The biological actions of the insulin-like growth factor (IGF) axis are well defined, and include profound effects on the growth and differentiation of most cell types (Hwa et al., 1999). The established components of the IGF system include IGFs (IGF-I and IGF-II), type I and type II IGF receptors, a family of six secreted IGF-binding proteins (IGFBPs), and IGFBP proteases (Fig. 1). IGF-I and IGF-II are very similar small peptides of approximately 7 kDa and similar to insulin in structure. IGFs are expressed ubiquitously and are important mitogens affecting cell growth and metabolism. In addition to endocrine effects exerted by circulating IGFs, locally produced IGFs exert paracrine, as well as autocrine, effects on cell proliferation (Jones and Clemmons, 1995). The IGFs interact with specific cell surface receptors, designated type I and type II IGF receptors, and can also interact with the insulin receptor. The mitogenic effects of IGFs are mediated mainly through interactions with the type I IGF receptor, which has tyrosine kinase activity. The type II IGF receptor is structurally distinct, binds primarily IGF-II, but also serves as a receptor for mannose-6-phosphate-containing ligands (Nissley and Lopaczynski, 1991). The role(s) of the type II receptor in mediating IGF action is less well defined (Kornfeld, 1992).

In biological fluids, IGFs are normally bound by members of a family of secreted IGFBPs of approximately 30 kDa. There are, to date, six well characterized mammalian IGFBPs, designated IGFBP-1 to -6. Since the affinity constants of the IGFBPs are 2–50-fold greater for binding IGFs than that of the IGF type I receptor, they can modulate IGF action by preventing the insulin-like effects of IGFs, controlling the half-life of IGFs in blood, acting as carrier proteins for IGFs and determining the distribution of IGFs between tissues and extracellular fluids (Hwa et al., 1999). The affinity of IGFBPs for IGFs is controlled by phosphorylation, glycosylation and specific proteolysis (Clemmons, 1998). In the prostate, thyroid and mammary glands, the expression of a particular binding protein, IGFBP-5, coincides with a stage during tissue remodelling known as involution, when many cells are removed by programmed cell death (apoptosis), which has been shown to be important in sculpting embryonic tissues, and, in particular, the developing limb bud. In addition, the very early onset of expression of various IGF family members in chicken embryos further emphasizes the fundamental importance of this system in development. This article reviews the work that has been carried out in this area in the context of current understanding of the IGF system.

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There is increasing evidence that IGFBPs not only regulate IGF bioavailability, but that they also have their own receptors that mediate IGF-independent actions. Cell surface receptors for IGFBP-1 (Jones et al., 1993), IGFBP-2 (Rauschnabel et al., 1999), IGFBP-3 (Oh et al., 1993a) and IGFBP-5 (Andress, 1995, 1998) have been described, although, to date, none of these proteins have been cloned. Direct IGF-independent effects of IGFBPs include modulation of bone cell proliferation (Mohan et al., 1995) and growth arrest of breast and prostate cancer cells (Oh et
However, owing to the diverse range of properties imparted by the IGFs and IGFBPs, their role during organ and whole animal development has been difficult to unravel.

The IGF axis in development

The mRNA and protein expression patterns of the various components of the IGF axis have been studied extensively in the developing embryo using in situ hybridization techniques and immunohistochemistry.

Mammalian development

For many years it was considered that there was a change from IGF-II to IGF-I during fetal to adult growth, as IGF-II is expressed more widely in the embryo (Bhaumick and Bala, 1987). The IGF-II gene is also a member of a small family of genes that have been shown to be subject to genomic imprinting (Reik et al., 1996; Moore et al., 1997). An imprinted gene is expressed primarily from one specific parental allele and such genes have been shown to exert important effects, primarily on fetal development. The use of homologous recombination technology proved conclusively that IGF-II was required for normal embryonic development, as IGF-II null mice (requiring only the disruption of the paternal allele due to parental imprinting) were 60% smaller than their wild-type littermates (DeChiara et al., 1990, 1991; Fig. 2). These growth-deficient animals were otherwise apparently normal and fertile, demonstrating that IGF-II protein is not essential for development and survival. The phenotype of the null mutant mice was surprising and difficult to reconcile with the fact that IGF-II had been shown to have a specific expression pattern in

Fig. 1. Components of the insulin-like growth factor (IGF) axis. IGF-binding protein 3 (IGFBP-3) is the main circulatory binding protein and is transported in serum complexed with a glycoprotein. The binding protein and glycoprotein together make up a 150 kDa complex. IGFBPs are secreted proteins and all six have been shown to have autocrine effects in a variety of different cell types. Cell surface receptors have been identified for IGFBP-1, -2, -3 and -5, and several have been shown to bind to the extracellular matrix.
many different tissues throughout the embryo (Wood et al., 1990; Pintar et al., 1991; Streck et al., 1992). However, later experiments showed that mice with both copies of the IGF-I gene knocked out were not only reduced in size to a similar extent, but also displayed severe muscle dystrophy and most (> 95%) of these mice died at birth (Powell-Braxton et al., 1993; Fig. 2). These findings clearly demonstrated that IGF-I is essential for correct embryonic development in mice. The reduction in size of both types of IGF knockout mice indicates a role for IGFs as cell survival factors. In support of this contention, studies in vitro with cells derived from null mutants for IGF-II (Lamm and Christofori, 1998) and the type I IGF receptor (Cui et al., 1997) demonstrated an increased number of apoptotic cells, whereas animals transgenic for IGF-I (Neuenschwander et al., 1996; Leri et al., 1999), IGF-II (Petrik et al., 1999) and the type I IGF receptor (Steller et al., 1996) demonstrated a reduced number of apoptotic cells.

Subsequently, the mRNA expression patterns of the other five IGFBPs were established (Cerro et al., 1993; Schuller et al., 1993; Green et al., 1994); for example, the expression of IGFBP-5 in a rat embryo at day 11.5 is shown (Fig. 3). All of the IGFBPs are expressed at least as early as day 14 in rat embryos, and each IGFBP has a unique pattern of expression, indicating that the members of this family have

**Fig. 2.** Insulin-like growth factor I (IGF-I) and IGF-II knockouts cause growth deficiency during embryonic development in mice. (a) Chimaeric mice containing a targeted disruption of one IGF-II allele (DeChiara et al., 1990, 1991). Germline transmission of the inactivated gene from male chimaeras yields heterozygous mutants (wt, grey mouse) that are 60% the size of wild-type (wt, white mouse) littermates. In contrast, when the disrupted gene is transmitted maternally, the heterozygous offspring are phenotypically normal, indicating that the maternal IGF-II allele is silenced by genetic imprinting. (b) Results of crossing chimaeras containing a disrupted IGF-I allele (Powell-Braxton et al., 1993). Heterozygous offspring (grey mouse) are 10–20% smaller than wild-type littermates (white mouse), whereas homozygous mutants (black mouse) are < 60% the bodyweight of wild-type mice. In addition, > 95% of the homozygous mutant pups die perinatally (red cross).
distinct and tightly regulated functions in the development of specific tissues. However, IGFBP-2 and -5 display complementary expression patterns that often overlap or occur in adjacent cell populations (Cerro et al., 1993; Schuller et al., 1993; Green et al., 1994). This finding may be indicative of complementary developmental roles for these two binding proteins in specific tissues. Recent immunohistochemical studies using antisera raised against IGFBP-1 to -6 enabled the comparison between IGFBP protein and mRNA localization in mouse embryos (van Kleeffens et al., 1998). This finding is consistent with the function of IGFs as potent cell survival signals and of IGFBPs as regulators of this function.

Avian development

In later stages of development in chicken embryos, IGF peptides have been localized immunohistochemically using a polyclonal antibody to human IGF-I, which recognizes both IGF-I and -II (Ralphs et al., 1990). Specific spatiotemporal patterns of expression are similar to those observed in mouse embryos. The cDNA for chicken IGF type I receptor has been cloned and used to generate an anti-sense probe for in situ hybridization, which demonstrated considerable overlap of receptor mRNA expression and the location of the IGF peptides (Holzenberger et al., 1996). In addition, the cDNAs for both chicken IGFBP-5 (Allander et al., 1997) and IGFBP-2 (Schoen et al., 1995a) have also been cloned. In situ hybridization experiments using the chicken IGFBP-2 cDNA as an antisense probe have outlined the expression of IGFBP-2 mRNA in the embryonic eye, brain, branchial arches, somites and limb buds of chicken embryos 3.5 days after mating (Schoen et al., 1995b). The expression patterns are similar to those observed for IGFBP-2 in mouse and rat embryos, indicating that this IGFBP is likely to perform similar functions in both avian and mammalian development.

Linkage of IGFBP and Hox genes

Each of the human IGFBP genes are localized to the same chromosomal regions as a specific homeobox (Hox) gene family (Allander et al., 1995); for example, IGFBP-2 and -5 are linked to the Hox D gene family on human chromosome 2 in opposite transcriptional orientations. The most likely mechanism to explain this linkage is that IGFBP and Hox genes have co-evolved from a single ancestral genetic locus to multiple loci via the process of chromosomal duplication and translocation (Fig. 4). Whether this tight linkage between the two gene families has any functional significance is unknown, but it is interesting to note that vertebrate Hox genes are well known for their important functions during embryonic development (Krumlauf, 1994).

Limb bud development

The developing limb bud is an attractive model for studying tissue morphogenesis since a number of key developmental processes are understood at both the cellular and molecular level (Tickle, 1996; Cohn and Bright, 2000; outlined in Fig. 5). In vertebrates, limbs develop from paired buds that appear at appropriate points along the main antero–posterior body axis. At early stages, these buds consist of undifferentiated mesenchyme cells encased in ectoderm. At first, limb buds are small mounds (Figs 3 and 5), but these soon elongate. As the buds continue to grow out, they
become broader at the tip as digits begin to develop. Later, cells between the developing digits undergo apoptosis. A prominent feature of limb bud development is the reciprocal signalling between the apical ectodermal ridge (AER), which is a region of specialized pseudostratified epithelium covering the distal edge of the developing limb bud, and the underlying undifferentiated mesenchymal cell population, known as the progress zone. The AER is essential for outgrowth of the limb during development, when the mesodermal cells in the progress zone divide more rapidly. Experiments in vitro have confirmed that the AER has a mitogenic effect on limb mesoderm (Tickle, 1996). The polarizing region is an additional signalling region at the posterior margin of the limb mesenchyme, which controls the antero–posterior patterning of the limb bud (Cohn and Bright, 2000). Many experiments have addressed the role of the IGF system during limb development, and these are discussed below.

Expression of the IGF axis in the mammalian embryonic limb bud

The location of IGF-I and -II expression in the developing limb bud appears to be species dependent. For example, early in rat limb development, IGF-I and -II are expressed throughout the limb mesoderm, but later their expression becomes confined to the proximal part of the bud (Streck et al., 1992). These results indicate that, at least during the early stages of rat limb outgrowth, IGF peptides are available in the region of rapidly proliferating undifferentiated mesoderm at the distal tip of the limb, owing to production within or near that region. It is possible that locally produced IGF peptides are still present in this region during later stages as well, depending on the half-life of the proteins. However, only a proximal location of IGF mRNA in mouse limb buds has been detected (van Kleffens et al., 1998) and an immunohistochemical study has shown that this location coincides with that of IGF peptides (van Kleffens et al., 1999). This discrepancy between rat and mouse limb buds may reflect differences in embryonic stages or the techniques used for detection, and it is possible that, at some point, IGFs are also expressed in the mouse progress zone. In support of this contention, growth of undifferentiated mouse limb bud mesodermal cells has been shown to be stimulated by both IGF-I and IGF-II in vitro (Kaplowitz et al., 1982).

The mouse and rat limb buds provide the most obvious example of the overlap of IGFBP-2 and -5 mRNA expression, with both binding proteins displaying strong expression along the whole length of the AER and weaker expression in a proximal region of the bud (Pintar et al., 1991; Streck et al., 1992; Wood et al., 1992; Allan et al., 1998; van Kleffens et al., 1998; Fig. 3). In fact, IGFBP-2 and -5 are the only two of the six binding proteins expressed in the AER (van Kleffens et al., 1998), and their expression at this site is consistent with them having a role in the directional outgrowth of limb mesenchyme. In addition to IGFBP-2 and -5, several members of the fibroblast growth factor (FGF) family, including FGF-2, -4 and -8 are expressed by the AER (Tickle, 1996). Other factors known to be involved in the apical–subridge mesoderm interaction are the TGFβ family
members known as bone morphogenetic proteins (BMP)-2, -4 and -7, the homeobox containing transcription factors Msx-1 and -2, as well as some members of the retinoic acid family.

After removal of the AER by apoptosis, the morphology of the digits has to be established. Apoptosis is an important mechanism in sculpting limb morphology and is responsible for removing inter-digital tissue (Milaire, 1992). In addition, programmed cell death may play a role in the positioning of muscle precursors in the limb (Amthor et al., 1998). The BMPs play a major role in the specification of digital and interdigital regions of the limb and may act as apoptotic signals in the presence of Msx and growth factors (Ganan et al., 1996). During this later stage in mouse limb bud development (13.5 days after mating), it was found that most of the IGF system co-localized with areas of apoptosis; IGFBP-2, -4 and -5 were found in the interdigital zone, whereas IGFBP-3 and IGF-I bordered this region (Allan et al., 1998; van Kleffens et al., 1998; Fig. 6 shows expression of IGFBP-5 in the interdigital zone of a mouse forelimb bud 13.5 days after mating). In addition to the limb bud, the IGF system co-localizes to apoptotic regions in the head–neck region of mouse embryos (van Kleffens et al., 1999).

Expression of the IGF axis in the avian wing bud

Most of the cellular interactions involved in limb morphogenesis are believed to be common for all vertebrates. The later development of the chicken bud is analogous to that of the rat limb bud, in that IGF-I and its receptor are expressed below the AER (Geduspan et al., 1992; Dealy and Kosher, 1995). Although IGF-I does not appear to be expressed in the chicken AER itself, IGF peptides have been detected in the AER by immunohistochemistry (Ralphs et al., 1990), indicating that IGF-I expressed in the subridge mesoderm may be transmitted to the AER. As described above, the mouse and rat AER are sites of strong overlapping expression of both IGFBP-2 and -5.

Owing to the accessibility of the chicken embryo in the egg, it is feasible to carry out limb bud experiments that involve physical and molecular manipulation. Experiments of this kind have shed further light on the potential role of the IGF axis in limb development. Several lines of evidence point to an IGF-1 autocrine regulatory loop in AER–subridge mesoderm interaction. First, both IGF-I and insulin are able to induce a thickened ridge-like structure in the distal anterior ectoderm, raising the possibility that IGF-I or insulin may be involved in the initial induction of AER formation in the limb bud (Dealy and Kosher, 1995). Second, exogenous IGF-I and insulin can promote the outgrowth of limb mesoderm in the absence of the AER (Dealy and Kosher, 1995). In addition, IGF-I expression in the subridge mesoderm is dependent on FGF expression in the AER, and the ability of these FGFs to
promote the outgrowth and proliferation of limb mesoderm is dependent on IGF-I activity (Dealy et al., 1996). Other lines of evidence link the IGF axis to genes expressed in the AER. First, IGF-I appears to regulate the expression of the homeobox containing transcription factor Msx-2 in the AER (Dealy and Kosher, 1996). Second, studies in bone cell cultures have demonstrated that BMP-2 can enhance IGF-I and -II synthesis and inhibit IGFBP-5 synthesis (Gabbitas and Canalis, 1995).

**Alteration of IGF axis expression in Hypodactyly limb buds**

Study of abnormal limb development in mutants has proved very valuable, as it has shed light on the patterning and differentiation processes in limb morphogenesis. During limb development, programmed cell death is essential for the modelling and fine tuning of limb shape (Milaire, 1992), and it is interesting that an alteration in the shape of limb buds is often correlated with changes in the pattern of cell death. The semi-dominant lethal mouse mutant Hypodactyly (Hd), which is caused by a deletion in the Hoxa13 on chromosome 6, develops pointed limb buds that are unlike the paddle shape common to wild-type mice (Hummel, 1970; Mortlock et al., 1996). The Hoxa13 mutation may be a gain of function, as it actually leads to the expression of a mutant HOXA13 protein (Post et al., 2000). Homozygous (Hd/Hd) pups have only a single 'digit' on both forelimbs and hindlimbs, and usually die in utero. While limb initiation in Hd embryos appears to be normal, the AER is more pronounced, persists for longer and does not appear to be closely associated with the underlying mesenchyme (Robertson et al., 1996). This abnormal AER may result in a change in reciprocal signalling between ridge and mesenchyme, which could account for the massive increase in cell death in the progress zone observed in Hypodactyly (Robertson et al., 1996). The concomitant reduction of both anterior and posterior mesenchyme in the distal part of the bud results in the distinctive pointed Hd limb bud. The expression patterns of some of the important limb bud signalling molecules, including sonic hedgehog (Shh), FGF-4, Hoxd13 and Hoxd11, have been analysed in Hd limb buds (Robertson et al., 1997), and it was found that the pattern of transcripts for genes in the mutant did not differ markedly.

We were interested in what other genes might be operating downstream of Hoxa13 that could lead to the phenotype observed in Hypodactyly. As described above, an increase in cell death is observed in Hd limb buds, indicating that factors involved in apoptosis during limb development may play a part in the manifestation of the Hd phenotype. The results of our studies using both in situ hybridization and bead implantation, and the detailed study of van Kleeffens et al. (1998), indicate strongly that members of the IGF system are involved in the regulation of apoptosis during development of the limb bud. For this reason, we decided to determine whether there was any alteration in the expression patterns of IGF-I, IGFBP-2 and -5 in heterozygous and homozygous Hd embryos using in situ hybridization. IGFBP-2 and -5 expression patterns have been found to be altered in the AER in Hd limb buds, and this is the first example of ridge markers that are affected in this mutant (Allan et al., 2000). IGFBP-2 expression is completely downregulated in the AER as early as 11.5 days after mating, whereas the expression of the gene at more proximal sites was not affected. This would imply that IGFBP-2 is only downregulated in regions where there was mutated Hoxa13 gene transcription. In contrast, IGFBP-5 was only downregulated in specific regions of the AER in Hd mutants. This alteration in expression indicates that IGFBP-2 and -5 are involved in the manifestation of the mutant phenotype displayed in Hd limb buds, and adds support to the contention that members of the IGF axis are involved in AER–subridge mesoderm interactions and programmed cell death during limb bud development. In addition, these findings indicate that IGFBP-2 and -5 lie somewhere downstream of Hoxa13 in a signalling pathway. This conclusion is particularly intriguing when the genetic linkage between IGFBP and Hox genes is considered.

**The IGF axis in early embryonic development**

The chicken embryo model has the advantage of being accessible from the period of gastrulation through neurrulation and organogenesis until hatching. Studies on the expression pattern of the IGF system in early chicken development have been confined to ligand-binding analysis of the IGF type I receptor in chick embryos during gastrulation and neurrulation (Bassas et al., 1985; Girbau et al., 1989). An alternative explanation for these expression patterns of the labelled IGFs is that the probe is bound by IGFBPs that are expressed early in development, as the binding proteins have a much greater affinity than the receptor for IGFs (Hwa et al., 1999). However, confirmation that the IGF type I receptor is also expressed at these developmental stages is provided by the findings that IGF-I-dependent tyrosine kinase activity was present in membrane preparations from whole day 2 embryos (Girbau, 1989) and that receptor mRNA was detected by polymerase chain reaction (PCR) analysis in chicken blastoderms at day 0 (Scavo et al., 1991). In addition, PCR analysis has demonstrated that IGF-I is expressed in chicken embryos during organogenesis (Serrano et al., 1990). Expression of the IGF type I receptor in chicken blastoderm indicates a potential role for IGF signalling before neurrulation or even gastrulation. Since the chicken embryo possesses neither blood circulation nor a liver at these early stages, conceptually, any effects of IGF-I mediated by its receptor are autocrine–paracrine effects.

**Conclusions**

Members of the IGF axis have unique temporal and spatial mRNA and protein expression patterns in embryos from different vertebrate species, indicating that these genes have distinct and tightly regulated functions in the development of specific tissues. These expression patterns often co-
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