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Cellular Localization of Somatomedin (Insulin-Like Growth Factor) Messenger RNA in the Human Fetus

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The somatomedins or insulin-like growth factors (IGFs) are synthesized in many organs and tissues, but the specific cells that synthesize them in vivo have not been defined. By in situ hybridization histochemistry, IGF I (somatomedin C) and IGF II messenger RNAs were localized to connective tissues or cells of mesenchymal origin in 14 organs and tissues from human fetuses. IGF messenger RNAs were localized to perisinusoidal cells of liver, to perichondrium of cartilage, to sclera of eye, and to connective tissue layers, sheaths, septa, and capsules of each organ and tissue. All of the hybridizing regions are comprised predominantly of fibroblasts or other cells of mesenchymal origin. Because these cells are widely distributed and anatomically integrated into tissues and organs, they are ideally located for production of IGFs, which may exert paracrine effects on nearby target cells.

THE SOMATOMEDINS OR INSULIN-like growth factors (IGFs) are peptide mitogens that are structurally homologous to proinsulin (*1*). In man, two major IGFs, IGF I (somatomedin C) and IGF II, have been characterized by amino acid sequence analysis of peptides purified from serum (*2*) and by isolation of complementary DNAs (cDNAs) encoding precursor forms of these peptides (*3, 4*). Traditionally, the liver has been thought to be the primary site of IGF synthesis, but evidence from a number of studies now suggests that the IGFs are synthesized in many mammalian tissues: (i) immunoreactive IGFs are secreted by cultured explants of a number of mouse organs (*5*) and by monolayers of fibroblasts derived from several human and rat tissues (*6*); (ii) concentrations of immunoreactive IGF I (*7*) and abundance of IGF I messenger RNA (mRNA) (*8*) is regulated by growth hormone in a variety of adult rat tissues; (iii) immunoreactive IGF I can be extracted from multiple midgestation human fetal tissues (*9*); and (iv) mRNA encoding IGF I and IGF II can be detected in a number of fetal and adult rat tissues and human fetal tissues (*10*).

The mitogenic actions of the IGFs on cultured cells from fetal tissues of several mammalian species and the presence of specific IGF receptors in many fetal tissues and cells (*1, 11*) point to a role for the IGFs in fetal growth. These findings and the evidence that IGFs are synthesized in many fetal tissues (*5–10*) support the hypothesis that IGFs act locally to elicit the biologic response (*5, 7*). Information about the cellular sites of synthesis of IGFs in the fetus should provide insight into their potential role as paracrine fetal growth factors and, toward this goal, we have investigated the cellular localization of IGF mRNA in human fetal tissues.

Synthetic oligodeoxyribonucleotides (oligomers) complementary to portions of human IGF I (*3*) and IGF II (*4*) mRNA (Fig. 1, A to C) were used as probes for in situ hybridization histochemistry to localize IGF mRNA in sections of human fetal tissues. The 31-nucleotide (nt) IGF II oligomer and cDNAs encoding human IGF I and II precursors were first tested in Northern blot hybridizations with polyadenylated RNA from human fetal tissues. The IGF II oligomer hybridized with five distinct mRNAs in human fetal liver and muscle that are the same size as mRNAs detected with the IGF II cDNA, but different in size from two mRNAs detected in these tissues with the IGF I cDNA (Fig. 1D). These data, plus observations that the 31-nt IGF II oligomer and a second 39-nt IGF II oligomer that corresponds to a different region of human IGF II mRNA do not hybridize with human IGF I cDNA, provide evidence for the specificity of the IGF II oligomers for human IGF II mRNA. Similarly, the three IGF I oligomers—IGF IA (20 nt), IGF IA (36 nt), and IGF IB (Fig. 1, A and B)—showed no hybridization with human IGF II cDNA or IGF II mRNA, providing evidence that these oligomers are specific for human IGF I mRNA.

Human fetal tissues were collected from prostaglandin-induced abortuses of 16 to 20 weeks gestation with prior approval of the Committee for the Protection of Rights of Human Subjects at the University of North Carolina at Chapel Hill. Sections prepared from fixed human fetal tissues (*12*) were hybridized with IGF IA, IGF IB, or IGF II oligomers labeled at the 5′ end with ^{32}P (Fig. 1). All human fetal tissues tested, except cerebral cortex, showed hybridization with IGF IA, IGF IB, and IGF II oligomers. With each IGF probe and in all tissues showing positive hybridization, the hybridization signal was localized to connective tissues or to cells of mesenchymal origin (Fig. 2 and Table 1). In liver, IGF oligomers hybridized to perisinusoidal cells and showed no detectable hybridization to hepatocytes. Although the precise identity of these hybridizing cells could not be conclusively established with the resolution achieved, perisinusoidal cells are composed of mesenchymal cells, such as endothelial and reticuloendothelial cells, and fibroblasts. In costal cartilage, hybridization of IGF oligomers was to perichondrium and fibrous sheath and not to chondrocytes. In the eye, hybridization was to sclera and not to neuroretina, choroid, or lens. In heart, thymus, lung, skeletal muscle, skin, spleen, kidney, adrenal, stomach, and small intestine, IGF

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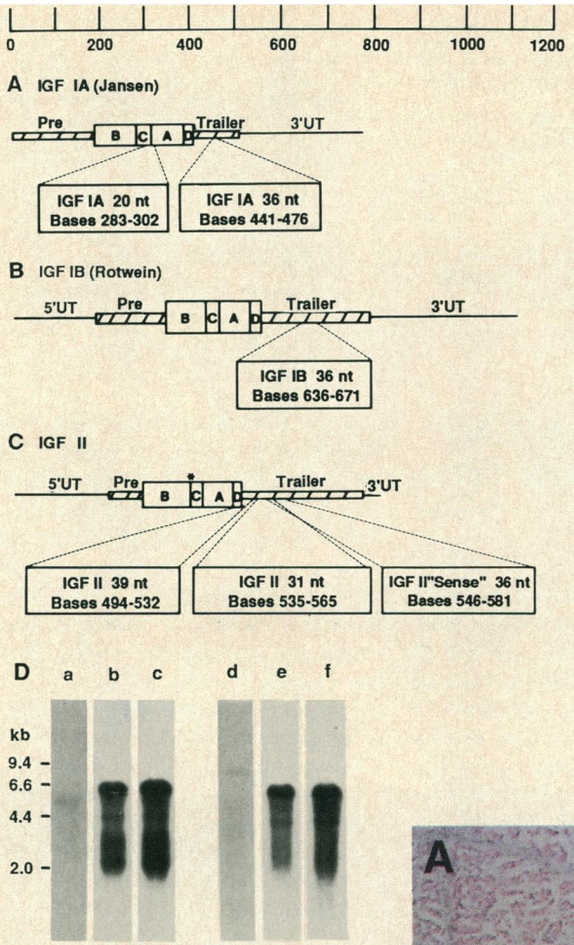
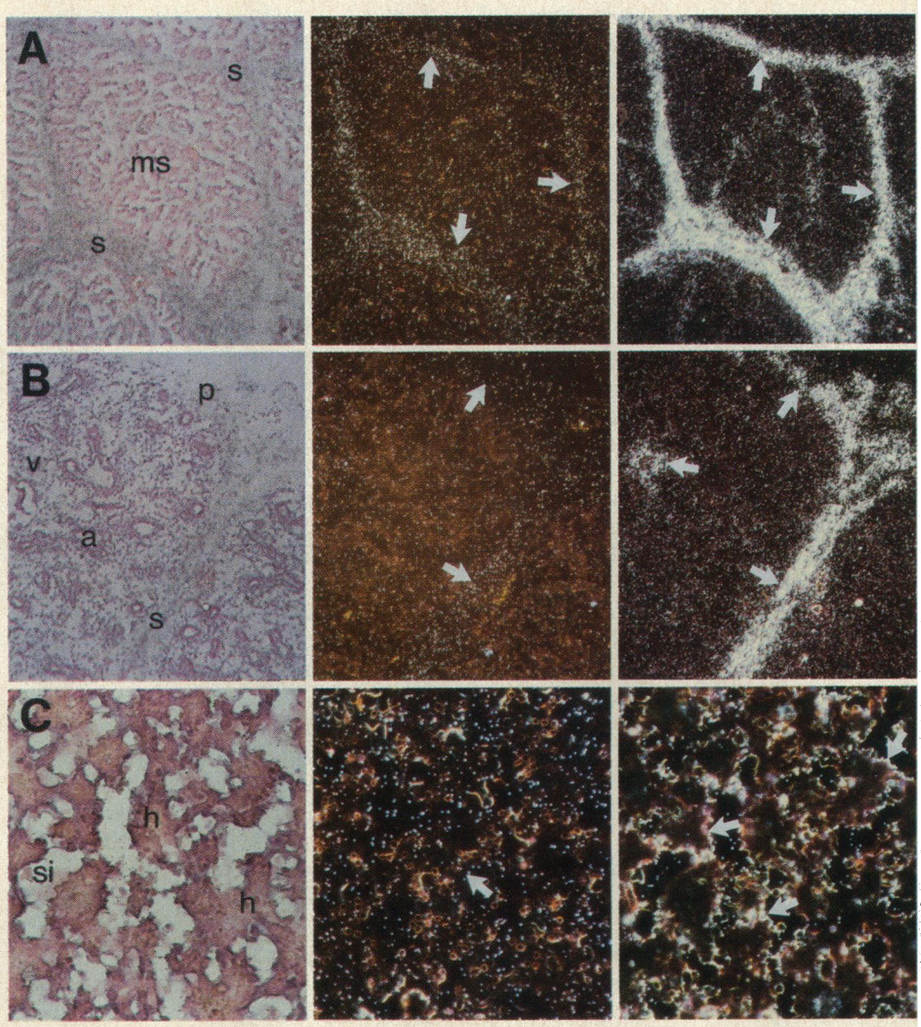


Fig. 1. Schematic diagram of human IGF I and IGF II mRNAs, their precursors, and synthetic oligonucleotide probes (oligomers). (**A** and **B**) The structures of two human IGF I mRNAs and encoded precursors derived from nucleotide sequence analyses of cDNAs (*3*). The IGF IA mRNA described by Jansen *et al.* and the IGF IB mRNA described by Rotwein have the same 5′ coding sequences for the prepeptide and IGF IB, C, A, and D domains. These mRNAs appear to be variants in splicing of the same IGF I gene transcript with different coding sequences for a portion of the carboxyl terminal trailer peptide and 3′ untranslated regions. The IGF I cDNA we used in this study corresponds to the IGF IA mRNA variant as reported by Jansen *et al.* The 36-nt IGF IA oligomer (3′-CCC-TCA-CGT-CCT-TTG-TTC-TTG-ATG-TCC-TAC-ATC-CTT-5′) and the 36-nt IGF IB oligomer (3′-ACC-GGT-TTC-TGT-GTA-GGT-CCT-CCC-CTT-GTC-TTC-CTC-5′) are each complementary to regions of the trailer peptide coding sequences of IGF IA and IB mRNAs that show no homology with each other or with reported human IGF II mRNA. The 20-nt IGF IA oligomer (3′-AG-CAC-CTA-CTC-ACG-ACG-AAG-5′) is complementary to a conserved region in both IGF IA and IGF IB mRNAs that encodes a portion of the A domain. (**C**) The structures of human IGF II mRNA and its encoded precursor derived from cDNA sequences (*4*) are similar to the IGF I mRNAs and precursors. Several groups have isolated cDNAs that encode the human IGF II sequence predicted from analyses of serum IGF II. A variant IGF II cDNA has also reported by Jansen *et al.* (*4*) that encodes IGF II with a four amino acid insertion replacing the serine residue at position 29 of the B domain. This variant appears to be derived from a mRNA splicing variant with a 9-nt insertion at the splice junction for the exons encoding the B and C domains (asterisk shows the position of this insertion). The IGF II cDNA used here corresponds to the IGF II variant mRNA. The 31-nt IGF II oligomer (3′-G-AAG-GGG-TCT-ATG-GGG-CAC-CCG-TTC-AAG-AAG-5′) and the 39-nt IGF II oligomer (3′-CTC-TCC-CTG-CAC-AGC-TGG-GGA-GGC-TGG- CAC-GAA-GGC-CTG-5′) are complementary to different regions of the IGF II mRNA coding sequence for the trailer peptide. These regions are identical in both of the reported IGF II mRNA variants and show no detectable homology with IGF I mRNAs. A 36-nt IGF II sense oligomer (3′-A-GGT-CCA-CAG-TAT-AAC-CTT-CTT-GAA-CGG-GTG-CCC-CA-5′) was used as a negative control and the first 20 nt of this oligomer are complementary in sequence to a portion of the IGF II 31-nt oligomer. (**D**) Autoradiograms of Northern blots hybridized with ^{32}P-labeled IGF I or IGF II cDNAs and oligomers. Polyadenylated RNA from human fetal liver (lanes a to c) and human fetal muscle (lanes d to f) hybridized with IGF I cDNA (lanes a and d), IGF II cDNA (lanes b and e), IGF II 31-nt oligomer (lanes c and f). To allow direct comparison of mRNA of different sizes on the same blots, Northern blots were probed first with IGF I cDNA (lanes a and d) followed by exposure to x-ray film, stripping of hybridized probe by incubation in 0.01× SSC and 0.1% SDS, at 100°C, then hybridization with IGF II 31-nt oligomer for 48 hours and exposure to x-ray film (lanes b and e). A similar procedure was used for the IGF II cDNA probe and IGF IA and IGF IB oligomers. Hybridization signals were barely detectable with the IGF I oligomers but there was no cross-hybridization with IGF II mRNA. Polyadenylated RNA extraction and Northern blot hybridization were performed as described (*10*). Oligomers were labeled at the 5′ end with ^{32}P to specific activities of 1×10^6 to 2×10^6 cpm/pmol with polynucleotide kinase and [^{32}P]adenosine triphosphate followed by purification on 15% polyacrylamide gels containing *7M* urea (*18*). The cDNA probes were labeled with ^{32}P by nick translation (*19*) to specific activities of 10^7 to 10^8 cpm/μg.



oligomers hybridized to organ capsules and to connective tissue layers, sheaths, and septa (Fig. 2 and Table 1). Results that support the specificity of in situ hybridization of probes to IGF mRNA include: (i) a similar localization of hybridization signals when oligomers complementary to different regions of both IGF I or IGF II mRNAs (Fig. 1) were used on selected human fetal tissue sections; (ii) elimination of hybridization signals by incubation of tissue sections with ribonuclease (10 μg/ml) (Boehringer Mannheim Biochemicals, Indianapolis, Indiana) for 30 minutes at 37°C prior to hybridization with labeled probes, or when hybridization was performed in the presence of excess unlabeled IGF I or IGF II cDNA inserts; (iii) no detectable hybridization signal with an oligomer corresponding to the "sense" strand of IGF II cDNA, of which 20 nt were complementary in sequence to the 31-nt IGF II "antisense" oligomer (Fig. 1); and (iv) no detectable hybridization of a 35-nt oligomer complementary to tyrosine hydroxylase mRNA (*12*). In addition, with the same fixation and hybridization procedures used for localization of IGF mRNA, a 20-nt oligomer complementary to human proglucagon mRNA (*12*) hybridized with glucagon-producing A cells in human fetal pancreas, thus demonstrating the integrity of mRNA in pancreatic nonmesenchymal cells. This suggests that mRNA of nonmesenchymal cells is not degraded during fixation and hybridization procedures even in pancreas, a tissue with high ribonuclease content.

Localization of mRNA to the same cells or tissue regions that contain immunoreactive peptide is commonly accepted as evidence of specificity of mRNA localization (*12*). In liver, our localization of IGF mRNA is consistent with a recent report of immunocytochemical localization of IGF I to rat liver perisinusoidal cells (*13*) but apparently discrepant with reports of IGF production by primary hepatocyte cultures (*14*) and established cell lines of presumed hepatocyte origin (*15*). In adult rat tissues other than liver (*13*) and in our studies of human fetal tissues (*16*) IGF immunoreactivity was not always localized to mesenchymal cells or connective tissues as we found for IGF mRNAs, but instead was localized to a wide variety of cells that are in close proximity, such as proliferating chondrocytes, renal tubular cells, intestinal epithelial cells, and myoblasts. Although our data on mRNA localization do not directly address these discrepancies, IGF immunoreactive cells may represent those that accumulate IGFs, possibly by receptor binding and internalization. Also, we cannot exclude the possibility that IGF mRNA is present in nonmesenchymal cells including hepatocytes, but its abundance may be lower than in mesenchymal cells and fibroblasts and below the detection limit of in situ hybridization procedures (*17*). A lower abundance of IGF mRNA in nonmesenchymal cells could reflect either a low level of IGF gene expression or a short half-life of IGF mRNA.

Hybridization signals obtained with IGF I oligomers were much weaker than those obtained with IGF II oligomers (Fig. 2 and Table 1). This was particularly evident with the IGF IA oligomer where we used five to ten times higher probe concentration and longer exposure times to detect the hybridization signal. IGF I mRNA, thus, appears to be less abundant than IGF II mRNA in

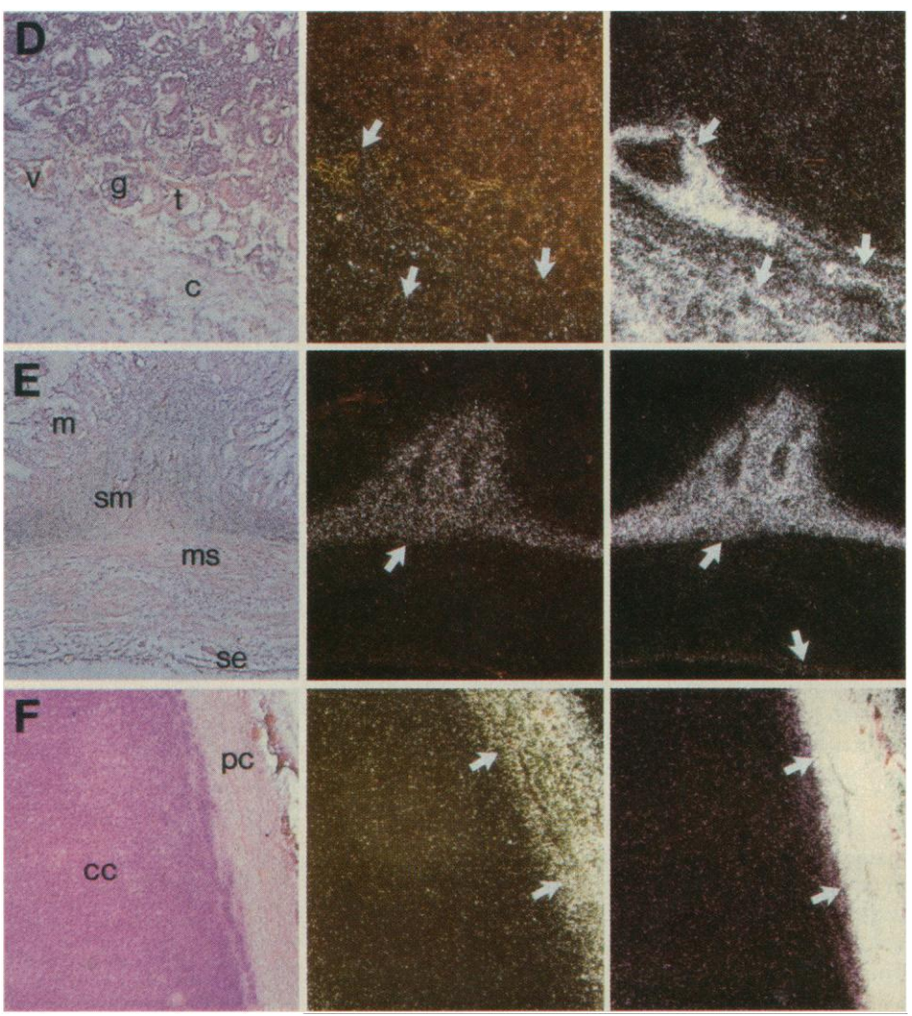


Fig. 2. Localization of IGF IA and IGF II mRNAs in human fetal tissues by in situ hybridization histochemistry with ^{32}P-labeled IGF oligomers. Bright-field (left panels) and dark-field (middle and right panels) photomicrographs of human fetal tissue sections hybridized with ^{32}P-labeled IGF IA oligomer (0.5×10^7 cpm/ml) (middle panels) and ^{32}P-labeled IGF II oligomer (0.5×10^7 cpm/ml) (right panels) for 48 hours. After washing the sections, they were exposed to NTB3 photoemulsion (Kodak) for 21 days (IGF I) and 7 days (IGF II), developed with D19 developer (Kodak), and counterstained with hematoxylin and eosin. (**A**) Skeletal muscle; ms, muscle fibers; s, muscle sheath and septa. (**B**) Lung, the periphery of the lung at the interlobular septum is shown; p, visceral pleura; s, interlobular septum; a, primitive airway (terminal bronchiole); v, pulmonary arteriole. (**C**) Liver; h, hepatocyte; si, sinusoid. (**D**) Kidney, the pelvicalyceal junction is shown; v, blood vessel; c, calyx; g, glomerulus; t, tubule. The nephrogenic zone of the renal cortex (not shown here) did not hybridize with IGF probes. (**E**) Stomach; m, mucosa; sm, submucosa; ms, smooth muscle layers; se, serosa. (**F**) Costal cartilage; cc, hyaline cartilage; pc, perichondrium. The growth plate of the costal cartilage showed no hybridization. Scale bars, 100 μm. White arrows indicate regions of strong hybridization. Note that the bright-field photomicrographs in the left panels are of the tissue sections hybridized with IGF II oligomer. Bright-field photomicrographs of adjacent tissue sections hybridized with IGF IA oligomer (middle panels) may not correspond exactly to the ones shown in the left panel. Tissues were prepared and processed for in situ hybridization as described (*12*).

Table 1. Localization of IGF I and IGF II mRNAs in human fetal tissues. +, Weak hybridization, where the signal was observed only in thick (15 μm) sections with high probe concentrations (4×10^7 cpm/ml) and after prolonged exposure (14 to 21 days) to photoemulsion; ++, moderate hybridization; +++, strong hybridization, where the signal was observed in thin (5-μm) sections with low probe concentrations (0.5×10^7 cpm/ml) after short exposure (5 to 7 days); −, negative hybridization, autoradiographic grain density on the tissues not higher than background. The hybridization signal intensity assigned to each tissue and region is based on subjective comparison of 12 sections of each tissue obtained from three different fetuses, hybridized with similar probe concentrations, and exposed for three different exposure times.

Tissue	Region	IGF IA	IGF IB	IGF II
Skin	Epidermis	−	−	+
	Dermis	+	++	+++
Muscle	Perimysium and epimysium	+	++	+++
	Muscle fibers	−	−	−
Thymus	Capsule and interlobular septa	+	++	+++
	Thymocytes	−	−	−
Heart	Epicardium and coronary vessel walls	+	++	+++
	Myocardium and endocardium	−	−	−
Lung	Pleura, interlobular septa, pulmonary vessel walls	+	++	+++
	Airway epithelium	−	−	−
Liver	Perisinusoidal cells	+	++	+++
	Hepatocytes	−	−	−
Stomach and intestine	Lamina propria, submucosa, serosa	+	++	+++
	Mucosa and muscle	−	−	−
Pancreas	Retroperitoneal tissue	++	+++	+++
	Acinar, ductal, and islet cells	−	−	−
	Lymphoid and ganglionic tissues	−	−	−
Spleen	Capsule and septa	+	++	++
	Pulp	−	−	−
Adrenal	Capsule	+	+++	+++
	Cortex	+*	++*	++*
	Medulla	−	−	−
Kidney	Capsule and calyces	+	++	+++
	Interstitium of inner cortex and medulla	+	++	++
	Nephrogenic zone, glomeruli and tubules	−	−	−
Costal cartilage	Perichondrium	++	++	+++
	Chondrocytes (including growth plate)	−	−	−
Eye	Sclera	+	+++	+++
	Cornea	+	+	+
	Choroid, retina, and lens	−	−	−
Cerebral cortex		−	−	−

*Weak to moderate hybridization was observed in cells of the permanent or definitive adrenal cortex. Cells of the fetal cortex showed no hybridization.

human fetal tissues, a conclusion that is supported by Northern blot hybridization studies (*10*) (Fig. 1). The intensity of hybridization with each probe also varied among tissues (Table 1). This variation may reflect tissue-specific differences in the abundance of IGF I or IGF II mRNA, but should be interpreted with caution as quantitative in situ hybridization procedures for measuring IGF mRNA are not yet validated and differences in hybridization signal intensity could result from many variables including different base composition of oligomer probes.

As stated originally by the "somatomedin hypothesis" (*1*), IGFs have been considered to be growth hormone–dependent circulating growth factors that are synthesized primarily in the liver and that mediate, at least in part, growth hormone actions on skeletal growth (*1*). Recent evidence (*5–11*) suggests that this view may be too restrictive. The production of IGFs in multiple tissues in addition to the liver and their biologic actions on a wide variety of cell types are consistent with their synthesis in widely distributed cells that are anatomically integrated in many organs and are situated near potential target cells. Fibroblasts and mesenchymal cells within connective tissues, shown here to be the predominant sites of localization of IGF I and IGF II mRNAs, are ideally situated to provide a source of IGFs for paracrine actions on multiple cell types in human fetal tissues.

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nol series. They were exposed to x-ray film (XAR-5, Kodak Laboratories) with intensifying screens for 16 to 18 hours at −70°C to give a guide to intensity of hybridization. Slides were dipped in NTB-3 photoemulsion (Kodak Laboratories) diluted 1:1 with distilled water, air-dried, and exposed in sealed boxes for 5 to 21 days to 4°C. Photoemulsion was developed with D19 developer (Kodak), fixed, washed, air-dried, stained with hematoxylin and eosin, dehydrated in an ascending ethanol series and then xylene, and mounted and cover slips applied with Permount. The hybridization signal was visualized with the bright- and dark-field optics of a Leitz photomicroscope.

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A Chimeric, Ligand-Binding v-*erb*B/EGF Receptor Retains Transforming Potential

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Comparison of amino acid sequences from human epidermal growth factor (EGF) receptor and avian erythroblastosis virus *erb*B oncogene product suggests that v-*erb*B represents a truncated avian EGF receptor gene product. Although both proteins are transmembrane tyrosine kinases, the v-*erb*B protein lacks most of the extracellular ligand-binding domain and a 32–amino acid cytoplasmic sequence present in the human EGF receptor. To test the validity of the proposed origin of v-*erb*B and to investigate the functional significance of the deleted extracellular sequences, a chimeric gene encoding the extracellular and the transmembrane domain of the human EGF receptor joined to sequences coding for the cytoplasmic domain of the avian *erb*B oncogene product was constructed. When expressed in Rat1 fibroblasts, this reconstituted gene product (HER-*erb*B) was transported to the cell surface and bound EGF. Its autophosphorylation activity was stimulated by interaction with the ligand. Expression of the HER-*erb*B chimera led to anchorage-independent cell growth in soft agar and EGF-induced focus formation in Rat1 monolayers. Thus, it appears that v-*erb*B protein sequences in the chimeric receptor retain their transforming activity under the influence of the human extracellular EGF-binding domain.

UNTIL RECENTLY, THE NORMAL biological role of cellular homologs of transforming genes was poorly understood. Some of these genes have now been shown to encode growth factors or growth-factor receptors, which when constitutively expressed or structurally altered may result in loss of normal growth control and thus oncogenesis. Two members of the tyrosine kinase oncogene family, v-*erb*B and v-*fms*, are highly homologous to receptors for epidermal growth factor (EGF) (*1, 2*) and macrophage colony-stimulating factor CSF-1 (*3*), respectively. The chemically activated *neu* oncogene product has also been shown to be structurally similar to the epidermal growth factor (EGF) receptor and to v-*erb*B (*4–6*) and probably represents another cell surface receptor for an unknown ligand with unknown biological activity.

Amino acid sequence comparison suggested that v-*erb*B was derived from the avian EGF receptor gene by gene truncation as a consequence of the recombination event that created the avian erythroblastosis virus (*2*). Both molecules are transmembrane tyrosine kinases; when aligned with the human EGF receptor sequence, v-*erb*B lacks the signal peptide for membrane translocation (24 amino acids), most of the extracellular ligand-binding domain (amino acids 1–555), and 32 carboxyl-terminal amino acids. We have been interested in characterizing the structural alteration that led to the generation of an oncogene from a normal growth factor receptor gene. Therefore, we have replaced the EGF binding region missing from the v-*erb*B protein to determine whether the presence of this domain is sufficient to regenerate a normal, ligand-responsive receptor from a transforming protein. The polypeptide generated by this gene fusion consists of normal human extracellular ligand-binding and transmembrane domains linked to oncogenic avian cytoplasmic sequences.

Cloned complementary DNA (cDNA) sequences coding for the human EGF receptor (HER) ligand-binding and transmembrane domains (residues −24 to 646) (*2*) were fused in vitro without sequence alteration with cytoplasmic domain sequences of the v-*erb*B oncogene (AEV-H strain) (*7*). A 2-kb Sac I–Nar I restriction fragment coding for the complete extracellular and transmembrane domain of the EGF receptor was combined with a 1.7-kb Aha II–Stu I restriction fragment coding for the complete cytoplasmic portion of (AEV-H)-*erb*B and cloned into Sac I/Sma I double-digested pUC12 (*8*). A 3.7-kb Sac I–Xmn I restriction fragment from this subclone containing the entire coding region of the chimeric receptor was then introduced into an expression vector downstream of a 640-bp Sma I–Sac I restriction fragment with Harvey sarcoma virus long terminal repeat promoter sequences and upstream of 3′-untranslated sequences of the gene coding for the hepatitis B virus surface antigen (*9*) providing transcription stop and polyadenylation signals. The vector also contained a dihydrofolate reductase (DHFR) gene under SV40 early promoter control to permit gene amplification by methotrexate selection (*10*) in addition to a neomycin resistance gene (*11, 12*) for the initial selection of transfectants. Sequences of the *Escherichia coli* plasmid pBR322 allowed plasmid DNA replication in *E. coli*. Expression plasmids were purified from *E. coli* HB101 by the alkaline extraction procedure (*13*) followed by a gel filtration purification step. Normal Rat1 fibroblasts were transfected by the calcium phosphate precipitation method (*14*) and selected for neomycin and methotrexate resistance.

The ability of transfected cells to bind increased levels of ^{125}I-labeled EGF was used to determine whether mutant EGF receptors were properly synthesized and transported to the cell surface of Rat1 recipient cells. Two transfected cell lines used for subsequent experiments displayed dramatically increased ^{125}I-labeled EGF surface binding relative to control Rat1 cells (1×10^4 receptors per cell). The HER-*erb*B1 line expressed about 8×10^4 and HER-*erb*B2 about 6×10^5 chimeric molecules per cell, which compares with

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