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IGF-II Expression in Human Tumor Tissues and Human IGF-II Ribozyme Action

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IGF-II EXPRESSION IN HUMAN TUMOR TISSUES AND HUMAN IGF-II RIBOZYME ACTION

by

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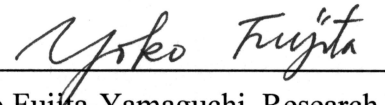

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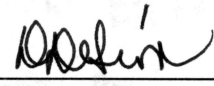

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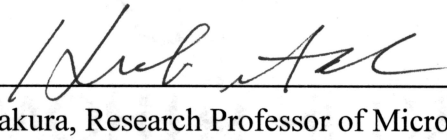

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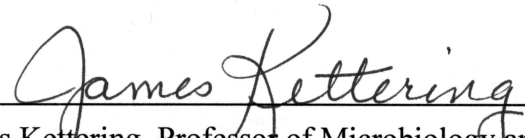

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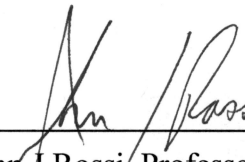

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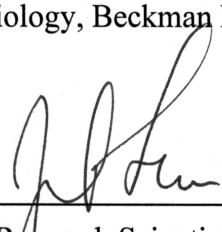

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ABBREVIATIONS

ALP	Alkaline phosphatase
BSA	bovine serum albumin
bp	base pair
cAMP	cyclic adenosine monophosphate
Ci	curies
CM	conditioned medium
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DU145	human prostate cell
EGF	epidermal growth factor
FCS	fetal calf serum
FSH	follicle stimulating hormone
G418	Genecitin
GH	growth hormone
H & E	hematoxylin and eosin
IHC	immunohistochemistry
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
ISH	in situ hybridization
kb	kilobase pair
kDa	kilodalton
LNCaP	human prostate cell
MAb	monoclonal antibody
MCSF	macrophage colony stimulating factor
MOPS	3-N-morpholino propanesulfonic acid
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PC-3	human prostate cancer cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PSA	prostate specific antigen
PTH	parathyroid hormone
P85,P110	phosphatidylinositol-3 kinase subunits
QCPCR	Quantitative competitor polymerase chain reaction
RIA	radioimmunoassay
rpm	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reaction
Rz	ribozyme
SD	standard deviation
SDS	sodium dodecyl sulfate
SE	standard error
SFCM	Serum free conditioned medium
SK-N-AS	human neuroblastoma cell

SV40	simian virus 40
TGF	transforming growth factor
TEMED	N,N,N1, N-tetramethylethylene diamine
TH	thyroid hormone
Tm	melting temperature
5' UTRs	5' untranslated regions

ABSTRACT

IGF-II EXPRESSION IN HUMAN TUMOR TISSUES AND HUMAN IGF-II RIBOZYME ACTION

by

Zhaodong Xu

Insulin-like growth factors (IGFs) are potent mitogens for a variety of cancer cells *in vitro*. In breast, prostate and neuroblastoma cancer cells, it has been suggested that IGF-II plays a paracrine/autocrine role. However, information on cell-type -specific IGF-II expression *in vivo* is limited. *In situ* hybridization and immunohistochemistry were carried out to determine the cell type expressing IGF-II in different tumor tissues. Both IGF-II mRNA and protein were localized to malignant cells, and expression in the stroma was minimal in all the tumors. The data are consistent with the hypothesis that cancer cell growth is regulated by IGF-II, and therefore IGF-II is a potential target for cancer therapy.

RNA enzymes (ribozymes) which selectively cleave RNA targets via base-pairing interactions can serve as therapeutic agents. We constructed IGF-II ribozymes and stably transfected them in prostate cancer PC-3 cells. Single- and double-hammerhead ribozymes were synthesized and cloned into the pTZU6+27 or pcDNA vectors. *In vitro* studies showed that both single- and double-ribozymes cleaved the ~140 bases and /or ~1 kilo bases IGF-II RNA substrates examined, while the mutant

ribozymes did not. Kcat/Km for cleaving the shorter IGF-II substrate by double ribozyme and single ribozyme was 4772 and 1546 $M^{-1} S^{-1}$, respectively, suggesting that double ribozyme was ~3 fold more efficient in cleaving the IGF-II substrate than the single ribozyme *in vitro*. PC-3 stable transfectants expressing single ribozyme or double ribozyme, under control of U6 promoter, reduced the endogenous IGF-II mRNA and cell growth compared to mutant ribozyme transfectants. Similiarly, PC-3 stable transfectants expressing single ribozyme, under control of CMV promoter, reduced the endogenous IGF-II mRNA and IGF-II protein secretion compared to vector-control cells. Furthermore, PC-3 cells expressing single ribozyme grew poorly under serum-free or 2% FCS conditions as judged by growth curves, supporting our hypothesis that IGF-II plays a critical role in prostate cancer cell growth, and thus provides a basis for developing a potential gene therapy for cancer.

I. INTRODUCTION

A. Insulin-like Growth Factor (IGF) System

The insulin-like growth factors (IGF-I and IGF-II) are single polypeptides with structural homology to proinsulin (Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b). They regulate proliferation and differentiation of a multitude of cell types and are capable of exerting insulin-like metabolic effects (Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b). For example, IGFs promote important metabolic processes such as neurite outgrowth (Hepler and Lund, 1990), steroidogenesis in ovarian granulosa cells (Rechler and Nissley, 1990), and erythropoiesis (Aron, 1992). Unlike insulin, they are produced by most tissues of the body and are abundant in the circulation. Thus the IGFs have the potential to act by endocrine as well as autocrine or paracrine mechanisms.

IGFs do function in conjunction with a complex IGF regulatory system. This IGF regulatory system includes IGFs, IGF receptors, IGF binding proteins (IGFBPs) (Nissley et al., 1984), and IGFBP proteases. The IGFs exert their effects at the cellular level by binding to and initiating signal transduction cascades through the type I IGF receptor, the type II IGF receptor and the insulin receptor. IGFBPs either enhance or inhibit the interaction of the IGFs with these receptors (Baxter et al., 1986). These interactions are in turn regulated by IGFBP proteases, which are important mediators of IGFBP activity in biological fluids. During the last decade, there has been increasing recognition of the role of IGFs, IGF-I receptor and IGFBPs

in the control of the proliferation of cancer cells (Rechler and Nissley, 1990; Cullen et al., 1992). The introduction of this dissertation provides an overview of the IGF regulatory system, especially the role of IGF system with the tumors and subsequently focuses on developing a potential gene therapy using an IGF-II ribozyme.

1. Insulin-like growth factors

(1) Insulin-like growth factor-I (IGF-I)

The human IGF-I gene is located on chromosome 12 and is approximately 90 kb in length (Tricoli et al., 1986; Sussenbach et al., 1989). Two mRNA transcripts (IGF-IA and IGF-IB) produced by alternative splicing are expressed in a defined pattern in most tissues during pre- or post-natal development. The highest expression is observed in adult liver (Han et al., 1988). Growth hormone (GH) is a major regulator, enhancing IGF-I gene expression in liver and cartilage (Hynes et al., 1987). In addition to GH, other hormones and trophic factors affect IGF-I expression. Thyroid hormone (TH) (Wolf et al., 1989), EGF (Rogers et al., 1991), parathyroid hormone (PTH) (Linkhart et al., 1989), cortisol (McCarthy et al., 1989) affect IGF-I expression or GH-induced IGF-I expression. IGF-I stimulates proliferation and differentiation of cells in many tissues, including rat and human bone (Rosen et al., 1994).

(2) Insulin-like growth factor-II (IGF-II)

The human IGF-II gene contains nine exons distributed over 30 kb DNA and is located on the short arm of chromosome 11 (Nielson et al., 1995; Sussenbach et al.,

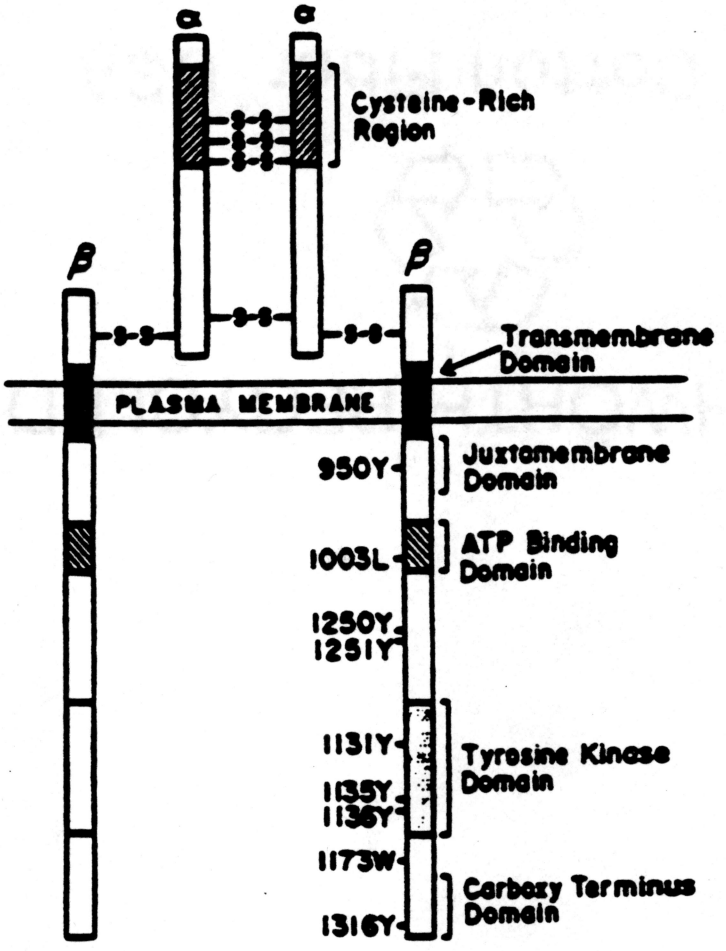
1989). Like IGF-I, the IGF-II gene is transcribed and processed into several different mRNA species (4.8, 5.3, 6.0 kb) resulting from alternative promoter usage and alternative RNA splicing (Daughaday et al., 1989). The human IGF-II gene generates multiple mature transcripts with different 5'untranslated regions (5'UTRs) but identical coding regions and 3'UTRs. In rats and mice, the level of IGF-II in serum is higher during fetal than during post-natal life (Brown et al., 1986), while in humans and guinea pigs, a substantial concentration of IGF-II remains in adult serum (Daughaday et al., 1986). Hormones and tissue-specific factors regulate IGF-II gene expression in selected tissues. Human ovarian granulosa cells in culture accumulate IGF-II RNA and secrete IGF-II in response to follicle stimulating hormone (FSH), chorionic gonadotropin and prolactin (Voutilainen et al., 1987). Primary human adrenal cells respond to adrenocorticotrophic hormone (ACTH) by increasing steady-state levels of IGF-II mRNA(Voutilainen et al., 1987). The mechanisms involved in the regulation of IGF-II expression are not completely understood. Nielson et al. (1990) have shown that among several IGF-II mRNA species, only mRNAs containing the non-coding exon 6 are translated into polypeptide, suggesting that translational competency for IGF-II is determined by the 5'-untranslated region (5'-UTR). IGF-II promotes cellular proliferation, differentiation and glucose metabolism (Rosen et al., 1994).

2. IGF Receptors

The biological effects of IGFs are mediated through IGF receptors on the cell surface. There are two structurally and functionally distinct IGF receptors.

(1) Type I IGF receptor

The type I IGF receptor binds to IGF-I and IGF-II with equally high affinity (Sakano et al., 1991; Casella et al., 1986). The type I IGF receptor is a disulfide bond linked heterotetrameric protein ($\alpha_2\beta_2$) in which the α subunit (~130 kDa) contains the ligand binding domain and the β subunit (~90 kDa) contains a transmembrane domain and an intracellular domain with tyrosine kinase activity (Ullrich et al., 1986; LeBon et al., 1986; Rubin et al.,1995) (Fig. 1). The type I IGF receptor gene is located on chromosome 15 (Ullrich et al., 1986). From the cDNA sequence, it was found that the insulin and IGF-I receptor tyrosine kinase domain shared 84% sequence identity (Ullrich et al., 1985). Several lines of evidence suggest that the IGF-I receptor can mediate the effects of both IGF-I and IGF-II (Rechler et al., 1990). Comparison of dose-response curves of IGF-I and IGF-II for stimulating cell proliferation in cultured human cells has revealed that higher concentrations of IGF-II are required to produce a response equivalent to that produced with IGF-I, suggesting that the majority of responses to IGFs are mediated through the IGF-I receptor rather than the IGF-II receptor (Rechler et al., 1990). A monoclonal antibody (αIR-3) against the IGF-I receptor blocks either IGF-I -stimulated or IGF-II-stimulated [^{3}H]-thymidine incorporation into DNA in human fibroblasts in culture (Flier et al.,


α
α
Cysteine-Rich Region
β
β
S-S
S-S
S-S
Transmembrane Domain
PLASMA MEMBRANE
950Y
Juxtamembrane Domain
1003L
ATP Binding Domain
1250Y
1251Y
1131Y
1135Y
1136Y
Tyrosine Kinase Domain
1173W
1316Y
Carboxy Terminus Domain

1986;Van-Wyk et al., 1985). Sakano et al., (1991) used site-directed mutagenic procedures to express mutants of recombinant IGF-II, and the mutant IGF-II specific to IGF-IR, not the ones specific to IGF-IIR, stimulate DNA synthesis and glycogen synthesis. These results indicate that both IGF-I and IGF-II can act through the IGF-I receptor to stimulate cell growth.

(2) Type II IGF receptor

The type II IGF receptor binds IGF-II with a 100 fold higher affinity than IGF-I and does not bind to insulin (Czech et al., 1989). The type II IGF receptor is distinct from the type I and insulin receptor in that it consists of a single polypeptide chain (~220 kDa) and is structurally identical to the mannose-6-phosphate (Man-6-P) receptor (Oshima et al., 1988) which recognizes mannose-6-phosphate residues on acid hydrolases and targets these enzymes to the lysosomes (Lobel et al., 1988).

The IGF-II/Man-6-P receptor is a transmembrane glycoprotein. The receptor does not have intrinsic kinase activity but can be phosphorylated (Mogan et al., 1987). Evidence indicates that N-linked glycosylation is required for proper folding of the protein portion of the receptor and the carbohydrate can be removed without adversely affecting the IGF-II binding function (MacDonald et al., 1985). Treatment of cells with insulin increases the cell surface IGF-II receptor number by causing a shift in receptors from a large intracellular pool to the cell surface in adipocytes (King et al., 1982). The IGF-II/Man-6-P receptor cycles continuously between the cell surface and intracellular compartments. The expression of IGF-II/Man-6-P receptors

in various rat tissues is developmentally regulated and similar to the developmental pattern of serum IGF-II (Moses et al., 1980) and tissue IGF-II mRNA expression (Brown et al., 1986), suggesting an important role for IGF-II and the IGF-II/Man-6-P receptor in fetal growth and development.

3. IGFBPs

There is a family of proteins that specifically bind to the IGFs. It has been proposed that binding to the IGFs increases their stability and modulates their biological activities (Baxter et al., 1986). The first indication that IGFBPs existed came from the observation that the IGFs circulate as part of a 150 kDa complex in human plasma. This complex could be dissociated by acid treatment into an IGF and a 50 kDa IGFBP. The function of the 150 kDa IGF:IGFBP complex in adult human plasma was to prolong the half-life of IGFs in the circulation (Nissley et al., 1984). In the 1970s, it was directly demonstrated that the IGFs in most body fluids and in media conditioned by cultured cells were complexed to proteins that bound IGF-I and IGF-II (Hintz et al., 1977; Hintz et al., 1980; Zapf et al., 1975; Megyesi et al., 1975). In the mid 1980s, a powerful technique, called ligand blotting, was introduced which greatly facilitated the identification of more IGFBPs (Hossenlopp et al., 1986). To date, six IGFBPs (designated IGFBP-1 through IGFBP-6) have been purified and characterized from body fluids and from conditioned media in different cell lines. cDNA clones and genomic clones for six IGFBPs have been isolated and characterized (Shimasaki et al., 1991). The protein sequences determined directly or

deduced from cDNA nucleotide sequence demonstrated that the IGFBPs are a family of closely related proteins. Amino acid sequences of the N- and C-terminal regions of the six IGFBPs are highly conserved (Shimasaki et al., 1991). Promoter regions of the human IGFBP-1, -2, -3, -5 and rat IGFBP-1, -2, -3, -4, -5 and -6 genes have been cloned and partially characterized (Suwanichkul et al., 1990; Brown et al., 1990; Binkert et al., 1992; Cubbage et al., 1990; Allander et al., 1994; Zhu et al., 1993).

The binding affinities of each IGFBP for IGF-I or IGF-II are different. IGFBP-1, -3, -4 bind to IGF-I and IGF-II with similar affinity, and IGFBP-2, -5, -6 bind to IGF-II with higher affinity (Martin et al., 1986; Mohan et al., 1989; Rechler et al., 1990; Roghani et al., 1991). Among these IGFBPs, IGFBP-3 has an inhibitory effect which is IGF-independent (Oh et al., 1998), whereas its stimulatory effects are associated with facilitation of IGF action (El-Badey et al.,1989; Van den Hooff et al., 1991; Ellis et al., 1994; Cullen et al., 1995; Christofori et al., 1994; Rogler et al., 1994; Bates et al., 1995). A recent study done by Rajah et al., (1997) showed that IGFBP-3 induces apoptosis through a novel pathway independent of either p53 or the IGF receptor-mediated cell survival pathway and that IGFBP-3 mediates TGF-β1 induced apoptosis in PC-3 cells. By binding with these proteins, serum IGFs have a half life of 12-15 hours compared with 10-12 min for the free peptides. The large complex probably functions as a serum IGF reservoir. This is important because unlike other hormones, IGFs are not stored in cells, but are secreted as soon as they are synthesized.

(1) IGFBP-1

IGFBP-1 was purified from human amniotic fluid (Drop et al., 1984), placenta (Bohn et al., 1980), endometrium (Bell et al., 1986) conditioned medium from Hep G2 hepatoma cells (Rechler et al., 1990), etc. IGFBP-1 cDNA clones were isolated from libraries established from human decidua, placenta, liver and Hep G2 cells (Rechler et al., 1990). The complete amino acid sequence of human IGFBP-1 was deduced from the cDNA sequence and confirmed by direct sequencing of the purified IGFBP-1 protein. The human IGFBP-1 is a 25.3 kDa protein with 234 amino acids (Lee et al., 1988; Brinkman et al., 1988; Luthman et al., 1989). Sequence analysis demonstrated that IGFBP-1 contains 18 cysteine residues which are conserved in IGFBPs 1-5 and are thought to stabilize the tertiary structure. The human IGFBP-1 gene is located on chromosome 7 and is 5.2 kb long (Brinkman et al., 1988). The transcribed mRNA from this gene is a single 1.55 kb mRNA transcript (Alitalo et al., 1989) in human liver (Brewer et al., 1988), and some breast cancer cells (Clemmons et al., 1990). The human IGFBP-1 promoter has been well characterized. It contains a TATA sequence, a CAAT box and a response element for the transcription factor, hepatic nuclear factor 1 (HNF-1) (Suwanichkul et al., 1990).

(2) IGFBP-2

IGFBP-2 was purified from MDBK bovine kidney cells (Rechler et al., 1990), human serum (Zapf et al., 1990), etc. cDNA clones encoding IGFBP-2 have been isolated from libraries established from adult rat liver (Margot et al., 1989), bovine

MDBK cells (Upton et al., 1990), Hep G2 hepatoma cells (Zapf et al., 1990), etc. The human and rat IGFBP-2 amino acids are 80% similar and the molecular masses are 31.4 and 29.6 kDa for human and rat, respectively (Brown et al., 1989). IGFBP-2 gene is located on chromosome 2 and is 40 kb in length (Agarwal et al., 1991; Ehrenborg et al., 1991). mRNA from this human gene is a single 1.38 kb transcript. IGFBP-2 has been detected in adult human brain and liver (Binkert et al., 1989), adult rat brain, testes, ovary (Margot et al., 1989). The IGFBP-2 promoter contains three SP1 binding sites (Binkert et al., 1992).

(3) IGFBP-3

IGFBP-3 has been purified from multiple sources including porcine serum (Walton et al., 1989), rat serum (Zapf et al., 1988), human plasma (Martin et al., 1986), etc. Human IGFBP-3 is a 38.5-41.5 kDa to 45 kDa N-glycosylated protein (Zapf et al., 1988; Yang et al., 1989). The human IGFBP-3 gene is located on chromosome 7 and spans 8.9 kb (Ehrenborg et al., 1990). The gene encodes a single 2.5 kb mRNA (Wood et al., 1988). The IGFBP-3 promoter has been cloned and contains a TATA box, AP-2 and SP1 sites (Cubbage et al., 1990). IGFBP-3 is the main carrier of IGF-I and -II in serum. At least 95% of the total concentration of IGF-I and IGF-II in the serum is bound to IGFBP-3 (Baxter et al., 1989). The remainder is bound to IGFBP-1, -2, and -4. Less than 1% of the total is free under normal conditions. IGFBP-3 can either enhance or inhibit the metabolic or mitogenic effects of the IGFs, depending on the cell type and the study conditions (Mohan,

1993; De Mellow et al., 1988). In addition, it may increase the half-life of IGFs by serving as a local reservoir from which IGFs can be continuously released into the local environmnent by autocrine or paracrine actions (Bautista et al., 1990; Schmid et al., 1989).

(4) IGFBP-4

IGFBP-4 is a 24 kDa binding protein purified from the conditioned medium of human prostate carcinoma PC-3 cells (Perkel et al., 1990), human colon carcinoma HT-29 cells (Culouscou et al., 1991), human glioblastoma T-98G cells (Camacho et al., 1992), human serum (Kiefer et al., 1991), etc. IGFBP-4 cDNA clones were isolated from rat liver (Shimasaki et al., 1990), human placenta (Shimasaki et al., 1990), human osteosarcoma TE89 (LaTour et al., 1990) cDNA libraries. Mature IGFBP-4 contains 233 (rat) or 237(human) amino acid residues. There are 18 cysteine residues and two additional cysteine residues that are not present in other IGFBPs. The human IGFBP-4 gene is located on chromosome 17 and is 15 kb long. The gene has a 1.8 or 2.2 kb mRNA transcript (LaTour et al., 1990; Shimasaki et al., 1991; Camacho et al., 1992). The function of IGFBP-4 is inhibitory. It inhibited the binding of IGF-II to the type II IGF receptor (Perkel et al., 1990) and cell proliferation (Amarnani et al., 1993) in MC3T3 mouse osteoblasts.

(5) IGFBP-5

IGFBP-5 is a 29 kDa protein that was purified from human bone (Bautista et al., 1991), cerebrospinal fluid (Binoux et al., 1991) and glioblastoma T98G cells

(Camacho et al., 1992). The human and rat IGFBP-5 contains 252 amino acid residues and shares 97% amino acid sequence identity. IGFBP-5 cDNA clones were isolated from human placenta (Shimasaki et al., 1991), osteosarcoma (Kiefer et al., 1991). The gene was located on human chromosome 2 (Shimasaki et al., 1991), the transcripts are multiple, ranging from 5 to 6 kb.

(6) IGFBP-6

IGFBP-6 was purified from normal and transformed human lung fibroblasts (Martin et al., 1990; Forbes et al., 1990), human serum (Zapf et al., 1990) and rat serum (Shimasaki et al., 1991). The protein from fibroblast is 32-34 kDa (Martin et al., 1990), 30-32 kDa from CSF (Roghani et al., 1991) and 28-30 kDa from human serum (Zapf et al., 1990). IGFBP-6 cDNA was isolated from rat and human osteosarcoma libraies (Kiefer et al., 1991). IGFBP-6 is expressed in human breast cancer cells (Sheikh et al., 1993), transformed and untransformed fibroblast cells (Martin et al., 1994), human adult liver, human osteosarcoma cells and human brain (Kiefer et al., 1991). The expression of IGFBP-6 is increased by TGF-β and agents that increase intracellular cAMP concentrations. A distinguishing characteristic of IGFBP-6 is its 20 to 100 fold preferential affinity for IGF-II over that for IGF-I (Martin et al., 1990; Forbes et al., 1990; Roghani et al., 1991; Bach et al., 1992; Bach et al., 1993), suggesting that IGFBP-6 has a special role in modulating IGF-II actions.

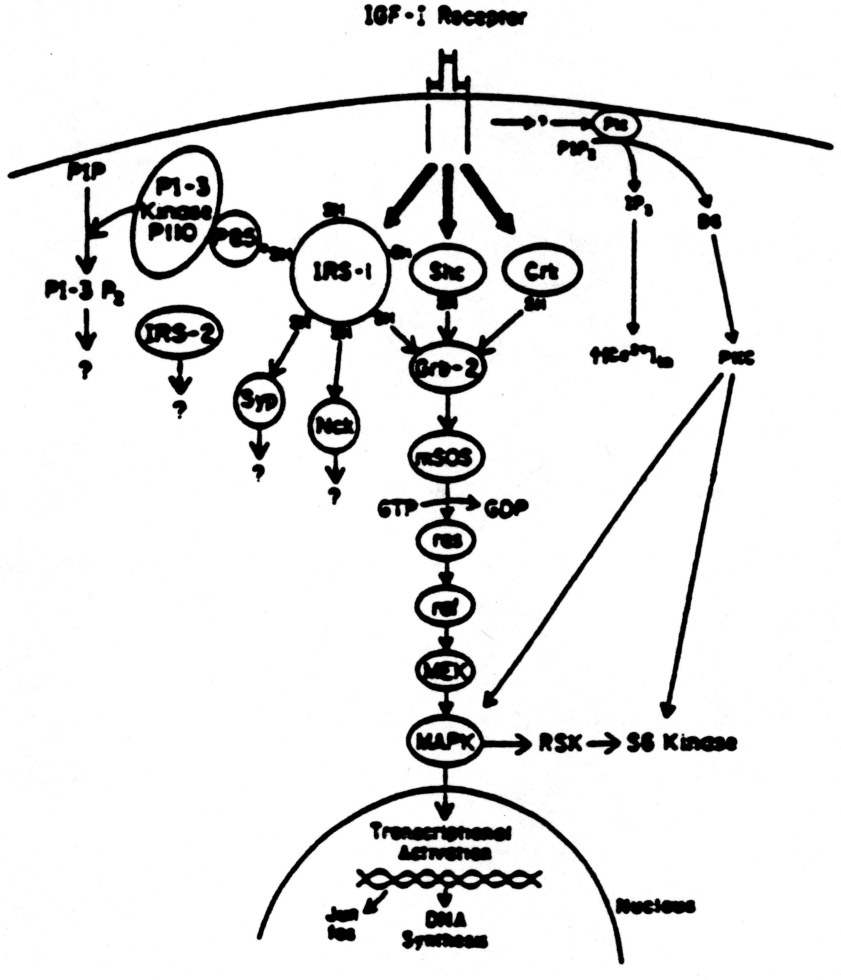
B. IGFs System and tumors

(1) Type I and Type II IGF Receptors

It is increasingly recognized that the IGF-I receptor (IGF-IR) plays a fundamental role in cell growth control and malignant transformation and is an important inhibitor of apoptosis (Baserga et al., 1997). IGF-IR is highly expressed in a variety of human tumors including breast cancer (Papa et al., 1993). Expression of IGF-IR is required for transformation by SV40 T antigen, bovine papillomavirus, activated ras, raf, and v-src. When activated by IGFs, IGF-IR transmits a signal to at least four major substrates, IRS1, IRS2, Grb10, Shc (Rubin et al., 1995) (Fig. 2). Other downstream effectors include ras, raf-1, and mitogen-activated protein kinase (MAPK).

Strategies using dominant negative mutants of IGF-IR, antibodies to IGF-IR, or antisense directed against IGF-IR mRNA have shown that decreased or aberrant receptor expression is associated with a reversal of the transformed phenotype with induction of apoptosis (Werner et al., 1997; Rubin et al., 1995).

Overexpression of the normal human IGF-IR in NIH 3T3 cells leads to ligand-dependent alteration in cell morphology, colony formation in soft agar and tumorigenicity in nude mice (Kaleko et al., 1990). IGF-IR deficient mice show severely diminished growth (Liu et al., 1993). Whereas mouse fibroblasts that overexpress IGF-IR have reduced growth factor requirements *in vitro* and demonstrate decreased susceptibility to apoptosis (Sell et al., 1995).


IGF-I Receptor
PIP
PI-3 Kinase P110
PI-3 P2
IRS-1
IRS-2
Shc
Crk
Grb-2
Syp
Nck
mSOS
GTP
GDP
Ras
Raf
MEK
MAPK
RSK
S6 Kinase
Transcriptional Activation
Jun fos
DNA Synthesis
Nucleus

An involvement of type II IGF receptor in cancer is less clearly understood. The IGF-II receptor (IGF-IIR) is a single-chain transmembrane protein identical to the mannose-6-phosphate receptor. It is clear that this receptor can mediate uptake and degradation of IGF-II. By this means, it can indirectly affect IGF-II/IGF-IR mitogenic response. De Leon et al. (1996) hypothesized that, in a cell overexpressing IGF-II, the routing of lysosomal enzymes might be altered based on a previous observation that IGF-II inhibits the cellular uptake of a lysosomal enzyme by inhibiting binding to the IGF-II/mannose 6-phosphate receptor. Another recent study showed that the IGF-II receptor may be directly involved in carcinogenesis. Ishiwata et al. (1997) found that the IGF-IIR is overexpressed in a significant number of human pancreatic cancers, indicating that IGF-IIR may contribute to the pathobiology of pancreatic cancer.

(2) IGF expression in tumors

IGFs may play a part in neoplastic transformation and metastasis. Strong evidence exists that a variety of tumors have the molecular machinery for the production of IGFs. By dot-blot and Northern blot analysis, investigators have found much higher concentrations of IGF-II messenger RNA in Wilms' tumors than in normal kidney (Reeve et al., 1985; Scott et al., 1985). Subsequent studies have established that increased IGF-II mRNA is associated with a variety of human tumors, including embryonic (Wilms' nephroblastoma, rhabdomyosarcoma, hepatoblastoma) (Reeve et al., 1985; Scott et al., 1985; Hoppener et al., 1988;

Haselbacher et al., 1987; Daughaday et al., 1988; Daughaday et al., 1989; Koufos et al., 1984), mesenchymal (fibrosarcoma, leimyosarcoma, hemangiopericytoma, liposarcoma) (Yee et al., 1988; Reeve et al., 1985; Little et al., 1987; Tricoli et al., 1986; Ron et al., 1989), neuroendocrine (pheochromocytoma, neuroblastoma), and epithelial (colon, hepatic, breast and prostate) tumors (Lowe et al., 1989; El-Badry et al., 1989; Cariani et al., 1988; Su et al., 1989; Tennant et al., 1996). The increased expression of IGF-II mRNA in tumors is generally assumed to result in increased synthesis of bioactive IGF-II protein. However, in some cases, IGF-II and IGF-II mRNA are discoordinate. Haselbacher et al. (1987) reported that Wilms' tumors expressed high levels of IGF-II mRNA, but that levels of immunoreactive IGF-II in the tumor did not differ from those in normal kidney. By contrast, pheochromocytomas had 20-fold increased IGF-II protein but only 4-fold increased IGF-II mRNA. Schofield et al. (1989) described a sarcoma expressing high levels of IGF-II mRNA, but having reduced levels of immunoreactive IGF-II in cystic fluid from the tumor or in plasma. The basis for these differences remains to be elucidated.

Experimental systems using transgenic mice have provided valuable information on the potential role of IGF-II in carcinogenesis. In transgenic mice expressing the simian virus-40 large T-antigen (Tag) under the control of the insulin gene regulatory region, the initial proliferative switch is correlated with focal activation of IGF-II. Reduced IGF-II expression by transfection with an antisense oligonucleotide to the IGF-II mRNA impairs tumor cell growth *in vitro*, and transgenic mice homozygous

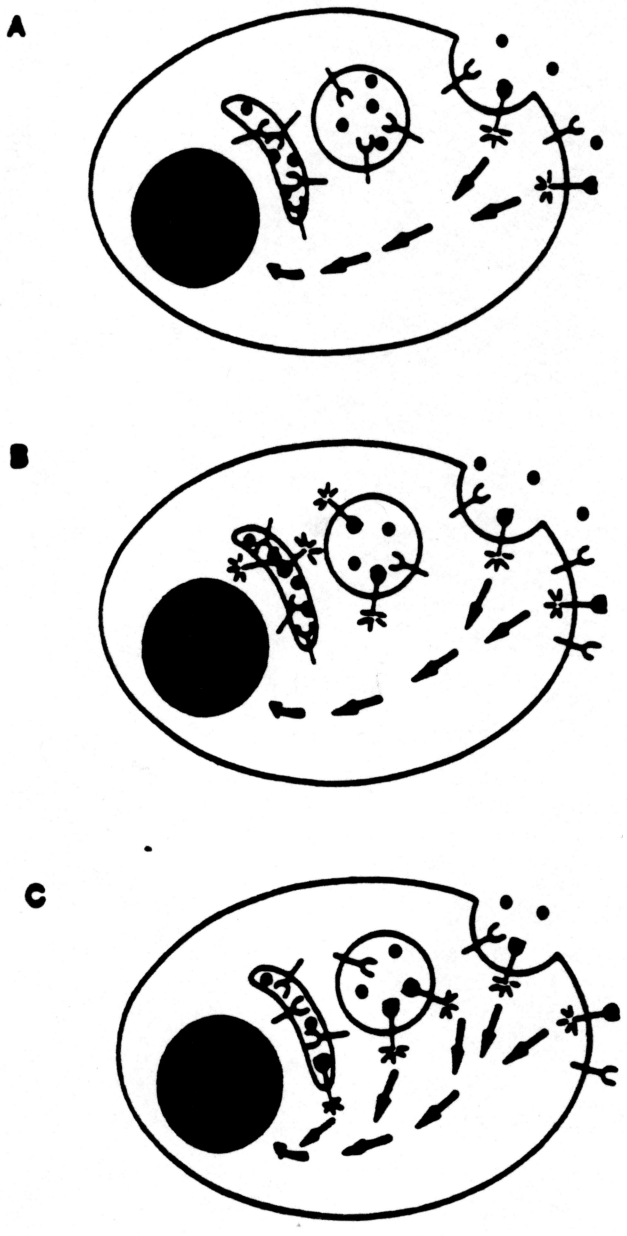
for a disruption of the IGF-II gene develop tumors with reduced malignancy and a higher incidence of apoptosis *in vivo* (Christofori et al., 1994). Until now, most studies have focused on the importance of IGFs in controlling the proliferation of previously transformed cells, however, evidence is now starting to accumulate that components of the IGF signal transduction system may play a role in the transformation process itself. One report involving transgenic mice, in which IGF-II expression was targeted to the liver under the control of the major urinary protein promoter, showed that the mice developed diverse varieties of tumors after 18 months of age (Rogler et al., 1994). Transgenic mice, in which expression of IGF-II was targeted to the mammary gland by placing it under the control of the sheep β-lactoglobulin promoter, were shown to develop an excess of mammary tumors (Bates et al., 1995). These two studies are highly in agreement and are the first *in vivo* experiments to suggest a direct role for IGFs in the malignant transformation of cells. Although the evidence that components of the IGF signal transduction system can be involved in cell transformation is compelling, the mechanisms involved are not known. Two possible explanations are that: the main function of IGF-II is to increase cell survival thereby allowing somatic mutations to accumulate in oncogenes or tumor suppressor genes, and that the IGF signal transduction system itself could be responsible for increased proliferation and the malignant transformation of cells.

(3) Autocrine /paracrine actions of IGFs

IGFs are potent mitogens for a wide variety of tumor cell types and have been identified as major autocrine or paracrine growth factors in a number of cancers (Daughaday et al., 1990). That is, tumor cells with functional IGF receptors may be able to enhance their own growth by synthesis of endogenous IGFs or by exogenous IGFs secreted from stromal cells surrounding tumor cells (Fig. 3). For example, it has been suggested that IGF-II may serve as a growth promoter in breast cancer in an autocrine/paracrine manner while IGF-I may serve as a paracrine regulator in normal and cancerous breast tissues (Singer et al., 1995).

Overexpression of IGFs is thought to contribute to tumor cell proliferation in an autocrine manner, which has been suggested by the experiment that mitogenic activity of cancer cells can be inhibited by monoclonal antibody αIR-3 against the IGF-I receptor. These cell lines include small cell lung carcinoma (Gansler et al 1988), rhabdomyosarcoma cells, and others (El-Badry et al., 1990). This provides evidence that the production of IGFs by these cells is the critical factor in stimulating mitogenesis in serum-free medium.

A paracrine role of IGFs has also been suggested for some cancers including breast and prostate cancers. The prominent stromal reaction seen in many breast carcinomas led to the speculation that stromal cells play a role in breast cancer pathogenesis (Van den Hooff et al., 1991). The identification of genes that are selectively expressed in the stroma of malignant breast lesions has recently provided


A
B
C

new insights into the molecular basis of stromal-epithelial interactions. It has been suggested that IGFs mediate stromal-epithelial interactions in human breast cancer (Ellis et al., 1994; Cullen et al., 1995). Similarly, Byrne et al. (1996) reported that IGF-II produced by prostatic stromal cells act on epithelial cells, leading to increased proliferation. The role of IGFs in the development and maintenance of prostate cancer is less clearly defined.

(4) IGFBPs

IGFBP and IGFBP protease may also play an important role in cancer development. Kanety et al. (1993) found that IGFBP-2 is increased while IGFBP-3 is decreased in patients with prostate cancer, with a good correlation with serum prostate-specific antigen, which is a IGFBP-3 protease. The results suggest that IGFBPs may be involved in growth modulation of prostate malignancy and that alterations in their serum levels may act as a marker for prostate cancer. Ho et al. (1997) found that in active prostate carcinoma, serum IGFBP-2 level is elevated while in benign prostatic hyperplasia (BPH), IGFBP-2 levels are not. Serum IGFBP-2 levels closely parallel those of prostate-specific antigen and probably reflect the prostate carcinoma burden. IGFBP-3 is known to block IGF action and inhibit cell growth. IGFBP-3 has been proposed to act by sequestering free IGFs or, possibly, act via a novel IGF-independent mechanism (Oh et al., 1998). Supporting its role as a primary growth inhibitor, IGFBP-3 production is increased by cell growth-inhibitory agents, such as transforming growth factor-beta (TGF-beta), and the tumor

suppressor gene p53. Rajah et al. (1997) demonstrated that, for the first time, a novel function of IGFBP-3 as an apoptosis-inducing agent and showed that this action is mediated through the IGF receptor-independent pathway. In the p53 negative prostate cancer cell line, PC-3, the addition of recombinant IGFBP-3 resulted in a dose-dependent induction of apoptosis. These membrane-associated molecules may serve as receptors that mediate the direct effect of IGFBP-3 on apoptosis

(5) IGF system in benign and cancerous prostate tissues

Prostate cancer is the most common cancer in adult men in the United States. The incidence increases with age, and about 80% of prostate cancers are diagnosed after the age of 65 years (Carter et al., 1990). Although an autocrine role of IGF-I in prostate cancer cell growth was originally suggested (Pietrzkowski et al., 1993), there has been substantial evidence which suggests that IGF-II, but not IGF-I, may play an important role in paracrine or autocrine regulation of normal and hyperplastic prostate growth (Dong et al., 1997) as well as prostate cancer cell growth. Angelloz-Nicoud et al. (1995) reported that the autocrine role of IGF-II via the type I IGF receptor in PC-3 cell proliferation. IGF-II bioavaiability in this system is modulated by limited IGFBP-3 proteolysis. Similarly, Figueroa et al. (1995) found that IGFBP-1, which binds IGF-II with high affinity, inhibited IGF-II-induced monolayer growth and both baseline and IGF-II-induced anchorage-independent growth in prostate cancer cell line, DU145. The data suggest IGF-II is an autocrine growth factor instead of IGF-I in DU145 cells, and that the inhibition of this autocrine loop may represent a new

therapeutic strategy for prostate cancer. In an effort to understand the role of IGFs in prostate cancer cell growth, Fujita-Yamaguchi's group characterized the IGF system components produced by human prostatic cancer cell-lines, LNCaP, DU145, and PC-3, grown in serum-free medium (Kimura et al., 1996). They found that mRNA for IGF-II and receptors for IGF-I and IGF-II were detected in all three cell-lines. In contrast to another published study (Pietrzkowski et al., 1993), only LNCaP cells expressed a trace amount of IGF-I mRNA. RIA revealed that all three cell-lines produced measurable IGF-II protein but not IGF-I protein. DNA synthesis in PC-3 cells was significantly inhibited by the IGF-I receptor-specific monoclonal antibody. Further, we showed that by *in situ* hybridization significant levels of IGF-II mRNA expression were present in 63% of prostate tumors examined, and that IGF-II mRNA was localized to malignant cells (Li et al., 1998). Tennant et al. (1996) also reported that IGF-II mRNA is increased in human prostate carcinoma compared to benign prostatic hyperplasia (BPH) while protein and mRNA for the type I IGF receptor is decreased. It has been demonstrated that the levels of IGF-I, -II, and IGFIR mRNAs in the prostate of old human subjects were 2- to 3-fold higher than those of young control subjects, suggesting that the observed increase may also be an age-related process (Bonnet et al., 1993). It is possible that the abundant presence of IGF-II in the adult prostate may cause subtle alterations over time, leading to the development of separate tumor foci.

BPH is the most common benign proliferative disorder of unknown etiology. Boudon et al. (1996) suggested that IGF-II and the secreted forms of IGFBPs, depending on anatomical location within the organ, were important for the autocrine regulation of normal and hyperplastic prostate growth. Dong et al. (1997) found that IGF axis is critical for prostate cell growth and is abnormal in BPH. The elevated expression of IGF-II and type I IGF receptor genes in BPH is associated with the decreased expression of Wilms' tumor gene WT-1, indicating that IGF system components may also be involved in the development of prostatic hyperplasia.

(6) Loss of imprinting in cancer

Genomic imprinting is defined as a gamete-specific modification causing differential expression of the two alleles of a gene in somatic cells (Mitsuyoshi et al., 1996). There are seven endogenous genes showing parental-specific monoallelic expression. IGF-II (DeChiara et al., 1991), small nuclear ribonucleoprotein-associated polypeptide (Leff et al., 1992), Ins 1, 2 (Giddings et al., 1994), U2afbp-sr (Hatada et al., 1993) are expressed from the paternal allele whereas IGF-II receptor (Barlow et al., 1991) and H19 (Bartolomei et al., 1991) are expressed from the maternal allele. WT1 is expressed from the maternal allele or both (Jinno et al., 1994). Recent evidence suggests that loss of imprinting (LOI) is associated with gene activation in cancer (Yun et al., 1998).

Preferential loss of heterozygosity (LOH) of a specific parental allele in several childhood tumors provides indirect evidence for genomic imprinting in cancer and

has led to a popular hypothesis that an aberrant imprint inactivates one allele of a tumor suppressor. LOH was first discovered in retinoblastoma and in Wilms' tumor (Fearon et al., 1984; Reeve et al., 1984). The gene associated with Wilms' tumor (nephroblastoma) is a potential tumor suppressor gene wt1. This gene encodes a DNA binding protein, WT1, which binds to the major fetal IGF-II promoter and functions as a potent suppressor of IGF-II transcription (Dong et al., 1997; Werner et al., 1993; Werner et al., 1995; Qing et al., 1996; Vincent et al., 1996). IGF-II thus appears to be part of a rate-limiting step in multistage oncogenesis, promoting tumor growth and malignancy.

Both the IGF-II and H19 gene are located on chromosome 11p15.5 in close proximity to each other, and are imprinted on different parental alleles. LOI leading to the biallelic expression of IGF-II and H19 genes has recently been reported in a variety of tumors (Wu et al., 1997; Kim et al., 1997) and in Beckwith-Wiedemann syndrome (Joyce et al., 1997). These data suggest that LOI leading to overexpression of IGF-II plays an important role in carcinogenesis. Jarrard et al. (1995) reported that in prostate, both the cancer and the normal peripheral zone tissue have a pronounced biallelic expression of IGF-II gene. They hypothesized that this loss of imprinting has a good correlation with the development of prostate cancer. Sun et al. (1997) introduced a IGF-II transgene into the mouse genome by using embryonic stem cells, which led to transactivation of the endogenous IGF-II gene. Overexpression of IGF-II resulted in most of the symptoms of Beckwith-Wiedemann syndrome,

including polyhydramnios, prenatal overgrowth, fetal and neonatal lethality, and disproportionate organ overgrowth. These studies provide new insights into how IGF-II gene expression is affected by LOI under pathophysiological conditions.

C. Ribozymes

1. Discovery of Ribozymes

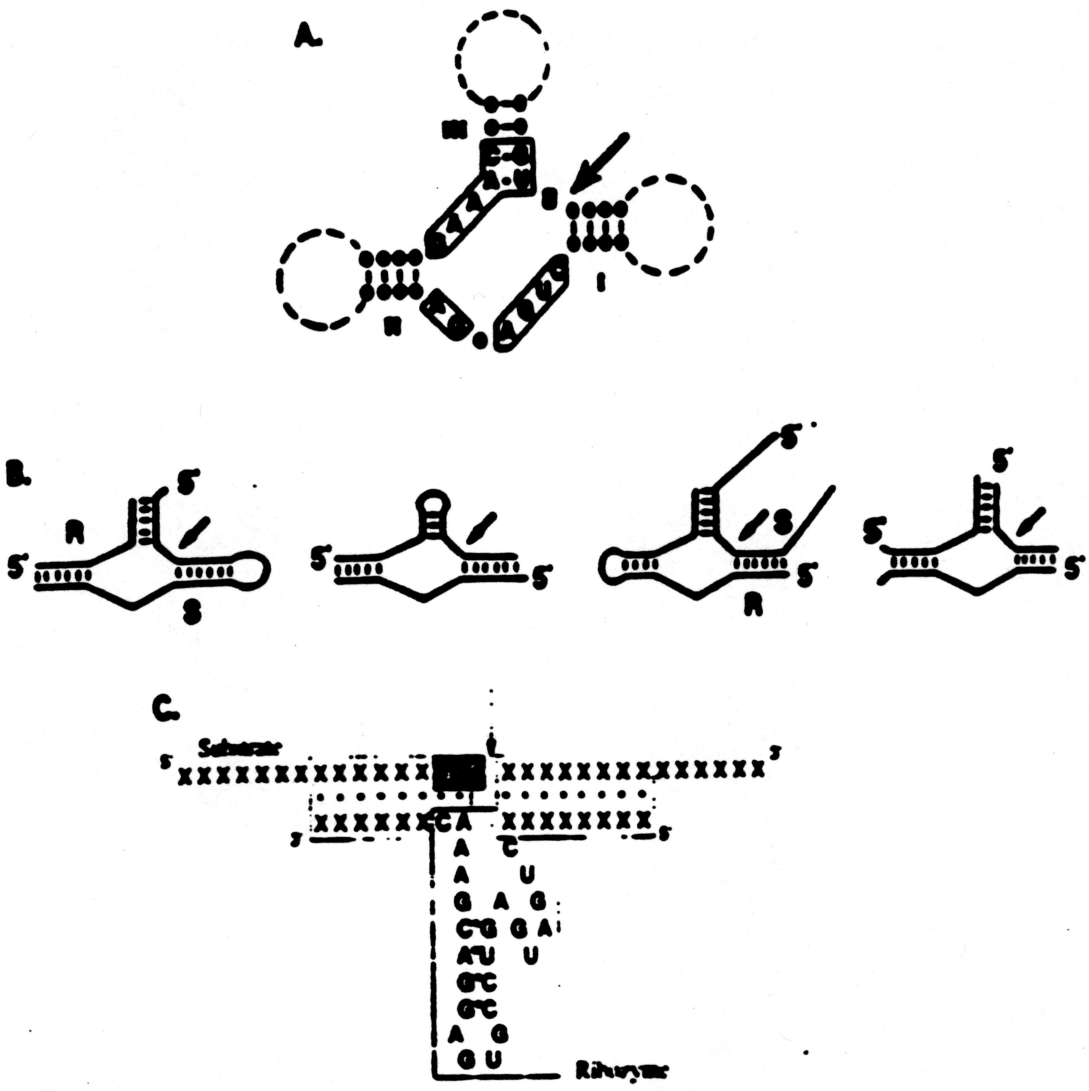
Cech and colleagues (1982) discovered an intervening sequence or intron, in the precursor of RNA of T. Thermophila could excise itself from a pre-RNA and in the process ligate the flanking exon sequences together to generate a mature rRNA. The RNA intron accomplishes splicing without aid from proteins, and the excised intron retains the ability to create or break phosphodiester bonds present in RNA. Altman and colleagues demonstrated that the RNA component of the ribonucleoprotein enzyme RNase P is the catalytic subunit of this tRNA processing enzyme. The RNase P ribozyme processes precursor tRNAs with multiple turnover and is not changed during processing. Thus, catalytic RNAs are not limited to single turnover intramolecular reactions but, like protein enzymes, are capable of multiple turnover.

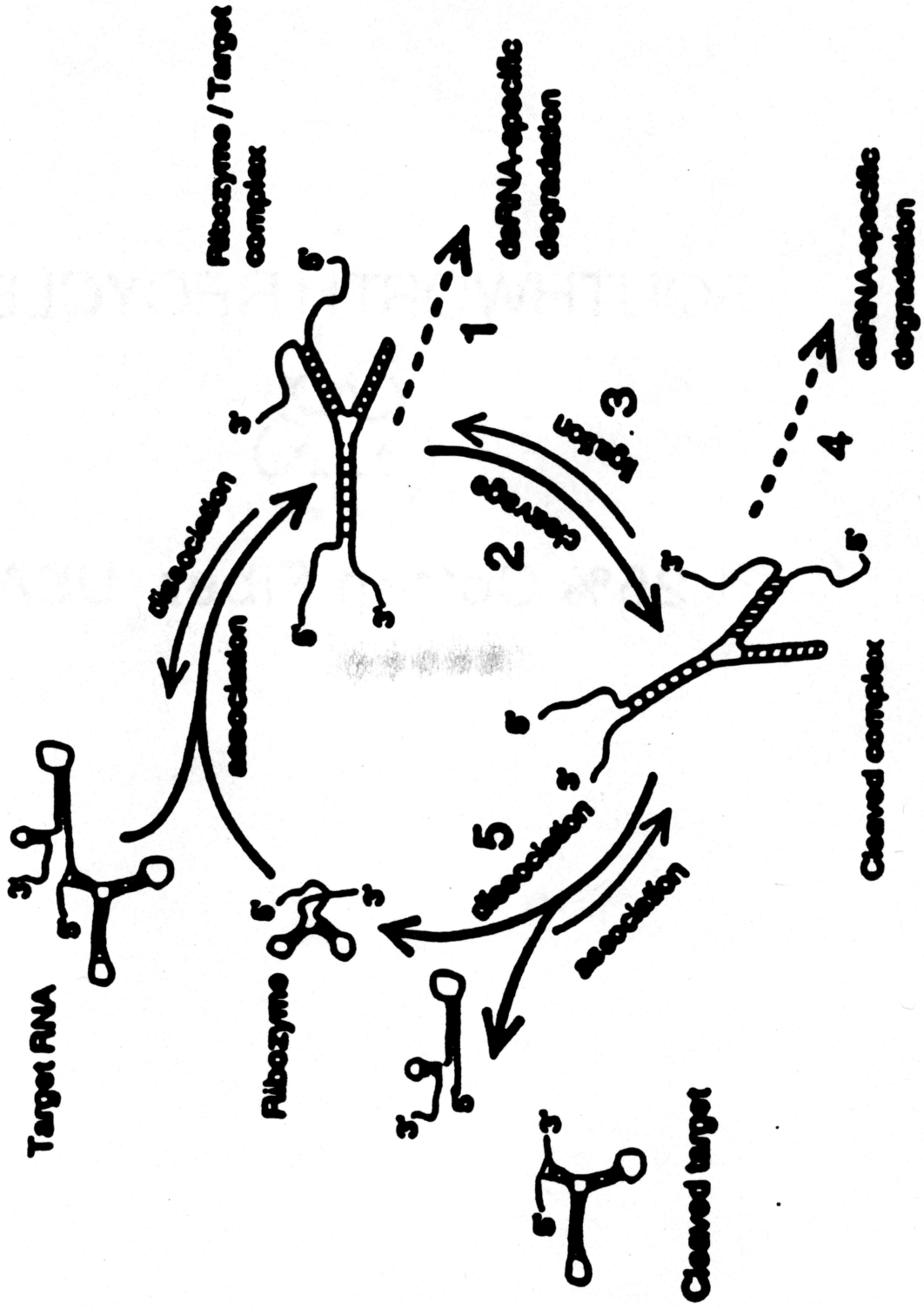
2. Ribozyme classification

(1) Hammerhead Ribozymes

This group of RNAs share a two-dimensional structural motif known as the "hammerhead" which has been shown to be sufficient to direct site-specific cleavage. The hammerhead structure consists of three base-paired stems (helices I, II, and III), which flank the susceptible phosphodiester bond, and two single-stranded regions,

which are highly conserved in sequence (Forster et al., 1987; Unlenbeck et al.,1987). Extensive mutagenesis has revealed the important nucleotides and functional groups for efficient catalysis. Haseloff and Gerlach (1988) proposed a model whereby the hammerhead domain is separated such that the substrate RNA contains just the cleavage site, and the ribozyme contains the other conserved nucleotides of the catalytic core. Mutagenesis has revealed that the target site can be any NUX sequence where X = A, C, or U (IUB system) and N is any nucleotide (Haseloff et al., 1988; Perriman et al., 1992) (Fig. 4). The sequence of the arms of the ribozyme aligns the catalytic core to the target site via complementary base pairing. A minimal catalytic cycle of a hammerhead ribozymes has been proposed (Fig. 5). McCall et al., (1992) have determined the minimum core sequence required for catalytic activity which has resulted in the "minizyme". The model of Haseloff and Gerlach has allowed the design of specific endoribonucleases. This work encouraged others to design trans-acting ribozymes. There are two main applications for the ribozymes' ability to cleave the RNA and thereby inhibit the expression of a specific gene selectively. One is to work as a tool for molecular biology (*in vitro* manipulation of RNAs) and the other is to inactivate gene transcripts *in vivo*, for example, as antiviral agents. The use of ribozymes for both applications has become possible with the development of chemical synthesis of RNA, with or without modified nucleosides and the identification of suitable expression vectors.


A.
B.
C.
Substrate
Ribozyme


Ribozyme / Target complex
deRNA-specific degradation
1
2
3
4
5
dissociation
association
Target RNA
Ribozyme
Cleaved target
Cleaved complex

(2) Hairpin Ribozyme

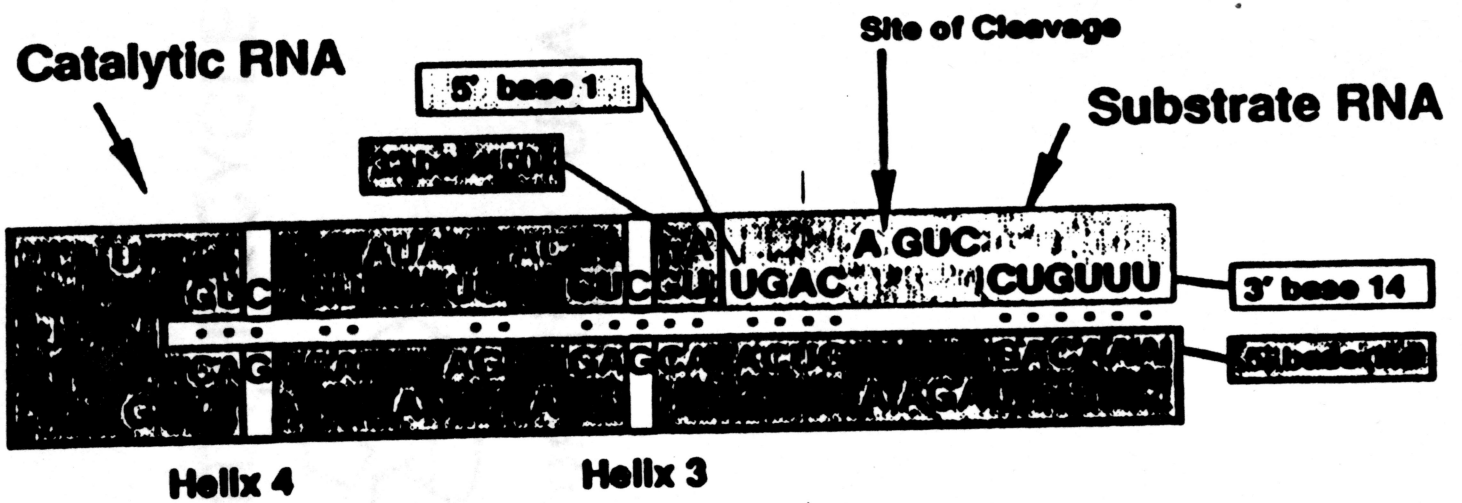
A second small catalytic domain is the "hairpin'structure, which has four helical domains and five loops (Hampel et al., 1989; Hample et al., 1990; Berzal-Herranz et al., 1993). Two helices of the hairpin domain form between the substrate and ribozyme, and this allows the design and specificity of binding for trans-acting hairpin ribozymes. The hairpin ribozyme has a more complicated substrate requirement than the hammerhead ribozyme, but despite this, any RNA of interest is expected to have numerous potential target sites. The summary of the base requirements for a hairpin ribozyme is shown in Fig. 6.

(3) Hepatitis delta virus

HDV genomic and antigenomic RNAs contain a self-cleavage site hypothesized to function during rolling circle replication (Wu et al., 1989). The sites in HDV are postulated to have a related secondary structure, three models of which have been proposed: cloverleaf (Wu et al., 1989); pseudoknot (Perrotta et al., 1990), and axehead (Branch et al., 1991), none of which is similar to the catalytic domains previously described. Like the other ribozyme motifs, the HDV ribozymes require a divalent cation (Wu et al., 1989), and cleavage results in products with 2', 3'-cyclic phosphate and 5'-OH termini (Branch et al., 1991). Investigation of trans-cleavage with the HDV ribozymes are limited.

(4) Group I intron

Group I intron self-splicing (*in vitro*) in the absence of protein was first observed

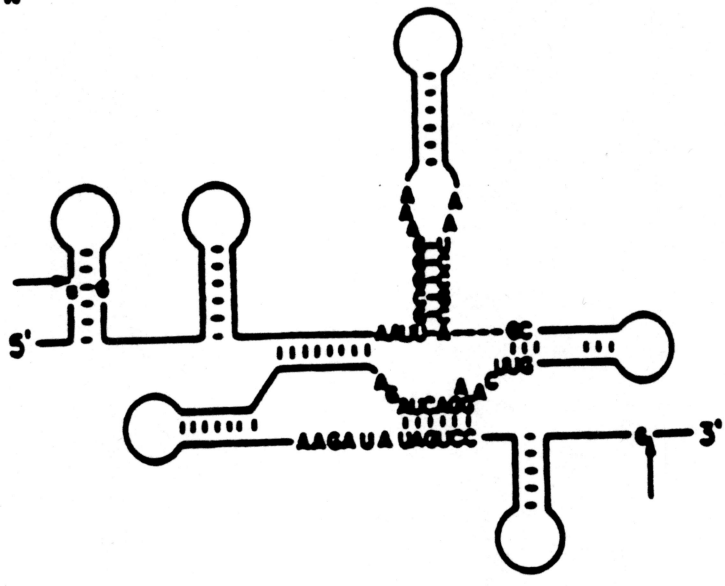

Catalytic RNA
5' base 1
Site of Cleavage
Substrate RNA
3' base 14
Helix 4
Helix 3

for the intervening sequence of the nuclear 26S rRNA gene in Tetrahymena thermophila (Kruger et al., 1982). Splicing proceeds by two consecutive trans-esterification reactions (Zaug et al., 1983). The reaction is initiated by a nucleophilic attack by the 3'-hydroxyl of a guanosine (or a phosphorylated derivative: GMP, GDP,or GTP) at the phosphodiester bond between the 5'-exon and the intron 5'-splice site). The new 3'-hydroxyl group of the 5'-exon than initiates a second nucleophilic attack, on the phosphodiester bond between the 3'-exon and the intron (the 3'-splice site). This results in ligation of the exons and excision of the intron. Shortened versions of the Tetrahymena IVS have been shown to be true enzymes *in vitro*, for example, as a restriction endoribonuclease (Azug et al., 1986) and as a template-dependent polymerase (Kay et al., 1987). The structure of group I intron is shown in Fig. 7.

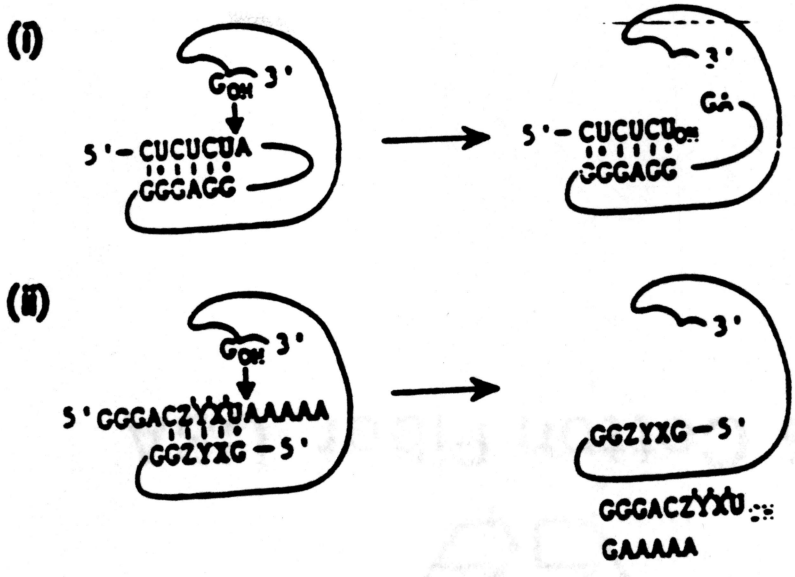
(5) Group II introns

Group II introns splice by way of two successive phosphate transfer reactions. In the first step, the 2'-OH group of an intramolecular branch point adenosine attacks the phosphodiester bond at the 5'-splice site, producing the free 5'-exon and a splicing intermediate, the intron-3'-exon. The second step involves cleavage at the 3'-splice site by the 3'-OH of the 5'-exon. Simultaneously, the exons are ligated and the intron lariat, with a 2', 5'-phosphodiester bond , is released. The ability of the group II introns to bind the 5'-exon specifically has been exploited to encourage the IVS to catalyze reactions on exogenous substrates. It has been shown that a group II intron

A.


5'
3'

B.


(i)
3'
5'-CUCUCUA
GGGAGG
3'
5'-CUCUCU
GGGAGG
(ii)
3'
5'GGGACZYXUAAAAA
GGZYXG-5'
3'
GGZYXG-5'
GGGACZYXU
GAAAAA

can ligate ssDNA to RNA, and another cleave a ssDNA substrate (Augustin et al., 1990).

(6) Ribonuclease P

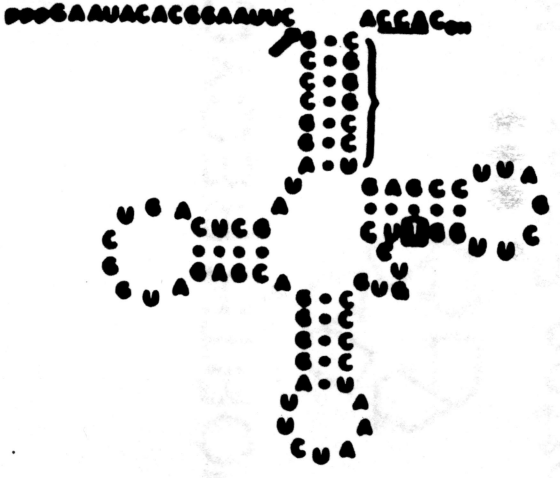
Ribonuclease P (RNase P) is the ubiquitous endoribonuclease that processes the 5'-end of precursor tRNA molecules (Darr et al., 1992). It cleaves specific bonds to produce 5'-phosphate and 3'-OH termini, and *in vitro* requires a divalent metal ion. RNase P consists of a protein moiety and an RNA moiety. It was discovered that, at least in bacteria, it is the RNA moiety that is the catalyst (Guerrier-Takada et al., 1983). Several studies have now shown that RNase P can be modified into cleaving any desired substrate (Forster et al., 1990) (Fig. 8).

3. Ribozyme delivery

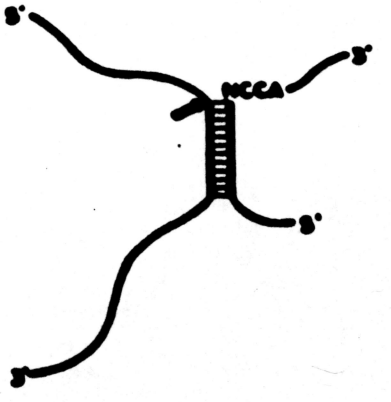
Ribozymes must be introduced into the target cell. There are now two general mechanisms that exist for introducing catalytic RNA molecules into cells, one is to exogenously deliver the preformed ribozymes, and the other is endogenously express the ribozymes from a transcription unit.

There are at least three methods for the delivery of preformed ribozymes into the cells, they are liposomes, electroporation, or microinjection. Efforts have been made to overcome the lack of stability of introduced RNAs by using modified nucleotides, 2'-fluoro- and 2'-amino- (Pieken et al., 1991), or 2'-O-methyl- (Paolella et al., 1992), mixed DNA/RNA molecules (Snyder et al., 1993), or by the addition of terminal sequences (such as the bacteriophage T7 transcriptional terminator) at the 3'-end of

A.



B.



the RNA to protect against cellular nucleases (Sioud et al., 1992). Endogenous expression can be achieved by inserting ribozyme sequences into the untranslated regions of genes transcribed by RNA polymerase II (pol II), which have strong promoters, such as the SV40 early promoter (Cameron et al., 1989), CMV promoter (Mahieu et al., 1994), the actin promoter (Sarver et al., 1990), or a retroviral long terminal repeat (LTR) (Koizumi et al., 1992). Ribozymes have also been inserted into the anticodon loop of tRNA transcribed by RNA polymerase III (pol III) as well as linked to downstream of promoter sequences derived from the human U1 snRNA and U6 snRNA (Bertrand et al., 1997).

4. Ribozymes as antineoplastic agents

Ribozymes can be used to target the replication of oncogenic DNA virus, for example, ribozymes have been shown to interrupt the LMP gene of EBV, which is necessary for persistence, episomal replication, and transformation of infected B-cells (Joske et al., 1993). Other viruses included HBV (Yoffe et al., 1992), papillomavirus and the newly discovered γ herpesvirus associated with kaposi's sarcoma (Chang et al., 1994). In the last 3-4 years while our ribozyme projects were in progress, an increasing number of studies describing neoplastic conditions being targeted by vectors expressing ribozymes have been published. For example, the bcr-abl translocation messages present in Philadelphia chromosome-positive adult chronic myelogenous leukemia has been targeted by ribozymes *in vitro* (Leopold et al., 1995; Leopold et al., 1994). A recent report by Wright et al., (1998) demonstrated a 53%

decrease in bcr-abl mRNA levels in a clone induced to express Rz, compared with its uninduced control. Phenotypic analysis of this clone also revealed a 63% decrease in colony-forming ability and a 43% inhibition of cell proliferation following ribozyme expression. Morphological analysis of the cells showed a slight increase (2.5% to 15%) in the number of cells undergoing apoptosis. These results suggest that ribozyme was effective in suppressing bcr-abl gene expression within a cellular environment and altering the leukemic nature of a CML cell line. Dorai et al., (1998) reported development of a hammerhead ribozyme against BCL-2. II. Ribozyme treatment sensitized prostate cancer LNCaP cells to apoptotic agents. Ribozyme transfection and subsequent application of apoptotic stimuli, such as, serum starvation or phorbol ester treatment, caused a 30% increase in cell death by apoptosis compared with these apoptotic stimuli alone. The results obtained strongly support the ability of a potential anti-bcl-2 ribozyme therapy to synergize with other agents in inducing apoptosis of hormone-resistant human prostate cancer cells. Ribozymes have also been shown to inhibit H-ras transformation of fibroblasts *in vitro*, as well as *in vivo* (Feng et al., 1995; Funato et al., 1994; Ohta et al.,1994; Kasgani-sabet et al., 1994; Tone et al., 1993; Kashani-sabet et al., 1992), as evidenced by reduction in tumor growth and aggressiveness in murine models. Yokoyama et al. (1997) also reported using a hammerhead ribozyme to modulate the c-fms-oncogene in an ovarian carcinoma cell line. Co-expression of macrophage colony-stimulating factor (M-CSF) and its receptor (c-fms) is often found in ovarian epithelial carcinoma,

suggesting the existence of autocrine regulation of cell growth by M-CSF. In order to block this autocrine loop, Yokoyama et al. (1997) have developed hammerhead ribozymes against c-fms mRNA. The ribozymes were able to cleave an artificial c-fms RNA substrate in a cell-free system, and in TYK-nu cells that expressed M-CSF and its receptor. The transfectant showed a reduced growth potential. The expression levels of c-fms protein and mRNA in the transfectant were clearly decreased with the expression of ribozyme RNA compared with that of an untransfected control or a transfectant with the vector without the ribozyme sequence.

Other strategies for using ribozyme-expressing vectors in the treatment of cancer include the introduction into malignant cells of transgenes designed to obviate resistance to antineoplastic drugs, to prevent resistance or restore susceptibility to conventional chemotherapeutic agents. Ribozymes against the human multidrug resistance gene (mdr) have been described (Bertram et al., 1995; Kobayashi et al., 1994a; Kobayashi et al., 1994b). Czubayko et al. (1996) used a hammerhead ribozyme targeted to secreted growth factor pleitrophin and successfully modulate melanoma angiogenesis and metastasis. Another *in vivo* experiment using an anti-CAPL ribozyme demonstrated reversal of the metastatic phenotype of human tumor cells (Maelandsmo et al., 1996).

5. Factors affecting hammerhead ribozyme cleavage *in vitro*

A consensus sequence defined as the catalytic core of hammerhead ribozymes has been described by Forster and Symons, (1987). Single changes in the consensus

region have shown some effect on catalytic efficiency on every base within the 14 conserved sites (Ruffner et al., 1990). Any change in the consensus sequence of the ribozyme catalytic core causes some, often significant, loss in catalytic activity (Ruffner et al., 1990).

Magnesium is needed to stabilize the 5'-oxygen leaving group (Taira et al., 1990). The reason for that is probably its ability to stabilize the tertiary structure of the hammerhead complex. Dahm et al. (1991) found that spermidine can lower the concentration of magnesium required for cleavage, suggesting spermidine can replace magnesium in stabilizing the active ribozyme structure.

Looking for the most favorable sequences for nonconserved bases in the ribozyme structure has not been successful. Ruffner et al. (1989) have shown that ribozyme activity can vary up to 1000-fold due to the changes in nonconserved bases. The difference between ribozyme activity with differing numbers of bases in stems and /or loops are difficult to generalize as they may be sequence-specific (Ruffner et al., 1989; Heidenreich et al., 1992).

6. Problems underlying the effective use of ribozymes as therapeutic agents

Intracellular experimentation with ribozymes leaves many factors uncontrolable. Many factors, such as magnesium concentration, cellular pH, degradation of the ribozyme, co-localization of the target and ribozyme, secondary or tertiary structure of endogenous target RNA or ribozyme , as well as RNA-binding proteins, can affect

the activity of ribozymes *in vivo*. This indicates that the application of this echnology is not as straightforward as simply expressing a complementary sequence *in vivo*.

Given the complexity of eukaryotic gene expression, there are numerous factors that can impact on ribozyme efficacy, including the rate of transcription of ribozyme genes, the stability of the ribozyme, the *in vivo* rate of hybridization, the location of the ribozyme gene relative to the target gene in the host genome, and the site at which the ribozyme functions (Denhardt et al., 1992). Furthermore, the cleavage capacity of the ribozyme provides an additional complexity related to achieving ideal conditions for both substrate RNA cleavage and release of products. Traditional efforts to enhance the efficiency of ribozyme-mediated suppression have been directed towards increasing the intracellular concentration and stability of ribozymes, with the aim of maintaining high ratios of these sequences relative to the target mRNA. There are two major approaches to overcome the degradation of ribozymes, and hence enhance the stability of ribozymes. One is to chemically modify the RNA, thereby decreasing degradation *in vivo* and the other is to add sequences or structures that naturally increase transcript stability. Other strategies have attempted to determine the general regions of the target mRNA accessible to ribozyme regulation and the optimal lengths for the flanking arms of the ribozymes (Murray et al., 1992). These efforts to improve upon the efficiency of ribozymes have resulted in some success.

With the recent advances in techniques for *in situ* detection of specific DNAs or RNAs inside intact cells, much attention has been focused on the location of

individual genes and mRNAs and the organization of the machinery required for transcription, processing, and transport within the eukaryotic nucleus (Carter et al., 1994). Genomic DNA sequences may occupy specific sites within the nucleus (Blobel et al., 1985; Marshall et al., 1997) and mRNAs have been shown to be localized to specific foci (Spector et al., 1993) and transported along specific tracks in both the nucleus (Lawrence et al., 1989; Xing et al., 1993) and the cytoplasm (Lawrence et al., 1986). Moreover, some specialized mRNAs undergo cellular localization via specific cis-acting RNA sequences and cellular localization machinery (Mowry et al., 1995). Co-localization of target RNA and ribozyme is crucial to ribozyme activity within cells. Localization of RNA within the cell depends on compartmentalization. Fulton et al. (1982) and Spector et al. (1993) have respectively presented evidence for a compartmentalized organization of the nucleus and cytoplasm. Targeting of a ribozyme to its substrate that are compartmentalized in the nucleus and/or cytoplasm remains to be overcome before establishment of ribozyme therapy.

D. Overview

In the United States, prostate cancer is the most common solid tumor malignancy in men and second to lung cancer as the leading cause of cancer deaths in this group. Prostate cancer progression involves the shifting of cells from androgen-dependent growth to an androgen-independent state, sometimes with the loss or mutation of the androgen receptors in prostate cancer cells. Both autocrine and paracrine pathways are up-regulated in androgen-independent tumors and may replace androgens as primary growth stimulatory factors in cancer progression. These growth factors include fibroblast growth factor, IGFs, EGF, TGF alpha, retinoic acid, vitamin D3, and the TGF beta families. Even though prostate cancer is responsible for 40,000 deaths per year, a screening program like the prostate-specific-antigen (PSA) test is a matter of controversy because scientific evidence is lacking that early detection decreases morbidity and mortality. Furthermore, PSA test detects the signal of cancers already under way, but it can not identify men at high risk of getting prostate cancer before they develop the disease. Recent epidemiology studies reported that IGF-I levels in plasma are associated with prostate cancer risk (Chan et al., 1998). Drugs which can lower IGF-I levels may thus help lower a man's risk of prostate cancer. Decisions of treating prostate cancer are difficult to make because of the nature of prostate cancer and because it tends to occur in older men who often have multiple, competing medical illnesses. In general, localized cancer is best treated with surgical removal of the prostate gland or radiotherapy. Hormone deprivation

therapy is the primary method of controlling metastatic prostate cancer. At present, chemotherapy can be used to treat disseminated prostate cancer. However, there is no effective method of treating hormone-independent prostate cancer. Gene therapy using molecular tools to down-regulate autocrine and paracrine pathways of growth stimulatory factors, like IGFs, in androgen-independent tumors may thus lead to a logical promising strategy.

Dr. Fujita-Yamaguchi's Laboratory has been interested in insulin and IGFs and the relationship between IGFs and tumors. They have laid ground work by examining the structure of insulin and IGF-I receptors. IGFs have been previously reported to promote prostate cell and neuroblastoma cancer cell growth via the IGF-1 receptor in an autocrine/paracrine manner. Dr. Fujita-Yamaguchi's group and others recently showed that IGF-II, but not IGF-I, is produced in established human prostate cancer cell lines, and suggested an autocrine regulation of DU145 and PC-3 cell growth by IGF-II (Figueroa et al., 1995; Angelloz-Nicoud et al., 1995; Kimura et al., 1996). Since IGF-II expression in prostate tumors and other tumors is not quite clear, it was necessary to determine whether epithelial or stromal cells express IGF-II in prostate tumors *in vivo* in order to understand the possible autocrine role of IGF-II in prostate cancer growth.

Li et al. (1998), recently used *in situ* hybridization and immunohistochemistry methods to determine whether epithelial or stromal cells express IGF-II in prostate tumors *in vivo* (chapter II). The results showed that IGF-II is expressed in over

50% of prostate, breast, and bladder cancer tissues. In all positive cases, the expression is localized to malignant cells. These data are consistent with the hypothesis that cancer cell growth may be regulated by IGF-II. This information has led us to consider IGF-II as a target for molecular manipulation toward the establishment of novel cancer therapy.

Of new molecular technologies developed recently, we have chosen the ribozyme strategy to manipulate IGF-II gene expression. Ribozymes have been increasingly used in treating human diseases, including HIV (Rossi, 1998). To test our hypothesis that prostate cancer cell growth is regulated by IGF-II, ribozymes against human IGF-II were constructed and intracellularly expressed in the androgen-independent prostate cancer PC-3 cell lines.

E. Goals and Objectives of the Project

Ribozymes are catalytic RNAs that can be useful for manipulation of gene expression, which may lead to a useful tool in gene therapy. Ribozymes have two properties, first, site-specific recognition of RNA, similar to antisense oligonucleotides, and second, RNA substrate destruction via site-specific cleavage. Hammerhead ribozymes are small, thus easy to handle. Successful application of hammerhead ribozymes to target genes of interest have been reported. The goals of my dissertation were 1) to evaluate IGF-II expression in different tumor tissues, breast, prostate, bladder, and paraganglioma by *in situ* hybridization and immunohistochemistry, 2) to design , construct and express an IGF-II ribozyme, 3) to compare cleavage kinetics of different ribozymes, and 4) to determine whether anti-IGF-II ribozymes can delay growth in cancer cell lines.

In this dissertation, two human cancer cell lines were utilized, human prostate cancer cell line PC-3, and human neuroblastoma cell line SK-N-AS. Both cell lines can express IGF-II as an autocrine factor.

II. EXPRESSION OF INSULIN-LIKE GROWTH FACTOR (IGF)-II IN HUMAN PROSTATE, BREAST, BLADDER, AND PARAGANGLIOMA TUMORS

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2. Principal investigator
3. Responsible for Western Blot and Histochemistry
4. Responsible for construction of probe plasmid, initially started *in situ* hybridization of human prostate, and breast tumors

5, 7, 8, 9 Collaborators

6. Responsible for Immunohistochemistry

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Expression of insulin-like growth factor (IGF)-II in human prostate, breast, bladder, and paraganglioma tumors *

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Heading title: IGF-II expression in human tumors

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A. Abstract

Insulin-like growth factors (IGFs) are potent mitogens for a variety of cancer cells in vitro. A paracrine/autocrine role of IGF-II in breast and prostate cancer cell growth has been suggested. Information on cell-type specific IGF-II expression *in vivo* in the breast and prostate is, however, limited. Thus, cell types expressing IGF-II mRNA and protein in tumors were identified by *in situ* hybridization and immunohistochemistry. Of 36 prostate, 17 breast, and 10 bladder cancers as well as 9 paraganglioma tissues examined, IGF-II was expressed in more than 50% of prostate, breast, and bladder tumors and in 100% of paraganglioma tumors. Expression levels of IGF-II were highest in the paraganglioma and bladder followed by prostate and breast tumors. In all the tumors expressing IGF-II, both mRNA and protein were localized to malignant cells and expression in stroma was minimal. Furthermore, since previous studies indicated that an incompletely processed form of 15 kDa IGF-II exhibits higher mitogenic potency than the completely processed 7.5 kDa IGF-II, the quantity and size of IGF-II proteins expressed in these tumors were analyzed by Western immunoblotting. Greater expression of 15 kDa relative to 7.5 kDa IGF-II was clearly demonstrated in all six prostate cancers and in half of the two breast and four bladder cancers examined. The results were consistent with the hypothesis that the 15 kDa form of IGF-II expressed in cancerous cells may contribute to autocrine cancer cell growth *in vivo*.

B. Introduction

Insulin-like growth factor (IGF)-II is a single chain polypeptide of 67 amino acid residues, that has amino acid homology with IGF-I and proinsulin (Humbel, 1990). Both IGF-I and IGF-II bind to the IGF-I receptor with high affinity, which initiates mitogenic responses in the cell (Daughaday and Rotwein ,1989; Perdue et al., 1991; Baserga, 1995). IGFs are potent mitogens of breast cancer cells, and an autocrine/paracrine regulation of breast cancer cell growth has been suggested (Huff et al., 1986; Osborne et al., 1989; Cullen et al., 1990). Most of the established breast cancer cell lines do not express IGF-I or IGF-II (Yee et al., 1988), and it has been proposed that IGFs expressed in tumor stroma stimulate the growth of neighboring tumor epithelium by paracrine mechanisms (Hogan et al., 1987, 1988; Manni et al., 1994; Singer et al., 1995). Despite the strong emphasis on paracrine actions of IGF-II in breast stromal-epithelial interaction *in vivo*, stromal IGF-II expression has not been clearly demonstrated by previously published *in situ* hybridization (ISH) studies (Manni et al., 1994; Singer et al., 1995; Giani et al., 1996).

It was originally reported that IGF-I is responsible for autocrine growth of human prostate cancer cell lines including androgen-dependent LNCaP as well as hormone-independent DU145 and PC-3 cells (Pietrzkowski et al., 1993). We and others more recently showed that IGF-II, but not IGF-I, is produced in those established human prostate cancer cell lines, and suggested an autocrine regulation of DU145 and PC-3

cell growth by IGF-II (Kimura et al., 1996; Figueroa et al., 1995; Angello-Nocoud and Binoux, 1995). It is now necessary to determine whether epithelial or stromal cells express IGF-II in prostate tumors *in vivo* towards understanding a possible autocrine role of IGF-II in prostate cancer growth.

The objective of this study was to identify cell types expressing IGF-II mRNA and protein in tumors, particularly in breast and prostate cancers. We also included bladder cancer as well as paraganglioma since IGF-II expression in the former has not been analyzed and since high levels of IGF-II protein expression in the latter have previously been demonstrated by immunohistochemistry (IHC) (Suzuki et al., 1989). We then analyzed the size and the level of IGF-II protein expressed in prostate, breast, and bladder tumors by Western immunoblotting to determine whether high Mr IGF-II is expressed in these tumors. The 15kDa IGF-II has been found in most patients with nonislet cell tumors that develop hypoglycemia (Daughaday 1990; Daughaday and Trivedi, 1992; Enjoh et al., 1993). The present study provides evidence that cancerous cells, but not stromal cells, specifically express IGF-II *in vivo,* and suggests that the high Mr IGF-II is more abundant than 7.5 kDa IGF-II in these cancers than benign tissues.

C. Materials and Methods

Multi-tumor blocks (MTBs) and cell cultures: MTBs of human prostate, breast, bladder cancers and paragangliomas used were formalin-fixed, paraffin-embedded

archives which had been prepared and stored by the Department of Anatomic Pathology, City of Hope Medical Center (Battifora, 1986). Human prostate cell lines, LNCaP, DU145, and PC-3, were obtained from American Type Culture Collection (Rockville, MD), and cultured in RPMI1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. Human neuroblastoma cell line, SK-N-AS, which can grow continuously in serum-free medium by autocrine growth stimulation by IGF-II, was kindly provided by Dr. Carol Thiele, Pediatric Branch, NCI, and were grown as described (El-Badry et al., 1991). Cells grown in a 75 cm^2 flask were collected, formalin-fixed, and paraffin-embedded, from which sections were made for ISH experiments.

In situ hybridization: An approximately 1 kb fragment of cDNA encoding the human precursor IGF-II (911 to 2067 nt., Dull et al., 1984) was subcloned into pBluescriptII KS(+) (Stratagene, San Diego). ^{35}S-Labeled sense and antisense probes were transcribed from the plasmid in the presence of ^{35}S-UTP using Stratagene's RNA transcription Kit (Stratagene, San Diego) and purified with NucTrap push columns (Stratagene, San Diego). IGF-I cRNA probe of 413 bases was also prepared from pBluescript vectors containing cDNA fragments of 130 to 542 nt. (Jansen et al., 1983). The probes were reduced to approximately 150 nucleotides by alkaline hydrolysis.

ISH was performed essentially as described by Angerer et. al.(1987). Briefly, sections on sialinized slides were baked at 80 °C for 1 h, deparaffinized in xylene and rehydrated in graded ethanols. Following proteinase K treatment (30 min at 37 °C in 100mM Tris HCl, pH 8.0, 50 mM EDTA, 1 μg/ml proteinase K [Boeringer Mannheim]), the tissue was acetylated in 0.1M triethanolamine-HCl at pH 8.0 at room temperature for 10 min. They were briefly washed in 2X SSC and dehyrdated in ethanol. Twenty five μl of the hybridization mix (50% formamide, 0.3M NaCl, 20mM Tris HCl, pH 8.0, 1mM EDTA, 1X Denharts, 10% dextran sulfate, 100 mM DTT, 500 μg yeast tRNA/ml, 1 μg of probe [10^6 dpm] were added to each slide. The slides were covered with plastic cover slips, sealed with rubber cement, and placed in a humidified chamber at 46°C overnight. After removal of the coverslip, the slides were rinsed 3 times with 4X SSC at room temperature, and then treated with RNase solution (20 μg/ml RNase in 0.5M NaCl, 10mM Tris HCl, pH 8.0, 1mM EDTA) at 37 °C for 30 min. Following washing for 30 min at 37 °C in RNase A buffer (0.5M NaCl, 10mM Tris HCl, pH 8.0, 1mM EDTA), the slides were rinsed with 2 X SSC for 30 min at room temperature and then in 0.1 X SSC at 55 °C. The slides were then dehydrated through graded alcohols and air dried. Autoradiography was performed using Kodak NTB-2 nuclear track emulsion, exposed for 3 weeks, and then following developing, lightly counterstained with hematoxylin (H).

Monoclonal antibodies (mAbs): Anti-IGF-II mAb which was kindly provided by Dr. K. Nishikawa of Kanazawa Medical University is IgG_1 against rat IGF-II (Tanaka et al., 1989). This is the same mAb as Amano mAb used by Enjoh et al. (1993) and anti-IGF-II mAb used by others (Tang and Rogler, 1991; Tennant et al., 1996: Giani et al., 1996). It binds to human IGF-II, human IGF-I, and human insulin with affinities of 100%, < 10%, and < 0.01%, respectively. Unless otherwise stated, this antibody was used for IHC. Three mAbs against human IGF-II, 2H11, 2B11, and 1D4, which do not crossreact with either human IGF-I or rat IGF-II (Enjoh et al., 1993) were kindly provided by Dr. K. Sakano of Daiichi Pharmaceutical Co. Ltd.

Immunohistochemistry: Studies were carried out with mAbs that are reactive in routine paraffin sections. Four μm paraffin sections mounted on Probe-on slides (Biotek Solutions; Vantana, Tucson, AZ) were baked for 1 hr at 55 °C and then dried overnight at 48 °C. They were subsequently deparaffinized in xylene, rehydrated in graduated alcohols to distilled water, and loaded into a Techmate™ Slide Holder and placed into 10 mM citrate buffer, pH 6.0. Antigen retrieval was performed by the HIER (Heat Induced Epitopy Retrieval) method (Battifora et al., 1995). The slides were steamed in 10 mM citrate buffer for 20 min and then allowed to cool for 5 min using a household Black and Decker Steamer (model #HS90). The primary IGF-II antibody was used at concentrations of 0.1-10 μg/ml as indicated, and incubated overnight in a humid container. A modified ABC (Vector Elite Kit; Vector Lab.

Burlingam, CA) technique was used for detection with DAB as the chromogen and the slides were lightly counterstained with Mayer's hematoxylin. All staining was performed using a Bioteck Techmate 1000 Immunostainer (Biotek Solutions) with a ABC detection system (Biotek Solutions).

Data analysis: The localization of hybridization or immunoreactivity in the tissues after ISH or IHC experiments was examined by three individuals including one of the experienced pathologists (G.K, Y.S., or S.W.). The intensity of hybridization was scored on a 5-point scale according to the judgement of the same pathlogist (S.W.), where 0 = no hybridization or staining, ± = some hybridization or staining but not definitely positive, 1+ = weak hybridization or staining, 2+ = moderate hybridization or staining, and 3+ = strong hybridization or staining.

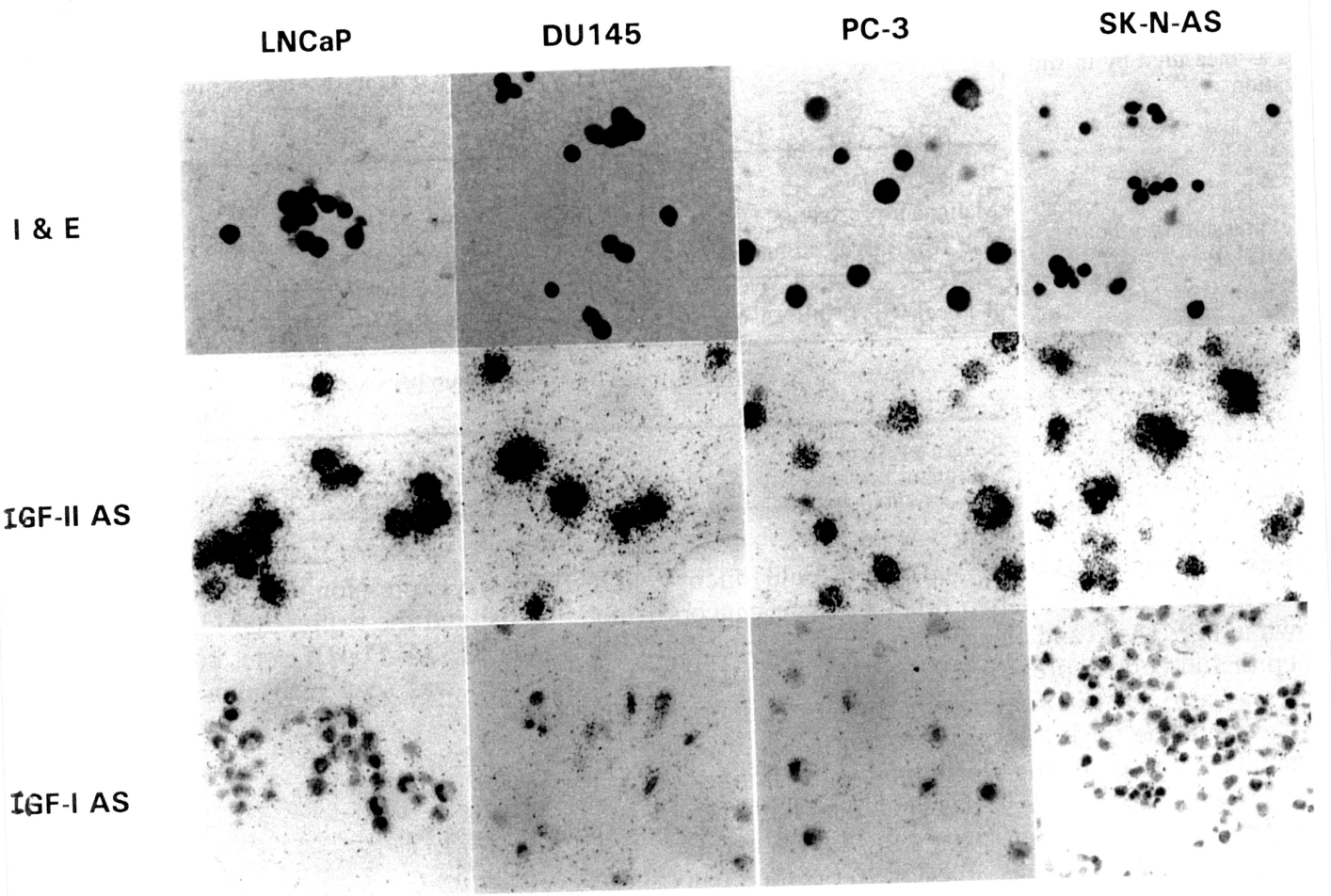
Immunoaffinity concentration of IGF-II in extracts of tumors: Immunoaffinity resins were prepared by coupling anti-IGF-II mAb 2H11 to either formyl-cellulose or CNBr-activated Sepharose 4B as previously described (Enjoh et al. 1993; Fujita-Yamaguchi et al., 1983). Immunoaffinity concentration of IGF-II from tumors was carried out as previously described (Enjoh et al. 1993). Briefly, ~0.2 g of frozen tumor tissues were extracted with 4 vol 87.5% (vol/vol) ethanol/12.5% (vol/vol) 2N HCl to release IGF-II from IGF-II/IGF binding protein complexes. The supernatants were lyophilized, dissolved in 1 ml of PBS containing 0.02% Tween, and incubated at 25°C for 3 hr with 30 µl of mAb 2H11-conjugated resin. After extensive washing

with PBS containing 0.02% Tween 20 and 1M NaCl, IGF-II was eluted from the resin with ~20 μl sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer under nonreducing conditions.

Western immunoblot analysis: IGF-II extracted from tumors (~0.1g tissue equivalent) was resolved by SDS-PAGE on a 15% gel under nonreducing conditions and electrotransfered to PVDF membranes as described (Enjoh et al. 1993). The membranes were blocked overnight at 4°C with 5% (wt/vol) skim milk in PBS containing 0.05% Tween 20, incubated with 2 μg/ml mAb 2H11, and then with biotinylated sheep anti-mouse IgG and streptavidin-HRP. The IGF-II-mAb complexes were detected using the ECL system (Amersham, Arlington Heights, IL), followed by exposure to X-ray films.

D. Results

IGF-II, but not IGF-I, is expressed in prostate cancer cell lines: IGF-II and IGF-I mRNA expression in three prostate cell lines and neuroblastoma SK-N-AS cells as positive control was examined by ISH. The results of ISH using the IGF-II cRNA antisense probe and IGF-I cRNA antisense probe shown in Fig. 9 demonstrate that IGF-II mRNA, but not IGF-I mRNA, is expressed in prostate cancer LNCaP, DU145, and PC-3 cells as well as neuroblastoma SK-N-AS cells. The results confirmed our previous study, in which both reverse transcriptase-polymerase chain reaction and radioimmunoassay (RIA) were used (Kimura et al., 1996), and suggested potential


LNCaP
DU145
PC-3
SK-N-AS
I & E
IGF-II AS
IGF-I AS

pitfalls only associated with estimating IGF levels by RIA (Pietrzkowski et al., 1993).

IGF-II expression in prostate tumors: Of 36 prostate cancer specimens examined, IHC experiments revealed that 22 specimens expressed significant levels of IGF-II mRNA (>1+) with the IGF-II cRNA probe (Table 1A). Fig. 10 shows H and E staining (A) and darkfield photographs of a typical prostate tumor analyzed by ISH using sense (B) and antisense (C) probes. While the sense probe only revealed background signals, cancerous epithelial cells showed significantly elevated hybridization signals with the antisense probe. Darkfield and brightfield photographs of ISH results of two other prostate cancers are shown in Fig. 11A-D, which clearly demonstrate that cancerous epithelial cells express IGF-II mRNA. IGF-II expression was also seen in some of the benign epithelial cells adjacent to the infiltrating cancer (Figs. 10C, 11E, and 11F). Of particular interest is the very intense staining of IGF-II mRNA as seen in Figs. 11E-H. This focal IGF-II expression, as indicated by open arrows in Fig. 11E and G, was observed in benign glands adjacent to prostate cancers (22% of 36 cancers examined) as well as one of four benign prostates examined (Table IB). Figs. 11E-H represent typical focal IGF-II expressions in benign epithelial cells in a prostate cancerous tissue and a benign prostate. A similar observation to this one, the IGF-II expression in focal groups of cells in neoplastic nodules of woodchucks, was also reported by Yang and Rogler (1991).

Immunostaining with anti-IGF-II mAb (10 μg/ml) indicated that 11 of 19 prostate

Table 1. Expression of IGF-II mRNA in benign and malignant prostates as measured by *in situ* hybridization.

A. IGF-II mRNA expression in benign and malignant prostate.

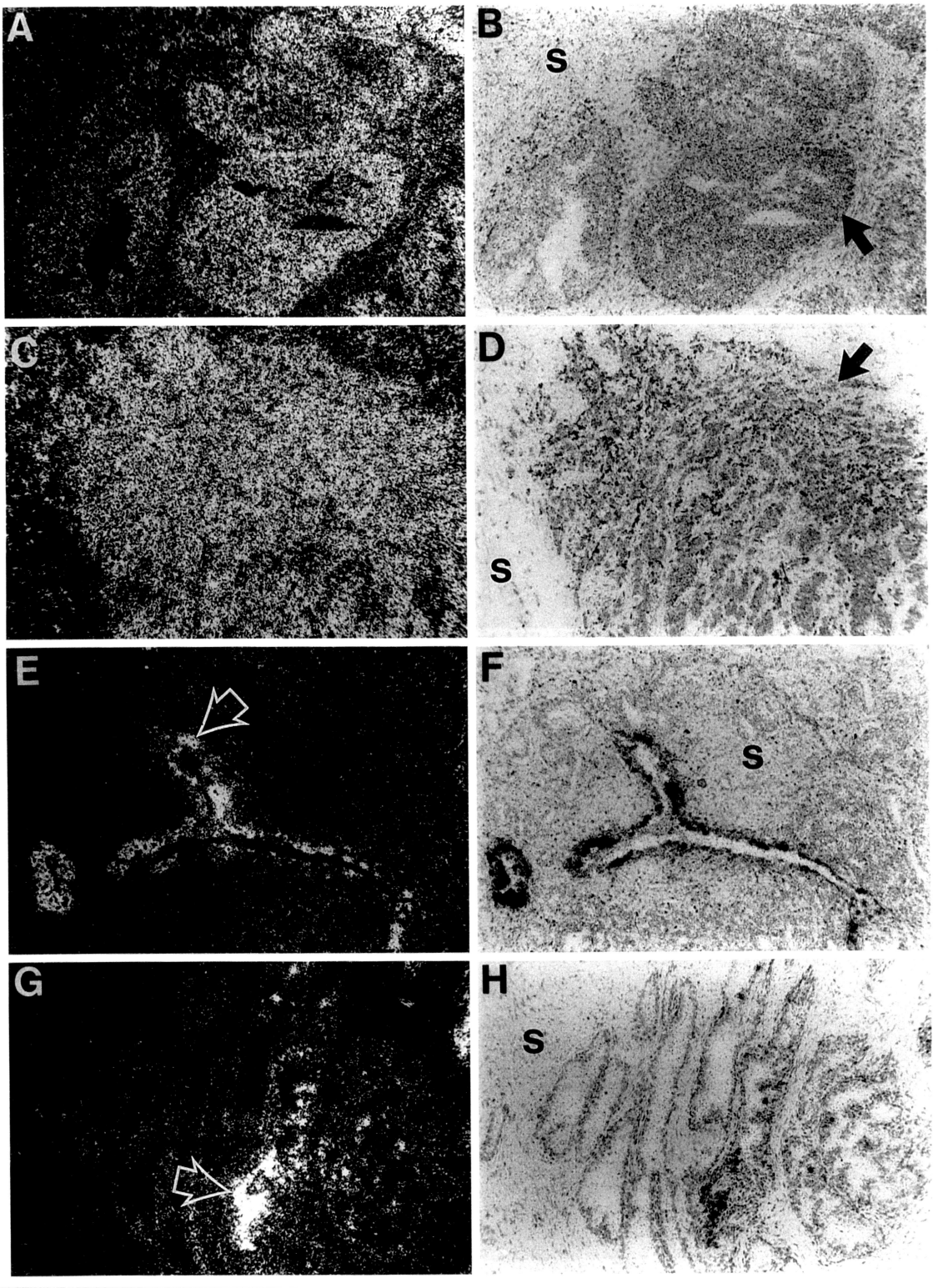
Number of samples	IGF-II mRNA expression level				
	–	±	1+	2+	3+
Benign prostate: 4	2	1	0	1*	0
Malignant prostate: 36	2	12	8	9	5
IGF-II positivity in malignant prostates	14(39%)		22(61%)		

* Focal expression (see below and Fig. 3E and G).

B. Focal IGF-II mRNA expression in benign epithelial cells in benign and malignant prostate.

	Total number examined	Focal IGF-II mRNA expression
Benign prostate	4	1 (25%)
Malignant prostate	36	8 (22%)

C
F
B
E
S
S
S
S


A
B
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D
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G
H
S

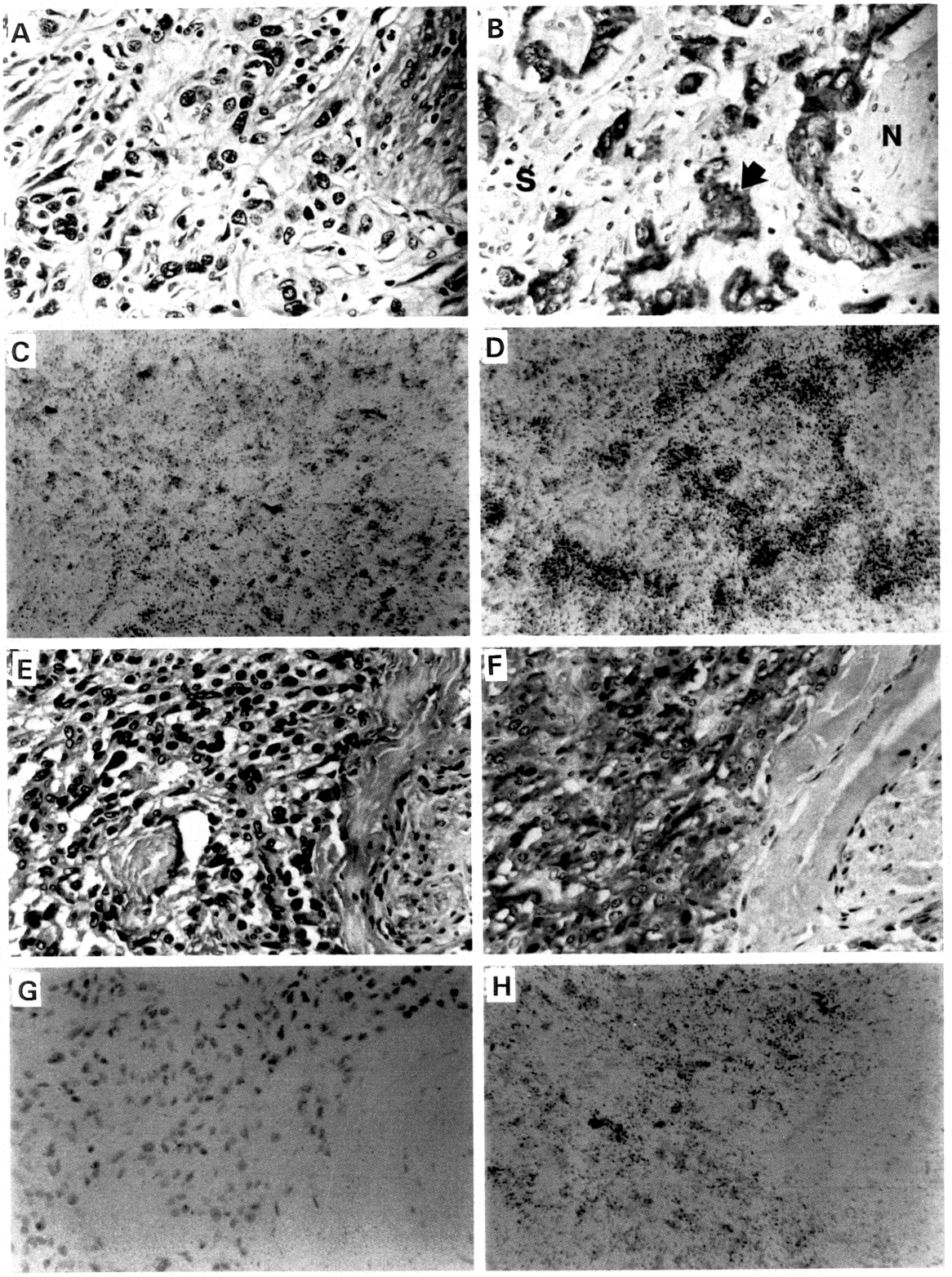
cancers examined showed the presence of IGF-II in good accordance with IGF-II mRNA expression (data not shown). In summary, high levels of IGF-II mRNA expression was detected in cancerous epithelial cells and the expression in stroma was minimal (Figs. 10C and 11A-D). Of note, however, was that IGF-II expression was seen in benign epithelial cells (Figs. 10C and 11E-H).

IGF-II expression in breast cancer: Of 17 breast cancers examined, nine showed positive for IGF-II mRNA expression by ISH (Table II). One set of results showing H and E staining, ISH with sense or antisense IGF-II probe demonstrate that IGF-II mRNA is clearly expressed in cancerous epithelial cells but not in stromal cells (Fig. 10D-F). Relatively high expression of IGF-II was also detected in benign epithelial cells of breast cancers as seen in Fig. 10F. Immunostaining with anti-IGF-II mAb using 10 μg/ml revealed the presence of IGF-II protein in five of nine specimens examined. The protein staining was seen in good accordance with IGF-II mRNA expression (data not shown).

IGF-II expression in bladder cancer and paraganglioma: Ten bladder cancers were examined for expression of IGF-II by ISH and IHC. Although only three bladder cancers were definitely positive for IGF-II mRNA by ISH, IHC using anti-IGF-II mAb at 0.1 μg/ml revealed that IGF-II protein was detected in five bladder cancers including two tissues that were negative for IGF-II mRNA. Fig. 12A, B, C, and D represent one set of results, *i.e.,* H and E staining, IHC, ISH using the sense probe,

Table 2. Expression of IGF-II mRNA in breast tumor tissues.

Breast cancer	Number of samples	IGF-II mRNA expression level –	±	1+	2+	3+
infiltrating ductal	14	5	2	2	4	1
Lobular	2	1		1		
Intraductal	1					1
IGF-II positivity	17(100%)	8(47%)		9(53%)		


A
B
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H

and ISH using the antisense probe, respectively. The results not only clearly revealed abundant expression of IGF-II mRNA and protein in cancerous cells not in stroma, but also demonstrated that IGF-II mRNA and protein expression in bladder cancers was higher than in prostate and breast cancers.

Consistent with the previous observation by Suzuki et al. (1989), we found that of nine paragangliomas examined, all were positive for IGF-II protein by IHC using anti-IGF-II mAb at a concentration of 0.1 μg/ml. ISH experiments revealed good expression levels of IGF-II mRNA in 8 of the specimens. Results of one paraganglioma specimen are shown in Fig. 12E, F, G, and H (H and E staining, IHC, ISH using the sense probe, and ISH using the antisense probe, respectively). IGF-II protein and mRNA expression is seen in tumor cells by both IHC (Fig. 12F) and ISH (Fig. 12H), respectively.

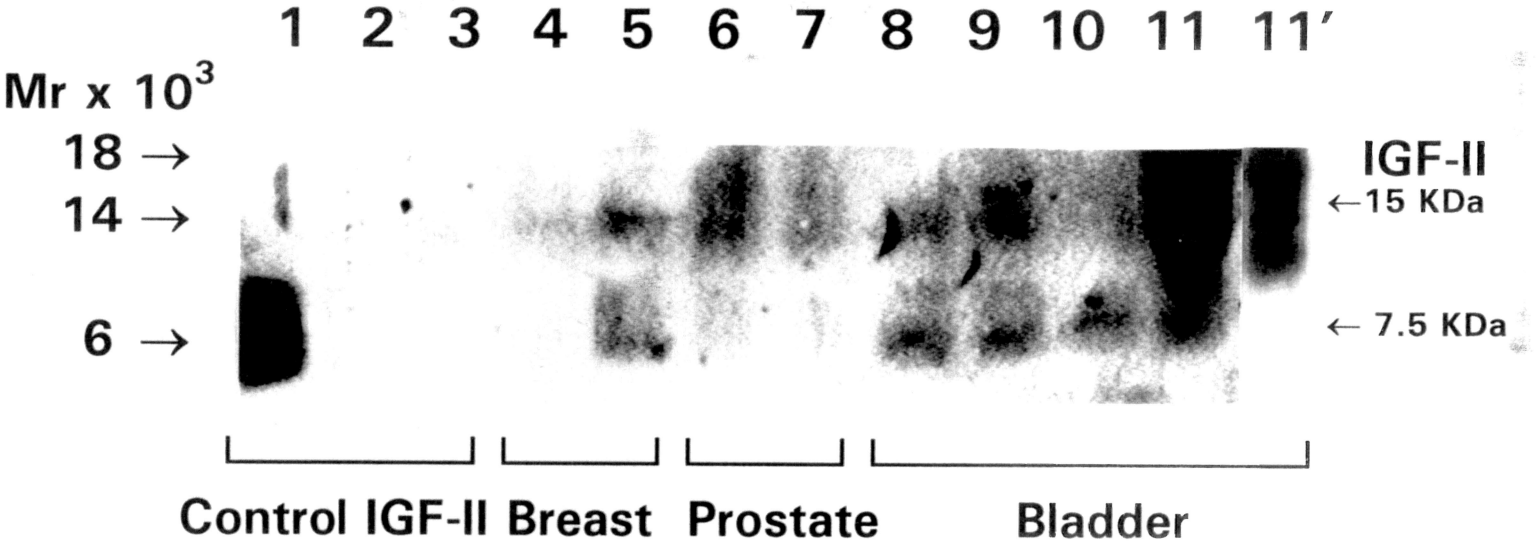
Neutralization of IGF-II immunostaining by exogenously added IGF-II: In order to verify that the positive signal obtained with IHC while using the anti-IGF-II mAb is in fact due to IGF-II expressed in the cancerous cells, the IHC experiments were performed in the presence of IGF-II (100 times in molar excess of the antibody). Both paraganglioma and bladder cancers that were positive by IHC did not show any positivity in the presence of excess IGF-II, confirming that the cells stained with this antibody express the IGF-II protein.

IHC using other anti-IGF-II mAb: Although the anti-IGF-II mAb used in this study, as well as other studies (Suzuki et al., 1989; Yang and Rogler, 1991; Tennant et al.,

1996; Giani at al., 1996), binds to human IGF-II, it is known to be specific for rat IGF-II and also crossreacts with human IGF-I to some extent. In contrast, newly introduced mAbs 2B11, 2H11, and 1D5 were shown to be specific for human IGF-II and not to crossreact with IGF-I at all (Enjoh et al., 1993). In addition, their antigen-specificities may differ from that of the original anti-IGF-II mAb. We thus examined whether the new mAbs detect the IGF-II protein expressed in breast, prostate, bladder and paraganglioma differently from those detected with the original anti-IGF-II mAb. Immunostaining with these mAbs at a concentration of 2 μg/ml revealed positive for IGF-II protein in bladder and paraganglioma. The cells stained were basically similar to those achieved by the original anti-IGF-II mAb at a concentration of 0.1 μg/ml. The intensity of the staining was, however, significantly lower than that stained with the original anti-IGF-II mAb even using 2B11 which gave the highest intensity among the three new mAbs. Under this condition, prostate and breast epithelial cells were not stained probably due to much lower affinities of those mAbs for IGF-II than that of the orignal anti-IGF-II mAb.

IGF-II Western immunoblotting of IGF-II isolated from prostate, breast, and bladder tumors: Increases in the ratio of high Mr IGF-II to 7.5K Mr IGF-II occur in most patients with nonislet cell tumors that develop hypoglycemia (Daughaday, 1990; Daughaday and Trivedi, 1992; Enjoh et al., 1993). In order to determine the abundance and size heterogeneity of IGF-II protein expressed in the tumor tissues

described above, 6 prostate, 2 breast, and 4 bladder cancers, 2 benign prostate, one prostate BPH, and 2 benign bladder tissues were subjected to Western immunoblotting. Tissues were acid-treated to remove IGF binding proteins and concentrated by immunoaffinity adsorbants (2H11-Sepharose) prior to Western immunoblot analysis according to the procedures described by Enjoh et al. (1993). The results including 5 cancerous and 3 benign tissues are shown in Fig. 13. The amounts of IGF-II protein detected by this procedure appeared to be between 13 and 130 ng since 130 ng, but not 13 ng, of IGF-II was seen in the control experiments (Fig. 13, lanes 1 and 2, respectively). Thus, except one tissue shown in Fig. 13 lane 11 which contained >130 ng IGF-II per ~0.1 g wet tissue, all the positive IGF-II signals from the tissue samples are assumed to have contained 13-130 ng IGF-II per ~0.1 g wet tissue. In addition to the completely processed 7.5 kDa IGF-II, high Mr IGF-II with apparent Mr 15K was always detected in all the cancerous tissues examined except one breast cancer (Fig. 13, lane 4), which showed a very weak signal probably due to low protein level in this predominantly fatty specimen. Fig. 13, lanes 9 and 10 represents the results of cancerous and benign tissues of the same bladder, respectively, which are consistent with the hypothesis that cancerous tissues express higher levels of high Mr IGF-II. It should be noted that the prostate BPH shown in Fig. 13 lane 7 clearly expressed a high Mr IGF-II.


1 2 3 4 5 6 7 8 9 10 11 11′
Mr x 10³
18 →
14 →
6 →
IGF-II
←15 KDa
← 7.5 KDa
Control IGF-II Breast Prostate Bladder

In summary, relatively more abundant expression of high Mr IGF-II to that of 7.5K Da IGF-II was clearly seen in all six prostate cancers, one BPH, one of two benign prostates, one of two breast cancers, and two of four bladder cancers but not in two benign bladders examined.

E. Discussion

In the present study, we analyzed expression of IGF-II in human prostate, breast, bladder, and paraganglioma by ISH, IHC, and immunoblotting. All the results are tablulated in Table III. We showed ; i) IGF-II was expressed in >50% of breast, prostate, and bladder cancer tissues as well as 100% of paragangliomas that have been examined by ISH and IHC; ii) in all positive cases, the expression of IGF-II was localized to malignant cells; iii) in prostate and breast cancers, IGF-II mRNA expression was localized to epithelial cells. IGF-II protein was detected weakly by ISH in epithelial cells expressing IGF-II mRNA; iv) in bladder cancers, both IGF-II protein and mRNA were highly expressed; v) in paraganglioma, high levels of IGF-II protein were detected, while mRNA levels were moderate; and vi) an incompletely processed 15 kDa IGF-II, which is thought to be more mitogenic than 7.5 kDa IGF-II (Perdue et al., 1991), was expressed in almost all of the cancerous tissues and the higher ratio of 15 kDa/7.5 kDa IGF-II was seen in >50% of those tissues as detected by Western immunoblot analysis. Thus, we have not only confirmed the previous observation by Suzuki et al. (1989) that IGF-II protein is highly expressed in paraganglioma, but also added new information on IGF-II mRNA expression in this tumor. To the best of our knowledge, possible involvement of IGFs in bladder cancer has not been reported. The present study revealed that ~50% of bladder cancers

Table 3. Summary of IGF-II expression in prostate, breast, bladder, and paraganglioma tumors as detected by ISH, IHC, and Western Immunoblotting.

	IGF-II mRNA detected by ISH	IGF-II protein detected by IHC	Higher ratio of 15 kDa/7.5 kDa IGF-II detected by Immunoblotting
Prostate cancer	22/36,61%	11/19, 58%	5/5, 100%
Breast cancer	9/17, 53%	5/9, 56%	1/2, 50%
Bladder cancer	3/10, 30%	5/10, 50%	2/4, 50%
Paraganglioma	8/9, 89%	9/9, 100%	Not determined

expressed IGF-II at the highest expression levels among the four different tumors that we examined. Furthermore, this study showed the ratio of 15 kDa and 7.5 kDa IGF-II proteins expressed in breast, prostate, and bladder cancer tissues which have not been previously reported.

The measurement of IGF-II in tissue samples is much more difficult as compared to that in sera of patients with nonislet cell tumor hypoglycemia as previously reported (Daughaday and Trivedi, 1992). This is mainly due to the presence of IGF binding proteins which affect an accurate estimation of IGF-II protein levels as well as low IGF-II protein levels in tissue homogenates. To overcome these problems, acid treatment followed by IGF-II concentration by IGF-II mAb affinity resins was necessary before Western blot analysis. Enjoh et al.(1993) previously analyzed the quantity and ratio of 15 kDa and 7.5 kDa IGF-II proteins in sera and tumor tissues of patients with nonislet cell tumor hypoglycemia by RIA and Western immunoblotting, respectively. They showed ~300 ng of IGF-II, of which more than 80% account for 15 kDa IGF-II, in ~300 μl serum or ~0.1 g tumor tissue from 12 different patients with nonislet tumor hypoglycemia including one patient each with breast and prostate cancer. Their Western blot results revealed much higher quantities of the 15 kDa IGF-II than our results, shown in Fig. 13, lanes 4-10. Approximately 10-fold higher levels of 15 kDa IGF-II were detected in their samples compared to ours, except that one bladder cancer specimen showed a high level of 15 kDa IGF-II protein (Fig. 13, lane 11) which appears to be similar to the levels they detected. The low level of

IGF-II protein expression we observed is probably due to the difference in the sources of cancer tissues used since the sera or tissues they analyzed were only from cancer patients who were known to contain high levels of circulating IGF-II.

In breast cancer tissues, IGF-II mRNA was clearly detected in malignant epithelial cells as well as in some benign epithelial cells. Previous studies, however, have suggested that the overexpression of IGF-II in breast stroma influences the growth of cancerous epithelial cells by paracrine mechanisms (Hogan et al. 1987, 1988; Manni et al., 1994; Singer et al., 1995). Evidence to support this stromal epithelial interaction includes *in vitro* as well as *in vivo* studies. For example, increased establishment of tumor and increased growth of MCF-7 cells occurred when the tumor cells were coinoculated with breast fibroblasts in athymic mice as compared to injection with MCF-7 cells alone (Hogan et al., 1987). More recently, Singer et al.(1995) and Gianti et al. (1996) reported that there were high levels of IGF-II expression in stroma of many invasive breast cancers but not in normal breast tissues by ISH. In our ISH experiments, malignant epithelial cells had stronger signal than surrounding stromal cells, and IGF-II expression in the stroma was scattered and focal. The morphology of the tumors illustrated in Fig. 1B and E of Giani et al. (1996) that did not express IGF-II are relatively well differentiated tumors. Their more poorly differentiated tumors (Fig. 1F and G) did show silver grains over tumor similar to what we found. It is thus plausible that the differences in our results and their results are due to the differentiation state of the tumors that we studied.

In the case of prostate cancer, our results are consistent with the findings by others. Jarrard et al. (1995) showed intense staining of the prostate epithelium with a biotinylated IGF-II oligonucleotide probe while showing that stromal cells appeared to react with the IGF-II probe to a lesser extent than epithelial cells. Tennant et al. (1996) showed that IGF-II mRNA was localized primarily to epithelial cells and that IGF-II expression was significantly increased in adenocarcinoma compared to that in benign epithelium. These observations with IGF-II expression *in vivo* are also consistent with previous reports (Kimura et al., 1996; Figueroa et al., 1995; Angello-Nocoud and Binoux, 1995), which suggested autocrine roles of IGF-II in human prostate cancer DU145 and PC-3 cells. We noticed a peculiar focal expression as well as an elevated expression of IGF-II in benign epithelium of the prostate. It should be noted, however, that the IGF-II expressing cancerous and benign epithelial cells that we found were in the prostate of patients at ages 68 ± 6.8 years (n=38). Since the levels of IGF-I, IGF-II, and the IGF-I receptor mRNAs in the prostate of advanced-age subjects were shown to be 2- to 3-fold higher than those of young control subjects (Bonnet et al., 1993), it is also likely the observed increase in IGF-II expression may be an age-dependent process.

Although prostate and breast cancers expressing IGF-II mRNA were positive for IGF-II protein by IHC, the IGF-II mAb concentration required was 100 times higher than stained IGF-II protein in paraganglioma and bladder tumors. Tennant et al. (1996) also used the same conditions as ours to stain prostate tissues. This in fact

indicates a possibility that the IGF-II protein level is either very low, or unstable in prostate and breast cancers. Alternatively, it is possible that the elevated IGF-II mRNA expression in prostate and breast cancers may not lead to abundant IGF-II protein expression. The human IGF-II gene consists of four promoters and nine exons. The major promoters in fetal and nonhepatic adult tissues are P3 and P4, and activation of each promoter seems to be dependent on the cell type of origin. De Moor et al. (1994) examined differential polysomal localization of human IGF-II mRNAs in five of the human tumor cell lines and in fetal liver, and reported that the P3-derived mRNA is the most abundant whereas P2- and P4-derived mRNAs were much lower in abundance. Interestingly, while P2- and P4-derived mRNAs were found in the polysome fractions only, the most abundant P3-derived mRNA was found predominantly in the untranslated fractions. Further investigation will be thus needed to identify the IGF-II promoters used in cancerous and benign epithelial cells by using specific probes for respective 5'UTR of IGF-II transcripts.

In summary, our data is not only consistent with the hypothesis that cancer cell growth may be regulated by IGF-II in an autocrine manner, but also supports the idea that IGF-II may be a potential target for cancer therapy.

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III. MATERIALS AND METHODS

A. Materials

1. Cells.

The human prostate cancer cell lines PC-3, LNCaP, DU145, were obtained from the American Type Culture Collection (Rockville,Md.), human neuroblastoma cell line, SK-N-AS was kindly provided by Dr. Carol Thiele, Pediatric Branch, NCI.

2. Reagents

Acrylamide, bis-acrylamide and Bradford-dye reagent , TEMED (N,N,N'N', -Tetramethylenediamine) and ammonium persulfate were purchased from Bio-Rad Laboratories (Richmond, CA). Bacto-yeast extract and Bacto-tryptone were obtained from DIFCO (Detroit, MI). Bovine Serum Albumin (BSA) were obtained from Calbiochem Corp. (La Jolla, CA). Fetal calf serum was purchased from Irvine Scientific. Inc. (Irvine, CA). G418 was purchased from GIBCO/BRL-life technologies Inc. (Gaithersburg, MD). Guanidinium isothiocyanate was obtained from Fluka Chemicals (Ronkonoma, NY). Klenow fragment of DNA Polymerase I was from Pharmacia (Piscataway, NJ) or Promega (Madison, WI). MEGAscriptTM kit were purchased from Ambion Inc. (Austin, Tx). Oligo primers were synthesized by DNA/Peptides core facility of City of Hope medical Center. (Duarte, CA). Proteinase K was purchased from Boeringer Mannheim. RNase- free DNase I was obtained from GIBCO/BRL-life Technologies Inc.(Gaithersburg,MD). RNA

transcription kits were purchased from Stratagene (San Diego, CA). RNA Trizol solution were purchased from GIBCO/BRL-life Technologies Inc. (Gaithersburg, MD). RPMI 1640 medium was obtained from GIBCO (Santa Clara, CA). Seaplaque agarose was purchased from FMC BioProducts (Rockland, ME). Superscript TM II Reverse Transcriptase was obtained from GIBCO/BRL Life Technologies Inc.(Gaithersburg, MD). T3 RNA polymerase, T7 RNA polymerase, T4 Polynucleotide Kinase, RNasin, restriction enzymes and buffers were from Promega (Madison, WI) and GIBCO/BRL life Technologies Inc. (Gaithersburg, MD). Trypsin-EDTA solution was purchased from Irvine Scientific. Inc. (Irvine, CA). Other molecular biology reagents were obtained from Sigma Chemical Company (St. Louis, MO), USB (Cleveland, OH) and Fisher Scientific (Tustin, CA). α-[^{32}P]-dCTP (3,000 Ci/mmol), ^{35}S-UTP (3,000 Ci/mmol) and γ-[^{32}P]-ATP (7,000 Ci/mmol) were purchased from ICN Biomedicals Inc. (Costa Mesa, CA). Vent DNA polymerase was from New England Biolabs Inc.(Beverly, MA).

3. Molecular Biology Solutions

Deionized formamide: Formamide was melted at room temperature and 50 ml was deionized with 5 g AG 501 x 8 resin (BioRad) by stiring for 30 min at room temperature. The solution was filtered twice with Whatman # 1 paper, aliquoted and stored at -20 °C.

Denhart's 50 x stock: 1% ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin. The solution was then filtered through a disposable 0.22 micro Nalgene filter.

DEPC water: 0.1% diethyl-pyrocarbonate was added to double-distilled water overnight. The treated water was then autoclaved.

Luria broth (LB): 0.1% Bacto-tryptone, 0.05% bacto-yeast extract, 0.1% NaCl, pH7.1.

Methylene blue solution: 0.02% methylene blue and 0.3 m sodium acetate, pH 5.5, stored in a dark bottle.

10 x MOPS: 0.2 M 3-(N-morpholino) propanesulfonic acid, 5 mm sodium acetate, 5 mM EDTA, pH 7.0.

Northern sample loading buffer: 720 µl deionized formamide, 320 µl 10 x MOPS, 260 µl formaldehyde solution (37% stock solution), 100 µl glycerol, 4 mg bromophenol blue, 4 mg xylene cyanol and 100µl DEPC water.

Pre-GIT solution: 4 M Guanidinium thiocyanate, 0.025 m sodium citrate and 0.5% n-lauryl sarcosine.

GIT solution: 360µl of 2-mercaptoethanol in 50 ml pre-GIT solution.

5 x first strand RT buffer: 0.25 M Tri-HCl, pH 8.3, 0.375 M KCl, 0.015 M MgCl2

10 x NEB Vent buffer: 0.1 m KCl, 0.2 M Tris-HCl, pH 8.8, 0.1 M (NH4)2 SO4, 0.02 m MgSO4, 1% Triton X-100.

20 x SSPE: 3.6 M NaCl, 0.03 m NaH2 PO4, 0.02 M EDTA, pH 7.4.

20 x SSC: 3.0 M NaCl and 3.0 M sodium citrate, pH 7.0.

50 x TAE: 2 M Tris-acetate and pH 8.0, 0.05 M EDTA.

10 x TBE: 0.4 M Tris-HCl, pH 8.0, 0.4 m boric acid and 0.012 M EDTA, pH 8.3

TBE loading buffer: 20% glycerol, 80% 10 x TBE, 0.4% bromophenol blue and 0.4% xylene cyanol.

TE buffer: 0.01 M NaOH, 0.01 M Tris-HCl, pH 8.0, 1 mM EDTA and 0.1% SDS

10 xTMDSE: 0.01 M Tris-HCl, pH 7.6, 0.1 M MgCl2, 0.01 M DTT, 1 mM spermidine and 1 mM EDTA

4. Miscellaneous Materials

MagnaGraph nitrocellulose membrane was purchased from Micro Separations Inc. (Westborough, MA). Nytran nylon membrane was purchased from Schleicher & Schuell Inc. (Keene, NH). ART tips were purchased from Molecular Bio-products Inc. (San Diego, CA). dNTP and NTP were purchased from Pharmacia (Piscataway, NJ). Chroma spin-10 column were purchased from Clontech Inc. (Palo Alto, CA). NucTrap push columns were purchased from Stratagene (San Diego, CA). WizardTM Miniprep DNA purification columns were obtained from promega (Madison, WI). Qiagen Plasmid maxi kits were purchased from Qiagen Inc. (Chatsworth, CA). Culture dishes and polypropylene tubes were obtained from Corning Glass Works (Corning, NY). Multi-well plates were from Costar

(Cambridge, MA). pBluescriptIISK(+) plasmid was purchased form Stragene (San Diego). pTZU6+27 was kindly provided by Dr. J. J. Rossi of City of Hope, Duarte, CA. Plasmid pcDNA3 was purchased from Invitrogen (San Diego, CA).

B. Methods

1. Plasmid Preparation

A single bacteria colony from an LB plate (1.5% agar in LB broth with appropriate antibiotic) was inoculated into 10 ml of LB broth containing ampicillin (100 μg/ml) and was grown overnight at 37°C with shaking. Overnight bacterial suspension (10 ml) was then added to 10 to 500 ml of fresh LB containing ampicillin and was incubated at 37°C overnight with vigorous shaking. The bacteria were pelleted by centrifugation at 5,000 x g for 15 min at 4°C in a 250 ml polypropylene bottle (100 to 500 ml) or 50 ml disposable tube (10 to 50 ml) in a Sorvall RC-2B centrifuge (Du Pont, Newtown, CT). Plasmid DNA was recovered using an alkaline, phenol extraction method or with Qiagen Kits.

(1) Alkali lysis preparation

Plasmid DNA preparation was prepared as described by Titus (1992). Briefly, bacteria was pelleted by centrifugation from 100 ml of the overnight culture, resuspended in 5 ml of TENS buffer by shaking until it became sticky. To this, 2.5 ml of 3.0 M sodium acetate, pH 5.2 was added and shaken until the lysate cleared. The cell lysate was centrifuged at 10,000 x g for 20 min at 4°C and the supernatant was treated with RNase A (10μg /ml) at 37°C for 10 min. To the supernatant, 10 ml

of 0.1 M Tris-HCl (pH 8.0)-saturated ethanol/chloroform/IAA (24:24:1) was added. The mixture was shaken, placed on ice for 5 min, and centrifuged as described above. The supernatant was removed and 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol (-20°C) was added to the upper aqueous phase. The solution was mixed and placed on crushed dry ice for at least 1 h to precipitate the DNA. Plasmid DNA was collected by centrifugation at 10,000 x g for 15 min at 4°C. The pellet was washed with 70% ethanol and resuspended in sterile deionized water. The yield was 2 μg/ml bacterial culture.

(2) Wizard mini-preparation

To check for the presence of the appropriate plasmid within a given colony, a small scale (<10 ml) plasmid DNA preparation was carried out using the Wizard mini-preparation kit according to manufacturer's protocol. Four ml of a saturated culture of bacteria were pelleted by centrifugation and resuspended in 200 μl of cell suspension solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA and 100μg/ml RNase A). 200 μl of cell lysis solution (0.2 M NaOH, 1% SDS) was added and mixed to lyse the cells. 200 μl of neutralization solution (2.55 M potassium acetate, pH 4.8) was added and mixed. The cell lysate was spun at 14,000x g for 5 min and the supernatant was collected. One ml of DNA purification resin was added to the supernatant and loaded onto a mini-column by applying a vaccum. The column was washed by the addition of 2 ml column wash solution (200 mM NaCl, 20 mM

Tris-HCl, pH 7.5, 5 mM EDTA) and the plasmid DNA was eluted with 50 µl of water preheated to 65°C. The yield was about 1-2 µg DNA/ml bacterial culture.

(3) Qiagen maxi-preparation

For large amounts of plasmid DNA (100µg), which was used in plasmid transfection experiments, the Qiagen plasmid preparation kit was employed according to the manufacturer's protocol. Bacterial cells were collected by centrifuging 500 ml of bacteria at 5,000 x g for 10 min at 4°C. The bacteria pellet was resuspended in 10 ml of P1 buffer (50mm Tris-HCl, pH 8.0, 10 mM EDTA and 100 µg/ml RNase A) and 10 ml of freshly prepared P2 buffer (200 mM NaOH and 1% SDS), mixed and the mixture was incubated at room temperature for 5 min. 10 ml of ice cold P3 buffer (3.0 M potassium acetate, pH 5.5) was added, mixed and incubated on ice for 20 min. The samples were centrifuged at 30,000 x g for 30 min at 4°C. The Qiagen-500 tip was equilibrated with QBT buffer (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% ethanol and 0.15% Triton X-100). The supernatant was loaded onto the Qiagen-tip and the tip was washed twice with 30 ml of QC buffer (1.0M NaCl, 50 mM MOPS, pH7.0, 15% ethanol). The plasmid DNA was eluted from the Qiagene column with 15 ml of QF buffer (1.25 M NaCl, 50 mM Tris-HCl, pH 8.5, and 15% ethanol) and precipitated with 0.7 volume of isopropanol. The plasmid DNA pellet was collected by centrifugation at 15,000 x g at 4°C for 30 min and washed twice with 70% ethanol. The pellet was air dried for 5 min and redissolved in distilled water. The yield was

about 1 μg/ml bacterial culture and the DNA was stored at 4°C for immediate use or stored at -85°C as aliquots for later use.

2. Sequencing

Sequencing was done with the commercial kit Sequenase 2.0 (USB) which employs the dideoxy- chain termination method (Sanger et al., 1977) and a modified bacteriophage T7 polymerase. The procedure used was as recommended by the manufacturer for sequencing double-stranded DNA templates. Double-stranded plasmid DNA was obtained using the mini-plasmid prep procedure described before. To denature the DNA for sequencing, 10 pmoles of plasmid DNA in 18 μl of water and 2 μl of freshly prepared 2 M NaOH were incubated for 5 minutes at room temperature. The DNA was precipitated with 8 μl of 5 M ammonium acetate pH 7.5 and 100 μl of cold absolute ethanol were added, mixed well and incubated at -20°C for 30 minutes. The tube was centrifuged for 30 minutes at 10,000 x g at 4°C and the supernatant was discarded. The pellet was rinsed with 500 μl of cold 80% ethanol by inversion and centrifuged for 1 minute. All traces of ethanol were removed and the pellet was stored at 4°C for up to one week. Denatured plasmid DNA (3 pmoles) in 6 μl of sterile water was annealed to 1 pmole of either the universal M13 primer or the T7 primer by adding 2 μl of reaction buffer and 2 μl of a 0.5 pmole/μl primer solution, incubating the mixture at 37°C for 20 minutes and at room temperature for an additional 15 minutes. During this incubation , 2.5 ml of each termination mix (ddNTPs) were aliquoted to labeled microfuge tubes and placed on ice. To the

annealing reaction tube, the following were added in order: 2 μl of diluted (1:5) labeling mix, 1μl DTT, 0.5 μl of [^{35}S] α-dATP (12.4 μCi/μl) and 2 μl of diluted Sequenase (1:8). The labeling reaction was incubated for 2 minutes at room temperature, and immediately 3.5 μl were transferred sequentially to each of the four prewarmed termination tubes. Incubation was carried out for 5 minutes at 37°C and 4 μl of stop solution were sequentially added to the four reaction tubes. The reaction mix was placed at -20°C until analyzed. The reaction products were analyzed on a denaturing TBE, 6M urea, 8% polyacrylamide gel. The gel was run for 2.5 to 4 hours at 50 constant watts. Gels were fixed in 1.5 L of 10% methanol, 10% acetic acid for 30 minutes and subsequently dried for 60 minutes at 80°C. Dried gels were exposed to Fuji-X-Ray film without a screen for 1-5 days at room temperature.

3. Oligonucleotide probe labeling and purification

The Oligonucleotide probes were end-labeled with T4 kinase by the transfer of γ-[^{32}P]-phosphate to the 5-hydroxy group of the oligonucleotide probe as described (Ausubel et al., 1994). The 10 μl labeling reaction mixture contained 50-100 ng of oligonucleotides, 1μl 10 x TMDSE, 200 μCi γ-[^{32}P]-ATP and 1 unit of T4 polynucleotide kinase. The reaction mixture was incubated at 37°C for 45 min and the labeled oligonucleotide was purified with a Chroma spin-10 column (Clontech inc., Palo Alto, CA). The Chroma spin-10 was first spun at 15,000 x g for 4 min and the labeled reaction mixture was applied into the column. The column was spun for 4 min.

4. RNA Extraction

Total RNA was extracted from cells using a single step acid guanidinium-thiocyanate phenol-chloroform method as described (Chomczynski et al., 1987) or a new and substantially improved version of the single-step method, RNAzol (Cinnai/Biotecx laboratories International Inc). For the Guanidinium-thiocyanate phenol-chloroform method, the culture medium was removed from each 100 mm^2 dish of cultured prostate PC-3 cells and 1 ml of GIT solution was distributed over each dish to lyse the cells. The cell extract was collected and stored at -80°C or used to isolate total RNA immediately. For RNA extraction, 0.1 volume of 2 M sodium acetate (pH 4.0), 1 volume of DEPC water-saturated phenol and 0.2 volume of DEPC water saturated-Chloroform/IAA (49:1) were added to the cell extracts sequentially with complete mixing. The extraction mix was vortexed vigorously and incubated on ice for 15 min and then centrifuged at 10,000 x g for 20 min at 4°C. The upper aqueous layer containing the total RNA was transferred to a new tube and RNA was precipitated by adding 1 volume of isopropanol and storing at -20°C overnight. The RNA was collected by centrifugation at 10,000 x g for 20 min at 4°C. The RNA pellet was redissolved in 500 µl of GIT solution and reprecipitated by adding an equal volume of isopropanol and storing the solution at -20°C overnight. The RNA was pelleted for 10 min at 14,000 x g at 4°C and washed twice (-20°C 80% ethanol). The RNA pellet was dried by heating at 65°C for 5 min and dissolved in DEPC water. The concentration and quality of the RNA was determined by reading the OD

at 260 nm and 280 nm, respectively. The isolated RNA was used for RT-PCR, and QC-PCR.

For the RNA Trizol method, briefly, culture medium was aspirated, then 1 ml RNA Trizol reagent was directly added into the flask, and cells homogenized with repetitive pipetting. The lysis solution was transferred to a fresh Eppendorf tube and stored for 5 min at room temperature, then 0.2 ml of chloroform was added and centrifuged at maximum speed (Eppendorf centrifuge 5415) at 4°C for 15 min. The aqueous phase was collected and mixed with 0.5 ml of isopropanol. The RNA was precipitated and dissolved in 10-15 μl of DEPC-treated water. The concentration of RNA was determined by absorption at 260nm on the spectrophotometer. The yield of total RNA is 3-7 $\mu g/10^6$ PC-3 cells.

5. Design, construction, and expression of IGF-II ribozymes

(1) Design of IGF-II ribozymes

The importance of hammerhead ribozymes is based on their ability to irreversibly inactivate their targets and to turn-over *in vitro* (Haseloff et al., 1988), hammerhead ribozymes work in cis (intramolecularly) in nature, and trans-hammerhead ribozymes (intermolecularly), also can site-specifically cleave substrate RNA. It consists of two parts, a catalytic core formed by 13 conserved nucleotides and a stem loop structure (stem II) (Fig. 4). Two variable sequences, which together form the specific sequence, located on each side of the catalytic core. The sequence allows the enzyme to hybridize to a complementary target sequence present in the substrate RNA,

Ribozyme/substrate hybrid restores the 2 stems (I and III), which is the hammerhead structure. After cleavage, the products can dissociate from the ribozyme, allowing turn-over (Jeffries et al., 1989), so the ribozyme can bind to another substrate molecule and cleave it as well. Hammerhead ribozymes were designed to target the human IGF-II mRNA. Cleavage sites are based on the finding that sequences near the translation initiation site are good targets for blockage of IGF-II expression (Christofori et al., 1994). The target RNAs for hammerhead ribozymes require the sequence NUX in which N is any nucleotide, and X can be A, C, U. The optimum length for ribozyme 2 arms (stem I and III) appears to be -14 bp by taking advantage of facilitation by the endogenous cellular protein hnRNP A1. The two hammerhead ribozyme sequences which we designed are follows: 1h and 2h (Fig. 14). Complementary to the sequence around the translation initiation site of human prepro IGF-II mRNA, nucleotide 16-30 and 16-46, respectively. The structure of single- and double- ribozymes are shown in Fig. 15. For the control ribozymes, ribozyme activity was lost in each ribozyme list above by introducing a point mutation of G to A (Fig. 15). These ribozymes were subcloned into PTZU6+27 (Fig. 16A), and pcDNA3 plasmids (Fig. 16B).

Single Ribozyme

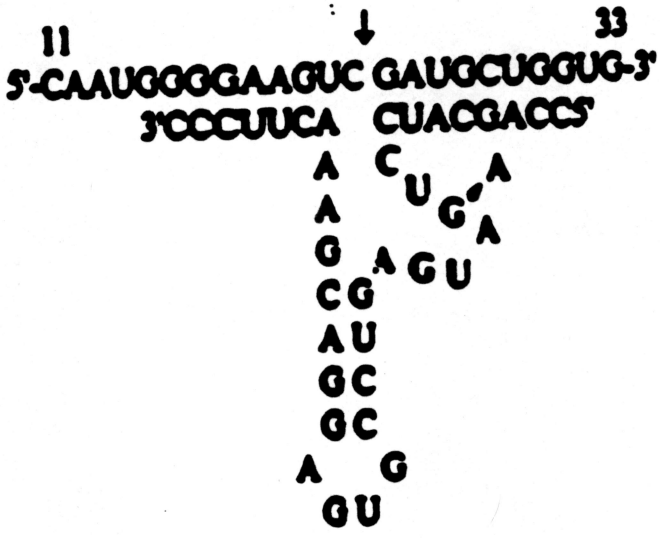
5' ACCAGCAUCCUGAUGAGUCCGUGAGGACGAAACUUCCCC 3'

Double Ribozyme

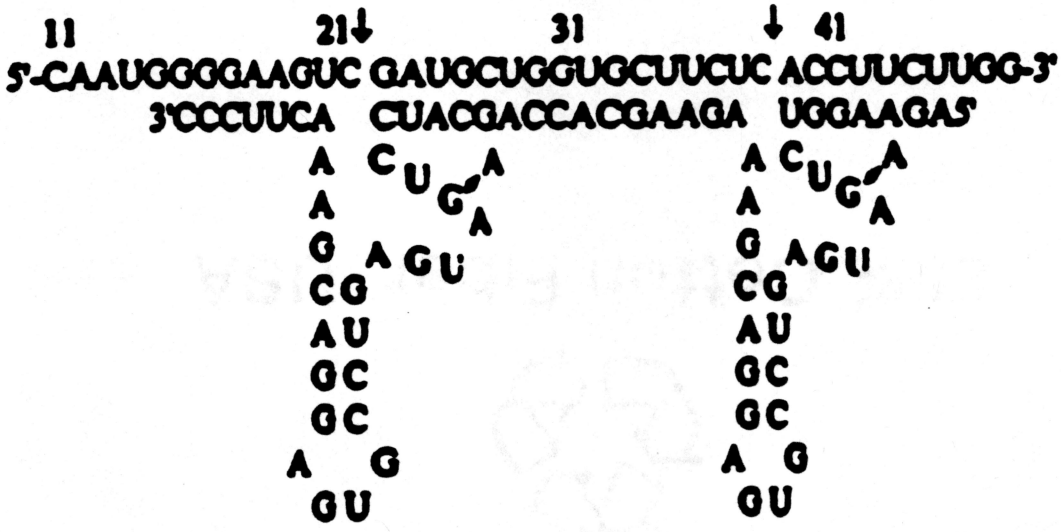
5'AGAAGGUCUGAUAGUCCGUGAGGACGAAAGAAGCACCAGC

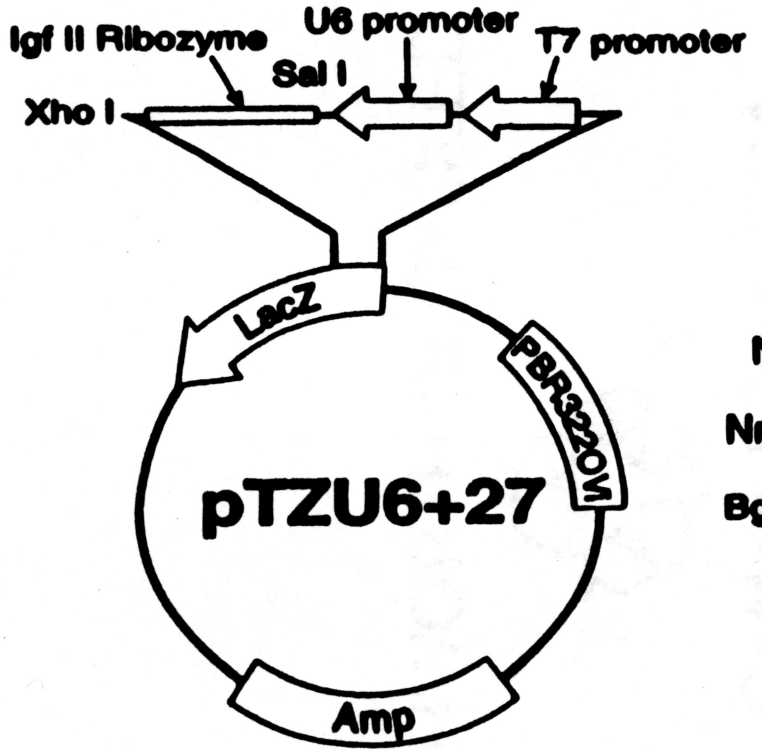
AUCCUGAUGAGUCCGUGAGGACGAAACUUCCC 3'

Single-ribozyme

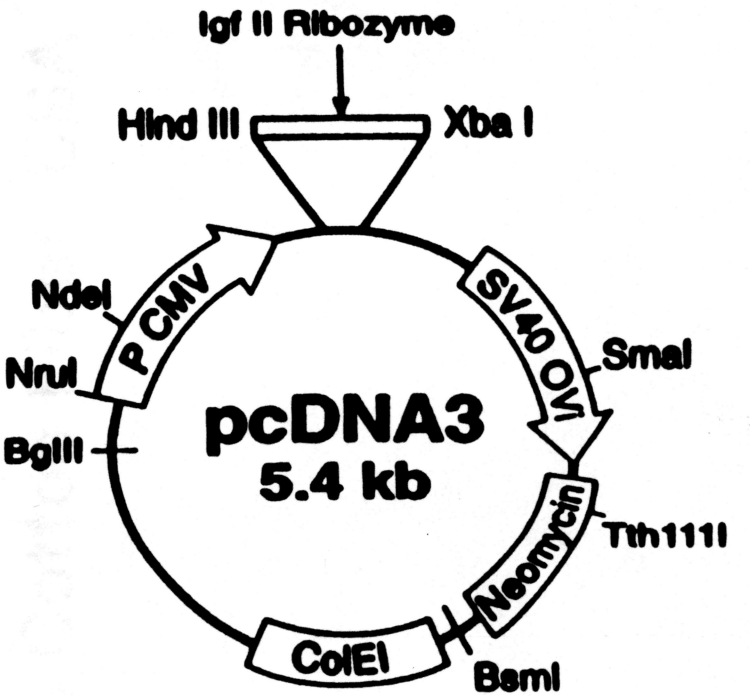


Double-ribozyme




Igf II Ribozyme
U6 promoter
T7 promoter
Sal I
Xho I
LacZ
PBR322ORI
pTZU6+27
Amp

(A)


Igf II Ribozyme
Hind III
Xba I
P CMV
NdeI
NruI
BglII
SV40 ORI
SmaI
pcDNA3
5.4 kb
Neomycin
Tth111I
ColEI
BamI

(B)

(2) Subcloning of a IGF-II substrate

A template for human IGF-II RNA (-6 to +74) was prepared by PCR from the full-length IGF-II cDNA (Bell et al., 1994) and subcloned into pBluescript. The primers for the PCR were: 1) 5'CGGAATTCCGACACCAATGGGAATCCC 3' and 2) 5'CGGGATCCCGGCAGGCAGCAATGCAGCACGA3', which contained restriction enzyme cutting sites for BamHI and EcoRI, respectively. The PCR was performed as followed: 95°C for 3 minutes, and then 30 cycle amplification at 95°C for 1 min (denaturing), 55°C for 1 min (annealing), and 72°C for 1 min (extension). After PCR, an aliquot of the PCR products was subjected to acrylamide gel electrophoresis to check the quality of PCR products. The PCR products were cleaned up for subcloning, and digested with BamHI and EcoRI. At the same time, a pBluescript KS plasmid was digested with BamHI and EcoRI. Restriction enzyme-digested PCR products and the plasmid were isolated by low melting agarose gel electrophoresis. The specific bands desired were cut out and incubated at 70°C for 5-15 min. For the ligation reaction, melted agarose containing insert and vector were mixed in ligation buffer with T4 ligase, and incubated at 15°C overnight. Transformation of the ligation mixture was achieved by electroporation. Briefly, 3 μl of the ligation mixture were electroporated into E. coli XL1 blue cells. After SOC medium was added, the bacteria were incubated at 37°C for 1 hour to express the antibiotic-gene, and plated on LB agar containing Ampicillin, X-gal, and IPTG at 37°C overnight. Plasmids were prepared from white colonies and their inserts

examined by BamHI/EcoRI double digestion and gel electrophoresis. Colonies containing the right size insert were selected. To confirm the authenticity of the IGF-II substrate sequence, plasmid DNAs were prepared and subjected to DNA sequencing using Maxim-Gilbert USB methods.

(3) Construction of IGF-II ribozymes

Template DNAs for ribozymes and mutant inactivated ribozymes were prepared by PCR. The primers for the single head ribozyme (equal to ribozyme-1h , mentioned above) were as follows:

1) 5' ACG CGT CGA CCA GCA TCC T(A/G)A TGA GTC CGT GAG 3', 34 bases,

2) 5' GCT CTA GAG CGG GGA AGT TTC GTC CTC ACG GAC TC 3', 35 bases, which contains restriction enzyme SalI and XbaI sites respectively. The primers used for the double head ribozymes (equal to ribozyme-2h) were: 1) 5' ACG CGT CGA CAG AAG GTC T(A/G)A TGA GTC CGT GAG GAC GAA AGA AGC ACC AGC AT 3', 53 bases, 2) 5'GCT CTA GAG CGG GAA GTT TCG TCC TCA CGG ACT CAT (C/T)AG GAT GCT GGT GTT 3', 52 bases, which contained restriction enzymes SalI and XbaI sites respectively. Note: in (/), each nucleotide was 50% incorporated in order to prepare both active and inactive ribozymes at the same time. The template DNA for the ribozymes were prepared by PCR. The condition of PCR for signal head ribozyme was 5 cycles of PCR, 94°C for 1 min, 37°C for 1.5 min, 72°C for 1.5 min. The condition of PCR for double head ribozyme was 5 cycles of PCR, 94°C for 1 min, 33°C for 1.5 min, and 72°C for 0.5 min. After the PCR,

the quality of PCR products was examined by polyacrylamide gel electrophoresis. The PCR products were cleaned up and digested with SalI and XbaI. At the same time, a pTZU6+27 vector was digested with SalI and XbaI (pTZU6+27 contains the human U6 promoter and pUC19 multiple cloning site. The plasmid containing the ribozyme can be used for both in vitro transcription of ribozymes as well as expression of the ribozymes in mammalian cells which use the Pol III RNA polymerase). After digestion, a ligation reaction was carried out, and the ligation products were electroporated into E. coli XL-1 blue cells, the bacteria were plated on the LB agar which contains ampicillin, X-gal and IPTG. Plasmids were prepared from white colonies and their inserts examined by SalI/XbaI digestion and gel electrophoresis. Colonies containing the right size inserts were selected. To confirm authenticity of the ribozymes, plasmid DNAs were prepared and subject to DNA sequencing. Single ribozyme (R) and its mutant (M), and double ribozyme (RR), its mutant (MM), and double ribozymes with one mutant (RM) and (MR) had been isolated. Similarly, a pcDNA3 vector which has a CMV promoter was used as the vector for single ribozyme and mutant ribozyme.

(4) Preparation of IGF-II mRNA substrate by in vitro transcription

The plasmid which contains IGF-II substrate was linearied by BamHI digestion and used as a template for *in vitro* transcription. Transcription reaction was carried at 37°C for 1 h in 40mM Tris-HCI buffer, pH 7.9 containing-0.2 μg DNA template, 0.5 unit/μl of T3 RNA polymerase, 20 mM $MgCl_2$, 10mM NaCl, 10mM DTT,

0.5 mM each of ATP, GTP, and UTP, 0.05mM CTP, 10μCi of [α-^{32}P] CTP and 1 unit/μl of RNase. After transcription, the RNAs were treated with RNase-free DNase I for 15 min, followed by gel purification in 6% denaturing polyacrylamide gel for 1 hr at 200V. Before gel purification, a small aliquot of RNAs was taken out for calculating the specific activity. After gel electrophoresis, the gel was exposed to a Kodak XRP film for 1 min and the film was developed. The appropriate region of the gel containing the desired RNA band was cut out and crushed until it became fine pieces. The IGF-II substrate RNA was eluted in elution buffer overnight. The aqueous phase was removed and mixed with phenol:chloroform:isoamyl alcohol to extract the RNA. The substrate RNA was precipitated with ethanol, redissolved in 20 μl DEPC H_2O and stored at -70°C.

(5) In vitro transcription of IGF-II ribozymes

The plasmids containing ribozyme DNAs were linerarized by XbaI digestion and used as templates for transcription of IGF-II ribozymes as described for preparation of the substrate RNA, except that a trace amount of the radioisotope was used, and also that T7 RNA polymerase was used instead of T3 RNA polymerase. Transcription reaction was carried at 37°C for 1 h in 40mM Tris-HCI buffer, pH 7.9 containing -0.2 μg DNA template, 0.5 unit/μl of T7 RNA polymerase, 20 mM $MgCl_2$, 10mM NaCl, 10mM DTT, 0.5 mM each of ATP, GTP, and UTP, 0.05mM CTP, 10μCi of [α-^{32}P] CTP and 1 unit/μl of RNase. After transcription, the RNAs were treated with RNase-free DNase I for 15 min, followed by gel purification in 6% denaturing

polyacrylamide gel for 1 hr at 200V. Before gel purification, a small aliquot of RNAs was taken out for calculating the specific activity. After gel electrophoresis, the gel was exposed to a Kodak XRP film for 1 min and the film was developed. The appropriate region of the gel containing the desired RNA band was cut out and crushed until it became fine pieces. The ribozyme RNA was eluted in elution buffer overnight. The aqueous phase was removed and mixed with phenol:chloroform:isoamyl alcohol (25:24:1) to extract the RNA. The substrate RNA was precipitated with ethanol, redissolved in 20 μl DEPC H_2O and stored at -70°C.

(6) Ribozyme cleavage assays

Ribozyme assays were performed as previously described (Bertrand et al., 1994; Hertel et al., 1994). Briefly, ribozymes and substrate were heated independently for 1 min at 90°C in water. After cooling to 25°C, the reaction buffer was added to a final concentration of 10mM $MgCl_2$, 140 mM KCl, and 50 mM Tris-HCl, pH 7.5. Ribozymes and substrate were then combined and incubated at 37°C. The reaction was stopped by adding an equal volume of stop solution (0.5% of SDS/25mM EDTA) and then with 100 μl of phenol. The aqueous phase was brought to 100μl, extracted, and precipitated with ethanol. The RNAs were analyzed by electrophoresis in 6% polyacrylamide/urea gels. Radioactive bands were visualized by autoradiography and quantitated by a Phosphor Imager (Molecular Dynamics). Ribozyme/substrate ratios and time course were determined.

6. Kinetic analysis of single and double IGF-II ribozymes

The single turnover experiments with ribozyme in excess over substrate were used to determine the first-order rate constant for cleavage of the substrate (Hertel et al., 1995). The initial cleavage velocities under single turnover conditions was determined at a concentration of 1 nM substrate and several different ribozyme concentrations over a 2 hour reaction time following initiation of the reaction. The rate constants for the reaction (Kcat/Km) were determined from the slope of semilogarithmic plots of the fraction of the ^{32}P-labeled substrate verus time according to the equation log[A] = (-k/2.303)t.

The methods for the kinetic study were similar to the ribozyme cleavage reaction as described above. First, both ribozymes and substrate were separately transcribed by using T7 RNA polymerase and T3 RNA polymerase, respectively, in the presence of α-^{32}P CTP according to the methods described in the ribozyme cleavage reaction. The transcripts were purified on a 6% polyacrylamide/8M urea gel. The ^{32}P-labeled transcripts were detected by autoradiography. The transcripts were eluted from gel areas showing the sizes expected, by extraction buffer. The RNAs were precipitated at -20°C overnight or at -70°C for 20 min by adding 1µl of 5 mg/ml tRNA and 2.5 vol. ethanol. The RNAs were recovered by centrifugation in a microcentrifuge for 30 min at 4°C. Each RNA pellet was washed twice with 70% ethanol, 2 mM EDTA to remove traces of SDS, which would otherwise inhibit the ribozyme reaction. The RNA pellets were dried in a speed-vac concentrator, the yields were quantified by

Cerenkov counting. The final concentrations were adjusted by dissolving the RNA pellets in the appropriate volumes of TE buffer. Different combinations of the substrate and ribozymes were heated at 90°C for 1 min, and then cooled to 25°C for 5 min. The cleavage buffer was added and incubated at 37°C for 1 hr. The ribozyme reaction was stopped by adding 5 μl of formamide-dye mixture, and the products of the ribozyme reaction were separated on a 6% polyacrylamide/8M urea gel. The RNA products were quantified using phosphor imager.

7. Expression of IGF-II ribozymes in cancer cells

The purpose of this aim was to examine whether the IGF-II ribozymes which were active in vitro can work in cancer cells (*in vivo*). Unlike *in vitro* cleavage reactions, when ribozymes are introduced into cells, additional factors which interfere the ribozyme action may exist. These included i) co-localization of ribozymes with the target RNAs, ii) ribozyme stability, iii) ribozyme expression level, iv) secondary or tertiary structure of endogenous target RNA or ribozyme, as well as v) RNA-binding proteins.

(1) Selection of cell lines

Ribozyme activity was evaluated in cells that express IGF-II mRNA such as human prostate cancer cell line PC-3 and human neuroblastoma SK-N-AS cell line. SK-N-AS cells overexpressed IGF-II which is an autocrine growth factor for SK-N-AS cells (El-Badry et al., 1989). PC-3 cells, epithelial cells derived from a human prostate adenocarcinoma whose growth was androgen-independent (Kaighn et al.,

1979), were shown to be capable of slow proliferation in serum-free medium and in the absence of added growth factor for 7 days. These cells secrete insulin-like growth factor-II (IGF-II) but no detectable IGF-I. IGF-II, produced by these cells plays a role in proliferation because cell growth could be inhibited dose dependently by up to 80% in the presence of monoclonal antibodies directed against IGFs or the type 1 IGF receptor (Angelloz-Nicoud et al., 1995; Jungwirth et al., 1997).

(2) Determination of optimum concentrations of G418 for selection of transformants

Since the amount of G418 required for obliteration of parental cells was likely to be different among different cell types, the optimal concentration of G418 for selection of transformed cells must be determined for each cell type. One ml of single cell suspension at a concentration of 3.0×10^5 cells/ml was plated into two 6-well tissue-culture plates containing 3 ml RPMI 1640 medium with 10% FCS and incubated at 37°C overnight. A series of two-fold dilutions of G418 from 75 μg/ml to 1.2 mg/ml in the medium were prepared. The medium was aspirated from the wells and 4 ml of each dilution of G418-containing medium were placed in duplicate. The medium without G418 was added to one set of wells. Plates were incubated at 37°C for about 10 days until colonies formed. When colonies had grown to approximately 1-3 mm in diameter, the medium was aspirated, and the wells were rinsed with 2 ml of PBS. Colonies were incubated with 1 ml of 0.5% methylene blue for 20 min, rinsed with water to remove excess dye, and air-dried. The lowest G418

concentration resulting in 100% obliteration of cells was determined. For the PC-3 cells, the concentration was determined to be approximately 0.35 mg/ml. For the SK-N-AS cells, the concentration was determined to be approximately 0.7 mg/ml.

(3) Cell culture and transfections

For pTZU6+27/Rz (Pol III promoter) and or pcDNA/Rz (Pol II promoter) expression vectors, cells were transfected by calcium-phosphate precipitate method or Electroporation method, respectively. The former vector was cotransfected with neo vector whereas the later vector which contained neo gene was transfected by itself.

a) Calcium Phosphate

The method for calcium-phosphate precipitate was as follows (Sambrook et al., 1980). Briefly, 24 hours before transfection, exponentially growing cells were harvested by trypsinization, and replated into 90-mm tissue culture dishes. Ten ml of RPMI 1640 medium with 10% FCS were added, and cells were incubated overnight at 37°C in a humidified incubator in an atmosphere 0f 5-7% CO_2. To those cells, were added 0.5 ml of 0.25 M $CaCl_2$ containing 18 μg of superhelical plasmid ribozyme DNA and 2 μg neo Vector DNA, and 0.5 ml of 2x BES-buffered saline. The cells were incubated for 10-20 minutes at room temperature. RPMI 1640 medium with 10% FCS were added dropwisely to the cells in the dishes, the dishes were swirled gently, and were incubated for 15-24 h at 37°C in a humidified incubator in an atmosphere of 3% CO_2. The calcium phosphate-DNA complex formed slowly in the medium under conditions of low pH and precipitateed gradually

onto the cells during the incubation in an atmosphere containing low concentrations of CO_2. The medium was removed by aspiration, and cells were rinsed twice with medium. Ten ml of fresh medium were added, and cells were incubated for 24 h at 37°C in a humidified incubator in an atmosphere of 5% CO_2. Following 18-24 h incubation in nonselective medium to allow expression of the transferred genes to occur, the cells were trypsinized and replated in medium containing the appropriate concentration of G418. The medium was changed every 2-4 days for 2-3 weeks to remove the debris of dead cells and to allow colonies of G418-resistant cells to grow. Individual G418-resistant colonies were picked up, grown, and screened for expression of the ribozymes. The ribozyme-expressing cells were analyzed for IGF-II mRNA and protein levels, cell growth, morphological changes, etc.

b) Electroporation

Cells were grown to 70% confluence, trypsinized in the minimum time possible (1-3 minutes) centrifuged in the table-top centrifuge, and resuspended in HEBS buffer at a cell density of 1-10 x 10^7 cells per ml. This suspension was preincubated with 5 to 20 μg of DNA on ice for 10 minutes with mixing, transferred to cuvette and immediately pulsed with a range of settings. A 2 mm gap chamber was used in a BTX electroporator (San Diego, CA). The settings were first adjusted to include parameters used to successfully transfect SK neuroblastoma cells. These were T= 500 V, Capacitance=800-900 μF, Resistance=R4 (72 ohm), Charging voltage= 350 Volts, Electric Field Strength (EFS)=1.0 kV/cm, and designated pulsed length

=0.2-10 mSec. The sample volume was 400 μl in all cases. After pulsing, the cells were left at room temperature for 10 minutes, transferred back into the petri dish, and selected using G418 for at least 1 month.

(4) Expression of the hammerhead ribozyme in mammalian cells

To detect ribozyme expression in transfected G418-resistant clones, RT-PCR was used (Funato et al., 1994). For pTZU6+27 clones, a set of primers for detection of ribozyme was prepared. One primer (Rz5') contains a sequence corresponding to the junction between U6 promoter and the ribozyme. The other primer (Rz3') contains a transcription termination sequence. The sequences of them are as follows: 5'primer, 5' TCG CTT CGG CAG CAC GTC GAC, 3'primer, 5'GGG AAG TTT CGT CCT CAC GGA. For pcDNA3 clones, a set of primers are as follows: 5'primer, 5'CCC ACT GCT TAC TGG CTT ATC GA, 3'primer, GGA CAG TGG GAG TGG CAC CTT C. RT reaction products, equivalent to 200 ng of total RNA from each clone, were processed through 30 cycles of PCR with denaturation at 94°C for 1 min, annealing at 47°C for 2 min, synthesis at 72°C for 3 min, in a final volume of 20 μl for pTZU6+27 clones and through 30 cycles of PCR with denaturation at 94°C for 30 second, annealing at 67°C for 1 min, synthesis at 72°C for 1 min, in a final volume of 20 μl for pCDNA3 clones. Half of the reaction product was analyzed in a 2% agarose gel and blotted to a nylon membrane. Blots were hybridized to a specific ^{32}P-labeled oligonucleotide probe (ribozyme catalytic domain). The sequence of the

probe was as follows, 5'ACC AGC ATC CTG ATG AGT. The ribozymes were visualized by autoradiography and quantified by Phosphor Imager.

8. Effect of IGF-II ribozyme expression on cancer cell lines

(1) Quantitation of Ribozyme target IGF-II abundance by QC-PCR

A competitive PCR was developed for quantitative analysis of ribozyme IGF-II RNA, this method showed very good specificity and sensitivity, allowing quantitation in a large linear range. (Payan et al., 1997; Revillion et al., 1997; Fasco, 1997; Lewohl et al., 1997).

a) Construction of QC-RTPCR internal control (competitor) plasmids

pBluescript KS/IGFII, pBluescript KS(+) (Stratagene, San Diego) containing approximately 1 kb EcoRI fragment encoding the human precusor IGF-II (911 to 2067 nt., Dull et al., 1984), was used to generate a new plasmid which encodes a competitor IGF-II sequence. Construction of the plasmid encoding the competitor IGF-II is schematically presented in Fig. 20. pBluescript KS/IGFII was digested by EcoRI and the 1.0 kb EcoRI fragment was ligated into the EcoRI-digested pBluescript SK vector. *E. coli* was transformed with the ligated plasmids and clones which contained the insert in the opposite direction to the original IGF-II clone were selected by restriction enzyme analysis using XhoI and a combination of XhoI and PvuII. Since the 1.0 kb EcoRI/EcoRI insert as well as multi-cloning sites contained unique SalI and XhoI sites, the pBluescript SK/opIGFII plasmid was digested by XhoI, from which the larger XhoI/XhoI fragment was separated from the small

XhoI/XhoI fragment. Self ligation of the larger XhoI fragment resulted in pBluescriptSK/IGFIIS encoding the shortened IGF-II sequence which had a unique SalI site. This was confirmed by restriction enzyme analysis. While pBluescript SK/IGFIIS yielded one linear band with SalI digestion and three bands of 1506, 683, 353 bp with PvuII digestion, XhoI and SalI digestion resulted in only a 174 bp band. This plasmid was digested by SalI, to which a 110bp SalI-SalI fragment was inserted. The new plasmid, pBluescript SK/IGFIIS/C, showed restriction enzyme maps of 1506, 683, and 463 bp whereas the original pBluescriptSK/IGFIIS showed restriction enzyme maps of 1506, 683, and 353 bp with PvuII digestion.

b) Synthesis of QC-RTPCR internal control (competitor) RNA

The pBluescript SK/IGFIIS/C was linearized by XhoI digestion, and used as the templates for *in vitro* transcription. Transcription reaction was carried out at 37°C for 1 h in 40 mM Tris-HCl buffer, pH 7.9, containing ~0.2 μg DNA template, 0.5 unit/μl of T3 RNA polymerase, 20 mM $MgCl_2$, 10 mM NaCl, 10 mM DTT, 0.5 mM each of ATP, GTP, UTP, CTP and 1 unit/μl of RNasin. After transcription, the RNAs were treated with RNase-free DNase I for 15 min. The RNA mix then was stopped by adding 115 μl of RNase-free dH_2O and 15 μl of Ammonium Acetate Stop Solution. The reaction mix was then extracted with an equal volume of water- or buffer-saturated phenol/chloroform, and once with an equal volume of chloroform, the RNA was precipitated by adding 1 volume of isopropyl alcohol at -20°C for at least 15 min,

centrifuged for 15 min at maximum speed, the aqueous phase was removed and the RNA was resuspended in RNase-free dH_2O and stored at -70°C.

c) Quantitative analysis of control (competitor) RNA

A series of RT-PCR were performed at 47°C for 30 min by using 50ng, 5ng, 0.5ng, 0.05ng, 0.005ng control (competitor) RNA and 10 pmol of the 3' primer, IGFII3', the sequence of it is as follows, 5' GTA TCT GGG GAA GTT GTC CGG AAG CAC GGT C, in 50 mM Tris-HCl, pH 7.5, containing 10 mM DTT, 75 mM KCl, 3 mM $MgCl_2$, 1 mM of each deoxynucleoside triphosphate (dNTP), 800 u of superscript II reverse transcriptase (RT), and 20 u of human placental ribonuclease inhibitor. To inactivate the RT, the reaction mixture was incubated at 65°C for 10 min. One quarter of the cDNA obtained from the RT reaction was incubated in presence of 2 u of Taq polymerase in 0.25 mM of each dNTPs, 10 pmol of 5' end ^{32}P γ-ATP labeled primers, IGFII 5' primer, the sequence of it is as follows, 5'CCA GCA CCA ATG GGA ATC CCA ATG GGG AAG, and 10 pmol 3' end primers, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, and 0.1% Triton-X-100. Reaction mixtures were overlaid with mineral oil and placed in a DNA thermal cycler for 25 cycles (94°C for 1 minute, 68°C for 30 Seconds, 72°C for 1 minutes). The PCR-amplified products were analyzed by electrophoresis in a 3.5% acrylamide gel in TBE Buffer. After the electrophoresis, the gel was transferred to a Whatman membrane, then the membrane was dried and exposed to film. The PCR products were

visualized by autoradiography and quantitated using a Phosphor Imager. An internal control titration curve was plotted as total RNA amount vs band intensity.

d) Analysis of control (competitor) RNA vs PCR cycles

RT-PCR was performed by using 0.5 ng internal control competitor RNA as described as above under 16, 20, 24, 28, 32 cycles. A 10 μl reaction product was removed from the reaction and analyzed by gel electrophoresis, and the PCR products were visualized by autoradiography and quantitated using a Phosphor Imager. The cycle curve was plotted as cycle numbers vs band intensity.

e) Quantitation of IGF-II abundance in ribozyme expressing clones

RT-PCR was performed at 47°C for 30 min by mixing 4 μg of total RNA from the transformants (total RNA amount was modified by ribosomal RNA) with a certain amount of internal control competitor RNA and 10 pmol of the 3' primer (same as above), in 50 mM Tris-HCl, pH 7.5, containing 10 mM DTT, 75 mM KCl, 3 mM $MgCl_2$, 1 mM of each deoxynucleoside triphosphate (dNTP), 800 u of superscript II reverse transcriptase (RT), and 20 u of human placental ribonuclease inhibitor. To inactivate the RT, the reaction mixture is incubated at 65°C for 10 min. One quarter of the cDNA obtained from the RT reaction was incubated in presence of 2 u of Taq polymerase in 0.25 mM of each dNTPs, 10 pmol of 5' end ^{32}P γ-ATP labeled primers and 10 pmol 3' end primers, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, and 0.1% Triton-X-100. Reaction mixtures were overlaid with mineral oil and placed in a DNA thermal cycler for 25 cycles (94°C for 1 minute, 68°C for 30 Seconds, 72°C

for 1 minutes). The PCR-amplified products were analyzed by electrophoresis in a 3.5% acrylamide gel in TBE Buffer. After electrophoresis, the gel was transfered to a whatman membrane, then the membrane was dried and exposed to film. The PCR products were visualized by autoradiography and quantitated by using a Phosphor Imager.

(2) Measurements of IGF-II protein level in PC-3 transfectants

The level of IGF-II protein secreted into conditioned medium and in cells were analyzed by RIA assay using IGF-II mAb. For each clone, a duplicate of 10^5 cells were seeded in T-25 flask and incubated at 37°C overnight. The next day, The medium was aspirated and the cells were washed three times using 1 x PBS, then the serum free medium was added and the cells were incubated for 2 more days without changing the medium. The medium were collected and analyzed by RIA assay using IGF-II mAb (Mohan et al., 1990). This part of study was done in collaboration with Dr. Mohan's group, Loma Linda University VA Hospital.

(3) Effect of ribozyme expression on PC-3 cell growth

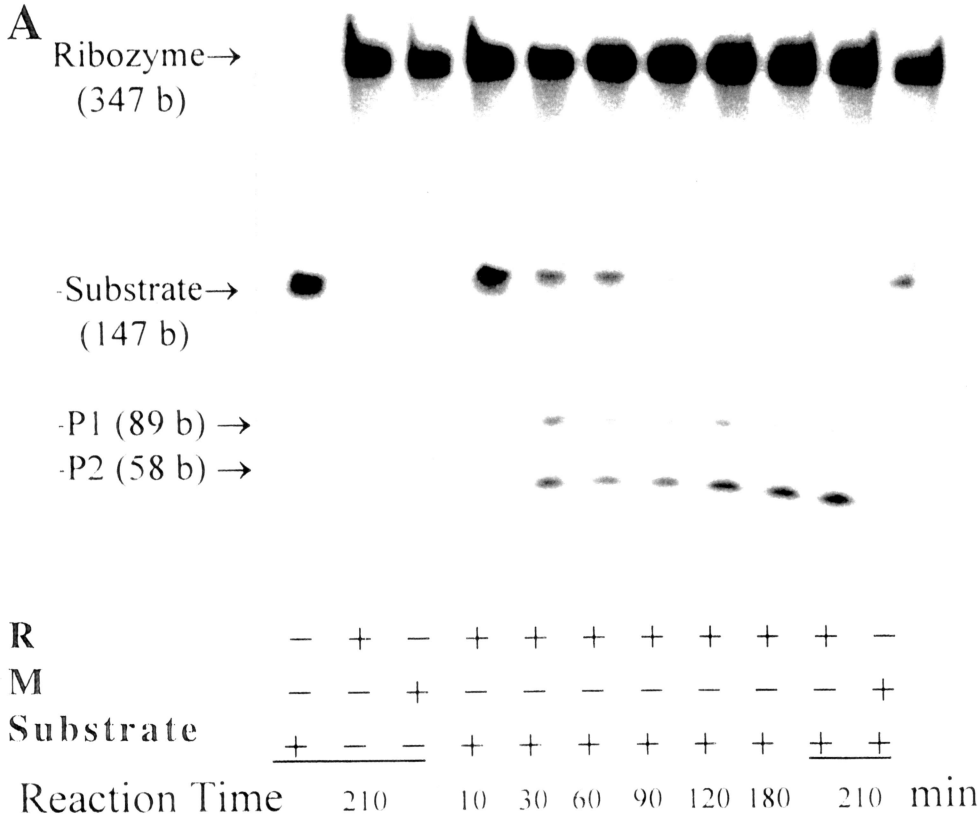
a) MTT Assay

Growth curve was done by MTT Assay, the cleavage and conversion of the soluble yellow dye to the insoluble purple formazan has been used to develop an assay system alternative to the conventional ^{3}H-thymidine uptake and other assays for measurement of cell proliferation. Active mitochondrial dehydrogenated from living cells will cause this conversion. Dead cells do not cause this change, this has been

used measuring cell cytotoxicity and cell number. PC-3 parental cells, vector clone cells, and ribozyme clone cells were seeded in 96 wells at $1x10^4$ in triplicate and cultured for 4 days. Each day, 20 μl MTT(5mg/ml) was added to a set of cells, and the cells were continued to be incubated at 37°C for 4 hours, and the medium was then removed and replaced with 100 μl isopropyl alcohol supplemented with 0.05 N HCL. The samples were read at 540nm. The growth curve was plotted as OD vs time.

b) Time-lapse microscopy

Cell division of PC-3 cells, vector-transfected cells, and R-expressing PC-3 cells was measured revealing doubling time. Approximately 10^5 Cells of each clone was seeded in a T-25 flask and incubated at 37°C overnight, and then placed in a special incubator under microscopy. A good microscopy view of isolated cells was selected and the cells were continued to incubated for at least four days, cells' doubling time was recorded. For each clone, at least eight individual cells' doubling time was observed and the average was taken as the doubling time for this clone.



-6 IGF-II RNA 76

Ribozyme	Substrate ~~~~~~~~~~ ↑	Products (bases)
R		89 + 58
M		147

RNA substrate was cleaved into two fragments of 213 bases and 944 bases by single ribozyme (R), while double ribozyme (RR) cleaved the substrate into 3 fragments of 213 bases, 928 bases and a small fragment of 16 bases (which was not seen on this 3% acrylamide gel) after a 16-hr digestion. The time course experiments showed that double- ribozyme (RR) cleaved the human prepro IGF-II RNA in a time-dependent manner, while the single ribozyme (R) showed a less cleavage activity than RR (Fig. 18).

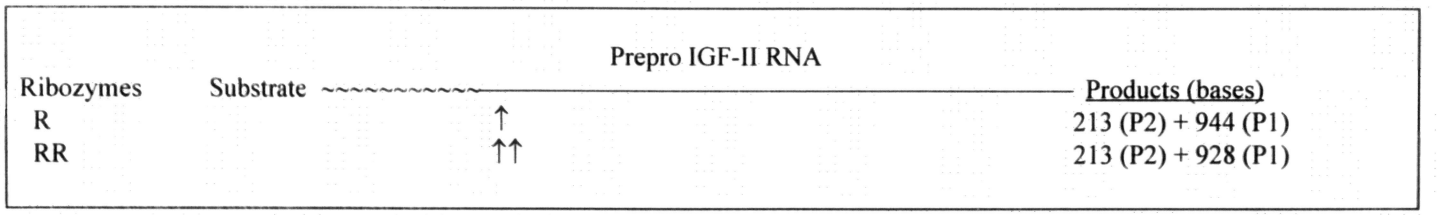
2. Kinetic analysis of single and double IGF-II ribozymes

The single turnover experiments with ribozyme in excess over substrate were used to determine the first-order rate constant for cleavage of the substrate (Hertel et al., 1995). The kinetic analysis of both single R and double RR ribozymes is shown in Fig. 19 and summarized in Table 4; Kcat/Km for single ribozyme R was 1546 $M^{-1}S^{-1}$, while Kcat/Km for double ribozyme RR was 4772 M^{-1} S^{-1}. The results clearly show that the double ribozyme is more efficient than the single ribozyme in *in vitro* experiments.

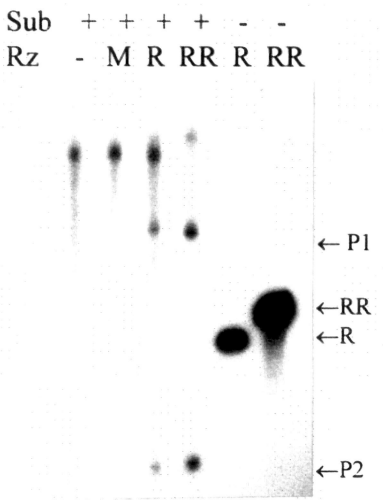
B. Quantitation of IGF-II mRNA by QC-RTPCR

1. Synthesis of internal control IGF-II

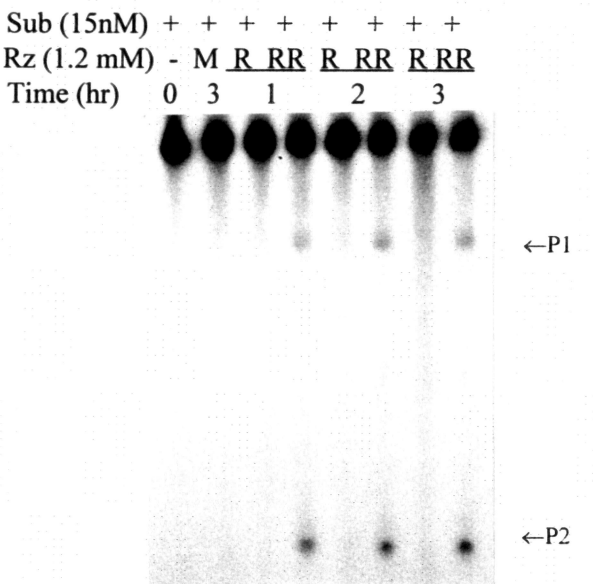
Internal control (competitor) plasmid was constructed according to the method described above. Construction of the plasmid is schematically presented in Fig. 20. Internal control (competitor) RNA was synthesized using T3 RNA polymerase.



A. 16-hr digestion (R & RR)



B. Time-course (R & RR)



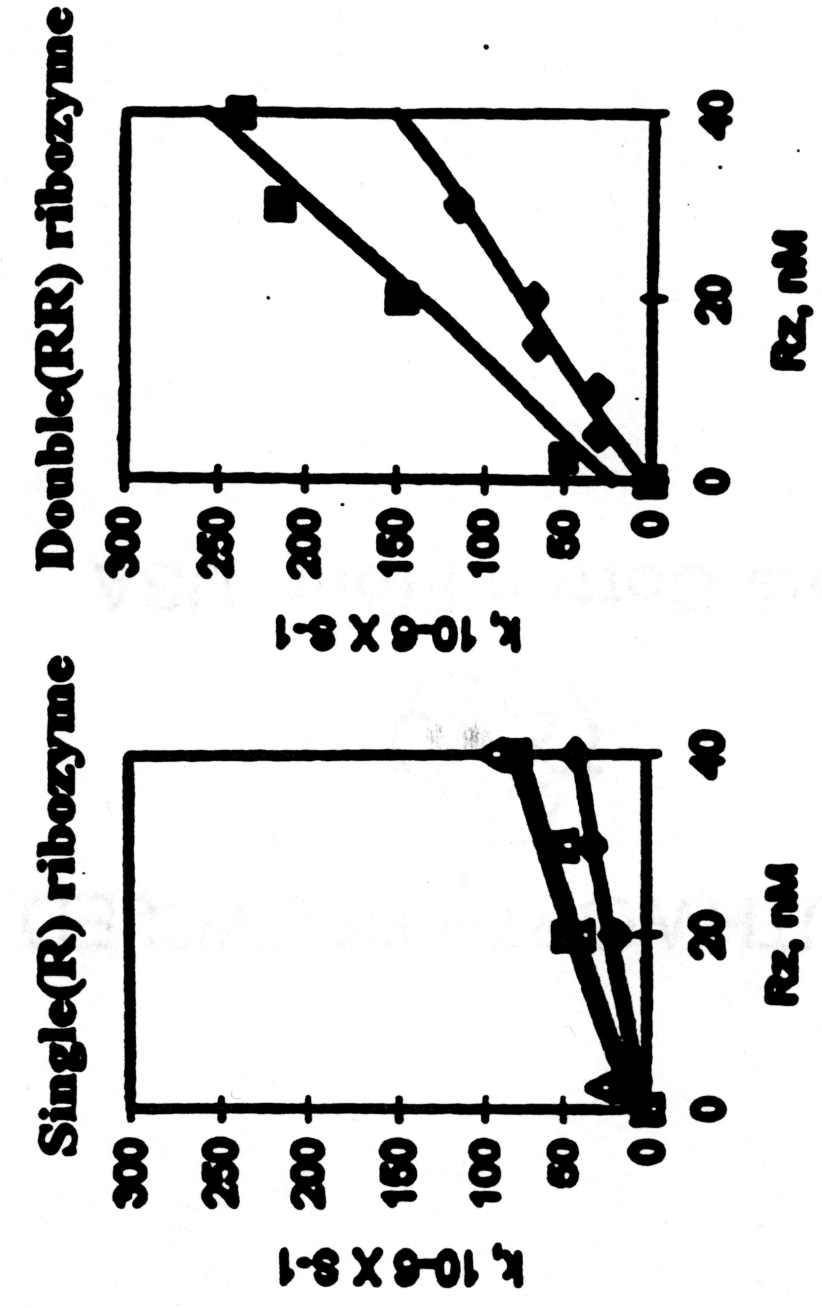
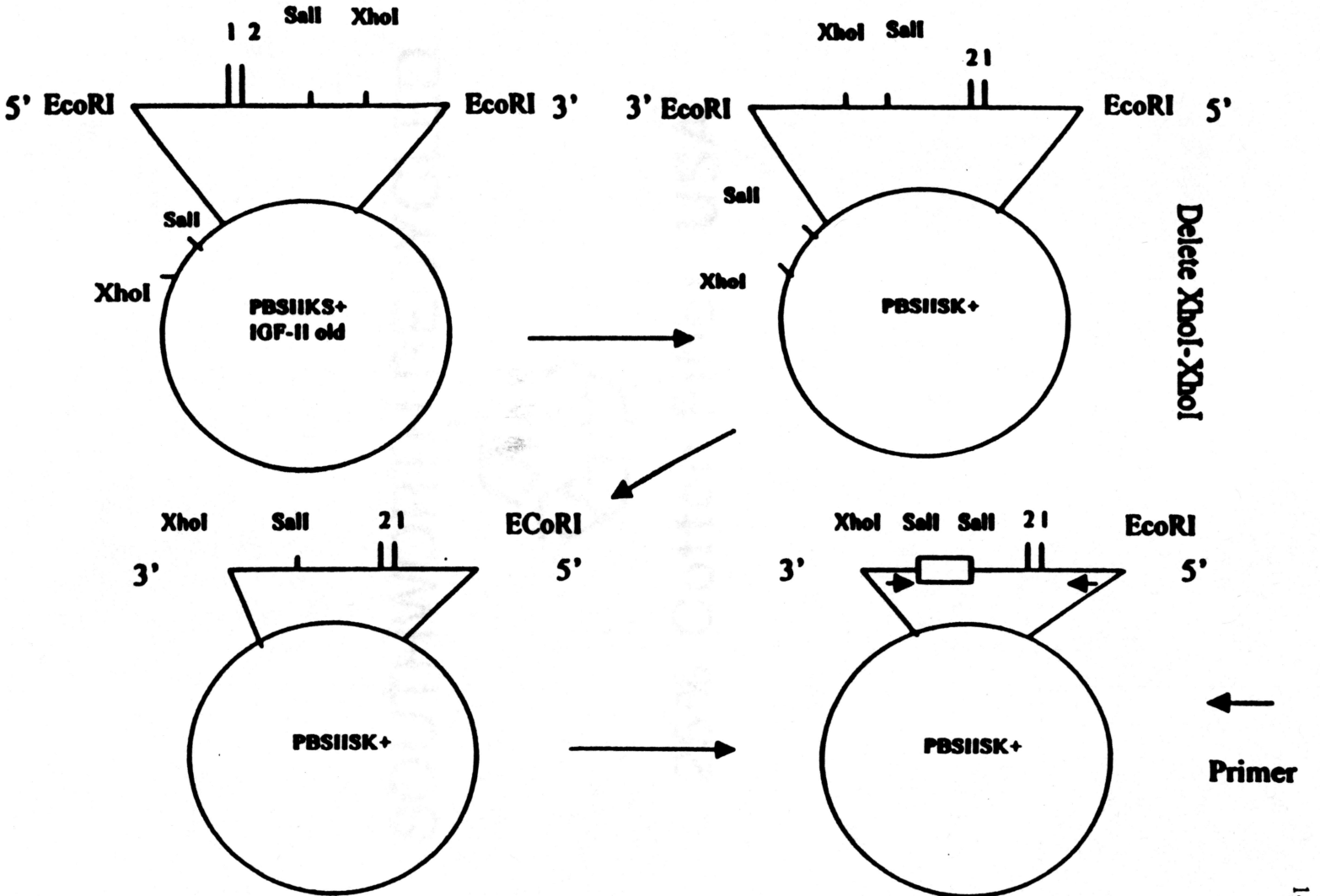

Double(RR) ribozyme
Single(R) ribozyme
k, 10-6 X S-1
300
250
200
150
100
50
0
0
20
40
Rz, nM

Table 4. Kinetics of single- and double-ribozymes.

	Kcat/Km, M^{-1} S^{-1}	
	R	RR
Experiment 1	1870	5840
Experiment 2	965	3704
Experiment 3	1803	
Average	1546 ± 411	4772


1 2
SalI XhoI
5' EcoRI
EcoRI 3'
SalI
XhoI
PBSIIKS+
IGF-II old
XhoI SalI
2 1
3' EcoRI
EcoRI 5'
SalI
XhoI
PBSIISK+
Delete XhoI-XhoI
XhoI SalI 2 1
3'
ECoRI
5'
PBSIISK+
XhoI SalI SalI 2 1
EcoRI
3'
5'
PBSIISK+
Primer

2. Quantitative IGF-II mRNA analysis

A serial dilution of the internal control RNA was reverse-transcribed, and then PCR product was amplified using primers specific to IGF-II (Fig. 20). The internal control titration curve as shown in Fig. 21A revealed good linearity within this range of internal control RNA. A 0.5 ng internal control (competitor) RNA was reverse-transcribed, then subjected to different PCR cycles as indicated in Fig. 21 B. The PCR product and cycle number were linear up to 28 cycles.

C. IGF-II ribozyme action in cancer cells

1. Expression of IGF-II ribozyme in human prostate cancer cells under the control of Pol III promoter

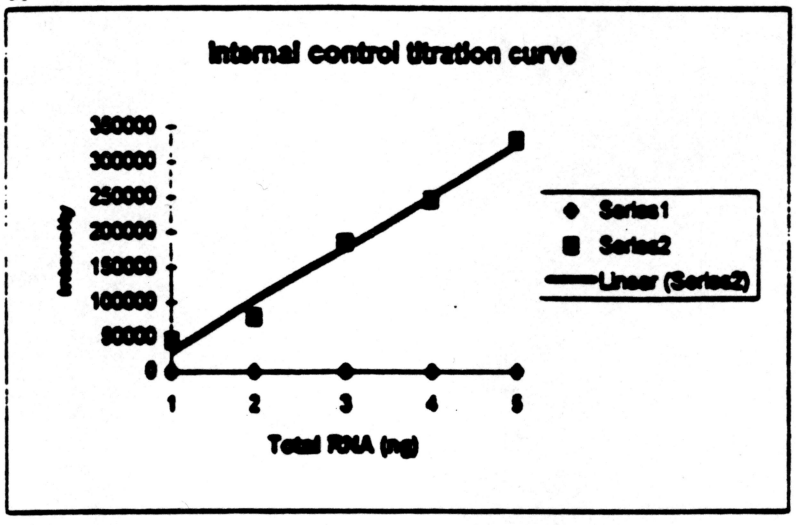
(1) Ribozyme expression in PC-3 cell transfectants

Genecitin (G418)-resistant stable clones were screened by RT-PCR to check whether ribozyme was expressed. From transfection with pTZU6+27 vector, 4 single ribozyme (R) clones, 2 double ribozyme (RR) clones, and 2 mutant ribozyme (M) clones were isolated. Expression of ribozyme in these clones was further confirmed by Southern hybridization using a ribozyme-specific probe as shown in Fig. 22A, B.

(2) Effect on IGF-II mRNA levels

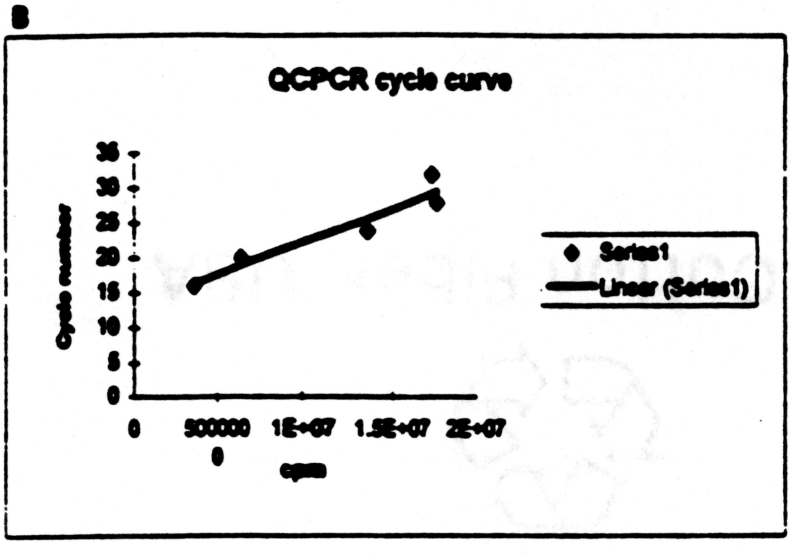
The flow chart of quantitative RT-PCR is shown in Fig. 23. The levels of IGF-II mRNA in ribozyme and mutant expressing PC-3 clones were determined by QC-RT-

A

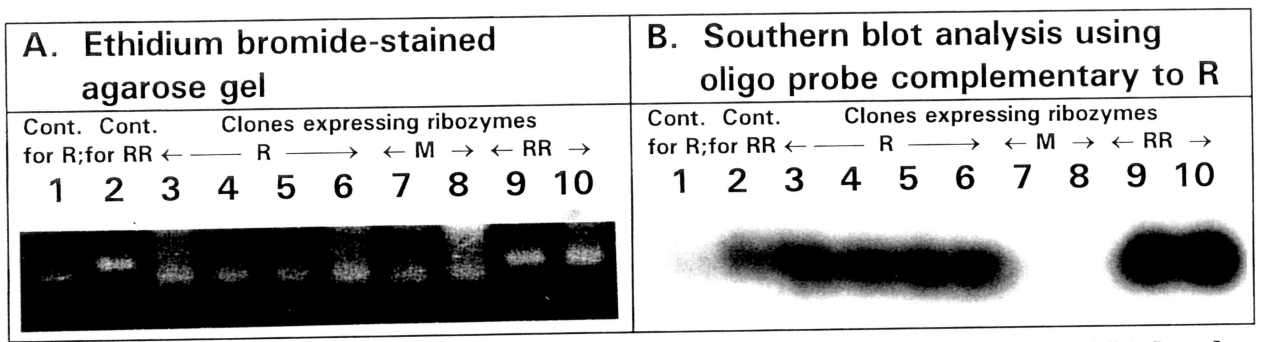


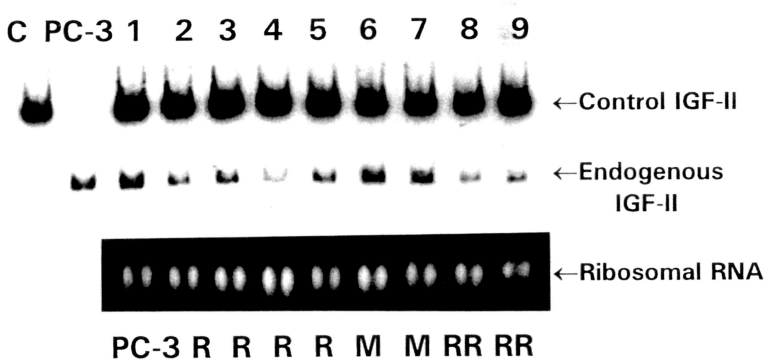
Total RNA (ng)	Intensity
0.005	44758
0.05	79200
0.5	184977
5	246543
50	330477

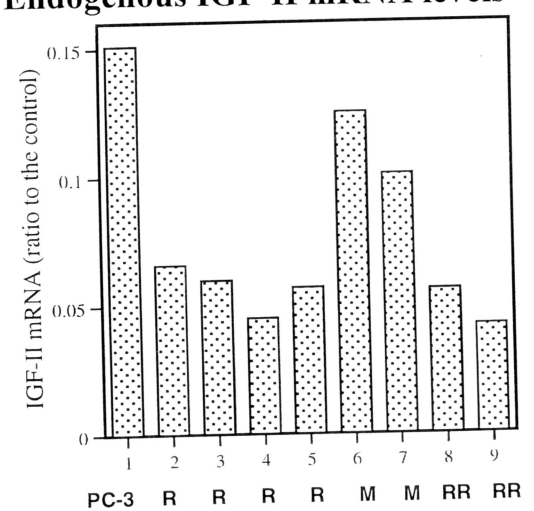
B



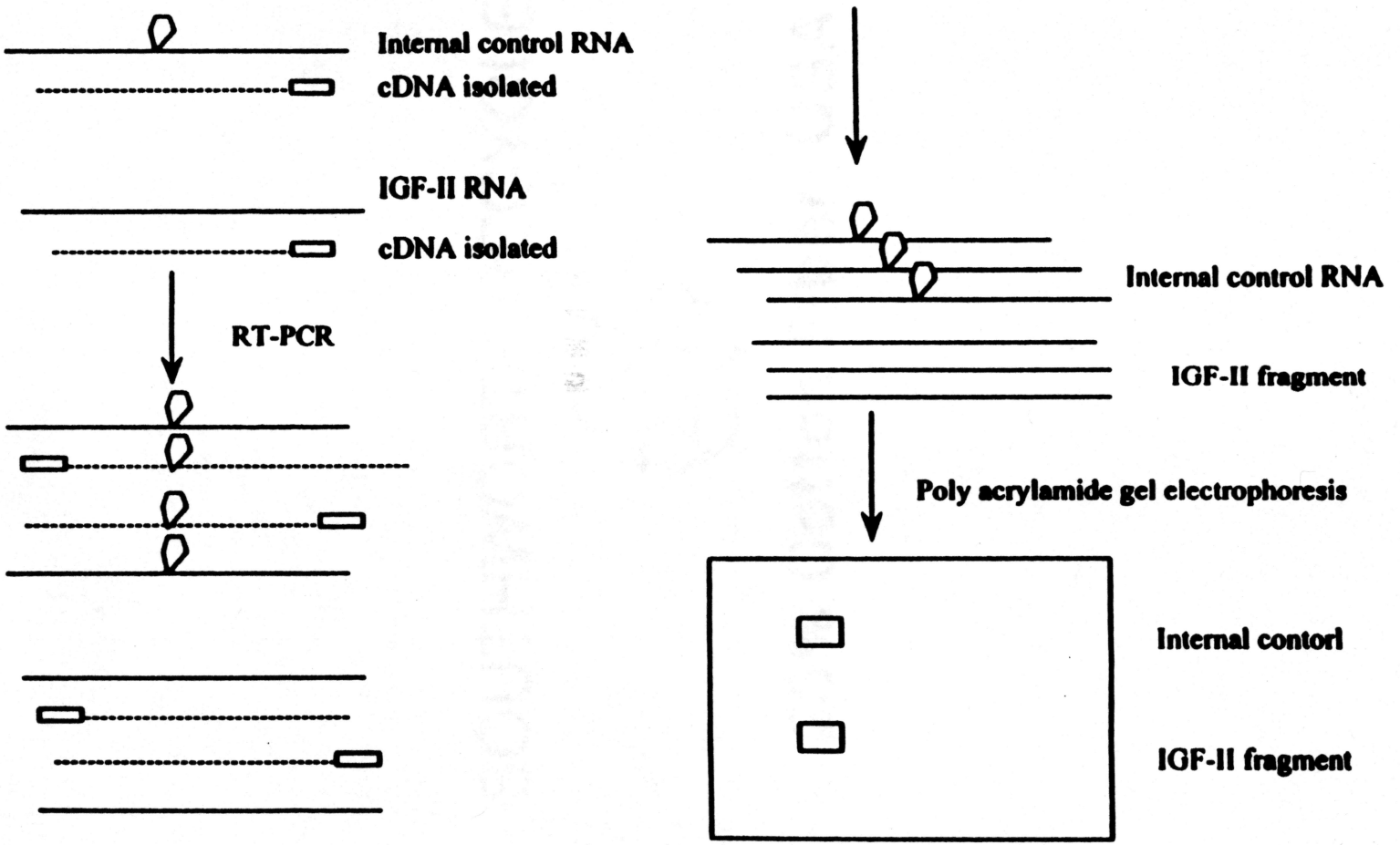
cpm	Cycle number
3612661	16
6457851	20
13560419	24
17697714	28
17396246	32


A. Ethidium bromide-stained agarose gel
Cont. Cont. Clones expressing ribozymes
for R;for RR ← R → ← M → ← RR →
1 2 3 4 5 6 7 8 9 10
B. Southern blot analysis using oligo probe complementary to R
Cont. Cont. Clones expressing ribozymes
for R;for RR ← R → ← M → ← RR →
1 2 3 4 5 6 7 8 9 10


C. Quantitative PT-PCR
C PC-3 1 2 3 4 5 6 7 8 9
←Control IGF-II
←Endogenous IGF-II
←Ribosomal RNA
PC-3 R R R R M M RR RR


D. Endogenous IGF-II mRNA levels
IGF-II mRNA (ratio to the control)
0.15
0.1
0.05
0
1 2 3 4 5 6 7 8 9
PC-3 R R R R M M RR RR

Flow Chart of Quantitative RT-PCR



PCR. As shown in Fig. 22C, in each lane, two bands are seen in each lane, the upper band (internal control) with ~ 400 bases and the lower band (endogenous IGF-II) with ~300 bases, the sizes of which were consistent with the predicted size. The relative IGF-II mRNA expression levels in different clones were expressed as the ratio of endogenous IGF-II/internal control. The results shown in Fig. 22D clearly demonstrate that both single and double ribozymes significantly reduce the IGF-II mRNA levels while mutant ribozyme had little effect on IGF-II mRNA expression compared to the PC-3 parental cells.

(3) Effect of IGF-II ribozyme expression on cell growth

The results of pTZU6+27 PC-3 clones are shown in Table 5. While cell numbers of parental PC-3 cells or M-expressing cells increased after 2 days in serum free medium, cell numbers of R- or RR- expressing cells (pTZU6+27) decreased, which indicates that ribozyme expression may have caused cell death.

2. Expression of IGF-II ribozyme in human prostate cancer cells under the control of Pol II promoter

(1) Ribozyme expression in PC-3 cell transfectants

Genecitin (G418)-resistant stable clones were screened by RT-PCR to check whether the ribozyme was expressed. From transfection experiments with pcDNA3 vectors, eleven G418-resistant clones were isolated. Among those eleven clones, four clones expressed single ribozyme (R) as shown in RT-PCR Fig. 24A.

Table 5. Effect of ribozyme (pTZU6+27) expression on PC-3 cell growth.

	Relative cell growth after 2 days in SFM Mean ± SE
parental PC-3	1.23 ± 0.02 (n=2)
R-expressing PC-3	0.58 ± 0.13 (n=5)
RR-expressing PC-3	0.4 (n=1)
M-expressing PC-3	1.61 ± 0.33 (n=2)

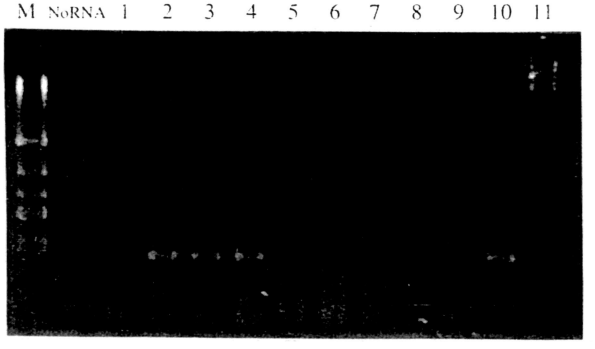
(2) Effect of IGF-II ribozyme on IGF-II mRNA levels

The levels of IGF-II mRNA in pcDNA3 stable ribozyme and vector PC-3 clones were determined by QC-RT-PCR. As shown in Fig. 24B, in each lane, there are two bands, the upper band (internal control) with ~ 400 bases and the lower band (endogenous IGF-II) with ~300 bases, the sizes of which were consistent with the predicted size. The relative IGF-II mRNA expression levels in different clones was expressed as the ratio of endogenous IGF-II/internal control. The relative IGF-II mRNA levels in different R-expressing clones were reduced as compared to the vector only clones and the PC-3 parental cells (Fig. 24B).

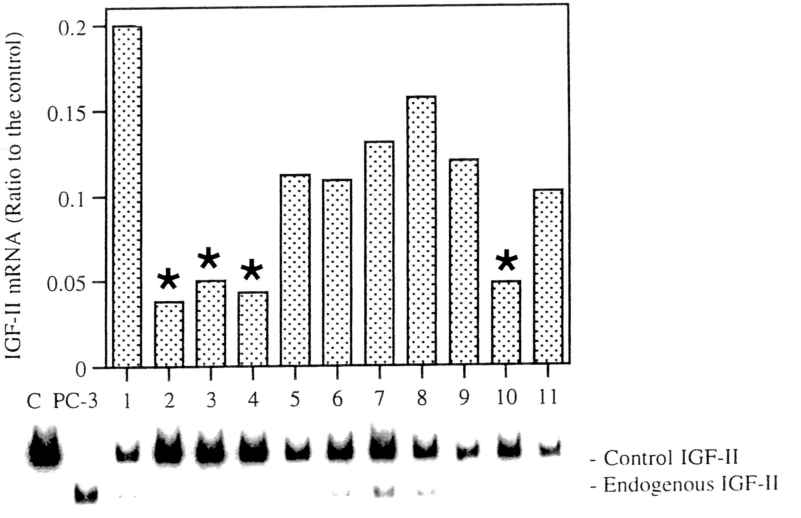
(3) Effect of IGF-II ribozyme on IGF-II protein levels

For the pcDNA3 ribozyme clones, the level of IGF-II protein secreted into conditioned medium was analyzed by radioimmunoassay. This was done in collaboration with Dr. Mohan's group, Loma Linda University VA Hospital (Mohan et al., 1990). The IGF-II protein levels among different clones are shown in Fig. 24C. Ribozyme expressing PC-3 clones showed relatively lower IGF-II protein levels, consistent with the lower IGF-II mRNA levels.

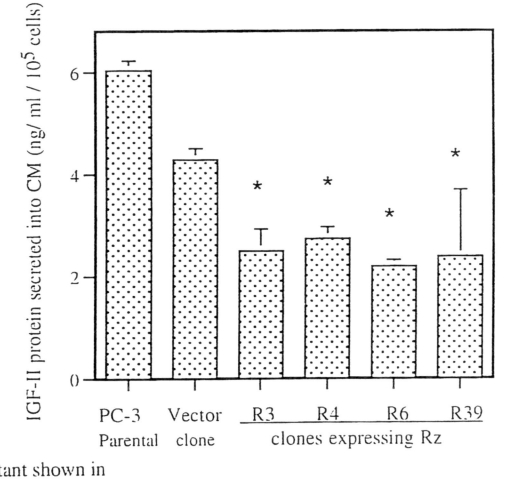
A. Ribozyme expression



B. IGF-II mRNA level



C. IGF-II protein level



(4) Effect of IGF-II ribozyme on cell growth

a) MTT assay

Effect of ribozyme expression (pcDNA3 vector) on PC-3 cell growth was measured by MTT assay, the result is shown in Fig 25. Ribozyme-expressing transfectants showed a significant decline in cell growth compared to the parental PC-3 cells, and vector expressing transfectants both in serum-free medium and 2% FCS medium.

b) Time-lapse microscopy.

Effect of ribozyme expression on doubling time of PC-3 cell grown in the presence of 10% FCS is shown as Table. 6. Analysis of cell division of parental PC-3 cells, vector-transfected cells, and R-expressing PC-3 cells (R4, R6, and R39) revealed doubling times (mean±SEM) of 28.3 ± 0.39 (n=8), 32.3 ± 1.0 (n=3), 37 ± 1.1 (n=6), 38.6 ±2.3 (n=5), and 42.5 ±2.8 (n=2) hr, respectively. Compared to the parental PC-3 cell, ribozyme-expressing clones showed a significantly prolonged doubling time while the doubling time of vector-transfected PC-3 cells was not significantly different from that of the parental cells (Table 6).

A (SFM)
B (2% FCS)

Table 6. Effect of ribozyme expression (pcDNA3) on PC-3 cell growth as measured by time lapsed microscopy

	R-expressing transfectants			Vector transfectant
	R4	R6	R39	
Doubling time ± SEM (hr)	37 ± 1.1	38.6± 2.3	42.5± 2.8	32.3 ± 1.0
Compared to parental PC-3 cells (%) [PC-3: 28.3 ± 0.39 hr]	131 P<0.01	136 P<0.001	150 P<0.001	114 P>0.05

D. Summary

In summary , this study demonstrated:

1) Single (R)- and double (RR)-ribozymes were catalytically active *in vitro*. RR was more active than R *in vitro*.

2) Both R and RR ribozymes similarly suppressed IGF-II mRNA levels in PC-3 cells (~40%) as compared to the level in parental PC-3 cells.

3) Pol II and Pol III promoter-driven ribozymes similarly suppressed IGF-II mRNA levels.

4) Suppression of IGF-II mRNA levels are correlated with suppression of IGF-II protein levels.

5) R- (or RR-) expressing cells showed reduced cell growth and prolonged doubling time as compared to those of parental or M-expressing cells.

V. GENERAL DISCUSSION

A. IGF-II expression in human tumor tissues

In our published studies included in Chapter II, we used *in situ* hybridization and immunohistochemistry methods to determine IGF-II expression in tumor tissues *in vivo*. The results showed that IGF-II expression is confined to epithelial cells and not stromal cells, implicating that cancer cell growth may be regulated by IGF-II in an autocrine manner, and also provided the basis that IGF-II is a pertinent target for ribozyme manipulation.

It should be noted, however, that we also observed a peculiar focal expression as well as an elevated expression of IGF-II in benign epithelium of the prostate. We must consider a possible contribution of stromally expressed IGF-II to the development of prostate cancer and BPH. With respect to IGF-I, recent tissue recombinant studies by Cunha's group (1998) demonstrated that the IGF-I pathway plays a critical role in prostatic development. In their studies, local IGF-I expression appears to play the role. In contrast, elevated IGF-I levels in plasma (systemic) are associated with a higher risk of prostate cancer (Chan et al., 1998). The roles of IGFs in prostate physiology and cancer remain important areas for future investigation.

B. IGF-II ribozyme action

Ribozymes have been used as a molecular tool to inactivate gene expression in some studies (Kashani-Sabet et al., 1992; Koizumi et al., 1992; Dorai et al., 1997).

A couple of studies using ribozymes to modulate oncogene expression have been reported previously. These included a ribozyme specifically targeting a mutant oncogene codon in which the mutation was suitable for ribozyme activity (Kashani-Sabet et al., 1992). Another study showed that an anti-p53 ribozyme designed to cleave the p53 pre-messenger RNA (mRNA) can efficiently reduce the level of endogenous mutant p53 mRNA (De Weiet al., 1995). Our studies demonstrated that IGF-II ribozymes are catalytically-active *in vitro*, and capable of reducing IGF-II mRNA and protein levels in prostate cancer cell lines.

There are several good reviews describing the optimal design for ribozymes (Deshler et al., 1992; Castanotto et al.,1995). The maximum optimal design is, however, achieved basically by a trial and error process. A computer program may provide some structural information on secondary or tertiary structures of the substrate which might impair ribozyme activity (Zuker et al., 1981; Sun et al., 1994). Recently, Pachuk et al. (1994) developed a potential novel design, which used a ribozyme anchor to overcome an obstruction caused by secondary structure around a cleavage site. In our study, we have used a hammerhead ribozyme motif because of its small size and ease of manipulation. The IGF-II ribozyme cleavage site near the translation initiation codon was chosen based on the results of antisense oligonucleotide experiments which were performed in Dr. Fujita-Yamaguchi's laboratory (unpublished).

Single- and double-hammerhead ribozymes were analyzed in vitro and cloned into

the pTZU6+27 or pCDNA vector. The *in vitro* result showed that the double ribozyme is more efficient than the single ribozyme as judged by single turnover kinetics as well as cleavage reactions using the prepro IGF-II mRNA as a substrate. This is probably because the double ribozyme has two cleavage domains while the single-headed ribozyme has only one cleavage domain.

Ribozyme gene delivery was achieved by two methods, calcium phosphate or electroporation. Both mothods seemed to work equally well in this study. Establishment of quantitative assays for the target RNA is necessary for the detection of ribozyme cleavge reactions *in vivo* (in cell lines). Previous studies used Northern blot analysis, RNase protection assays, and RT-PCR (Funato et al., 1994; Efrat et al., 1994). In our study, the QC-PCR method was used because it is very sensitive, specific, and quantitative (Beaudry et al., 1995). QC-PCR uses one pair of primers for both endogenous and competitor RNAs in contrast to most of the RT-PCR methods which use two pairs of primers. The competitor IGF-II RNA shares the same sequences for primer binding as the target endogenous IGF-II mRNA, but contains an additional 110 base-long insert which will give a PCR product larger than the PCR product generated from the target endogenous IGF-II mRNA. This 110 base difference in size is sufficient for easy separation of the PCR products on denaturing polyacrylamide gels and for quantitation of the radioactive bands with a Phosphor Imager.

We must acknowledge that although *in vitro* studies of ribozymes are essential steps for learning their catalytic mechanisms, *in vitro* studies cannot be used to

predict the efficiency of the ribozymes when expressed in cells. For example, Domi et al., (1996) designed a small anti-HIV hammerhead ribozyme, the ribozyme was active in the cell, but inactive *in vitro*. Interestingly, in our study, single- and double-ribozymes showed almost the same level of efficacy in reduction of IGF-II mRNA in cells. This observation can be explained in the following ways: 1) due to secondary or tertiary structure, interaction of IGF-II mRNA with the double-headed ribozyme makes one of the cleavage domains unable to anneal with the substrate, thus there is no cleavage reaction for that domain; 2) within the cell, mRNA is stabilized by the structure of 5' Cap and 3' poly A tails, if one cleavage domain anneals with the substrate, and subsequently cleaves the substrate mRNA, then the broken IGF-II mRNA is easily degraded by a variety of ribonucleases within the cell; 3) the ribozyme cell clones are those selected by the cell which is able to moderately reduce IGF-II to a point which does not cause cell death. The results tell us that, compared to *in vitro* experiments, ribozyme activity is likely to be altered in the cellular environment.

Other factors which may interfere with ribozyme action need to be considered. These factors include ribozyme expression level, co-localization of ribozyme with the targets, location of the ribozyme gene relative to the target gene in the host genome, sites at which the ribozyme functions, secondary structure of the target RNA or ribozymes, cofactors or inhibitors like proteins, Mg^{2+} concentration, pH, etc. Due to the complexity of eukaryotic gene expression, and also these multiple factors, we

must acknowledge that this technology is not straightforward as simply expressing a complementary sequence *in vivo*. If ribozyme expression is not highly efficient, the level of ribozymes may be too low to cleave the target RNA. Even though ribozymes are efficiently expressed, if ribozymes are not in the same cell compartment with the target RNA, ribozyme activity would be dramatically decreased. One common observation in ribozyme/antisense studies is the extreme variation in the effectiveness of ribozymes produced from the same gene located at different chromosomal positions (Kim et al., 1985; Van der krol et al., 1988; Cannon et al., 1990). In general, the end result is that only a small proportion of transgenic organisms transformed with any one DNA construct show an effect. The effectiveness of the antisense/ribozyme produced appears to be unrelated to either the number of antisense/ribozyme genes or the steady-state levels of the antisense/ribozyme RNA. In addition, unlike most situations, there are systems in which an antisense RNA functions effectively when expressed at a lower steady-state level than the target mRNA (Nishikura et al., 1987; Sheehy et al., 1988). In contrast, there are examples in which massive overexpression of antisense/ribozymes, relative to the target mRNA, have failed to down-regulate expression of the target gene (Kerr et al., 1988). One explanation given is the influence of position effect on the expression of integrated antisense/ribozyme genes. Arndt et al. (1997) gave a more complex explanation, it is possible that the location of the antisense/ribozyme gene relative to the target gene may be even more important. They defined this as location effect,

which refers to the influence of the antisense/ribozyme gene position relative to the target gene on the extent of inhibition mediated by the expressed antisense/ribozyme. The extent of the observed location effect can be adjusted by the influence of classical position effects, wherein the flanking chromosomal DNA at the site of integration of the antisense/ribozyme gene can affect the level and timing of expression of the antisense/ribozyme. It may be that integration of antisense/ribozyme genes into different sites in transgenic mammalian cell lines leads to different local concentrations of antisense/ribozymes at the position of the target gene or in the vicinity of the target mRNA. Within this scenario, there is no need for a correlation between the concentration of antisense RNA and the extent of inhibition.

The secondary structure of the target RNA or ribozymes is also very important. Since ribozymes cleave the target RNA by first complementary binding to the target RNA, if the secondary structure of target RNA or ribozymes interfere with the binding of ribozyme and target, ribozyme activity will be reduced. Also, proteins may bind to portions of the RNA substrate or to the ribozyme itself, and may facilitate or interfere with the catalysis. A limited number of reports demonstrated an enhancement of ribozyme cleavage reactions by RNA-binding proteins like hnRNP A1 or the capsid protein NC7 of HIV-1 (Bertrand et al., 1994; Tsuchihashi et al., 1993; Herschlag et al., 1994). In our study, pTZU6+27 vector and pcDNA were used, pTZU6+27 has an advantage for directing the expression of short, defined transcripts in high copy number under control of RNA polymerase III promoter. These RNAs

are relatively stable, both in nucleus and cytoplasm, but mainly in the nucleus (Good et al., 1997; Bertrand et al., 1997; Michienzi et al., 1996;). pcDNA vector (CMV promoter) can be ubiquitously expressed under the control of RNA polymerase II. The ribozyme transcript will be mainly expressed in cytoplasm. Both Pol II and Pol III driven vectors have worked successfully in our ribozyme studies. Pol II promoters, including viral promoters, which are used naturally for mRNA synthesis, can allow tissue-specific expression of ribozymes. Despite differences in the amount of ribozyme transcripts obtained from different promoters, their ability to confer resistance to HIV-1 replication is similar (Zhou et al., 1996). A disadvantage of Pol II promoters is their requirement for long coding sequences, so that the ribozyme sequence may be placed in long RNA molecules. This can interfere with ribozyme conformation. In contrast, Pol III promoters, which naturally drive tRNA and snRNA synthesis, are good for very-high-level, non-tissue-specific expression of short RNA. For example, Thompson et al. (1995) used a tRNA-based RNA polymerase III promoter driven ribozyme, and found that high accumulation of recombinant pol III ribozyme transcripts was observed in all cell lines tested, and that the ribozyme activity was readily detectable in total RNA extracted from stably transduced human T cell lines. Michienzi et al. (1996) used U1 small nuclear RNA chimeric ribozymes with substrate specificity for the Rev pre-mRNA of human immunodeficiency virus, and showed that this construct caused more efficient reduction of Rev pre-mRNA in vivo. Kawasaki et al. (1996) reported that by using a Pol III driven vector, the

adenoviral-E1A-associated 300-kDa-protein expression in HeLa cells was inhibited. In our study, both Pol II and Pol III driven vectors similarly suppressed IGF-II mRNA levels in PC-3 cells. Suppression of IGF-II mRNA levels are correlated with reduced IGF-II protein levels. Ribozyme expressing cells showed reduced cell growth and prolonged doubling time as compared to those of parental or M-expressing cells. These results supported our hypothesis that prostate cancer cell growth is regulated by IGF-II in an autocrine manner.

C. Observation

One interesting observation in our study was that when I stably transfected SK-N-AS cells with pTZU6+27/Rz, first, some clones grew. These clones were picked up and placed in 96 well plates, and then in T-25 flasks. These clones, however, never continued growing, but eventually died off. In contrast, SK-N-AS cells transfected with pTZU6+27/mutant Rz could continue to grow even into T-75 flasks. This suggests that since SK-N-AS cells are more dependent on IGF-II for their growth than PC-3 cells, IGF-II ribozyme-expressing clones could not survive. An experiment was carried out using pcDNA vectors encoding IGF-II ribozyme (R) and mutant ribozyme (M). SK-N-AS cells were stably transfected with these vectors. After one month culture in selection medium containing G418, I found far less R-expressing clones than M-expressing clones. This supports the fact that IGF-II plays an important role in SK-N-AS cells and suggests that an alternative strategy for designing an IGF-II ribozyme is necessary, that is, using an inducible vector, so that we can turn off

ribozyme expression first to get stable clones and then turn on ribozyme expression to see the effect of ribozyme on the growth of cancer cells.

D. Mechanisms by which suppression of IGF-II RNA and protein affects cancer cell growth

In this study, IGF-II ribozymes targeted to the IGF-II mRNA were evaluated in prostate cancer PC-3 cell line. The result showed that both IGF-II mRNA and IGF-II protein levels were decreased, and that prostate cancer cell growth was delayed. This clearly demonstrated that IGF-II was important in supporting PC-3 cell growth. The potential mechanisms by which suppression of IGF-II RNA and protein affects cancer cell growth are presented below:

1. Mitogenic response in prostate cancer cells is mainly via IGF-II binding to IGF-I receptor. The IGF-I receptor mediates mitogenic and antiapoptotic effects on normal and transfromed prostate eptithelial cells (Cohen et al., 1991; Rajah et al., 1997). By decreasing IGF-II levels, the mitogenic signal is decreased and more prostate cancer cells go through programmed cell death, apoptosis.

2. M6P/IGF-II receptor has recently been implicated in fetal development and carcinogenesis. The mitogenic response through IGF-II binding to IGF-II receptor may also be blocked by IGF-II ribozyme by decreasing IGF-II levels.

3. It is possible that the IGF-II receptor indirectly affects mitogenic responses of the IGF-I receptor. Resulting in decreased availability of IGF-II to the IGF-I receptor. Similarly, reduction of IGF-II levels may increase IGFBP expression, which in turn

binds IGF-II, resulting in decreased availability of IGF-II to the IGF-I receptor. Likewise, IGFBP proteinases may be downregulated in R-expressing cells, which may result in increased IGFBPs, in turn binding IGF-II, resulting in decreased availability of IGF-II to the IGF-I receptor.

4. In the cell growth experiments, ribozyme expressing PC-3 cells showed growth inhibition in both serum-free medium and 2% FCS medium. Parental and vector expressing PC-3 cells continued to grow in 2% FCS, but showed slower growth in serum free medium. This may indicate that other factors in serum may also be important for PC-3 cell growth. Since serum-deprivation is a stimuli for apoptosis, ribozyme action and apoptosis through serum-deprivation both make contributions to delaying cancer cell growth in these experiments. Further studies are thus required to understand mechamisms by which ribozyme expressing cells undergo apoptosis.

E. Prostate cancer therapy

Prostate cancer represents a heterogeneous disease entity with varying degrees of behavior, aggressiveness, patterns of metastasis, and response to therapy. Progressive metastatic prostate cancer is associated with a formidable array of morbidity that ultimately contributes to death of the patient. Androgen ablation remains the primary systemic therapeutic modality for this disease, yet the intense delineation of mechanisms involved in tumor cell metastasis has lead to new therapeutic strategies, ranging from cytotoxic to cytostatic, including immunomodulators. Among those strategies currently being studied are granulocyte-macrophage colony-stimulating

factor-transduced prostate cancer vaccines, inducers of apoptosis, antimetastatic agents, angiogenesis inhibitors, radiation therapy (local and systemic), systemic approaches targeted at prostate cancer morbidity and gene therapy. We must acknowlege that cancer is a very complex disease, usually not due to one lesion, but many lesions accumulated through the aging process. It is more like network breakdown, causing chaos of cells. Accordingly, since treatment of cancer can not be achieved by a single therapy, combined therapy usually prevails. With more understanding of molecular mechanisms, gene therapy such as ribozymes may play a role in cancer therapy, providing an additional promising modality.

F. Conclusion

In recent years, IGF-II has been shown to be an important factor for prostate cancer cell growth. Thus, IGF-II provides a good target for gene manipulation. This work presents the first comprehensive investigation of the role of IGF-II in androgen-independent prostate cancer cell growth using IGF-II ribozymes. It provides support for the hypothesis that prostate cancer cell growth is regulated by IGF-II, and that IGF-II is required for prostate cancer cell growth. In summary, ribozyme gene therapy can be used to decrease IGF-II levels in prostate cancer cells. Since IGF-II reduction decreases tumor growth and stimulates apoptosis, it represents a promising therapeutic target to treat prostate cancer patients.

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