ANALYSIS OF THREE LEVELS OF REGULATION OF EXPRESSION OF THE HUMAN BASIC FIBROBLAST GROWTH FACTOR GENE: mRNA ACCUMULATION, UPSTREAM REGULATION, AND BASAL PROMOTION

by

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A DISSERTATION

Presented to the Department of Cell Biology & Anatomy
and the Oregon Health Sciences University
School of Medicine
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

July 1993

APPROVED:

[redacted]

Professor in Charge of Thesis

[redacted]

Chairman, Graduate Council

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LIST OF ABBREVIATIONS

aa	amino acids
act-D	actinomycin D
aFGF	acidic fibroblast growth factor
ATP	adenosine triphosphate
BCE	bovine corneal endothelial cells
bFGF	basic fibroblast growth factor
bp	base pairs
CAT	chloramphenicol acetyl transferase
cDNA	complementary deoxyribonucleic acid
chx	cycloheximide
cpm	counts per minute
CTP	cytosine triphosphate
DMEM	Dulbecco's modification of Minimal Esssential Medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DSE	dyad symmetry element
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOME	human omental microvascular endothelial cells
Ifn	interferon
IL-1	interleukin-1

Inr	initiator
IPCR	inverse polymerase chain reaction
IVT	*in vitro* transcription
kb	kilobases
kD	kiloDaltons
KGF	keratinocyte growth factor
LDL	low density lipoprotein
mRNA	messenger ribonucleic acid
NFF5	normal foreskin fibroblast strain 5
NRE	negative regulatory element
NRO	nuclear run on
pCB	pCAT-Basic
pCP	pCAT-Promoter
pCE	pCAT-Enhancer
PCR	polymerase chain reaction
PDD	phorbol 12,13-didecanoate
PDGF	platelet-derived growth factor
PMA	phorbol 12-myristyl 13-acetate
Py	pyrimidine
RNA	ribonucleic acid
RNase	ribonuclease
SDS	dodecyl sulfate, sodium salt
SMC	smooth muscle cell
SRE	serum response element
SV40	simian virus 40
t1/2	half-life
TdT	terminal deoxynucleotidyltransferase

TGF-β	transforming growth factor-beta
TNA	total nucleic acid
Tris	tris(hydroxymethyl)aminomethane
UTP	uridine triphosphate
v/v	volume/volume

ACKNOWLEDGEMENTS

A number of people deserve acknowledgement for giving me their help, support and inspiration over the last several years. It would be impossible to mention all of the people who have contributed to my arrival at this point in my career and so I would like to thank *en masse* those Thesis Committee members, faculty, staff, co-workers and fellow students whose day to day interactions helped to educate me and helped to facilitate my passage through graduate school.

Certain people have had special positive impact on my life and education and bear special thanks. These people include:

My brother, Raymond, and sister, Leslie, who have provided enduring support throughout my long sojourn in graduate school.

Jill Hendrickson, Ethan Strauss, Blake Allen, and Winnie Keeble, each of whom provided special inspiration in their own special way.

And finally, Dr. John H. Sinclair and Dr. Keith Iams who provided me with my first laboratory experience and who first opened my eyes as to what scientific research was all about.

ABSTRACT

Basic Fibroblast Growth Factor (bFGF) is a polypeptide found in vertebrates which has been shown to have a variety of activities on cells *in vitro* and on tissues *in vivo*. bFGF has been implicated to act in some of the most fundamental developmental decisions in the oocyte and early embryo and is thought continue to be a local mediator of tissue development and homeostasis throughout development and throughout somatic existence. bFGF is also being examined for its capacity to intervene in a variety of pathological conditions, particularly those involving vascular supply, wound healing, and myo- or neurodegenerative disease. It is the wealth of roles that bFGF is likely to be playing *in vivo* as well as the possible benefits its medicinal use might provide that underlies the significance of work on what controls its expression in human cells.

The work reported in this thesis has had as its central aim the collection of more information on how the expression of bFGF is controlled. The work has focused on three areas of gene expression: (1) the control of cellular bFGF mRNA content by extracellular effectors (2) the control of bFGF transcription in cells in response to extracellular agents, and (3) the control of the transcriptional initiation event from the human bFGF gene.

A variety of polypeptide growth factors and other agents including tumor promoters were examined for their capacity to cause normal human fibroblasts to elevate their bFGF mRNA level. The agents: fetal calf serum, phorbol didecanoate, platelet derived growth factor, transforming growth factor beta, and the non-phorbol tumor promoter thapsigargin were all shown to be able to exert an inductive effect on fibroblast bFGF mRNA as measured by a sensitive solution hybridization technique. It was found that many of these agents could act in concert to give synergistic inductions of bFGF messenger levels. The magnitude and pattern of the effects produced by combinations of inducers suggested that

numerous pathways exist by which extracellular agents may act on cells to influence their bFGF mRNA.

The second part of my research sought to examine the region upstream from the human bFGF gene for regulatory elements which were involved in the control of bFGF expression by extracellular agents. In the pursuit of this aim, I isolated a genomic clone harboring bFGF sequence from the region upstream from the first coding exon and I examined the first 2000 base pairs of this upstream sequence for the capacity to support regulated expression of a reporter gene. The results indicated the absence of any strongly inducible agonist regulated elements in this area and suggested that these regulatory loci may lie in the far upstream region or in the intronic or downstream regions of the human bFGF gene.

The third part of my research was directed at dissecting out the minimum basal promoter sequences for the human bFGF gene. Using bFGF sequences linked to a reporter gene it was found that as little as 9 bp at the transcriptional start site are sufficient to promote significant transcription.

Chapter 1 General Introduction

Introduction

The central problem that my research has sought to address is, under what conditions and by what mechanisms does expression from the human basic fibroblast growth factor gene occur. This thesis is segmented into four chapters with the first serving as a general introduction and the other three chapters each introducing, describing, and discussing a discrete part of the research that I have conducted at OHSU. The general introduction which comprises Chapter 1 will describe and review some of the biology of bFGF and will seek to establish the potential long-term significance of the research reported here.

General and Structural Information

Basic fibroblast growth factor (bFGF) is a peptide growth hormone initially discovered in the early 1970s as a component of extracts of bovine brain and pituitary which supported the growth of fibroblasts in tissue culture [Baird *et al.*, 1986]. This factor was purified to homogeneity by Gospodarowicz in 1975 [Gospodarowicz, 1975] and eventually sequenced by Esch in 1985 [Esch *et al.*, 1985]. A cDNA for this factor was first isolated and described by Abraham in 1986 [Abraham *et al.*, 1986].

The primary structure of bFGF has been revealed by a large amount of work carried out from 1985 through the present [Baird *et al.*, 1986]. Active bFGF can be found in forms ranging in molecular weight from 22.5 kD to 14 kD, corresponding to forms with 210 to 131 amino acids [Klagsbrun *et al.*, 1987; Gospodarowicz, 1989; Prats *et al.*, 1989; Renko *et al.*, 1990]. The variation in the size of this factor occurs at the amino terminus and is believed to result from multiple translational initiations from bFGF mRNA giving rise to

primary translation products (in human cells) of 210, 201, 195, and 155 amino acids as well as from post translational amino terminal clipping of these forms to generate the 146 and 131 amino acid forms commonly found in biological samples [Klagsbrun *et al.*, 1987; Prats *et al.*, 1989]. While all of these forms of bFGF have mitogenic activity, it has been found that the 210, 201, and 195 amino acid species contain a nuclear targeting sequence [Bugler *et al.*, 1991]. These forms are found to localize preferentially in cell nuclei, and they possess some unique biological activities [Couderc *et al.*, 1991; Quarto *et al.*, 1991].

The mRNA for bFGF is also detected in a number of sizes. In humans, the predominant forms have the apparent sizes of 7kb and 3.7 kb [Schweigerer, 1987; Murphy, 1988]. Highly sensitive northern blot analysis can reveal other forms: 4.0, 2.2, 2.1, and 1.5 kb [Sternfeld *et al.*, 1988; Sato *et al.*, 1989]. The mRNA size heterogeneity is thought to derive from differential choice of transcriptional termination site [Prats *et al.*, 1989].

bFGF is a member of a family of factors, the Fibroblast Growth Factor or FGF family, which presently numbers at least 7: acidic FGF (aFGF), bFGF, int-2, hst, FGF5, FGF6, and keratinocyte growth factor (KGF) [Gimenez-Gallego *et al.*, 1985; Abraham *et al.*, 1986a ; Dickson and Peters, 1987; Yoshida *et al.*, 1987; Zhan *et al.*, 1988; Finch *et al.*, 1989; Marics *et al.*, 1989; Baird and Klagsbrun, 1991a]. A new nomenclature for this family has recently been suggested [Baird and Klagsbrun, 1991b]. In this scheme these seven factors are renamed FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, and FGF7, respectively. This family is defined by primary sequence homology in core regions of the proteins and by conserved intron /exon boundaries in the genes. FGF family members share 30 to 55% amino acid homology with bFGF/FGF2 (hereafter referred to as bFGF) in the conserved regions and all 7 FGF family members have three coding exons which occur in the same relative position to the conserved amino acid sequences [Baird and Klagsbrun, 1991a; Baird and Klagsbrun, 1991b]. The cytokines interleukin

1 alpha and beta (Il-1α and Il-1β) [Thomas and Gimenez-Gallego, 1986] and the newly discovered growth factors, androgen inducible growth factor (AIGF) [Tanaka *et al.*, 1992] and FGF9 [Miyamoto *et al.*, 1993], share a lesser homology with the FGFs and while related, they are not presently included in the FGF gene family [Baird and Klagsbrun, 1991b]. As more information on the gene structures of AIGF and FGF9 is obtained this may change. Figures 1.1 and 1.2 summarize some of this information about the FGF gene family.

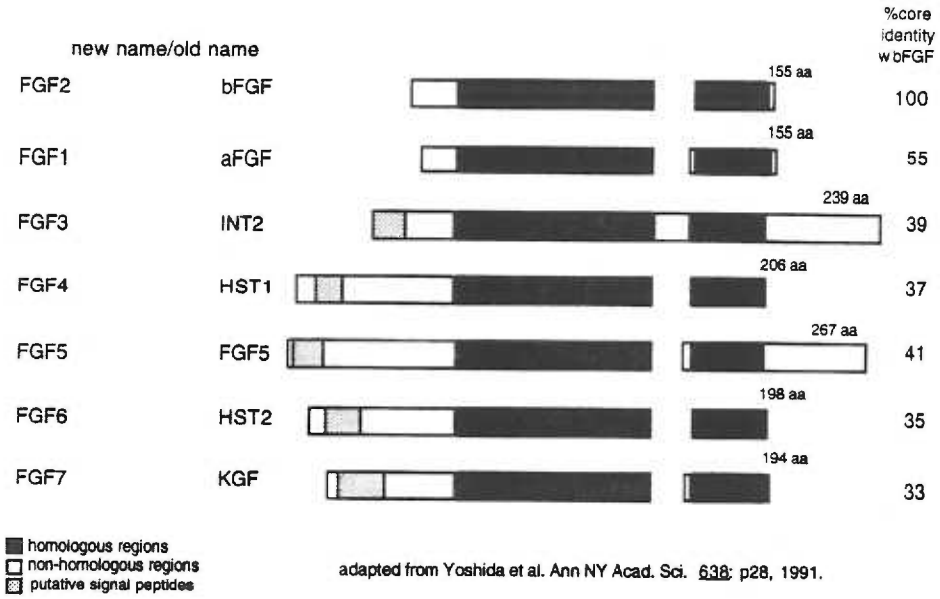


Figure 1.1 The FGF Family: protein alignment This is a schematic representation of the FGF family of proteins. The regions in black are conserved among the 7 members. The % core identity is the % of amino acids in these regions which are identical with those in FGF2/bFGF.

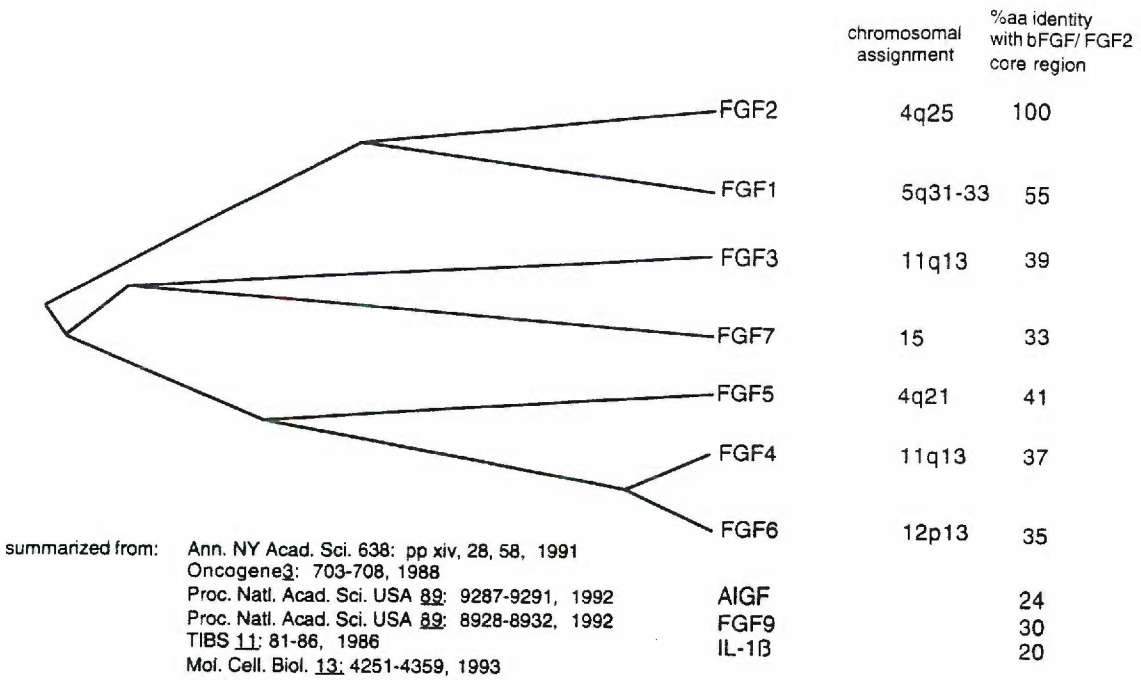


Figure 1.2 Phylogenetic tree for the FGF gene family This is one scheme of arranging the FGF family of proteins according to their evolutionary relatedness. This figure is based on a similar figure drawn by Coulier *et al.* Ann. NY Acad. Sci 638: p58, 1991. AIGF and FGF9 are candidate members in the FGF family and IL-ß also shows some primary sequence homology with the FGFs.

bFGF Gene Organization

The organization of human bFGF gene was originally investigated by Abraham *et al.* [Abraham *et al.*, 1986b] and later looked at by Shibata *et al.*[1991] The overall structure is shown in Figure 1.3. The gene consists of three exons containing the coding regions separated by two large introns which are ≥16kb and 16 kb in size. The gene's 3' transcribed untranslated region is at least 5823 bp in length and the 5' untranslated region is 318 bp [Prats *et al.*, 1989]. The region downstream from the translational stop codon contains a number of consensus sites for poly-adenylation, AATAAA. Several of the consensus polyadenylation sites are thought to be used giving rise to the multiple species of bFGF mRNA seen in human cells and tissues [Prats *et al.*, 1989].

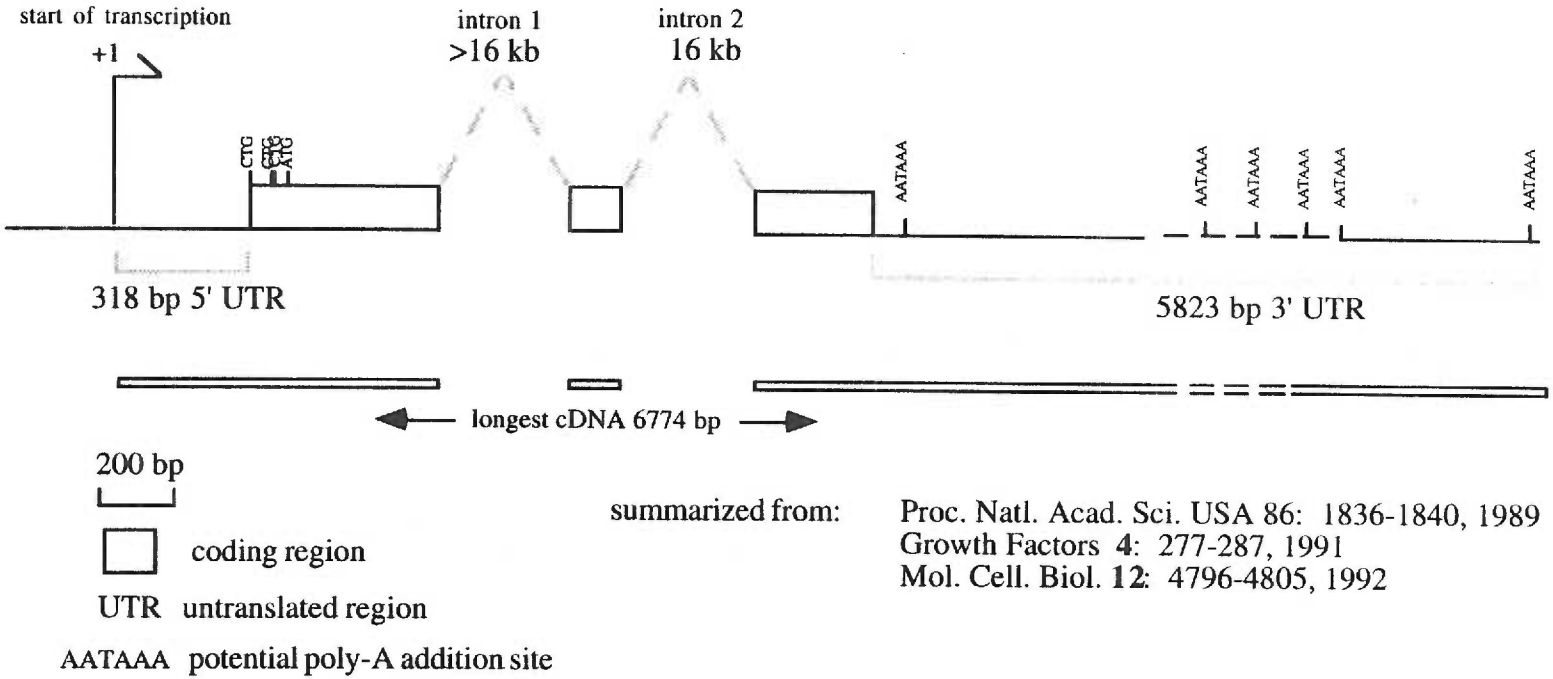
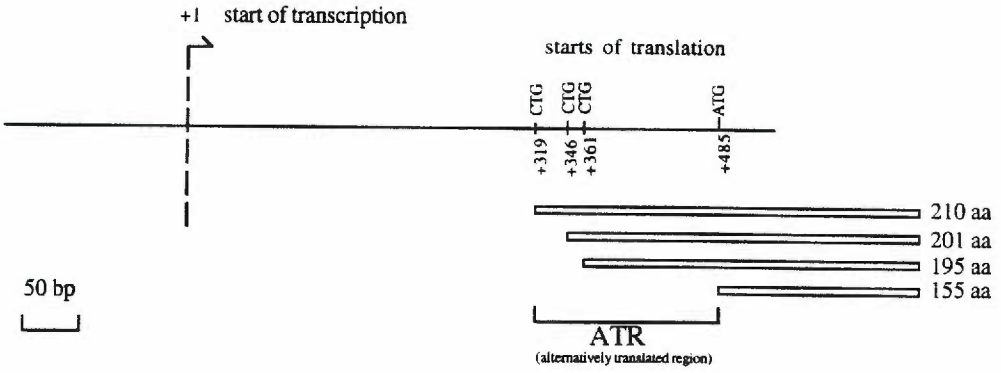


Figure 1.3 Human bFGF gene organization This is a representation of the organization of the human bFGF gene. The coding region is contained in three exons divided by two large introns. A very long 3' untranslated region has been characterized. This region contains numerous concensus sites for poly-adenylation, several of which are thought to be used to generate the various bFGF mRNA species found in human cells. The 5' untranslated region is 318 bp long.

Start of Translation

The region immediately upstream from the coding region of the human bFGF gene is diagrammed in Figure 1.4. The region in which bFGF translation begins is quite interesting. The region between the start of transcription (+1) and the first AUG in a translatable context is 483 nucleotides in the human gene. Two groups have shown that synthetic RNAs containing this upstream region plus the bFGF coding region can *in vitro* or in cells give rise to not one, but four primary translation products [Florkiewicz and Sommer, 1989; Prats *et al.*, 1989]. These four species have also been detected in cells and tissues [Florkiewicz and Sommer, 1989; Prats *et al.*, 1989]. One of these species corresponds to a translational start at the predicted AUG start codon and the other three correspond to species which have resulted from translational start at CUG codons located upstream from the AUG. The CUG which gives the largest primary translation product is located 165 bp upstream from the AUG start site and so the region in which translation starts is 165 bp wide. This region has been termed the alternately translated region, ATR,



summarized from: Growth Factors **4:** 277-287, 1991
Mol. Cell. Biol. **12:** 4796-4805, 1992

Figure 1.4 Human bFGF gene immediate upstream region This is a representation of the relative location of the transcriptional and translational start sites of the human bFGF gene. The four different primary translation products are also shown.

and it is thought that some regulation of expression is specified within this region [Prats *et al.*, 1992]. The 5' transcribed untranslated region is 318 bp in length.

Start of Transcription

Shibata *et al.* [1991] determined by primer extension analysis that the transcriptional start site (the +1 site) in the human gene occurred at a point 485 bases upstream from the AUG translational start and 318 bases upstream from the most upstream CUG translational start. A single band was reported from the primer extension analysis suggesting that bFGF transcription occurs accurately and that, at least in the two cell types that provided the source RNA for the primer extensions, only a single promoter is being used.

The Activities of bFGF

aFGF and bFGF were the first members of the FGF family to be isolated and the activity of these factors is still the best studied. In this regard they continue to be the prototype members of this family. The range of tissues in which bFGF has been detected is extensive and this factor is detectable at specific sites through-out vertebrate development from the oocyte to the adult [Gospodarowicz, 1989; Gospodarowicz, 1991]. The activities that bFGF and aFGF exhibit have with a few exceptions been shown to be typical of the rest of this family. The range of biological activities shown by bFGF is probably the greatest and these activities are listed in Table 1.1 and will be briefly reviewed.

bFGF was isolated by virtue of its mitogenic activity for mouse fibroblasts. It has been subsequently found that bFGF is a potent mitogen for a vast number of cell types. Virtually all mammalian cells of mesodermal and neuroectodermal origin (for example: fibroblasts, large vein and microvascular endothelial cells, myoblasts, melanocytes, and keratinocytes) that grow in cell culture will, under permissive conditions, respond to

bFGF by either entering the cell cycle from a quiescent state or by increasing their rate of cell division if already in a cycling state [Gospodarowicz, 1989; Gospodarowicz, 1991]. Certain cell types derived from the ectodermal and endodermal germ layers are also seen to divide in response to this factor, for example: lens cells, thyroid cells, prostatic cells, and pancreatic cells.

Another activity of bFGF is its capacity in selected cell systems to influence the developmental state. bFGF has been associated with either enhancing differentiation (chondrocytes, melanocytes, endothelial cells, neurons)[Gospodarowicz, 1989; Murphy *et al.*, 1989; Gospodarowicz, 1991; Stocker *et al.*, 1991; Brill *et al.*, 1992] or by delaying differentiation (myocytes, glial cells, adipocytes)[Clegg *et al.*, 1987; Navre and Ringold, 1989; Wolswijk and Noble, 1992]. On an organismal level, bFGF has also been implicated in the primary mesodermal induction event in *Xenopus* [Slack *et al.*, 1987] and in normal development of the male reproductive tract in the rat [Liu and Nicoll, 1988].

bFGF has been shown in a large number of studies to act as a neurotrophic factor [Gospodarowicz, 1989]. bFGF will enhance the survival of various types of neurons in culture [Unsicker *et al.*, 1987; Ferrari *et al.*, 1989], it can enable the survival of neurons that have been lesioned *in vitro*, and treatment of glial cells with bFGF can cause these cells to in turn support the survival of lesioned neurons [Anderson, 1988; Petroski *et al.*, 1991]. In this regard it is interesting that brain was the tissue in which FGF activity was initially detected and that it is found in large quantity in this relatively non-mitotic tissue.

Angiogenesis activity can be operationally defined as the capacity of a substance to foster new vessel (capillary) growth towards a point source of that substance. This activity is important in tissue development and homeostasis and also important in the process of tumor growth in that most tumors require an extensive blood supply and need to actively recruit new vessels to satisfy this requirement [Thomas and Gimenez-Gallego, 1986; Gospodarowicz, 1989]. bFGF is a potent angiogenesis factor when tested in both

the chick chorioallantoic membrane and the rabbit cornea assays of angiogenesis [Thomas and Gimenez-Gallego, 1986; Gospodarowicz, 1989].

The most recent activity that has been attributed to bFGF is in a sense the most unique. bFGF has been shown by two groups to be able to act specifically on transcription reactions performed *in vitro* to either repress or augment the transcription of specific genes [Bouche' *et al.*, 1987; Nakanishi *et al.*, 1992]. These reports take added significance with the observations that the larger forms of bFGF have a nuclear targeting sequence [Bugler *et al.*, 1991], are seen to accumulate in cell nuclei *in vivo* [Shiurba *et al.*, 1991] and are seen to be differentially expressed in development [Giordano *et al.*, 1992]. A direct activity on gene expression is quite distinct from the normal mode of action of polypeptide growth factors. Most growth factors, including bFGF, are thought to act as extracellular signaling molecules and to transduce their effects into the cell interior by binding to and activating specific transmembrane receptors. The FGF family of proteins has a cognate family of FGF receptors of the tyrosine kinase class and many of the effects of bFGF have been shown to act through these receptors [Amaya *et al.*, 1991; Bellot *et al.*, 1991; Rapraeger *et al.*, 1991; Yayon *et al.*, 1991; Givol and Yayon, 1992]. A direct nuclear role for bFGF in addition to the effects transduced through the transmembrane receptors is suggested by this new work.

A number of activities have been ascribed to bFGF that are of a more complex nature and that probably derive from some combination of the activities described above. Examples of these types of activities are: the action of intravenously administered bFGF to lower blood pressure in rats [Cuevas *et al.*, 1991], the ability of bFGF delivered to the site of experimentally occluded retina in chicken embryos to promote retinal regeneration [Park and Hollenberg, 1991], the immortalizing activity of etopically expressed high molecular weight forms of bFGF [Couderc *et al.*, 1991], and the linkage of over expression of bFGF or its receptors with certain types of cancer in humans [Murphy *et al.*, 1989; Adnane *et al.*, 1991; Albino *et al.*, 1991; Takahashi *et al.*, 1991].

Table 1.1 The activities of basic fibroblast growth factor

activity	description	reference
mitogenic activity	bFGF is mitogenic for virtually all cells derived from the mesoderm and neuroectoderm and from selected cell typesderived from embryonic endo- and ectoderm	Gospodarowicz, 1989 Gospodarowicz, 1991
developmental activity	bFGF can suppress differentiation of myoblasts, glial cells, adipocytes	Clegg *et al.*, 1987 Wolswijk *et al.*, 1992 Navre *et al.*, 1989
	bFGF stimulates the differentiation of chondrocytes, melanocytes, endothelial cells, neurons	Gospodarowicz, 1989 Gospodarowicz, 1991 Stocker *et al.*, 1991 Brill *et al.*, 1992
neurotrophic activity	bFGF supports the survival of a variety of types of neucrons in culture	Gospodarowicz, 1989 Ferrari *et al.*, 1989 Unsicker *et al.*, 1989 Anderson *et al.*, 1988
	bFGF causes glial cells to promote the survival of certain neurons in culture	Petroski *et al.*, 1991
angiogenesis activity	bFGF test postive in chick chorioallantoic membrane and rabbit cornea tests for angiogenesis	Thomas *et al.*, 1986
	bFGF is used an an angiogenic therapeutic agent in duodenal ulcer	Folkman *et al.*, 1991
transcription factor activity	bFGF added to in vitro transcription reactions specifically stimulated transcription of the gene Pgk-2, and inhibited the transcription of Pgk1	Nakanishi *et al.*, 1992
misc. complex activities	bFGF injected into rats has hypotensive activity	Cuevas *et al.*, 1991
	bFGF iserted into occluded retina augments regeration	Park *et al.*, 1991
	ectopic expression of high molecular weight forms of human bFGF can immortalize, but not transform, cells	Couderc *et al.*, 1991
	bFGF or its receptor has been associated certain types of cancer	Albino *et al.*, 1991 Adnane *et al.*, 1991 Murphy *et al.*, 1989 Takahashi *et al.*, 1991

The activities of bFGF and by analogy other of the FGFs are both diverse and distinct. Since bFGF is thought not to be a circulating hormone [Gospodarowicz, 1989], bFGF is a factor that almost certainly is a key local regulator of cell and tissue dynamics in development and of cell and tissue homeostasis in the adult. The significance to science of research into how bFGF's expression is regulated is multiplied by the number of roles bFGF is playing *in vivo*. The significance to medicine lies in the enhanced understanding of and in the enhanced opportunity for intervention in processes like, wound healing, neurodegenerative disease, myodegenerative disease, tumorigenesis, and conditions characterized by hyperproliferation or hypoproliferation. Indeed, medical involvement of bFGF in the analysis and treatment of various diseases is already in progress. Two examples of this are found in a recent study implicating overexpression of bFGF in the etiology of Dupuytren's Contracture [Gonzalez *et al.*, 1992], and in another study which shows the utility of bFGF as a therapeutic agent in the treatment of duodenal ulcer [Folkman *et al.*, 1991].

Levels of Regulation of Expression of bFGF

It would be logical to predict that a factor with many biological activities and that was playing a variety of roles at a large number of sites and at distinct developmental stages would possess a very complex apparatus for regulation. The study of how the expression of bFGF is controlled has been going on for several years and Table 1.2 summarizes some of what is known about the complexity of this regulation.

Regulation of bFGF gene expression has been seen to take place in different systems at the levels of: transcription, mRNA half-life, mRNA processing, translation, post-translational processing, and secretion. It has been the focus of my thesis research to gain information about the first of these areas, the regulation of transcription by looking at what agents can cause cells to alter their bFGF mRNA levels (Chapter 2), by

looking for what DNA regulatory elements may be involved in this alteration (Chapter 3), and by looking at what DNA elements are involved in the basalexpression of this factor (Chapter 4).

Table 1.2 Known levels of regulation of bFGF gene expression

level of regulation	example(s)	references
transcription on/off	Only certain cell types have bFGF mRNA for example, BHK cells do not transcribe bFGF.	Gospodarowicz, 1989
termination of transcription	Multiple bFGF mRNAs are seen which derive from different poly-A addition sites. Different distributions of the different mRNA forms are seen as a function of cell type and treatment.	Prats *et al.*, 1989 Gay & Winkles, 1991
transcription rate	TGF-β and PMA have been shown to cause human lung fibroblasts to increase the rate of bFGF transcription.	Goldsmith *et al.*, 1991
mRNA accumulation	FCS, TGF-β, PMA, PDBu, TNF, and Il-1 have been shown to cause various cell types to accumulate bFGF mRNA (probably by altering the rate of bFGF transcription). High glucose, glucosamine, cLDL, thrombin, and cycloheximide have also been shown to cause certain cell types to increase their bFGF mRNA levels. Dexamethasone and Ifn-γ have been shown to cause certain cell types to reduce or block bFGF mRNA accumulation.	Murphy *et al.*, 1988a,b Sternfeld *et al.*, 1988 Winkles & Gay, 1991 Gay & Winkles, 1991 Okamura *et al.*, 1991 Weich *et al.*, 1991 McClain *et al.*, 1992 Kraemer *et al.*, 1993

mRNA t1/2	Cycloheximide has been shown to significantly increase bFGF mRNA t1/2 in a couple of cell types.	Murphy *et al.*, 1990
post-transcriptional mRNA processing	A protein encoded in an antisense orientation within intron2/exon3 of the *xenopus* bFGF gene processes bFGF mRNA by converting selected adenine residues to inosines. This process is thought to be regulated.	Kimmelman *et al.*, 1989
translation	Translation can occur from 4 distinct sites within human bFGF mRNAs. The choice of the sites used is thought to be regulated as a function of development.	Prats *et al.*, 1992 Giordano *et al.*, 1992
secretion (suspected)	None of the protein forms of bFGF contains a classical signal sequence. Some cell types appear to be able to actively secrete it and to vary secretion as a function of treatment. Other cells appear to lack the capacity to secrete bFGF.	Kandel *et al.*, 1991 Mignatti *et al.*, 1991

Chapter 2 Work on bFGF mRNA Accumulation

Introduction

As described in the general introduction bFGF is a multifunctional signaling molecule whose expression and action are almost certainly tied to a great number of biological events and processes *in vivo*. The studies that I will describe in the second chapter of this thesis were undertaken from early in 1988 through mid-1990 and occurred before many of studies on bFGF mRNA regulation reviewed below had been reported. The work that I carried out followed on the heels of the study by Sternfeld *et al.* in 1988 [Sternfeld *et al.*, 1988] which showed that FCS, TGF-β and chx could all affect bFGF gene expression in primary cultures of human dermal fibroblasts. This report along with the early reports of Murphy *et al.* [1988a, 1988b] describing similar effects by extracelluar agents on a human astrocytoma cell line provided the first indications that bFGF gene expression could be altered by exposure of cells to extracellular agents. It was the purpose of the work that I will present in this chapter of the thesis to extend the work of Sternfeld *et al.* and to further describe the phenomenology of the regulation of bFGF mRNA accumulation in primary human dermal fibroblasts in response to various agents.

What is Known About the Regulation of bFGF mRNA Levels

Only one laboratory to date has demonstrated directly that bFGF gene expression can be regulated by changing the rate of transcription. Goldsmith *et al.* [1991] used nuclear run on analysis (NRO) to examine bFGF expression in human lung fibroblasts and found that transforming growth factor-beta (TGF-β) and phorbol esters exerted their positive effects on bFGF mRNA level (a 3 to 4-fold induction) via increasing the rate of

bFGF transcription.

The majority of studies on bFGF gene expression have looked at the regulation of bFGF expression by measuring the accumulation of bFGF mRNA in cells. These studies have employed indirect means to assess whether the observed regulation was being brought about by changing transcription or by post-transcriptional events.

Astrocytoma/Glioblastoma Cells

Murphy and co-workers were among the first to show regulation of bFGF expression at the mRNA level [Murphy *et al.*, 1988a,b]. These researchers studied bFGF mRNA accumulation in two human brain tumor lines, U87-MG and T98-G both of which have been shown to respond mitotically to their endogenously produced bFGF [Takahashi *et al.*, 1990]. The U87-MG astrocytoma line responded to fetal calf serum (FCS), phorbol esters, and platelet-derived growth factor (PDGF) by increasing bFGF mRNA levels by 4 to10-fold over a 3 to 6 hour period [Murphy *et al.*, 1988a; Murphy *et al.*, 1988b]. When the bFGF mRNA half-life (t1/2) was measured it was found that in unstimulated cells the half-life was considerably greater than 5 hours [Murphy *et al.*, 1990]. The changes in mRNA levels caused by FCS, phorbol esters and PDGF in 3 to 6 hours were therefore concluded to be due to increases in bFGF transcription. When the same group studied the bFGF mRNA metabolism in the T98-G glioblastoma cell line they found that the t1/2 in unstimulated cells was 10 minutes and that this could be increased to 120 minutes with the application of chx (chx) [Murphy *et al.*, 1990]. This message stabilization resulted in a 10-fold increase in bFGF mRNA level and this result showed that bFGF mRNA accumulation is regulable in some circumstances by alteration of message half-life. It was seen, however, that even in this cell type FCS and phorbol esters were exerting some of their effect on bFGF mRNA level by a means not related to message half-life, and again an effect on bFGF transcription was implicated.

Smooth Muscle Cells (SMCs)

Three groups have examined bFGF mRNA regulation in smooth muscle cells. Winkles and Gay [Gay and Winkles, 1991; Winkles and Gay, 1991] have shown that in human SMCs bFGF mRNA levels could be increased significantly by exposure of these cells to a variety of agents: FCS, phorbol 12-myristyl 13-acetate (PMA), PDGF, TGF-β, Il-1α, Il-1β, tumor necrosis factor alpha (TNF-α), and the combination of epidermal growth factor (EGF) and insulin-like growth factor 1. The patterns of induction by these agents were similar. Increases in bFGF mRNA started at around 2 hours after exposure and peaked at 6 hours. All of the inductions were eliminated by the co-addition of actinomycin D (act-D) indicating that new transcription was probably involved, but none were eliminated by exposure of the cells to chx suggesting that new translation was not necessary. bFGF mRNA t1/2 measurements in the human SMCs indicated that chx increased the t1/2 in unstimulated cells from 5 hours to greater than 16 hours. Since the agonist mediated changes in bFGF mRNA accumulation in SMCs were seen to take place within 2-6 hours of exposure and given the effect of act-D, it was concluded that the major component of bFGf mRNA accumulation in response to these agonists in this cell type was probably due to changes in the rate of transcription.

In the case of rat smooth muscle cells, McClain *et al.* [1992] showed that treatment of these cells for 3 days with either elevated glucose or with glucosamine, resulted in a 2-3 fold increase in bFGF mRNA.

Kraemer *et al.* [1993] studied rabbit and bovine smooth muscle cells and showed that treatment of these cells for 7 days with cholesterol/LDL or with 25-hydroxycholesterol lead to a 3 to 5-fold increase in the steady state bFGF mRNA levels.

Endothelial Cells

A number of studies have shown that various types of endothelial cells express

bFGF protein or mRNA [Gospodarowicz, 1989]. A few of these studies have looked at regulation. Weich *et al.* [1991] showed that bovine capillary endothelial cells (BCE) would respond to two agents, thrombin and the 18 kD form of bFGF, with a transient 2 to 4-fold elevation in bFGF mRNA level peaking at two hours after exposure. They also found that PMA could cause a transient 3-fold elevation of bFGF mRNA level peaking at four hours after exposure.

Okamura *et al.* [1991] looked at TNF-α induction of bFGF mRNA in human microvascular endothelial cells derived from the omentum (HOME cells). They found that TNF-α caused a 5-fold increase in bFGF mRNA over a 12 hour exposure. Another group studying the same cell type noted that these cells have a high constitutive expression of bFGF and showed that PMA gives very little increase in bFGF mRNA level [Bikfalvi *et al.*, 1990].

Fibroblasts

Several groups have looked at bFGF mRNA regulation in fibroblasts. In the earliest of these studies reported in 1988, Sternfeld *et al.* [1988] showed that TGF-β and FCS could produce either a 3-fold or 10-fold increase in bFGF mRNA in human foreskin-derived fibroblasts over a 4 hour exposure. Also, it was seen that in these cells chx treatment produced a 4-fold increase. This group showed no induction of bFGF mRNA in human fibroblasts in response to bFGF itself. This observation stands in contrast to the report of Weich *et al.* [1991] which indicated that bFGF could induce an increase in its own mRNA in the mouse embryonic fibroblast line NIH 3T3 as well as in BCE cells

Winkles *et al.* [1992] showed that a different strain of human dermal fibroblasts (strain GM 2037) responded to the phorbol esters PMA and phorbol didecanoate (PDD) as well as the non-phorbol tumor promoter mezerein by increasing bFGF mRNA levels 4-fold over a 4 hour exposure. Il-lα was also shown to provide a substantial increase in

bFGF mRNA levels in this same cell strain [Gay and Winkles, 1991].

And, as mentioned above, Goldsmith *et al.* [1991] demonstrated that TGF-β and phorbol esters can act to increase bFGF transcription in human lung fibroblasts resulting in mRNA levels which are 4-fold higher than in unstimulated cells.

Table 2.1 summarizes the literature reports on agonist stimulated accumulation of bFGF mRNA. A general pattern of regulation of bFGF mRNA level appears in these studies. In most cases the elevations that any single effector causes in bFGF mRNA level are modest, less than 10-fold. In most cases, notably excepting the work with glucose and cholesterol, the inductions are transient and take place from 2 to 6 hours after which the bFGF mRNA levels gradually fall such that by 24 post-treatment they are only slightly above the levels in untreated cells. In most systems, the protein synthesis inhibitor chx causes a mild induction in bFGF mRNA level, with the exception being in the T98-G cells where it causes a large increase in bFGF mRNA levels due to a stabilization of a particularly short lived bFGF mRNA in these cells. In most cases where phorbol esters or growth factors are acting the data from experiments using actinomycin D suggest that the most likely mode of regulation is the stimulation of new bFGF transcription. This last idea is corroborated in the specific case of human lung fibroblasts by the results of Goldsmith *et al.* [1991] which demonstrate directly that TGF-β and PDGF can cause an increase in the rate of bFGF transcription.

Table 2.1 Previous studies on bFGF mRNA accumulation

cell type	treatment	fold induction	length of treatment	reference
U87MG, astrocytoma, human				
	FCS	2.5	6hr	Murphy *et al.*, 1988a,b
	replating	7	24 hr	
	PDBu	2.5	6 hrs	
	PDGF	5	6 hrs	Murphy *et al.*, 1988b
	OAG	2.5	6 hrs	
	chx	1.3	6 hrs	Murphy *et al.*, 1990
T98G, gliobalstoma, human				
	FCS	substantial	6 hrs	Murphy *et al.*, 1990
	PDBu	3	4 hrs	
	chx	10	4-6 hrs	
SMC, human (saphenous vein)				
	FCS	14	4-6 hrs	Winkles & Gay, 1991
	TGF-β	3	6 hrs	
	PMA	~6	6 hrs	
	TGF-β,EGF,IGF-1	9	6 hrs	
	EGF/IGF-1	5	6 hrs	
	EGF	1	6 hrs	
	IGF-1	1	6 hrs	
	IL-1α,β	4.5	4 hrs	Gay & Winkles, 1991
	TNF-α	1.3	4 hrs	
	chx	~3	4 hrs	
SMC, rat (aortic)				
	glucose	3	3 days	McClain *et al.*, 1992
SMC, rabbit, bovine				
	cLDL	3	7 days	Kraemer *et al.*, 1993
	25hydroxycholesterol	3-5	7 days	
BCE, adrenal				
	PMA	2.6	4 hrs	Weich *et al.*, 1991
	bFGF	4.3	4 hrs	
	thrombin	2.4	2 hrs	
HOME	TNF-α	5	12 hrs	Okamura *et al.*, 1991
	TPA	δ	1.5–8 hrs	Bikfalvi *et al.*, 1990
HUVE	TPA	substantial	8 hrs	Bikfalvi *et al.*, 1990

cell type	treatment	fold induction	length of treatment	reference
fibroblasts, rat				
	PMA	6-23	3(9) hrs	Lowe *et al.*, 1992
	OAG	5	9 hrs	
	thrombin	5-6	4-12 hrs	
	bradykinin	2.8	4 hrs	
	chx	.57	9 hrs	
fibroblasts, mouse (NIH3T3)				
	bFGF	≥4.3	4	Weich *et al.*, 1991
fibroblasts, human dermal (pooled primary cultures)				
	FCS	10	4	Sternfeld *et al.*, 1988
	TGF-β	3	4	
	chx	4	4	
fibroblasts, human dermal (GM2037)				
	PMA	4	4	Winkles *et al.*, 1992
	PDD	~4	4	
	mezerein	~4	4	
	Il-1α	substantial	4	Gay &Winkles, 1991
fibroblasts, human lung (CCD-19Lu and -8Lu)				
	FCS	4	10	Weich *et al.*, 1991
	TGF-β	4	6	
	PDGF	4	2	

Experimental Approach

The experiments described below were conducted to gain more information about which substances were acting on human fibroblasts in culture to cause them to alter their bFGF mRNA levels and to gain some insight into the mechanisms by which these agents may be acting. As mentioned, these studies were an extension of the work reported by Sternfeld [Sternfeld *et al.*, 1988] described above. While the previous researchers had used northern blot analysis for their characterization of changes in bFGF mRNA levels, the primary tool used in these studies was the quantitative solution hybridization method of Durnam and Palmiter [Durnam and Palmiter, 1983]. This technique was used because the quantity of cells and other reagents required in the studies of Sternfeld and co-workers would have expanded to prohibitive levels in the type of analyses that were planned. The solution hybridization technique offered a sensitive detection of bFGF mRNA level with vastly reduced material and processing requirements per experimental point.

The studies described in this Chapter had as their initial aim the identification of more extracellular agents and conditions that would affect bFGF mRNA accumulation. The interesting combinatorial effects in the actions of the multiple agonists that were encountered became another focus of this work. A third aim of the work was to set the stage for the work reported in Chapter 3.

Materials and Methods

Cell Culture

Normal human foreskin-derived fibroblast strain number 5 (NFF5) was originated in this laboratory by primary isolation from a single donor neonatal foreskin. Foreskin tissue was subjected to overnight digestion with 0.17% trypsin to remove the

epidermis after which the dermis was treated for 2 hours in a 0.25% collagenase in saline solution to disperse the cells. Cells were plated in medium MCDB 202a [Hammond *et al.*, 1984] with 10% FCS. Subsequent cultures were grown in MCDB 202a with 5% FCS. Cells used in these experiments were at approximate population doubling levels of 8-20. Cells were plated for RNA analysis into 10 cm dishes at a density of 2-5 x 10^3 cells/cm^2, and then allowed to grow to confluence in MCDB 202a with 5% FCS. The cultures were then washed free of FCS and maintained for 2 days in unsupplemented MCDB 202a at which point experimental manipulation took place as described. In some studies culture medium DMEM was used in place of MCDB 202a.

SK-hep-1 cells were originally derived from a human hepatic carcinoma [Fogh *et al.*, 1977]. They were grown in McCoy's 5A medium supplemented with 10% FCS and were not serum deprived prior to RNA harvest.

Northern Blot Analysis

Poly-A^+ RNA was prepared as previously described [Sternfeld *et al.*, 1988]. Poly-A^+ RNA and RNA size markers (0.24-9.5 kb RNA ladder, Bethesda Research Laboratories) were electrophoresed through 1.2% agarose gels containing 2.2 M formaldehyde. Capillary transfer of the poly-A^+ RNA onto nylon membranes (Schleicher & Schuell Nytran+) was carried out overnight according to the manufacturer's instructions. Hybridization of blots with *in vitro* transcribed cRNA probes was conducted overnight at 62°C using 1-5 x 10^6 cpm /ml of probe in a hybridization mix consisting of: 50% formamide, 250 µg/ml herring sperm DNA, 10X Denhardt's solution (2 mg/ml polyvinylpyrrolidone, 2 mg/ml bovine serum albumin, 2 mg/ml Ficoll 400), 50 µg/ml oligo(dA), 0.5% SDS, 0.75 M NaCl, and 0.075 M sodium citrate. Post-hybridization washes were carried out over 2-3 hours at 65°C with several changes of 0.1% SDS, 0.1 x SSC (1x SSC is 0.15 M NaCl, 0.015 M sodium citrate). Autoradiography was carried out using Kodak XAR-5 film with one intensifying screen

at -80°C for the time indicated.

Solution Hybridization Analysis

Quantitation of bFGF mRNA was accomplished by the solution hybridization technique of Durnam and Palmiter [1983] with slight modifications. NFF5 cells were harvested by scraping in 1% SDS, 10 mM Tris pH 7.5, 5 mM EDTA. Total Nucleic Acid (TNA) was extracted from the lysate by brief sonication followed by digestion with proteinase K (50-100 µg/ml for 1 hour at 45°C), phenol/chloroform extraction and ethanol precipitation. TNA samples were hybridized to [^{32}P]-labeled bFGF cRNA probes at 68°C overnight in a solution consisting of: 667 mM NaCl, 11 mM Tris pH 7.5, 4.6 mM EDTA, 44% formamide, and 0.033% SDS in a total volume of 30 µl. Following hybridization, 1 ml of a solution containing 30 µg/ml ribonuclease A (Sigma), 2 µg/ml ribonuclease T_1 (Sigma), 100 µg/ml herring sperm DNA, 600 mM NaCl, 10 mM Tris pH 7.5, and 4 mM EDTA was added to each sample and RNA digestion was carried on for 45 minutes at 37° C. RNA protected from digestion was precipitated with trichloroacetic acid, collected onto glass fiber filters and counted in a liquid scintillation counter. In each solution hybridization experiment samples were included which contained known amounts of unlabelled *in vitro* generated coding-strand RNA. The cpm given by these known coding-strand RNAs were used to generate a standard curve relating cpm measured on filters and mass of bFGF mRNA in a given sample. The total amount of bFGF mRNA a sample contained was divided by a measure of the number of cell equivalents that sample represented obtained either by measuring the amount of DNA fluorimetrically [Labarca and Paigen, 1980] or by measuring the absorbance of the sample at 260 nm. The data from the solution hybridization experiments are expressed in picograms of coding-strand equivalent per µg of nucleic acid or pg/µg. Error bars indicate the standard deviation of the mean of duplicate hybridizations of a given sample.

Nuclear Run On Transcription

Nuclear Run On transcription (NRO) was carried out by a modification of the procedure reported by Eriksson *et al.* [1991]. The nuclei of NFF5 cells which had been treated for either 2 or 4 hours with various combinations of FCS, TGF-β, and PDD were harvested by the procedure reported by Gough [1988] and then washed and resuspended and frozen at -70 °C in the buffers reported by Eriksson *et al* [1991]. At the time of NRO, the nuclei (approximately 2 x 10^6) were thawed on ice, centrifuged and resuspended in transcription buffer. Transcription was carried out at 30 °C for 30 minutes in a 150 µl reaction containing: 20 mM Tris pH 8.0, 2 mM $MgCl_2$, 180 mM KCl, 0.67 µM DTT, 667 µM ATP,667 µM GTP, 6.7 µM UTP, 6.7 µM CTP and 100 µCi each 3000 Ci/mMole [^{32}P]-UTP and [^{32}P]-CTP. After the 30 minute reaction, 100 µl of DNase I buffer (10 mM tris pH7.4, 500 mM NaCl, 50 mM $MgCl_2$ and 2 mM $CaCl_2$) was added along with 150 units of DNase I and the reaction was allowed to continue for 15 minutes at 30 °C. At this point 120 µl of proteinase K solution (1.25% SDS, 25 mM EDTA, 25 mM Tris pH 7.5 and 2 µg/ml proteinase K) was added and the mixture was incubated at 37°C for another 30 minutes. The mixture was then extracted twice with 1:1 phenol/chloroform and precipitated with isopropanol in the presence of 2 M ammonium acetate. The precipitated nucleic acids were collected by centrifugation, washed twice with 70 % ethanol, and resuspended in 20 µL water of which 1 µL was counted in a scintillation counter. Filters for NRO were prepared by applying 2 µg of linearized denatured plasmid to Nytran + membranes (Schleicher & Schuell) using a Hybri-slot filtration manifold (Bethesda Research Laboratories). The labeled nuclear transcripts, 2 X 10^6 cpm, were hybridized to the membranes in glass scintillation vials the manner described by Greenberg and Bender [1990], in 4 mL of a hybridization mixture containing: 50% formamide, 5X SSC, 5X Denhardt's solution, 200 µg/ml denatured fish sperm DNA, 50 µg/ml E coli tRNA, and 0.2% SDS. Hybridization was undertaken for 2 days with shaking at 42 °C. Post-hybridization washes were done with several changes

of 2X SSC, 0.1% SDS at 60 °C over two hours. Autoradiography was carried out using Kodak XAR-5 film with one intensifying screen at -80°C for the time indicated.

Probes

The cRNA probe for bFGF used in this study was synthesized from plasmid pGb530 which consists of the 0.53 kb Bal I/EcoRI restriction fragment from human bFGF cDNA clone λHFL-1 (kindly provided by Dr. Judy Abraham of Scios Nova Inc. and described in Abraham *et al.*, 1986b) inserted into the Sma I site of the vector pGEM4z (Promega). This fragment contains the first 530 nucleotides of sequence 3' to the translational stop codon of the human bFGF gene. This region was chosen to serve as a probe in an attempt to minimize the possibility of inadvertent cross-hybridization with transcripts from other members of the FGF gene family. *In vitro* transcription reactions were carried out using linearized pGb530 essentially according to the method reported by Melton *et al.* [1984] to generate coding-strand bFGF RNA to serve as a standard in the solution hybridization technique or to generate [^{32}P]- labeled complementary strand bFGF RNA to use as a probe in the solution hybridization and in northern blot analyses. Probe specific activities of 1-2 x 10^9 cpm/μg were generated.

The plasmids used for NRO were: pGb530, pGa479, KC3, pGEM4z, and pβ-actin. pGb530 is described above. pGa479 contains 479 bp of the coding region for human acidic fibroblast growth factor inserted into the vector pGEM4z. pGk320 contains 320 bp from exon 3 of the human gene for keratinocyte growth factor (KGF) inserted into the vector pBS KS II (Stratagene). pGEM4z is an insertless vector (Promega). pβ-actin is a vector which contains the coding regions of the human β-actin gene and was the gift of Dr. Paul Woloshin (Oregon Health Sciences University).

Chemicals and Reagents

TGF-β1 was purified from human platelets in our laboratory using techniques previously described [Assoian *et al.*, 1983]. Actinomycin D, PMA, PDD, 4αPDD, and chx were obtained from the Sigma Chemical Corporation. PDGF AB was obtained from Upstate Biotechnology. Thapsigargin was purchased from LC Services. FCS was purchased from JR Scientific and Hyclone Laboratories. MCDB 202a was prepared in our laboratory from component chemicals as described [Hammond *et al.*, 1984]. DMEM was obtained from GIBCO.

Results

Northern Blot Analysis

Northern blot analysis was done to verify that the probe to be used in solution hybridization was binding to previously identified forms of bFGF mRNA. Fibroblasts were grown to confluence, changed to serum-free medium for two days, and then exposed for four hours to effective doses of various candidate inducer substances. Poly-A^+ RNA was extracted, electrophoresed, blotted onto nylon membranes and probed with [^{32}P]-labeled cRNA generated from plasmid pGb530, all as described above. Two major bands are seen in most lanes in Figure 2.1. These bands have the approximate sizes 7.1 and 3.7 kb and correspond to the major bFGF species identified by Sternfeld in another strain of human dermal fibroblast [Sternfeld *et al.*, 1988] and by others in other human cell lines [Schweigerer *et al.*, 1987; Murphy *et al.*, 1988b; Goldsmith *et al.*, 1991] using double stranded DNA probes spanning the coding region. Thus, the bFGF mRNAs detected using the 530 base anti-sense RNA probe are the same as those detected using DNA probes made from sequences in the translated region.

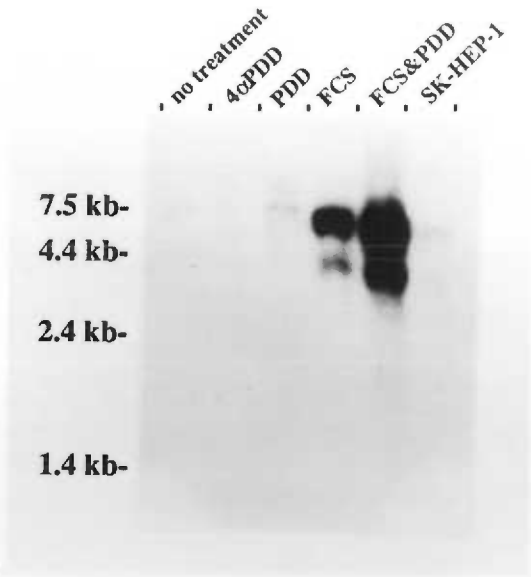


Figure 2.1 Northern Blot Analysis: bFGF mRNA accumulation in NFF5 cells following exposure to serum, and phorbol ester

Confluent human foreskin fibroblasts (strain 5 or NFF5) were washed free of serum and maintained in serum-free MCDB 202a for 2 days and then given either no treatment or a 4 hour treatment with an effector. SK-hep-1 cells were grown to confluence in serum containing medium (DMEM). Poly-A+ RNA was isolated from both cells types as described in the Materials and Methods section. 1.5 μg of poly-A+ RNA per lane was electrophoresed through 1.2% agarose containing 2.2 M formaldehyde and then transferred onto a nylon membrane. The membrane was hybridized with a bFGF cRNA probe made from plasmid pGb530 as described. Lanes 1-5 show NFF5 RNA from cells that were treated for 4 hours with the following: lane 1, no addition; lane 2, 100 ng/ml 4αPDD; lane 3, 100 ng/ml PDD; lane 4, 5% v/v FCS; and Lane 5, 100 ng/ml PDD & 5% v/v FCS. Lane 6: 1.5 μg RNA from SK-hep-1 cells growing in serum containing medium.

Also observable in Figure 2.1 is the difference in bFGF mRNA levels as a function of treatment. Exposure of human fibroblasts to either FCS, PDD or to a combination of these agents resulted in an increased accumulation of bFGF mRNA (lanes 3, 4, 5 compared to lane 1). Lane 2 shows the bFGF mRNA levels in cells treated for 4 hours with 4αPDD. This stereoisomer of PDD which is not active as a tumor promoter and does not activate protein kinase C did not act to raise bFGF mRNA levels (compare lanes 1 and 2). Lane six shows the bFGF mRNA levels in a similar amount of poly-A+ RNA from a human liver tumor cell line, SK-hep-1, previously shown to express bFGF mRNA [Abraham *et al.*, 1986b]. It can be seen that human fibroblasts grown in culture and stimulated by either FCS or FCS+PDD have considerably more bFGF mRNA than do SK-hep-1 cells (compare lane 4 or 5 with lane 6).

Effects of FCS, PDD, or TGF-β on Induction of bFGF mRNA

The capacity of FCS, PDD, and TGF-β to stimulate fibroblasts to increase their bFGF mRNA level was studied quantitatively by the solution hybridization method of Durnam and Palmiter [1983]. Figure 2.2 shows the graphs relating the amount of bFGF mRNA accumulation to the amount of FCS, PDD, or TGF-β added to the cells in a 4-hour exposure. Panel A shows the FCS dose response. A maximum effect was reached at about 5% v/v with the half maximum being around 3% v/v. 5% v/v FCS was used for subsequent experiments. Panel B shows a PDD dose response. Maximum induction of bFGF mRNA accumulation by PDD was seen at 10 ng/ml (15nM) and full effect was maintained at concentrations of up to 1000 ng/ml in 4-hour exposures. A dose response study was also done for another active phorbol, phorbol 12,13-myristyl acetate (PMA), and this yielded similar results (data not shown). A concentration of 100 ng/ml was chosen for subsequent experiments with phorbol esters to assure a maximum effect. The inactive stereoisomer of PDD, 4αPDD, produced no increase in bFGF mRNA levels when used at concentrations of up to 100 ng/ml (Figure 2.1 and other data). The dose

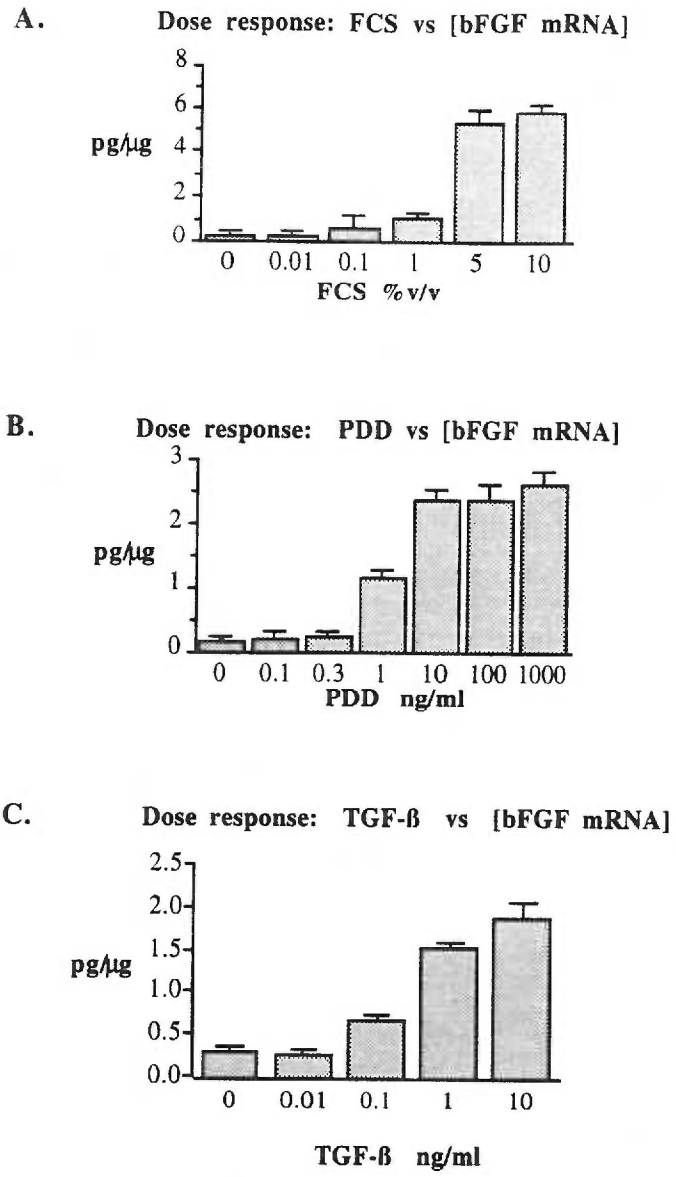


Figure 2.2 Dose response curves for FCS, PDD, and TGF-ß vs bFGF mRNA level

Confluent human fibroblasts were maintained for 2 days in unsupplemented MCDB 202a and then given the indicated concentrations of FCS, PDD, or TGF-ß. At 4 hours after the addition of these agents, total nucleic acids were harvested and examined by solution hybridization as described in the Materials and Methods section. Error bars represent the standard error of the mean of two independent solution hybridization analyses done on a given total nucleic acid sample.

response relation for TGF-β is seen in panel C. A maximum effect of TGF-β to cause bFGF mRNA accumulation was seen between 1 and 10 ng/ml. A concentration of 5-10 ng/ml was used for subsequent experiments.

Time Course of bFGF mRNA Accumulation

The time course of induction of bFGF mRNA accumulation by FCS or PDD was examined to determine the optimum time of exposure of cells to these agents and to determine if they were acting with similar kinetics. Figure 2.3 (panel A) shows the time course of mRNA accumulation in response to FCS. At one hour after serum addition, a rapid increase in bFGF mRNA level began, with maximum levels being seen between 3 and 4 hours post-addition. The peak mRNA levels in this experiment were about 18 times higher than those measured in untreated cells. Twenty-four hours after serum addition, the cellular bFGF mRNA levels were back to levels only slightly above basal levels.

Panel B of Figure 2.3 shows a similar analysis done for the stimulation of bFGF mRNA accumulation by 100 ng/ml PDD. The accumulation of bFGF mRNA following PDD exposure shows a pattern similar to that following serum exposure with a response shifted to slightly later times.

Additivity of the effects of FCS, PDD, and TGF-β

Experiments were conducted to assess whether the effects of FCS, PDD, and TGF-β were non-additive, additive, or synergistic in the induction of bFGF gene expression. Cells were exposed for four hours to maximally effective doses of each factor alone or to various combinations of the three factors. The nucleic acids were then isolated and the bFGF mRNA content quantitated by solution hybridization. The results of a representative experiment are shown in Figure 2.4. When added singly, PDD or TGF-β showed a small but definite capacity to increase bFGF mRNA levels while FCS

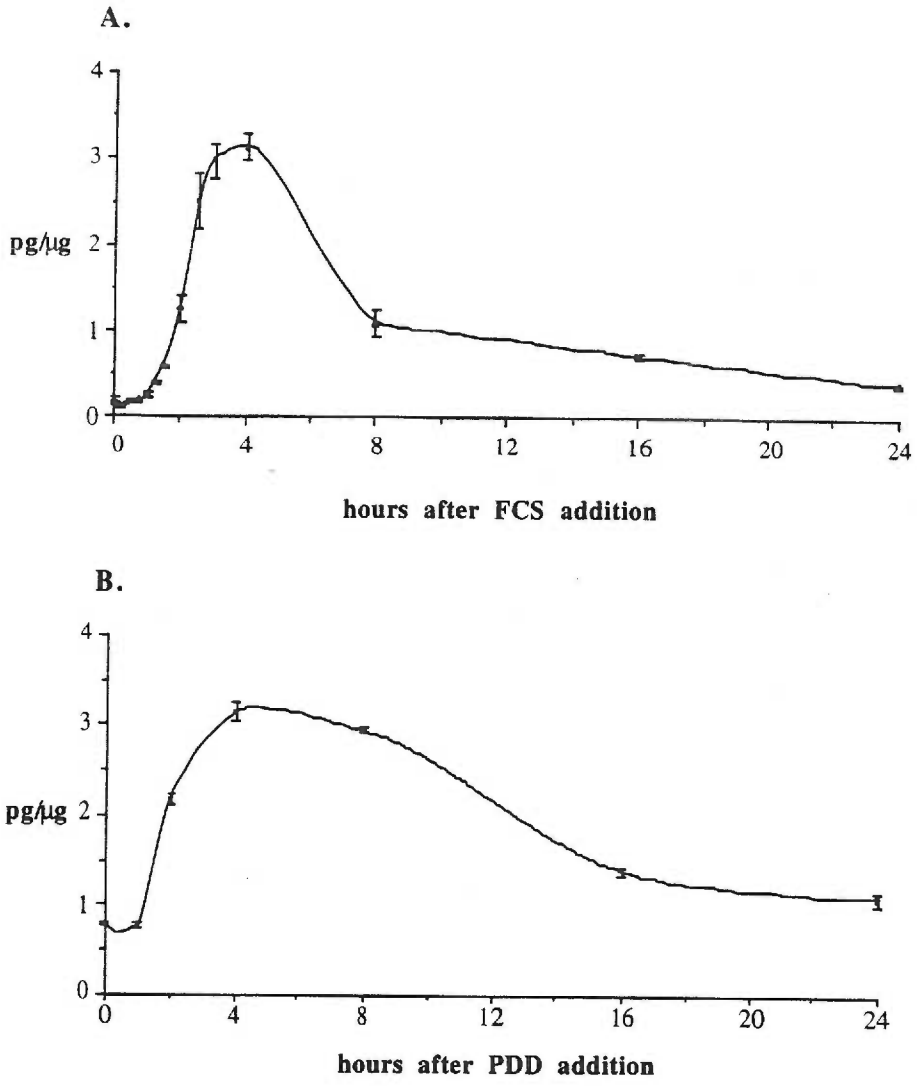


Figure 2.3 Time course of bFGF mRNA accumulation in response to serum and phorbol esters Confluent human fibroblasts (strain NFF5) were maintained for 2 days in unsupplemented MCDB 202a and then given either 5% v/v FCS or 100 ng/ml PDD. At various times after addition of either agent, total nucleic acids were harvested and examined by solution hybridization as described in the Materials and Methods section. Values are the average of 2 solution hybridization analyses done on a given sample and error bars represent the standard error of the mean.

added alone produced a more sizable increase in bFGF mRNA abundance. When added in concert the effect on mRNA accumulation produced by any pair of inducers was greater than the sum of the individual effects. In this experiment, the addition of all three agents resulted in only marginally greater bFGF mRNA accumulation than was produced by the co-addition of any two of the agents. This was a consistent finding.

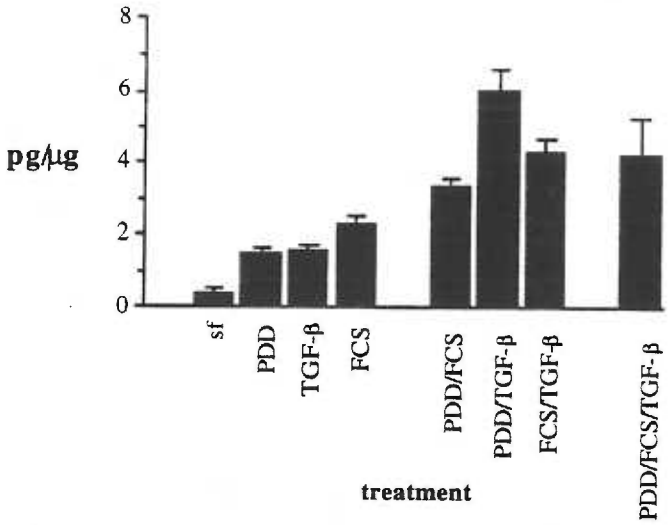


Figure 2.4 Additivity of effect of agonist action
Confluent human fibroblasts (strain NFF5) were maintained for 2 days in unsupplemented medium and then given either no addition (sf) or given various combinations of the agents: PDD, TGF-ß, and FCS. After 4 hour exposures to these agents total nucleic acids were isolated and examined for bFGF mRNA content by solution hybridization as described in the Materials and Methods section. The values shown are the average of 2 solution hybridization analyses done on a given sample and error bars represent the standard deviation of the mean.

Figure 2.5 shows the effects of adding the same combinations of agents described above to cells that had been pre-treated for 24 hours with 100 ng/ml PDD. This pretreatment was designed to produce a downregulation of cellular protein kinase C activity and thus to allow assessment of which of the inductive effects caused by FCS, PDD, and TGF-β were dependent on this activity. The solid bars in this figure show the

effect of adding the same agonist combinations as used in the previous figure to cells given no pretreatment. The open bars show the effect of applying these treatments to cells which had been given a 24 hour PDD pretreatment. PDD pretreatment completely eliminated the capacity of an acute dose of PDD to alter bFGF mRNA accumulation. PDD pretreatment almost completely eliminated the ability of FCS to cause accumulation of bFGF mRNA. PDD pretreatment had little apparent effect on TGF-β stimulation of bFGF mRNA accumulation.

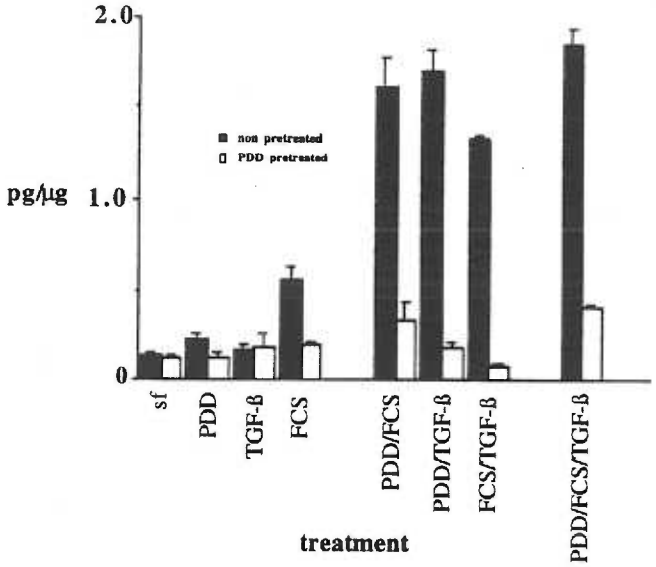


Figure 2.5 Additivity of effect of agonist action and the effect of chronic phorbol ester exposure

Confluent human fibroblasts (strain NFF5)were maintained for 2 days in unsupplemented medium. One group of cells (open bars) was given 100 ng/ml PDD for the second day of this period. After two days in unsupplmented medium cells here then given either no addition (sf) or given various combinations of the agents PDD, TGF-ß, and FCS. After 4-hour exposures to these agents total nucleic acids were isolated and examined for bFGF mRNA content by solution hybridization as described in the Materials and Methods section. The values shown are the average of 2 solution hybridization analyses done on a given sample and errors bars represent standard deviation of the mean value.

Effect of Cycloheximide

To further categorize and distinguish the actions of FCS, PDD and TGF-β,

experiments were performed using the protein synthesis inhibitor cycloheximide (chx). Confluent serum deprived cells were treated with 15 μg/ml chx for 1 hour and then exposed for 4 hours to other factors in the continued presence of chx. The nucleic acids were then isolated and the bFGF mRNA levels were measured. Figure 2.6 displays the results of a representative experiment. Cycloheximide, when added in the absence of other agents, caused an increase in bFGF mRNA levels by a factor of 2 (the range of increase over a number of experiments being 2-6). Pretreatment with chx eliminated the capacity of PDD to increase bFGF mRNA levels. In fact, the bFGF mRNA levels that resulted from the addition of PDD in the presence of chx were consistently lower than those produced by chx alone. In contrast, the combination of chx pretreatment and TGF-

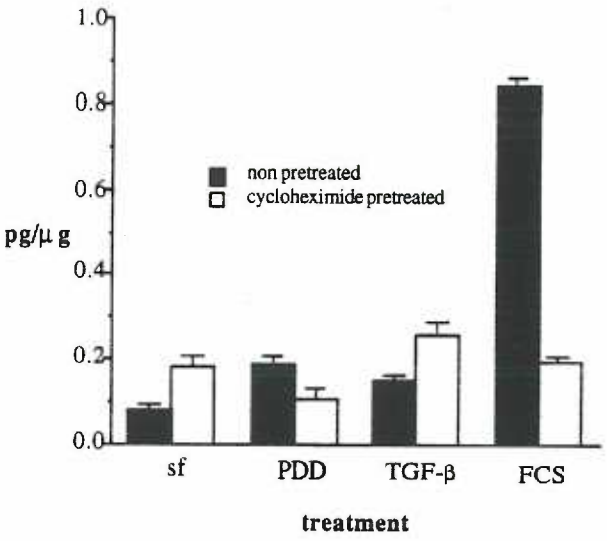


Figure 2.6 The effect of cycloheximide on agonist stimulation of bFGF mRNA level

Confluent human fibroblasts (strain NFF5) were maintained for 2 days in unsupplemented medium. At the end of this period one group of cells (filled in bars) was given a one hour pretreatment with 15 μg/ml chx. Following this, groups of cells were given either no addition (sf) or 100 ng/ml PDD, 5 ng/ml TGF-ß, or 5% v/v FCS. After 4 hours, total nucleic acids were isolated and examined for bFGF mRNA content by solution hybridization as described in the text. The values shown are the average of 2 solution hybridization analyses done on a given sample and error bars represent the standard deviation of the mean value.

β treatment resulted in higher levels of bFGF mRNA than produced by either agent alone. The effect of chx on serum-treated cells was variable but in most cases chx substantially decreased but did not eliminate the capacity of FCS to cause increased bFGF mRNA accumulation (as displayed in Figure 2.6).

Effect of Actinomycin D

Actinomycin D (act-D) is an agent which intercalates into a DNA duplex and which has been shown to inhibit RNA synthesis [Atwater *et al.*, 1990]. Act-D was applied in two ways in these investigations. One way was to add 5-10 μg/ml act-D along with agents like FCS or PMA and then to look at bFGF mRNA accumulation at 4 hours. act-D co-addition along with such agonists eliminated their inductive effect, Figure 2.7.

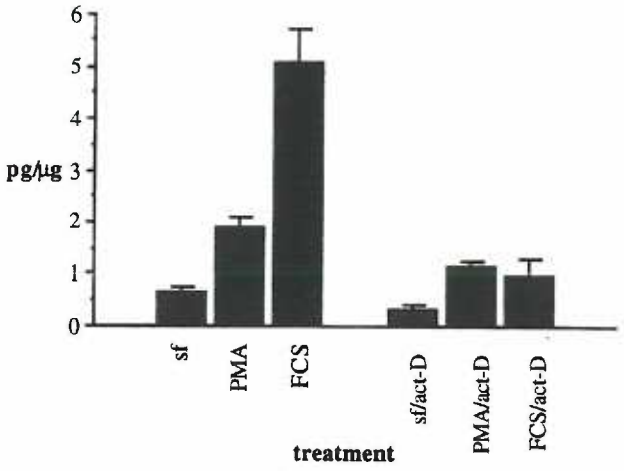


Figure 2.7 The effect of actinomycin-D on bFGF mRNA induction by phorbol esters and serum

Confluent human fibroblasts (strain NFF5) were maintained for 2 days in unsupplemented medium. At the end of this period some groups of cells (bars on the right) were given a one half hour pretreatment with 10 μg/ml act-D. Following this, groups of cells were given either no addition (sf), 100 ng/ml PMA, or 5% v/v FCS. After 4 hours, total nucleic acids were isolated and examined for bFGF mRNA content by solution hybridization as described in the text. The values shown are the average of 2 solution hybridization analyses done on a given sample and error bars represent the standard deviation of the mean value.

In another type of experiment, act-D was added 4 hours after the addition of candidate agonist combinations, at the time of their maximum effect. At various times after act-D addition, the bFGF mRNA content of the cells was analyzed by solution hybridization. Because act-D is predicted to stop new bFGF mRNA synthesis, the plot of bFGF mRNA level over time after act-D exposure should indicate bFGF message stability. If an agonist or agonist combination were acting to change bFGF message stability this type of experiment should show this as an agonist dependent change in the slope of the curve of bFGF mRNA level as a function of time. Several such experiments were undertaken and Figure 2.8 shows the results from a representative example. No clear trend of agonist effect on the bFGF message stability was observable in such

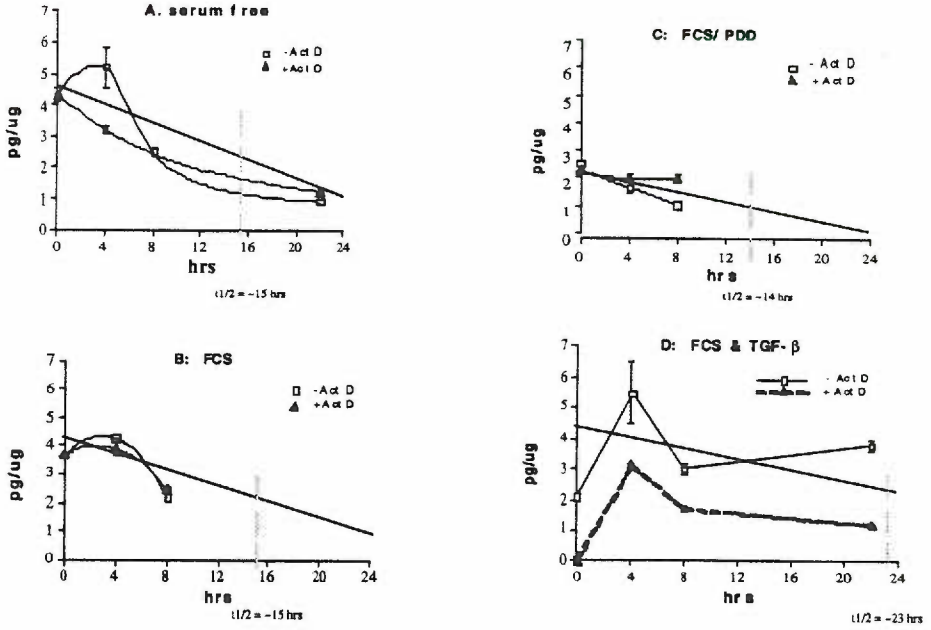


Figure 2.8 bFGF mRNA level after actinomycin-D treatment
Confluent human fibroblasts (strain NFF5) were maintained for 2 days in unsupplemented medium. They were then given either no addition (panel A) or additions of 5% v/v FCS (panel B), 5% v/v FCS and 100 ng/ml PDD (panel C), or 5% v/v FCS and 10 ng/ml TGF-ß (panel D). At 4 hours after addition of these agents act-D was added to some of the dishes (▲) to 10 μg/ml. At various times after act-D addition cells from all groups were harvested and their total nucleic acids examined for bFGF mRNA content by solution hybridization as described in the text. The values shown are the average of 2 solution hybridization analyses done on a given sample and error bars represent the standard deviation of the mean value.

experiments. The data in all such experiments displayed the variations seen in Figure 2.8. Another characteristic of these experiments is that no consistent effect of act-D could be detected on bFGF mRNA level. What is seen, as shown in Figure 2.8, is that a natural decay in the bFGF mRNA level does occur with time with or without act-D treatment. The indicated mRNA half-life calculated from the data shown in Figure 2.8 is 14 to 23 hours although the variation in the data from all such experiments tends to limit the reliability of these values. Another limitation of these experiments is that the expression of a second gene with a known t1/2 was not examined as a function of act-D exposure to allow the evaluation of the efficacy of the act-D treatment.

Nuclear Run On

Nuclear run on (NRO) transcription was attempted with the NFF5 cells in order to gain direct evidence as to whether agonists like FCS, TGF-β, and PDD were causing elevation of bFGF transcription. Figure 2.9 shows the result of the most successful NRO experiment. In this figure, which corresponds to an autoradiogram exposed for 10 days, only the slot corresponding to β-actin had a detectable signal. No bFGF transcription was detectable from nuclei isolated from agonist treated or untreated cells in this or in other experiments. Likewise, no transcription was measurable from either the aFGF or KGF genes, genes known to be expressed in this cell type [Finch *et al.*, 1989; Cook *et al.*, 1990]. The most successful NRO detected only modest expression from the human β–actin gene which has been shown to give good NRO signal in this cell type [Quinones *et al.*, 1989](Figure 2.9) and so the sensitivity of this procedure as attempted was concluded to be insufficient to detect the transcription of the bFGF gene.

Other Effectors

I examined a number of substances for capacity to affect bFGF gene expression in human fibroblasts. The positive results obtained to date from solution hybridization

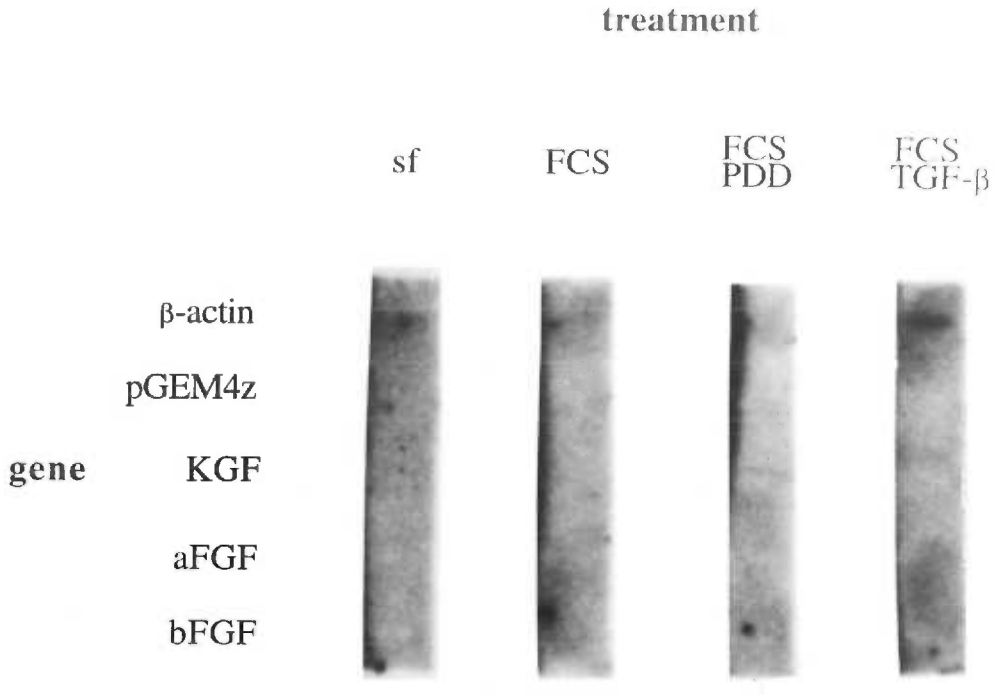


Figure 2.9 Nuclear Run-On Analysis

Nuclear Run-On transcription (NRO) carried out according to Eriksson *et al.*, 1991. Approximately 2 x 10^6 nuclei were isolated from NFF5 cells treated for 4 hours with either, no exogenous agent (sf), 10% v/v fetal calf serum (FCS), 10% FCS and 100 ng/ml PDD, or 10% FCS and 10 ng/ml TGF-β, and then subjected to transcription *in vitro* in the presence of [32P]-labeled nucleotides. Total RNA was isolated and hybridized for 2 days tonylon filters which had affixed to them 2 μg each of plasmids containing coding regions for the listed genes. Following hybridization the filters were washed and autoradiography was carried out for 10 days at -80 ^{o}C using Kodak XAR-5 film with 1 intensifying screen.

experiments are summarized in Table 2.2. Where multiple experiments with an agent have resulted in different values, the range has been listed. The range of values point out the variability in effect of these agents from experiment to experiment, a phenomenon which can also be detected by comparison between Figures 2.1-2.5.

As seen in Table 2.1, FCS produced the largest increases in bFGF mRNA level of any single agent, giving up to 20-fold increases over a 4 hour exposure (routine stimulations were on the order of 10-fold). PDD, TGF-β, and chx produced effects of 2-10 fold, 2-6 fold, and 2-6 fold, respectively.

Table 2.2 Substances showing capacity to effect bFGF mRNA levels in human fibroblasts

substance	fold bFGF mRNA increase
fetal calf serum (FCS) 5% v/v	5-20
phorbol 12,13-didecanoate (PDD) 100 ng/ml	2-10
phorbol 12,13-myristyl acetate (PMA) 100 ng/ml	2- 5
transforming growth factor beta (TGF-β) 5 ng/ml	2- 6
thapsigargin 2 μM	1- 2
platelet-derived growth factor (PDGF AB) 10 ng/ml	2- 5
cycloheximide (chx) 10-15 μg/ml	2- 6

Confluent human fibroblasts were maintained for 2 days in unsupplemented medium and then given a 4 hour exposure to the substances listed. Total nucleic acids were isolated and bFGF mRNA content was assessed by solution hybridization as described in the text. Values for fold bFGF mRNA increase were calculated by dividing the bFGF mRNA level measured in treated cells by the bFGF mRNA level measured in untreated cells in the same experiment. The range of values reported is the range seen over several experiments.

Two other substances were shown to increase bFGF mRNA levels in fibroblasts, platelet-derived growth factor heterodimer (PDGF AB) and the non-phorbol tumor

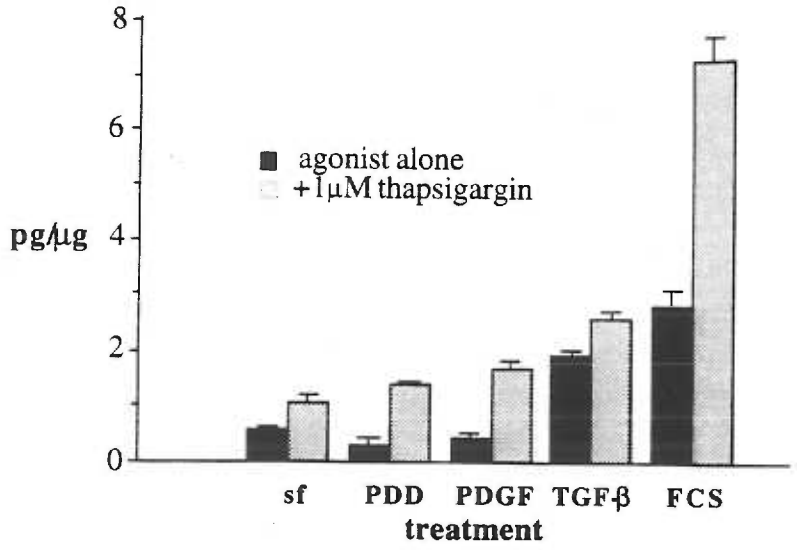


Figure 2.11 Additivity of the effect of thapsigargin and other agonists on bFGF mRNA accumulation

Confluent human fibroblasts were maintained for 2 days in unsupplemented medium. They were then given no treatment (sf) or treated with 100 ng/ml PDD, 5 ng/ml PDGF, 10 ng/ml TGF- β, or 10 % v/v FCS either in the absence (black bars) or presence (grey bars) of 1 µM thapsigargin. After 4 hours, the total nucleic acids were isolated and examined for bFGF mRNA content by solution hybridization as described in the text. The values shown are the average of 2 solution hybridization analyses done on a given sample and error bars represent the standard deviation of the mean value.

PDGF, or FCS with the most pronounced effect being the combination of thapsigargin and FCS.

Substances Which Showed No Effect on bFGF mRNA Level

Table 2.3 shows the substances which were screened for effect in this system and which did not, as used, give any reproducible alteration in NFF5 bFGF mRNA. The trial exposures of these compounds were done for 4 hours and the concentrations selected were based on literature values of the concentrations of these substances which have been shown to be effective in other cases. The qualifications on this data are that only a

small number of trials were done with test agonists which gave a negative result and that no internal positive measure of each agonists activity in the NFF5 strain was obtained.

Table 2.3 Substances showing no consistent capacity to affect bFGF mRNA levels in human fibroblasts

substance	concentrations examined
basic fibroblst growth factor (bFGF)	2 ng/ml
acidic fibroblast growth factor (aFGF)	1 ng/ml
epidermal growth factor (EGF)	10 ng/ml
insulin	1 μg/ml
insulin-like growth factor I (IGF-I)	10 ng/ml
tumor necrosis factor (TNF)	10 ng/ml
8-Br cAMP	250 μM
estradiol	10^{-5}-10^{-9} M

Confluent human fibroblasts were maintained for 2 days in unsupplemented medium and then given a 4 hour exposure to the substances listed. Total nucleic acids were isolated and bFGF mRNA content was assessed by solution hybridization as described in the text. None of the substances listed gave any effect on bFGF mRNA accumulation when delivered at the specified concentrations in a 4 hour exposure.

Discussion

The Shipley laboratory originated the study of the regulation of bFGF mRNA accumulation in human foreskin-derived fibroblasts in response to extracellular agents. Sternfeld *et al.* [1988] showed that bFGF mRNA levels in this cell type could be increased by exposing cells to FCS, TGF-β or to chx. The work that I have done in

partial fulfillment of my Ph.D. thesis has extended these observations to include time courses of induction and dose response relations for FCS and TGF-β. I have also demonstrated that a variety of other substances can affect bFGF gene expression in these cells and I have characterized some potentially important combinatorial effects of these substances.

Time Course of bFGF mRNA Accumulation

The time courses of bFGF mRNA accumulation after exposure to FCS or PDD are similar to those displayed by the "immediate-early" response gene *c-myc* in response to serum [Dean *et al.*, 1986]. Both of the time course curves in Figure 2.3 show that the stimulation of increased bFGF mRNA levels by these agents is transient. In the case of PDD, this is consistent with the findings reported by Winkles *et al.* [1992] using human dermal fibroblasts, by Weich *et al.*[1991] using bovine capillary endothelial cells, and by Lowe *et al.*[1992] using rat dermal fibroblasts. This transience of phorbol ester effect on bFGF mRNA accumulation was not seen by Murphy *et al.* [1988b] using the U87-MG astrocytoma cell line. Although these researchers examined times of up to 12 hours after phorbol ester exposure, they found no decrease in bFGF mRNA level.

The effect of FCS on bFGF mRNA accumulation was also shown to be transient, with a maximum effect being manifest at 4 hours and bFGF mRNA levels returning to levels just slightly greater than those in unstimulated cells by 24 hours after acute FCS exposure, Figure 2.3A. The transience of the serum effect is in agreement with the results obtained by Goldsmith *et al.* [1991] using lung fibroblasts and with results of Winkles and Gay [1991] using human vascular smooth muscle cells. In contrast, Murphy *et al.* [1988a] showed that the bFGF mRNA level in the U87-MG astrocytoma cells remained elevated even at 24 hours after FCS exposure. An even more striking contrast is provided by work from the Shipley laboratory done with strains of human foreskin derived fibroblasts similar or identical to the NFF5 strain that I used. Two

studies reported that rapidly dividing human fibroblasts maintained a FCS elevated bFGF mRNA level for 24 hours or longer [Sternfeld *et al.*, 1988; Cook *et al.*, 1990].

A major difference in the experiments reported by Sternfeld and by Cook and my studies is that cells in the present experiments were quiescent prior to serum addition, while cells in the experiments of Sternfeld *et al.* [1988] and Cook *et al.* [1990] were continuously and asynchronously cycling. One hypothesis which could be advanced to reconcile the FCS time course data is that some aspect of the quiescence-to-cycling transition that occurs after acute serum exposure limits the serum-induced bFGF mRNA accumulation and that this phenomenon is not seen in asynchronous cycling populations.

The apparent non-transience in the effect of both phorbol esters and FCS in the case of the U87-MG cells, cells which like the fibroblasts used in my studies had been quiescent prior to agonist exposure, points to a cell type dependent difference in the response to these agonists or to some other difference in the metabolism of bFGF mRNA in these cells.

A transience in the elevation of bFGF mRNA level in response to extracellular agonists has also been seen for Il-1 [Gay and Winkles, 1991], thrombin [Weich *et al.*, 1991], and bFGF [Weich *et al.*, 1991]. Other agents like glucose [McClain *et al.*, 1992] and cholesterol [Kraemer *et al.*, 1993] have been seen to produce a more stable elevation in bFGF mRNA level although it can be pointed out that the stable elevation is quite modest in both cases.

bFGF mRNA metabolism

The question of the stability or transience in the agonist mediated elevation in bFGF mRNA level raises the greater issue of how these increases are achieved. As reviewed briefly above, it has been demonstrated in isolated cases that changes in bFGF mRNA level can be brought about either by changing the rate of transcription or by alteration of the mRNA t1/2. The NRO experiments that I attempted in the course of

these studies failed on the technical grounds of insufficient sensitivity to give direct information as to whether FCS, PDD, and TGF-β are acting to alter bFGF transcription in NFF5 cells. The experiments with act-D suggest, but do not prove, that the action of these agonists is on bFGF transcription in these cells. Act-D co-addition prevented FCS or PMA from inducing an increase in bFGF mRNA (Figure 2.7). And, the estimated mRNA t1/2 of >13 hours from experiments like those in Figure 2.8 was too long to allow for the rapid changes in bFGF mRNA accumulation seen in response to FCS, PDD or TGF-β to occur via message stabilization. That is, it is difficult to explain how increasing the bFGF mRNA t1/2 to a value longer than 14 hours could result in the bFGF mRNA accumulations seen in response to various agonists at 2-4 hours. This conclusion is similar to that reached by Winkles and Gay [1991] from their work on human vascular smooth muscle cells and to that of Murphy *et al.* [1990] from their work with the U87-MG cells.

While it seems clear that transcriptional regulation is playing a role in the regulation of bFGF gene expression in response to various agonists, it is also clear that several questions remain with respect to bFGF mRNA metabolism, particlularly in the area of message stability. Protein synthesis inhibitors can change the apparent t1/2 of bFGF message drastically from 10 minutes to 2 hours or from 5 hours to 16 hours depending on the cell type [Murphy *et al.*, 1990]. One can hypothesize that what protein synthesis inhibitors are effecting is a system for actively regulating bFGF message level via degradation. In this context a reexamination of the data in Figure 2.3A shows an interesting effect. The decrease in bFGF mRNA level that is seen after the maximum accumulation is achieved at approximately 4 hours exposure to FCS would appear to be faster than predicted by the t1/2 estimates. Taking the curve shown Figure 2.3A and assuming that all transcription of bFGF mRNA stops at 4 hours after exposure to FCS, one arrives at a t1/2 drawn from this data closer to 3 hours than the 15 hours estimated from act-D experiments. If the assumption that transcription has stopped at 4 hours after

FCS exposure is incorrect then an even shorter half-life would be indicated. It is possible that in these experiments act-D is altering a system for the active clearance of bFGF mRNA and that the t1/2 values obtained using act-D represent an artificial or exceptional case of bFGF mRNA metabolism. Such an interference of protein or RNA synthesis inhibitors on the measurement of message half life has been noted in other cases and is an important issue in the use of these substances in the measurement of messenger RNA stability [Atwater *et al.*, 1990].

One specific hypothesis that would fit the FCS and PDD time course data would be the existence of an agonist-stimulated bFGF mRNA clearance mechanism that acts to limit agonist stimulaton of bFGF mRNA to a single pulse of expression and that this mechanism is interfered with by RNA synthesis inhibitors and possibly by protein synthesis inhibitors. One could imagine that in certain transformed cell types like U87-MG this post-stimulation suppression response is not functioning, giving rise to enhanced autocrine growth and to an experimental FCS effect on bFGF mRNA accumulation which lacks any down-regulation.

The outstanding issues on bFGF mRNA metabolism could be looked at with the combination of a successful NRO analysis and a more sensitive acrylamide gel-based RNase protection assay done on nuclei and RNA from cells which had been treated with the combinations of agonists and inhibitors used in the above analysis.

Evidence for Multiple Pathways

The question of what signal transduction mechanisms a given agent employs to bring about a change in bFGF expression is a complicated one. At this point only circumstantial evidence exists to predict what a full mechanistic dissection will find. Evidence displayed here leads to the prediction that at least three pathways exist in human fibroblasts to bring about bFGF mRNA accumulation in response to extracellular effectors. The most compelling evidence comes from the additivity studies of the type

shown in Figures 2.4 and 2.5. The fact that the bFGF mRNA accumulation caused by a maximally effective dose of FCS, TGF-β, or PDD is much less than that caused by the co-addition of any pair of these factors suggests that each of these factors exerts its effect through a pathway which is, at least in some of its elements, distinct. If, for example, FCS was acting solely or even mostly through the same response pathway as PDD, then one would expect no additivity of effect from exposing human fibroblasts simultaneously to FCS and PDD at their maximally effective concentrations. It is notable that Goldsmith *et al.* [1991] found that TGF-β and PDGF had an additive effect in inducing bFGF mRNA levels in human lung fibroblasts. I do not believe that this represents the same effect as that seen between TGF-β and FCS in our cells for reasons discussed below.

The interesting observation that the simultaneous addition of FCS, PDD, and TGF-β results in no or little more mRNA accumulation than does the addition of any two of these agents points perhaps to some common control stage that is maximally engaged when two pathways are being used or alternatively to a secondary effect caused by one or more of these agents that acts to limit expression.

The attenuated response of fibroblasts to FCS, PDD or TGF-β in chronically PDD treated, protein kinase C depleted, cells (Figure 2.5, open bars) indicates one of two situations: (1) intact cellular protein kinase C activity is important to all of the signal transduction pathways that these agents employ to alter bFGF mRNA level or (2) protein kinase C activity is important to the general maintenance of higher bFGF mRNA levels. Since phorbol esters used at their optimal concentrations do not give a maximum accumulation of bFGF mRNA (phorbol esters alone give at most 6-fold increases while up to 40-fold increases can be obtained with some combinations of inducers) it can be concluded that while a protein kinase C requiring step is an important part of whatever mechanisms are operating to control bFGF mRNA level, other important parts exist and contribute to regulation.

The data from experiments performed using chx treatment (Figure 2.6) also

give evidence for multiple routes of action. The ability of FCS to augment bFGF mRNA levels is partially (and variably) diminished by chx, while the effects of PDD and chx are actively antagonistic and the effects of TGF-β and chx appear additive.

Other Inducers

The observation that PDGF can stimulate the accumulation of bFGF mRNA in these cells is in line with previous observations with other cell types [Murphy *et al.*, 1988a; Goldsmith *et al.*, 1991]. I believe that it is unlikely that a significant amount of the effect of FCS is due to the presence of PDGF in serum. The PDGF predicted to be delivered in 10% FCS would be approximately 0.1 ng/ml [Bowen-Pope *et al.*, 1989]. This amount of PDGF is nearly two orders of magnitude lower than the minimum amount required to influence bFGF in my experiments with cultured human fibroblasts (Figure 2.10).

Thapsigargin is a sesquiterpene lactone which has been shown to be a mild tumor promoter in mouse skin [Hakii *et al.*, 1986]. Its mechanism of action is distinct from that of phorbol ester tumor promoters PDD and PMA and apparently involves the release of intracellular calcium into the cytoplasm from intracellular stores [Thastrup *et al.*, 1990]. Its action to cause accumulation of bFGF mRNA is consistent with recent reports of its capacity to increase the rate of transcription from viral promoter/enhancer constructs [Lenormand *et al.*, 1990]. My preliminary experiments with this compound show that it acts synergistically with FCS or PDD to increase bFGF mRNA accumulation (Figure 2.11). This apparent synergy of the action thapisgargin points perhaps to a fourth distinct route to control of bFGF gene expression. The action of thapsigargin, by itself a very modest inducer but in combination with other agents giving very substantial effects, again underscores the fact that single agonists cause only small changes in bFGF gene expression and that the orchestration of a number of inductive effects is required for

large, 20-40 fold, increases in bFGF mRNA levels.

I find it particularly interesting that two different tumor promoters, thapsigargin and PDD, apparently have synergistic effects on the gene expression of a potent growth and differentiation factor like bFGF. The report of Winkles *et al.*[1992] adds a third distinct tumor promoter, mezerin, to this list of bFGF expression agonists as well.

Summary

In Chapter 2 of this thesis I have reported that serum, phorbol esters, thapsigargin, TGF-β, PDGF, and chx can modulate the levels of bFGF messenger RNA in human foreskin-derived fibroblasts. I have shown that combinations of inducers can produce bFGF mRNA levels far in excess of those produced by the most potent single inducer, FCS. Circumstantial evidence leads to the prediction that these changes in mRNA level are being caused by agonist effects on the rate of transcription from the bFGF gene. The patterns of modulation of bFGF gene expression caused by these agents suggest that there are multiple pathways for controlling bFGF mRNA level in human fibroblasts. I hypothesize that these pathways are distinct at their initial points and that some of them converge to share distal transduction elements. I feel that the multiple pathways indicated for the control of bFGF gene expression being activated by the diverse set of agents I and others have identified reflects the diversity of functions that bFGF is likely to be playing *in vivo*.

Chapter 3 Work on the bFGF upstream region

Introduction

Chapter 3 of this thesis concerns work done to examine the sequences near the start of the human bFGF gene for regulatory elements, particularly elements which may be transducing the effects of FCS, TGF-β, phorbol esters, and thapsigargin, on bFGF transcription. This examination was limited to the region from -2000 to +483 relative to the start of bFGF transcription as determined by Shibata *et al.* [1991].

Upstream Regulatory Sequences

The sequences associated with a gene which are responsible for the regulation of its expression as a function of tissue, developmental status, exposure to extracellular factors or cell state can be termed the regulatory sequences of a gene [Dynan and Tjian, 1985; Sassone-Corsi and Borrelli, 1986; Marriott and Brady, 1989]. Regulatory sequences or elements usually consist of small stretches of DNA 7 to 40 bp long which are thought to specifically bind effector proteins which are involved in the regulatory process [Marriott and Brady, 1989]. Regulatory elements may be located either upstream of the coding region, in introns, within the coding region itself, or downstream from the coding region [Sassone-Corsi and Borrelli, 1986]. Among genes characterized to date the most common location of regulatory sequences is in the region upstream from the start of transcription. It was the purpose of the work reported in Chapter 3 of this thesis to analyze the bFGF upsream region for regulatory elements.

What is Known about the bFGF Promoter and Upstream Regulatory Sequences

Shibata *et al.* [1991] were the first to report an analysis of human bFGF gene for regulatory loci. These researchers demonstrated by primer extension done on RNA samples from two different cell types that bFGF transcription appeared to be initiated accurately at a point 318 bp upstream from the most upstream site of translational initiation. The region surrounding the +1 site identified by primer extension was examined for known functional elements and for functional promoter activity. With respect to known elements, Shibata noted the absence of TATA box or CAAT box motifs which are associated with many eukaryotic promoters and normally localized to the region -25 and -60 bp upstream from the start of transcription [Dynan and Tjian, 1985]. The only known elements that were identifiable in the region near to the +1 site were 5 binding sites for transcription factor Sp1 located at positions -166, -139, -83, -65, and +45 relative to the +1 transcriptional start. These are displayed in Figure 3.1.

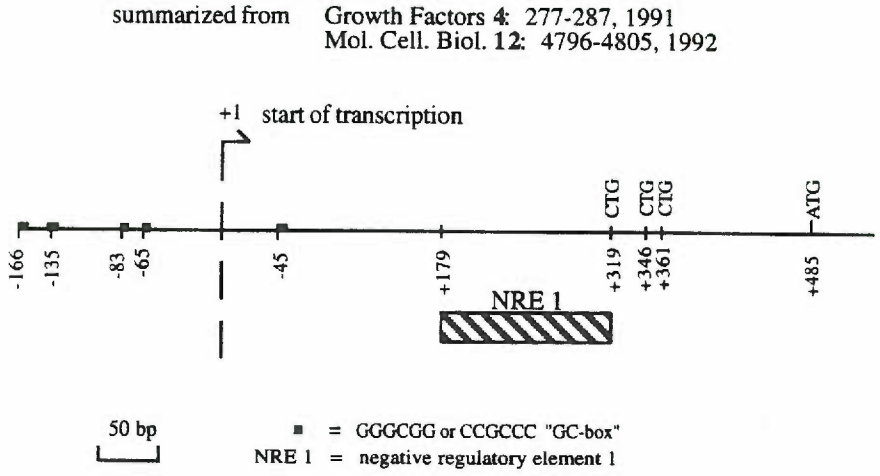


Figure 3.1 Human bFGF gene immediate upstream region
This is a representation of the region around the transcriptional and translational start sites of the human bFGF gene. A putative negative regulatory element, NRE 1, and 5 potential binding sites for transcription factor Sp1 are indicated.

Promoter activity

This same laboratory, led by Dr. Robert Florkiewicz, also attempted to localize the bFGF promoter by a functional analysis. The approach that was used was to insert various amounts of bFGF sequence from the region surrounding the +1 site into a vector with the coding region for the gene for chloramphenicol acetyl transfersase (CAT). These constructs were transfected into Te671 cells and then extracts from the transfected cells were examined for CAT enzyme activity. In such assays one expects to see CAT activity only if the heterologous sequences that have been inserted into the CAT vector contain a promoter. The amount of CAT activity that is seen is proportional to the strength of the inserted promoter.

Figure 3.2 displays results of an analysis reported by Shibata *et al.* of promoter activity contained within bFGF sequences surrounding the +1 site. A CAT construct which contained bFGF sequences -480 to +173 gave the greatest expression of CAT. Constructs with more upstream sequence , -780 to +173, -1000 to +173 and -1800 to

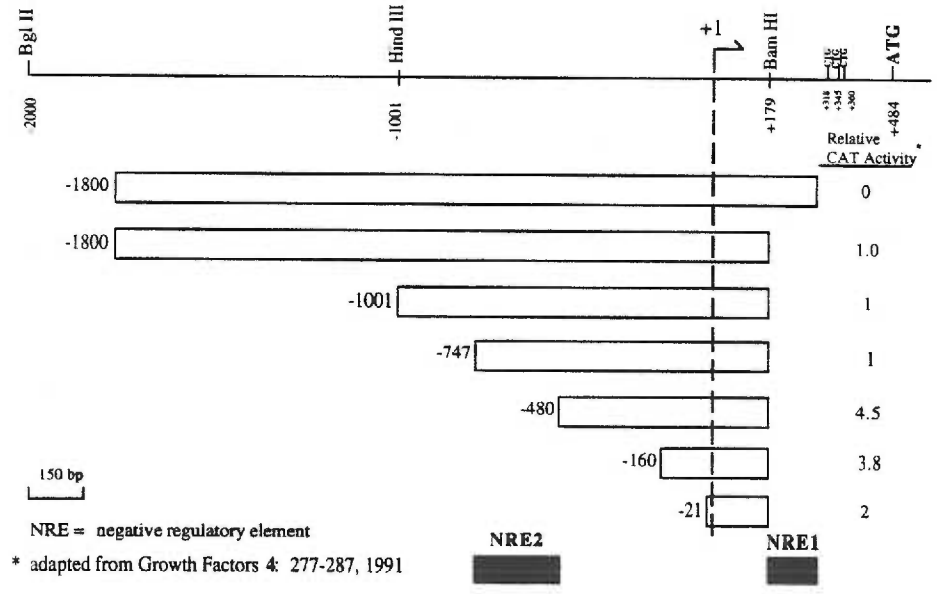


Figure 3.2 data from Shibata *et al.* on promoter activity contained in the region upstream from the +1 site of the human bFGF gene
This figure is redrawn from data reported by Shibata *et al.* [1991]. It displays the relative CAT activity supported by a series of 5' deletion constructs containing various amounts of sequence from the human bFGF promoter region in transient expression assays done using Te671 cells.

+173, gave considerably less expression of CAT and this was attributed to the probable presence of a negative regulatory element, termed NRE2, located between -780 and -480.

Constructs which contained -21 to +173 gave one half of the maximum activity conferred by the -480 to -173 construct suggesting that positive regulatory elements were contained in the region -480 to -21. The amount of CAT expression supported by the -21 to +173 construct was significant with respect to background and indeed was twice that supported by the -1800 to +173 species. This data indicated that the -21 to +173 region contains functional promoter activity for the bFGF gene.

Interestingly, Shibata *et al.* reported that constructs which contained sequences -1800 to +317 gave no significant expression of CAT. This was attributed to the presence of an additional negative regulatory element, termed NRE1, in the region +173 to +317 which was acting either to repress transcription or to interfere with translation. Another group subsequently analyzed the bFGF +1 to +483 region and their conclusions based on a study of the *in vitro* translatability of various parts of this region were that this region contained elements which inhibited translation and which were responsible for alternative translational start codon choice [Prats *et al.*, 1992]. Their data did not directly resolve the question of whether sequences in the NRE1 region were also acting to repress transcription from the bFGF promoter. The bFGF sequences from -1 to +539 did not, however, appear to affect the efficiency of transcription from a cytomegalovirus promoter that was used in their studies [Prats *et al.*, 1992].

Known Upstream Elements Appearing in the bFGF Upstream

Two sequences present in the bFGF upstream region were discussed by the Florkiewicz group. The sequence TGAGTCA located at -243 to -237 is similar to the consensus binding site for the transcription factor AP-1. AP-1 sites have been shown to be able to confer phorbol ester responsiveness to genes [Angel *et al.*, 1987] and in one case responsiveness to TGF-β [Kim *et al.*, 1989]. The other sequence discussed by these

authors is a region of near-dyad symmetry which is located at -597 to -565 (see Figure 3.6). This dyad symmetry element (DSE) lies within the region identified as containing NRE2 and is homologous to a region in the c-myc gene that is thought to be involved in negative regulation of that gene [Hay *et al.*, 1987]. These authors failed to note that this DSE contains at its core the sequence GGATTTATCC which is an inverted copy of the CArG box consensus sequence, $CC(AorT)_6GG$ [Miwa *et al.*, 1987] . The CArG box element is thought to provide the core of a functional serum response element or SRE which can transduce the effect of serum treatment into increased transcription [Treisman, 1985; Liu *et al.*, 1991; Parker *et al.*, 1992]. In the case of the proximal fos CArG box this sequence has also been shown to be able to confer transcriptional responsiveness to bFGF [Parker *et al.*, 1992]. As discussed above, the bFGF gene has been shown to be transcriptionally responsive to TGF-β, to phorbol esters and to serum and it has been shown that in certain cases bFGF can act to elevate its own mRNA level so the presence of the AP-1 and CArG-like sequences in the bFGF gene are of potential significance. Recently, another inverted CArG box, termed a GArC box, was discovered and shown to be a regulatory site in the gene for human cardiac heavy chain myosin [Mably *et al.*, 1993].

In summary, the present knowledge of the organization of the promoter and upstream regions for the human bFGF gene is as follows. A single unique start of transcription is located 320 bp upstream from the most upstream translational start site. A functional promoter for the bFGF gene is located within the sequences -21 to +173. Elements with positive affect on expression, possibly including 4 Sp1 binding sites, are located in the region -480 to -21. Elements with negative effects on expression, NREs 1 and 2, are located in the regions +173 to +317, and -780 to -480. And, elements with homology to the consensus AP-1 binding site, to the c-myc DSE, and to the CarG/GArC box elements are located in the bFGF upstream sequence and may be involved in the regulation of this gene in response to serum or growth factors.

This preliminary characterization of the human bFGF gene's upstream region leaves many questions unresolved. As described in Chapter 2 of this thesis, a large amount of data exists to predict that bFGF transcription is regulated at the level of transcription in response to the extracellular agents: FCS, TGF-β, phorbol esters, PDGF, IL-1α, IL-1β, TNF-α and perhaps others. DNA sequence elements have been described which can in other genes transduce the effect of these extracellular agents into increased transcription [Treisman, 1985; Angel *et al.*, 1987; Kim *et al.*, 1989; Ray *et al.*, 1989]. It was the purpose of the work described in Chapter 3 to localize and identify the DNA sequence elements resident in the upstream region of the bFGF which are responsible for transducing the effect of some of these agents into increased transcription of the bFGF gene.

Materials and Methods

Cell Culture

Te671 cells were obtained from the American Type Culture Collection (ATCC, Rockville Maryland) culture #ATCC CRL 8805, TE671 subline No. 2. U87-MG cells were obtained from ATCC culture #ATCC HTB 14. Normal human foreskin-derived fibroblast strain 5 (NFF5) was isolated as described in Chapter 2. The Rat-1 fibroblast cell line was originated as described by Prasad *et al.* [1976]. The Rat-1 subline used in these experiments is the same as that reported in the studies of Lenormond *et al.*[1990] . All cell strains and lines were grown in DMEM (GIBCO) supplemented with 10% calf serum (Hyclone) in a 5% CO_2 humidified incubator.

Transfection

Transfection of the above cells was most commonly done using the calcium

phosphate precipitate technique described in Current Protocols in Molecular Biology [Kingston, 1987b]. The cells were grown to 80-90% confluence in 60 mm dishes and 5 μg of CAT plasmid DNA was added in 0.5 ml of a HEPES buffered calcium phosphate suspension prepared as described. In most experiments each 5 μg of CAT plasmid was co-transfected in the same precipitate with 0.5 μg of a luciferase expression vector, either pGL-Promoter or pGL-Control (Promega). Three plates received equal aliquots of the same calcium phosphate/DNA precipitate. The DNA was allowed to remain on the cells overnight (12-18 hours) after which the cells were given fresh medium and allowed to recover for 12 hours to 1 day. In most experiments the cells were at this point washed free of serum containing medium and allowed to remain in serum-free DMEM for 2 days at which point they were challenged with various agonists.

In the case of NFF5 cells transfection was attempted also by use of the DEAE-dextran method reported by Holter *et al.* [1989], by electroporation using a BTX 4000 electroporation device (BTX) according to manufacturer's instructions, and by the use of a cationic lipid reagent, Transfectam (Promega), according to manufacturer's instructions.

Creation of Cell Lines Stably Expressing CAT

Rat-1 cells were co-transfected with various bFGF constructs along with a plasmid pSV2neo, which contains the gene for neomyosin resistance linked to the SV40 early gene promoter and which confers on cells resistance to the antibiotic Geneticin or G418 (GIBCO). DNAs were co-transfected using the calcium phosphate method described above at a 20:1 ratio of test CAT plasmid to pSV2neo plasmid. Following recovery from transfection, cells were replated into medium containing 700 μg/ml G418 and cultured for a couple of weeks until G418 resistant colonies of cells grew out. Selected clones of cells were isolated and grown up in the presence of G418 for assay of CAT activity.

CAT Assays/Luc Assays

Chloramphenicol acetyl transferase (CAT) activity in cellular extracts was assayed by the two phase technique reported by Neumann *et al.* [1987] with the modification that 1 ml of scintillation fluid (Econofluor II, NEN) was used. Cellular extracts were made in two ways which appear to have yielded equivalent results. The first way was to collect the cells by scraping in cold DMEM, to pellet the cells by centrifugation and then to resuspend them in a small volume of 0.5 M Tris pH 7.4/O.25% triton X-100 (Tris/Triton solution). The second way was to aspirate all of the culture medium from the cells and then to add and scrape in a small volume, e.g. 100 µl, of Tris/Triton solution. Once in the Tris/Triton solution via either route, the extracts were stored at -20°C until assayed. Prior to CAT assay the extracts were heat inactivated at 70°C for 10 minutes and the particulate matter was spun down. 10 to 50 µl of the clarified heat-inactivated extracts was added to scintillation vials for CAT assay.

The controls used in CAT assays were: mock CAT assays done with Tris/Triton solution without the addition of cellular extracts, CAT assays done with extracts from cells transfected with the promoterless plasmid pCAT-Basic (Promega), and CAT assays done with extracts from cells transfected with either pCAT-Control (Promega), a plasmid that contains the strong promoter and strong enhancer regions from the SV40 early gene region driving CAT expression or pCAT-Promoter (Promega), a plasmid that contains the promoter region of the SV40 early gene region driving CAT expression.

CAT data is expressed as cpm [^{3}H] acetyl-CoA transferred into the organic layer (scintillation fluid layer) per hour. This was calculated by subtracting the cpm counted in a given tube at time 1, t_1, from the cpm counted in the same tube at time 2, t_2, and then dividing this by t_2-t_1, the time between the two counting events. Data points are the average of the values given by three scintillation reactions with each scintillation reaction being performed with extract independently harvested from a single 60 mm dish of

transfected cells. Error bars represent the standard deviation of the mean value. No error bars were applied to Figure 3.9 because the CAT assays were not done in replicate. The large number of experimental conditions made replicate CAT assays impractical and the extracts from multiple wells of cells were physically combined, hence in a sense physically averaged, a process that did not allow for the calculation of error.

In most cases the CAT data has been normalized to the expression of a co-transfected luciferase (Luc) expression vector. Since within a given experiment, a constant amount of luciferase vector was transfected into all dishes, the variation in Luc expression within an experiment was taken as a measure of the transfection efficiency and as the basis for normalization. For a given DNA precipitate which went onto three dishes of cells, the Luc expression was averaged. Within each experiment a Luc data point was selected as a basis for normalization. Usually this was a point with a value near to the median Luc expression of the experiment and with near the lowest standard deviation of the mean error value. The Luc average values for all of the independent precipitates were divided by this value to obtain a normalization factor. Average CAT values and their standard deviation values were divided by this normalization factor to give a normalized CAT value with a normalized error value. In most cases the changes in the data trends were slight after the normalization process.

The assay for luciferase (Luc) activity was carried out using a method based on the Luciferase Assay System of the Promega Corporation and described in Promega technical bulletin #101 and references found within. Cellular extracts were made as described above for the CAT assay. Prior to the heat inactivation step in the CAT assay protocol, an aliquot of extract was removed for Luc assay and stored at -20°C until assayed. 5-10 µl was assayed in 12 x 75 mm borosilicate tubes. Extract was delivered to the tubes and then the tubes were loaded into an AutoLumat LOB 953 Luminometer (EG&G Berthold). At the time of analysis the machine automatically injected 300 µl of a solution containing: 20 mM tricine, 1 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$,

0.1 mM EDTA, 33 mM DTT, 270 μM coenzyme A, 470 μM luciferin, and 530 μM ATP. The light emission from each sample was measured for 25 seconds and recorded by the luminometer in arbitrary light units.

Library Screening

A human genomic DNA library in the vector EMBL 3 was obtained from Dr. Michael Forte (Oregon Health Sciences University). It contained inserts of approximately 16-20 kb of human genomic sequence made from a partial SauIIIa digest of human genomic DNA inserted at the BamHI site of the EMBL 3 vector. This library was propagated and plated in bacterial strain LE392. Plaque lifts were done on nylon membranes (Colony Plaque Screen, NEN) according to manufacturer's instructions. Various human bFGF sequences were used as probes to screen this library (described below). Successful screening was done using an EcoRI/Sty I fragment of human genomic DNA which was contains in plasmid pGEbFGF obtained from Dr. Judy Abraham (Scios Nova). pGEbFGF is a plasmid which contained human bFGF upstream sequences from -478 to +479 relative to the start of transcription along with the cDNA region derived from bovine mRNA and a downstream flanking region derived from human growth hormone all inserted into the EcoRI site of vector pUC8. The 140 bp fragment excised from pGEbFGF for use in screening the genomic library constitutes the most upstream part of the genomic clone λMG4 described by Abraham *et al.* [1986b] and lies 962 to 822 bp upstream from the ATG translation initiation codon of the human bFGF gene. The 140 bp fragment was labeled by random hexamer extension in the presence of [^{32}P] CTP. Hybridization was done in a solution containing: 1 x 10^6 cpm/ml denatured probe, 25 mM Na_2HPO_4, 5X SSC, 1X Denhardt's solution (2 mg/ml polyvinylprrolidone, 2 mg/ml bovine serum albumin and 2 mg/ml ficoll 400), 50 μg/ml denatured fish sperm DNA, 50% v/v formamide, and 1% SDS for 19 hours at 42°C. Post-hybridization was carried out for 90 minutes at 42°C with four changes of 2X

SSC/2%SDS (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate). Autoradiograms of the hybridized filters was done at room temperature without screens for 2 days using Kodak XAR-5 film.

Secondary and tertiary screening of positive λ clones was done in the same manner with a 195 bp EcoRI/Nhe I fragment of pGEbFGF (described below) which consists of the most upstream 195 bp of the genomic clone λMG4 described by Abraham *et al.* [1986b].

Mapping

Preliminary mapping of the λ6.1 bFGF genomic clone was done using restriction endonuclease digestion according to established protocols. Southern blot analysis of the restricted λ6.1 DNA was done on nylon membranes according to the manufacturer's instructions (Nytran+, Schlieicher & Schuell) using the same 195 bp probe described above in the secondary and tertiary screening of positive λ clones.

Cloning

Subcloning from the bFGF genomic clone λ6.1 was done using established techniques, such as those reported in Current Protocols in Molecular Biology (Wiley Interscience, 1992). bFGF sequences were subcloned into the vectors pBSKSII+(Stratagene), pCAT-Basic (Promega) and pTKp. pTKp is a vector constructed by Dr. Phillipe Lenormonde while in the Magun laboratory (OHSU, Portland, OR) and which contains promoter sequences -109 to +16 relative to the transcriptional start site of the herpes simplex virus thymidine kinase gene inserted into the vector pCAT-Basic.

Nomenclature

CAT constructs containing bFGF promoter sequences were named according to the 5'-most base contained in that sequence. For example pCB1000 (or pCB1k) contains bFGF sequences from -1000 to +173. All 5' deletion constructs had the 3' endpoint of

+173 except for pCB103 which had sequences -103 to +17. Internal deletion constructs were named according to parental plasmids followed by ∂ indicating deletion and the first letter of the restriction enzymes used to make the deletion. pCB1k∂HN denotes a plasmid made by deleting a Hind III-Nhe I fragment from pCB1k. Cassette constructs of bFGF upstream sequence in plasmid pTKp, a derivative of pCAT-Basic containing the herpes simplex virus thymidine kinase (HSV TK) promoter, were named according to the restriction enzymes used to excise the bFGF cassette. pTKp RR denotes a plasmid with a Rsa I-Rsa I fragment from the bFGF upstream region. One cassette construct, pTKp285p was named after the 285 bp size of its bFGF insert which was lifted out of plasmid pGEbFGF using PCR with the primers F5, 5'-CCCGCACCCCAAACCACGT-3' a primer complementary to sequences in the upstream region of the bFGF gene -191 to -171 relative to the +1 site, and the vector, and βGAL1, 5'-CATCTATGACCATGATTACG-3' a primer complementary to vector sequences in pCAT-Basic, pUC8, and pBSKSII+ (Stratagene) flanking the multiple cloning sites of these vectors.

Sequencing

DNA sequence analysis on subcloned fragments of λ6.1 was done using a Sequenase 2.0 Kit (USB) according to manufacturer's instructions. The sequencing primers used were: RevCAT, 5'-CTGAGCTCCTGAAGATCTCGCCAAGCTC-3', a primer complementary to sequences in the CAT gene of pCAT-Basic, βGAL1 (described above), or F1, 5'-ACAGAGGCTGAAGTCGTCTACCAAC-3' a primer complementary to sequences in the upstream region of the bFGF gene -461 to -437 relative to the +1 site.

PCR

Polymerase chain reaction (PCR), both preparative and analytical, was done in a Coy Model 50 Programmable Incubator (Coy Laboratory Products) using Taq DNA

polymerase and polymerase buffer from Promega. The standard cycle that was used was 96°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds.

IPCR

Inverse polymerase chain reaction (IPCR) was attempted as described by Triglia *et al.* [1988]. Human genomic DNA was isolated from NFF5 cells by a procedure described by Strauss [1990]. After isolation, the genomic DNA was restricted with either BamHI or Pst I. Restricted DNA was diluted to less than 1 μg/ml and then ligated overnight at 22 °C. The temperature/time settings used for IPCR were: 94°C-90 seconds, 50°C for 90 seconds, 72°C for 4 minutes for 40 cycles. Primers F1 (described above) and F2, 5'-GCTTCTCCGTTTTGAAACGCTAGCG-3', were used.

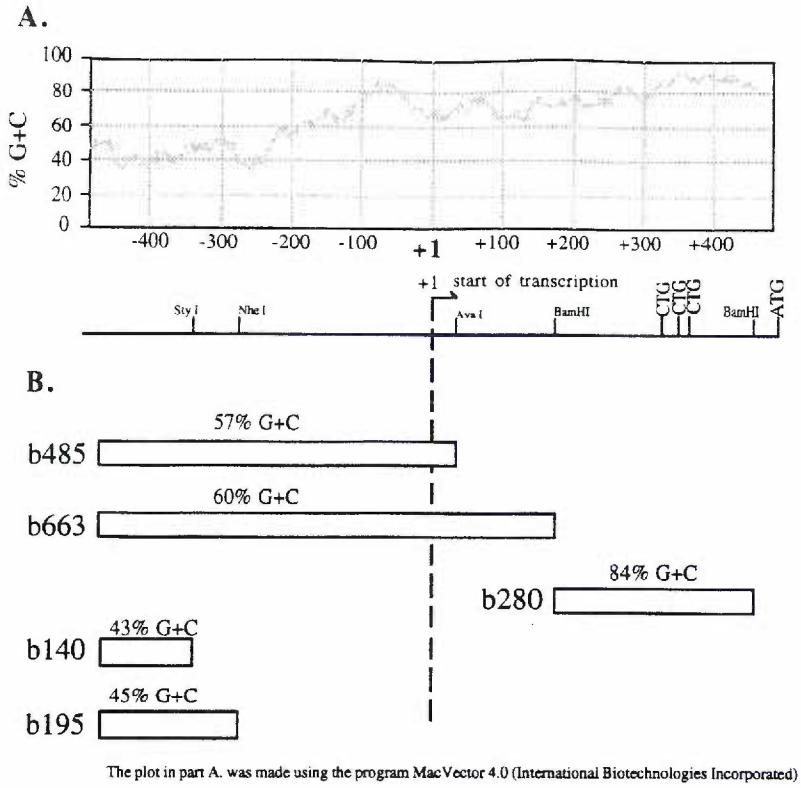
Chemicals

PMA and PDD and all chemicals with an unspecified source were obtained from Sigma Chemical Corporation. Enzymes for DNA manipulation were obtained from Boeringer Mannheim, New England Biolabs, or Bethesda Research Laboratories. DEAE-dextran was obtained from Pharmacia. SDS was obtained from Biorad. Luciferin and acetyl-CoA were obtained from Boeringer Mannheim. TGF-β1 was purified from human platelets in our laboratory using techniques previously described [Assoian *et al.*, 1983]. Thapsigargin was purchased from LC Services. FCS was purchased from JR Scientific and Hyclone Laboratories.

Results

Although at the beginning of this work I had obtained the vector pGEbFGF which contained human bFGF sequences as far upstream as 479 bases 5' to previously

determined the start of transcription it was desirable to obtain considerably more upstream sequence for the purpose of systematically examining the human bFGF upstream region for regulatory loci. The initial attempt at cloning more human bFGF upstream sequence was made using the inverse polymerase chain reaction (IPCR) technique reported by Triglia *et al.* [1988]. In this technique one uses information within a cloned region to enable PCR from an adjacent uncloned genomic DNA region. In this attempt, genomic DNA was restricted with BamHI or Pst I, purified, and then religated at dilute concentration. IPCR was then attempted as described above. The result of several attempts was that no IPCR product was obtained. It is notable that no IPCR product was obtained using plasmid pGEbFGF as the template. This plasmid contains bFGF upstream sequences -479 to +478 relative to the start of transcription. Homologous sites for both the F1 and F2 primers used in the IPCR procedure existed in this region and so pGEbFGF should have provided a template similar in form to that ideally yielded by the genomic circularization ligations. Given these failures, the technique was abandoned and attempts to clone more bFGF flanking sequences were made by the more conventional approach of screening a human genomic DNA library (see below). Subsequent to my attempts at IPCR with either plasmid or religated genomic DNA, I found that even normal, non-inverse, PCRs attempted with some templates containing sequences upstream from the bFGF coding region also failed to give a product. This problem was solved by raising the standard melting temperature to 96°C or higher. The explanation is apparently that the high % G+C "islands" found in the sequences proximal to the bFGF gene (see Figure 3.3A) make necessary the use of a higher than normal melting temperature during the PCR cycle. As the library screening method was in progress and yielding results no subsequent attempt was made at IPCR using an elevated melting temperature.



The plot in part A. was made using the program MacVector 4.0 (International Biotechnologies Incorporated)

Figure 3.3 %G+C in the bFGF immediate upstream region and in probes made from this region

Panel A is a representation of the % G+C content of the region surrounding the +1 site of the human bFGF gene. Panel B diagrams the regions that were used as probes in screening a human genomic DNA library to isolate genomic sequences proximal to the +1 site of the human bFGF gene.

Library Screening

A genomic DNA library was screened for clones which contained human bFGF sequences upstream from the first coding exon. This procedure was attempted several times before 7 positive plaques were obtained. I hypothesized that the reason that the first 5 attempts failed to give any positive clones was related to the high %G+C content found in the region immediately upstream from the ATG translational start codon. Figure 3.3A shows the %G+C content of the bFGF sequences -479 to +478 contained in plasmid pGEbFGF and Figure 3.3B shows G+C content of the bFGF upstream regions used to probe the EMBL3 DNA library. As can be seen, although the average G+C

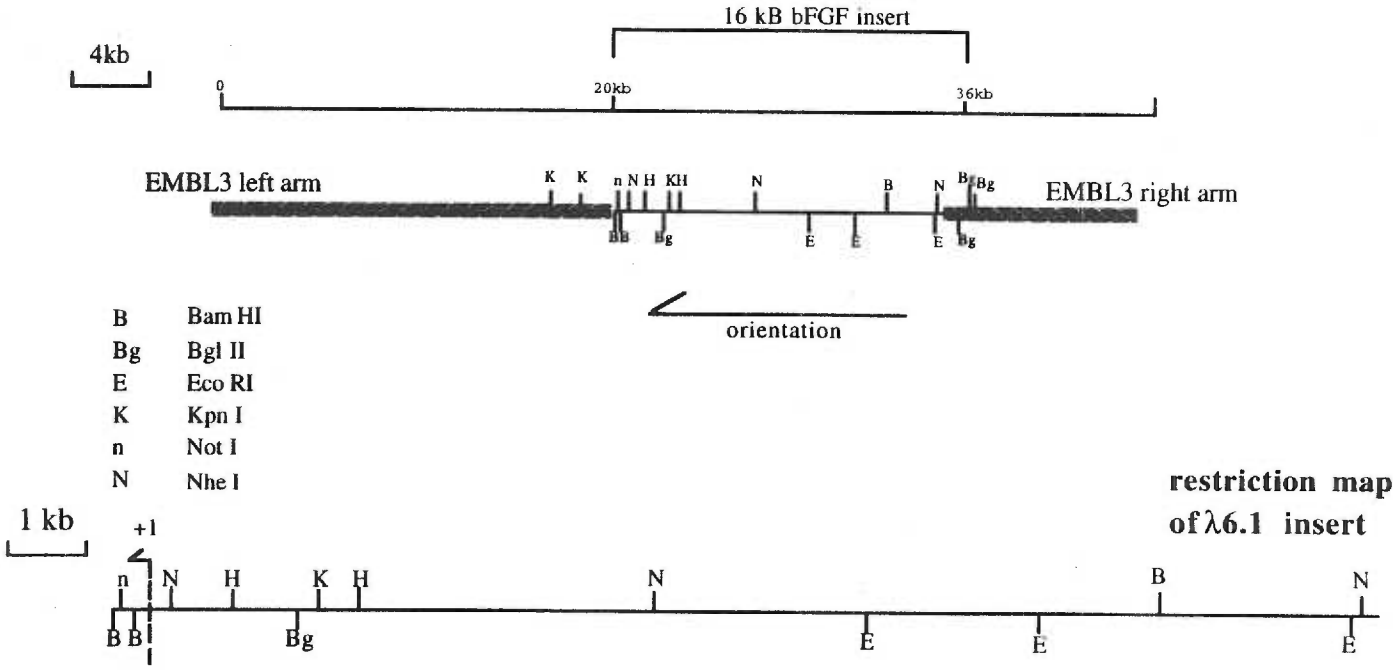


Figure 3.4 Human genomic clone λ6.1

This is a representation of clone λ6.1 which contains sequences proximal to the transcriptional start site (+1) of the human bFGF gene. The upper panel shows the map of the entire phage with insert while the lower panel displays a restriction map of the human bFGF sequences inserted into λ6.1. The arrow in the lower panel indcates the start and orientation of transcription relative to the restriction map.

content of some of these probes was not extreme (*e.g.* b663 had a 60 % G+C content), all but the b140 probe used in primary screening and the b195 probe used in the secondary screening contained regions of very high % G+C. Examination of the initial failures in library screening indicated that a large amount of probe was binding non-specifically to the filters even after the hybridization temperatures were adjusted to match the average G+C content of the probes used. The resulting strategy was to use as a probe a part of the upstream region which did not contain any islands of extremely high G+C and the b140 fragment was tried. Using this fragment as a probe, 7 apparently positive plaques were identified, 4 of which were shown to hybridize to the b195 fragment upon secondary and tertiary plaque purifications.

The four purified clones obtained from the library screen were grown up and subjected to restriction mapping/southern blot analysis with a number of restriction enzymes. All four clones showed the same patterns of restriction and the same pattern of southern blot hybridization. Clone λ6.1 was picked for subsequent work. Figure 3.4 shows the restriction map of this clone which was determined by restriction analysis and southern blot analysis. It consists of bFGF flanking sequences starting at 453 bases downstream from the +1 transcriptional start and progressing further upstream approximately another 16 kb.

Subcloning from λ6.1

Two fragments were isolated for subcloning into CAT expression vectors. One was a 6 kb Nhe I fragment containing bFGF sequences from -6 kb to -293 relative to the +1 transcriptional start site. Another was a 2 kb Bgl II-BamHI fragment containing bFGF sequences from about -2 kb to +173 relative to the +1 site. Figure 3.5 shows the fragments that were isolated for subcloning into plasmid vectors. Of the two fragments, only the 2kb Bgl II-BamHI fragment yielded bacterial colonies containing recombinant molecules when ligations were attempted into vectors pCAT-Basic and pBSKSII+.

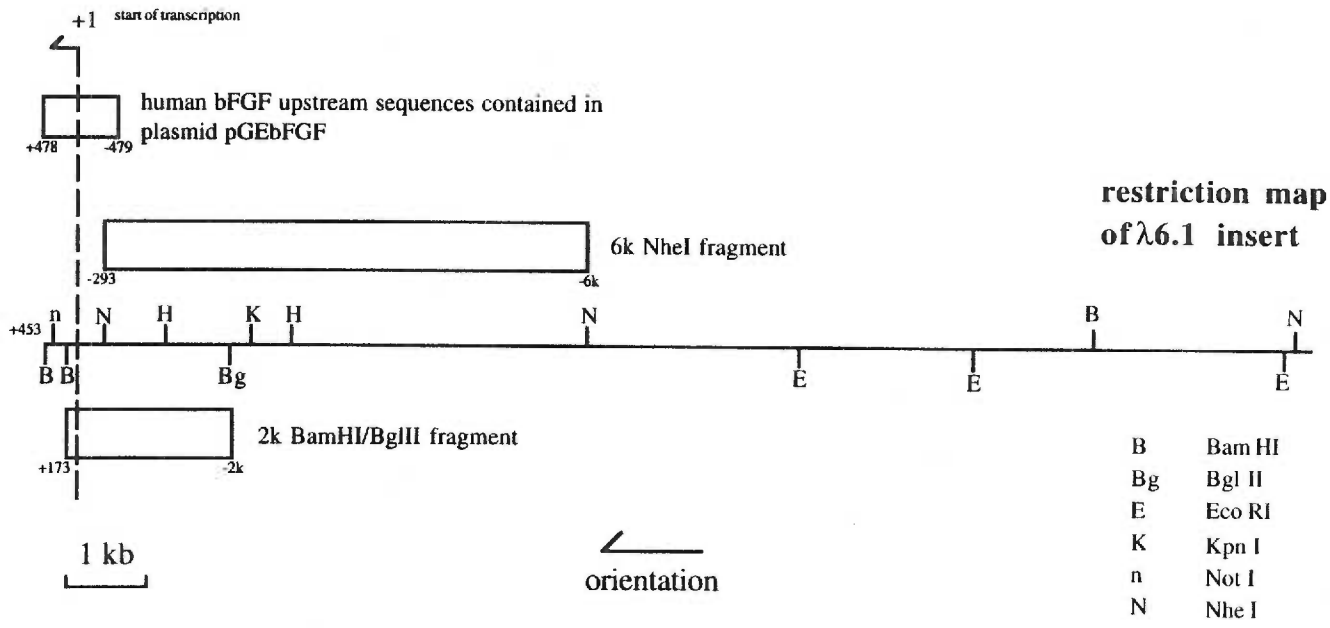


Figure 3.5 Location of fragments used in subcloning from the human bFGF locus

This figure shows the location, relative to the transcriptional start (+1), of three subsequences from the human bFGF locus that were used in various parts of the work described in the text. The bFGF promoter proximal sequences that were subcloned into vectors with the reporter gene chloramphenicol acetyl transferase (CAT) were derived from the 2 kb Bam HI-Bgl II fragment.

Although a large number of ligations of the 6kb Nhe I fragment were attempted into a variety of vectors under a variety of ligation conditions and using a variety of bacterial species as hosts for transformation of these ligations, no successful subclonings of the 6kb fragment were obtained. When some of the 6kb/vector ligation reactions were run on an agarose gel it was observed that a molecular weight shift did occur after ligation indicating that the ligation step *per se* was not inhibited. The conclusion made from the analysis of all of these failed attempts at subcloning the 6kb fragment was that it seemed likely that part of the region -6kb to -2kb was in some way inhibitory to cloning. Subsequent work concentrated on the analysis of activities contained in the 2 kb Bam HI-Bgl II fragment.

The 2kb Bgl II/BamHI fragment was cloned into the vector pBSKSII+ and then into pCAT-Basic and limited DNA sequencing was done to verify the identity of the clone with published sequences. Figure 3.6 displays a partial comparison of my initial sequence analysis of the subcloned 2 kb fragment with human bFGF flanking sequences reported by Shibata *et al.*[1991]. These sequences are virtually identical verifying that the λ6.1 clone contains sequence from the human bFGF locus. The small number of differences seen between my sequence and the published sequence I attributed to my reading errors due to inexperience in reading sequence information.

A number of constructs were made by taking subsequences from the bFGF 2kb fragment and subcloning them into the promoterless reporter plasmid pCAT-Basic. Another series of constructs was made by subcloning parts of the 2kb region into pTKp, a derivative of pCAT-Basic which has promoter sequences from the herpes simplex virus thymidine kinase gene. Figure 3.7 diagrams these constructs.

Transfections

As stated above, the purpose of the work constituting Chapter 3 of this research was to localize any sequences in the human bFGF gene's upstream region which may be

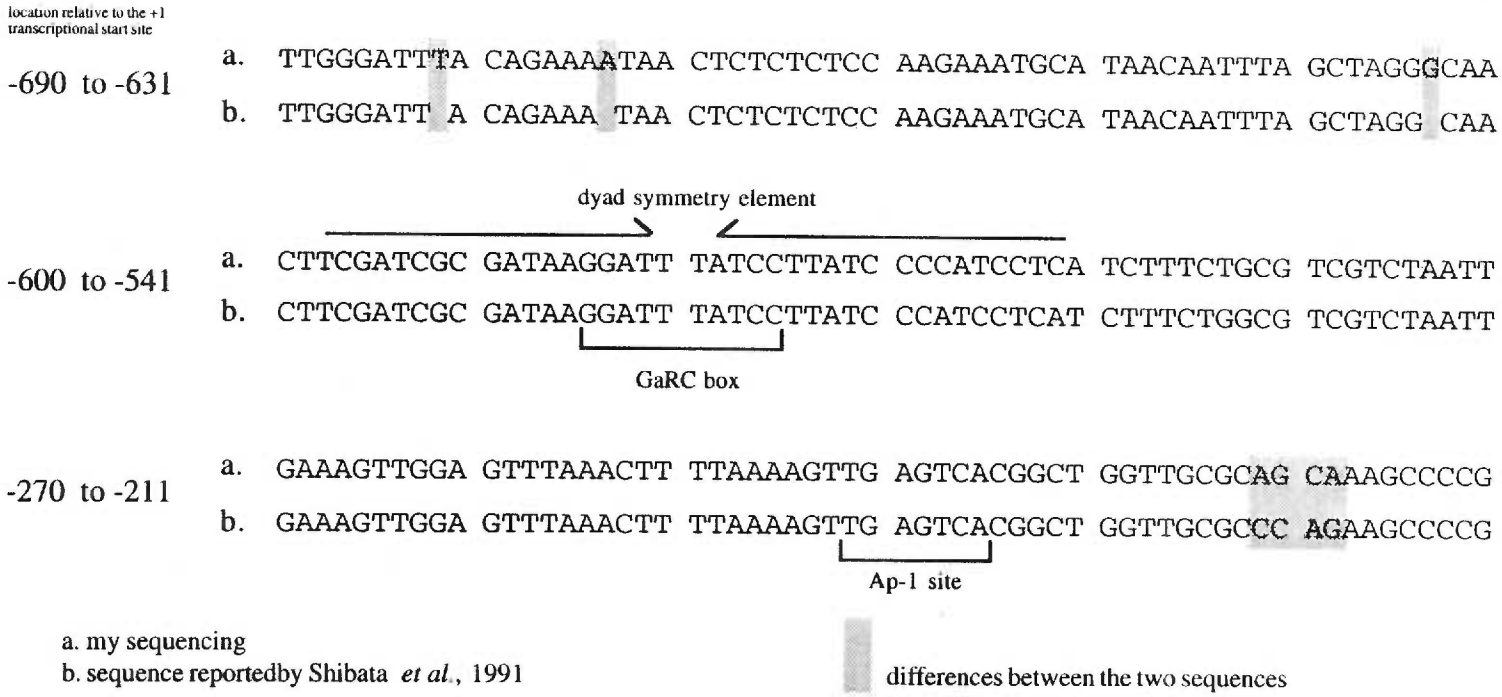


Figure 3.6 Comparison of selected sequences derived from λ6.1 with published human bFGF sequence

This figure displays sequences from the original analysis done on λ6.1 to assess its identity with sequences known to occur at the human bFGF locus. The sequences are aligned for best match. The upper (a.) sequence was derived from my sequencing and the lower (b.) sequence is that reported by Shibata *et al.*, [1991]. Also displayed in this figure are the dyad symmetry element/ GaRC box motif and the Ap-1 site contained in the sequence upstream from the +1 site of the human bFGF gene.

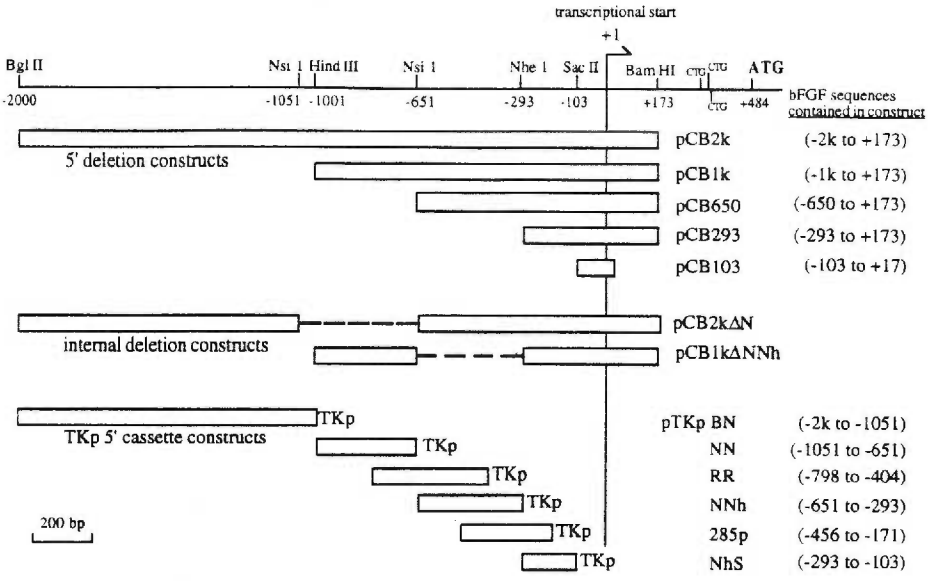


Figure 3.7 Human bFGF regions subcloned into pCAT-Basic or pTKp

This is a representation of the sequences from the human bFGF gene that were inserted into reporter vectors containing the CAT gene. The 5' deletion series and the internal deletion series of constructs were made using the promoterless vector pCAT-Basic. The TKp 5' cassette series of constructs had the indicated segments of human bFGF gene sequence inserted into the vector pTKp which contained the HSV thymidine kinase promoter.

involved in changes in bFGF gene expression in response to the agents identified in my previous work (see Chapter 2), agents like FCS, phorbol esters, TGF-β, and thapsigargin. As all of the work on agonist stimulated bFGF gene expression had been carried out using strains of primary human fibroblasts, primarily strain NFF5, a continuity of approach mandated that the transfections of the CAT constructs and the subsequent examination for regulatory elements be attempted in this same cell type.

I attempted to transfect NFF5 cells with a variety of CAT constructs including pCAT-Basic, pCAT-Control, pTKp, and pCB2k by a variety of methods. Even in the best experiments the NFF5 cells were seen to have only modest* CAT activity when transfected with pCAT-Control using the DEAE-dextran technique, Figure 3.8A (*routine transfections performed in our laboratory using Rat-1 cells were obtaining

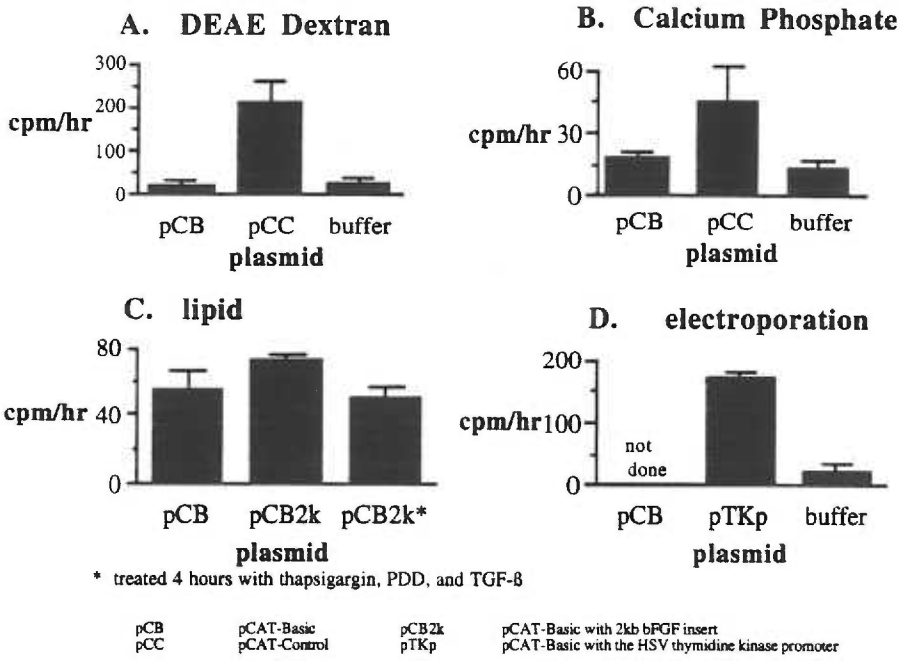


Figure 3.8 Attempts to transfect NFF5 cells with CAT constructs
Human fibroblasts (strain NFF5) were transfected with the indicated constructs by the means shown as described in the text. Extracts of these cells were prepared and analyzed for CAT activity as described. The CAT activity shown, expressed in cpm/hr of [^{3}H] acetyl coenzyme A transported into the organic layer in the scintillation vial, is the average value given by extracts from three dishes of cells transfected with the same plasmid. Error bars are the standard deviation of the mean value.

extracts with 2-4 orders of magitude more activity than given by the NFF5 cells). Attempts to transfect NFF5 cells by two other techniques, calcium phosphate and cationic lipid, gave no expression of control plasmids, see Figure 3.8B,C. In the case of electroporation although some modest CAT activity was finally acheived, as seen in Figure 3.8D, the success was not reproducible over several experiments, and this amount of successful expression (still two orders of magnitude lower than might be expected from a facilely transfectible cell line like Rat-1) required ~10^7 cells per experimental point which made it impractical with the non-imortalized NFF5 strain.

The conclusion taken from these attempts was that NFF5 cells could not, by practical means at my disposal, be made to efficiently take up and express plasmid sequences. The mRNA work and attempts at NRO had indicated that expression from the bFGF promoter was likely to be weak. It was decided not to attempt to dissect a predictedly weak promoter using a host cell strain which did not give strong expression from a plasmid like pCAT-Control which contains both a strong promoter and a strong enhancer. NFF5 cells were therefore abandoned as a host cell type for this work.

Two cell lines were used for most of the subsequent work. Rat-1 cells were in use in the Magun laboratory and had been demonstrated to be both transfectible and responsive to most of the agonists to which bFGF gene expression was responsive to [Lenormand *et al.*, 1990]. One study had shown that these cells express bFGF [Iberg *et al.*, 1989]. U87-MG, a human astrocytoma derived cell line, had been extensively studied for bFGF mRNA accumulation in response to FCS and phorbol esters [Murphy *et al.*, 1988a; Murphy *et al.*, 1988b], agents that I had shown affected bFGF expression in NFF5 cells. These cells were found to be readily transfectible using the $CaPO_4$ transfection technique. Figure 3.9 shows data from an early experiment done to compare the expression of a bFGF/CAT construct, pCB1k in NFF5 cells, U87-MG cells and in Rat-1 cells after transfection using the calcium phosphate technique. As can be seen both the U87-MG and Rat-1 cell lines expressed the pCB1k construct considerably more

efficiently than did the NFF5 cells. Most subsequent experiments were done in both of these cell lines and equivalent results were obtained. In addition, a couple of experiments were done using cell line Te671, a medulloblastoma cell line. This was the cell line used in the studies reported by Shibata *et al.* [1991] and thus was expected to take up and express CAT constructs with bFGF promoter sequences.

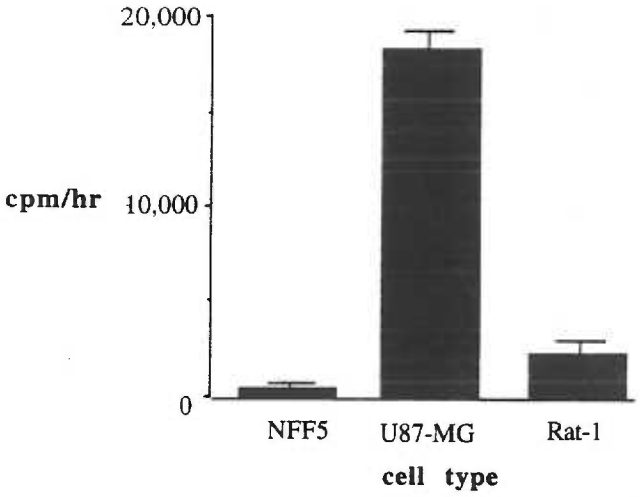


Figure 3.9 CAT expression of pCB1k in three cell types following $CaPO_4$ transfection

The three indicated cell types (NFF5, U87-MG, and Rat-1) were transfected with plasmid pCB1k using the calcium phosphate technique as described in the text. The resulting CAT activity is displayed. CAT activity values, expressed in cpm/hr, are the average of the values given by extracts from three dishes of cells transfected with a common DNA precipitate. The error bars represent the standard deviation of the mean value.

CAT Activity Supported by a 5' Deletion Series of bFGF/CAT Constructs

Figure 3.10 shows the result of transfections of a 5' deletion series of CAT constructs made with bFGF upstream sequence in either U87-MG cells, Rat-1 cells, or Te671 cells. This data supports the finding of Shibata that promoter activity is contained in the region immediately upstream from the longest cDNAs isolated for human bFGF. Another trend that can be observed, particularly in panels B and C, is that no progressive loss of expression was seen with deletions up to the -293 point in the bFGF constructs. Some loss of activity was seen when deletion was carried to the -103 site (see for

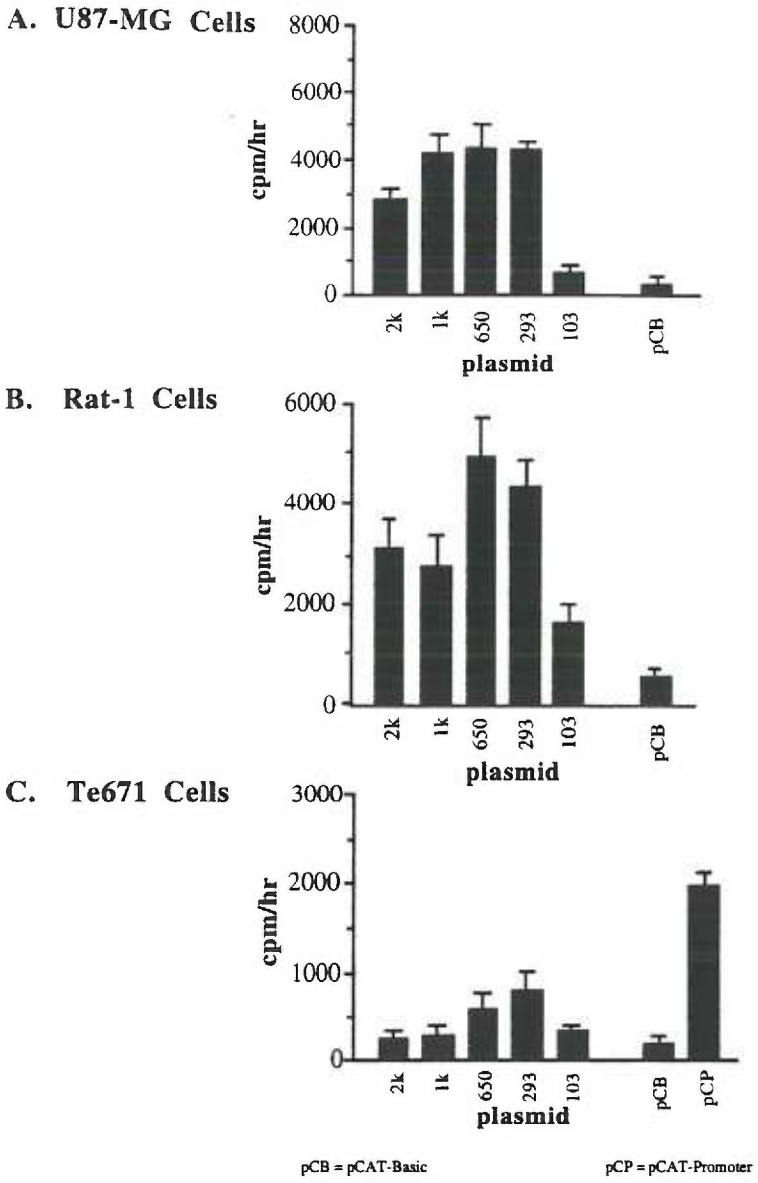


Figure 3.10 CAT activity of 5' deletion series constructs

This figure displays the results of calcium phosphate transfection of 5' deletion series of bFGF/CAT constructs into three cell types U87-MG, Rat-1 and Te671. The constructs (described in the text and illustrated in Figure 3.7) are named according the 5'-most extent of human bFGF sequence they contain. CAT activity values are the average of the activities given by extracts from three dishes of cells receiving the same DNA precipitate. The error bars represent the standard deviation of the mean value.

example in Figure 3.10B) and this may reflect the presence of a positive element resident in the -293 to -103 region. {The observation seen in panel A in which plasmid pCB103 shows virtually no activity, which was also seen in Figure 3.11 was attributed to a preparation of the pCB103 plasmid which was degraded.}

The overall result was that deletions revealed the presence of little activity, positive or negative, in the region -2000 through -293, and they revealed the possible presence of an element with up to a 2 fold effect in the -293 to -103 region.

In Figure 3.10 B and C a mild increase in activity was seen in constructs which had the endpoints -650 and -293. This increase is reminiscent of a similar effect noted by Shibata *et al.*[1991]. It is notable that this deletion associated increase in activity was slight, ≤ 1.5 fold, and that it in general fell within the range of variation seen in the transient expression assays.

Agonist Effect on CAT Expression from bFGF/CAT Constructs

A different question is addressed by experiments of the type seen in Figure 3.11. In this experiment the cells which had been transfected with bFGF/CAT constructs were either given no treatment (filled in bars) or given a combination of phorbol 12,13-didecanoate (PDD), thapsigargin and fetal calf serum (FCS)(open bars). As can be seen, the treatment with this agonist mixture did not have any effect on CAT activity driven by the bFGF promoter constructs. Previous work had shown that this agonist mixture was able to produce up to 20-fold increases in bFGF mRNA accumulation in NFF5 cells as described in Chapter 2. This experiment was repeated several times and no consistent effect of any agonist mixture on CAT activity of any bFGF/CAT construct was seen. Similar experiments were conducted in three separate cell types: U87-MG, Rat-1, and Te671 and equivalent results were obtained.

Included in Figure 3.11 is data from the plasmid 3.4. This plasmid represented a more radical deletion of the bFGF proximal sequences and contained bFGF sequences

from -24 to +49 inserted into pCAT-Basic. In this experiment this plasmid showed activity comparable to that given by pCB2k and also showed no induction by the agonist mixture. The observation that the 73 bases contained in plasmid 3.4 could promote activity near, and in other experiments greater than, that supported by the >2000 bases contained in pCB2k was an interesting finding and provided the starting point for the

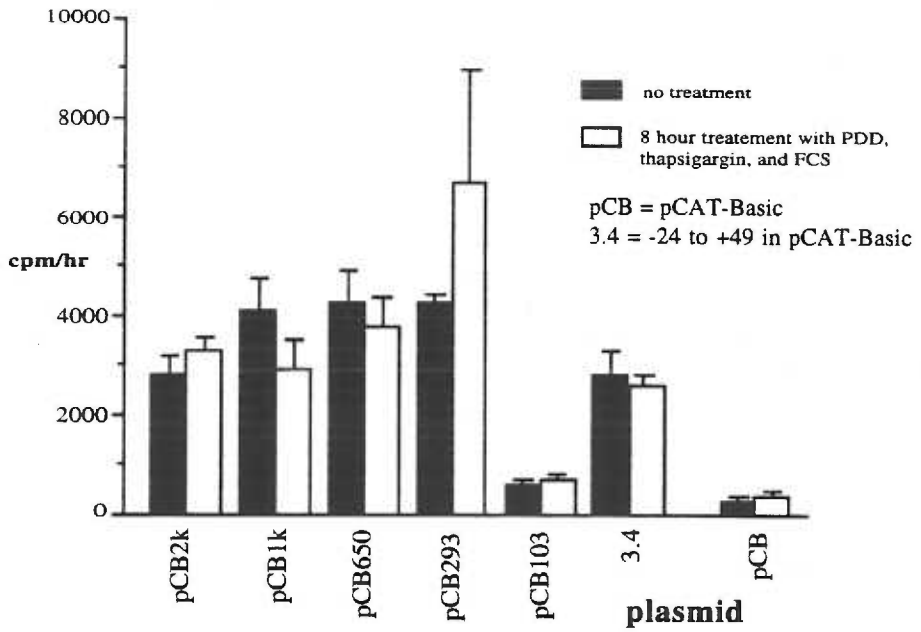


Figure 3.11 The effect of agonists on CAT expression from bFGF 5' deletion constructs

Six dishes of U87 MG cells were transfected with the indicated plasmids using the calcium phosphate technique as described in the text. Eight hours prior to harvest, 3 dishes of each group (open bars) were given a combination of PDD (100 ng/ml), thapsigargin (1 μM), and FCS (10% v/v). The other three dishes of each group received no addition (filled in bars). After 8 hours, the dishes were harvested for CAT extract which was assayed for activity as described. CAT activity values are the average of the values given by extracts from three dishes receiving the same precipitate and treatment. Error bars represent the standard deviation of the mean value.

studies described in Chapter 4 of this thesis.

Hypotheses on Lack of Agonist Effect on CAT Expression

Several hypotheses were advanced to explain this lack of agonist effect on CAT expression. The simplest hypothesis was that the agonist responsive elements which mediate bFGF gene expression in response to agents like FCS, phorbol esters, TGF-β.and thapsigargin are located in regions which lie outside the tested region, either further upstream than -2000 bp or further downstream than +173. With the exception of looking at the region +173 to +478, discussed below, this hypothesis was not practically assailable given the time and resources at my disposal. Although I had in the λ6.1 clone as much as 14 kb more of upstream sequence, almost 6 months of effort had failed to achieve subcloning of fragments further upstream than -2000 bp, and so the sequences further upstream than -2000 were in a practical sense out of reach. With respect to sequence downstream of the +485 site I had no genomic clones with sequence downstream from the +485 in hand and so the testing of these sequences for regulatory elements would have required new isolation, characterization, and subcloning of genomic sequences which was not practical in the available time.

In order to examine the narrow band of sequence between +173 and +478 constructs were made with sequences spanning the region either -228 to +309 (Fsp I to Xho I) or -228 to +478 (Fsp I to Nco I) subcloned into pCAT-Basic. These constructs supported no CAT activity over CAT assay background either in the presence or absence of agonists. This finding suggested that this region is not responsible for agonist mediated stimulation of bFGF expression. It also corroborated the finding of Shibata *et al.*[1991] that sequences in this region had a negative effect on CAT expression, see Figure 3.2.

Interspersed Negative and Positive Elements

A second hypothesis was formulated to explain a lack of agonist action on the subsequence constructs from the 2k region. In this hypothesis, agonist regulated

elements are located in this region but they are interspersed with negative elements which are masking the effect of the agonist regulated expression. This hypothesis was motivated by the report of Shibata *et al.* [1991] which indicated that a negative element, NRE2, was located in the region -780 and -480 relative to the +1 transcriptional start and by the example provided by the human urokinase plasminogen activator gene [Verde *et al.*, 1988] and the rat catalase gene [Sato *et al.*, 1992] both of which had been shown to have interspersed positive and negative elements in their upstream regions.

This hypothesis was examined by making two different types of constructs. One was deletion constructs which removed internal sequences from the plasmids pCB2k and pCB1k. The second type of construct which was made in part to approach this issue of negative elements masking positive ones was cassette constructs. These were made by putting small parts of the bFGF upstream region, remote from the +1 site and probably not containing the basal promoter machinery for this gene, into the vector, pTKp, which contains the HSV TK gene promoter inserted into pCAT-Basic. Both types of construct sought to isolate parts of the bFGF upstream region away from other parts of this region so as to isolate individual activities from one another. If a negative effect were masking a positive effect then one might predict that an internal deletion in a plasmid like pCB2kΔNN that eliminated the masking negative element would show an agonist stimulation when the pCB2k undeleted construct would not. In the case of the cassette constructs one would predict that a cassette that contained only a positive element and not a potentially masking negative element would show an agonist stimulated response. Figure 3.12 shows the comparison of the activity, basal and agonist stimulated of the two internal deletion constructs. pCB2kΔN is based on pCB2k and has deleted sequences -1051 through -651 and pCB1kΔNNh is based on pCB1k and has deleted sequences -651 through -293. The deleted regions in these two plasmids span the region -747 to -450 within which Shibata *et al.* had localized NRE 2. As can be seen in Figure 3.12 the effect of the internal deletions is small. The apparent loss of activity seen in the

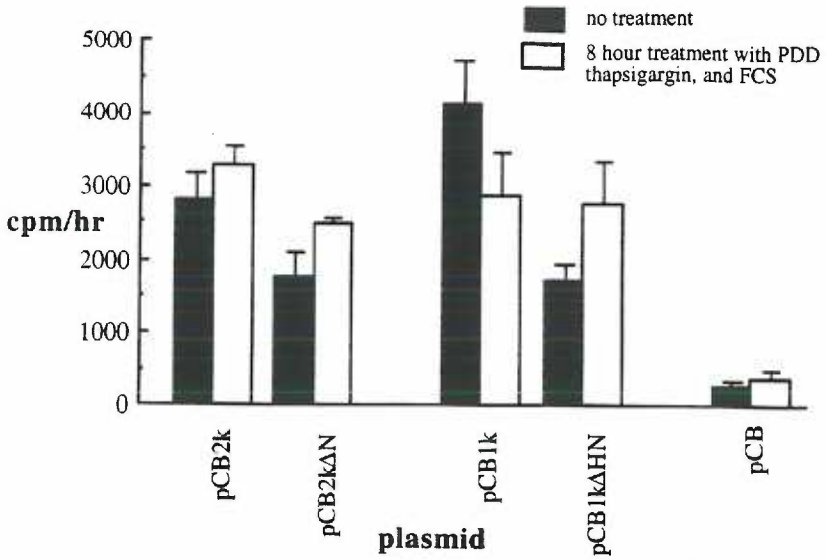


Figure 3.12 CAT activity of internal deletion constructs
Six dishes of U87 MG cells were transfected with the indicated plasmids using the calcium phosphate technique as described in the text. Eight hours prior to harvest, 3 dishes of each group (open bars) were given a combination of PDD (100 ng/ml), thapsigargin (1 μM), and FCS (10% v/v). The other three dishes of each group received no addition (filled in bars). After 8 hours, the dishes were harvested for CAT extract which was assayed for activity as described. CAT activity values are the average of the values given by extracts from three dishes receiving the same precipitate and treatment. Error bars represent the standard deviation of the mean value.

pCB1kΔNNh plasmid relative to pCB1k in the untreated case may represent a weak positive activity in the region -651 to -293 although the magnitude of the decrease was within interexperimental variation. In no case in Figure 3.12 or in other experiments did a deletion plasmid exhibit a significant agonist responsive activity.

Figure 3.13 shows the activity of various cassette constructs with respect to the parent plasmid pTKp. None of the cassette constructs have augmented activity with respect to the parent plasmid, pTKp, either with or without agonist. In almost all cases the pTKp plasmids containing bFGF cassettes had less activity than the parent pTKp vector. The apparent agonist mediated stimulation of constructs pTKpNNh and pTKpNhS cannot be taken to be a sign of specific induction mediated by their bFGF

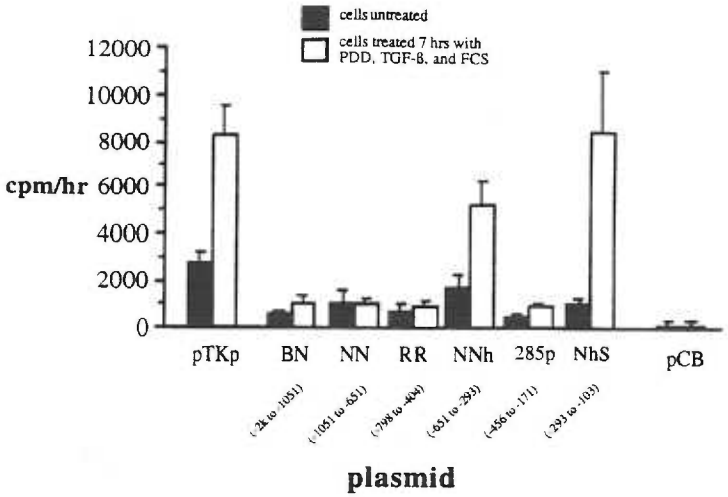


Figure 3.13 CAT activity of bFGF cassette constructs in pTKp
Six dishes of U87-MG cells were transfected with the indicated plasmids using the calcium phosphate technique as described in the text. Seven hours prior to harvest, 3 dishes of each group (open bars) were given a combination of PDD (100 ng/ml), TGF- β (10 ng/ml), and FCS (10% v/v). The other three dishes of each group received no addition (filled in bars). After 7 hours, the dishes were harvested for CAT extract which was assayed for activity as described. CAT activity values are the average of the values given by three dishes receiving the same precipitate and treatment. Error bars represent the standard deviation of the mean value.

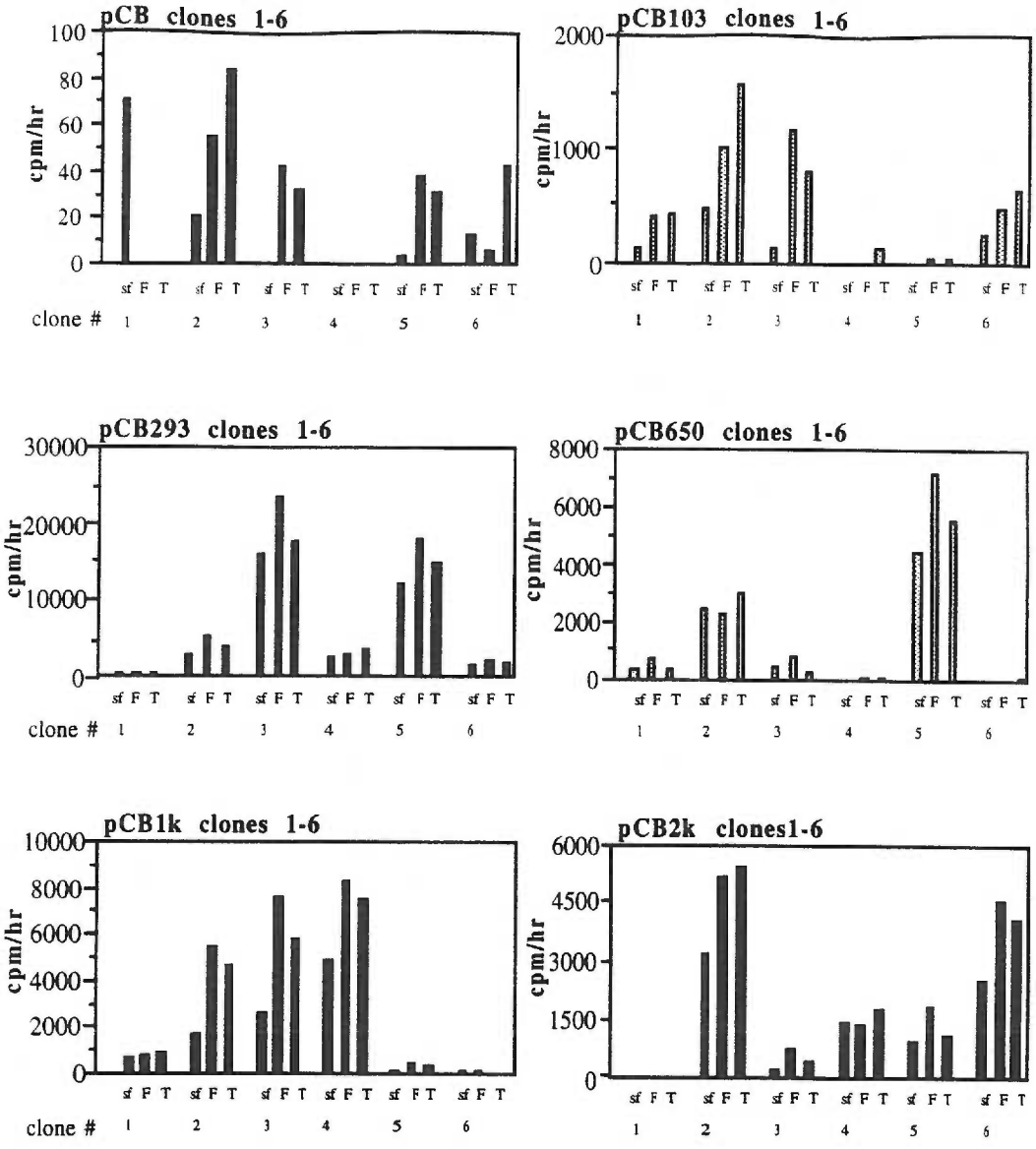
sequences since a similar induction is seen in the pTKp parent vector. It is unknown why the inclusion of a variety of exclusive bFGF sequences decreased expression from plasmid pTKp. It is possible but would seem unlikely that negative regulatory elements are the explanation in that the degree of non-overlap among the bFGF inserts would require at least four separate elements and that two of the cassettes NN and NNh correspond closely or exactly to the sequences internally deleted in constructs pCB2kΔN and pCB1kΔNNh (see Figure 3.7). Neither of these internal deletions exhibited an increased activity with respect to their undeleted counterpart as would be predicted if they contained a negative regulatory element.

Although this cassette approach did not yield evidence for isolated positive regulatory elements of a constitutive or agonist regulated nature the fact that all of the inserts appeared to decrease the activity of the HSV TK promoter reduced the confidence

in this analysis. Also limiting was the inherent inducibility of the HSV TK promoter by the test agonists. Were this approach to be repeated, a different heterologus promoter with lower intrinsic activity and no inducibility would be desirable. A candidate for this type of promoter would be the initiator element from the mouse lymphocyte-specific terminal deoxynucleotidyl transferase gene [Smale and Baltimore, 1989] or perhaps the basal promoter for the bFGF gene, see Chapter 4.

Is An Aspect of Transient Expression Masking Agonist Induction?

Another testable hypothesis was that agonist regulated elements do exist in the 2kb region but that the calcium phosphate transfection protocol was either giving rise to spuriously deregulated expression or globally upsetting the signal transduction pathways required for the various agonists to work. This hypothesis was addressed by creating Rat-1 cell lines which contained stably integrated copies of the constructs: pCB2k, pCB1k, pCB650, pCB293, pCB103, and the promoterless vector pCAT-Basic. Expression patterns from stably integrated copies of these plasmids would not be expected to be affected by the transfection procedure since several weeks separated the transfection and the analysis of CAT activity. Five to six independent clones for each plasmid used were isolated and tested for basal and agonist mediated expression of CAT. Figure 3.14 shows the result of such an experiment. Significant variability is seen from clone to clone consistent with expected heterogeneity in the sites of and in the multiplicity of integration of the plasmids into the genomic DNA. Some consistent variation is seen in the intensity of expression between clones with the pCB293 clones taken as a group out-expressing the other clones and with pCB103 having the least expression of the bFGF promoter-containing clones. Some or all of this effect could be due to differences in DNA quality. It is clear that the stable clones which had taken up the promoterless pCAT-Basic construct had significantly less CAT activity than clones expressing any of the bFGF promoter containing constructs by at least an order of



sf - no treatment F - 8 hrs exposure to FCS T - 8 hrs exposure to PMA, TGF-ß, thapsigargin

Figure 3.14 CAT expression from clones of Rat-1 cells stably expressing bFGF/CAT constructs

Rat -1 cells were stably transfected with plasmids of the bFGF 5' deletion series as described in the text. Six clones of cells resistant to G418 were selected for each input plasmid. These clones were either given no treatment (sf), given 10% v/v FCS (F), or given the combination of 100 ng/ml PMA, 10 ng/ml TGF-ß, and 1 µM thapsigargin (T). After 8 hours exposure to these treatments, cells were harvested for CAT extracts which were assayed as described. The CAT activity values are the activities measured from the pooled extracts from two wells of cells for each clone/treatment group.

magnitude.

It would appear that in some cases two different agonist conditions, FCS denoted by "F", or the combination of PDD, thapsigargin, and TGF-β, denoted by "T", did induce CAT activity in bFGF promoter/CAT constructs (see for instance pCB2k clone 2, pCB1k clones 2 and 3, or pCB103 clone 3). However, these stimulations are of a similar magnitude as seen in some pCAT-Basic containing clones (see pCB clones 2 and 5). Taken *in toto,* this data on the CAT expression of clones of cells stably expressing bFGF promoter/CAT constructs does not indicate any agonist specific effect due to the inserted bFGF promoter sequences.

Discussion

The work described in this Chapter sought to examine the region -2000 bp through +485 relative to the +1 transcriptional start site of the human bFGF gene for elements which contribute to the expression of this gene, particularly in response to certain extracellular effectors. The data collected in transient expression assays as well as in assays of CAT expression by stably integrated constructs does not indicate the presence of any significant positive regulatory elements, either constitutive or agonist-responsive.

The Comparison of This Work with that of Shibata *et al.* [1991]

Previous investigation into regulatory elements contained in the upstream region of the human bFGF gene was carried out by Shibata *et al.*[1991]. Figure 3.15 is redrawn from data contained in this report. As summarized earlier, some of the conclusions that this group reached were: the promoter is contained in the upstream sequence proximal to

the first coding exon, an element with a negative effect on expression is contained in the region -747 to -480, an element or elements with some accessory positive effect is

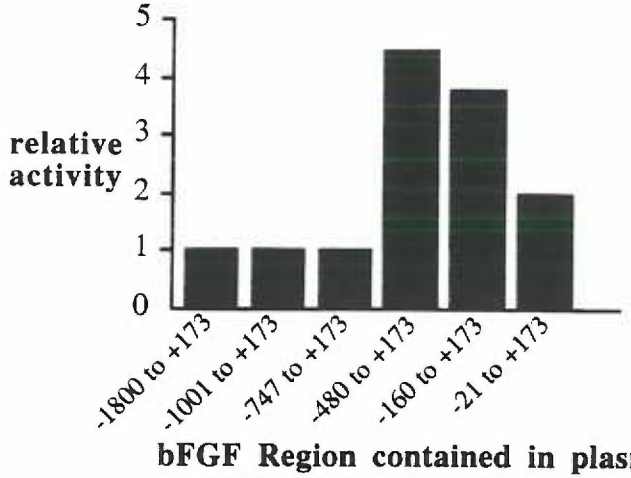


Figure 3.15 Data from Shibata *et al.* [1991] CAT activity of bFGF 5' deletion constructs

This figure is redrawn from data reported by Shibata *et al.* [1991]. It displays the relative CAT activity supported by a series of 5' deletion constructs containing various amounts of sequence from the human bFGF promoter region in transient expression assays done using Te671 cells.

contained in the region -160 and -21, and significant promoter activity is contained in the region -21 to +173.

Although the exact deletions that were made and analyzed in my experiments and in the experiments of Shibata *et al.* were not the same, the general trends of the data portrayed in Figure 3.15 are in agreement with trends seen in the data shown in Figure 3.10B and C. This may be seen better in Figure 3.16 which contains a superposition of the data from the Shibata paper and the data seen in Figure 3.10B. The magnitude of the changes both positive and negative are muted in comparison with the Shibata data, for example the increase in activity as a function of deleting to -293 was nearer to 1.5 fold

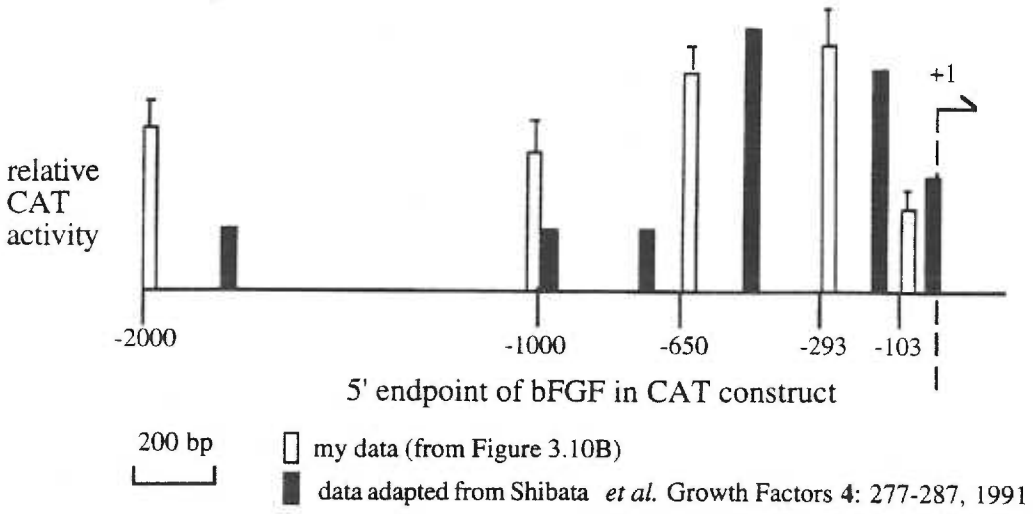


Figure 3.16 Comparison of the CAT activity supported by my 5' deletion series with the CAT activity supported by a 5' deletion series of Shibata *et al.* (1991)

This figure is redrawn from data reported by Shibata *et al.* [1991] and from my data displayed in Figure 3.10B. Both sets of data display the CAT activity supported by a series of human bFGF 5' deletion constructs. The CAT activities have been expressed relative to the activity given by the most active plasmid in each series which have been given roughly the same value. Error bars from the data in figure 3.10 B were derived as described. No error bars were reported by Shibata *et al.*

than 4.5 fold but the patterns are similar. The drop in activity that Shibata report in deleting the -160 to -21 region was also seen in muted form in the data in Figure 3.7 in the comparison between plasmids pCB293 and 3.4 (plasmid 3.4 having the 5' endpoint -24). The drop in activity seen upon deleting sequences from -293 to -103 is possibly another reflection of the same drop in activity that Shibata saw when deleting from -160 to -21. This may indicate the presence of a positive activity in the -160 to -103 segment of sequence or it may it may indicate more than one activity spread between -293 and -24. It is interesting to note that four potential Sp1 binding sites are found in this region (see Figure 3.1).

Given a general, albeit muted, similarity between the two data sets one must ask what the data indicate about the presence of regulatory elements in this region. The

trends shown in my data although tending to support the observations of Shibata are of an insufficient magnitude relative to the inherent variability of transient expression assays to independently establish the presence of any regulatory elements. It is unclear why the magnitude of the effects seen by Shibata *et al.* was not reproduced in my studies. Differences in the exact sequences contained in the various plasmids employed by Shibata and by me could be a part of the explanation. Another possibility is that the two-phase liquid scintillation form of the CAT assay used in these studies could be giving a slightly different relative quantitation than given by the thin layer chromatography-based CAT assay used by Shibata. Weighing the outcomes of numerous different experiments (e.g. 5' deletion series, cassette constructs, internal deletion constructs, and different host cell lines) done in the present study, I would conclude that if the positive and negative elements suggested by Shibata *et al.* are present, their contribution to the regulation of bFGF gene expression is minor.

Lack of Agonist Effect on CAT Activity

A more important issue to consider is what conclusions may be drawn from the lack of stimulation of CAT activity by treatment of the transfected cells with FCS, PDD, TGF-β or thapsigargin since the search for agonist responsive elements was the purpose for the work reported in this chapter. As introduced above, the data admit to a number of possible explanations. The first possibility is that DNA sequence elements which act to stimulate expression in response to FCS, TGF-β, phorbol esters and thapsigargin are located outside the regions that were examined, -2000 to +485 relative to the transcriptional start. Numerous examples of this exist in the literature including the TPA responsive element of the urokinase plasminogen activator gene which lies 2400 bases upstream from the transcriptional start [Rorth *et al.*, 1990] and the TPA responsive element of the IL-1β gene which is located 2800 bases upstream from that gene's +1 site [Bensi *et al.*, 1990]. This possibility is difficult to evaluate until the location of agonist

regulated elements which are transducing the effect of these substances are located or until it is demonstrated that such agents are not acting to stimulate bFGF gene expression at the level of transcription. The first step towards examining this possibility lies in cloning more downstream bFGF sequence and in the successful subcloning of human bFGF sequences upstream from the Bgl II site at -2000. Once these are in hand then a variety of approaches might be used including DNase hypersensitivity analysis or continued CAT expression assays.

A second explanation for the lack of agonist effect on the expression of CAT in the test plasmids could be that agonist regulated elements do exist in -2000 to +173 region but that the CAT assays are not resolving the agonist stimulation. The fact that the same assay procedures including one of the same cell lines (Rat-1), protocols and reagents for CAT assay has worked to demonstrate phorbol and thapsigargin responsiveness of sequences within the mouse VL30 locus [Lenormand *et al.*, 1990], show that the assay as used is capable of detecting such effects. It may be that the magnitudes of the activities present in the human bFGF sequences are too low to be detected in the CAT assay. This would be particularly likely if only one such agonist regulated element existed in the -2000 to +173 region. As was shown in Chapter 2, single agonists like phorbol esters, TGF-β, or thapsigargin have only a modest (2 to 6-fold) effect on mRNA accumulation. Assuming that an element responsible for a mild effect on mRNA accumulation produced a similarly mild stimulation of CAT expression in response to agonist, then it is possible that the presence of one element was not detected due a lack of sensitivity of the CAT assay. As mentioned above two candidate elements, an AP-1 sequence, and an inverted CArG box motif, are found in the bFGF upstream region. It is conceivable, for example, that the AP-1 site located at -243 to -237, is responsible for the stimulation of transcription of the human bFGF gene in response to phorbol esters but that the effect of its action is sufficiently mild in the artificial genetic contexts used here so as to be undetectable using the CAT assay. It is

difficult to evaluate the likelihood of this possibility with the data at hand. Evaluation could come from experiments employing new constructs which took a single phorbol responsive element from another gene and put it in front of a core bFGF promoter to see if the action of a single known regulatory element acting in the present genetic context was detectable using this CAT assay system. If sensitivity were thought to be a limiting problem this might be addressed by using a more sensitive primary reporter gene like luciferase, by extending the analysis of the behavior of cells with a wider variety of stably integrated bFGF promoter constructs, and by creating a new series of cassette constructs with a promoter which has a lower basal activity and less inducibility than shown by the HSV TK promoter used in this study.

Yet another class of explanation for the lack of effect of agonist mixtures on CAT expression from bFGF promoter/CAT constructs is that while agonist regulated elements are present in the region -2000 to +173, their function requires the presence of other sequences which are located outside this region. Examples of this type of necessary cooperation between widely separated elements have been described. The bipartite steroid-dependent regulatory element of the ovalbumin gene is one example [Schweers *et al.*, 1990]. A second example is seen in the carp β-actin gene in which two CArG box motifs, one in the region proximal to the promoter and the other in the first intron, are required for full activation of the promoter [Liu *et al.*, 1991]. The investigation of this possibility in the case of the human bFGF gene would require the cloning of further bFGF sequence downstream from the first exon and the successful subcloning of sequences further upstream than the Bgl II site at -2000. Once this was accomplished then the previously cited approaches of DNase hypersensitivity or DNase I footprinting analysis and the analysis of CAT expression driven by very large bFGF sequence cassettes could be attempted.

Summary

The work reported in Chapter 3 of this thesis has examined the region of the human bFGF gene located -2000 to +173 relative to the start of transcription for activities which affect the amount of expression from this gene. This has entailed: the isolation and mapping of a genomic clone, λ6.1, containing sequences extending at least 16 kb upstream from the first coding exon of the human bFGF gene; the subcloning of various parts of approximately 2 kb of this sequence into reporter plasmids containing the coding region of the CAT gene; and the subsequent transfection of these plasmids into various cell lines for the examination of CAT activity as a function of the amount of bFGF sequences contained and of exposure of the host cells to various agonists previously shown to affect bFGF mRNA accumulation. The positive evidence from this analysis indicates that bFGF sequences -24 to +49 relative to the transcriptional start contain sufficient information to support significant CAT activity. The sum of the investigations indicates that outside these sequences surrounding the +1 site, no elements with strong, i.e. >2 fold effect on CAT expression appear to be contained in the -2000 to +173 region. The data do not support a view of bFGF promoter architecture that would place cis-acting regulatory elements responsible for elevating bFGF mRNA levels in response to FCS, TGF-β, phorbol esters, and thapsigargin solely within the -2000 to +173 region, but the presence of one or more of these elements is not excluded by this data if their action were sufficiently weak so as to be below the level of detection afforded by the CAT expression system used.

Chapter 4 Work on the human bFGF basal promoter

Introduction

Experiments reported in Chapter 3, conducted in the context of looking for regulatory loci in the bFGF upstream region led to the observation that constructs with as few as 73 bp of bFGF sequence surrounding the +1 site were sufficient to drive the expression of the CAT gene at significant levels. The expression levels supported by the 73 bp of bFGF sequence were as great or greater than were given by constructs which contained over 2000 bases of bFGF sequence. This was a striking finding and suggested that the bFGF promoter was quite compact, perhaps to an exceptional extent. These findings motivated the work described in Chapter 4 of this thesis. The point of this work was the dissection of the functional architecture of the human bFGF gene's promoter region to find which DNA sequences were minimally sufficient for expression and to locate other sequences in the immediate vicinity which were contributing to expression from this locus.

Promoter Architecture

A promoter for a gene can be operationally defined as the sequence or set of sequences proximal to the gene which are sufficient and necessary to enable significant and accurate initiation of transcription from that gene. The general architecture of the promoters of higher eukaryotic genes was described in the 1980's as covering approximately the 100-120 bp of sequence located immediately 5' to the transcriptional start site [Sassone-Corsi and Borrelli, 1986]. Two types of elements were described that participate in promoter function: a TATA box element which acts to determine the site of transcriptional initiation 28±2 bp downstream from itself, and one or more upstream

elements located in the region -110 to -40 which act to increase the magnitude of expression. Among the more thoroughly characterized of these upstream elements are the binding site for the transcription factor Sp1, the GGGCGG or GC box motif, and the binding site for the transcription factor NF-1 (also known as CPB or CTF), the CCAAT-box [Dynan and Tjian, 1985; McKnight and Tjian, 1986; Sassone-Corsi and Borrelli, 1986].

Recent investigation into promoter function has focused on the region from -30 to +10 relative to the transcriptional start site. This region has been termed the "basal promoter" or the "core promoter" region and while this region can benefit from the effects of near upstream elements like the GC-box or CCAAT-box, it appears to be sufficient in most cases to specify accurate basal expression [Bhargava and Chatterji, 1992; Ham *et al.*, 1992; Weis and Reinberg, 1992]. The basal promoter appears to contain two functional domains that in most cases cooperate to give accurate transcriptional initiation, a domain centered at -30, and a domain centered at +1. The elements that occupy these sites have been termed selector elements in that their function is to select the point where transcription is initiated [Weis and Reinberg, 1992; Kollmar and Farnham, 1993].

-30 elements

The most common and the best characterized selector element occupying the -30 site is the TATA-box. This element directs transcriptional initiation to occur about 30 bp downstream from itself and appears to work through the binding of a complex group of proteins called TFII-D (transcription factor II-D)[Kollmar and Farnham, 1993]. A number of genes have been isolated which lack a TATA-box at the -30 site [Weis and Reinberg, 1992]. In some of these cases it has been demonstrated by titration and inactivation studies that the action of TFII-D is still necessary for function even in the absence of an efficient binding site in the -30 region. It has been suggested that TFII-D

is binding to some sort of adapter molecules that make direct contact with the +1 site [Weis and Reinberg, 1992]. It is also clear from other cases that sequences other than the TATA consensus will act at the -30 site as selector elements. In the gene for ribosomal protein S16, for example, a complex other than TFII-D was detected binding to the non-TATA consensus sequences at -30 [Hariharan and Perry, 1990]. It has also been found the binding site for the transcription factor Sp1 placed at the -30 site can give TATA-like selector function [Smale *et al.*, 1990].

Initiator elements

Initiator elements (Inr) constitute another class of basal promoter elements which have been described and can be thought of as selector elements which specify the start of transcription somewhere within their own sequence [Weis and Reinberg, 1992; Kollmar and Farnham, 1993]. The existence of such an element was first suggested by the observation of a loose consensus, 5'PyPyCAPyPyPyPyPy-3', at the start of transcription of a number of TATA-box containing genes [Weis and Reinberg, 1992; Kollmar and Farnham, 1993]. An active role for the Inr in specifying the start of transcription was first demonstrated in 1989 with the report of Smale and Baltimore that showed that the sequence from -6 to +11 relative to the start of transcription of the gene for lymphocyte specific terminal deoxynuclotidyltransferase (TdT) were sufficient to specify significant and accurate transcription *in vitro* [Smale and Baltimore, 1989]. The TdT gene lacks a TATA-box at the -30 site and the 17 bp TdT Inr which was shown to be transcriptionally active *in vitro* did not require any wild-type sequence at the -30 site. The lack of strong consensus at the +1 site of genes shows this to be a very heterogeneous class of functional element. This is also reflected by the large differences in the activity seen among isolated Inr elements. When the Inr regions are removed from their context and examined for capacity to support transcriptional initiation *in vitro*, some Inr regions will support accurate transcription of varying intensities in the absence of TATA-box or other

-30 elements, while others show no activity in the absence of a good selector like a TATA-box or an Sp1 binding site [Smale and Baltimore, 1989; Beaupain *et al.*, 1990; Blake *et al.*, 1990; Smale *et al.*, 1990; Weis and Reinberg, 1992].

It is unclear at this time how Inr elements specify transcriptional initiation. Some Inrs appear not to directly bind specific proteins [Smale and Baltimore, 1989; Weis and Reinberg, 1992; Kollmar and Farnham, 1993] while in other cases a variety of distinct proteins have been reported to bind [Blake and Azizkhan, 1989; Hariharan *et al.*, 1989; Pierce *et al.*, 1992; Basu *et al.*, 1993]. In several cases Inr elements have been found to contain binding sites for known transcription factors usually associated with upstream activation [Kollmar and Farnham, 1993]. An NF-E1 site (also known as ∂ factor or YY-1) is located within the Inr of the gene for cytochrome oxidase c subunit Vb [Basu *et al.*, 1993] and a binding site for transcription factor E2F is contained within the Inr of the dihydrofolate reductase gene [Blake and Azizkhan, 1989].

Many Ways to Build a Basal Promoter

The picture that is emerging from these studies is that transcriptional initiation can be directed by a large number of combinations of elements acting at the -30 to +10 sites. The simplest cases exist where very strong elements like the consensus TATA-box or the TdT self-sufficient Inr elements are acting. In the case of a strong TATA-box it is likely that an Inr of some minimum nature is required for accurate initiation. In the case of a strong Inr, like the TdT Inr, it is clear that TFII-D or something that complements its function is still required for activity even in the absence of TFII-D binding to an element at -30 [Beaupain *et al.*, 1990; Hariharan and Perry, 1990; Weis and Reinberg, 1992; Wiley *et al.*, 1992; Kollmar and Farnham, 1993]. More complex and subtle cases exist where weak elements exist at the -30 site and at the +1 site. In these cases the -30 to +10 region cannot be subdivided without loss of all activity [Blake *et al.*, 1990]. The combination of -30 and Inr elements of varying strengths would appear to provide a

means of specifying basal promoter strength over a wide range of intensities. The involvement of binding sites for transcription factors like Sp1, E2F, and NF-E1 could provide a means of regulation of the transcriptional initiation event.

The Promoter Region of the Human bFGF Gene

The territorial/functional limits of the human bFGF promoter have been only preliminarily looked at and many unanswered questions remain as to its functional architecture. As described above, the sole published investigation on this topic demonstrated that the region -21 to +173 relative to the start of transcription was sufficient to specify significant expression of the heterologous gene CAT [Shibata *et al.*, 1991]. Removal of sequences upstream from -21 eliminated the wild-type sequence at the -30 site (although as noted above, no TATA-box is resident in this region). The fact that this construct had significant activity suggested that the -30 site is not dominant in the basal promoter of the bFGF gene and hinted that this gene possesses a strong Inr. The -21 to +173 region contains only one recognizable element, an Sp1 binding site located at the +45 position. Shibata *et al.* identified a single distinct transcriptional start site by performing primer extension on RNA from two different cell lines [Shibata *et al.*, 1991]. The sequences surrounding the identified +1 site do not fall into even the most general Inr consensus, and the +1 site itself falls on a C residue which is somewhat atypical (see Figure 4.17). No analysis of accuracy of initiation was done with the -21 to +173/CAT construct or other CAT constructs reported by this group and so the question of which sequences are necessary to support accurate initiation of transcription from the human bFGF gene remains open. In addition, no further truncation of the bFGF promoter region was reported beyond the -21 to +173 region and so the question of which sequences within this 194 base pair region are contributing to bFGF gene expression remains open as well. It is this last question that I sought to address in the research described in Chapter 4 of this thesis.

Materials and Methods

Cell Culture

Te671 cells were obtained from the American Type Culture Collection (ATCC, Rockville Maryland) culture ATCC CRL 8805, TE671 subline number 2. U87-MG cells were obtained from ATCC, culture ATCC HTB 14. The Rat-1 fibroblast cell line was originated as described by Prasad *et al.* [1976]. The subline of Rat-1 cells used in these experiments is the same as that reported in the studies of Lenormand and coworkers [Lenormand *et al.*, 1990]. Hela cells were obtained from the Magun laboratory (Oregon Health Sciences University) with an indeterminate subline history. All cell strains and lines were grown in DMEM (GIBCO) supplemented with 10% calf serum (Hyclone) in a humidified incubator with a 5% CO_2/95% air atmosphere.

Transfection

Transfection of the above cell types was done using the calcium phosphate precipitate procedure as described by Kingston [1987b]. The cells were grown to 80-100% of confluence in 60 mm dishes and 5 μg of CAT reporter plasmid DNA along with 0.5 μg of a Luciferase reporter plasmid DNA, either pGL-Promoter or pGL-Control (Promega), was added in 0.5 ml of a HEPES buffered calcium phosphate suspension prepared as described. Most experiments examining the CAT activity of bFGF promoter constructs contained a set of cells transfected with pCAT-Basic, a promoterless vector, and another set of cells which were transfected with either pCAT-Promoter or pCAT-Control, vectors which contain the promoter or the promoter and enhancer regions of the SV40 T-antigen gene. The cotransfected luciferase expression vectors, either pGL-Control or pGL-Promoter were included for the purpose of monitoring transfection efficiency as described in Chapter 3. The DNA was allowed to remain on the cells overnight (12-18 hours) after which the cells were given fresh medium and allowed to

recover for 12 hours to 1 day. In most experiments the cells were at this point washed free of serum containing medium and allowed to remain in serum-free DMEM for 2 days at which point they were harvested. In some experiments no serum deprivation was done and cells were harvested 24-48 hours after the transfection recovery period.

CAT Assays/Luc Assays/Chemicals

CAT and Luc assays were carried out as described in Chapter 3. Treatment of data from these assays was also described in Chapter 3. Chemicals were obtained from the sources described in Chapter 3.

Plasmid Nomenclature/Linker Scanning (LS) Constructs

All of the LS constructs were made using the promoterless vector pCAT-Basic and so unless otherwise indicated in a plasmid name, the LS bFGF sequences are inserted into the Pst I/XbaI sites of this vector. As depicted in Table 4.1, the region -24 to +49 surrounding the transcriptional start site of the human bFGF gene has been for the purpose of nomenclature divided into sections of 8-9 bp each and these sections have been given a letter designation C through L. An upper case letter denotes a wild-type sequence while a lower case letter indicates that all of the bases in that region have been mutated. These sections were also referred to as "scans" or "scan regions". For example, e-scan refers to the mutated version of the sequences -8 to -1. A lower case letter in a LS plasmid name indicates wild-type sequence in all sections but the listed one. For example, construct 1e denotes a plasmid (based on pCAT-Basic) which contains bFGF wild-type sequence in all but the E region and which contains a non- wild-type base at each site within the E region, hence e. The LS constructs were made in two different contexts of bFGF sequence. Those which contain bFGF sequences from -24 to +49 were based on a plasmid, plasmid 3.4, which contains this wild-type bFGF region cloned into pCAT-Basic PstI/XbaI sites. The LS constructs based on this context are

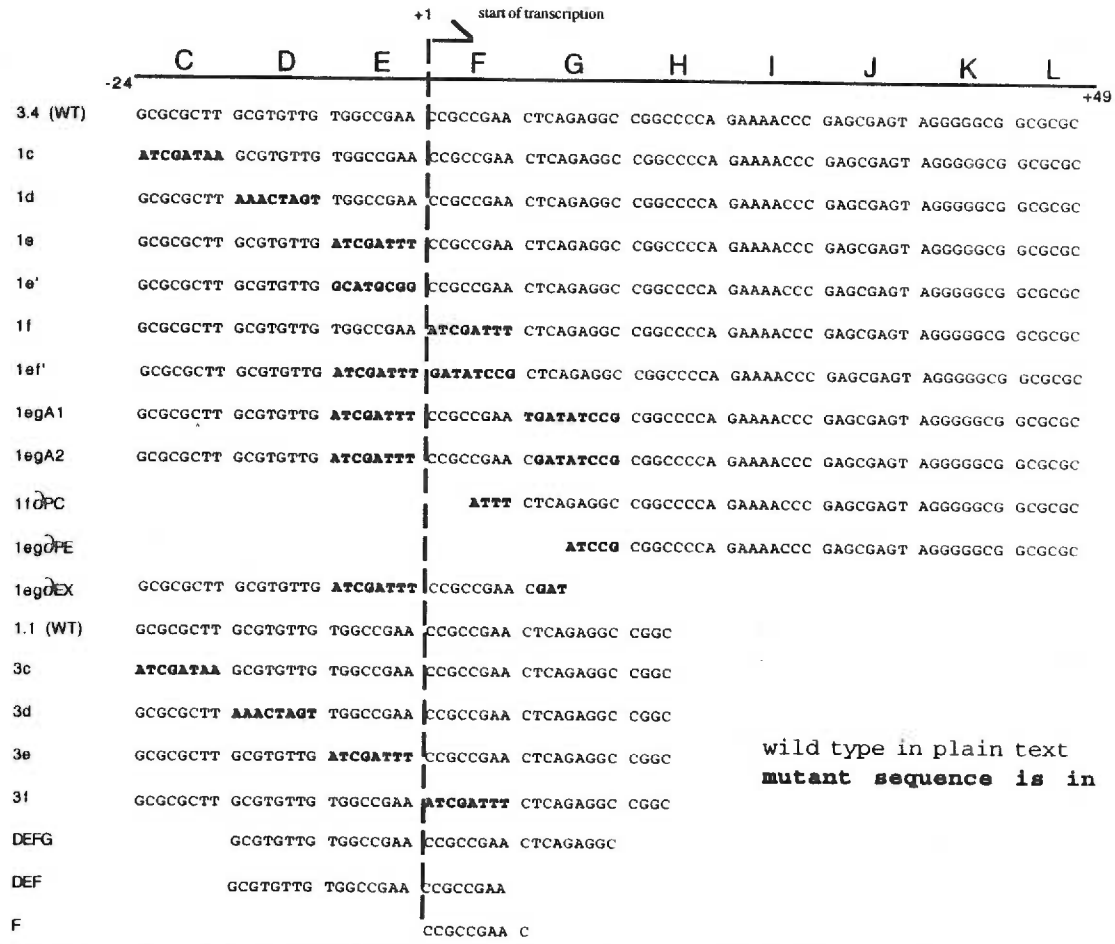


Table 4.1 bFGF sequences inserted into pCAT-Basic

All sequences are inserted into the Pst I/XbaI sites of pCAT-Basic and have the same vector sequences flanking them with the exception that the deletion constructs (∂) have slightly different vector sequences flanking them. Wild type sequence is in plain text, mutant sequence is in bold type.

named 1.x, where x is the mutated section. In the example of construct 1e cited above, the "1" denotes a -24 to +49 context, and the "e" denotes a replacement of the wild-type sequence in the E region with a mutated sequence. Some LS constructs have the context -24 to +17 and are based on the wild-type plasmid 1.1 which contains these regions inserted into pCAT-Basic. The LS constructs with this context are named 3.x, where x is the mutated section. Plasmid 3c denotes a -24 to +17 context inserted into pCAT-Basic with mutant bases in the C region and wild-type bases in all other regions.

Cassette constructs shorter than the 1.1 construct with -24 to +17 ends are referred to by listing all of the scan regions they have within them, again with wild-type designated by upper case and LS mutant regions being represented by lower case. Examples are construct DEF which contains wild-type regions -24 to +8 or construct F which contains wild-type regions +1 to +9.

Deletion constructs are named by the parent plasmid with a suffix, ∂, denoting that a deletion has been carried out followed by upper case letters after the ∂ taken from the first letter of the restriction enzymes that were used to make the deletion. For example, 1eg∂EX denotes a deletion done from parent construct 1eg (denoting wild-type sequence in all but the e and g regions and with a -24 to +49 context) using the restriction enzymes EcoRV and Xba I.

Table 4.1 contains a list of the LS, cassette, and deletion constructs used in the experiments reported below along with the bFGF sequences they contain.

Subcloning

Subcloning was carried out using established protocols, for example those contained in Current Protocols in Molecular Biology (Wiley Interscience, 1992).

Linker scanning (LS) constructs were made using the polymerase chain reaction technique (PCR) with one primer containing the mutant sequence and the other containing sequences homologous to vector sequences in the parent plasmid. In most

cases the mutation-specific primer also carried a convenient restriction site such that the PCR product could be conveniently ligated back into pCAT-Basic. Table 4.2 lists the primers used in the creation of the LS series of various bFGF-CAT plasmids. In most cases, the first primer listed in Table 4.2 was the mutation specific primer and the second primer, either RevCAT or #86, was a general primer which was homologous to sequences in pCAT-Basic downstream from the multiple cloning site.

The small cassette constructs DEFG, DEF, and F are also listed in Table 4.2. DEFG was made using PCR and two wild-type primers, #227 and #229, which had Pst I and Xba I restriction sites in them respectively to allow easy subcloning back into pCAT-Basic. DEF was made via PCR using a similar pair of wild-type primers #227 and #228. The F cassette was created by directly annealing two oligonucleotides, #230 and #231 to form a double stranded small cassette containing bFGF sequences from +1 to +9 with Pst I/XbaI compatible recessed ends. The F cassette was inserted into the Pst I/Xba I sites of both pCAT-Basic and into pCAT-Promoter (both from Promega).

Oligonucleotide primers were synthesized in the laboratory using a Cyclone Plus DNA Synthesizer (MilliGen/Biosearch). Several constructs, including the prototypic bFGF promoter/CAT plasmids 3.4 and 1.1, were made by using restriction enzymes to delete sequences from a wild-type or LS series construct. Table 4.3 lists the restriction-based deletion constructs along with the enzymes used to generate them and the parent plasmids from which they were derived.

PCR

Polymerase chain reaction (PCR), both preparative and analytical, was done in a Coy Model 50 Programmable Incubator (Coy Laboratory Products Inc.) using Taq DNA polymerase and polymerase buffer from the Promega Corporation. The standard cycle that was used was 96°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds.

name	primers used in construction	PCR template	bFGF region included
1c	#190 5' GAAATCTGCAGGTCATCGATAAGCGTGTTGTG RevCAT 5' CTGAGCTCCTGAAGATCTCGCCAAGCTC	3.4	-24 to +49
1d	#191 5' GAAATCTGCAGGTCGCGCGCTTAAACTAGTTGGCCGAACC RevCAT 5' CTGAGCTCCTGAAGATCTCGCCAAGCTC	3.4	-24 to +49
1e	#193 5' GAAATCTGCAGGTCGCGCGCTTGCGTGTTGATCGATTTCCGCCGAACT RevCAT 5' CTGAGCTCCTGAAGATCTCGCCAAGCTC	3.4	-24 to +49
1e'	#214 5' GAAATCTGCAGGTCGCGCGCTTGCGTGTTGGCATGCGGCCGCCGAACT RevCAT 5' CTGAGCTCCTGAAGATCTCGCCAAGCTC	3.4	-24 to +49
1f	#194 5' GAAATCTGCAGGTCGCGCGCTTGCGTGTTGTGGCCGAAATCGATTTCTCAGAGGCCC RevCAT 5' CTGAGCTCCTGAAGATCTCGCCAAGCTC	3.4	-24 to +49
1ef '	#215 5' GAAATATCGATTTGATATCCGCTCAGAGGCC RevCAT 5' CTGAGCTCCTGAAGATCTCGCCAAGCTC	1e	-24 to +49
1egA1	#216* 5' GAAATATCGATTGCCGCCGAAGGATATCCGCGGCCCCAGA RevCAT 5' CTGAGCTCCTGAAGATCTCGCCAAGCTC	1e	-24 to +49
1egA2	#216* 5' GAAATATCGATTGCCGCCGAAGGATATCCGCGGCCCCAGA RevCAT 5' CTGAGCTCCTGAAGATCTCGCCAAGCTC	1e	-24 to +49
3c	#190 5' GAAATCTGCAGGTCATCGATAAGCGTGTTGTG #86 5' CCATTATAAGCTGCAATAAACAAG	1.1	-24 to +17
3d	#191 5' GAAATCTGCAGGTCGCGCGCTTAAACTAGTTGGCCGAACC #86 5' CCATTATAAGCTGCAATAAACAAG	1.1	-24 to +17
3e	#193 5' GAAATCTGCAGGTCGCGCGCTTGCGTGTTGATCGATTTCCGCCGAACT #86 5' CCATTATAAGCTGCAATAAACAAG	1.1	-24 to +17
3f	#194 5' GAAATCTGCAGGTCGCGCGCTTGCGTGTTGTGGCCGAAATCGATTTCTCAGAGGCCC #86 5' CCATTATAAGCTGCAATAAACAAG	1.1	-24 to +17
DEFG	#227 5' TCGCCTGCAGGCGTGTTG #229 5' TCTGTCTAGAGCCTCTGAG	3.4	-16 to +16
DEF	#227 5' TCGCCTGCAGGCGTGTTG #228 5' CGGCTCTAGACTTCGGCGG	3.4	-16 to +9
F	#230 5' GCCGCCGAAC #231 5' CTAGGTTCGGCGGCTGCA	oligos 230/231 directly annealed	+1 to +9

* oligo #216 was made with one degenerate position

Table 4.2 Creation of bFGF linker scanning and small cassette constructs

This table lists the oligonucleotide primers used in the construction of the linker scanning mutation and small cassette series of plasmids. All inserts except the F plasmid insert were constructed using PCR with the listed template. The insert in the F plasmid was created by directly annealing two synthetic oligonucleotides. The bFGF sequence inserts generated either by PCR or direct annealing were ligated into the Pst I/Xba I sites of the plasmid pCAT-Basic. The F insert was also ligated into the Pst I/Xba I sites of the plasmid pCAT-Enhancer.

name	restriction enzymes used in construction	parent construct(s)	bFGF region included
3.4	BssHII	pGEbFGF pCAT-Basic	-24 to +49
1.1	NaeI Xba I	pGEbFGF pCAT-Basic	-24 to +17
1f∂PC	Pst I Cla I	1f	+9 to +49
1eg∂PE	Pst I Eco RV	1egA2	+18 to +49
1eg∂EX	Eco RV Xba I	1egA2	-24 to +9

Table 4.3 Creation of deletion constructs from the bFGF promoter region

This table lists the restriction enzymes used to create several deletion constructs. In the cases of plasmids 3.4 and 1.1 the indicated enzymes lifted the indicated bFGF sequences from the first parent construct listed and the excised sequences were then ligated into pCAT-Basic. In the remaining plasmids the indicated enzymes were used to excise internal sequences after which the plasmids were religated.

In Vitro Transcription/Primer Extension

Coupled *in vitro* transcription (IVT) and primer extension reactions were attempted as described by Zenzie-Gregory *et al.* [1992] and references therein with the slight changes that the ribonuclease inhibitor RNasin (Promega) was added to the IVT reactions to a final concentration of 0.5 units/μl and that in some experiments the *in vitro* transcripts were purified by the technique of Gough [1988] rather than that reported by

Jones *et al.* [1985]. The primer used in the primer extension reactions was RevCAT (see Table 4.2) which contains sequences homologous to nucleotides 2280 through 2305 of the pCAT-Basic vector. The 2280 site is 12 bases downstream from the Xba I site of this vector into which the majority of the bFGF sequences were inserted. The primer extended products were therefore predicted to be of a size 40 bp plus any bases contributed by the bFGF insert.

RevCAT was labelled for use in primer extension reactions using T4 polynucleotide kinase and γ^{32}P-ATP as described by Kingston [1987a]. Annealing of RevCAT and the *in vitro* transcribed RNA from various CAT plasmids was done according to Jones *et al.* [1985] or according to Kingston [1987a].

Primer extension products were run on standard 0.4 mm thickness sequencing gels along with sequencing reactions which allowed an absolute sizing of the primer extension products.

Run-off IVT

Run-off IVT which generated directly labelled *in vitro* transcripts was attempted according to the protocols given in Promega Technical Bulletin #123. The Promega IVT protocols were similar to those reported by Zenzie-Gregory *et al.* [1992] which were used in the coupled IVT/primer extension reactions. The minor differences between the two types of IVT reaction lay mainly in subtle differences in the concentrations of $MgCl_2$, HEPES, and KCl and in the addition of α^{32}P-CTP to the labelled IVT reactions. The 25 µl IVT labeling reactions contained 400 µCi of α^{32}P-CTP, as well as 0.64 µM unlabelled CTP and 40 µM each unlabelled ATP, GTP and UTP.

In some of the run-off IVT experiments the newly transcribed RNA was purified according to Promega technical bulletin #123, which lists a procedure essentially the same as that reported by Jones *et al.*[1985], while in other run-off IVT experiments the RNA was purified by the procedure reported by Gough [1988].

Run-off IVT reaction products were sized on a 0.4 mm thickness DNA sequencing gel using DNA sequencing reactions as size standards.

Nuclear Extracts

The nuclear extracts used in both the coupled IVT/primer extension and the run-off IVT procedures were made from HeLa cells. One batch of extract was made in our laboratory according to the procedure reported by Zenzie-Gregory *et al.* [1992] and references within [Dignam *et al.*, 1983; Briggs *et al.*, 1986], while another batch of nuclear extract used for these experiments was commercially prepared, HelaScribe Nuclear Extract purchased from Promega.

Results

Initial Observations

Figure 4.1 shows the result of a transient expression assay comparing the CAT activity supported by some of the macro-deletion constructs described in Chapter 3 with that supported by the more radical deletions contained in constructs 3.4 and 1.1. These plasmids contain only very small amounts bFGF promoter proximal sequences, 73 bp and 41 bp respectively, inserted into pCAT-Basic. In this experiment and in most subsequent experiments these two small segments of wild-type sequence surrounding the start of transcription, the +1 site determined by Shibata *et al.*[1991], had as much or slightly greater promoter activity than did constructs like pCB2k which contained sequences -2000 to +173. This was a striking finding and it motivated the further investigation into what sequences proximal to the human bFGF +1 site were necessary and sufficient for promoter activity at this locus.

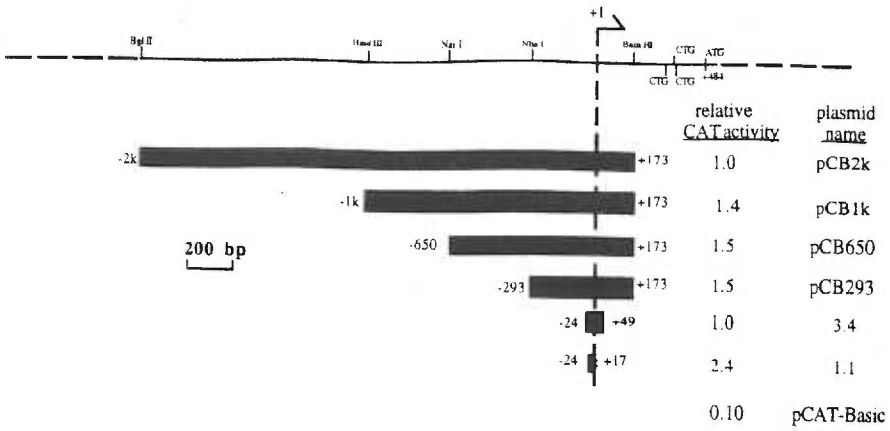


Figure 4.1 CAT activity of progressive 5' deletions of the bFGF upstream region

Three dishes of U87-MG cells were transfected with the indicated plasmids by the calcium phosphate technique as described. Three days after transfection CAT extracts were isolated and assayed as described. The relative CAT activity was calculated as the average CAT activity for a particular sample divided by the average CAT activity in extracts from cells transfected with pCB2k.

The First Linker Scanning Series of Mutations

Two distinct kinds of perturbation of the wild-type sequence near the human bFGF transcriptional start were done. The first type was linker scanning (LS) mutagenesis. The first group of LS constructs, series c through f, mutated 8 bp contiguous non-overlapping segments of sequence in the region -24 through +8 in both -24 to +49 and -24 to +17 contexts. Figure 4.2 shows the bFGF sequences contained in the first series of LS constructs. The data from a transient expression assay done using these constructs is seen in Figure 4.3 and a diagrammatic rendering of this data is seen in Figure 4.4.

A number of interesting things are seen in this data. The most dramatic result is the activity of the e-scan which changed all bases from -8 through -1. This change resulted in a dramatic increase in promoter activity in both contexts. In the -24 to +49 context, the e-scan resulted in a 17.8 fold increase in activity and resulted in an activity which was nearly twice that conferred by the SV40 early region promoter contained in the plasmid pCAT-Promoter. In contrast, the effect in this context of the other LS

Figure 4.2 bFGF sequences contained in the first linker scanning series

This figure shows the bFGF sequences containing the first linker scanning series of mutations, inserted into the vector pCAT-Basic. The inserts were all placed into the Pst I/Xba I sites of pCAT-Basic. The vector sequences flanking these inserts are the same at the 5' end and vary slightly at the 3' end between the 3.4 and 1.1 contexts. Wild-type sequence is in plain text and mutant sequence is in bold type.

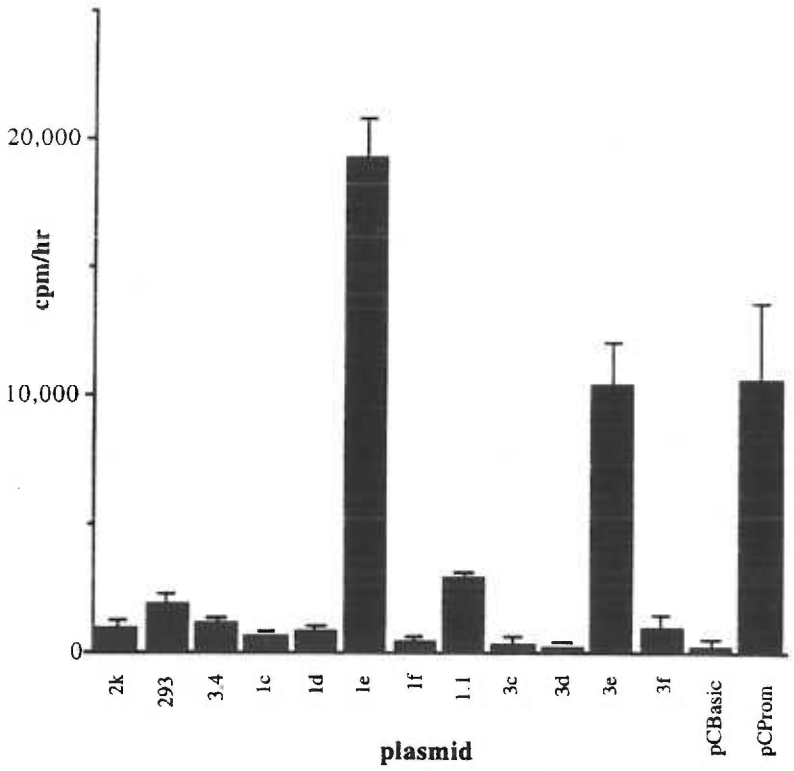


Figure 4.3 CAT activity of the first series of linker scanning mutations of the bFGF promoter region

U87 MG cells were transfected with the indicated plasmids by the calcium phosphate technique as described. Three days after transfection CAT extracts were prepared and assayed as described in the text. Values for CAT activity, in cpm/hr, represent the average of the CAT activities contained in extracts from three dishes of cells which received the same DNA precipitate. Error bars represent the standard deviation of the mean value. Plasmids 2k and 293 are also named pCB2k and pCB293 and are described in Chapter 3. pCBasic and pCProm are plasmids pCAT-Basic and pCAT-Promoter, respectively. pCAT-Basic is a promoterless plasmid upon which the other constructs are based. pCAT-Promoter contains the strong promoter from the early region of SV40.

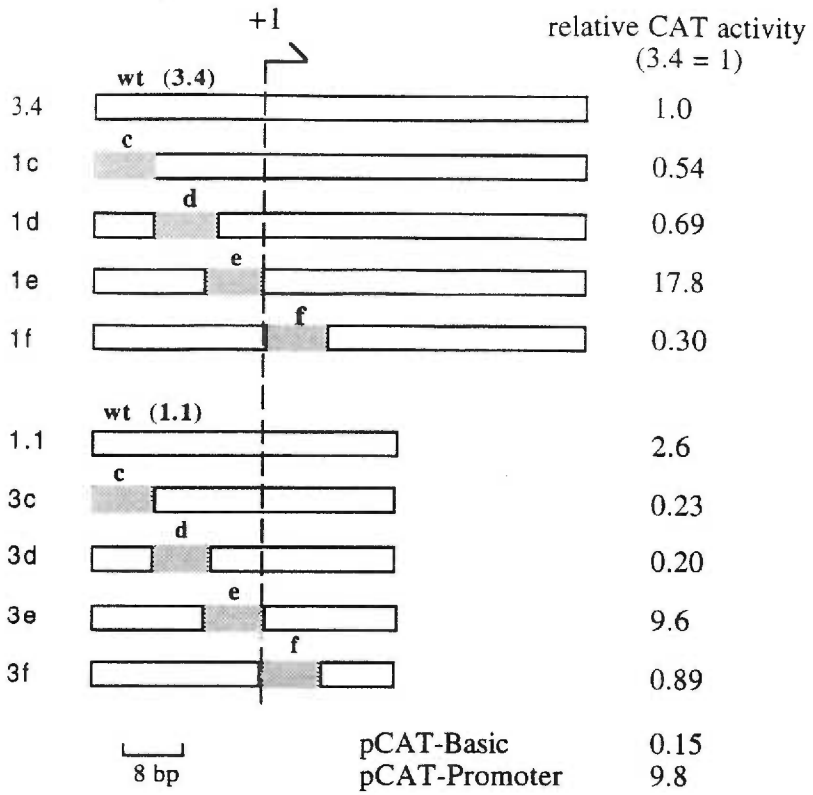


Figure 4.4 Relative CAT activity of the first series of linker scanning mutations of the bFGF promoter region

The data in this figure is derived from the data shown in Figure 4.3. The relative CAT activity was calculated as the average CAT activity given by a particular construct divided by the average CAT activity in extracts from cells transfected with plasmid 3.4. The horizontal bars indicate the regions surrounding the +1 site of the human bFGF gene that are contained in the plasmids. Open bars indicate wild-type sequence and shaded bars indicate mutated sequence.

mutations, c, d, and f, was to decrease promoter activity to 0.54, 0.69, and 0.30 of the wild-type. These values were still above those given by the promoterless vector pCAT-Basic, particularly in the 1c and 1d cases, and so these mutations while deleterious were not catastrophic in effect on promoter activity.

The same set of mutations made in the shorter, -24 to +17, context gave similar trends. It is notable the -24 to +17 wild-type construct (plasmid 1.1) had slightly greater activity than the -24 to +49 wild-type construct (plasmid 3.4). This was consistently observed (see also Figures 4.1 and 4.13). The activating nature of the e-scan mutation was seen in the shorter context, but the activation was far less than in the longer context. The e-scan in the -24 to +17 context resulted in only a 3.7-fold increase in activity relative to wild-type, plasmid 1.1, and resulted in an activity which was comparable to pCAT-Promoter. The c, d, and f scans were again deleterious in the shorter context just as in the longer, with the c and d scans bringing activity to levels very close to zero.

It was an apparent paradox that while the unperturbed 1.1 plasmid gave rise to consistently greater CAT activity than did the unperturbed 3.4 plasmid, any parallel change in the wild-type sequences of the two plasmids reversed this relation. That is, seemingly any change to plasmid 1.1, even the activating e-scan mutation, gave a plasmid with less activity than given by the same change to plasmid 3.4. This will be discussed below.

The Methylation Hypothesis

At this point two specific hypotheses which sought to explain the exceptional e-scan result were prompted by inspection of the DNA sequence. The first hypothesis was inspired by the observation that while both the e and f scans had identical insertions of ATCGATTT, each with an ATCGAT Cla I restriction site in the mutated scan region, only the f scan constructs in either context would actually cut with Cla I. I hypothesized that the most likely explanation of this was that the host strain of bacteria that these

plasmids had been grown in had methylated the e but not the identical f-scan region due to a difference in the bases flanking the scan region. The Methylation Hypothesis was framed to answer the question of whether the exceptional nature of the e-scan with respect to promoter activity was somehow tied to the observation that the e-scan constructs would not restrict with the enzyme Cla I. Figure 4.5 displays this hypothesis.

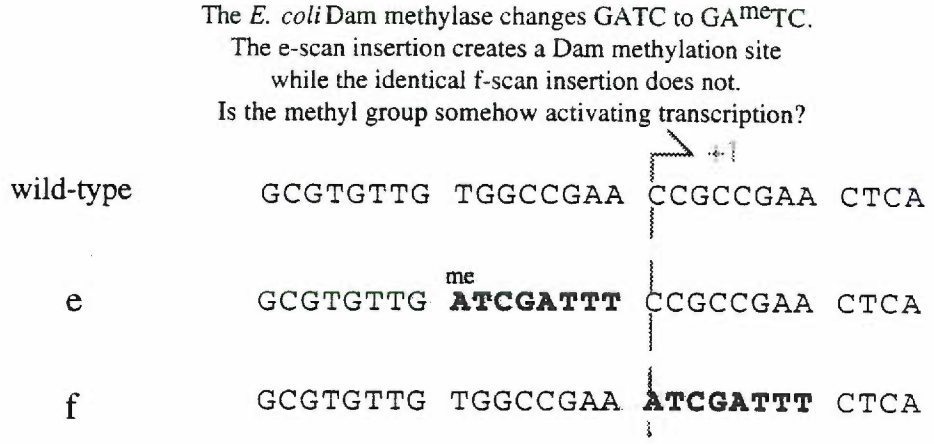


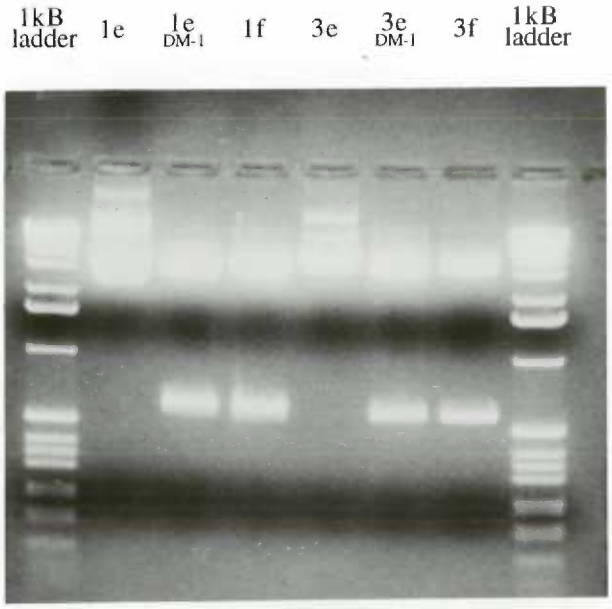
Figure 4.5 The Methylation Hypothesis
This figure shows that construct 1e contains a site for *E. coli* Dam methylation near the +1 site while the wild type and f-scan sequences do not. The Methylation Hypothesis seeks to test the link between this difference in the e- and f-scans with the difference in their CAT activity.

The Methylation Hypothesis was testable in two steps. The first step was to verify that methylation was indeed occurring at the e-scan site. The 1e constructs along with other plasmids were transformed into a host strain of bacteria which lack the Dam methylation system predicted to be responsible for the e-scan methylation. *E. coli* strain DM-1 (Bethesda Research Laboratories) which lacks the Dam methylation system was used for this purpose. Plasmids were then isolated from this host strain and examined for their sensitivity to restriction with Cla I. Figure 4.6 is a photograph of an agarose gel displaying the result of restriction reactions done on e-scan constructs grown in methylating or non-methylating strains of bacteria. If one compares lanes 2 and 3 of this

gel one can see that only the 1e construct which had been grown in the DM-1 non-methylating strain of bacteria, lane 3, gave the predicted Cla I-Nco I band at 563 nucleotides. In lane four was run a Cla I-Nco I restriction reaction of the 1f plasmid grown in the normal strain of bacteria (DH5α) with its Dam methylation system intact. This lane also contains the Cla I-Nco I product showing that as originally seen, the Cla I site in the f-scan was not being methylated even in a methylating strain. The first part of the hypothesis, the demonstration that bacterial methylation was responsible for the original difference in restriction sensitivity displayed by the e-scan and f-scan constructs, was supported by this data and by that from other similar restriction reactions.

The second part of the hypothesis sought to link the methylation differences demonstrated between the 1e and 1f constructs with the differences seen in their ability to support CAT expression. If the greatly increased activity of the e-scan containing constructs was due to some aspect of the methylations taking place at the -8 position of the e-scan, then one would predict that 1e plasmids grown in DM-1 bacteria should show little greater activity than the wild-type or f-scan constructs. Figure 4.7 shows the result of a CAT assay done with either methylated or non-methylated e-scan LS constructs. The passage of plasmids through the DM-1 host bacterial strain (designated with the suffix "-d") appears to have resulted in slightly less promoter activity. Note for example that pCAT-Promoter shows a reduced activity after having been passed through the non-methylating strain of bacteria. It can be seen, however, that the non-methylated e-scan constructs are still activated to a large degree in comparison to their wild-type counterparts. The CAT activity supported by the unmethylated 1e plasmid (1e1-d) at about 2300 cpm/hr is still much greater than that supported by the unmethylated 3.4 plasmid (3.4-d) at 300 cpm/hr. Therefore, in its simplest form the Methylation Hypothesis was not supported by the data.

The observation that most if not all plasmids appeared to have slightly less



DM-1 indicates the plasmid has been grown in bacterial strain DM-1 which lacks a Dam methylation system

Figure 4.6 Agarose gel electrophoresis of restriction digests done to assess plasmid Dam methylation state

The indicated plasmids were restricted with the enzymes Cla I/Nco I and electrophoresed through a 1.2% agarose gel. Plasmids which had been isolated from bacterial strain DM-1 and thus predicted to have no Dam methylation are indicated. 1kb ladder size standards were purchased from Bethesda Research Laboratories.

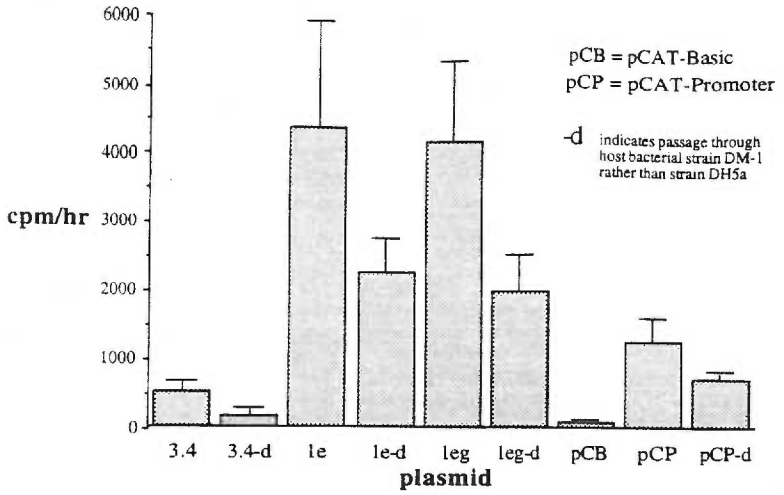


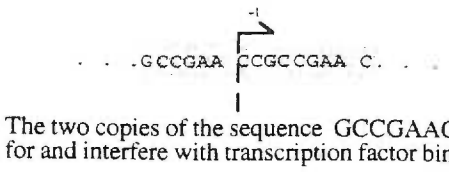
Figure 4.7 The effect of plasmid methylation on CAT activity
The indicated plasmids were transfected into U87-MG cells by the calcium phosphate technique as described in the text. Three days after transfection CAT extracts were isolated and assayed as described in the text. The indicated CAT activity, expressed as cpm/hr, is the average of the activities found in extracts from three dishes of cells transfected with the same DNA precipitate. Error bars represent the standard deviation of the mean value. The suffix "-d" indicates that the plasmid had been grown in the DM-1 bacterial strain, a strain which lacks the Dam methylation system.

activity after being passed through the DM-1 strain was not predicted. Since all of these plasmids had been purified twice on cesium chloride gradients it did not seem likely that cellular contaminants specific to the DM-1 strain were the cause. Both pCAT-Basic and pCAT-Promoter plasmids contain 18 intrinsic GATC sites at which methylation could take place in cells with a competent Dam methylation system. It is possible that the methylated form of these plasmids is somehow more stable *in vitro* or in the transfected mammalian cells. Although outside the scope of my investigations, I found this is to be an interesting observation potentially worthy of study.

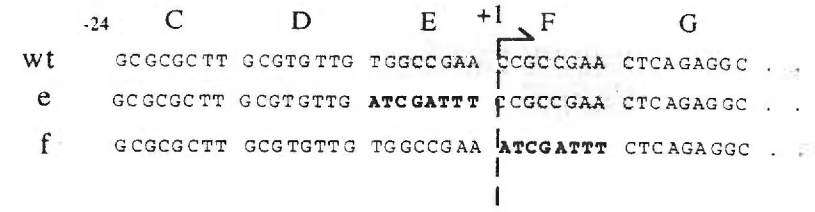
The Initiation Interference Hypothesis

A second hypothesis as to the e-scan's activity was suggested by inspection of the wild-type sequence surrounding the +1 site. Figure 4.8 diagrams this hypothesis. A direct repeat of the sequence GCCGAAC is seen to occur around the +1 site. When the e and f scans are examined for their disruption of the wild-type sequence it is seen that the

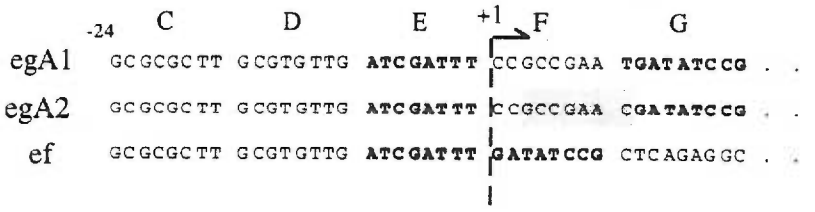
Figure 4.8 The Initiation Interference Hypothesis



A. The e scan leaves one GCCGAAC, the f scan does not.



B. egA2 leaves one GCCGAAC, egA1 and ef leave none.



e-scan cleanly removes the upstream copy of this repeat and leaves the downstream copy intact. The f-scan removes the downstream copy but also removes part of the upstream copy. The f-scan replaces the base which lies at +1 which might be predicted to be an important base. The Initiation Interference Hypothesis predicts that the wild-type sequence is a poor template for transcriptional initiation because factors compete to bind at the two adjacent repeats and that the competition results in poor initiation. In this scheme the e-scan could be understood to activate transcription by removing one site completely, thereby removing the interference. The f-scan fails to activate because it fails to leave at least one functional binding site.

While this hypothesis also has more subtle forms that postulate that the repeated elements are non-identical due to position effects, the simplest form was analyzed by the creation and testing of several more LS constructs, the ef' scan and two distinct eg scans. Figure 4.8B depicts these LS constructs and their full sequence is also listed in Table 4.1. In plasmid 1ef' the wild-type region in the region -8 to +8, sections E and F, has been

replaced with non-wild-type sequence. This change removes both of the repeated GCCGAAC motifs. Implicit in the Initiation Interference Hypothesis is that these directly repeated elements are important for transcriptional initiation and so the hypothesis would prompt the prediction that removing both of them would drastically reduce promoter activity. Figure 4.9 shows data from an experiment examining the CAT activity supported by the 1ef construct. What is seen is that while the ef' construct is impaired with respect to the activity of the e construct, it is still quite activated with

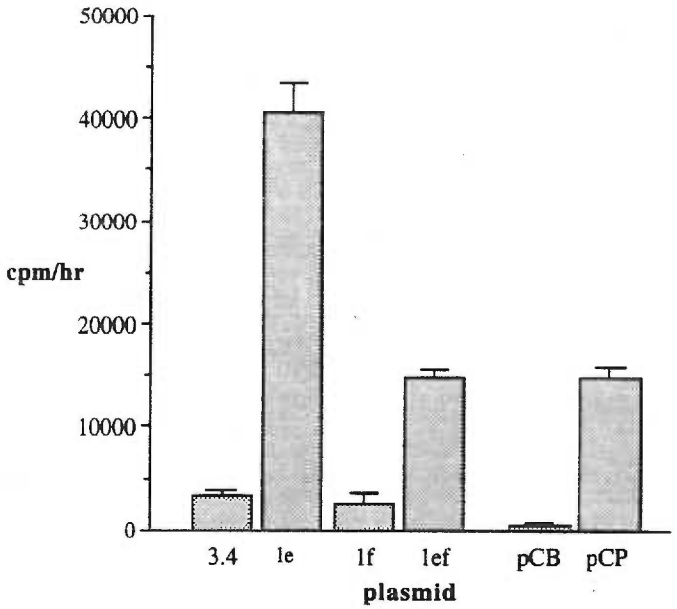


Figure 4.9 CAT activity of linker scanning constructs 1e,1f, and 1ef'

The indicated plasmids were transfected into Te671 cells by the calcium phosphate method as described in the text. Three days after transfection CAT extracts were isolated and these were assayed as described in the text. The indicated CAT activity, expressed as cpm/hr, is the average of the activities found in extracts from three dishes of cells transfected with the same DNA precipitate. Error bars represent the standard deviation of the mean value. pCB and pCP are plasmids pCAT-Basic and pCAT-Promoter.

respect to the wild-type 3.4 construct. This indicates that neither GCCGAAC motif is essential for promoter activity and tends to discount the simplest form of the Initiation Interference Hypothesis.

The eg scans were another series of constructs which were created in part to

examine the Initiation Interference Hypothesis. The impact of the eg scan mutations on the GCCGAAC direct repeats may be seen in Figure 4.8B. The eg LS constructs were designed to simultaneously replace the wild-type sequences in both the E and G sections of the bFGF promoter in such a way as to either mutate or leave intact the single GCCGAAC motif contained in the 1e plasmid. Two forms of the 1eg construct were made. In one form, 1egA1, all of the wild-type bases in the G section were replaced. This resulted in the removal of the terminal C residue from the single GCCGAAC motif and left the 1egA1 plasmid with no intact copies of this motif, much like the 1f plasmid. In the second form, 1egA2, one wild-type base was retained in the G section. This allowed 1egA2 to retain an intact GCCGAAC motif, like the parental 1e plasmid. The original Initiation Interference Hypothesis would prompt the prediction that the activity supported by the 1egA1 plasmid with its imperfect single copy of the GCCGAAC motif, would be greatly reduced with respect to the activity supported by the 1egA2 plasmid which retained an unaltered GCCGAAC motif. Figure 4.10 shows data from an

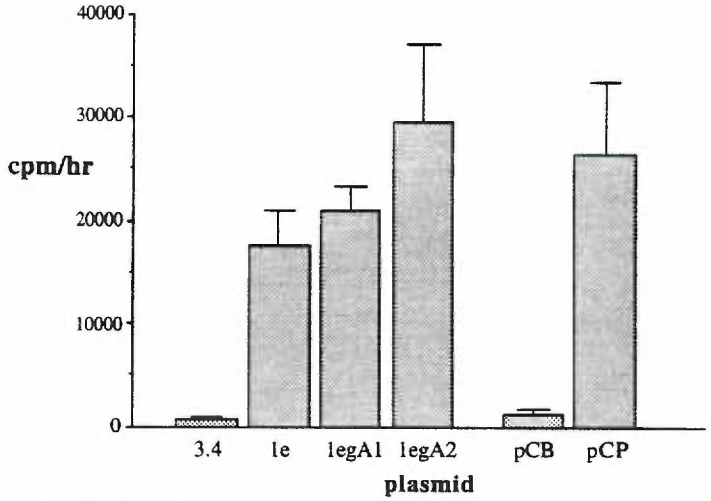


Figure 4.10 CAT activity of linker scanning constructs 1e, 1egA1, and 1egA2

The indicated plasmids were transfected into Te671 cells by the calcium phosphate method as described in the text. Three days after transfection CAT extracts were isolated and these were assayed as described in the text. The indicated CAT activity, expressed as cpm/hr, is the average of the activities found in extracts from three dishes of cells transfected with the same DNA precipitate. Error bars represent the standard deviation of the mean value. pCB and pCP are plasmids pCAT-Basic and pCAT-Promoter.

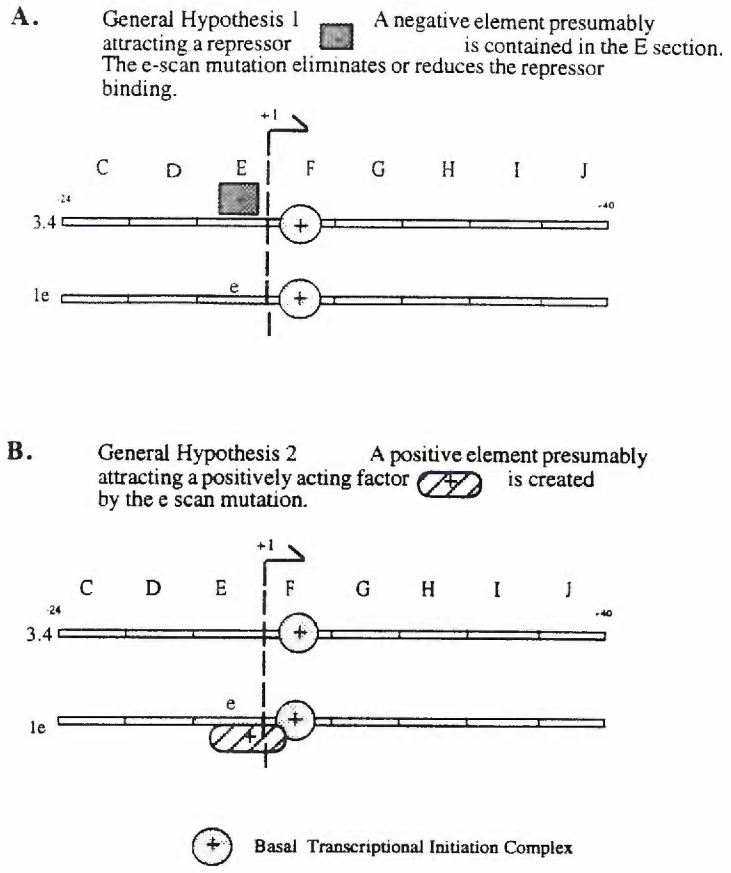
experiment testing the CAT activity supported by the two types of eg mutation. The two versions of the 1eg plasmid have virtually the same activity. This data, like the data seen Figure 4.9, does not support the simplest version of the Initiation Interference Hypothesis. Also observable in this data is the phenomenon that the two forms of the 1eg plasmid have the same activity as the 1e plasmid. This implies that nothing in the G region, +9 to +17, is contributing to promoter activity.

Two General Hypotheses as to e-Scan Activation

One more significant LS construct was made and tested in an effort to gain insight into the activating nature of the e-scan mutation. A second e-scan mutation, e', was made such that the bases in the E section of the bFGF sequences in this new plasmid were neither wild-type nor e-type. The 1e' plasmid was made in an attempt to distinguish between two general hypotheses put forth to explain the e activation effect. These hypotheses are diagrammed in Figure 4.11. General Hypothesis 1 postulates that the E region harbors a negative regulatory element and would explain e activation as being due to the deletion of this negative element. General hypothesis 2 postulates that the e mutation has serendipitiously created a positive element that is not present in the wild-type sequence. General Hypothesis 2 is further constrained by the observation shown in Figures 4.3 and 4.4 that the f-scan plasmids, which contain the identical insertion as the e-scan plasmids, had activity which was reduced instead of increased with respect to wild-type plasmids. A gain of activity being supplied by the e-scan but not by the f-scan would have had to arise from the junction between the inserted mutated sequence and the contextual wild-type sequence or from a highly unusual position effect.

The 1e' plasmid would be predicted to be activated with respect to wild-type under General Hypothesis 1 because a second non-wild-type scan in the E section would be predicted to similarly disrupt the sequence of E which was responsible for the repression. Under General Hypothesis 2, the 1e' plasmid would be expected to either not

Figure 4.11 **Two General Hypotheses as to the nature of the e-scan activation**



be activated or to have reduced activity with respect to wild-type. This is because it would appear improbable that two different mutations at the E-region would by chance provide the same positive regulatory element or provide two different positive regulatory elements. Figure 4.12 shows CAT data from an experiment conducted with the 1e' plasmid. The activity of 1e' is seen to be significantly less than the activity of 1e scan but it is seen to be activated with respect to the wild-type. Over a number of experiments the activity of 1e' was seen to be variable but these trends were maintained, that is, 1e' is activated with respect to wild-type but is far less active than 1e. This result is intermediate of the results predicted by the two hypotheses and as such does not strongly support either one. Of the two framed General Hypotheses the first is more supported by

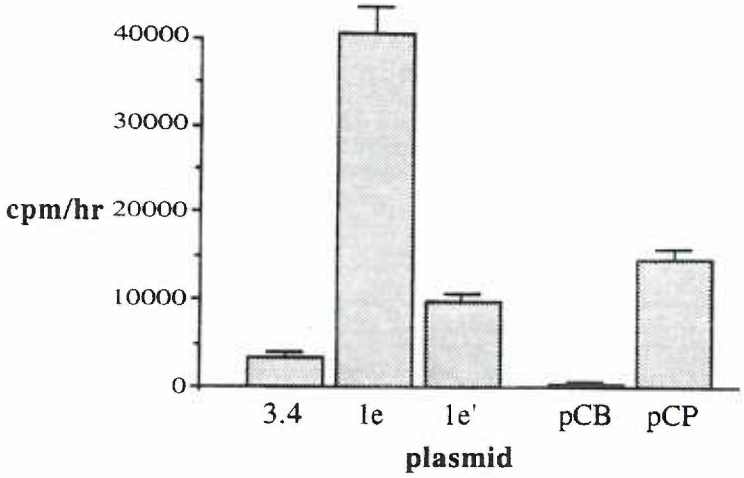


Figure 4.12 CAT activity of linker scanning constructs 1e and 1e' The indicated plasmids were transfected into Te671 cells by the calcium phosphate method as described in the text. Three days after transfection CAT extracts were isolated and these were assayed as described in the text. The indicated CAT activity, expressed as cpm/hr, is the average of the activities found in extracts from three dishes of cells transfected with the same DNA precipitate. Error bars represent the standard deviation of the mean value. pCB and pCP are plasmids pCAT-Basic and pCAT-Promoter.

this data due to the fact that it seems more probable that two different mutations of the E region would destroy or partially destroy a wild-type binding site than the case where two different mutations e and e' would both create a novel binding site but of different strengths. In the context of General Hypothesis 1, however, it is dissatisfying that the magnitude of the e'-scan activation is far less than the e-scan activation.

Deletion Mutations and Small Cassette Constructs

In an effort to obtain more information on the minimum sequence requirement for activity from the bFGF promoter region several constructs were made which contained substantially less bFGF promoter proximal sequence than contained by the 73 bp of plasmid 3.4 or by the 41 bp of plasmid 1.1. A series of deletion constructs was made by removal of restriction fragments from LS constructs or by the use of synthetic oligonucleotides as described in the methods section. Tables 4.1, 4.2, and 4.3 display the bFGF sequences inserted into these constructs as well as details of their creation. Figure

4.13 shows the CAT activity supported by this series of bFGF promoter sequences inserted into pCAT-Basic.

Plasmids 1f∂PC and 1eg∂PE which contain wild-type bFGF sequences +9 to +49 and +18 to +49, respectively, support no more CAT activity than does the promoterless host vector pCAT-Basic. This points to sequences further upstream than +9 as being necessary for bFGF promoter activity. Constructs like the wild-type 1.1 plasmid and the deletion plasmid 1eg∂EX with bFGF sequences -24 to +17, and -24 to +9, respectively, have as much activity as plasmids like 3.4 which contains sequences from -24 to +49. This suggests that sequences further downstream from +9 are not essential for normal levels of activity.

The focus on finding what promoter activity may reside in sequences further upstream than +9 was taken to its extreme in the small cassette series of constructs the CAT activities of which are also seen in Figure 4.13. Constructs DEFG and DEF containing bFGF sequences -16 to +16 and -16 to +8 appear to support diminished activity with respect to plasmids 3.4 or 1.1. This is consistent with the diminished activity supported by the c-scan constructs and would suggest that some positive elements reside in the -24 to -16 C region. Paradoxically, the smallest cassette construct, the F scan, which lacks the implicated C region and which contains only the wild-type bFGF sequences from +1 to +9, supported CAT activity at the same or at slightly higher levels than the 3.4 and 1.1 plasmids. This was consistently observed. In the experiment shown in Figure 4.13, the isolated F sequence supported activity which was twice that of plasmid 3.4 and which was 45% of that given by plasmid pCAT-Promoter containing the SV40 early region's strong promoter.

Another aspect of the results seen in Figure 4.13 is that while the 1eg∂EX deletion construct had activity comparable to plasmid 1.1 or 3.4, it did not have activity comparable to 1e which would have been predicted if no contributing activities were located in the +9 to +49 region.

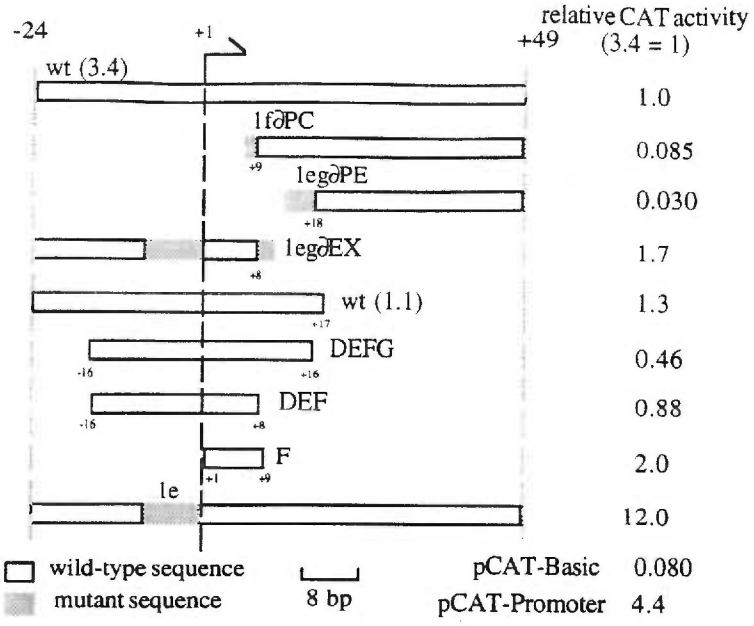


Figure 4.13 Relative CAT activity of selected deletion/cassette constructs

Te671 cells were transfected with the indicated plasmids by the calcium phosphate technique as described. Three days after transfection CAT extracts were isolated and assayed as described. The relative CAT activity was calculated as the average CAT activity obtained from a particular plasmid divided by the average CAT activity in extracts from cells transfected with plasmid 3.4. The horizontal bars indicate the regions surrounding the +1 site of the human bFGF gene that are contained in the plasmids. Unfilled bars indicate wild-type sequence and shaded regions indicate mutated sequence.

Collaboration of the F sequence Activity with an Upstream Element

The wild-type F sequence cassette which consisted of bFGF sequences from +1 to +9 relative to the start of transcription was then tested to see if its activity was responsive to that of an exogenous enhancer. The F sequence cassette was inserted into the vector pCAT-Enhancer (Promega) which is a derivative of plasmid pCAT-Basic that contains the strong enhancer from the SV40 early gene region. The context of the F sequence insertion into pCAT-Basic and pCAT-Enhancer was identical and the only

differences in the two plasmids was the presence in the latter of the SV40 enhancer which was inserted at a location 1650 bp downstream from or 2700 bp upstream from the insertion site of the F sequence. Figure 4.14 displays the results of one of a couple of experiments which examined the activity supported by the F sequence in these two plasmid contexts. The activity of the F sequence in pCAT-Basic, plasmid pCB-F, is significantly greater than that given by pCAT-Basic, pCB, alone (compare columns 1 and 2). The insertion of the F region into pCAT-Enhancer resulted in a plasmid, pCE-F, which also supported dramatically greater activity than was supported by the host plasmid alone (compare columns 3 and 4). The level of activity shown by pCE-F in this experiment is quite significant, being nearly twice that shown by the plasmid pCAT-

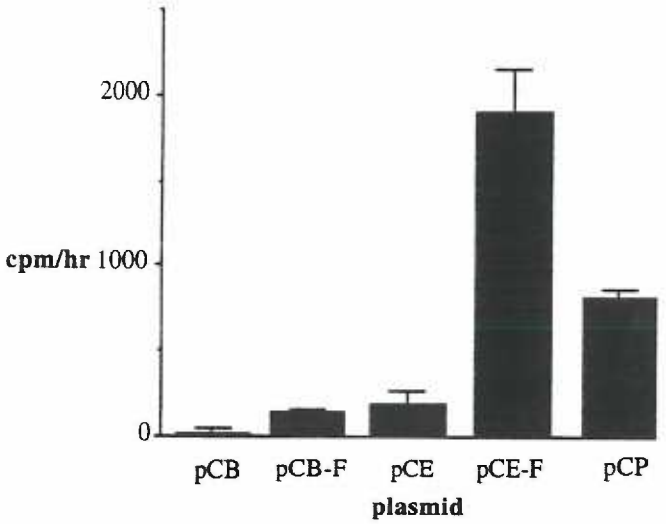


Figure 4.14 CAT activity of the wild-type F region inserted into either pCAT-Basic or pCAT-Enhancer

The indicated plasmids were transfected into HeLa cells by the calcium phosphate method as described in the text. Three days after transfection CAT extracts were isolated and these were assayed as described in the text. The indicated CAT activity, expressed as cpm/hr, is the average of the activities found in extracts from three dishes of cells transfected with the same DNA precipitate. Error bars represent the standard deviation of the mean value. pCB, pCP, and pCE are plasmids pCAT-Basic, pCAT-Promoter and pCAT-Enhancer respectively. The suffix "-F" indicates that a given plasmid contains the wild-type F region of the human bFGF gene (+1 to +9) inserted into its Pst I/Xba I sites.

Promoter (pCP, column 5). The activity of F was therefore potentiated to support higher levels of expression by the remote presence of the SV40 early region enhancer. In this regard the F sequence appears to be operating as one would expect a normal basal promoter to operate.

Attempts to Map the Start of Transcription from bFGF/CAT Constructs

In order to assess whether the promoter activity demonstrated by the various bFGF/CAT constructs represented accurate initiation of transcription from the +1 site determined by Shibata *et al.* [1991] two types of *in vitro* transcription experiments were attempted. The first was a coupled *in vitro* transcription (IVT)/primer extension experiment. In this procedure one incubates plasmids in the presence of nuclear extracts, buffer, and unlabelled nucleotides. If conditions are correct, accurate and promoter-dependent transcription can occur from plasmid constructs containing eukaryotic promoters (see for example Kollmar *et al.*[1992]). The unlabelled transcripts are then subjected to primer extension using specific labeled oligonucleotide primers and the size and uniformity of the primer-extended products, judged by their migration on sequencing gels, are taken as measures of the specificity and accuracy of transcriptional initiation supported by the plasmid-borne sequences.

The second type of IVT experiment attempted was the run-off IVT procedure. In this procedure the plasmid template is first linearized at a point slightly downstream from the candidate promoter. This linearized plasmid serves as the template in the IVT reaction which is conducted with at least one ^{32}P-labeled nucleotide in the reaction mixture. Transcription from the promoter sequences in the plasmids used for run-off IVT terminates at the restricted end hence the name "run-off". The labeled products of the IVT are directly sized by analysis on a sequencing gel. It is the size and uniformity of the products of the labeled IVT reaction which are taken as the measures of the specificity and accuracy of the transcription supported by the plasmid sequences.

The plasmids 3.4, 1e, pCAT-Basic, and pTKp were subjected to both types of IVT procedure. pCAT-Basic was included as a template which lacked a good promoter and pTKp, which contains the strong HSV TK promoter (see Chapter 3) was included as a template which contained a known strong promoter. Several attempts at using both types of procedure yeilded no specific *in vitro* transcript by any of the plasmids, including pTKp. This resulted in no information being gained as to whether the bFGF constructs were accurately specifying transcriptional initiation.

The lack of positive and specific results from the IVT experiments with plasmid pTKp suggested that the protocols as attempted were not permissive for transcription from even a strong promoter, so the lack of a specific IVT product from the 3.4 and 1e plasmids was not taken as significant. It has been demonstrated previously that certain templates require IVT conditions which are distinct from the standard IVT protocols [Kollmar *et al.*, 1992]. Since no reports exist demonstrating IVT using either the HSV TK promoter or the bFGF promoter it is possible (if not likely) that the conditions under which IVT was attempted here were simply inappropriate for these templates. Since the issue of accuracy of transcriptional initiation is a critical one for the aims of this research, one of the first steps in the continuation of this work would be to get one form of the IVT procedure to work. A first step in this would be to obtain another promoter whose successful IVT had been reported and then to carry out an optimization procedure (like the one reported by (Kollmar *et al.*[1992]) on the IVT done using bFGF/CAT constructs. Alternatively, cell lines that stably express the various bFGF/CAT constructs could be made. Then, primer extension could be conducted using the RNA from these cell lines. A time limitation prevented me from pursuing either of these alternative avenues.

Discussion

The significant indications in the results reported in Chapter 4 are that: (1) the 9 bp of the F sequence, CCGCCGAAC, are able to support a wild-type level of promoter activity, (2) the e-scan mutation of the region -8 to -1 dramatically increases promoter activity via an unclear mechanism, and (3) sequences located somewhere within both the -24 to -16, and the +16 to +49 regions appear to (at least under certain circumstances) augment expression.

The F region's Activity

As introduced above, the core promoter of higher eukaryotic genes is thought to consist of a variety of selector elements situated at -30 and +1 relative to the start of transcription [Ham *et al.*, 1992]. In the case of the human bFGF gene it would appear that the sequence $^{+1}$CCGCCGAAC^{+9} which constitutes the F region falls best into a class of selector elements called initiators which are situated at the transcriptional start site and which are thought to participate in the selection of the start site. In the case of the F region of the human bFGF gene this short sequence is sufficient to confer significant promoter activity, up to 50% of the activity of the strong promoter from the SV40 early region, on the promoterless vector pCAT-Basic (see Figure 4.13).

The data from Figure 4.14 show that the expression supported by the F region can be modulated upward by the remote addition of the strong enhancer from the SV40 early gene region. The F cassette thus shows another feature of a native Inr element, the capacity to functionally interact with upstream regulatory elements to give increased transcription.

Is F Necessary?

While the sufficiency of the F element for significant expression is clear, the question of whether it is necessary for promoter function in the natural context of the bFGF gene is somewhat less clear. Many of the mutant plasmids which lack this sequence, the plasmids 1f∂PC, 1eg∂PE, 3f and 1f show little or no promoter activity (Figures 4.3 and 4.13), indicating that the wild-type F sequences may be necessary for significant activity even when other bFGF promoter proximal sequences are present. This feature would be consistent with its identification as an Inr element.

The 1ef' Construct

The CAT activity supported by plasmid 1ef' presents a potential problem to the identification of the F sequence as an essential Inr element. Figure 4.9 shows that the 1ef' construct, which lacks wild-type sequence in the F region, supports good activity in relation to its totally wild-type equivalent, plasmid 3.4. In this special case, that is in the presence of the highly activating e-scan mutation, the presence of the wild-type F sequence would appear not to be necessary for promoter activity. Alternatively, one can assess the activity of 1ef' not in relation to the totally wild-type plasmid 3.4, but to plasmid 1e, see Figure 4.9. By this measure, since the activity of 1ef' is far less than that of 1e, the wild-type sequence in the F-region is seen to be necessary for full activity.

One explanation for the apparent lack of requirement of the F sequence seen in the 1ef' case is that the activating e-scan is influencing the intensity of expression but not the accuracy. A good example of this can be found in work from on the mouse ribosomal protein S16 Inr. Hariharan and Perry demonstrated that the S16 Inr element embedded in a longer context could be completely mutated, much as the F is in the 1ef' construct, and the longer context still specified 30% of total activity but at imprecise transcriptional initiation sites [Hariharan and Perry, 1990]. This behavior is quite similar to the pattern of expression supported by the bFGF/CAT plasmids used here. Using the

S16 Inr element as a model, I would predict that the 1ef' construct would not support accurate initiation and that F is the bFGF gene's essential Inr element.

Is F a Self-Sufficient Initiator?

Fit into the model of basal promoter architecture described above and contingent on an analysis that demonstrates which if any of these constructs are supporting accurate transcriptional initiation at the previously determined +1 site, it would appear that the F sequence falls into the class of strong Inr elements, like the TdT Inr, which do not require a strong selector at -30. This conclusion is based on the observations that the truncation of bFGF sequences distal to the -24 site in these experiments or to the -21 site in the report by Shibata *et al.* [1991] either did not affect or only modestly affected expression and that cassette constructs with small amounts of sequence surrounding the +1 site also appeared to support significant expression in the absence of known accessory elements.

Is pCAT-Basic Contributing an Element at -30?

One assumption that was made in this analysis was that no exogenous selector elements were being supplied by the vector sequences in pCAT-Basic. An initial examination of the vector sequences flanking the bFGF inserts in this vector indicated that no TATA-box or Sp1 binding sites or other known motifs were present in the vector sequences. This was corroborated by the observation that unmodified pCAT-Basic supported very low CAT activity. With the advent of recent reports by Singer *et al.* [1990] and Zenzie-Gregory *et al* . [1993] which demonstrated that in certain cases sequences unrelated to the TATA-box consensus could bind TFII-D and activate transcription, a reexamination of the sequences at the -30 site in the native bFGF gene and in various bFGF/CAT constructs was carried out. Figures 4.15 and 4.16 show the results of the comparison at the -30 site of the alternate TFII-D binding sites demonstrated by Singer *et al.* [1990] or Zenzie-Gregory *et al.* [1993] with the -30

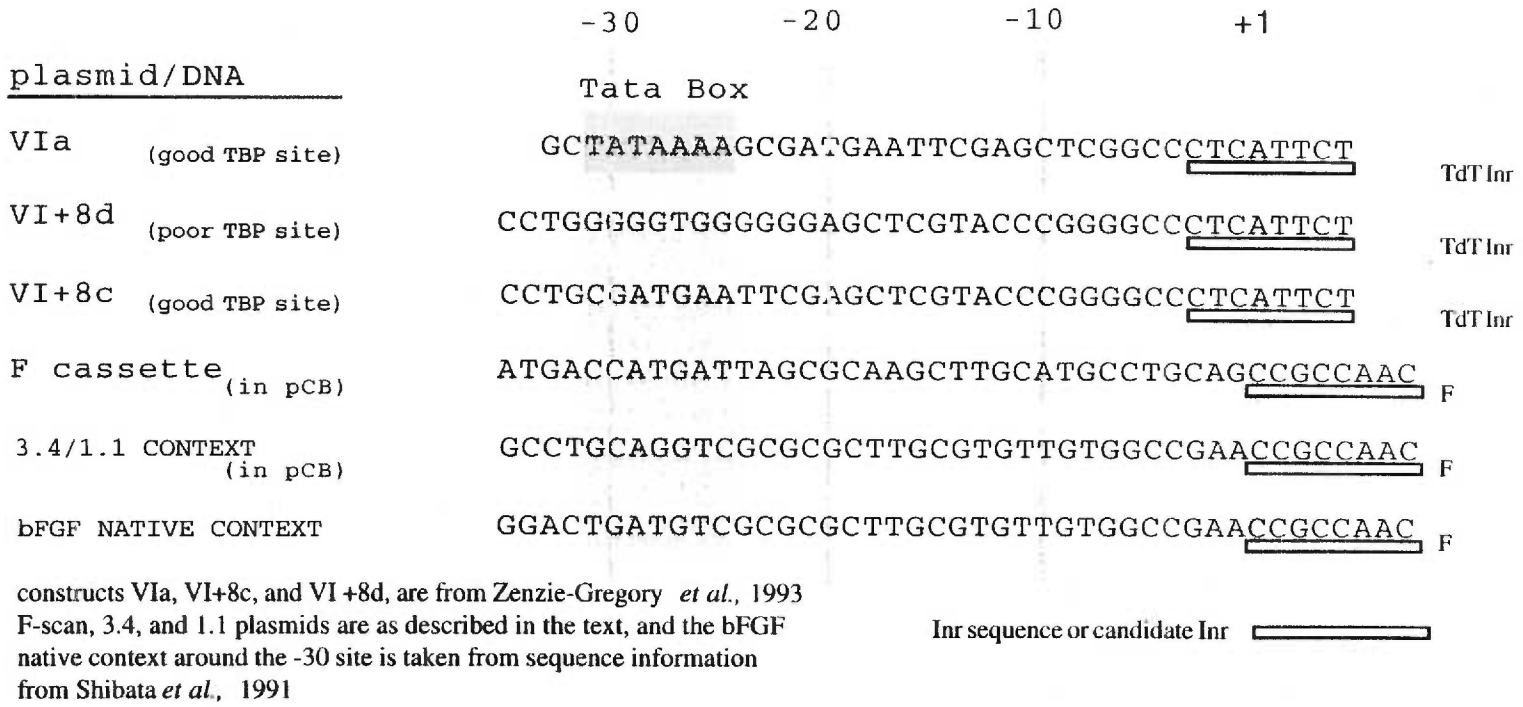


Figure 4.15 Comparison of the -30 region of constructs with a TATA-box or an alternate TBP binding site with the -30 region of various bFGF constructs

This figure shows the sequences from -35 to +5 that are contained in three synthetic Inr constructs made by Zenzie-Gregory et al. and in various bFGF/CAT constructs and in the native human bFGF gene. The shaded region around -30 show where a selector element is normally located. The sequences at -30 for the F region and the LS constructs based on 3.4 and 1.1 are contributed by the vector pCAT-Basic. The point of this figure is to show that the vector contribution to the F cassette plasmid at -30 has some similarity to construct VI+8c of Zenzie-Gregory. The non TATA sequence at -30 contained by construct VI+8c did prove to be able to bind TPB (TATA binding protein, a major componant of TFII-D) and to act as a selector element [Zenzie-Gregory *et al.*, 1993].

-31 -25

A.

TATAAAA	TATA-box
GTCGCCT	VIc
GATATCA	VIe
CGATGAA	VI+8c
CCATGAT	F region
GCAGGTC	3.4/1.1
TGATGTC	native bFGF

VIc, VIe, VI+8c taken from Zenzie-Gregory *et al.*, 1993
F-scan, 3.4, 1.1 are as described in the text, native bFGF sequence is as described in Shibata *et al.*, 1991

B.

TATAAA	T_R
GTCCGC	R2
ACCGCA	R6
TACTAT	R7
ATAAAA	R16
GGCCTT	R72
CCATGA	F region
GCAGGT	3.4/1.1
TGATGT	native bFGF

T_R, R2, R6, R7, R16, and R72 taken from Singer *et al.*, 1990
F-scan, 3.4, 1.1 are as described in the text, native bFGF sequence is as described in Shibata *et al.*, 1991

Figure 4.16 Comparison of the -30 region of the bFGF gene or bFGF/CAT constructs with a variety of functional TBP binding sites

This figure shows a variety of alternate sequences which, when located at the -30 site, can bind TBP (the componant of TFII-D which recognizes the TATA box) and function as selector elements. Panel A shows alternate TBP binding sites reported by Zenzie-Gregorye *t al.* [1993] along with bFGF/CAT and native bFGF gene -30 sequences. Panel B shows alternate TBP binding sites reported by Singer *et al.* [1990] along with bFGF/CAT and native bFGF gene -30 sequences.

sequences contained in either the native bFGF gene or contributed by pCAT-Basic to various bFGF/CAT constructs.

It can be seen and was noted by Singer *et al.* [1990] that the sequences which can substitute for a TATA-box element in selected cases fall into no clear or predictable pattern. None of these alternate TFII-D-binding elements is an exact match for the bFGF native -30 site or for the -30 site in any of the bFGF/CAT constructs. It should also be reiterated that the context at -30 for all of the LS mutants was the same as the 3.4 and 1.1 -30 context and this shows no similarity to any described alternate TFII-D-binding site. The closest match occurs between the VI+8e plasmid used by Zenzie-Gregory and the F plasmid used in these studies with a match of 5 of 7 bases. This may indicate that the vector contributed -30 site in the F cassette construct could have some TFII-D binding and selector activity. Since the match with the alternate TFII-D-binding site is not exact and since apparently no algorithm exists to predict which sequences may be better or worse TFII-D-binding sites this possibility will have to be experimentally examined. This could be done in a manner much like that reported by Zenzie-Gregory *et al.* by inserting into the F plasmid a variety of sequences between the +1 site and the vector contributed sequences at the -30 site such that a variety of new sequences, some with good TFII-D binding and some with poor TFII-D binding, are placed in the -30 site. The activity of these plasmids along with their requirement for TFII-D for activity could then be monitored to examine the role of TFII-D and/or vector sequences at -30 in transcriptional initiation from the F region.

If the -30 sequence contributed by the vector to the F cassette construct was providing an alternate (and weak) TFII-D-binding site (again, strong TFII-D binding is not suggested due to the low expression of CAT from unmodified pCAT-Basic) then it is possible that some of the F construct's activity was contributed by the TFII-D effect. This scenario might explain the observation that the activity of the F cassette construct appears to be greater than plasmids containing more sequence, like 1.1 or DEF, since the

-30 context of no plasmids other than the F cassette shows similarity to any alternate TFII-D-binding sites. Were a vector encoded cryptic TFII-D-binding site to be demonstrated, the behavior of the presumptive bFGF Inr in F would be mirroring the behavior of the TdT Inr in some of the plasmids used by Zenzie-Gregory *et al.* [1993].

The question of whether cryptic selector sequences are in play in transcription from the F construct remains open at this point. The unexpected degeneracy in TFII-D-binding sites may make the existence of cryptic selectors an open issue in any experiments conducted to examine Inr activity. On the other hand, as suggested by Singer, the apparent commonness of non-TATA-box TFII-D binding sites may indicate that it is the Inr element which is the key player in the TATA-less genes and that a weak binding site for TFII-D is by itself not sufficient for expression [Singer *et al.*, 1990; Weis and Reinberg, 1992].

Is F Related to Any Known Inr?

A general consensus derived from the study of known initiator elements is displayed in Figure 4.17. The F region 5'-CCGCCGAAC-3' is a poor match for this already loose consensus. The sequences of a variety of other initiator elements or sites of transcriptional initiation are also listed in Figure 4.17. When aligned to the +1 site of each there would appear to be a poor match between any of these and the bFGF F region. A few of these sequences produce a better match with the bFGF F region if alignment is made disregarding the +1 sites. This is shown in Figure 4.18. The matches between the initiators of the dihydrofolate reductase (DHFR) gene or the gene for cytochrome c oxidase subunit Vb (COX Vb) and the bFGF F-Scan region are particularly good with a 77% identity each.

It is difficult to interpret the meaning of any similarities between the bFGF F region and the initiators of the DHFR or COX Vb genes without information mapping the start of transcription promoted by the F region. It certainly possible that the F region

Figure 4.17 Comparison of the human bFGF gene's F region with other transcriptional start sites

The sequences surrounding +1 for a variety of genes is listed along with a concensus of sequences associated with transcriptional initiation. Sequences were aligned according to their +1 sites (dotted line).

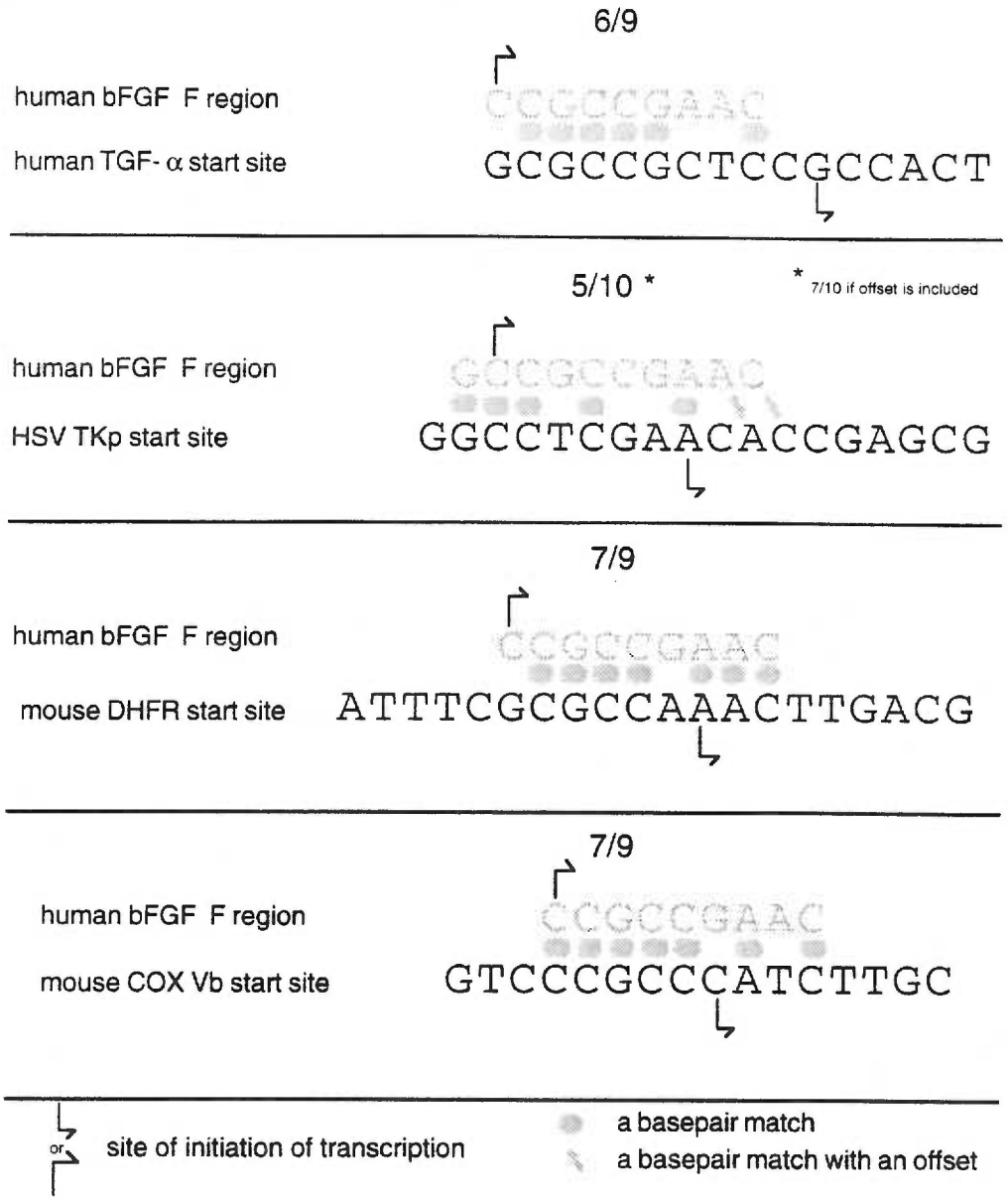


Figure 4.18 Alignment of the human bFGF gene's F region with other transcriptional start sites

This figure shows the best aligment of some of the Inr elements listed in Figure 4.17 with the F region of the human bFGF gene. In this figure the +1 sites have not been used to align the sequences.

shares a binding site for a specific trans-acting factor with one or both of these two genes and that this factor is either sufficient or at least important in attracting a transcriptional initiation complex. Candidate trans-acting factors would be either NF-E1 (also known as YY-1 or δ) which has been shown to be acting at the COX Vb initiator [Basu *et al.*, 1993], or E2F which is thought to be acting at the DHFR initiator [Blake and Azizkhan, 1989]. In any future studies of the bFGF core promoter, it would be desirable to devise strategies that test the hypothesis that one or both of these factors are at work in the bFGF +1 region.

Accuracy of Initiation

The question of the accuracy of transcriptional initiation from any of the bFGF promoter constructs was not resolved here due to the failure of the preliminary attempts at conducting IVT. The absence of this type of data limits the conclusions that can be made about whether F is an initiator since part of the definition of an Inr element is the specification of accurate initiation. Also, as discussed above in the case of the 1ef' construct and as will be touched on below in the case of the various possible explanations of the e-scan's effect, the lack of this information limits the amount of mechanistic insight the tested constructs might give as to how the bFGF basal promoter might be functioning. Any future studies of the bFGF basal promoter will necessarily include analysis of the accuracy of transcriptional initiation using either IVT or primer extension done on RNA from cell lines stably expressing bFGF promoter constructs.

The Nature of the Activation Caused by the e-scan Mutation

The mechanism of activation of promoter activity by the e-scan mutation is still unclear despite preliminary attempts to investigate it. The lack of IVT data is again a significant handicap in the analysis of what may be taking place and the results of successful IVT or primer extension experiments may well resolve the matter. In the

absence of more conclusive data the most plausible general guess is articulated by General Hypothesis I, Figure 4.11, which posits that the wild-type E region either has intrinsic negative activity or it contains the binding site for a factor which acts to repress transcriptional initiation at the +1 site. The location of the E region immediately upstream from the +1 site makes the latter model both simple and attractive. One can imagine that a factor binds to this region and occludes the +1 site, thereby preventing access of the transcriptional initiation complex to this region.

As noted above, the data on this issue are not perfectly in accordance with the simplest view of this hypothesis. Figure 4.12 shows that e', a different mutation in the E region, causes only a modest increase in activity relative to wild-type. In the context of General Hypothesis 1 this would mean that the negative effects of the E region could be partially conferred by sequences other than wild-type and that the e-scan sequence, ATCGATTT , is much better than the e'-scan sequence, GCATGCGG , at removing the negative effect conferred by the wild-type sequence, TGGCCGAA. In terms of a hypothetical repressor, R , binding in this region, one must postulate that the strength of binding would be ER>e'R>>>eR.

Other explanations of the e-scan effect are possible. General Hypothesis 2 provides such an alternate view. One version of this hypothesis would have the junction of the e-scan and either the D or F sections giving rise to a binding site for a trans-acting factor, P, with positive effect on transcription. In this case one would postulate that the strength of binding of P to the E section would be eP>>>e'P>EP.

Among the experiments which might help to determine how the e-scan acts would be gel shift and methylation protection analyses. The most likely versions of General Hypothesis 1 would predict that plasmids containing the wild-type E region, would bind at least one more protein, probably a unique protein, than plasmids that lack it. Gel shift experiments and methylation protection studies could demonstrate the fact of a repressor binding to the E but not the e-scan region and detail the sites at which it

was binding. Conversely, if a factor with positive effect on transcription were binding to e and e' then gel shift and methylation analyses might be expected to indicate that the e sequence is shifted more efficiently than the E sequence and that the e-scan harbors sites that are protected from methylation.

The action of the e-scan region either to derepress or to activate may not be mediated by the binding of a unique repressor or activator molecule. It is possible that the positive effect of mutating the E region is due to sequence specific changes in the conformation of the nucleic acids and that conformational changes are acting to augment or diminish the action of transcriptional initiation complexes working at the +1 site in the F region. Sequence dependent conformational effects on promoter activity have been recently implicated in the case of the promoter for the dihydrofolate reductase gene [Pierce *et al.*, 1992]. If this were the mode of action in the bFGF promoter, then the gel shift/methylation protection approaches suggested above might not be revealing. If an E- or e-scan specific conformational change were acting to influence the access of a transcriptional initiation complex to F then gel shift analysis might reveal this by showing an E or e-scan dependent difference in gel shifts only in the context where the F region is also present. This would prompt the inclusion in any gel shift experiments of plasmids which contained the E- and e- scans in isolation. An E or e-scan specific shift seen with plasmids 3.4 or 1e but not in the corresponding plasmids bearing the E or e regions in isolation might suggest a conformational mode of action.

Territorial Limits of the Activity in the human bFGF Promoter Region

The results like those shown in Figure 4.13 from experiments done with deletion mutants give some clear indications as to the localization of promoter activity within the bFGF proximal region. Constructs which do not contain sequences further upstream than the +9 position are basically without activity. The F construct which contains no sequences upstream of +1 has full activity. This suggests that the essential activity for

expression from the human bFGF promoter region resides in the F sequence, not further downstream and not further upstream.

Corroborating results come from various wild-type plasmids and LS mutations. The legA1 plasmid has the same activity as the 1e plasmid (Figure 4.10) suggesting that the G region located from +9 to +17 is without activity. The 1.1 plasmid had deleted all sequences downstream from +17 and shows full activity (for example see Figure 4.4) indicating that nothing further downstream from +17 is essential. The data obtained with the early LS scan series c, d, and e (Figure 4.4) indicated that each of these regions was dispensable without catastrophic loss of activity. These results corroborate the conclusion made from the analysis of the activity of the F cassette that no wild-type sequences upstream from +1 are essential.

Accessory Activities: the +17 to +49 Region

While the sequences -24 to -1 and +10 to +49 were concluded to be dispensable without a catastrophic effect on promoter activity, other data indicated that these sequences were not without effect on promoter activity. The first indication of this was in the analysis of the effect of the LS mutations c through f made in two different wild-type contexts. As pointed out above, it appeared as if any change made to plasmid 1.1 with its -24 to +17 context, resulted in a plasmid with less activity than one obtained by making the same change to plasmid 3.4 with its -24 to +49 context (see figures 4.3 and 4.4). This result was in contrast to the observation that the CAT activity supported by the 1.1 plasmid was generally greater than that supported by the 3.4 plasmid. The conclusion prompted by these observations is that an activity is resident in the +17 to +49 region with the characteristics that it is silent in a wild-type background and it is active when region C, D, or E is mutated. This would appear to be an accessory activity or a latent activity and would imply some sort of cooperativity or complementation exists between an element or elements contained in the +17 to +49 region and elements

contained in the C, D, and/or E regions.

Accessory Activities: the -24 to -9 Region

The fact that some but not all promoter activity was lost by mutating the C and D regions to give plasmids 1c and 1d (Figures 4.3 and 4.4) suggests that some sort of non-essential activity is resident in the region -24 to -9. This was also seen in the activity of the small cassette constructs DEFG, and DEF, both of which had less activity than plasmid 1.1 which contained regions CDEFG. In the simultaneous absence of both the upstream C or D regions and the downstream +17 to +49 regions, the F cassette condition, activity is not compromised. As discussed above, this may reflect a serendipitous contribution of a selector element by the vector sequences in the F cassette plasmid. Alternatively, as discussed above in the case of elements contained in the +17 to +49 region, the activity in the C and D region may require cooperation with downstream sequences.

A Model of bFGF Promoter Architecture

A very general model of how the bFGF promoter might function is diagrammed in Figure 4.19. This diagram is less a true model than a compilation of the features suggested by the data that has been gathered to this point. This synthesis was done to see if all of the hypothesized features could be incorporated into a single physical representation.

The features are: 1) The F sequence, 5'-CCGCCGAAC-3', is a self sufficient initiator for the bFGF gene and acts to efficiently direct the binding of the transcriptional initiation complex

2) The E sequence, 5'-ATCGATTT-3', acts to attract a factor which represses transcription.

3) An accessory activity is resident in regions C and D. This activity may cooperate with the activities contained in regions G through L.

4) An accessory activity is resident in the regions G through L. It has no effect if wild-type sequences C to E are present. This activity presumably is manifest by cooperating with elements in the C to E region.

Summary

The work described in Chapter 4 of this thesis corroborates the initial study by Shibata *et al.* that indicated the basal promoter of the human bFGF gene resides in the sequences surrounding the transcriptional start of this gene. In addition, the present studies have identified within the region -24 to +49, several subsequences which have significant effect on transcription from this region. These subsequences include: the F region located +1 to +9 and which has many properties of a strong Inr element and is predicted to be the Inr of the human bFGF gene; the E region located -8 to -1 which when mutated to a different sequence, e, gives rise to a dramatic increase in transcription; and less well defined and less critical activities in the regions -24 to -9 and +16 to +49 which under certain circumstances appear to mildly increase the transcription from the bFGF promoter.

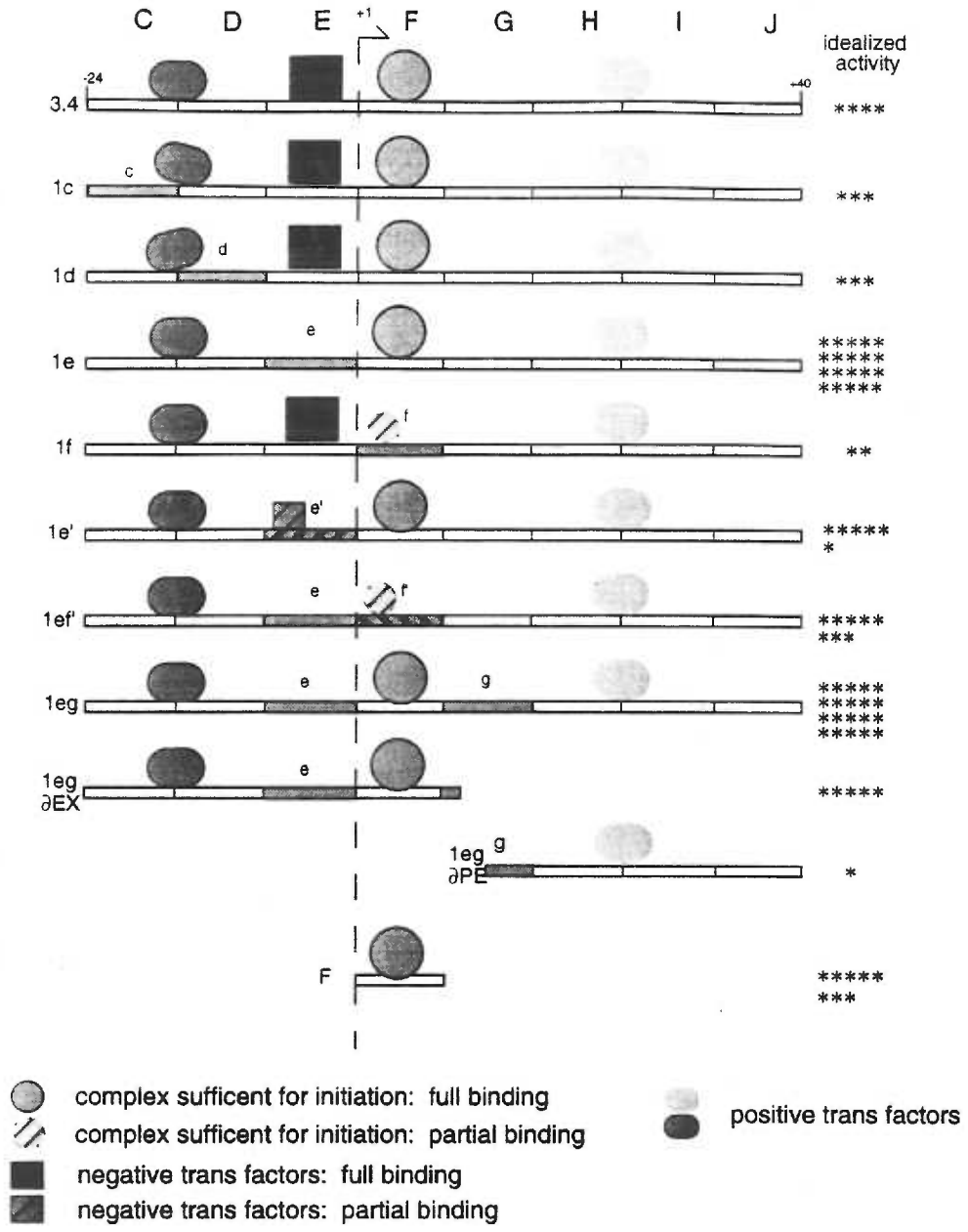


Figure 4.19 A general model of bFGF promoter architecture

This figure represents one possible model of the function architecture of the human bFGF gene's basal promoter. It is based on the CAT assay results described in the text.

REFERENCES

1. Abraham, J.A., Mergia, A., Whang, J.L., Tumolo, A., Friedman, J., Hjerrild, K.A., Gospodarowicz, D. and Fiddes, J.C. (1986a). Nucelotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science* **233**: 545-548.

2. Abraham, J.A., Whang, J.L., Tumolo, A., Mergia, A., Friedman, J., Gospodarowicz, D. and Fiddes, J.C. (1986b). Human basic fibroblast growth factor: nucleotide sequence and genomic organization. *EMBO J* **5**: 2523-2528.

3. Adnane, J., Gaudray, P., Dionne, C.A., Crumley, G., Jaye, M., Schlessinger, J., Jeanteur, P., Birnbaum, D. and Theillet, C. (1991). *BEK* and *FLG*, two receptors to members of the FGF family, are amplified in subsets of human breast cancers. *Oncogene* **6**: 659-663.

4. Albino, A.P., Davis, B.M. and Nanus, D.M. (1991). Induction of growth factor RNA expression in human malignant melanoma: markers of transformation. *Cancer Res.* **51**: 4815-4820.

5. Amaya, E., Musci, T.J. and Kirschner, M.W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in Xenopus embryos. *Cell* **66**: 257-270.

6. Anderson, K.J. (1988). Basic fibroblast growth factor prevents death of lesioned cholinergic neurons in vivo. *Nature* **332**: 360-361.

7. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rhamsdorf, H.J., Jonat, C., Herrlich, P. and Karin, M. (1987). Phorbol ester-inducible gene contain a common cis element recognized by a TPA-modulated trans-acting factor. *Cell* **49**: 729-739.

8. Assoian, R.K., Komoriya, A., Meyers, C.A., Miller, D.M. and Sporn, M.B. (1983). Transforming growth factor in human platelets. Identification of a major storage site, purification and characterization. *J. Biol. Chem.* **258**: 7155-7160.

9. Atwater, J.A., Wisdom, R. and Verma, I.M. (1990). Regulated mRNA stability. *Annu. Rev. Genet.* **24**: 519-541.

10. Baird, A., Esch, F., Mormedge, P., Ueno, N., Ling, N., Bohlen, P., Ying, S., Wehrenberg, W.B. and Guillemin, R. (1986). Molecular characterization of fibroblast growth factor: distribution and biological activities in various tissues. Recent Progress in Hormone Research. Academic Press, Inc. 143-205.

11. Baird, A. and Klagsbrun, M. (1991a). The fibroblast growth factor family. *Ann. N. Y. Acad. Sci.* **638**: xi-xii.

12. Baird, A. and Klagsbrun, M. (1991b). Nomenclature meeting, report and recommendations. *Ann. N. Y. Acad. Sci.* **638**: xiii-xvi.

13. Basu, A., Park, K., Atchison, M.L., Carter, R.S. and Avadhani, N.G. (1993). Identification of a transcriptional initiator element in the cytochrome c oxidase subunit Vb promoter which binds to transcription factors NF-E1 (YY-1, ∂) and Sp1. *J. Biol. Chem.* **268**: 4188-4196.

14. Beaupain, D., Eleouet, J.F. and Romeo, P.H. (1990). Initiation of transcription of the erythroid promoter of the porphobiniogen deaminase gene is regulated by a cis-acting sequence around the cap site. *Nucl. Acids. Res.* **18**: 6509-6515.

15. Bellot, F., Crumley, G., Kaplow, J.M., Schlessinger, J., Jaye, M. and Dionne, C.A. (1991). Ligand-induced transphosphorylaton between different FGF receptors. *EMBO J.* **10**: 2849-2854.

16. Bensi, G., Mora, M., Raugei, G., Buonamassa, D.T., Rossini, M. and Melli, M. (1990). An inducible enhancer controls the expresion of the human interleukin 1ß gene. *Cell Growth & Diff.* **1**: 491-497.

17. Bhargava, P. and Chatterji, D. (1992). DNA intervention in transcriptional activation. *FEBS Let.* **307**: 245-248.

18. Bikfalvi, A., Alterio, J., Inyang, A.L., Dupuy, E., Laurent, M., Harmann, M.P., Vigny, L., Raulais, D., Courtois, Y. and Tobelem, G. (1990). Basic fibroblast growth factor expression in human omental microvascular endothelial cells and the effect of phorbol ester. *J. Cell. Physiol.* **144**: 151-158.

19. Blake, M.C. and Azizkhan, J.C. (1989). Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene *in vitro* and *in vivo*. *Mol. Cell. Biol.* **9**: 4994-5002.

20. Blake, M.C., Jambou, R.C., Swick, A.G., Kahn, J.W. and Azizkhan, J.C. (1990). Transcriptional initiation is controlled by upstream GC-box interactions in a TATAA-less promoter. *Mol. Cell. Biol.* **10**: 6632-6641.

21. Bouche', G., Gas, N., Prats, H., Baldin, V., Tauber, J., Teissie', J. and Amalric, F. (1987). Basic fibroblast growth factor enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing G_0-G_1 transition. *Proc. Natl. Acad. Sci. USA* **84**: 6770-6774.

22. Bowen-Pope, D.F., Hart, C.E. and Seifert, R.A. (1989). Sera and conditioned media contain different isoforms of platelet-derived growth factor (PDGF) which bind to differnt classes of PDGF receptor. *J. Biol. Chem.* **264**: 2502-2508.

23. Briggs, M.R., Kadonaga, J.T., Bell, S.P. and Tjian, R. (1986). Purification and biochemical characterization of the promoter-specific transcription factor, Sp1. *Science* **234**: 47-52.

24. Brill, G., Vaisman, N., Neufeld, G. and Kalcheim, C. (1992). BHK-21-derived cell lines that produce basic fibroblast growth factor, but not parental BHK-21 cells, initiate neuronal differentiation of neural crest progenitors. *Development* **115**: 1059-1069.

25. Bugler, B., Amalric, F. and Prats, H. (1991). Alternative initiation of translation determines cytoplasmic or nuclear localization of basic fibroblast growth factor. *Mol. Cell. Biol.* **11**: 573-577.

26. Clegg, C.H., Linkhart, C.A., Olwin, B.B. and Hauschka, S.D. (1987). Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. *J. Cell Biol.* **105**: 949-956.

27. Cook, P.W., Coffey, R.J., Jr., Magun, B.E., Pittelkow, M.R. and Shipley, G.D. (1990). Expression and regulation of mRNA coding for acidic and basic fibroblast growth factor and transforming growth factor-alpha in cells derived from human skin. *Mol. Endocrinol.* **4**: 1377-1385.

28. Couderc, B., Prats, H., Bayard, F. and Amalric, F. (1991). Potential oncogenic effects of basic fibroblast growth factor requires cooperation between CUG and AUG-initiated forms. *Cell Regulation* **2**: 709-718.

29. Cuevas, P., Carceller, F., Ortega, S., Zazo, M., Nieto, I. and Gimenez-Gallego, G. (1991). Hypotensive activity of fibroblast growth factor. *Science* **254**: 1208-1210.

30. Dean, M., Levine, R.A., Ran, W., Kindy, M.S., Sonenshein, G.E. and Campisi, J. (1986). Regulation of c-myc transcription and mRNA abundance by serum growth factors and cell contact. *J. Biol. Chem.* **261**: 9161-9166.

31. Dickson, C. and Peters, G. (1987). Potential oncogene product related to growth factors. *Nature* **326**: 833.

32. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**: 1475-1489.

33. Durnam, D.M. and Palmiter, R.D. (1983). A practical approach for quantitating specific mRNAs by solution hybridization. *Anal. Biochem.* **131**: 385-393.

34. Dynan, W.S. and Tjian, R. (1985). Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. *Nature* **316**: 774-778.

35. Eriksson, A., Nister, M., Leveen, P., Westermark, B., Heldin, C.-H. and Claesson-Welsh, L. (1991). Induction of platelet-derived growth factor alpha- and beta-receptor mRNA and protein by platelet-derived growth factor BB. *J. Biol. Chem.* **266**: 21138-21144.

36. Esch, F., Baird, A., Ling, N., Ueno, N., Hill, F., Denoroy, L., Klepper, R., Gospodarowicz, D., Bohlen, P. and Guillemin, R. (1985). Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine brain acidic FGF. *Proc. Natl. Acad. Sci. USA* **82**: 6507-6511.

37. Ferrari, G., Minozzi, M.-C., Toffano, G., Leon, A. and Skaper, S.D. (1989). Basic fibroblast growth factor promotes the survival and development of mesencephalic neurons in culture. *Dev. Biol.* **133**: 140-147.

38. Finch, P.W., Rubin, J.S., Miki, T., Ron, D. and Aaronson, S.A. (1989). Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. *Science* **245**: 752-755.

39. Florkiewicz, R.Z. and Sommer, A. (1989). Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons. *Proc. Natl. Acad. Sci. USA* **86**: 3978-3981.

40. Fogh, J., Fogh, J.M. and Orfeo, T. (1977). One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J. Natl. Cancer Inst.* **59**: 221-230.

41. Folkman, J., Szabo, S., Stovroff, M., McNeil, P., Li, W. and Shing, Y. (1991). Duodenal ulcer: Discovery of a new mechanism and development of angiogenic therapy that accelerates healing. *Ann. Surg.* **214**: 414-427.

42. Gay, C.G. and Winkles, J.A. (1991). Interleukin 1 regulates heparin-binding growth factor 2 gene expression in vascular smooth muscle cells. *Proc. Natl. Acad. Sci. USA* **88**: 296-300.

43. Gimenez-Gallego, G., Rodkey, J., Bennett, C., Rios-Candelore, M., DiSalvo, J. and Thomas, K. (1985). Brain-derived acidic fibroblast growth factor: complete amino acid sequence and homologies. *Science* **230**: 1385-1388.

44. Giordano, S., Sherman, L., Lyman, W. and Morrison, R. (1992). Multiple molecular weight forms of basic fibroblast growth factor are developmentally regulated in the central nervous sytem. *Devel.Biol.* **152**: 293-303.

45. Givol, D. and Yayon, A. (1992). Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. *FASEB J.* **6**: 3362-3369.

46. Goldsmith, K.T., Gammon, R.B. and Garver, R.I., Jr. (1991). Modulation of bFGF in lung fibroblasts by TGF-beta and PDGF. *Am. J. Physiol.* **261**: L378-L385.

47. Gonzalez, A.-M., Buscaglia, M., Fox, R., Isacchi, A., Sarmientos, P., Farris, J., Ong, M., Martineau, D., Lappi, D.A. and Baird, A. (1992). Basic fibroblast growth factor in Dupuytren's contracture. *Amer. J. Pathol.* **141**: 661-671.

48. Gospodarowicz, D. (1975). Purification of a Fibroblast Growth Factor from Bovine Pituitary. *J. Biol. Chem.* **250**: 2515-2520.

49. Gospodarowicz, D. (1989). Fibroblast Growth Factor. *Critical Reviews in Oncogenesis* **1**: 1-26.

50. Gospodarowicz, D. (1991). Biological activities of fibroblast growth factors. *Ann. N. Y. Acad. Sci.* **638**: 1-27.

51. Gough, N.M. (1988). Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. *Anal. Biochem.* **173**: 93-95.

52. Greenberg, M.E. and Bender, T.P. (1990). Indentification of newly transcribed RNA: nuclear runoff transcription in mammalian cells. *Curr. Protocols in Mol. Biol.* **Suppl. 9:** 4.10.1-4.10.9.

53. Hakii, H., Fujiki, H., Suganuma, N., Nakayasu, M., Tahira, T., Sugimura, T., Scheuer, P.J. and Christensen, S.B. (1986). Thapsigargin, a histamine secretagogue, is a non-12-O-tetradecanoylphorbol-13-acetate (TPA) type tumor promoter in two-stage mouse skin carcinogenesis. *J. Cancer Res. Clin. Oncol.* **111**: 177-181.

54. Ham, J., Steger, G. and Yaniv, M. (1992). How do eukaryotic activator proteins stimulate the rate of transcription by RNA polymerase II? *FEBS Let.* **30**: 81-86.

55. Hammond, S.L., Ham, R.G. and Stampfer, M.R. (1984). Serum-free growth of human mammary epithelial cells: Rapid clonal growth in define medium and extended serial passage with pituitary extract. *Proc. Natl. Acad. Sci. USA* **81**

56. Hariharan, N., Kelley, D.E. and Perry, R.P. (1989). Equipotent mouse ribosomal protein promoters have similar architecture that includes internal sequence elements. *Genes & Devel.* **3**: 1789-1800.

57. Hariharan, N. and Perry, R. (1990). Functional dissection of a mouse ribosomal protein promoter: significance of the polypyrimiding initiator and an element in the TATA-box region. *Proc. Natl. Acad. Sci. USA* **87**: 1526-1530.

58. Hay, N., Bishop, J.M. and Levens, D. (1987). Regulatory elements that modulate expression of human c-myc. *Genes Dev.* **1**: 659-671.

59. Holter, W., Fordis, C.M. and Howard, B.H. (1989). Efficient gene transfer by sequential treatment of mammalian cells with DEAE-dextran and deoxyribonucleic acid. *Exp. Cell Res.* **184**: 546-551.

60. Iberg, N., Rogelj, S., Fanning, P. and Klagsbrun, M. (1989). Purification of 18- and 22-kDa forms of basic fibroblast growth factor from rat cells transformed by the ras oncogene. *J. Biol. Chem.* **264**: 19951-19955.

61. Jones, K.A., Yamamoto, K.R. and Tjian, R. (1985). Two distinct transcription factors bind to the HSV thymidine kinase promoter in vitro. *Cell* **42**: 559-572.

62. Kim, S.-J., Denhex, F., Kim, K.-Y., Holt, J.T. and Sporn, M.B. (1989). Activation of the second promoter of the transforming growth factor-beta1 gene by transforming growth factor-beta1 and phorbol ester occurs through the same target sequences. *J. Biol. Chem.* **264**: 19373-19378.

63. Kingston, R.E. (1987a). Primer Extension. *Curr. Protocols in Mol. Biol.* **Suppl.17**: 4.8.1-4.8.3.

64. Kingston, R.E. (1987b). Transfection of DNA into eukaryotic cells: calcium phosphate transfection. *Curr. Proto. Mol. Biol.* **Suppl. 14**: 9.1.1-9.1.9.

65. Klagsbrun, M., Smith, S., Sullivan, R., Shing, Y., Davidson, S., Smith, J.A. and Sasse, J. (1987). Multiple forms of basic fibroblast growth factor: amino-terminal cleavages by tumor cell- and brain cell-derived acid proteinases. *Proc. Natl. Acad. Sci. USA* **84**: 1839-1843.

66. Kollmar, R. and Farnham, P.J. (1993). Site-specific initiation of transcription by RNA polymerase II. *Proc. Soc. Exp. Biol. Med.* **in press**

67. Kollmar, R., Londstrom, M.J. and Farnham, P.J. (1992). Heat sensitivity and Sp1 activation of complex formation at the syrian hamster carbamoyl-phosphate synthase (glutamine-hydrolysing)/aspartate carbamoyltransferase/dihydroorotase promoter *in vitro*. *J. Biol. Chem.* **267**: 385-391.

68. Kraemer, R., Pomerantz, K.B., Joseph-Silverstein, J. and Hajjar, D.P. (1993). Induction of basic fibroblast growth factor mRNA and protein synthesis in smooth muscle cells by cholesterly ester enrichment and 25-hydroxycholesterol. *J. Biol. Chem.* **268**: 8040-8045.

69. Labarca, C. and Paigen, K. (1980). A simple, rapid and sensitive DNA assay procedure. *Anal. Biochem.* **102**: 344-352.

70. Lenormand, P., Muldoon, L.L., Eslen, H., Rodland, K.D. and Magun, B.E. (1990). Two tumor promoters, 12-O-tetradecanoylphorbol-13-acetate and thapsigargin, act synergistically via distinct signaling pathways to stimulate gene expression. *Cell Growth & Differentiation* **1**: 627-635.

71. Liu, L. and Nicoll, C.S. (1988). Evidence for a role of basic fibroblast growth factor in rat emybronic growth and differentiation. *Endocrinol.* **123**: 2027-2031.

72. Liu, Z., Moav, B., Faras, A.J., Guise, K.S., Kapuscinski, A.R. and Hackett, P. (1991). Importance of the *CArG* box in regulatin of B-acting-encoding genes. *Gene* **108**: 211-217.

73. Lowe, W.L., Jr., Yorek, M.A., Karpen, C.W., Teasdale, R.M., Hovis, J.G., Albrecht, B. and Prokopiou, C. (1992). Activation of protein kinase-C differentially regulates insulin-like growth factor-I and basic fibroblast growth factor messenger RNA levels. *Mol. Endocrinol.* **6**: 741-752.

74. Mably, J.D., Sole, M.J. and Liew, C.-C. (1993). Characterizatio of the GArC motif. *J. Biol. Chem.* **268**: 476-482.

75. Marics, I., Adelaide, J., Raybaud, F., Mattei, M.-G., Coulier, F., Planche, J., de Lapeyriere, O. and Birnbaum, D. (1989). Characterization of the *HST*-related *FGF.6* gene, a new member of the fibroblast growth factor gene family. *Oncogene* **4**: 335-340.

76. Marriott, S.J. and Brady, J.N. (1989). Enhancer function in viral and cellular gene regulation. *Biochimica et Biophysica Acta* **989**: 97-110.

77. McClain, D.A., Paterson, A.J., Roos, M.D., Wei, X. and Kudlow, J.E. (1992). Glucose and glucosamine regulate growth factor gene expression in vascular smooth muscle cells. *Proc. Natl. Acad. Sci. USA* **89**: 8150-8154.

78. McKnight, S. and Tjian, R. (1986). Transcriptional selectivity of viral genes in mammalian cells. *Cell* **46**: 795-805.

79. Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl. Acids Res.* **12**: 7035-7056.

80. Miwa, T., Boxer, L.M. and Kedes, L. (1987). CarG boxes in the human cardiac α-actin gene are core binding sites for positive trans-acting regulatory factors. *Proc. Natl. Acad. Sci. USA* **84**: 6702-6706.

81. Miyamoto, M., Naruo, K., Seko, C., Matsumoto, S., Kondo, T. and Kurokawa, T. (1993). Molecular cloning of a novel cytokine cDNA encoding the ninth member of the fibroblast growth factor family, which has a unique secretion property. *Mol. Cell. Biol.* **13**: 4251-4259.

82. Murphy, P.R., Guo, J.Z. and Friesen, H.G. (1990). Messenger RNA stabilization accounts for elevated basic fibroblast growth factor transcript levels in a human astrocytoma cell line. *Mol. Endocrinol.* **4**: 196-200.

83. Murphy, P.R., Myal, Y., Sato, Y., Sato, R., West, M. and Friesen, H.G. (1989). Elevated expression of basic fibroblast growth factor messenger ribonucleic acid in acoustic neuromas. *Mol. Endocrinol.* **3**: 225-231.

84. Murphy, P.R., Sato, R., Sato, Y. and Friesen, H.G. (1988a). Fibroblast growth factor messenger ribonucleic acid expression in a human astrocytoma cell line: regulation by serum and cell density. *Mol. Endocrinol.* **2**: 591-598.

85. Murphy, P.R., Sato, Y., Sato, R. and Friesen, H.G. (1988b). Regulation of multiple basic fibroblast growth factor messenger ribonucleic acid transcripts by protein kinase C activators. *Mol. Endocrinol.* **2**: 1196-1201.

86. Nakanishi, Y., Kihara, K., Mizuno, K., Masamune, Y., Yoshitake, Y. and Nishikawa, K. (1992). Direct effect of basic fibroblast growth factor on gene transcription in a cell-free system. *Proc. Natl. Acad. Sci. USA* **89**: 5216-5220.

87. Navre, M. and Ringold, G.M. (1989). Differential effects of fibroblast growth factor and tumor promoters on the initiation and maintenance of adipocyte differentiation. *J. Cell Biol.* **109**: 1857-1863.

88. Neumann, J.R., Morency, C.A. and Russian, K.O. (1987). A novel rapid assay for chloramphenicol acetyltransferase gene expression. *BioTechniques* **5**: 444-447.

89. Okamura, K., Sato, Y., Matsuda, T., Hamanaka, R., Ono, M., Kohno, K. and Kuwano, M. (1991). Endogenous basic fibroblast growth factor-dependent induction of collagenase and interleukin-6 in tumor necrosis factor-treated human microvascular endothelial cells. *J. Biol. Chem.* **266**: 19162-19165.

90. Park, C.M. and Hollenberg, M.J. (1991). Induction of retinal regeneration *in vivo* by growth factors. *Dev. Biol.* **148**: 322-333.

91. Parker, T.G., Chow, K.-L., Schwartz, R.J. and Schneider, M.D. (1992). Positive and negative control of the skeletal alpha-actin promoter in cardiac muscle. *J. Biol. Chem.* **267**: 3343-3350.

92. Petroski, R.E., Grierson, J.P., Choi-Kwon, S. and Geller, H.M. (1991). Basic fibroblast growth factor regulates the ability of astrocytes to support hypothalamic neuronal survival *in vitro*. *Dev. Biol.* **147**: 1-13.

93. Pierce, A.J., Jambou, R.C., Jensen, D.E. and Azizkhan, J.C. (1992). A conerved DNA structural control element modulates transcription of a mammalian gene. *Nucl. Acids. Res.* **20** (24): 65830-6587.

94. Prasad, I., Zouzias, D. and Basilico, C. (1976). State of the viral DNA in rat cells transformed by Polyoma virus I. Virus rescue and the presence of nonintegrated viral DNA molecules. *J. Virol.* **18**: 436-444.

95. Prats, A.-C., Vagner, S., Prats, H. and Amalric, F. (1992). cis-Acting elements involved in the alternative translation initiation process of human basic fibroblast growth factor mRNA. *Mol. Cell. Biol.* **12**: 4796-4805.

96. Prats, H., Kaghad, M., Prats, A.C., Klagsbrun, M., Lelias, J.M., Liauzun, P., Chalon, P., Tauber, J.P., Amalric, F., Smith, J.A., et al. (1989). High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. *Proc. Natl. Acad. Sci. USA* **86**: (March): 1836-1840.

97. Quarto, N., Talarico, D., Florkiewicz, R. and Rifkin, D.B. (1991). Selective expression of high molecular weight basic fibroblast growth factor confers a unique phenotype to NIH 3T3 cells. *Cell Regulation* **2**: 699-708.

98. Quinones, S., Saus, J., Otani, Y., Harris, E.D., Jr. and Kurkinen, M. (1989). Transcriptional regulation of human stromelysin. *J. Biol. Chem.* **264**: 8339-8344.

99. Rapraeger, A.C., Krufka, A. and Olwin, B.B. (1991). Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science* **252**: 1705-1711.

100. Ray, A., Sassone-Corsi, P. and Sehgal, P.B. (1989). A multiple cytokine- and second messenger-responsive element in the enhancer of the human interleukin-6 gene: similarities with *c-fos* gene regulation. *Mol. Cell. Biol.* **9**: 5537-5547.

101. Renko, M., Quarto, N., Morimoto, T. and Rifkin, D.B. (1990). Nuclear and cytoplasmic localization of different basic fibroblast growth factor species. *J. Cell. Physiol.* **144**: 108-114.

102. Rorth, P., Nerlov, C., Blasi, F. and Johnsen, M. (1990). Transcription factor PEA3 participates in the induction of urokinase plasminogen activator transcription in murine keratinocytes stimulated with epidermal growth factor or phorbol-ester. *Nucleic Acids Res.* **18**: 5009-5017.

103. Sassone-Corsi, P. and Borrelli, E. (1986). Transcriptional regulation by trans-acting factors. *TIG* **2**: 215-219.

104. Sato, K., Ito, K., Kohara, H., Yamaguchi, Y., Adachi, K. and Endo, H. (1992). Negative regulation of catalase gene expression in hepatoma cells. *Mol. Cell. Biol.* **12**: 2525-2533.

105. Sato, Y., Murphy, P.R., Sato, R. and Friesen, H.G. (1989). Fibroblast growth factor release by bovine endothelial cells and human astrocytoma cells in culture is density dependent. *Mol. Endocrinol.* **3**: 744-748.

106. Schweers, L.A., Frank, D.E., Weigel, N.L. and Sanders, M.M. (1990). The steroid-dependent regulatory element in the ovalbumin gene does not function as a typical steroid response element. *J. Biol. Chem.* **265**: 7590-7595.

107. Schweigerer, L., Malerstein, B., Neufeld, G. and Gospodarowicz, D. (1987). Basic fibroblast growth factor is synthesized in cultured retinal pigment epithelial cells. *Biochem. Biophys. Res. Comm.* **143**: 934-940.

108. Shibata, F., Baird, A. and Florkiewicz, R.Z. (1991). Functional characterization of the human basic fibroblast growth factor gene promoter. *Growth Factors* **4**: 277-287.

109. Shiurba, R.A., Jing, N., Sakakura, T. and Godsave, S.F. (1991). Nuclear translocation of fibroblast growth factor during Xenopus mesoderm induction. *Devel.* **113**: 487-493.

110. Singer, V.L., Wobbe, C.R. and Struhl, K. (1990). A wide variety of DNA sequences can functionally replace a yeast TATA element for transcriptional activation. *Genes & Devel.* **4**: 636-645.

111. Slack, J.M., Darlington, B.G., Heath, J.K. and Godsave, S.F. (1987). Mesoderm induction in early *Xenopus* embryos by heparin-binding growth factors. *Nature* **326**: 197-200.

112. Smale, S.T. and Baltimore, D. (1989). The "initiator" as a transcription control element. *Cell* **57**:103-113.

113. Smale, S.T., Schmidt, M.C., Berk, A.J. and Baltimore, D. (1990). Transcriptional activation by Sp1 as directed through TATA or initiator: Specific requirement for mammalian factor IID. *Proc. Natl. Acad. Sci. USA* **87**: 4509-4513.

114. Sternfeld, M.D., Hendrickson, J.E., Keeble, W.W., Rosenbaum, J.T., Robertson, J.E., Pittelkow, M.R. and Shipley, G.D. (1988). Differential expression of mRNA coding for heparin-binding growth factor type 2 in human cells. *J. Cell. Physiol.* **136**: 297-304.

115. Stocker, K.M., Sherman, L., Rees, S. and Ciment, G. (1991). Basic FGF and TGF-B1 influence commitment to malogenesis in neural crest-derived cells of aviann embryos. *Development* **111** 635-645.

116. Strauss, W.M. (1990). Preparation of Genomic DNA from Mammalian Tissue. *Curr. Proto. Mol. Biol.* **Suppl. 13** 2.2.1-2.2.3.

117. Takahashi, J.A., Fukumoto, M., Kozai, Y., Ito, N., Oda, Y., Kikuchi, H. and Hatanaka, M. (1991). Inhibition of cell growth and tumorigenesis of human gliobalstoma cells by a neutralizing antibody against human basic fibroblast growth factor. *FEBS Let.* **288**: 65-71.

118. Takahashi, J.A., Mori, H., Fukumoto, M., Igarashi, K., Jaye, M., Oda, Y., Kikuchi, H. and Hatanaka, M. (1990). Gene expression of fibroblast growth factors in human gliomas and meningiomas: demonstration of cellular source of basic fibroblast growth factor mRNA and peptide in tumor tissues. *Proc. Natl. Acad. Sci. USA* **87**: 5710-5714.

119. Tanaka, A., Miyamoto, K., Minamino, N., Takeda, M., Sato, B., Matsuo, H. and Matsumoto, K. (1992). Cloning and characterization of an androgen-induced growth factor essential for the androgen-dependent growth of mouse mammary carcinoma cells. *Proc. Natl. Acad. Sci. USA* **89**: 8928-8932.

120. Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R. and Dawson, A.P. (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2+-ATPase. *Proc. Natl. Acad. Sci. USA* **87**: 2466-2470.

121. Thomas, K.A. and Gimenez-Gallego, G. (1986). Fibroblast growth factors: broad spectrum mitogens with potent angiogenic activity. *TIBS* **11**: 81-84.

122. Treisman, R. (1985). Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5' element and c-fos 3' sequences. *Cell* **42**: 889-902.

123. Triglia, T., Peterson, M.G. and Kemp, D.J. (1988). A procedure for *in vitro* amplification of DNA segments that lie outside the boundaries of known sequences. *Nucleic Acids Res.* **16**: 8186.

124. Unsicker, K., Reichert-Preibsch, H., Schmidt, R., Pettmann, B., Labourdette, G. and Sensenbrenner, M. (1987). Astroglial and fibroblast growth factors have neurotrophic functions for cultured peripheral and central nervous sytem neurons. *Proc. Natl. Acad. Sci. USA* **84**: 5459-5463.

125. Verde, P., Boast, S., Franze, A., Robbiati, F. and Blasi, F. (1988). An upstream enhancer and a negative element in the 5' flanking region of the human urokinase plasminogen activator gene. *Nucleic Acids Res.* **16**: 10699-10716.

126. Weich, H.A., Iberg, N., Klagsbrun, M. and Folkman, J. (1991). Transcriptional regulation of basic fibroblast growth factor gene expression in capillary endothelial cells. *J. Cell. Biochem.* **47**: 158-164.

127. Weis, L. and Reinberg, D. (1992). Transcription by RNA polymerase II: initiator-directed formation of transcription-competent complexes. *FASEB J.* **6**: 3300-3309.

128. Wiley, S.R., Kraus, R.J. and Mertz, J.E. (1992). Functional binding of the "TATA" box binding componant of transcription factor TFIID to the -30 region of TATA-less promoters. *Proc. Natl. Acad. Sci. USA* **89**: 5814-5818.

129. Winkles, J.A. and Gay, C.G. (1991). Serum, phorbol ester, and polypeptide mitogens increase class 1 and 2 heparin-binding (acidic and basic fibroblast) growth factor gene expression in human vascular smooth muscle cells. *Cell Growth & Differentiation* **2**: 531-540.

130. Winkles, J.A., Peifley, K.A. and Friesel, R.E. (1992). Tumor promoters induce basic fibroblast growth factor gene expression in human dermal fibroblasts. *Cancer Res.* **52**: 1040-1043.

131. Wolswijk, G. and Noble, M. (1992). Cooperation between PDGF and FGF converts slowly dividing O-2A^{adult} progenitor cells to rapidly dividing cells with characteristics of O-2A^{perinatal} progenitor cells. *J. Cell Biol.* **118**: 889-900.

132. Yayon, A., Klagsbrun, M., Esko, J.D., Leder, P. and Ornitz, D.M. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64**: 841-848.

133. Yoshida, T., Miyagawa, K., Odagiri, H., Sakamoto, H., Little, P.F.R., Terada, M. and Sugimura, T. (1987). Genomic sequence of *hst*, a transforming gene encoding a protein homologous to fibroblast growth factors and the int-2-encoded protein. *Proc. Natl. Acad. Sci. USA* **84**: 7305-7309.

134. Zenzie-Gregory, B., Khachi, A., Garraway, I.P. and Smale, S.T. (1993). Mechanism of initiator-mediated transcription: evidence for a functional interaction between the TATA-binding protein and DNA in the absence of a specific recognition sequence. *Mol. Cell. Biol.* **13**: 3841-3849.

135. Zenzie-Gregory, B., O'Shea-Greenfield, A. and Smale, S.T. (1992). Similar mechanisms for transcription initiation mediated through a TATA box or an initiator element. *J. Biol. Chem.* **267**: 2823-2830.

136. Zhan, X., Bates, B., Hu, X. and Goldfarb, M. (1988). The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. *Mol. Cell. Biol.* **8**: 3487-3495.