It may also explain the lack of success in ligating the middle fragment DNA into TA vector. For example, following PCR when the heteroduplex re-anneals, the 186 base region will loop out. This heteroduplex could be ligated into TA, but once transfected into E.coli the cells’ repair mechanism may consider the 186 base loop to be damaged DNA and either copy the 186 base region into the short strand, or excise the loop (Lewin, 1995). The TA vector would thereby contain either the alternatively spliced or the full length fragments. Therefore when performing test-PCR to check for insert, only the products from the amplification of the deleted or full length transcripts would be apparent. Indeed, this hypothesis has recently been supported by data obtained from single-stranded conformational polymorphism (SSCP - data not shown) which indicated that the intermediate fragment comprised two separate length of transcript which corresponded in size to strands of the full-length and alternatively spliced transcripts.

**Northern Analysis**

In order to determine the heterogeneity of the transcripts produced in whole bone to a greater extent than was possible using RT-PCR, Northern analysis was performed. However, it was first apparent that while the same concentration of total RNA from both tissues was initially used, the signal obtained from bone required a much longer exposure to obtain a similar intensity to that obtained with liver. Therefore, the level of IGF-I expression as a proportion of total RNA synthesised is much lower in bone than liver, which is consistent with studies on other extrahepatic tissues including lung and intestine (Lund *et al.*, 1986). Data from Northern analysis also demonstrated that IGF-I
transcripts varying in size between 1.9 and 7kb were present in whole bone. In contrast, the longer transcripts were not detected in liver.

As already described, IGF-I transcripts are heterogeneous in both the 5′- and 3′-ends (Daughaday and Rotwein, 1989; Adamo et al., 1991a and 1991b; Hoyt et al., 1992). For instance, not only are there multiple transcription start sites and alternate splicing of the 186 base region in the 5′-UTR, but many putative polyadenylation sites are predicted within exon 6. Furthermore, exon 5 can be alternatively spliced. However, since exon 6 is approximately 6 kb in length (Hoyt et al., 1992), alternate polyadenylation site usage results in great variation in transcript length. This will mask the smaller variations in length, of between ~20 to 800 bases, caused by the heterogeneous initiation from different start sites and alternative splicing within the 5′-region of the transcripts. Therefore, when examining differences in the total length of transcripts expressed by bone and liver, the variability in length observed from Northern analysis is predominantly due to variations in the 3′-UTR.

The data obtained is consistent with observations that the transcripts vary in size enormously (Lund et al., 1986; Shimatsu and Rotwein, 1987b; Foyt et al., 1991). Furthermore, studies have demonstrated that the longer 7 kb transcripts predominate in both rat extra-hepatic tissues, which is also evident from our data (Hoyt et al., 1992; Lund et al., 1986). Studies of polysomal association of the transcripts, however, found that the 7 kb transcripts were abundant in the total RNA but were virtually absent from the polysomal fraction and the cytoplasm (Foyt et al., 1991). However, these transcripts have also been found associated with polyribosomes, as well as in the cytosol, which suggests that they may be functional transcripts (Hoyt et al., 1992).
They may be unstable, for instance, multiple destabilising \((U)_nA, A(U)_nA, U\)-rich motifs and inverted repeats have been localised to the sequence encoding the 7-7.5 kb transcripts (Hepler et al., 1990; Hoyt et al., 1992). Indeed this hypothesis is corroborated by the observation that the 7 kb transcripts have much shorter half-lives than the 1-2 kb transcripts (Hepler et al., 1990).

Indeed the 7 kb transcripts have been implicated in post-transcriptional regulation of IGF-I synthesis, by regulating the stability of the transcripts during protein deprivation. During protein starvation, serum IGF-I has been observed to fall (VandeHaar et al., 1991; reviewed by Thissen et al., 1994). Concomitantly, there was a substantial decrease in hepatic IGF-I mRNA (VandeHaar et al., 1991) which has been attributed to both a fall in the rate of transcription of the IGF-I gene (Hayden et al., 1994) and a specific decline in the 7 kb IGF-I transcripts, considered to be differentially regulated (Straus and Takemoto, 1990; Thissen et al., 1991). Interestingly, all transcripts appear to be co-ordinately decreased in fasting and energy-restricted rats (Straus and Takemoto, 1990).

Furthermore, while the differently sized transcripts in liver, ovaries and adipose tissue appear to be equally down-regulated when rats were rendered GH-deficient and returned to normal upon supplementation with GH (Roberts et al., 1986; Vikman et al., 1991; Malozowski et al., 1995), differential regulation has been observed under other circumstances. For instance, during late pregnancy in rats, there is a dissociation between GH and IGF-I levels. In part this is probably mediated through a decrease in the levels of the 7 kb transcripts, while the smaller transcripts appeared to be unaffected (Travers et al., 1993).
Transcripts of sizes between 0.7 to 7 kb have been characterised in other bone cell systems, such as osteoblasts from foetal rat calvaria (Delany and Canalis, 1995; Ernst and Rodan, 1991) and rat osteoblast cell lines (Ernst et al., 1989). In these systems, the longer 7 kb transcripts were characterised as also having a shorter half-life than the 1-2 kb transcripts (Bichell et al., 1993). Furthermore while oestrogen increased the expression of all transcripts in foetal calvarial osteoblasts and cell lines (Ernst et al., 1989) and bFGF, TGF-β and PDGF BB lowered the expression of all transcripts, 1,25 dihydroxyvitamin D₃ affects one size of transcript predominantly in mouse osteoblasts (Scharla et al., 1991). 1,25 dihydroxyvitamin D₃ has been observed to mediate some of its action through the inhibition of IGF-I synthesis and release. In part, this is achieved in osteoblasts through the specific down-regulation of the 7 kb transcript. Therefore, taking these examples together, it would appear likely that the stability of the 7 kb transcript may be regulated by the cell, which could be achieved by the 3′-UTR interacting with a physiologically-sensitive binding protein, in a similar manner to that postulated for the post-transcriptional regulation of TSH-β mRNA by T₃. This is thought to involve labile protein(s) binding to the 3′-UTR and thereby regulate destabilisation of the TSH-β mRNA (Leedman et al., 1995). In this manner, IGF-I transcripts could be stabilised (thereby allowing translation to proceed) or destabilised as the need for IGF-I increases or decreases.

**RNase Protection Assays**

As already explained, the large variability in the 3′-UTR of IGF-I mRNA, as observed in the Northernns, masks the heterogeneity found in the 5′-UTR of these transcripts.
However, one of the main aims of the project was to elucidate the heterogeneous nature of the 5'-end of IGF-I transcripts expressed in osteoblasts and bone. Therefore, RNase protection assays were employed as a means of ascertaining transcription start site usage, in a quantitative manner. In conjunction with this, RT-PCR was used to confirm the identity of the protected bands.

However, one of the main obstacles to using RNase protection assays as described by Lowe et al. (1987) was the length of time the film had to be exposed before the bands could be measured densitometrically. However, the concentrations of RNase A and T1, and the duration of incubation were optimised as described by Lau et al. (1993). The most favourable conditions were found to be RNase A (10μg/ml), RNase T1 (0.5μg/ml) for 15 minutes, the use of which resulted in strong signals from a short exposure, while still completely digesting the control radiolabelled RNA.

**i) Differential Transcription Start Site Usage**

From analysis of RNase protection assays, exon 1-derived transcripts were observed to comprise a much larger proportion of IGF-I transcripts produced in bone and osteoblasts than in liver. In contrast, exon 2-derived transcripts are poorly expressed in bone and osteoblasts, whereas expression is four- to five-fold greater in liver. These results are consistent with studies performed on other extrahepatic tissues such as testes, lung, stomach, muscle, heart, fat and spleen (Shemer et al., 1992; Butler et al., 1994; Zanconato et al., 1994).

Data from RNase protection assays has also demonstrated that exon 1 start site usage differs markedly between bone/osteoblasts and liver. In bone and primary osteoblasts,
transcripts from start site 3 predominate comprising approximately 50% of the exon 1-derived transcripts with the remainder being expressed in roughly equal amounts from all the other start sites. Therefore, the majority of transcripts present in bone and osteoblasts have a long 5'-UTR of ~250 bases or more. In contrast, start site 3 is poorly expressed in liver and half of the transcripts containing leader exon 1 are initiated from start sites 1 and 2 from which the 186 base region has been excised. The majority of the remaining transcripts are initiated from start site 4. From these observations it is apparent that the transcripts expressed in liver predominantly have short 5'-UTRs of between approximately 45 to 150 bases in length.

It is interesting to note that in rat liver as well as bone and osteoblasts, the level of expression of full-length transcripts initiated from start site 1 was very low. However, while these transcripts are almost undetectable using RNase protection assays, we have previously demonstrated with RT-PCR that these transcripts are expressed in osteoblasts. As has previously been indicated, the technique of RT-PCR allows the amplification of transcripts which are present at very low concentrations, from which only the presence or absence of the transcripts within the tissue may be determined. In contrast, RNase protection assays provide the means of determining the relative abundance of the transcripts, although the sensitivity achieved is not as great as that obtained using RT-PCR.

Also, using this riboprobe which is complementary to exons 1, 3 and part of 4 of the full-length IGF-I transcript, one can not distinguish between the use of start site 1 or 2 for those transcripts from which the 186 bases region has been excised. This is due to the underlying principles of RNase protection assays, namely that any single-stranded
RNA is digested by RNases while the double-stranded RNA is protected from digestion. Therefore, when a full-length riboprobe anneals to an alternatively spliced transcript, the riboprobe may be able to bind to both the 3'- and 5'-ends, but the 186 bases in the middle may loop out, providing a site of digestion for the RNases. This results in the production of a fragment of 260 bases in length irrespective of the start site used. Simultaneously, fragments of approximately 70 and 110 bases in length will be produced from alternatively spliced transcripts initiated from start sites 1 and 2 respectively. However, these transcripts will be very difficult to visualise from autoradiographs for two reasons. In order to achieve adequate separation of the fragments derived from full-length transcripts initiated from start sites 2 and 3, the polyacrylamide gel must be run for a great length of time. Due to this, these small fragments are likely to have run off the gel. Furthermore, the size of the fragments is such that they are unlikely to have a high specific activity. Therefore, in order to visualise them, the autoradiograph would need to be exposed for a long period of time.

As regards changes in start site usage with age in bone, the major difference was a significant decrease in start site 4 usage in 50-day old rats in comparison to those at 28-days of age. Concomitantly, there was an increase in the expression of transcripts initiated from start site 3. In comparison there was no appreciable alteration in the relative proportion of the remaining species of transcripts. Furthermore, liver exhibited a similar trend, with a slight alteration in start site usage, resulting in a greater proportion of the transcripts containing a long 5'-UTR.
ii) Exon 1 Start Site Usage in Hepatic and Extrahepatic Tissues

The data obtained from RNase protection assays of osteoblast RNA is consistent with previous studies performed on other extrahepatic tissues and liver. For example, exon 1-derived transcripts have been found to be present in all tissues studied thus far, but the proportion with which each transcription start site is used varied between tissues. For example, kidney and brain tend to use start sites 2 and 3, whereas in testes and lung transcripts are initiated predominantly from start site 3 (Shemer et al., 1992). Simultaneously, it has been demonstrated that the proportion of alternatively spliced transcripts is greatest in liver out of those tissues studied, while the amounts of full-length start site 1-initiated transcripts are found to be consistently low (Shemer et al., 1992; Adamo et al., 1991b).

iii) Exon 2-Derived Transcripts in Extrahepatic and Hepatic Tissues

In contrast to the ubiquitous nature of exon 1-derived transcripts, those initiated from exon 2 start sites are expressed predominantly in the liver but are detectable in testes, lung and stomach, albeit poorly expressed (Shemer et al., 1992; Foyt et al., 1991; Adamo et al., 1991b). Expression of exon 2-derived transcripts in other extrahepatic tissues, such as muscle and kidney, was almost undetectable (Shemer et al., 1992; Adamo et al., 1989). In addition, the manner with which exon 2-derived transcripts are expressed in liver and kidney exhibits a marked temporal pattern and appears to rise concomitantly with growth hormone induction (Shemer et al., 1992; Adamo et al., 1991; Adamo et al., 1989). This would, therefore, suggest an endocrine role for the initiation of exon 2-derived transcripts in the regulation of IGF-I synthesis, while transcripts derived from exon 1 may be more important in autocrine/paracrine regulation.
iv) Growth Hormone and Hepatic Transcript Expression

The increase in abundance of exon 2-derived transcripts has been linked to stimulation by growth hormone (Adamo et al., 1991b). This has been well established using hypophysectomised rats resulting in the rat being unable to effect growth hormone-mediated growth. Under these conditions, there was a large down-regulation of transcript production in both a leader exon- and start site specific manner which was found to be at least partially reversible after growth hormone-replacement. Specifically, following hypophysectomy, the proportion of transcripts derived from exon 2 were found to be preferentially down-regulated in comparison to those initiated from within exon 1, when compared to sham-operated animals (63-fold decrease as opposed to a 33-fold decrease). Hypophysectomy also led to an increase in the proportion of transcripts initiated from start site 4 and those alternatively spliced, with a concomitant fall in the full-length form and the transcripts initiated from start site 3 (Foyt et al., 1991).

In contrast, growth hormone administration mediated only a 7-fold increase in exon 1-derived transcripts while those initiated from within exon 2 were augmented 14-fold the levels after hypophysectomy (Foyt et al., 1991). Furthermore, mirroring the decrease in exon 1-derived transcripts following hypophysectomy, the increase in exon 1-derived transcripts was observed to be mostly due to an rise in the proportion of those transcripts initiated from start site 3 and the full-length transcripts (Foyt et al., 1991).

The major rise in circulating endocrine IGF-I, predominantly synthesised by the liver, which occurs several weeks into rat development, has also been correlated with the...
increase in growth hormone during puberty, as suggested by Salmon and Daughaday (1957) in their somatomedin hypothesis (reviewed by LeRoith and Roberts, 1991). Simultaneous to this, the abundance of exon 2-derived transcripts rose rapidly, accompanied by a smaller increase in exon 1-derived transcripts as demonstrated by Adamo et al. (1991b) using RNase protection assays.

However, this differential regulation of start site usage in the liver described above, both following hypophysectomy and during development, may not be due wholly to GH. Studies were also performed using rats (dw) which have a defect in the growth hormone releasing factor, and thereby have depressed levels of GH in serum, commonly seven-fold lower than in normal rats. Basal hepatic IGF-I transcript expression was approximately 50% of that found in normal rats for all transcripts. Furthermore, when normal or dw rats were stimulated with GH, all hepatic transcripts were increased co-ordinately, irrespective of whether they were derived from exon 1 or 2. Therefore comparing this co-ordinate decrease in expression with the differential regulation observed following hypophysectomy would suggest that the specific up-regulation of exon 2-derived transcripts may be due to another pituitary-dependent factor than GH (Butler et al., 1994).

Furthermore, IGF-I transcripts, both exon 1- and exon 2-derived in rat liver, may also be co-ordinately regulated in catabolic states. For example, fasting and diabetes lead to the ubiquitous down-regulation of all IGF-I transcripts (Adamo et al., 1991b). Interestingly, it has been proposed that the co-ordinate decrease in IGF-I transcripts during fasting may be in part due to a decrease in growth hormone receptor levels, in particular the high affinity binding sites (Baxter et al., 1981) resulting in a lack of
responsiveness to growth hormone. Indeed, while the data from hypophysectomised rats would be in disagreement with this hypothesis, it would be concordant with the observations made from $dw$ rats. For instance, fasting and diabetes were accompanied by a ubiquitous down-regulation of hepatic transcripts initiated from all start sites, as they were in $dw$ rats when compared to normal rats.

**v) Growth Hormone and Extrahepatic Transcript Expression.**

More recently, the actions of GH have been demonstrated to mediated through the autocrine or paracrine production of IGF-I. Several studies have been performed to examine the effect of GH upon total IGF-I transcripts expressed in extrahepatic tissues, unfortunately without examining any possible changes in transcription start site usage. For instance, as already described, GH has pronounced stimulatory effects upon IGF-I mRNA abundance in the uterus of rats (Malozowski et al., 1995; Murphy and Friesen, 1988) and in rat adipose tissue and isolated rat adipocytes (Vikman et al., 1991). Butler et al. (1994) also demonstrated expression of IGF-I transcripts could be up-regulated in skeletal muscle and kidney.

However, Butler et al. (1994) also examined changes in start site usage in the kidneys of $dw$ rats when the rats were treated with GH. In contrast to the ubiquitous up-regulation of all start sites in liver, transcription appeared to be differentially regulated in the kidney. Specifically, the increase in IGF-I mRNA was mediated through an increase in expression of transcripts from start sites 3 and 4 predominantly, and to a lesser extent the alternately spliced transcripts. Transcripts derived from exon 2 were not significantly increased, which was surprising from the observations from liver. It
would therefore appear that the proportion with which each transcription start site is used may be tissue-specific.

**vi) Tissue-Specific Regulation of Transcription During Development**

Tissue-specific differences in the expression may be easily observed from an examination of the developmental profiles of the transcripts, as demonstrated by Shemer *et al.* (1992) and Adamo *et al.* (1991b). Initially, the expression of IGF-I mRNA from liver was studied, from which it was ascertained that exon 1- and exon 2-derived transcripts were differentially expressed. Using RNase protection assays, transcripts derived from exon 1 could not be visualised until postnatal day 8, while exon-derived transcripts could not be seen until 15 days postnatally. Specifically, all exon 1 start sites were used, the majority of which were initiated from start sites 2 and 3. The abundance of transcripts derived from exon 1 rose uniformly from day 8 until day 50 at which point the study was finished. In contrast, expression of exon 2-derived transcripts increased rapidly later during GH-dependent growth and was therefore differentially regulated (Adamo *et al.*, 1991b). However, stomach, lung and kidney all displayed different developmental profiles. For instance in lung and stomach, while the transcripts were preferentially expressed from start site 3, the relative expression of the various IGF-I transcripts appeared to remain constant. In contrast to this ubiquitous regulation of start site usage, transcripts in kidney were initially (postnatal day 1) expressed solely from start site 3. From day 8 to 22 there was a sharp rise in transcript expression from start site 2, with a concomitant rise in exon 2-derived transcripts. This was followed by an increase in the synthesis of alternatively spliced transcripts (Shemer *et al.*, 1992).
This regulation at RNA processing is similar to that observed in several other genes including rat growth hormone/growth hormone binding protein (Domené et al., 1995), the rat glucocorticoid receptor (Gearing et al., 1993) and IGF-II (de Moor et al., 1995; Teerink et al., 1994; Nielsen et al., 1990) to name but a few. For instance, The rat GH receptor protein is identical to the GH binding protein except that the binding protein lacks the transmembrane and intracellular domains of the GH receptor, and instead has a short hydrophilic sequence of amino acids. The rat GH receptor/GH binding protein are both encoded from transcripts which have been alternatively spliced (resulting in transcripts of ~4.5 and ~1.3kb respectively), originating from a single gene. However, this gene contains at least five transcription start sites which appear to be regulated in a tissue- and developmentally-specific manner resulting in the production of many transcripts with varying 5'-UTRs. Furthermore, the expression of the transcripts encoding the receptors and binding proteins is tissue-specific and studies by Domené et al. (1995) have correlated the alternative splicing involved with the synthesis of either the receptor or binding protein with differential start site usage. Therefore, it appears that the 5'-UTR may control the expression of the two types of proteins.

The IGF-II gene, by comparison, only codes for one type of protein and yet the IGF-II transcripts may be encoded from four start sites. Specific promoter usage is both developmentally-regulated and tissue-specific and appears to alter the translatability of the transcripts synthesised (de Moor et al., 1995; Teerink et al., 1994; Nielsen et al., 1990). This is described in more detail below.
vii) Regulation of IGF-I in Osteoblasts

IGF-I transcript expression has been poorly studied in bone. However, we have demonstrated that the expression of transcripts from the various start sites is specific for osteoblasts and bone, in comparison to liver but occurs in a similar manner to that observed by Shemer et al. (1992) and Adamo et al. (1991b) in other extrahepatic tissues. IGF-I expression in bone and in particular osteoblasts, however, is known to be regulated by a number of different factors as well as developmentally (Bickle et al., 1994). For example, thyroid hormones T₃/T₄ up-regulate IGF-I production by the rat osteosarcoma cell line UMR-106 (Lakatos et al., 1993), as does E₂ in the rat calvarial osteoblast cell lines RCT-1 and RCT-3 (Ernst et al., 1989). However, the effects of E₂ have since been observed to be mediated through an increase in IGF-I mRNA synthesis (Ernst and Rodan, 1991). Similarly, Bichell et al. (1993) have shown that PGE₂ stimulates IGF-I transcript expression. It may also be inferred that PTH increases IGF-I synthesis in osteoblasts from the observations that PTH stimulates an increase in cAMP production (Ernst et al., 1989), which has been demonstrated to induce IGF-I mRNA synthesis (McCarthy et al., 1990). This induction of IGF-I synthesis may thereby mediate the anabolic actions of PTH on bone via osteoblasts.

In contrast to this stimulation of IGF-I transcription, many factors have been shown to reduce IGF-I mRNA in rat and human osteoblastic cells and cells obtained from primary culture of foetal rat calvaria. These include bFGF, TGF-β, PDGF-AA and PDGF-BB (Canalis et al., 1993). Indeed the effect of PDGF on IGF-I concurs with the study by Tashjian et al. (1982), in which PDGF was observed to stimulate bone resorption. Also, 1,25 dihydroxyvitamin D₃, which induces differentiation and inhibits proliferation, has been demonstrated to decrease IGF-I synthesis in clonally-derived mouse osteoblasts.
(Scharla et al., 1991). Similarly, the glucocorticoid cortisol, which decreases osteoblast proliferation, mediates its action through inhibition of IGF-I expression (Delany and Canalis, 1995; Swolin et al., 1996). Furthermore, the anti-inflammatory drug indomethacin, which inhibits tibial fracture repair, mediates its action, at least in part, through the decrease in IGF-I synthesis (Edwall et al., 1992).

Possible Regulatory Mechanisms

Bearing in mind that IGF-I in bone mediates the actions of many hormones and growth factors, the manner in which it is regulated is likely to be highly complex. This could involve regulation at the transcriptional level, post-transcriptionally or post-translationally.

i) IGF-I Promoters

As yet, the promoters for the start sites have not been identified and characterised fully. Indeed, exon 1 does not contain the sequence for core promoter elements, such as GC-rich elements, CAAT and TATA boxes (Thomas et al., 1994). GC and TATA boxes have been well characterised as being important in positioning RNA polymerases to transcription start sites. Indeed, mutations in the sequence of TATA boxes often result in variations of the transcription start point from its usual precise location (Lewin, 1995). GC-rich sequences and CAAT boxes, though, appear to regulate the efficiency of the promoter, and may increase promoter strength while aiding localisation of the RNA polymerase to the general area from which transcription will be initiated. However, CAAT boxes have also been demonstrated to associate with a number of regulatory proteins, some of which (the CAAT-displacement
protein for instance) prevent basal transcription factors from binding, thereby down-regulating transcription.

The lack of core promoter elements adjacent to the exon 1 start sites may indicate the presence of tissue-specific factors which may be responsible for directing RNA polymerase II to the tissues' preferentially used start sites. These may act by altering the secondary structure of the chromatin, thereby regulating the accessibility of the start site either to the transcription factors and RNA polymerase. A similar mechanism has been implicated in the regulation of tissue-specific transcription start site usage in the α2(I) collagen gene (Beck et al., 1991) as well as the rat serum albumin gene (Babiss et al., 1986) as observed from differences in DNase I hypersensitive sites. Therefore, it is likely that transcriptional regulation of exon 1 start site usage of IGF-I may be mediated through similar tissue-specific proteins interacting with the 5'-flanking region to the IGF-I gene.

In contrast to exon I, potential TATA and CCAAT-like elements are positioned 5' to the preferentially used transcription start site with in exon 2 (~30 and ~80 bases upstream of the start site, respectively). While the elements upstream of the major start sites display a great deal of homology to the consensus TATA and CCAAT sequences (TTAA and CCAAAT), the variation, especially of the TATA box may lead to the initiation of transcription from a cluster of local sites (Adamo et al., 1991a). These postulated promoter elements are found in the promoter regions of the majority of genes. However, it has previously been shown that exon 2-derived transcripts are present in very low abundance, if at all, in some extrahepatic tissues (Shemer et al., 1992; Adamo et al., 1991b). In contrast, these transcripts are highly expressed in liver.
Therefore, this tissue-specific transcription may implicate transcriptional control by further regulatory elements. Another possible regulatory mechanism may involve post-transcriptional regulation of exon 2-derived transcripts in a tissue-specific manner.

Indeed, as mentioned above, the expression of the α2(I) collagen gene is tissue specific and although the transcripts are synthesised in both bone and cartilage, α2(I) collagen is only synthesised in bone. This was due to tissue-specific alternate leader exon usage, leading to the production of bone- and cartilage-specific transcripts (Bennett et al., 1989). The cartilage-specific transcript, which resulted in the synthesis of a non-collagenous protein, however, arose from sequences which were preceded by putative CAAT and TATA boxes, which were likely to have basal promoter activity (Bennett and Adams, 1990). The ability of bone cells to ‘switch off’ transcription from a basal promoter, taken together with the data from alterations in the DNase I hypersensitive sites flanking the gene (Beck et al., 1991) could indicate a mechanism whereby IGF-I exon 2-derived transcripts may be regulated. It could be envisaged that a tissue-specific protein may bind upstream of the transcription start sites within exon 2, thereby altering the conformation of the chromatin, leading to the inhibition of transcription from the basal promoter.

In a similar manner to α2(I) collagen, human nitric oxide synthase is transcribed from disperse sites, even though there is a TATA box in the promoter region (Chu et al., 1995). Nitric oxide synthase itself is critical in the synthesis of nitric oxide at site of inflammation. With this role, it is not surprising that the expression of this enzyme may be up-regulated by cytokines such as IL-1/-6 and TNF-α. The manner in which this
regulation is mediated may, at least in part, be conferred through consensus sequences for three interferon-γ response elements. Therefore, there may be as yet uncharacterised response elements upstream of the transcription start sites in IGF-I which, when the cell is stimulated with the appropriate factor, may dictate specific start site usage.

**ii) The Significance of Transcripts with Different 5′-UTRs**

While there are many possible mechanisms whereby the synthesis of IGF-I transcripts can be regulated differentially, the overall effect is the production of a heterogeneous population of transcripts varying in their 5′-UTR in a tissue-specific manner. In turn, the relative expression of the different types of transcripts may have repercussions on both the rate of synthesis of IGF-I peptide and possible post-translational regulation of its export.

**a) Translatability**

*Upstream Start Stop Codons* - Studies have demonstrated that the short 5′-UTR-transcripts with the deletion excised were twice as abundant on polysomes as those transcripts still containing the 186 bases (Foyt et al., 1991). At the extreme 5′-end of the 186 base region (Figure 3.32), upstream of the Met 48 start-codon (boxed), are a pair of translation initiation codons immediately adjacent to a pair of termination codons (underlined). Using the scanning model of translation (Kozak, 1991), it was predicted that this would inhibit some of the 40S ribosomes from reaching the Met 48 site, in the full length transcripts. In contrast, those transcripts with the 186 bases excised would have a larger proportion of the 40S ribosomes binding to the cap reaching the translation initiation codon (Foyt et al., 1991). This proposal could also be relevant for those transcripts initiated from start site 3 (the arrow), with the difference that the
Figure 3.32 - Putative in-frame start/stop codons within the 186 base deletion in exon 1. The upper sequence gives the consensus donor and acceptor splice sites, with the asterisks showing the conserved residues. The underlined sequences denote the putative start/stop codons, while the arrow indicates the transcription initiation start site 3. Adapted from Foyt et al., 1991.
efficiency of translation would only be reduced due to one pair of translation
initiation/stop codons, instead of the 2 pairs for the full length transcripts initiated from
start sites 1 and 2. However, the AUGUGA sequence upstream of start site 3 appears
to play only a minor role since there is little enrichment of start site 3-derived transcripts
in the polysomal fraction in comparison to those initiated from start site 1 and 2, which
may be due to the poor context of the upstream AUG codon (Foyt et al., 1991), the
exon 1-derived transcripts in osteoblasts and bone are predominantly those which are
putatively poorly translated.

Recently, a correlation has been found between enhanced translatability and exon 1-
derived translatability lacking the 186 base region when compared to the full-length
transcripts (Yang et al., 1995). Therefore it is likely that both up- and down-regulation
of IGF-I is mediated to some extent through the 5'-end of the transcripts. However,
transcripts initiated from start site 4, which is positioned downstream of both stop
codons, are more prevalent at 28-days than at 50. If the shorter exon 1-derived
transcripts are more efficiently translated as is suggested, this may represent the
increased need of the growing rat to express IGF-I. By comparison, the adult rat may
have declining growth potential and therefore not require as much IGF-I.

Also, surprisingly, reduction in growth hormone levels resulted in an increase in the
proportion of those exon 1-derived transcripts found to be strongly associated with
polysomes, while restoration of growth hormone had the opposite effect, leading to the
preferential increase in those transcripts initiated from start site 3 which are found to be
poorly associated with polysomes and which have low translatability (Foyt et al., 1991;
Yang et al., 1995).
Two studies have been undertaken to examine promoter function within exon 1 or 2 in the human IGF-I gene using a luciferase reporter system and deletion analysis (Kim et al., 1991; Jansen et al., 1992). Specifically, a construct containing DNA from the 5′-flanking region of IGF-I including sequence up to the major translation start sites within the exons was spliced into promoterless luciferase plasmids. These plasmids were transcribed and translated in a luciferase expression system and the resulting luciferase activity measured. However the variations due to the presence of multiple transcription start sites within the leader exons would have resulted in heterogeneous transcripts with differing length and sequence within their 5′-UTRs. The multiplicity of 5′-UTRs thus produced may, in turn, have affected translatability, making the comparison between luciferase activity and promoter function very difficult to ascertain.

The presence of upstream AUG codons short open reading frames has been observed to affect the translatability of several other genes including murine complement factor B (Garnier et al., 1995), retinoic acid receptor-β2 (RARβ2) (Zimmer et al., 1995) and murine tissue inhibitor of metalloproteinases (TIMP) (Waterhouse et al., 1990). Specifically, murine complement factor B may be transcribed in two forms differing in their 5′-UTR, Bf short and Bf long, with the short transcript having approximately twice the translatability of the long in vitro. Contained within the long transcript are four short open reading frames. When the AUGs were mutated, the Bf long transcript was almost as translatable in vitro as the short transcripts. Also, in a similar manner to IGF-I, the inhibition of translation was found to mediated through one AUG codon primarily. However, in vivo, the mutated Bf long transcripts were still translated with half the efficiency of the short transcripts, indicating that other post transcriptional
processes may be involved in the regulation of expression of the complement factor B protein.

Similarly, both RARβ2, TIMP and nitric oxide synthase (Chu et al., 1995) may express heterogeneous transcripts, the longer ones of which contain upstream open reading frames which may regulate tissue-specific expression of their respective proteins. This type of regulation is therefore well characterised and is involved in the control of expression of several genes. Therefore for IGF-I transcripts this may be important, especially in cells and tissues, such as osteoblasts and bone which predominantly express transcripts initiated from start site 3 which contain the putatively poorly translated transcripts.

Other Possible Mechanisms for the Regulation of Translation - Another well-studied regulatory mechanism involves proteins interacting with the 5′-UTR and thereby regulate translatability. For instance, translation of ferritin transcripts may be regulated without the need for alterations to the transcription of the ferritin gene. Briefly, ferritin is an intracellular iron storage protein which may whose translation is regulated by the level of iron, such that when iron is present in high concentrations, ferritin translation is stimulated. The manner in which this translation is regulated is thought to be mediated through an iron binding protein called the iron regulatory factor (IRF) which binds iron when iron is present in high concentrations. This protein-iron complex has low affinity for the ferritin transcript. However, in low iron concentrations, iron dissociates from the IRF thereby allowing the IRF to bind to a sequence within the 5′-UTR of the ferritin transcript, the iron response element (IRE). Once bound the protein is thought to stabilise secondary structure within the 5′-UTR and thereby prevent the 40S ribosomal
subunit from scanning through the transcript to search for the initiation codon. Translation is thus inhibited (reviewed by Melefors and Hentze, 1993).

Translation of IGF-II transcripts may also be regulated by RNA-protein interactions. IGF-II, as already mentioned, may be transcribed as five different transcripts varying with four different 5'-UTRs. These untranslated regions are derived from differential start site usage and alternate splicing of leader exons. The four promoters are expressed in a tissue-specific and developmentally regulated manner. However, an extra level of complexity is involved since the interaction of proteins regulating translation appears to be transcript-specific. Furthermore, it also seems to be developmentally regulated (Teerink et al., 1994; de Moor et al., 1995). This type of regulation, as described for IGF-II and ferritin, is described more extensively in Chapter 4.

Another possible regulatory mechanism is characterised by somatostatin synthesis (Danoff and Shields, 1988). Somatostatin (SRIF) is transcribed in two forms, SRIF I or SRIF II. SRIF II is known to be poorly translated in pancreatic islets in comparison to SRIF I. This was thought to be due to the presence of octamer repeats within the 5'-UTR of SRIF II, since their progressive removal led to a concomitant increase in translatability of the transcripts in vitro.

It is therefore possible that the tissue-specific expression of IGF-I transcripts, with varying 5'-UTRs, could have repercussions upon the efficiency with which the various transcripts are translated.
b) Alternate signal sequences

Another important consequence of the initiation of transcripts from multiple start sites within exons 1 and 2 is the production of different polypeptides initiated from many possible translation start codons (Figure 3.33). While each of these polypeptides contains the 70 amino acids of the IGF-I protein, each also encodes alternative signal sequences.

Translation of Exon 1-Derived Transcripts - Transcripts initiated from start sites 1, 2 or 3 may use the translation start site within exon 1. The propeptide produced would have a 48 amino acid signal sequence. However, propeptides made from transcripts initiated at start site 4 would have a 22 amino acid signal sequence derived from within exon 3. Interestingly, upstream of the Met 48 start codon is a nono-nucleotide sequence with a high degree of complementarity to the 3'-end of the 18S ribosomal subunit. It has been suggested that base pairing may occur between some eukaryotic mRNAs and the 3'-end of the 18S subunit, which may then play a role in translation initiation (Jansen et al., 1983).

Translation of Exon 2-Derived Transcripts - Transcripts derived from exon 2 may also be translated from a multiplicity of initiation codons resulting in propeptides of 22-72 amino acids in length. However, since the vast majority of the transcripts are initiated from start sites 2 or 3, the 32 or 22 amino acid signal sequences will predominate.

The consequence of the differences in signal peptides has not been shown, but it may lead to some translation products being differentially exported or stored. In the case of IGF-I, Yang et al (1995) have demonstrated that all the propeptides are processed into
Figure 3.33 - A diagrammatic representation of the positions of the many initiation codons within exons 1, 2 and 3 of IGF-I mRNA. The initiation codons (lines) are positioned with respect to the transcription start sites (arrows) and the alternatively spliced region within exon 1. These may result in the production of multiple signal sequences which may affect post-translational processing. Only exons 1-3 are represented. For a general view of the IGF-I gene see Figure 1.1.
the same mature protein. However, it may play a role in post-translational processing of IGF-I as has been described for glucose-induced insulin synthesis. Briefly, Welsh et al (1986) demonstrated that stimulation with glucose enhanced the interaction of the signal sequence of insulin with the signal recognition particle. The importance of this type of regulation is hard to gauge, though, since so few examples have been characterised.

Summary

In summary, multiple IGF-I transcripts are produced in bone and osteoblasts and are expressed in a manner particular to this tissue. The mechanism controlling the tissue-specific pattern of transcripts may partly involve the regulation of transcription factors and also other proteins, such as enhancers may also play a role in directing specific start site usage and thus determine the length of the 5'-UTR. In this manner, the different start site used may also determine which of the translation start sites is used, thereby determining the length and sequence of the signal sequence. The diversity of possible signal sequences may be important in regulating the post-translational modifications of IGF-I and its passage through the cell. The 3'-UTRs, in comparison, may be important in regulating the intracellular localisation of the transcripts or their half-life.

In this manner, while the different 5'-UTRs may determine the translatability of the transcripts, which is potentially poor in bone and bone cells due to start/stop codons predominating within their transcripts, the length of the 3'-UTR in bone and osteoblasts (from Northern analysis) would suggest that a large proportion of the transcripts either has a short half-life, or is localised in the nucleus, where it cannot be translated.
Therefore the protein may be poorly expressed. This is in contrast to liver transcripts and may reflect the paracrine/autocrine production of IGF-I by bone and osteoblasts.
Chapter 4

RNA-Protein Interactions in IGF-I
Introduction

As described in Chapter 3, the heterogeneity in the 5′-UTR of IGF-I transcripts is generated from alternate leader exon usage, multiple transcription start sites and alternate splicing. Also, the proportion with which each type of transcript is expressed is tissue-specific suggesting an important role for post-transcriptional regulation of IGF-I production. In Chapter 1, I described in detail the major post-transcriptional points at which translation of RNA transcripts may be regulated. This chapter focuses primarily on the role of the 5′-UTR in the regulation of translation and the role of RNA-binding proteins.

Translational Regulation

The rate of translation may be regulated in a number of ways, and this control is predominantly mediated through the regulation of initiation and elongation of polypeptide synthesis. For example, ferritin mRNA is one of many transcripts whose translatability is controlled at initiation of translation (reviewed by Melefors and Hentze, 1993). In a similar manner, insulin stimulation of adipocytes has been demonstrated to affect ribosomal associations with mRNA and thereby control protein synthesis in a global manner (Pause et al., 1994). Co-ordinate activation of dormant mRNA (Bachvarova, 1992), mediated through the stimulation of mRNA-ribosomal interactions is a further example of translation controlled at the initiation stage. In contrast, examples of the regulation of translation mediated through variations in the rate of elongation, have been implicated in the increased translational yield of insulin (Welsh et
Global and Transcript-Specific Control of Translational Efficiency

Regulation of the rates at which proteins are synthesised offers a flexible and rapid control mechanism, such that the cell can quickly enhance or reduce protein production. Control of this kind, though, may be divided into two regulatory systems - i) global control of translation; ii) mRNA-specific translational regulation.

i) Global Control

Regulation of this nature involves changes to translational initiation, elongation and accessory proteins. Since these factors tend to be ubiquitous to the translational machinery, their effects are felt upon all translational processes. For example, initiation is known to be a rate limiting step in translation and is thus an important regulatory point (Pause et al., 1994). Therefore, control mechanisms described to date usually involve modifications to translation initiation factors, such as eIF-2α and eIF-4E. For instance, it is known that eIF-2α is known to become phosphorylated following heat shock, viral infections and heme-depletion (Hershey, 1989). Translation may also be regulated by inhibiting the formation of the eIF-4F complex by, for example, dephosphorylation of eIF-4E as has been shown during serum starvation (Kaspar et al., 1990). Furthermore, a regulatory protein has been implicated which may reversibly bind eIF-4E and thereby inhibit cap-dependent translation (Pause et al., 1994). Regulation of

al., 1986), ornithine aminotransferase (Muekler et al., 1983), β-glucuronidase (Bracey and Paigen, 1987) and gonadotropin-releasing hormone receptors (Tsutsumi et al., 1995).
this kind tends to be mediated through the 5′-UTR, although the translational inhibition of maternal mRNA in oocytes involves ‘masking proteins’ binding to the 3′-UTR.

ii) mRNA-Specific Translational Control

The most characterised transcript whose translation is regulated post-transcriptionally through the 5′-UTR, is ferritin mRNA (reviewed by Melefors and Hentze, 1993). Other examples of such post-transcriptional regulation include IGF-II (Teerink et al., 1994), retinoic acid receptor-β2 (RAR-β2) (Zimmer et al., 1994) and murine tissue inhibitor of metalloproteinases (Waterhouse et al., 1990). The 5′-UTR has also been implicated in the auto-regulation of translation of, for example thymidylate synthase and dihydrofolate reductase (Standart and Jackson, 1992).

Regulation of translation by RNA-protein interactions in the 5′-UTR is now recognised as an important mechanism for control of protein synthesis. The critical role of RNA-binding proteins in the regulation of translation has been demonstrated for many genes, including IGF-II (de Moor et al., 1995), ornithine decarboxylase (Manzella and Blackshear, 1994) and thymidylate synthase (Johnson, 1994). Importantly, these regulatory proteins may be expressed in a tissue- and/or developmentally-specific manner.

The process by which these regulatory proteins affect translation may follow the paradigm of ferritin. Briefly, in low-iron concentrations, a protein which responds to iron concentrations, binds to secondary structure within the 5′-UTR of the ferritin mRNA which, in turn, prevents the 40S ribosomal subunit from scanning along the transcript and inhibits translation (Melefors and Hentze, 1993).
Methods for Studying RNA-Protein Interactions

Many methods have been employed to study these RNA-protein interactions, including gel shift and UV-protein crosslinking assays. Gel shift assays involve the incubation of cytosolic proteins with radiolabelled RNA. The samples are then separated on non-denaturing polyacrylamide gels and the bands visualised by autoradiography. Any proteins binding to the radiolabelled RNA would result in the retardation of the complex with respect to the unbound control RNA. However, the apparent molecular mass of the protein cannot be determined using this method and it has also previously been found, in our laboratory, that there is much non-specific binding of proteins to the RNA. This has made gel shift assays very uninformative in comparison to the other main technique, UV-crosslinking.

This assay also involves the incubation of cytosolic proteins with radiolabelled RNA. The proteins are crosslinked to the RNA using UV-radiation, after which the exposed or unprotected RNA bases are digested with RNase. In this manner, any bound protein is effectively tagged with radiolabel, and may be separated on SDS-PAGE and visualised by autoradiography. A major benefit of this method over gel shift is that the protein may be separated according to molecular mass, making the results easier to interpret.

Aims

As described in Chapter 3, the regulation of IGF-I synthesis is complex, and regulation of its synthesis may mediate the endocrine, paracrine and autocrine roles of many
hormones and factors. While regulation through alterations in IGF-I transcript production has been characterised, the possible role of specific, regulatory protein interactions with the various 5'UTRs has not been studied.

Therefore, the aims of this chapter were to elucidate any protein interacting with the 5'UTR of IGF-I. In order to achieve this UV-crosslinking was performed using liver cytosolic proteins and probes representing exon 1-derived IGF-I transcripts in the first instance. This was to be followed up with the use of cytosolic proteins from the rat osteosarcoma cell lines ROS 17/2.8 and UMR-106 in order to determine whether there was any tissue-specificity of regulatory protein expression. Furthermore, progressively truncated RNA probes, and one from which the 186 base region was excised, were to be employed, broadly representing the exon 1-derived transcripts present in rat cytosol. These were used in order to determine whether proteins bound to these sequences and whether they bound specifically to a particular 5'UTR. Excess unlabelled RNA was also added as competitor to determine the specificity of any interactions.
Methods and Results

In order to determine whether any proteins bind to the 5'-UTR of IGF-I mRNA, our aim was to UV-crosslink liver cytosolic proteins from 28-day old Sprague Dawley rats to radiolabelled riboprobes. The riboprobes synthesised would contain progressively less of the 5'-UTR so as to characterise the region to which the proteins may be binding. Subsequently, our aim was to examine the manner in which proteins from the rat osteosarcoma cell line, ROS 17/2.8, may bind to the radiolabelled transcripts, and compare the profile obtained to that obtained from liver cytosolic proteins.

Therefore, in order to achieve this, RT-PCR was performed to obtain fragments progressively truncated in the 5'-end. These fragments were ligated and subcloned into a vector from which radiolabelled transcripts could be transcribed. Cytosolic proteins were obtained, UV-crosslinked to the radiolabelled transcripts which were digested with RNase. The radiolabel-tagged proteins were separated according to size on SDS polyacrylamide gels. The manner in which this was performed is described in detail below.

Preparation of RNA Constructs

i) RT-PCR of Liver RNA

A range of fragments were amplified by RT-PCR of liver RNA. This was performed as described in Appendix I using the primers shown in Figure 4.1a. The constructs produced are shown diagrammatically in Figure 4.1b. Two constructs, both with and
Figure 4.1a - A diagrammatic representation of RT-PCR primers and their orientation. Primers 1, 2 and 3 were used in conjunction with primer 4 in order to amplify four fragments termed long, deletion, medium and short. These were ligated into pGEM*-3Z which contains a T7 promoter site from which sense RNA, both labelled and unlabelled, could be synthesised.

Key: → Primer direction
     † - Transcription start sites

Figure 4.1b - A schematic representation of the RNA constructs used in crosslinking experiments. The numbers shown indicate the distance in bases from the 5'-end of exon 1.
Figure 4.2 - RT-PCR amplification of liver RNA using upstream primer 3 and downstream primer 4. This is represented diagrammatically below. Marker sizes are given to the left, and the fragment size is shown to the right. Lanes 1 and 2 are molecular weight markers; lane 3 is a control RT-PCR lane with no RNA; lane 4 is the sample lane.

Key: → - Primer direction
    ↪ - Transcription start sites
without the alternatively spliced region, were amplified using upstream primer 1 and downstream primer 4 and were termed the long and deletion constructs respectively. Those constructs amplified using upstream primers 2 and 3 in combination with downstream primer 4 were referred to as medium and short constructs. An example of a fragment amplified using upstream primer 3 and downstream primer 4, separated on a 2% agarose gel is shown in Figure 4.2. Lanes 1 and 2 are DNA size markers, lane 3 is a control lane in the absence of RNA and lane 4 shows a fragment of 161 bases in length, corresponding to the amplification of the short construct.

ii) Cloning and Subcloning the Amplified DNA

The methods used in cloning and subcloning are given in more detail in Appendix I. Briefly, the fragments were ligated into the linearised pCR™II vector (Invitrogen) and transfected into HB101 competent cells which were grown on agar plates containing ampicillin in order to select for those cells containing the vector. The presence of insert was verified by PCR using primers flanking the multiple cloning site, which adds a further 241 bases onto the fragment size generated. An example of the PCR products thus amplified and separated on a 2% agarose gel is shown in Figure 4.3. Lanes 1 to 4 and 6 to 19 show products of 578 bases in length obtained from clones transfected with the deletion construct ligated into the pCR™II vector. In lane 5, a fragment of 241 bases has been amplified corresponding to a clone transfected with pCR™II vector into which no insert has been ligated. Lane 20 is a control PCR lane in the absence of DNA.

The TA vector is a convenient plasmid for cloning PCR fragments, due to the ease and simplicity of the methodology. However other plasmids are better designed for the purpose of transcribing a cloned insert. This is due to due to TA plasmid having a long
Figure 4.3 - PCR using primers flanking the multiple cloning site in order to verify the presence of insert within the TA plasmid. Representative marker sizes are given in base-pairs to the left, and the 'deletion' fragment size is shown to the right. Lanes 1 to 19 are samples; molecular weight marker VI (Boehringer Mannheim) is shown in lane M; lane C is a control PCR lane with no DNA added.
stretch of DNA between the T7 promoter and the multiple cloning site (MCS). This would be included in the transcript generated, and may thereby interfere with the binding of sequence-specific proteins. The use of plasmids, such as pGEM®-3Z (Promega) which contain only six bases between the Eco RI site in the MCS and the T7 promoter, would minimise this problem (see Appendix III for the maps of the plasmids).

Therefore, the insert was removed from the plasmid using Eco RI and an aliquot separated on a 2% agarose gel. An example of digestion of plasmid containing the long and medium constructs is shown in Figure 4.4. Lanes 1 and 2 contain DNA digested with Eco RI to produce 2 bands. The upper band of approximately 4 kb represents plasmid DNA, while the band of 538 bases in length comprises the long construct DNA. Molecular size markers are shown in lanes 3 and 4. Lanes 5 and 6 also contain 2 bands, the plasmid DNA and a smaller band of 351 bases representing medium construct DNA.

An approximately 3:1 molar ratio of insert to vector pGEM®-3Z (Promega), pre-digested with Eco RI and dephosphorylated, was used in the ligation, in which 500ng of vector was used. The ligation was performed in the presence of 1 x T4 ligation buffer and T4 DNA ligase (1 Weiss unit/10µl) and incubated for 16 hours at 4°C. Competent HB101 E.coli cells were transfected with ligated insert and vector (40ng) and grown on agar plates containing ampicillin.

Since the aim was to transcribe sense RNA from the T7 promoter, the orientation of the insert within the plasmid had to be verified. Therefore, PCR was performed using DNA from the isolated clones using an upstream primer complementary to the T7 site in
Figure 4.4 - Ligated insert-plasmid DNA digested with Eco RI and separated on 2% agarose gels to purify the insert DNA. Lanes 1, 2 - long DNA; 3, 4 - markers; 5, 6 - medium DNA.
pGEM®-3Z and the downstream primer, internal to the insert, primer 4. This allowed the identification of clones containing plasmid with the insert in the correct orientation.

Two examples of this selection method are shown in Figures 4.5a and 4.5b. In the upper panel (Figure 4.5a), the numbered lanes contain the products amplified by PCR from different clones transfected with long construct ligated into pGEM®-3Z. Fragments of 554 bases would be predicted if the insert was in the sense orientation, whereas no fragment would be amplified either if the insert was ligated in the antisense orientation or in the absence of any insert. Therefore, clones in lanes 1, 3, 4 and 6 to 9 contain the desired construct. The lower panel (Figure 4.5b) is a similar gel obtained by PCR of clones transfected with the medium construct ligated into pGEM®-3Z. The bands of 367 bases in length in lanes 3 to 5, 9 and 10, are of the correct size to represent clones containing medium insert ligated in the required orientation. The sequence of the clones was verified using Taq DyeDeoxy Terminal Cycle Sequencing (ABI/Perkin Elmer - see Appendix I).

Having confirmed the orientation, the plasmids (~ 2μg/μl) were linearised using Bam HI (1 unit/μl) in the presence of buffer B, incubated for 16 hours at 37°C and PIC- and IC-extracted twice and ethanol precipitated (Appendix I). The DNA pellet was resuspended in ddH2O to a final concentration of 500ng/μl.

**iii) RNA Transcription**

Radiolabelled IGF-I sense riboprobes were synthesised from the inserts ligated in pGEM®-3Z, from the T7 transcription promoter. In order to verify that the RNA constructs were intact, the radiolabelled constructs were separated on 3.5% non-
Figure 4.5 - PCR using T7 upstream primer within pGEM®-3Z vector and downstream primer 4 within insert in order to verify presence and orientation of insert. Lanes 1-12 (a) are long insert; lanes 13-24 (b) are medium insert; molecular weight markers V and VI (Boehringer Mannheim) are shown in lanes M. The fragment size is shown to the right.
denaturing PAGE, an example of which is shown Figure 4.6. Long, medium and short radiolabelled constructs are shown in lanes 1, 2 and 3 respectively. The intense bands indicated by arrows to the left represent the full-length constructs. The smaller fragments seen in each lane are due to premature termination of the transcription reaction. However, the intensity of the band representing the intact construct in comparison to the truncated products indicated that elution of the intact construct was unnecessary since the majority of the constructs were full-length.

For competition studies, unlabelled RNA was necessary, which was prepared in the same manner but in a total reaction volume of 100μl. All reagents were correspondingly increased, except 2μg of template DNA was used and all four unlabelled radionucleotides were present at the same concentration (0.5mM).

**RNA-Protein UV Crosslinking**

Cytosolic proteins were extracted from liver tissue (~7g) and rat osteosarcoma cells ROS 17/2.8, as described in Appendix I. Radiolabelled RNA (6ng, ~700,000cpm) was incubated with and without cytosolic proteins (liver - 50μg, ROS 17/2.8 2 - 8μg) for 20 minutes at 30°C in HEPES (12mM, pH7.9), KCl (15mM), tRNA (0.25μg/μl), dithiothreitol (DTT - 0.2mM) and glycerol (0.1 volumes) to a final volume of 20μl. The samples were irradiated with ultraviolet radiation for 5 minutes at 3000μW and 4°C in a Ultraviolet Crosslinker CL-1000 (UVP). The samples were digested with RNase buffer to the final concentration of EDTA (0.22mM, pH8.0), RNase A (0.43μg/μl) and RNase T₁ (0.22units/μl) at 37°C for 30 minutes, after which the proteins were separated on
Figure 4.6 - Radiolabelled long, medium and short riboprobes (lanes 1, 2 and 3 respectively). These were separated on a 3% denaturing polyacrylamide gel to verify the integrity of the probes prior to use.
12.5% SDS-PAGE with a 3% stacking gel. In competition experiments, unlabelled RNA was added in molar excess prior to cross-linking.

The ratio of RNA construct to cytosolic protein was optimised, from which it was ascertained that 50μg of liver cytosolic proteins cross-linked to 6ng of radiolabelled RNA resulted in the production of clearly-defined bands.

i) The Protein-Binding Profiles of the Short, Medium and Long Constructs

In order to ascertain where specific proteins may be binding, the binding patterns obtained from progressive deletion of RNA constructs (long, medium and short) were compared. Typical results are shown in Figure 4.7.

Lanes 1 and 2 were obtained using the long radiolabelled RNA construct. In the absence of any liver cytosolic proteins (lane 1), no bands were visible. Upon the addition of cytosolic protein, four bands were predominantly detected, with apparent molecular weights of 63 kDa, 48 kDa, 38 kDa and 28 kDa. The top and bottom bands at 63 and 28 kDa respectively were the most intense, while the 48 and 38 kDa bands required longer exposure times to be clearly seen. A very similar binding profile was obtained for medium RNA (lanes 3 and 4). In contrast, only two bands, representing 63 and 28 kDa proteins, could be detected after short exposure following addition of liver cytosolic proteins to short constructs (lane 6). However, longer exposure revealed the presence of two further proteins of 48 and 38 kDa.
Figure 4.7 - Comparison of autoradiographs obtained from crosslinking liver cytosolic proteins to long, medium and short radiolabelled RNA constructs. The amount of cytosolic protein is indicated along the top (µg) with the radiolabelled construct used. In the absence of liver cytosolic protein, no bands were apparent. However, addition of protein consistently resulted in the appearance of at least four bands. The apparent molecular weight of the proteins visible are marked to the right.
Therefore, of the three sequences of radiolabelled RNA synthesised, all bound four proteins of the same apparent molecular weight, but the binding characteristics of the proteins appeared to vary.

**ii) Competition Studies Using Liver Cytosolic Proteins**

Studies of the effects of adding increasing amounts of competitor sense unlabelled RNA, were performed in order to test the specificity of the RNA-protein interactions. If specific, a proportion of the liver cytosolic proteins binding to the sense labelled RNA probe would bind to the competitor instead, resulting in a decrease in the intensity of the band representing the protein. In addition, the molar excess of unlabelled RNA required to reduce binding would act as an indicator of the relative affinity of the protein for the RNA probe.

**iii) Determination of Specificity of Binding**

*a) Long labelled construct competed with long unlabelled RNA* - A typical example of an autoradiograph of long labelled competed with unlabelled RNA is shown in Figure 4.8. No bands are visible in the first lane, in the absence of any protein. In the presence of cytosolic protein with no competitor (lane 2), the four previously described bands were visualised, at apparent molecular masses of 63, 48, 38 and 28 kDa. Unlabelled competitor RNA was added in 1-, 10-, 50- and 100-fold molar excesses, as shown in lanes 3, 4, 5 and 6 respectively. Addition of increasing amounts of unlabelled RNA resulted in a marked diminution of the intensity of both the 63 and 48 kDa bands. Indeed, it is apparent that addition of 10-fold molar excess of competitor diminished the intensity of the 63 kDa band, while 50-fold excess almost completely ablated the signal representing the protein. The 48 kDa protein, in contrast, required a greater molar
<table>
<thead>
<tr>
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<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>0</td>
<td>1</td>
<td>10</td>
<td>50</td>
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</tr>
</tbody>
</table>

**Figure 4.8 - An autoradiograph of liver cytosolic proteins crosslinked to long radiolabelled probes.** Cytosol and molar excesses of long unlabelled RNA were present in the incubation as shown at the top of the photograph. In the absence of cytosolic protein (lane 1), no bands were visible. Addition of protein led to the production of four bands (lane 2). Increasing concentrations of long unlabelled RNA as competitor (lanes 3 to 6) resulted in a gradual disappearance of the 63 and 48 kDa bands while the 38 and 28 kDa bands appeared to be unaffected. The apparent molecular weights of the proteins are shown to the left.
excess of unlabelled RNA to compete for the labelled construct and the band was only markedly diminished between 50- and 100-fold excess. In contrast to this, the 38 and 28 kDa bands appeared to be unaffected by increasing amounts of competitor, even up to 500-fold excess (data not shown). Therefore the 38 and 28 kDa bands were proposed to be due to non-specific binding of proteins to the radiolabelled RNA.

b) Medium labelled construct competed with medium unlabelled RNA - An example of an autoradiograph obtained is shown in Figure 4.9. As described above, upon addition of the proteins (lane 2), in the absence of competitor, four bands were visible corresponding to proteins of apparent molecular mass 63, 48, 38 and 28 kDa. The 28 kDa band was the most intense, followed by the 63 kDa band while both the 48 and 38 kDa bands could only be visualised after long exposure.

Competitor was added in 1-, 10-, 50-, 100- and 1000-fold molar excesses as shown in lanes 3 to 8. In a similar manner to that of long labelled construct competed with long unlabelled RNA, there was rapid reduction in intensity of the 63 kDa band when the medium labelled RNA was used as the probe. Addition of between 1- and 10-fold molar excess markedly diminished the intensity of the band, and at 100-fold excess, the band could no longer be visualised. The behaviour of the 48 kDa protein, when competed, also produced similar results, but the band was still apparent at 100-fold excess. The remaining bands were unaffected by competition, except upon addition of large molar excess of competitor.

c) Short labelled construct competed with short unlabelled RNA - A typical autoradiograph obtained is given in Figure 4.10. Four bands representing proteins of
<table>
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<th>3</th>
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<th>5</th>
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<td>+</td>
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<td>0</td>
<td>1</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td>500</td>
<td>1000</td>
</tr>
</tbody>
</table>

![Image showing protein bands at 63 kDa, 48 kDa, 38 kDa, and 28 kDa]

**Figure 4.9** - *Medium unlabelled RNA was used to compete for liver cytosolic proteins binding to the medium radiolabelled probes. Increasing concentrations of medium competitor RNA resulted in a rapid decrease in intensity of the 63 and 48 kDa bands (lanes 3 to 8). The 38 kDa band was scarcely visible and appeared to be unaffected by competition except at 500-fold molar excess or more. The 28 kDa band appeared to be similarly unaffected. Molar excess is indicated along the top and the apparent molecular weights of the proteins are shown to the right.*
Figure 4.10 - Short unlabelled RNA constructs were used to compete for liver cytosolic proteins binding to short radiolabelled RNA. As previously observed, four bands were produced when liver cytosolic proteins were crosslinked to short radiolabelled RNA. Upon addition of competitor (lanes 3 to 8), there was a reduction in intensity of the band representing a 63 kDa protein. However, the 48 kDa band appeared to be unaffected by short competitor RNA except at 100-fold molar excess or more. The 38 and 28 kDa bands were unaffected by competition. Molar excess of competitor is indicated at the top and the apparent molecular weights of the proteins are given to the right.
63, 48, 38 and 28 kDa, were visualised in the absence of competitor, as previously seen with the long and medium constructs (lane 2). The relative intensities of the bands was also similar to those already described, with the 28 and 63 kDa band as the most prevalent, while the 38 kDa required further exposure for visualisation.

Short unlabelled RNA was added as competitor in 1-, 10-, 50-, 100-, 500- and 1000-fold molar excesses, as shown in lanes 3 to 8. The 63 kDa protein was readily competed, and the intensity of the band diminished rapidly between 10- to 100-fold excess. In contrast, the smaller bands (representing 48, 38 and 28 kDa proteins) appeared to be much less affected by the addition of competitor and were still visible at 500-fold excess, albeit less intense.

The bands visualised in the competition assays were measured densitometrically and the molar excess of competitor required to reduce binding to 50% of that in the absence of competitor was calculated (Table 4.1). Since, qualitatively, there was little or no reduction in band intensity for the 28 and 38 kDa proteins with increasing competition, calculations were only performed for the 48 and 63 kDa proteins.

For the 63 kDa protein, in all cases of competition when the same construct was used as probe and competitor, approximately 50-fold excess (45 to 70) of competitor was required. This would suggest that all three constructs contain domains to which the 63 kDa protein may bind. In contrast to this, the binding characteristics of the 48 kDa protein vary markedly. These range from the long being readily competed by 100-fold excess of competitor, while higher concentrations of competitor are required using the medium and short constructs (500- and >1000-fold excess respectively). Indeed, the
The percentage changes in intensity of the bands representing the 63 and 48 kDa proteins in cross-competition studies, comparing the bands obtained in both the presence and absence of competitor RNA.

### Long Radiolabelled RNA vs. Long Unlabelled RNA

<table>
<thead>
<tr>
<th>Molar excess Competitor</th>
<th>x 1</th>
<th>x 10</th>
<th>x 50</th>
<th>x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>63 kDa Protein</td>
<td>108%</td>
<td>62%</td>
<td>37%</td>
<td>16%</td>
</tr>
<tr>
<td>48 kDa Protein</td>
<td>108%</td>
<td>85%</td>
<td>55%</td>
<td>47%</td>
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### Long Radiolabelled RNA vs. Medium Unlabelled RNA

<table>
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<th>x 75</th>
<th>x 150</th>
<th>x 750</th>
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<td>50%</td>
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<tr>
<td>48 kDa Protein</td>
<td>77%</td>
<td>88%</td>
<td>83%</td>
<td>55%</td>
<td>28%</td>
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### Long Radiolabelled RNA vs. Short Unlabelled RNA

<table>
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<th>Molar excess Competitor</th>
<th>x 2.8</th>
<th>x 28</th>
<th>x 140</th>
<th>x 280</th>
<th>x 1400</th>
<th>x 2800</th>
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<tbody>
<tr>
<td>63 kDa Protein</td>
<td>149%</td>
<td>100%</td>
<td>73%</td>
<td>40%</td>
<td>16%</td>
<td>12%</td>
</tr>
<tr>
<td>48 kDa Protein</td>
<td>137%</td>
<td>88%</td>
<td>73%</td>
<td>52%</td>
<td>35%</td>
<td>22%</td>
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</tbody>
</table>

Examples of data obtained from cross-competition studies using long radiolabelled RNA and unlabelled long, medium and short RNA constructs. The percentage change in intensity of the bands was determined as described on page 251.
inability to compete for the 48 kDa protein with the short construct may be due to the interaction being non-specific. In order to characterise the relative binding affinities of the 63 and 48 kDa proteins for the three constructs, cross-competition experiments were performed, competing long radiolabelled constructs with both medium and short unlabelled RNA.

<table>
<thead>
<tr>
<th>Radiolabelled RNA versus Unlabelled RNA</th>
<th>~63 kDa Protein</th>
<th>~48 kDa Protein</th>
</tr>
</thead>
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<tr>
<td>Long versus Long</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Medium versus Medium</td>
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<td>500</td>
</tr>
<tr>
<td>Short versus Short</td>
<td>70</td>
<td>&gt;1000</td>
</tr>
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</table>

Table 4.1 - A summary of self-competition studies of the 63 and 48 kDa protein using both the labelled and unlabelled long, medium and short RNA constructs. The figures shown refer to the amount of unlabelled competitor required to reduce the protein-binding to 50%. The 28 and 38 kDa proteins are unrepresented since they appeared uncompeted upon visualisation, except at large molar excess.

d) Cross-competition of long labelled and medium unlabelled RNA - An example of an autoradiograph obtained is shown in Figure 4.11. In this figure, only the 63 and 48 kDa proteins are shown because, as already described, the 38 and 28 kDa proteins were consistently uncompeted, irrespective of the competitor. Medium RNA was added as a competitor in 1.5-, 15-, 75-, 150- and 750-fold molar excesses as shown in lanes 3 to 7. The intensity of the 63 kDa protein rapidly diminished, and was ablated with even a 15-fold excess of competitor. In contrast, the 48 kDa protein required higher
**Figure 4.11 - An autoradiograph of medium unlabelled RNA competing for liver cytosolic proteins prior to crosslinking to long radiolabelled RNA.** Four bands were visible in the absence of competitor, as previously described (lane 2). Addition of medium unlabelled RNA as competitor resulted in a reduction in the intensity of the bands representing proteins of 63 and 48 kDa (lanes 3 to 8). The binding of the 38 and 28 kDa proteins appeared to be unaffected, except with high molar excess of competitor. The apparent molecular masses of the proteins are indicated to the right, while the molar excesses of competitor used are shown along the top.
Figure 4.12 - An autoradiograph of long labelled RNA crosslinked to live cytosolic proteins, competed with short unlabelled RNA. The four bands described previously were obtained in the absence of competitor (lane 2). Addition of short unlabelled competitor RNA (lanes 3 to 8) resulted in the preferential decrease in intensity of the band representing a 63 kDa protein. In contrast, the binding of the 48, 38 and 28 kDa proteins was unaffected except at high molar excess. The molar excesses of competitor used is indicated along the top, while the apparent molecular masses of the bands visualised are shown to the right.
The densitometry data from self-competition studies of the 63 and 48 kDa proteins from which Table 4.2 is derived.

Long Radiolabelled RNA vs. Long Unlabelled RNA

<table>
<thead>
<tr>
<th>Molar excess Competitor</th>
<th>x 1</th>
<th>x 10</th>
<th>x 50</th>
<th>x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>63 kDa Protein</td>
<td>108% ± 41%</td>
<td>62% ± 26%</td>
<td>37% ± 16%</td>
<td>16% ± 2%</td>
</tr>
<tr>
<td>48 kDa Protein</td>
<td>108% ± 12%</td>
<td>85% ± 7%</td>
<td>55% ± 6%</td>
<td>47% ± 3%</td>
</tr>
</tbody>
</table>

Medium Radiolabelled RNA vs. Medium Unlabelled RNA

<table>
<thead>
<tr>
<th>Molar excess Competitor</th>
<th>x 1</th>
<th>x 10</th>
<th>x 50</th>
<th>x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>63 kDa Protein</td>
<td>94%</td>
<td>66%</td>
<td>37%</td>
<td>46%</td>
</tr>
<tr>
<td>48 kDa Protein</td>
<td>95%</td>
<td>104%</td>
<td>80%</td>
<td>65%</td>
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Short Radiolabelled RNA vs. Short Unlabelled RNA

<table>
<thead>
<tr>
<th>Molar excess Competitor</th>
<th>x 1</th>
<th>x 10</th>
<th>x 50</th>
<th>x 100</th>
</tr>
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<tr>
<td>63 kDa Protein</td>
<td>72%</td>
<td>58%</td>
<td>51%</td>
<td>52%</td>
</tr>
<tr>
<td>48 kDa Protein</td>
<td>72%</td>
<td>61%</td>
<td>64%</td>
<td>55%</td>
</tr>
</tbody>
</table>

Examples of results obtained by self-competition using both the labelled and unlabelled long, medium and short RNA constructs. Each band was measured densitometrically and compared to the bands obtained in the absence of competitor RNA. The data is presented as the percentage intensity of the individual bands obtained in the presence of competitor RNA compared to those visualised in the absence of competitor RNA. 'n.m.' represents samples which could not be quantitated.
concentrations of competitor, and the band was still visible at 75-fold excess. The data is quantitated in Table 4.2.

e) Cross-competition of long labelled and short unlabelled RNA - A typical autoradiograph obtained is shown in Figure 4.12. Lanes 3 to 8 were obtained after competing in 2.8-, 28-, 140-, 280- and 1400-fold molar excesses respectively. High concentrations of competitor were required before the intensities of the two bands were reduced. For example, even with 140-fold excess, the band corresponding to a protein of 63 kDa was still apparent and was only markedly diminished in 280-fold excess. In contrast, a 280-fold excess of competitor had little effect on the 48 kDa protein binding. The quantitated results are shown below (Table 4.2).

<table>
<thead>
<tr>
<th>Radiolabelled RNA versus Unlabelled RNA</th>
<th>~ 63 kDa Protein</th>
<th>~ 48 kDa Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long versus Long</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Long versus Medium</td>
<td>5</td>
<td>70</td>
</tr>
<tr>
<td>Long versus Short</td>
<td>270</td>
<td>&gt;1000</td>
</tr>
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</table>

Table 4.2 - Quantitative analysis of cross-competition studies of the 63 and 48 kDa proteins using both the labelled and unlabelled long, medium and short RNA constructs. The figures denote the molar excess of unlabelled competitor needed to reduce the binding of the protein by 50%. Since, as already described, the binding of the 28 and 38 kDa proteins is non-specific, the data for these proteins is not shown.
The figures above show some interesting variations; for example, the cross-competition data suggested that the three RNA constructs had differing binding affinities for the proteins. For instance the medium construct competed very efficiently for the 63 kDa protein, since only a 5-fold molar excess of competitor could reduce binding by 50%. In contrast, the short competed much less efficiently, requiring 270-fold molar excess of competitor.

Similarly, differential binding affinities were observed for the 48 kDa protein with the different constructs. In general, the 48 kDa protein bound to the medium and long constructs with much lower affinity than the 63 kDa protein, while the short RNA did not appear to bind specifically to the protein. In detail, the 48 kDa protein binding the long labelled construct could be competed by both long and medium RNA in low molar excess (100- and 70-fold respectively). It is surprising to note that self-competition of the medium construct required a large molar excess (500-fold) in order to reduce the intensity of the band by 50%. In contrast to this, both self-competition of the short RNA, together with cross-competition with long radiolabelled RNA, required over 1000-fold excess of competitor to reduce the intensity of the band. This suggests that the 48 kDa protein binds in a non-specific manner to the short construct. Therefore, this would support the hypothesis that the 48 kDa protein binds mostly further upstream within the 5'-end of the long construct.

iv) Comparisons of ROS 17/2.8, UMR-106 and Liver Cytosolic Proteins

Results from Chapter 3 demonstrate tissue-specific differences in transcript expression between osteoblasts and liver. It might therefore also be possible that different proteins would be present in osteoblast cytosol. In order to test this hypothesis, cytosolic
**Figure 4.13 - A comparison of the binding pattern of ROS 17/2.8 and UMR-106 cytosolic proteins.**

Long labelled RNA was used as the probe. From this autoradiograph, it is clear that ROS 17/2.8 and UMR-106 have similar banding patterns to that produced with liver. However, some differences are apparent such as the presence of a 90 kDa and a 75 kDa band which may represent bone-specific proteins. Also the 63 kDa band is visualised as a doublet. The amount of cytosolic protein (µg) is shown along the top, and the apparent molecular weight of the proteins visualised are indicated to the left.

<table>
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</table>
proteins from osteoblastic cells (ROS 17/2.8 rat osteosarcoma and UMR-106) were compared to liver cytosolic proteins in order to characterise any tissue-specific differences in the properties of those proteins binding IGF-I. Both UMR-106 and ROS 17/2.8 cytosolic proteins had similar binding patterns to liver cytosol (Figure 4.13), insofar as four bands representing proteins of apparent molecular mass, 63, 48, 38 and 28 kDa were visible. The predominant band was of 63 kDa once more, although clearly defined as a doublet, with a less intense band at 48 kDa. A noticeable difference was the enormous change in the amount of protein required between transformed osteoblast cell lines and liver. Approximately 50μg of liver cytosolic proteins was required to obtain band intensity to that obtained with between 2 to 8μg of bone cell protein. Furthermore, two proteins of approximately 75 and 90 kDa were faintly visible from the osteoblast lines but absent from liver cytosol, despite similar loading. These may therefore represent two bone-specific proteins, but further confirmation of this is needed.

v) Self- and Cross-Competition Using the Deletion Construct

Having shown that proteins of the same apparent molecular weight in cytosol from rat liver, ROS 17/2.8 and UMR-106 bind to the long, medium and short RNA, the construct representing the transcripts initiated from start site 1 from which the 186 base region has been excised (termed deletion) was used as probe. In this series of experiments, ROS 17/2.8 cytosolic proteins, were used. Importantly, while these cytosolic proteins appear to have similar binding patterns to those from liver, there are several differences, as described previously.

Long, medium, short and deletion unlabelled RNA was used in competition assays with deletion radiolabelled RNA (Figure 4.14 a) to d) respectively). In the absence of cytosol
<table>
<thead>
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<th>4</th>
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</table>

**Figure 4.14 - Competition studies using ROS 17/2.8 cytosolic proteins crosslinked to deletion labelled RNA competed in molar excess of a) long; b) medium; c) short and d) deletion unlabelled RNA. In all four competition experiments, only one band with an apparent molecular weight of 69 kDa was visible. This was readily competed at low molar excess. Molar excess of competitor is indicated at the top and the apparent molecular weight of the protein is given to the right.**
(lane 1 in all panels) no bands were visible. However, upon addition of ROS 17/2.8 cytosolic proteins only one band with the apparent molecular mass of 69 kDa was seen (lane 2) while none of the other cytosolic proteins which bound to the other probes were visible. Interestingly the results obtained when competed with the different constructs varied dramatically. Self- and cross-competition was performed in 1-, 10-, 25-, 50-, 75-, 100-, 300- and 500-fold molar excesses as labelled in Figure 4.14. Cross-competition with long, unlabelled RNA (panel a) resulted in a rapid decline in the intensity of the 69 kDa band so that even when present in equal concentrations, the intensity of the band was much reduced. In a 10-fold excess, the band was almost completely ablated. Similarly, when using medium unlabelled RNA as competitor (panel b), at 10-fold excess the band representing a 69 kDa protein was barely discernible. The use of short and deletion construct as competitors (panels c and d) resulted in similar competition profiles. In contrast to long and medium cross-competition, a decrease in intensity of the 69 kDa band was only visible at 25-fold excess. However, the band was almost completely ablated with 100-fold excess competitor.

Summary of Results

Therefore, these data demonstrate that four proteins, present in liver cytosol, bind to the 5'-UTR of IGF-I. Competition studies have shown that of these proteins, the 63 and 48 kDa proteins bind specifically. Furthermore, deletion analysis, using RNA constructs progressively truncated at the 5'-end, has demonstrated that these two proteins bind with differing affinities to the different lengths of RNA (summarised in Figure 4.15). Specifically, the 63 kDa protein preferentially binds the construct approximating to transcripts initiated from start site 3, while the 48 kDa protein appears to bind this
Figure 4.15 - A summary of the binding characteristics of the 48, 63 and 69 kDa proteins. The data obtained from competition studies indicate that the 63 kDa protein might bind to the short, medium and long RNA constructs. In contrast, the 48 kDa protein appears to bind to the long and medium RNAs, while binding non-specifically to the short construct. Interestingly, the 69 kDa protein only seems to bind to the deletion RNA. The numbers refer to the distance in bases from the 5' end of exon 1.
construct and the RNA equivalent to transcripts initiated from start site 1. Both the 63 and the 48 kDa proteins bound the shorter RNA, which is similar to transcripts initiated from start site 4 with low affinity.

ROS 17/2.8 cytosolic proteins gave a similar binding profile to that obtained with liver. However, the signal appeared to be more intense for the same amount of cytosolic protein used, and two extra bands, of 75 and 90 kDa were observed which might represent bone-specific proteins. Further characterisation of these proteins is required before this can be stated definitively.

Also, using a RNA construct equivalent to transcripts initiated from start site 1 from which the 186 bases have been excised, neither the 63 nor 48 kDa proteins were observed. Instead, a 69 kDa protein, which had not previously been seen, bound specifically, which surprisingly, could be competed using the other constructs.
Discussion

The data presented in this chapter would suggest that two cytosolic proteins from the osteoblast cell lines, ROS 17/2.8 and UMR-106, and liver, with apparent molecular masses of 48 and 63 kDa bind to the long IGF-I sequence, that is 1-523 bases (Figure 4.15). This is equivalent to full-length transcripts and covers the sequence from start site 1 to 140 bases within exon 3. Removal of the first 187 and 362 bases (that is in the medium and short RNAs) did not alter the protein binding profile, indicating that these proteins bind to the sequence 363-523. These two lengths of RNA correspond to sequences initiated from the middle of the alternatively-spliced region, approximating to start site 3, and transcripts initiated from start site 4, respectively. A further construct was used, termed deletion or alternatively-spliced RNA, and was equivalent to the long RNA from which the 186 base region had been excised between bases 115-302. Surprisingly, despite the deletion RNA apparently having the appropriate sequences (bases 363-523), neither the 48 nor the 63 kDa proteins bound. However, unlike the other constructs, a 69 kDa cytosolic protein from ROS 17/2.8 cells, which had not previously been seen, bound specifically.

Cross-competition experiments demonstrated that the 63 and 48 kDa proteins bound to the long, medium and short constructs with different binding affinities. This variation was most notable for the 63 kDa protein, which bound to the medium constructs with apparently 10-fold greater affinity than to the long RNA, and 50-fold greater affinity than to the short RNA. Similarly, from these studies it was ascertained that the 48 kDa protein bound to the long and medium RNA with approximately the same affinity,
whereas binding to the short RNA appears to be non-specific due to the lack of competition by long and medium RNA (>1000-fold excess required).

**Localisation of the Binding Sites**

Although the binding site for the 63 kDa protein is present in the long, medium and short RNA sequences, factors other than primary sequence must be important in the interaction. That is, since deletion analysis demonstrated that the binding site lies within the region 363-523, it is likely that the conformation adopted by the RNA within this region has a great affect on the protein-RNA interaction. For instance, proteins are often associated with stem-loop structures such as those within ferritin RNA, to which the iron response factor has been postulated to bind (Leibold and Munro, 1988). To examine the possible structures with which interactions with proteins may occur, computer predictions of the secondary structure adopted by the various lengths of RNA were made using RNA Draw (Version 1.0, by Ole Matzura, Dept. of Medical Biophysics, Karolinska Institute, Sweden). These are shown in Figure 4.16.

All the structures shown display characteristic features of secondary structure, such as stem-loops and hairpin-loops, and it is easily seen that the complexity of the conformation adopted by the constructs increases with the lengths of the RNA. However, there are regions present within each structure which remain well conserved, irrespective of the lengths of the constructs, which have been highlighted on each sequence. For instance, the long, medium and short RNAs contain stem-loop structures of 56- and 32-bases, at bases 444-499 (circled in black) and 379-410 (circled in red) respectively. A 133 base stem-loop structure is predicted at bases 218-350 (circled in...
Figure 4.16 - Predicted secondary structure of the long, medium, short and deletion RNA constructs, as generated by the program RNA Draw. Areas of similarity between the various constructs are circled and colour coded.
yellow) in both the long and the medium constructs but is absent in the short RNA. In contrast, at bases 363-428, a 66-base stem-loop (highlighted in blue) is predicted to be present only in the medium and short RNAs. Interestingly, the only regions of structural similarity between the RNA sequences described above and the deletion RNA is a 92-base hairpin-loop (circled in green) between bases 430-521 which is found only in the short RNA, within which lies the 56-base hairpin loop already described. Importantly, the 32-base stem-loop structure at bases 379-410 is present in the small, medium and large sequences but not in the alternatively-spliced RNA. Since this falls within the region which binds the proteins, it is a possible candidate protein-binding site. However, of course, it is possible that all these secondary structures are sites of interaction for proteins. For example, the 63 kDa protein could bind to the 56-base hairpin-loop which is a common structure in the long, medium and short RNA sequences but is also present in the alternatively-spliced RNA. Further practical analysis is required in order to ascertain exactly where the proteins bind.

Given these caveats, the context in which these structures are found within each construct vary greatly and thus, this is likely to affect the affinity of the proteins for the RNA. Therefore, a particular conformation of the local secondary structure may favour high-affinity binding. For instance, the long and medium RNAs, which bind the 63 kDa protein with higher affinity than the short RNA, are predicted to have a large 133-base multiple loop structure which is not present in the shorter RNA. If, as an example, it is assumed that the 63 kDa protein binds to the 32-base structure in all RNAs, the presence of the 133-base multiple loop structure may in some manner favour high-affinity binding to the long and medium RNAs compared to the short RNAs. In contrast, the differences in affinities of the proteins for the long and medium RNAs may
be due to steric hindrance by the large secondary structures in the long RNA, diminishing the protein-RNA interaction. Using a similar argument, although the 48 kDa protein bound to the short RNA, this was either with very low affinity or non-specific, and therefore this protein binds preferentially to structures common only to the long and medium RNAs. For example, a possible site of interaction could be the 133-base stem loop structure, not found in the short RNA.

Using these predicted conformations, it becomes clear that the alternatively-spliced RNA contains structures absent in all the rest, despite the homology in sequence. This explains how the 69 kDa protein may only bind to this RNA. Similarly, if the 32-base stem-loop structure is important for binding of the 48 and/or 63 kDa proteins in the small, medium and large RNAs, then the absence of this structure in the alternatively-spliced RNA is consistent with a lack of binding. Another possibility is that the 69 kDa protein has such high affinity for the alternatively spliced RNA, that it inhibits the 63 or 48 kDa proteins from binding.

Although this model appears to be logical and concordant with most of the data, there are limitations. For example, aside from the conformations being predicted, the competition studies in which the long, medium and short RNAs compete for 69 kDa protein, are inconsistent in parts. Therefore, it is clear that a great deal more work is required to fully elucidate the manner in which the RNA-protein interactions are mediated.
The Possible Significance of RNA-Protein Interactions

As described in detail in Chapter 3, IGF-I transcripts are initiated from a number of sites and Yang et al., (1995) demonstrated that these transcripts are translated with different efficiencies. Briefly, the transcripts with a long 5' -UTR were all poorly translated, while the alternatively-spliced transcripts, and those initiated from start site 4, were six- to ten-times as translatable. This variation in the translatability of the transcripts may be due, in part, to the presence of start/stop codons within the 186-base alternatively-spliced region, upstream of the translation start site and the possible implications have already been discussed in Chapter 3. However, regulation of translation is also likely to depend on RNA-protein interactions.

Post-transcriptional Regulation and the 3' -UTR

The untranslated regions have been found to be important in the regulation of many genes. To date, though, the majority of the studies have focused on the 3' -UTR. This region often contains elements regulating the stability of the mRNA transcripts occurring, for instance, in the genes for human interferon-β (Kruys et al., 1987), amyloid protein precursor (de Sauvage et al., 1992) and the AU-rich sequences of GM-CSF (Shaw and Kamen, 1986) and primate lactate dehydrogenase (Salehi-Ashtiani and Goldberg, 1995). However, the mechanism by which the stability of transcripts is regulated, generally involves protein interactions with specific sequences. Examples of genes in which this has been shown to occur include ribonucleotide reductase (Amara et al., 1993), thyroid stimulating hormone (Leedman et al., 1995) and angiotensinogen (Klett et al., 1995).
Post-transcriptional Regulation and the 5'-UTR

In contrast to the apparent role of the elements within the 3'-UTR, the 5'-UTR tends to regulate translation, as shown in genes encoding, for example ornithine decarboxylase (Manzella and Black Spear, 1992; Wallon et al., 1995) and thymidylate synthase (Johnson, 1994). Again, protein-RNA interactions within the 5'-UTR have been characterised as exerting an important role in altering the translation of some mRNAs, such as transcripts for parathyroid hormone (Vadher et al., 1996), insulin (Welsh et al., 1986; Knight and Docherty, 1992), IGF-II (Nielsen et al., 1990; Teerink et al., 1994; de Moor et al., 1995) and ferritin (Leibold and Munro, 1988; Rouault et al., 1988; Casey et al., 1989).

The importance of post-transcriptional regulation of gene-expression mediated through proteins interacting within the 5'-UTR may be exemplified by ferritin synthesis, which is amongst the most extensively studied. Ferritin is an intracellular iron-storage protein, whose translation is controlled by reversible RNA-protein interactions (Melefors and Hentze, 1993). For example, in vivo, haemin or iron has been found to stimulate an increase in translation from ferritin mRNA, which could be inhibited by desferal, an iron chelator. Studies within the last 10 years have demonstrated that a protein, the iron regulatory factor (IRF) reversibly binds to a short stem-loop structure within the 5'-UTR, called the iron response element (IRE) and that this interaction is iron-sensitive (Leibold and Munro, 1988; Rouault et al., 1988). In the absence of iron, the IRF binds to the IRE with high affinity leading to the repression of ferritin synthesis. However, when intracellular iron levels are restored, the iron binds to IRF which, in turn,
diminishes the affinity of the protein for the IRE, de-repressing translation and permitting protein synthesis (Haile et al., 1989).

A further example of post-transcriptional regulation of protein synthesis is given by the insulin gene. It has been shown that while prolonged exposure to elevated levels of glucose enhances transcription of the insulin gene, transient increases in glucose levels result in increased translation of the insulin mRNA (Welsh et al., 1986) and subsequent work by Knight and Docherty (1992) indicated a possible role for regulatory proteins binding to the 5′-UTR. Similarly, ornithine decarboxylase is regulated at this level as shown by the observation that enzymatic activity increases 20- to 30-fold in Ehrlich ascites tumour cells, despite only a 2- to 3-fold increase in transcription, and this is mediated through specific binding of a protein to the 5′-UTR (Manzella and Blackspear, 1992; Wallon et al., 1995).

The regulatory mechanisms described above involve genes transcribed as a single transcript type, whereas the human IGF-II gene is transcribed from four promoters, resulting in four types of transcripts varying in the length and sequence of their 5′-UTR (Nielsen et al., 1990; Teerink et al., 1994). A fifth transcript, differing in the 3′-UTR may also be transcribed, but is only a minor species (de Moor et al., 1995). The transcript expression is both developmentally regulated and tissue-specific. For instance, promoter 1 resulting in a 5.3 kb transcript, is only used in the adult liver, whereas promoters 3 and 4 (specifically the 6.0 and 4.8 kb transcripts) are expressed in foetal and certain extrahepatic tissues (Nielsen et al., 1990). These studies also demonstrated that the 4.8 and 6.0 kb transcripts were differentially translated in a rhabdomyosarcoma cell line. Specifically, the minor 4.8 kb transcript was associated with membrane-bound
polysomes and appeared to be responsible for the majority of the translation. In contrast, the major 6.0 kb mRNAs were associated with a protein in the cytosol, forming ribonucleoprotein particles and were therefore poorly translated. The authors also implicated the relative abundance of these readily translatable and untranslatable transcripts in the apparent discrepancy between the levels of IGF-II mRNA and protein synthesis in pheochromocytomas and Wilms' tumours.

Subsequently, a protein extracted from a human hepatoma cell line was shown to bind specifically to short sequences in the 5'-UTR of the 6.0 kb transcript. The region to which this protein bound was characterised, and the sequence ligated to a CAT construct so the effect of the protein on translation could be examined *in vitro*. Competition studies indicated that these sequences bind a regulatory protein which enhances translation (de Moor *et al.*, 1995). Therefore, it would appear that the translation of the 6.0 kb transcript is regulated by two proteins with opposite functions:- one of which forms an untranslatable complex (see above) and another which increases translational ability. It will be interesting to determine whether these are expressed in a tissue-specific fashion.

There is evidence that these RNA-binding proteins are developmentally-regulated. As previously mentioned, IGF-II transcripts initiated from promoter 1 (5.3kb) are solely expressed in adult liver, and translational efficiency of a CAT reporter gene construct containing the 5'-UTR of this transcript was very low compared to that of β-globin 5'-UTR-CAT mRNA. However, addition of cell extract from undifferentiated embryonal carcinoma cells greatly up-regulated translation of the IGF-II 5'-UTR mRNAs whereas in contrast, extracts from differentiated cells had no stimulatory effect. Therefore, it
would suggest a possible regulatory mechanism involving RNA-binding proteins in the production of IGF-II during development.

The paradigm of IGF-II is especially interesting, though, since IGF-I expression is similar in terms of the multiplicity of transcripts produced and the manner in which the transcription start site usage is both developmentally-regulated and tissue-specific. Therefore, the apparent interaction of the 69, 63 and 48 kDa proteins with particular transcripts, also with differing affinities, could be indicative of a mechanism to regulate the synthesis of IGF-I protein from particular transcripts. For example, by analogy with IGF-II, it is interesting to note that as regards IGF-I, the 69 kDa protein is associated with one of the more translatable transcripts, namely those initiated from start sites 1 and 2 from which the 186-base region has been excised (Yang et al., 1995). Therefore, this protein may possibly enhance the translatability of these transcripts, in a similar manner to the apparent action of the proteins interacting with the IGF-II transcripts (Nielsen et al., 1990; Teerink et al., 1994; de Moor et al., 1995).

Conversely, the 63 and 48 kDa proteins tend to bind with greatest affinity to those mRNAs corresponding to the less-translatable transcripts, that is those initiated from start sites 1, 2 and 3, but bind with least affinity to those initiated from start site 4 and did not bind to the alternatively spliced transcripts, both of which have greater translatability. Therefore, it is possible that these proteins may have a role in inhibiting translation of transcripts initiated from the upstream start sites. The mechanism whereby RNA-protein interactions could mediate this response may be similar to the ferritin model. As described above, the IRF is postulated to bind to the stem-loop structure of the IRE. While many hypotheses exist to explain the manner in which the protein
modulates translation, it is likely that the 48S ribosomal pre-initiation complex is unable to interact with the 5′-end of the ferritin transcript due to steric hindrance by the IRF/IRE complex (Melefors and Hentze, 1993; Standart and Jackson, 1994). However, the region to which the IRF binds the ferritin transcript is only 30 to 40 bases downstream from the 5′-end of the transcript, whereas the 63 and 48 kDa proteins are predicted to bind the IGF-I transcript over 300 bases downstream of transcription start site 1. It would, therefore, be difficult to envisage a mechanism whereby proteins positioned so far downstream may prevent access of the 48S pre-initiation complex to the 5′-cap.

Another possible mechanism is that translation may be inhibited due to an inability of the 43S pre-initiation subunit to scan through a protein-stabilised mRNA secondary structure. Experiments were performed by Goossen et al. (1990) to alter the position of the IRE within the 5′-UTR of ferritin mRNA, from which it was found that, while the ability of the protein to bind was relatively unaltered, the effectiveness of the IRF/IRE complex to inhibit translation was markedly diminished the further it was from the 5′-cap (Goossen et al., 1990; Goossen and Hentze, 1992).

The sites to which the 48 and 63 kDa proteins may bind, though, are all positioned further downstream than that which is effective for IRF/IRE-type of translational inhibition. However, the need for the proteins to bind near the 5′-cap in order to mediate down-regulation of translation is not a universal feature, as has been characterised in poly(A) binding proteins (PABP) mRNA and thymidylate synthase (TS) mRNAs. PABP auto-regulation of gene expression, decreases protein synthesis from PABP mRNA by binding to regulatory elements within the 5′-UTR. The
positioning of these elements though, unlike the IRE, is not critical and may function when at least 100 bases from the 5'-cap (reviewed by Standart and Jackson 1994). Similarly, TS inhibits its own translation *in vitro*, mediating this response by binding to a 35-base element which is thought to form a hairpin loop structure 94 bases from the 5'-end of the transcripts, spanning the initiation codon (Chu *et al.*, 1993). Therefore, it is possible that the proteins may bind 100 bases or more from the 5'-cap and reduce translation, as is postulated for the 63 and 48 kDa proteins with IGF-I mRNA.

**Possible Regulation of Translation Start Codon Usage**

The situation with the IGF-I gene is made more complex by the presence of two in-frame start codons at bases +320 in exon 1 and +16 in exon 3, encoding different signal sequences of 48- and 22-amino acids respectively (Figure 4.17). Therefore, transcripts initiated from start site 4 may be translated from only the Met-22 initiation codon, while those initiated from start sites 1, 2 and 3 may be translated from either start codon. However, the majority of the prepro-IGF-I peptides synthesised are initiated from the Met-48 start codon (Yang *et al.* 1995). The putative binding site for the 48 and 63 kDa proteins are positioned between the Met-48 and Met-22 codons. Therefore, another possible role for these proteins could be to regulate the translation start site usage and inhibit the use of the Met-22 initiation codon. In this manner, the protein could regulate the production of peptides with particular prepro-sequences, which in turn might alter IGF-I protein transport through the cell.
Figure 4.17 - The possible regions of interaction for the 48, 63 and 69 kDa proteins in relation to the transcription start sites and the translation initiation codons. This diagram summarises the results and shows that the 63 kDa protein probably interacts with RNA secondary structure downstream of the translation start codons (blue line), while the 48 kDa protein binds to sequence upstream of the start of translation (red line). The critical binding region for the 69 kDa protein probably involves the sequence to either side of the alternatively-spliced region, but may also involve any of the area encompassed by the green line.
Regulation of RNA-Protein Interactions

IGF-I is regulated by many hormones and growth factors. It could be envisaged that factors such as glucocorticoids, which are known to decrease IGF-I synthesis, may not only do so by decreasing the rate of transcription, but may also mediate their response through a protein specifically inhibiting the translation of IGF-I. Conversely, the response to GH could be two-fold, mediating an increase in the abundance of cytoplasmic mRNA transcripts, but also 'switching on' a protein which stimulates IGF-I translation.

However, the mechanism whereby regulatory proteins are induced to bind or dissociate from IGF-I mRNA remains unclear. Two systems have raised some possible mechanisms, namely the regulation of the IRF/IRE interaction and the insulin-induced translation in adipocytes. The IRF has high affinity for the IRE leading to the repression of translation but this is reversed by iron binding to an iron-sulphur cluster within the protein. This causes an alteration in the conformation of the protein to one which has low affinity for the IRE, although there is also some evidence that regulation may occur through protein turnover (Melefors and Hentze, 1993). In contrast, insulin-induced translation in adipocytes is dependent upon phosphorylation of a repressor protein which interacts with the translation initiation factor eIF-4E (Pause et al., 1994); the phosphorylated form of this protein dissociates from eIF-4E, allowing cap-dependent translation to proceed. Regulation of protein interactions by phosphorylation could be a likely candidate mechanism for the control of translatability of IGF-I. This is because many of the hormones and growth factors which regulate IGF-I synthesis are thought to mediate their actions through protein kinase A or C pathways.
Summary

In this chapter, a number of cytosolic proteins, present in both liver and the osteosarcoma cell lines ROS 17/2.8 and UMR-106, have been shown to bind to the 5' UTR of IGF-I. Of these, three proteins, with apparent molecular masses 69, 63 and 48 kDa, appear to bind specifically to transcripts varying in their 5'-UTR but with differing affinities. Furthermore, preliminary data suggests that two proteins of apparent molecular mass 75 and 90 kDa, which are present in ROS 17/2.8 and UMR-106 cytosol but not from liver, interact with the 5'-UTR of IGF-I mRNA.

Proteins interacting with the 5'-UTR of transcripts are often indicative of post transcriptional regulation of protein synthesis. Taking this into account, along with studies by Yang et al. (1995), it would appear that the 63 and 48 kDa proteins interact with those transcripts which are poorly translated, whereas the 69 kDa protein interacts with one of the more efficiently translated transcripts. It is therefore possible that these proteins alter the rate with which translation of the various IGF-I transcripts occurs and indeed, they may, in part, be responsible for the differences in translational efficiency. This level of regulation in addition to that at transcription would allow cells expressing IGF-I to rapidly alter IGF-I protein synthesis and thereby adapt to physiological changes without the need for de novo transcription. Furthermore, the possibility of tissue-specific regulatory proteins also raised in this chapter, could indicate a means whereby similar transcripts expressed in different tissues may be translated with differing efficiencies. However a great deal of work has yet to be performed in order to further characterise the interactions of the proteins and their effects upon translation.
Chapter 5

Conclusions
The aim of this study was to investigate the nature and potential significance of the IGF-I transcripts expressed in a population of bone cells enriched for osteoblasts. Therefore, in the first instance primary osteoblast cultures had to be prepared and characterised. Furthermore, I needed to determine methods for both quantitating the IGF-I transcripts and examining the possible mechanisms of regulation. Initially, therefore, primary bone cell cultures enriched for osteoblasts were obtained from rat long bones by sequential collagenase digestion, using a methodology which has been predominantly employed to isolate osteoblasts from foetal rat calvariae. However, the yield of cells achieved using this standard protocol was poor. Therefore, the extraction procedure was extensively optimised so as to obtain consistent osteoblast cultures from the postnatal long bones used in this study. The population of cells thus obtained was shown to have properties characteristic of osteoblasts, including the ability to form mineralising bone nodules in culture and positive staining for alkaline phosphatase, while the absence of contamination by osteoclasts was confirmed by negative staining for tartrate-resistant acid phosphatase. In addition, the osteoblast-enriched cultures were shown to produce IGF-I, as measured by RIA.

The presence of the expected IGF-I transcripts in osteoblasts was confirmed by RT-PCR and subsequently Northern analysis demonstrated that there were some differences in the IGF-I transcript expression in bone and liver. For example, a larger ~7kb transcript was present in bone which was absent from liver of rats of the same age. However, since my interest centred on the heterogeneity in the 5'-UTR of IGF-I mRNA, Northern analysis could not be used effectively due to the large variability in the length of the 3'-UTR. RNase protection assays, therefore, were optimised and employed to determine the pattern of expression in isolated bone cells. Transcription
start site usage in osteoblasts was found to differ to that in liver cells, confirming that the pattern of expression in bone cells was similar to that observed in other extrahepatic tissues such as lung and muscle.

Since heterogeneity in the 5'-UTR of transcripts is often associated with post-transcriptional regulation of gene expression and this is usually mediated through RNA-protein interactions, the third part of this project focused on the possibility of RNA-binding proteins interacting with the 5'-UTR of IGF-I mRNA to affect translation. Two proteins of 48 and 63 kDa were observed to bind differentially to three RNA species, corresponding to the 5'-UTR of exon 1-derived transcripts initiated from different start sites, indicating possible transcript-specific RNA-protein interactions.

It is clear that there are multiple mechanisms by which regulation of IGF-I synthesis occurs and which appear to be present in bone cells. These include regulation at: (a) transcription - for example the use of alternate promoters; (b) RNA processing - such as the alternate splicing of exon 1-derived transcripts and differential polyadenylation; (c) mRNA stability and (d) regulation of the efficiency of translation.

The existence of multiple parts in a regulatory system in fact permits a large degree of specificity in the control of protein synthesis, as certain factors may only utilise particular pathways in the process. This is obviously of great physiological significance with regards to IGF-I since it is produced in so many tissues; therefore there is a requirement for a degree of tissue-specific regulation. In many cases, part of this specificity will be due simply to the presence or absence of, for example, hormone receptors, but also IGF-I synthesis in response to a stimulus may vary in different
tissues with respect to, for instance the direction of change, time period etc. This diversity of response could be achieved by the action of regulatory proteins which may, in turn, be expressed in a tissue- or developmentally-specific manner. Such proteins may determine which start site is used, the rate of mRNA degradation or be involved in the regulation of mRNA translatability. The last effect would be supported by my work which suggests that proteins bind to IGF-I mRNA in a transcript-specific manner. Moreover, preliminary data indicates that there may be bone-specific proteins, supporting the hypothesis that regulation of IGF-I synthesis may involve tissue-specific proteins.

A number of questions arise as to the nature of the interaction between the regulatory proteins and the IGF-I gene, and how this alters in response to incoming stimuli and the various signal transduction pathways. For example, the actions of GH are mediated through a tyrosine kinase pathway, whereas the PTH induces cAMP production and it is possible that the downstream consequence of these pathways have contrasting effects upon the regulatory proteins.

There are clearly situations in which it is necessary to have a mechanism regulating IGF-I production which is common to all tissues, for example in co-ordinated growth. Conversely, other conditions can be envisaged in which for example, a change in IGF-I synthesis is desirable in selected tissues only. There are a number of hypothetical mechanisms to achieve this, but consider the switching from a poorly to a highly translatable transcript such as occurs when transcripts derived from exon 1 are alternatively spliced. My data in bone cells have shown that these transcripts comprise a small proportion of IGF-I mRNA compared to that in liver. While the measurements
were obtained from cells in culture, it is conceivable that the processes by which alternate splicing occurs are less-developed in bone cells. Therefore, given a suitable stimulus, the transcripts expressed in liver would be the more translatable species whereas this alternate splicing would occur to a lesser extent in bone cells so that potentially, the relative increase in protein synthesis in bone cells would be much less. Indeed, to date the majority of the studies examining changes to IGF-I mRNA synthesis have been performed using Northern analysis, from which these subtle alterations would not be observed. Therefore, it will be very interesting to use RNase protection assays to study this diversity of response in osteoblasts.

Therefore, several avenues of research are immediately apparent from my data, namely investigations into:

(1) the mechanisms underlying the choice of promoter used, in particular the differences between extrahepatic transcriptional control and that found in liver;

(2) the factors involved in regulating promoter usage in bone cells, including the study of the effects of hormones such as PTH, GH and E_2 and developmental age upon transcription start site use;

(3) the mechanisms involved in the regulation of alternate splicing of exon 1-derived transcripts leading to the deletion of 186 bases and the production of more translatable transcripts;

(4) the role of RNA-binding proteins in determining the translatability of the various IGF-I mRNA transcripts and their possible cell-specificity.
Conclusions

In conclusion, in this study it has been shown that IGF-I is synthesised by osteoblasts in culture, and that the transcripts are expressed from all start sites in a tissue-specific manner. Furthermore, the data indicate that in the basal state osteoblasts preferentially synthesise the less translatable transcripts. Also, several cytosolic proteins found in liver and bone have been observed to bind in a transcript-specific manner and are likely to be regulatory in nature. The work presented here, therefore, supports the hypothesis of multifaceted control of IGF-I synthesis and further investigations will help to elucidate and understand the complex nature of IGF-I gene expression.
Appendix I

General Methods
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A 1.1 - Cell Culture

**Cell Passaging**

The medium was discarded, and the cells were incubated in trypsin/EDTA in calcium- and magnesium-free phosphate-buffered saline (PBS) at 37°C briefly. Flasks were gently tapped to dislodge the cells, and the supernatant containing the cells transferred to a sterile centrifuge tube containing foetal calf serum (FCS) to a final concentration of 4%. Cells were collected at 1,500 x g for 5 minutes and the supernatant discarded. The cell pellet was gently resuspended in an appropriate volume of PBS and an aliquot (100μl) used for cell counting.

**Cell Counting**

An aliquot of cells (100μl) was added to cell-staining solution containing trypan blue to a final concentration of 0.02% w/v in PBS. The cells were counted in a haemocytometer. Cell viability was assessed by comparing the total number of cells with those which were blue and were therefore dead.

**Alkaline Phosphatase Staining**

The medium was discarded and the cells were washed in PBS. The cells were fixed in citrate-buffered acetone for 1-2 minutes, rinsed in water and air-dried for 10 minutes. Fast blue RR salt (5mg) was dissolved in 24ml ddH₂O for 10 minutes and filtered, followed by the addition of 1ml napthol AS-MX phosphate (0.25% w/v, pH8.6) and the stain used immediately. The cells were left in the dark at room temperature for 30 minutes and rinsed in ddH₂O.
**Tartrate-Resistant Acid Phosphatase (TRAP) Staining**

Fast garnet salt (240μg/ml) was dissolved in 46ml ddH₂O, 2ml acetate (2.5M, pH 5.2), tartrate (0.67M) and napthol AS-B1-phosphoric acid (12.5mg/ml in N,N,-dimethyl formamide) for 30 minutes and filtered. The cells were washed in PBS, fixed in formaldehyde (4% v/v) for 2 minutes and liberally rinsed in ddH₂O. Stain was added and the colour allowed to develop for 10-15 minutes at 37°C. The cells were rinsed again in ddH₂O and dehydrated in ethanol.

**von Kossa Staining**

The cells were fixed in formaldehyde (see TRAP staining) and hydrated. Silver nitrate (1%) was added to cover the cells, and illuminated intensely for 15 minutes. The cells were rinsed twice with ddH₂O and immersed in aqueous sodium thiosulphate (5% w/v) for 2 minutes. After rinsing in three changes of ddH₂O, the cells were dehydrated in ethanol.

**Alizarin-Red S Staining**

Alizarin red S (1g) was dissolved in 90ml ddH₂O and the pH adjusted to 6.4 using ammonia (0.28% v/v). The cells were fixed in formaldehyde, hydrated (see TRAP staining) and immersed in the stain for 2 minutes. After rinsing quickly in ddH₂O, fresh differentiating solution (ethanol 95% and concentrated HCl (0.01%)) was added for 15 seconds and the cells were dehydrated in ethanol.
A 1.2 - General Extraction and Precipitation Protocols

**Phenol-Isoamyl Alcohol-Chloroform Extraction**

Phenol saturated overnight at 4°C in 1 volume of TEN buffer (Appendix II.10) was mixed with an equal volume of isoamyl alcohol:chloroform (IC-1:24) to give phenol:isoamyl alcohol:chloroform (PIC-25:1:24). Samples to be extracted were mixed with an equal volume of PIC and centrifuged at 10,000 x g for 2 minutes at 20°C. The upper aqueous layer was retained and sometimes re-extracted in PIC (refer to individual protocols for details).

The PIC-extracted samples were added to an equal volume of IC, vortexed and centrifuged at 10,000 x g for 2 minutes at 4°C. This procedure was sometimes repeated, dependent upon the specific protocol.

**Ethanol Precipitation**

Samples were precipitated with 0.1 volumes of sodium acetate (3M, pH5.2) and 2.2 volumes of ethanol (-20°C) for 1 hour at -70°C. The DNA or RNA was recovered by centrifugation at 10,000 x g for 30 minutes at 4°C, the pellets washed with ethanol (70%, -20°C) centrifuged at 10,000 x g for 5 minutes at 4°C, dehydrated in 100% ethanol (-20°C) and recentrifuged at 10,000 x g for 5 minutes at 4°C. The samples were dried *in vacuo* and resuspended as designated by the specific protocol.
RNA Extraction

All equipment and solutions utilised in handling RNA were diethyl pyrocarbonate (DEPC)-treated (0.1% v/v) for 24 hours, followed by autoclaving. RNA extraction was performed as described by Chomczynski and Sacchi (1987). Liver or bone were ground in a DEPC-treated mortar in liquid nitrogen and homogenised with a glass Teflon homogeniser in 1ml of denaturing solution (Appendix II.3). Cells in culture were lysed with 2ml of denaturing solution and passed through a pipette ten times. 0.1 volumes of sodium acetate (2M, pH4.0) was added and mixed, followed by 1 volume of water-saturated phenol and 0.2 volumes of 49:1 chloroform:isoamyl alcohol, mixing thoroughly after each addition. The samples were incubated at 4°C for 15 minutes prior to centrifugation at 10,000 x g for 20 minutes at 4°C. The upper aqueous layer was retained and the RNA precipitated in 1 volume of isopropanol (100%) at -20°C for 1 hour. The samples were recentrifuged at 10,000 x g at 4°C for 20 minutes and the supernatant discarded. For liver and bone, the pellet was resuspended in lithium chloride (4M) and the RNA collected at 5,000 x g for 10 minutes. The RNA pellet was dissolved in denaturing solution and reprecipitated in 1 volume of isopropanol (100%) at -20°C for 1 hour. The samples were centrifuged for 10 minutes at 10,000 x g at 4°C, the supernatant discarded and the pellet resuspended in ethanol (75%), vortexed and incubated for 15 minutes at room temperature. The RNA was centrifuged at 10,000 x g for 5 minutes, dried in vacuo and dissolved in DEPC-ddH₂O prior to being quantified at 260nm.
Radiolabelled Riboprobes

Linearised cDNA clones (500ng) were incubated in transcription buffer, rNTPs (Appendix II.4), T7 polymerase (20U) and α\(^{32}\)P-labelled- and unlabelled-rUTP in a total reaction volume of 20µl. In order to obtain full length riboprobes the ratio of labelled:unlabelled UTP was optimised and found to be 0.125:0.6 nmol. The optimal incubation conditions were found to be 30°C for 1 hour. An aliquot (1µl) was retained in order to determine the specific activity of the riboprobe (see TCA precipitation). RQ1 RNase-free DNase (1U/µg template DNA) was added to the remainder for 15 minutes at 37°C to digest the template. The reaction volume was increased to 100µl with DEPC-ddH\(_2\)O and the riboprobes were PIC- and IC-extracted, prior to the removal of the unincorporated nucleotides using sephadex G50 columns (Quick Spin, Boehringer Mannheim). To assess the integrity of the probe, eluent (5µl) was mixed with 10µl formamide loading buffer, heated to 95°C for 3 minutes and separated on a 3.5% denaturing polyacrylamide gel * containing urea (8.3M) in TBE buffer (Appendix II.4). The remainder of radiolabelled riboprobe was ethanol precipitated.

TCA Precipitation and Measurement of Specific Activity.

The aliquot retained before PIC-extraction of the riboprobes was diluted 1:40 in DEPC-ddH\(_2\)O. Duplicate glass fibre filters (Whatman GF/A) were spotted with 1µl of the dilution, air-dried and the total amount of \(^{32}\)P determined using a scintillation counter. The amount of \(^{32}\)P-rUTP incorporated into riboprobes was determined by trichloroacetic acid (TCA) precipitation. In duplicate, tRNA (100µg) was added to the diluent (1µl), the total volume increased to 100µl with DEPC-ddH\(_2\)O and mixed with 500µl of ice-cold TCA (5% w/v). The samples were incubated at 4°C for 5 minutes and applied to wet (5% w/v TCA) GF/A filters under vacuum. After washing the filters with

* - Protean II Slab Gel (Bio Rad Laboratories, Watford, U.K.) using 1mm spacers.
5ml of ice-cold TCA (5% w/v), and rinsing with acetone, the filters were air dried and counted using a scintillation counter. The specific activity of the riboprobes was then determined.

**RNase Protection Assays**

Dry RNA (20μg) was resuspended in hybridisation buffer (30.5μl - refer to Appendix II.5 for solution composition). Radiolabelled riboprobe (~0.02pmol) in 0.5μl DEPC-H₂O was added and the mixture incubated at 85°C for 5 minutes followed by 16 hours at 45°C.

EDTA (pH7.0, 2μmol) was added prior to the addition of digestion buffer (270μl), containing RNase A (10μg/ml) and RNase T₁ (0.5μg/ml), to a total volume of 300μl and incubation at 30°C for 15 minutes. Sodium dodecyl sulphate (SDS) was added to 0.6% followed by proteinase K (50mg). The samples were incubated for 15 minutes at 37°C and then PIC and IC extracted. The RNA was recovered with tRNA (20mg) in 0.1 volume of sodium acetate (3M, pH5.2) and 2.2 volumes of ethanol (100%) for 1 hour at -70°C. RNA was pelleted and resuspended in 5ml of formamide loading buffer. The samples were heated to 95°C for 3 minutes and loaded onto a 6% denaturing polyacrylamide gel * with urea (8.3M) in 1 x TBE (Appendix II.4). The gel was run at 60W until the xylene cyanol dye reached the bottom. The gel was dried and exposed at -70°C to Amersham Hyperfilm-MP.

**Quantitation of Transcripts on Autoradiographs**

The bands visualised from autoradiographs of RNase protection assays and Northern were quantitated densitometrically (Quick Scan Jr., Helena Laboratories) taking the

* - Sequencing Rig G3-0620 (Flowgen Instruments Ltd., Staffordshire, U.K.) using 0.4mm spacers and a square-toothed comb.
length and the sequence of each of transcript into account. The mean and standard deviations were calculated from repeated experiments.

**Characterisation of RNase Protection Assay Bands**

When characterising the bands produced, six lanes of 40µg of liver RNA were used in the protection assay, and once run, the gel was wrapped in cling-film and exposed to film at room temperature. The bands produced from the RNase protection assay were detected by autoradiography, excised, eluted from the gel in 0.5ml elution buffer (Appendix II.6) and incubated for 1 hour at 65°C. The supernatant was collected, the gel vortexed in a further 0.25ml of elution buffer and the supernatants pooled, and purified on a sephadex G50 column (Quick Spin, Boehringer Mannheim). The eluent was separated into 4 aliquots and the RNA fragments were precipitated with 0.1 volumes of sodium acetate (3M, pH5.2) and 2.2 vol. ethanol at -70°C for 1 hour, centrifuged and dried in vacuo. Reverse transcription-polymerase chain reaction (RT-PCR) was performed in order to characterise the bands using an aliquot of eluted RNA per reaction and the appropriate primers. Amplified PCR fragments were ligated into TA vector (Invitrogen) which was used to transform HB101 competent *E.coli* cells and grown on agar plates containing ampicillin to select for those cells containing the vector. The presence of the insert was demonstrated by PCR and the colonies selected were grown overnight in LB broth. Plasmid DNA was extracted and sequenced using Taq Dyedexoxy Terminal Cycle Sequencing (ABI/Perkin Elmer). The individual protocols used are described in more detail below.
Northern Analysis

Northern Gels

Refer to Appendix II.7 for the exact composition of the solutions. RNA (20μg) was resuspended in 10μl of denaturing mixture with tRNA (0.6μg/μl), incubated at 50°C for 1 hour and cooled on ice. Glycerol (2.5μl) was added to the samples which were separated on a 1% agarose gel, concomitantly with a separate dye-marker, in sodium phosphate buffer (10mM) and the buffer recycled from positive electrode to negative electrode. Control lanes were stained in ethidium bromide (1mg/l) to elucidate the integrity of the RNA and also so as to be able to determine the size of any bands detected after blotting and probing.

Transfer of RNA to Membranes

Nitrocellulose membranes (0.45μm) were soaked in ddH₂O for 10 minutes and 20 x SSC for 30 minutes (Appendix II.7). RNA was transferred in 20 x SSC for 16 hours at 4°C and fixed at 80°C for 1 hour.

Hybridisation of Radiolabelled Riboprobe to Immobilised RNA

Membranes were incubated in prehybridisation buffer with denatured salmon-sperm DNA (0.1mg/ml) overnight at 42°C. The buffer was discarded and replaced with hybridisation buffer, with the addition of boiled radiolabelled riboprobe (1 x 10⁶ cpm/ml, ~0.05pg/ml), and incubated overnight at 42°C. The filters were then washed 4 x 15 minutes at room temperature in a high salt wash followed by 3 x 20 minutes at 50°C in a low salt wash. The filter was exposed at -70°C to Amersham Hyperfilm-MP.
**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

RNA (~1μg in 2μl) was incubated with reverse transcription mix, downstream primer (0.75μM - see Appendix III.1) and Molony-Murine Leukaemia Virus (M-MLV) reverse transcriptase (2.5U/μl - Gibco BRL) in a total reaction volume of 20μl. The samples were then incubated for 15 minutes at 42°C, 15 minutes at 99°C and 5 minutes at 5°C in a Perkin Elmer Cetus DNA Thermal Cycler.

PCR was performed in MgCl₂ (2mM), 1 x buffer, ddH₂O, Taq polymerase (25 mU/μl - Gibco BRL) and upstream primers (0.15mM) in a 100μl reaction. The Tₘ of the primers was calculated, using the approximation that Tₘ \( \approx 2 \times (A+T) + 4 \times (G+C) \); the Tₘ of both primers should preferably be the same. The incubation was as follows: 2 minutes at 95°C for 1 cycle; 1 minute at 95°C, 1 minute at (Tₘ - 5°C), 2 minutes at 72°C for 30 or 35 cycles; 7 minutes at 72°C for 1 cycle; 4°C soak.

DNA loading buffer and E buffer (1 x final concentration) was added to the samples which were separated on a 2% Nusieve agarose gel (Nusieve 3:1 (FMC)) run in 1 x E buffer and ethidium bromide (1μg/ml).

**A I.4 - Ligation and Transformation of DNA Fragments and Plasmid Purification**

**TA Cloning**

**Ligation**

The cloning was performed as described by the manufacturer (Invitrogen). Briefly, ligations into the linearised pCRᵀᴹII vector (50ng) were performed using two
concentrations of PCR product (1μl or 3μl), in 10 x ligation buffer (1μl) and T4 DNA ligase (4 Weiss units) and made up to a total reaction volume of 10μl with ddH2O. The samples were incubated at 4°C for 16 hours.

**Cell Transformation**

HB101 *E.coli* cells (200μl) were incubated with the ligation reaction (10μl) and transformation buffer (190μl) on ice for 25 minutes followed by an incubation for 5 minutes at 37°C. 1ml of pre-heated (37°C) LB-broth was added to the sample and incubated at 37°C for a further hour before being spread onto LB-bacteriological agar plates (1.2g agar/100ml LB-broth) in varying amounts (e.g. 50μl, 100μl and 200μl/plate), air dried, and incubated inverted at 37°C for 16 hours.

**PCR and Growing the Colonies**

PCR was performed using the M13-forward and -reverse primers (Appendix III.1) which span the insertion site, in order to detect whether ligation has occurred. The PCR was set up as follows: the primers (0.15mM) were added to MgCl₂ (1.25mM), 1 x PCR buffer, dNTPs (1mM of each) and Taq polymerase (25mU/μl) and made up to a total volume of 100μl with ddH2O. Individual colonies were picked and dipped into separate micro-reaction tubes containing the PCR mix, before being added to 10ml aliquots of LB-broth with ampicillin (100μg/ml) and incubated for 16 hours at 37°C in an orbital shaker. The PCR incubation was carried out at 94°C for 2 minutes for 1 cycle; 94°C for 1 minute, 51°C for 1 minute, 72°C for 2 minutes for 25 cycles; 72°C for 7 minutes extension; 4°C soak. The products were then separated on a 2% agarose gel (see RT-PCR).
Preparation of Competent HB101 E.coli

Competent HB101 E.coli cells (10μl) were grown in 10ml of LB-broth at 37°C for 16 hours in an orbital shaker. This was used to inoculate 100ml of LB-broth at 37°C and the growth of the culture was monitored by measuring the absorbance at 650nm. When the optical density had reached 0.6, the cells were collected by centrifugation at 1,500 x g at 4°C, after which the supernatant was discarded, the cells resuspended in storage buffer and frozen at -70°C (stock).

Extraction and Purification of Plasmid DNA

Mini-preparation (small scale)

An aliquot of cells were centrifuged at 8,800 x g for 10 minutes and resuspended in 500μl storage buffer and stored at -70°C. The remainder of the culture was centrifuged at 1,500rpm and 4°C in a Beckman Coolspin Centrifuge. The broth was discarded, the pellet resuspended in solution A (200μl), 400μl of solution B and solution C (200μl) (Appendix II.10) and mixed thoroughly. The samples were centrifuged at 10,000 x g for 10 minutes at 20°C after which the supernatant was collected and mixed with propan-2-ol (0.6 volumes) and centrifuged for 5 minutes at 10,000 x g and 20°C. The pellet was washed in 70% ethanol and respun for 5 minutes at 10,000 x g and 20°C, after which the pellet was resuspended in TE buffer and incubated with boiled pancreatic RNase A (to a final concentration of 20μg/ml) for 1 hour at 37°C. The samples were PIC- and IC-extracted twice and the DNA was ethanol precipitated. The DNA was dried in vacuo and resuspended in ddH2O or TE buffer as required.
Maxi-preparation (large scale)

10ml of LB-broth containing ampicillin (100μg/ml) were inoculated from stock and shaken vigorously for 16 hours at 37°C. These were used to inoculate 500ml of LB-broth containing ampicillin (100μg/ml) and incubated in an orbital shaker for 16 hours at 37°C. The cells were pelleted at 1500 x g for 30 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 25ml solution A, 50ml solution B, and 25ml solution C. The lysis was centrifuged at 6,000 x g for 20 minutes at 4°C and the supernatant filtered through cotton gauze. Propan-2-ol (0.6 volumes) was added and mixed well prior to centrifugation at 6,000 x g for 20 minutes at 20°C and the pellet was washed in ethanol (70%, 20°C).

Plasmid DNA was purified on CsCl gradients by equilibrium centrifugation, and visualised with ethidium bromide. The pellet was resuspended in TE (100:2) until the total weight was 9g. CsCl (9g) was added with 0.5ml of ethidium bromide solution (10mg/ml), mixed and transferred to 16 x 76 mm Beckman polyallomer quick seal tubes and topped up with paraffin. Centrifugation was performed in a near vertical rotor (NVT 65) at 202,000 x g for 13 hours at 20°C in a Beckman L70 ultracentrifuge. The lower band of closed circular plasmid was removed through the tube wall using a needle and syringe and made up to a final volume of 10ml with TE (100:2). The DNA was precipitated with 20ml ethanol (100%, 20°C), centrifuged for 20 minutes at 1,500 x g at 20°C, washed in ethanol (100%, 20°C) and recentrifuged at 1,500 x g for 10 minutes at 20°C. The pellet was dried in vacuo, resuspended in 1 x SSC containing boiled pancreatic RNase A (100μg/ml) and incubated for 1 hour at 37°C. The samples were PIC- and IC-extracted as for the Mini-preparation, after which the DNA was ethanol
precipitated and dried *in vacuo*. The pellet was resuspended in ddH$_2$O or TE as required and the DNA quantitated by measuring absorbance at 260nm.

### A 1.5 - Taq DyeDeoxy™ Terminator Cycle Sequencing

Double-stranded plasmid DNA (1µg) was added to reaction premix (9.5µl) and primers from which to sequence (3.2pmol) to a total volume of 20µl. The reaction mixture was overlaid with mineral oil (40µl) and immediately incubated in a Perkin Elmer Cetus Model 480 thermal-cycler pre-heated to 96°C. The samples were incubated as follows: 96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes for 25 cycles; 4°C soak.

Following amplification, the unincorporated terminators were removed using a specific form of phenol/chloroform extraction performed with reagents at room temperature. The reaction volume was increased to 100µl with ddH$_2$O to which chloroform (100µl) and homogeneous phenol:H$_2$O:chloroform (68:18:14 - 100µl) was added. The samples were vortexed, centrifuged and the lower organic phase discarded after which the aqueous phase was re-extracted with phenol:H$_2$O:chloroform (100µl). After centrifugation, the aqueous layer was transferred to a clean tube to which sodium acetate (2M, pH4.5 - 15µl) and ethanol (100% - 300µl) was added. The mixture was centrifuged for 15 minutes at 10,000 x g at room temperature, the pellet washed with ethanol (70%) and dried *in vacuo*. The samples were resuspended in gel loading buffer, denatured at 90°C for 2 minutes and placed on ice prior to loading.
A 1.6 - Purification of DNA fragments from agarose gels

DNA fragments were separated on agarose gels in 1 x E buffer and ethidium bromide (1µg/ml). The DNA bands were visualised under UV radiation at 302nm and compared to molecular weight markers. DNA fragments were cut out from the gel using sterile scalpel blades and the DNA eluted using a Gene Clean II kit (Bio 101, Inc.). Three volumes of sodium iodide solution (6M) was added to the agarose gel slices and incubated at 45-55°C until the gel had melted, after which silica matrix suspension (Glassmilk®, 10µl) was added, mixed thoroughly, and incubated at 45-55°C for a further 5 minutes. The silica matrix-DNA complex was pelleted by brief centrifugation at 10,000 x g for approximately 10 seconds and the supernatant discarded. The pellet was washed three times in NEW wash (NaCl, ethanol, water), briefly vortexing and centrifuging the samples between each addition. ddH₂O (10µl) was added and vortexed prior to incubation at 45-55°C for 5 minutes. The samples were centrifuged at 10,000 x g for 15 seconds at 20°C and the supernatant collected. A further 10µl of ddH₂O was added to the silica matrix, vortexed and centrifuged and the supernatant pooled. The yield of DNA could be ascertained by measuring absorbance at 260nm.

A 1.7 - Eco RI Digestion

Plasmid DNA (~5µg) was digested with Eco RI (30U) in 1 x buffer H, DTT (1mM) and spermidine (10µM) at 37°C for 16 hours. Following incubation, the DNA was extracted twice in PIC and IC and ethanol precipitated.
A.8 - Dephosphorylation of Plasmid Vectors

Dephosphorylation of plasmid vector was necessary to prevent self-ligating of the plasmid. The plasmids were incubated in dephosphorylation buffer with CIP (calf alkaline phosphatase - 1 Unit/100pmol of protruding 5'-termini) for 30 minutes at 37°C. The reaction was terminated by adding EDTA (pH8.0) to a final concentration of 5mM and incubation at 75°C for 10 minutes. The plasmid was purified using PIC- and IC-extraction and ethanol precipitated.

A.9 - Preparation of Cytosolic Proteins

The liver tissue (~7g) was ground in a mortar in liquid nitrogen and homogenised with a glass Teflon homogeniser in 10ml of homogenising buffer comprising Tris-HCl (10mM, pH7.4), KCl (2.5mM), dithiothreitol (DTT) (1mM) and phenylmethylsulphonylfluoride (PMSF) (1mM). Cells in culture were harvested using sterile cell scrapers and added to homogenising buffer (0.1 volumes). Samples were centrifuged at 10,000 x g for 10 minutes at 4°C, the supernatant transferred to ultracentrifuge tubes and filled to capacity with homogenising buffer. The cytosolic fraction was obtained after centrifugation at 100,000 x g for 1 hour at 4°C. The protein content of the supernatant was measured by Lowry assay, after which the remainder was aliquotted for storage at -70°C.
Protein samples (200μl) or BSA (bovine serum albumin) standards (0-100μg/ml) were mixed with 1ml of alkaline solution and incubated at 20°C for 10 minutes. Folin’s reagent (100μl of 1:4 dilution) was added and incubated at 20°C for 30 minutes, after which absorbance was measured at 510nm and the concentration of samples calculated from the standard curve generated.
Appendix II

General Solutions
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A II.1 - General Cell Culture Solutions

*Dulbecco's Phosphate Buffered Saline (PBS - Oxoid)*

8g/l NaCl  
1.15g/l NaHPO₄  
0.2g/l KCl  
0.2g/l KH₂PO₄

*PBS with Calcium and Magnesium (Sigma)*

9.6mM Na₂HPO₄  
4.9mM MgCl₂ • 6H₂O  
2.7mM KCl  
1.5mM KH₂PO₄  
0.9mM CaCl₂ • 2H₂O  
0.14M NaCl

*Trypsin Solution in PBS (Gibco BRL)*

0.15M NaCl  
0.54mM EDTA  
0.5g/l Trypsin (1:250)

*Storage Medium in Dulbecco's Modified Eagle Medium (DMEM) without Phenol Red*

20% Foetal Calf Serum  
10% DMSO  
2mM Glutamine  
100U/ml penicillin/streptomycin  
100U/ml nystatin  
0.25µg/ml amphotericin

A II.2 - Alkaline Phosphatase Staining Solutions

*Citrate working solution*

25.2mM citric acid  
4.8mM sodium citrate
Citrate Buffered Acetone

3 volumes acetone
2 volumes citrate working solution

A II.3 - Solutions for RNA Extraction

Denaturing Solution for RNA Extraction

4M guanadinium thiocyanate
0.1M β-mercaptoethanol
25mM sodium citrate (pH7.0)
0.5% v/v N-lauroylsarcosine

A II.4 - Solutions for Radiolabelled Riboprobes

Transcription Mix

40mM Tris-HCl (pH7.5)
10mM dithiothrietol (DTT)
10mM NaCl
6mM MgCl₂
2mM spermidine
0.5mM nucleotides (rATP, rCTP, rGTP)
1U/µl RNasin

Formamide Loading Dye

80% v/v deionised formamide
10mM EDTA (pH8.0)
1mg/ml xylene cyanol FF
1mg/ml bromophenol blue

TBE Buffer

89mM Tris-HCl
89mM boric acid
2mM EDTA
Polyacrylamide Gel

8.3M urea
89mM Tris-HCl
89mM boric acid
3.5% acrylamide
2mM EDTA
0.8% ammonium persulphate (APS)
0.18% methylene bisacrylamide
0.04% N,N,N',N'-tetramethylethylenediamine (TEMED)

A II.5 - Solutions for RNase Protection Assays

RNase Hybridisation Buffer

75% v/v deionised formamide
0.4M NaCl
20mM Tris-HCl (pH7.6)
6.5mM vanadium ribonucleoside complex (VRC)
1mM EDTA
0.1% sodium dodecyl sulphate (SDS)

RNase Digestion Buffer

300mM NaCl
10mM Tris-HCl (pH7.6)
5mM EDTA

A II.6 - Solution for Elution of RNA from PAGE

Gel Elution Buffer

0.5M ammonium acetate
10mM magnesium acetate
0.1% SDS
A II.7 - Solutions for Northern Analysis

Northern Denaturing Solution

50% v/v dimethyl sulphoxide (DMSO)
10mM sodium phosphate buffer (Na$_2$PO$_4$/Na$_2$HPO$_4$) (pH7.0)
7.2% v/v deionised glyoxal

RNA Loading Dye

50% v/v glycerol
50mM sodium phosphate buffer (pH7.0)
0.5mg/ml bromophenol blue

Northern Marker Dye

15.6µl Northern denaturing solution
5µl RNA loading dye
4.4µl DEPC-ddH$_2$O

20 x SSC

3M sodium chloride
0.3M sodium citrate (pH7.0)

1 x Denhardt's Solution

0.02% w/v polyvinyl pyrolidone
0.02% w/v ficoll 400
0.02% w/v bovine serum albumin

Northern Prehybridisation Buffer

50% v/v deionised formamide
50mM sodium phosphate buffer (pH7.0)
4 x SSC
1 x Denhardt's
**Northern Hybridisation Buffer**

4 volumes Northern prehybridisation buffer  
1 volume dextran sulphate (50% w/v)

**High Salt Wash**

2 x SSC  
1% w/v SDS

**Low Salt Wash**

0.1 x SSC  
1% w/v SDS

**A II.8 - RT-PCR Solutions**

**Reverse Transcription Buffer**

1 x PCR Buffer :  
50mM KCl  
10mM Tris-HCl  
5mM MgCl₂  
1mM dGTP, dCTP, dATP and dTTP  
1U/µl RNasin  
DEPC-treated ddH₂O

**1 x E Buffer**

40mM Tris-HCl (pH7.0)  
20mM sodium acetate  
2mM EDTA

**DNA Loading Buffer**

50% w/v glycerol  
5mM Tris-HCl (pH7.4)  
0.5mM EDTA  
0.2% w/v bromophenol blue
A II.9 - Solutions for Cloning into pGEM™ and TA® Vectors

10 x Ligation Buffer for TA® Vectors

70mM β-mercaptoethanol
60mM MgCl₂
60mM Tris-HCl (pH8.3)
50mM NaCl
20mM DTT
10mM spermidine
1mg/ml bovine serum albumin
1mM ATP

T4 Ligation Buffer for pGEM™

30mM Tris-HCl (pH7.5)
10mM DTT
10mM MgCl₂
1mM ATP

Transformation Buffer

0.25M KCl
0.1M CaCl₂
10mM RbCl
5mM MgCl₂
5mM Tris-HCl (pH7.6)

LB-Broth

10g/l NaCl
10g/l tryptone
5g/l yeast extract

Storage Buffer

Transformation buffer
50% v/v glycerol
A II.10 - Mini- and Maxi-Plasmid Preparation Solutions

Solution A

50mM glucose
25mM Tris-HCl (pH8.0)
10mM EDTA

Solution B

0.2M NaOH
1% w/v SDS

Solution C

3M potassium acetate (pH4.3)

TE Buffer

10mM Tris-HCl (pH7.4)
1mM EDTA

TE (100:2) Buffer

0.1M Tris-HCl (pH8.0)
2mM EDTA

TEN Buffer

0.1M NaCl
20mM Tris-HCl (pH7.6)
1mM EDTA

A II.11 - Solution for Dephosphorylation of Plasmid Vectors

Dephosphorylation Buffer

10mM Tris-HCl (pH8.3)
1mM MgCl₂
1mM ZnCl₂
A II.12 - Buffers for Restriction Digestion

Buffer B

100mM NaCl
10mM Tris-HCl (pH 8.0)
5mM MgCl₂
1mM β-mercaptoethanol

Buffer H

100mM NaCl
50mM Tris-HCl (pH 7.5)
10mM MgCl₂

A II.13 - Solutions for DyeDeoxy™ Terminator Cycle Sequencing

5 x Terminator Ammonium Cycle Sequencing (TACS) Buffer

400mM Tris-HCl
100mM (NH₄)₂SO₄ (pH 9.0)
10mM MgCl₂

Reaction Premix

1 x TACS Buffer
79μM dITP
16μM dATP
16μM dTTP
16μM dCTP
0.84U/μl AmpliTaq® DNA Polymerase
0.11μl/μl DyeDeoxy™ A Terminator
0.11μl/μl DyeDeoxy™ T Terminator
0.11μl/μl DyeDeoxy™ G Terminator
0.11μl/μl DyeDeoxy™ C Terminator

Loading Buffer

83% deionised formamide
8.3mM EDTA (pH 8.0)
A II.14 - Protein Assay Solutions

Alkaline Solution

Added in the following order:  
- 50 ml Na₂HCO₃ (2% w/v)
- 0.5 ml NaK tartrate (2% w/v)
- 0.5 ml CuSO₄ · 5H₂O (1% w/v)

A II.15 - SDS Polyacrylamide Gels and Solutions

12.5% SDS Polyacrylamide Gel

12.5% acrylamide  
0.375mM Tris-HCl (pH 8.8)  
0.33% methylene bisacrylamide  
0.1% SDS  
0.04% APS  
0.04% TEMED

3% SDS Polyacrylamide Stacking Gel

3% acrylamide  
0.125mM Tris-HCl (pH 6.8)  
0.1% SDS  
0.1% APS  
0.08% methylene bisacrylamide  
0.05% TEMED

SDS Polyacrylamide Gel Buffer

0.38M glycine  
0.1% SDS  
0.05M Tris

SDS Polyacrylamide Gel Loading Buffer

100mM DTT  
50mM Tris-HCl (pH 6.8)  
10% glycerol  
2% SDS  
0.1% bromophenol blue
Appendix III

Primers and Sequence of IGF-I
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<tr>
<td>III.2 IGF-I Sequence for Exons 1, 2 and 3</td>
<td>315</td>
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<td>III.3 pGEM® Vector</td>
<td>319</td>
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<td>III.4 TA™ Vector</td>
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### A III.1 - Primers Used

<table>
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<tr>
<th>Primer Number</th>
<th>Sequence</th>
<th>Up-/down-stream</th>
<th>Expected size with Primer 14</th>
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<tbody>
<tr>
<td><strong>Exon 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5' CACGGTGCCCCAAAAGTCCT3'</td>
<td>Up</td>
<td>523 &amp; 337 bases</td>
</tr>
<tr>
<td>2</td>
<td>5' CGATAACTTTGCCAGAAGAGG3'</td>
<td>Up</td>
<td>498 &amp; 312 bases</td>
</tr>
<tr>
<td>3</td>
<td>5' GAGAGAGAAGGCGAATGTCC3'</td>
<td>Up</td>
<td>473 &amp; 287 bases</td>
</tr>
<tr>
<td>4</td>
<td>5' CCCCTTTCTGCTTGCTAAATC3'</td>
<td>Up</td>
<td>389 bases</td>
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<tr>
<td>5</td>
<td>5' GCCTGCAGAATCGAACAAAG3'</td>
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<td>336 bases</td>
</tr>
<tr>
<td>6</td>
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<td>194 bases</td>
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<td>9</td>
<td>5' TGCCTCTGTGACTTCTTGAAG3'</td>
<td>Up</td>
<td>161 bases</td>
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<td><strong>Exon 2</strong></td>
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<td>12</td>
<td>5' ACCAAAATGAGCGCACACTCCA3'</td>
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<td><strong>Exon 3</strong></td>
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<td>14</td>
<td>5' GGTCACACACAGGAACCTGAAGA3'</td>
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<tr>
<td><strong>Primers Flanking MCS</strong></td>
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<tr>
<td>M13 (-20) F</td>
<td>5' CAGGAAACAGCTATGACCATG3'</td>
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</tr>
<tr>
<td>M13 R</td>
<td>5' ACGTTGAAAACGACGGCGCAG3'</td>
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<tr>
<td>T7</td>
<td>5' TAATACGACTCTACTATAGGG3'</td>
<td>Up</td>
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*Table A III.1 - Primer sequences and predicted fragment sizes*
A III.2 - IGF-I Sequence for Exons 1, 2 and 3

Exon 1 and Intrinsic Sequences

Key:  Red Arrows - Primers
Black arrow/Yellow letter - Transcription start site
Blue box/grey letters - Start codon
Green box - Stop codon
Blue letters - 186-base deletion

-111 -101 -91 -81 -71
atggcagcat cagttattta cgctgctttaa ccctgctcaga aacacacatt

-61 -51 -41 -31 -21
ctttaaaggg ggggaaaaaa aacgaccttg tgctcagtt tttaaaagca

-11 -1 10 20 30
aaggtatgat gttattttgccggtgccccctaaaaagctcttactcagtaa

90 100 110 120 130
CTTTGCCAi WtatttetfCScGGTGCCCA ÂAXXXGTCC

90 100 110 120 130
TCCTGTCTAC AGTGTCTGTG TTTTGTAGAT AAAGGG ATTTTCTCTA

90 100 110 120 130
AATCCCTTTT CTGCTTGCTA AATCTCACTG TCGCTGCTAATTCAGAGCA

190 200 210 220 230
GATAGAGCCT GCCCAATCGA AATAAAGTTAC TCAAAAATTTG ATG

240 250 260 270 280
TGCTCTAAACA TCTCCCATCT CTCTGAATT TCTTTGCT TCTATATCTT

290 300 310 319
GCCCACCAAT TCATTTCACACTTTTGCTACT TCAGAAGCCG AGG

330 340 350
AAA ATC AGC AGT CTT CCA ACT GCA
Lys Ile Ser Ser Leu Pro Thr Glu Leu

360 370
TTT AAG ATC TGC CTC TGT GAC TTC TTG
Phe Lys Ile Cys Leu Cys Asp Phe Leu

380 +7 +17 +27 +37 +47
AAG gtaaata tctcttact tttttttct ttttcctgca gtcggtggt

Lys
Exon 2 and Intrinsic Sequence

-431 cacgctgtgc
cggaaaagca
-421 gcagcggttct
ggccggctgc
tgcttaacct
-411 -381
ttcatttggga
aggggacctt
tggtggtgct
gagcttgggg
cttgatttc
-361 -331
ggtttggaggc
tctgcttta
gacaggtgtgc
gttaagttct
gttaggtga
-321 -311
tctgcttta
gacaggtgtgc
gttaagttct
gttaggtga
-301 -281
gtttggaggc
tctgcttta
gacaggtgtgc
gttaagttct
gttaggtga
-291 -231
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cgctgctgct
gctgctgcca
gagcttgggg
cttccttct
-281 -221
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actggaaccta
tttcagtcttt
tcattctcag
caaaattata
-271 -181
tcctgccaga
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caaaattata
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tcattctcag
caaaattata
-161 -131
accggctctct
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ttgccgga
gttaattctc
taaagatcc
-121 -81
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tttcagtcttt
tcattctcag
taaagatcc
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taaagatcc
-61 -31
caggcaaga
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tcattctcag
taaagatcc
-51 -1

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tttcagtcttt
tcattctcag
-21
gactttttct
tagcgcaagga
gtgggtgtgtg
tttcagtcttt
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-1

20 30 40 50 60
CCTTGTCAAG CACCTAATTG TGCCATCCGT GCGATGCCAG CCAGCTCCAT
Primer 10
70 80 90 100 110
CCACCTTACAGGAGTGAAGT CAGCGTTCCAT ATACGCTTGTC TTGTCTGGCA
120 130 140 150 160
GCTGAGATAG TGTTTCCCAA AGGGACTGTG GAATGTTACC TCAGCAGGCA
170 180 190 200 210
TTCATTTCCG GTTTGGAAAA TCGTCTCCAA ATGAACTTCC TTTCCGTGTGCT
220 230 240 250 260
GGGTCTGCAG AACATTTCAG AAGCGGAGCT AGCAATCTGC TTCAACTTTT
270 280 290 300 310
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Exon 3 and Flanking Regions:

-151 gccaggatac
-141 tccgggaccc
-131 ttcataccac
-121 agtgcgttcc
-111 taggataag
-101 agtttttaatga
-91 atgatagcaa
-81 ggagtttga
-71 tgtggttcaa
-61 acatatctct
-51 gccccacccct
-41 ttcaggacca
g-aaggatacc
-31 gaaggatacc
g-acacctcct
-21 ttgtgacag
-11 ttttttccc

-10 Primer 13
20 Met 22 Ser

TCT TCA CAT CTC TTC TAC CTG GCA CTC
Ser Ser His Leu Phe Tyr Leu Ala Leu

50 TGC TTG CTC ACC TTT ACC AGC TCG GCC
Cys Leu Leu Thr Phe Thr Ser Ser Ala

80 ACA GCC GGA CCA GAG ACC CTT TGC GGG
Thr Ala Gly Pro Glu Thr Leu Cys Gly

110 GCT GAG CTG GTG GAC GCT CTT CAG TTC
Ala Glu Leu Val Asp Ala Leu Gln Phe

130 GTG TGT GGA CCA AGG GGC TTT TAC TTC
Val Cys Gly Pro Arg Gly Phe Tyr Phe

AAC Exon 4 continues from here.

Asn
A III.3 - pGEM® Vector

T7 Promoter

EcoRI
SacI

KpnI
Aval
SmaI
BamHI
XbaI
SacI
Accl
HincII
PstI

SP6 Promoter

Promega

pGEM®-3Z

2.7 kb
A III.4 - pCR™ II TA Vector

$5'CAGGAA ACA GCT ATG ACC ATG ATT ACG CCA AGC TAT TTA GGT$

M13 Reverse Primer

SP6 Promoter

GAC ACT ATA GAA TAC TCA AGC TAT GCA TCA AGC TTG GTA CCG

NsiI HindIII KpnI

AGC TCG GAT CCA CTA GTA ACG GCC GCC AGT GTG CTG GAA TTC

Sacl Spel XmalII BstXI EcoRI

GGC TT Insertion A GCC GAA TTC TGC AGA TAT CCA TCA CAC TGG

A Site TT EcoRI BstXI NotI

CGG CCG CTC GAG CAT GCA TCT AGA GGG CCC AAT TCG CCC TAT

XmalII Xhol/Aval NsiI Xbal Apal

AGT GAG TCG TAT TAC AAT TCA CTG GCC GTC GTT TTA CAA CGT

T7 Promoter

M13 (-20) Forward Primer

CGT GAC TGG GAAAAC$3'$

M13 (-40) Forward Primer

Invitrogen

pCR™II

3.9 kb
References


Knight, S.W. and Docherty, K. (1992) RNA-Protein interactions in the 5'-untranslated region of preproinsulin mRNA. *Journal of Molecular Endocrinology* **8**:225-234.


341


Murrills, R.J., Stein, L.S., Fey, C.P. and Dempster, D.W. (1990) The effects of parathyroid hormone (PTH) and PTH-related peptide on osteoclast resorption of


Wong, G.L. and Cohn, D.V. (1975) Target cells in bone for parathormone and calcitonin are different: enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces. Cell Biology 72:3167-3171.


Addendum

Page 108 (Collagenase Digestion) - The shaker used was Titertek from Flow Laboratories Ltd., Irvine, U.K., on setting 3.

Pages 122 & 123 (Quantitation of Alkaline Phosphatase Staining) - The percentage of alkaline phosphatase-positive cells was determined by counting the number of cells staining for the presence of alkaline phosphatase as a proportion of the total number of cells visible in the field of view under light microscopy (x100). Three different fields of view were used at random per flask, and the mean calculated.

Page 130 (Cell Culturing for IGF-I RIA) - Primary cultures of cells enriched for the osteoblast lineage were grown to confluence, from stocks stored in liquid nitrogen, and collagenase fractions 2, 3 and 4 were trypsinised and pooled. The passage number and seeding density used were those previously optimised (pages 125-6). The cells were cultured in triplicate in 9cm² dishes for 48 hours in 2ml of DMEM without phenol-red containing FCS (10%), penicillin / streptomycin (100U/ml) and amphotericin (0.25µg/ml). The medium was discarded and the cells rinsed with PBS, prior to addition of fresh medium which did not contain any FCS. The cells were cultured for 24 hours, after which the medium was frozen at -70°C until assayed.

Pages 166 & 185 (Cell Culturing for RNase Protection Assays and Northern) - The fractions of cells released from collagenase digestions 2, 3 and 4 were grown to confluence from stocks, as described on page 109. The cells were trypsinised and pooled and four 175cm² flasks were seeded at the optimal density. The cells were cultured for five days in 25ml of DMEM without phenol-red containing FCS (10%), penicillin / streptomycin (100U/ml) and amphotericin (0.25µg/ml). The cells were rinsed in PBS and cultured for a further 24 hours in standard medium which was serum-depleted (2.5%). The medium was discarded and RNA extracted from the cells as described in Appendix I. This procedure was performed in duplicate for every experiment. The mean of the results obtained from the densitometric analysis of the RNase protection assays was calculated. The mean values obtained in discrete experiments (i.e. from different cell cultures) were then used to calculate the standard deviation.