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Expression of the c-fos proto-oncogene during normal and pathological bone development

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In memory of my mother,

with the hope that one day this research may, in some way, contribute to alleviate needless suffering.

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Contributions to Original Knowledge

The differential stimulation of the expression of different members of the fos and jun family by calcitriol support a role for these oncoproteins in bone metabolism and bone cell differentiation via transient proto-oncogene expression. This is the first report that studied the differential induction of AP-1 family members in a physiologically relevant target tissue. This study investigated the induction of AP-1 members in bone using a hormone that is central to the regulation of bone remodelling. It also studied some of the molecular mechanisms responsible for this response.

The work describes a novel vitamin D response element (VDRE) in the c-fos promoter. It is the first report of a VDRE in an immediate early gene. This required novel methods of detecting the transient vitamin D response. It establishes the element to have a novel DNA structure. It also identifies a new vitamin D receptor partner that is necessary for binding to this element.

Moreover, overexpression of c-fos was demonstrated in bone from patients with fibrous dysplasia. This was the first indication that aberrant c-fos expression contributes to a disease other than cancer. Although not a neoplastic disease, it classifies fibrous dysplasia into a preneoplastic state as well as a disease of hyperactive remodelling. It is the first report that points to the pathological

consequence of an activating G protein mutation resulting in the bone symptoms seen in fibrous dysplasia.

Abbreviations Used

ATF	activating transcription factor
ARF	activation resorption formation
AP-1	activating protein-1
CRE	cAMP response element
CREB	cAMP response element binding
DR	direct repeat
FCS	fetal calf serum
MAP	mitogen activating protein
OP	osteopontin
RAR	retinoic acid receptor
RXR	retinoic X receptor
SRE	serum response element
SRF	serum response factor
TCF	ternary complex factor
TR	thyroid receptor
VDR	vitamin D receptor
VDRE	vitamin D response element

Table of Contents

I. Résumé

Nous avons étudié l'expression du proto-oncogène c-fos durant le développement osseux normal et pathologique. La régulation de l'expression de c-fos dans les cellules osseuses par le calcitriol et sa contribution à l'étiologie de la dysplasie fibreuse ont été abordés. Nous avons démontré que le traitement d'ostéoblastes par le calcitriol, la forme active de la vitamine D, stimule l'expression transitoire de c-fos, fosB, c-jun, et junB de façon spécifique. L'expression de la protéine c-Fos parallèle l'expression du gène. Le mécanisme de la régulation de c-fos est transcriptionnel. Cependant, l'expression de c-jun et junB est controlée par un mécanisme post-transcriptionel n'impliquant pas la stabilité de l'ARN messager. Nous avons par la suite identifié un nouvel élément de réponse à la vitamine D dans le promoteur de c-fos. Cette région de 36 paires de base, qui est centrée environ 161 paires de base en amont du site d'initiation, répond au calcitriol dans les cellules osseuses. Le récepteur de la vitamine D lie cette séquence. La fonctionnalité de l'élément a été démontrée par une mutation qui inactive la réponse au calcitriol in vivo. L'analyse de la structure et de la fonction démontre que l'élément a une structure inhabituelle. Cette observation est supportée par le fait que le récepteur de la vitamine D requiert un nouveau partenaire afin de lier l'élément de réponse à la vitamine D. Entre autres, nous avons aussi démontré une expression de c-fos surélévée dans des lésions osseuses de patients atteints de dysplasie fibreuse. La surexpression de c-fos a été détectée seulement dans des

lésions de dysplasie fibreuse et non dans des biopsies qui proviennent de maladies osseuses exhibant un remodelage anormal ou un excès de tissus fibreux. Nos résultats supportent un rôle important pour c-fos dans l'étiologie de la dysplasie fibreuse.

II. Abstract

The expression of the c-fos proto-oncogene during normal and pathological bone development was studied. The regulation of c-fos expression in bone cells by calcitriol and its contribution to the etiology of fibrous dysplasia was addressed. We have shown that treatment of osteoblasts with calcitriol, the active form of vitamin D, transiently stimulated the expression of c-fos, fos-B, c-jun and jun-B in a specific and dose-dependent manner. The expression of the Fos protein correlated with the expression of the c-fos gene. Finally, calcitriol appeared to modulate c-fos transcription in osteoblasts, whereas it stimulated c-jun and jun-B expression by a post-transcriptional mechanism distinct from mRNA stabilization. The transcriptional stimulation of c-fos was explored further and we identified a novel vitamin D response element (VDRE) in the c-fos promoter. We report that a 36 bp sequence centered around position -161 upstream of the c-fos transcription start site is responsive to 1,25-$(OH)_2D_3$ in osteoblastic cells. This sequence binds the VDR, and mutations that abrogate binding to this element also abolish transcriptional activation by 1,25-$(OH)_2D_3$, demonstrating that we have identified a functional VDRE. Structure-function analysis revealed that the c-fos VDRE has an unusual structure that does not correspond to the classical direct repeat (DR)-type elements. Supporting this observation, our results show that the VDR requires a novel dimerizing partner to bind the c-fos VDRE. Finally, we report increased expression of c-fos in bone lesions from patients with fibrous dysplasia.

The elevated c-fos mRNA levels were detected only in fibrous dysplasia lesions and not in samples from patients with other bone diseases where high bone turnover and fibrous marrow tissue are present. Our results support the implication of c-fos in the etiology of fibrous dysplasia.

III. Introduction

A. Embryonic Bone Development

1. Bone Histogenesis

Embryonic bone development is well described in histological terms[1]. The histogenesis of bone into a functional tissue involves five closely integrated and complex processes. These coordinated processes include: (1) intramembranous ossification (flat bones), (2) endochondral ossification (long bones), (3) growth, (4) modelling, and (5) remodelling.

These processes are typically all observed when studying the histological changes during long bone development[2]. A long bone forms from an initial cartilaginous model. A direct periosteal bone collar (intramembranous ossification) then forms on the circumference of the prototype before the subsequent calcification of the cartilage model. Eventually, blood vessels and perivascular mesenchyme (periosteal bud) penetrate the calcified cartilage and form two growth plates (endochondral ossification). This process collectively forms the primary center of ossification. Secondary centers of ossification form at the epiphyseal cartilage. Eventually, bone slows in longitudinal growth and the growth plates fuse.

Bone histogenesis proceeds by the initial formation of a primary (woven) bone network, which is later remodeled into secondary (lamellar) bone or bone marrow. This process is essentially the same in both intramembranous and endochondral ossification. The lamellar bone skeleton forms the adult cortical and trabecular bone. The cortex fulfils the mechanical and protective function of bone, whereas the trabeculae fulfill the metabolic function of bone.

Interestingly, in situations where bone is formed extremely rapidly (such as during histogenesis, fracture healing, tumors, or in some metabolic bone disorders) woven bone is continually present. Collagen is deposited too quickly to allow for any preferential organization.

The process of intramembranous ossification[3] begins in a highly vascularized area of connective tissue. Mesenchymal cells proliferate and subsequently differentiate into preosteoblasts and then osteoblasts which secrete an immature bone tissue called woven bone. Woven bone is characterized by irregular bundles of randomly oriented collagen fibers. Due to delayed calcification there is an abundance of partially calcified immature new bone (osteoid). Calcification proceeds in disordered and irregularely distributed patches. Moreover, the bone tissue has a relatively low mineral content. Osteocytes, within these patches, are numerous and large. At the periphery of the patches, mesenchymal cells continue to differentiate thus enlarging the woven bone patch. Blood vessels get

incorporated between woven bone trabeculae and give rise to hematopoietic bone marrow. Eventually, as woven bone formation slows down, the tissue is progressively replaced by lamellar bone (remodelling).

The process of endochondral ossification begins in devascularized regions of connective tissue. Mesenchymal cells divide and differentiate into prechondroblasts and chondroblasts forming a cartilage model. These cells initially secrete a cartilagineous matrix that progressively surrounds them. Despite this gelatinous matrix, the cells continue to proliferate and differentiate to secrete more matrix. In addition to this interstitial growth however, appositional growth occurs at the periphery (perichondrium) with more mesenchymal cell proliferation and differentiation. These processes account for growth in the cartilage model.

As the avascular embryonic cartilage grows, the ring of woven bone around the midshaft is eventually invaded by vascular tissue. This vascular invasion is responsible for growth plate formation which maintains longitudinal bone growth. Growth plate chondrocytes appear in regular columns of isogenous groups that initially proliferate and then progressively hypertrophy. Just below the hypertrophic zone the cartilaginous matrix becomes mineralized. The chondrocytes then die. The transverse septae are resorbed by osteoclasts, followed by blood vessels, that are chemotactically attracted toward the hypertrophic chondrocytes. After resorption, osteoblasts differentiate from mesenchymal cells

that were brought with the invading vascular tissue. Woven bone is then deposited on top of the remaining calcified cartilage remnents of the longitudinal septa. Still lower in the growth plate, this mixture of woven bone and calcified cartilage is further remodelled and replaced by mature lamellar bone trabeculae.

Modelling is the process where bone is altered in size and shape by a coordinated change in formation and resorption at different surfaces and with different rates during growth. The continuous deposition of membraneous bone beneath the periosteum results in a growth of the shaft diameter. However, resorption does not immediately preceede formation in this case, instead it occurs in the lower metaphysis to transform it into a diaphysis. Resorption by osteoclasts is continuous and not directly coupled to formation.

Bone remodelling fulfils the endocrine function of bone, together with the kidney and intestine, in the maintenance of mineral ion balance[4]. Bone is continually turned over in a highly regulated sequence of coupled events called the activation-resorption-formation sequence (ARF sequence)[5]. It is a cyclical event that occurs in patches along a small portion of available bone surface. This means that in the adult, bone formation occurs only where bone resorption has previously occurred. A cementing line, produced during the "reversal phase" between bone resorption and formation, delineates the limit of new bone and old bone.

Bone remodelling differs from bone modelling except in the "activation step"[1]. Bone modelling is a continuous activation of either bone formation or resorption leading to a net gain in bone mass. On the other hand, bone remodelling is the cyclical activation of resorption and formation of new bone resulting in bone turnover. Both the modelling and remodelling coordinated processes activate resorption by a common mechanism. Morphogenic or physical signals to activate resorption are received by resting, flat bone-lining cells. These cells then shift to an osteoid destruction state. The cells secrete collagenase, change shape, and proliferate. They attract osteoclasts and detach from the bone surface to allow the osteoclasts to resorb the exposed mineral[1].

2. Bone Cell Differentiation

On a cellular level, bone histogenesis involves the differentiation and function of bone forming (osteoblast) and bone resorbing (osteoclast) cells. Differentiation along the osteoblast lineage as well as the establishment of the osteoblastic phenotype and function results from a tightly integrated and sequential pattern of specific gene expression[6,7]. Mesenchymal cell progenitors can differentiate into the bone forming cell lineage. They can become bone marrow stromal cells[8], chondrocytes[9] or osteocytes[6]. The bone forming lineage begins with a progressive differentiation of stromal cells to preosteoblasts to osteoblasts and to osteocytes. Committed preosteoblasts are central to bone formation, they proliferate and

express several growth-associated genes such as histones and proto-oncogenes like c-fos. They express genes encoding matrix proteins like type I collagen and regulate the process by which it mineralizes into a specific crystalline form. This proliferative phase is followed by the onset of the matrix maturation phase with the expression of alkaline phosphatase, a protein associated with the bone cell phenotype. Although the actual mechanism of osteoid maturation is not well understood, it remains a fact that the deposited collagen must "mature" before it can normally mineralize. Mineralization is controlled by the osteoblast at both the initiation and deposition stage of hydroxyapatite formation[10]. In fact, genes encoding non-collagenous bone matrix proteins, such as osteocalcin and osteopontin, are expressed in the matrix mineralization phase. Osteocalcin, a principal bone specific noncollagenous protein, is secreted in the bone matrix and may be involved in calcium binding and bone turnover[11]. Osteocalcin measurements are valuable markers of bone formation in metabolic disease states[12]. There are also many different autocrine regulators of bone cells[13,14] that are secreted into the matrix.

Several *in vitro* systems have been developed to study these processes at the cellular and molecular level. Many *in vivo* studies have validated the biological relevance of these observations. Various cell lines have been developed, including MC3T3-E1, ROS 17/2.8, and primary calvarial cultures. MC3T3-E1 cells were established from murine osteogenic cells that express a tissue specific

pattern[15,16] of gene expression toward differentiation to the osteoblastic phenotype with ultimate matrix mineralization[17]. Ros 17/2.8 cells were specifically isolated from rat osteosarcoma cells for their ability to support a vitamin D response in transient tranfection assays using heterologous promoter constructs[18]. Primary calvarial cultures of murine osteoblasts are extremely useful because they are not immortalized or transformed[19,20].

The osteoclast is responsible for resorption during the histogenic process[21,22]. It is a cell involved in bone remodelling[23]. It has distinct morphological features. The best known markers for osteoclast differentiation is tartarate-resistant acid phosphatase (TRAP) activity and presence of the calcitonin receptor. The molecular mechanism by which the cell resorbs bone is dependent on ion transport[24]. Central to this resorptive process is the attachment to bone matrix which is mediated by integrin molecules[25]. It is an extremely motile cell that is derived from the promonocytic lineage[26,27] and not the mesenchymal lineage[28]. The osteoblast lineage[29] cells (stromal cells[30]) regulate osteoclast formation and activation[31]. Defects in this pathway can lead to metabolic bone defects such as osteopetrosis[32,33].

Osteoclast formation (proliferation and differentiation) is regulated by systemic and local factors. Some better understood systemic factors are parathyroid hormone, calcitriol (1α25-dihydroxyvitamin D_3), and calcitonin. Calcitriol, the

active form of vitamin D, is an important stimulator of osteoclastic bone resorption. The effects on resorption of mature osteoclasts may be indirect via actions on bone lining cells. Fusion[34] from precursors[35,36], on the other hand, may be due to direct effects of the hormone on osteoclast precursors. Calcitriol may act directly on osteoclast precursors to enhance their ability to attach to bone by stimulating integrin expression[37]. Moreover, the effects of calcitriol on bone resorption are multiple and complex.

The local factors that regulate osteoclast formation are secreted by bone marrow stromal cells[38]. Factors generated in the microenvironment of bone are probably more important than systemic factors in the regulation of turnover since remodeling occurs in discrete and distinct patches throughout the skeleton. Factors such as IGF-1[39], interleukin-1, lymphotoxin, tumor necrosis factor, colony stimulating factor-1, osteoclastpoietic factor[40], gamma-interferon, and transforming growth factor-ß are local remodeling factors. Recently, immune factors like interleukin-6 are becoming better understood for their role in promoting osteoclast formation[41,42] and in bone pathology[43,44]. This relationship with the immune system is extended further by the findings that the third component of complement was found to regulate osteoclast formation[45,46]. Interleukin-4 was also shown to be important in osteoclast formation[47,48].

3. Regulation of Bone Remodelling

Hormonal regulation of bone remodelling occurs via an osteoblast mediated mechanism, a complex and not fully understood process[21]. The osteoblast has a significant role in the tight coupling of bone resorption and formation. Parathyroid hormone (PTH) and calcitriol are two of many important systemic signals[49], that regulate bone remodelling. Moreover, the osteoblast mediates the signal for the recruitment and activity of osteoclasts. In fact, only osteoblasts and non-mature osteoclasts have detectable calcitriol and PTH receptors. Bone-lining osteoblasts are thought to activate osteoclasts by the subsequent secretion of soluble activating factors. The release of activating humoral mediator(s) has been shown to be controlled, at least in part, by c-fos[50]. These factors regulate both the maturation and resorption activity of osteoclasts. In this manner, motile osteoclasts are recruited to adhere to the bone as the bone-lining cells (osteoblasts) move out of the way. The osteoclast will begin resorbing bone in a small area causing the release of the 10-15 local osteoblast growth factors mentioned above, from the bone. These local factors stimulate osteoblast activity and differentiation and recruitment to the site[49]. These local autocrine factors were initially deposited in bone by the osteoblast itself. In other words, the osteoblast secretes autocrine growth factors into the bone matrix that will restimulate bone formation after they are released by the activated bone resorbing osteoclast. This explains why almost all osteoclastic activity is followed by and tightly coupled to osteoblastic activity.

Bone is then redeposited in the resorbed pit together with more autocrine factors that will drive the next ARF sequence.

There are an enormous number of newly identified actions of the vitamin D endocrine system on a large variety of tissues. This hormone acts to regulate bone remodelling, modelling, and cellular differentiation[51]. The major physiologic role of calcitriol in bone metabolism is to increase calcium and phosphate absorption in the intestine[52,53]. In fact, calcitriol will enhance bone formation and suppress bone resorption when levels of calcium and phosphate are high in the organism. Calcitriol not only influences the bone remodelling process, it has also been shown to have a dose-dependent effect on growth and modelling of embryonic bone during development[54]. However, physiologic concentrations of the hormone will differentiate bone cells[5] and other cell types[55]. Furthermore, the effect of calcitriol on osteoblast function will vary, depending on its stage of maturation[56,57]. In fact, depending on the timing and dose administered, both PTH and calcitriol could produce net formation or net resorption. Calcitriol also directly exerts its effects on genes that are related to osteoblast function. It stimulates the expression of type I collagen, alkaline phosphatase, osteopontin, and osteocalcin. The response is dependent on the particular stage of differentiation of the osteoblast. This differential response to calcitriol in bone is observed in MC3T3-E1 cells[58,59].

B. Gene Regulation by Steroid Hormones

Cells differentiate by expressing various tissue specific proteins with specialized functions. One mechanism of controlling gene expression is regulation at the level of transcription. The cell regulates the abundance of mRNA to control the presence of specific proteins. Genes encoding eukaryotic proteins are transcribed by the basic RNA polymerase II transcriptional machinery[60,61]. This machinery involves a large number of general transcription factors that specifically interact at the basal promoter[62]. Basal transcription occurs by sequentially mounting protein complexes on the basal promoter[63]. A preinitiation complex is followed by the formation of an initiation complex that is competent to begin transcription[64]. The mRNA is initially capped and elongated[65] until termination and the formation of a poly-A tail. The regulation of basal transcription[66] is controlled by the interaction of sequence-specific DNA-binding nuclear proteins (transcription factors) with particular cis-acting modulator elements in the promoter region of a gene[67]. Basal transcription[68,69] is either enhanced or reduced. Transcription factors bind upstream modulator regions of the promoter. They then either directly interact with the basal machinery or indirectly interact with it through a coactivator. Coactivators bridge the gap between the transcription factor and the basal machinery.

The steroid/nuclear receptors are ligand-activated transcription factors[70,71]. The

recent cloning of members of this superfamily has allowed considerable understanding of their mechanism of action[72]. The superfamily is well conserved among different species[73]. This family of transcription factors is involved in differentiation, embryogenesis and neoplasia[74]. This superfamily of receptors fall into three sub-groups: the homodimeric binding steroid receptors, the heterodimeric binding nuclear receptors, and the monomeric binding receptors. Numerous orphan receptors exist for which the ligand and function is yet unknown.

Steroid receptors have conserved DNA binding domains. They all have homologous zinc finger regions which dictate specific recognition of half sites and which determine the dimerizing partner capabilities. It is possible to deduce a general consensus binding sequence. Steroid receptors recognize imperfect palindromic AGAACA sequences spaced by three basepairs. Nuclear receptors recognize imperfect direct repeats of the AGGTCA motif. Binding sites for nuclear receptors are determined by the organization of the response element rather than the particular half site composition.

Moreover, hormone responses are specified by a dynamic equilibrium of many factors. There can be some cellular selectivity once the ligand is secreted through target cell modification of the ligand[102]. A restricted pattern of receptor expression is another means of generating specificity. Selectivity for different members of

this family is also determined by the spacing between and organization of the half sites. In the absence of ligand, the subcellular localization of these receptors remains controversial. In the presence of ligand, steroid/nuclear receptors bind hormone response elements[75] to stimulate or repress[76] the basal level of transcription[77,78]. The local concentration of ligand may also be a determinant factor. In fact, the response to a ligand is determined by the presence of the appropriate nuclear receptor partners that recognize a particular response element structure within the context of a particular promoter. In addition, promiscuous dimerization and interactions with different sequence specific nuclear proteins can also be thought of as a mechanism of generating diverse responses to a particular ligand[79].

1. Transcriptional Activation by Calcitriol

The molecular mechanism of calcitriol action involves genomic and nongenomic pathways[80,81]. Most of the effects of 1,25-dihydroxyvitamin D, such as in maintaining calcium homeostasis or promoting cellular differentiation, are through the genomic actions of the vitamin D receptor[82,83]. The better characterized mechanism by which steroid receptors mediate gene expression is through transcriptional regulation. Various post-transcriptional and post-translational mechanisms are also regulated by calcitriol[84,85]. Nevertheless, transcriptional activation remains the most important mechanism by which calcitriol exerts its

genomic actions.

Calcitriol exerts these genomic actions following binding to a specific receptor that is a member of the steroid receptor superfamily[86]. The vitamin D receptor (VDR) has been cloned in chickens[87], humans[88], and rats[89]. It has been characterized as a transcription factor. Analysis of the receptor revealed that it is a member of the steroid receptor superfamily. The vitamin D receptor preserves a superfamily domain structure. This consists of an N-terminal DNA binding domain and a C-terminal ligand binding domain. However, it does not have a generally well conserved DNA binding domain as compared to other steroid/nuclear receptors. Despite this small overall divergence, the DNA binding domain contains eight highly conserved cysteine residues that form two zinc fingers. One finger recognizes the vitamin D receptor binding sequence (P box) and the other is involved in dimerization (D box). The ligand binding domain is responsible for specific, high affinity binding of calcitriol, the active form of vitamin D.

Expression of the receptor protein is itself regulated at several levels. Differential regulation of mRNA expression in osteoblasts is possible since there are three VDR alternatively spliced transcripts identified[90]. Phosphorylation status of the receptor prior to binding its target promoter sequence has also been shown to be important[91,92]. Casein kinase II[93] and protein kinase C[94] will phosphorylate

the vitamin D receptor on different serine sites. Furthermore, calcitriol will activate protein kinase C to phosphorylate a Raf kinase which itself translocates from the cell membrane to begin another separate phosphorylation signal transduction cascade[95].

Recent work revealed the complexity of the molecular mechanisms implicated in the vitamin D transcriptional regulatory pathway. The VDR is thought to bind DNA either as a homodimer[96,97] or together with nuclear accessory factors present in nuclear extracts[98]. At least one of these nuclear accessory factors has now been identified. The VDR forms heterodimers with retinoid X receptors (RXRs)[99,100], a family of nuclear receptors binding the retinoid 9-*cis* retinoic acid[101,102,103]. An as yet unidentified nuclear protein, termed RAF, is required for high affinity binding of the VDR to a VDRE[104,105]. This molecular cross-talk between the VDR and other nuclear proteins is supported by evidence showing that VDR heterodimerization can also occur with retinoic acid receptors (RARs)[106] and thyroid hormone receptors (T_3Rs)[107] on some response elements. On inverted palindromes it has been suggested that the action of these receptors is RXR-independent[108]. Interestingly, the VDR dimerizing partner seems to affect the binding affinity to and the transcriptional response from particular VDREs.

It is now becoming clear that other nuclear proteins may also be involved in

interacting with nuclear receptors. New proteins are continually being discovered to interact with members of the nuclear hormone superfamily. An endogenous liver protein was shown to heterodimerize with members of the nuclear receptor superfamily[109]. The orphan receptor COUP has been reported to heterodimerize with the thyroid receptor(TR), retinoic acid receptor(RAR), and retinoic X receptor(RXR)[110] on some types of hormone response elements[111,112]. In fact, the availability of a partner and the DNA element can have a role in dictating which partner a nuclear receptor can interact with. Moreover, Pit-1, a tissue specific factor, was shown to be absolutely dependent for retinoic acid receptor function on a novel tissue specific response element[113]. Some proteins may indirectly facilitate binding by bending DNA to allow nuclear receptors to bind target response elements better[114].

Following binding to its specific ligand, the ligand-receptor complex translocates to the nucleus and binds specific sequences in the promoter of target genes and activates their transcription[115]. This ligand-receptor complex can also repress transcription on some promoter sequences such as the parathyroid hormone gene[116]. In fact, for the osteocalcin promoter VDRE, which binds VDR-RXR, it was shown that calcitriol activates whereas 9-*cis* retinoic acid represses the calcitriol stimulated osteocalcin expression[117]. Interestingly, a report suggested that ligand modulated the conversion of a constitutively bound VDR homodimer to a heterodimer with RXR on the VDRE[118]. This was proposed as a hypothesis

to explain how a constitutively bound nuclear receptor complex could affect transcription in a hormone dependent fashion.

Since the vitamin D receptor DNA binding domain is quite divergent from other steroid/nuclear receptor superfamily members, it is not surprising that the target recognition sequence of this receptor may diverge from other nuclear receptor superfamily tandem repeats. In fact, binding studies using the VDR DNA binding domain have revealed the order of relative binding affinity for various hexameric core binding sites[107]. It was found that the following half sites bound the VDR DNA binding domain with decreasing order of affinity: GGTTCA > AGGTCA > AGGACA > GGGTGA.

A number of specific natural VDRE sequences have been identified from different promoter sequences. Subtle differences exist between specific VDRE hexameric half-sites. A synthetic consensus sites can be derived from these sequences[119]. However, it is important to study natural VDREs in the context of their promoter because there can be complex effects on transcription due to other elements present on a promoter[120].

These response elements consist of direct repeats (DR) of two hexameric core binding sites spaced by varying numbers of nucleotide residues. The DR3-type element, exampled by the mouse osteopontin VDRE[107], consists of two GGTTCA

hexameric core sites spaced by three residues. It has been shown to bind VDR homodimers with low affinity but to bind VDR-RXR and VDR-T_3R heterodimers with high affinity[97,100,96,99]. DR3-type elements have been described in the promoters of the rat osteocalcin gene[121], the rat calbindin D-9k gene[122], the avian integrin β_3 subunit gene[123] and the rat calcidiol 24-hydroxylase gene[124,125]. Amongst these, the avian integrin β_3 subunit gene VDRE has been directly shown to bind the VDR-RXR heterodimer.

A DR4-type element with two AGTTCA hexameric sites (on the non-coding strand) spaced by 4 residues has recently been described[113]. It is actually a composite retinoic acid and vitamin D response element called RDE. The transcriptional response to 1,25-$(OH)_2D_3$ mediated through the RDE was exceptionally high and the element was shown to bind VDR-RXR heterodimers, but not VDR homodimers. A DR4-like element has also been described in the promoter of the calbindin D-28k gene[126]. The VDR was shown to bind that element but the putative dimerizing partners involved were not characterized.

In contrast, the DR6-type element of the human osteocalcin VDRE consists of two GGGTGA hexameric consensus sites spaced by 6 residues[127,128]. It has been shown to bind preferentially to VDR-RAR and VDR-T_3R heterodimers[106], as well as to VDR homodimers, although with lower affinity. Other structural motifs that have been shown to mediate transcriptional induction by the VDR alone, and thus

inferred to bind VDR homodimers, include a palindrome of the same hexameric site spaced by 12 residues (IP12)[96].

C. The c-fos Proto-Oncogene

The c-fos proto-oncogene is the cellular counterpart of the transforming gene of the FBJ and FBR murine osteosarcoma viruses[129,130,131]. The c-fos gene product (Fos) is a nuclear phosphoprotein that interacts with the product of another recently identified nuclear proto-oncogene, c-jun[132,133,134]. Several related genes have been identified and cloned on the basis of sequence similarity and/or antibody cross-reactivity of their protein products[135,136,137,138,139]. Thus fos and jun represent multigene families which comprise to date c-fos, fos-B, fosB2, fra-1 and fra-2 on the one hand and c-jun, jun-B and jun-D on the other.

A number of elegant analyses from various laboratories has revealed the structural and functional details of the fos/jun interaction[140,141]. The fos family members heterodimerize with the members of the jun family through a structural motif called the leucine zipper[142,143,144,145,146,147,148]. The heterodimer can then bind DNA at a consensus site termed the AP-1 site and act as a transcription factor to modulate the expression of AP-1-responsive genes[149,150,151,152,153,154]. All the jun family members can also homodimerize to exert a similar function[25,26,155] (figure 1).

Following c-fos interaction with a dimerizing partner, the transcription factor complex will bind DNA sequences in promoters to activate or repress

transcription[156]. The DNA binding domain of the protein contains a high density of basic amino acids. The interaction between Fos and Jun with an AP-1 site results in a conformational change in the basic amino acid DNA-binding domain. A flexible hinge model was proposed for DNA binding where this basic DNA binding domain adopts an α-helical structure upon binding to DNA[157]. This change induces a corresponding change in the DNA helix. A deformation of the DNA helix allows for contact between transcription factors and the basal transcriptional machinery. Thus, the structure of DNA is also important in understanding gene regulation.

Numerous studies have looked at mitogens that induce fos/jun expression in numerous cell types[158]. However, few studies address fos/jun induction in pre-osteoblastic cells by growth factors relevant to bone. Hormones such as PTH or calcitriol, which are known modulators of bone remodeling and differentiation, have been shown to influence the expression of c-fos in osteoblasts. Administration of PTH was shown by in situ hybridization to induce a rapid, transient, and sequential in vivo expression of c-fos in bone cells and in MC3T3-E1 cells[159]. We have shown that calcitriol increased c-fos in murine osteoblasts[160]. Recent studies have shown that growth hormone stimulates the expression of c-fos, c-jun, and junB in murine primary osteoblast cultures[161,162]. In addition, insulin-like growth factor I and II (IGF-I and IGF-II) have also been shown to induce c-fos in osteogenic cells and primary cell

cultures[163]. That study, however, did not address any possible IGF-I and IGF-II effects on jun family members. Interestingly, retinoic acid was also recently shown to induce c-fos, c-jun and jun-B mRNA levels in preosteoblastic cells[164]. The stimulation of c-fos in particular, was shown to be at the transcriptional level. Tumor Necrosis factor-α, a potent osteotropic cytokine involved in bone remodeling, also stimulated fos and jun genes in MC3T3-E1 cells[165]. Moreover, general serum growth factors were shown to increase transcriptional activation of c-fos and c-jun in MC3T3-E1 cells[166]. Therefore a large number of bone growth and differentiation factors that are involved in remodeling have been shown to stimulate Fos and Jun family members in osteoblast-like cells. Presumably these factors, which control various aspects of bone metabolism, can do so through the c-fos protooncogene which subsequently controls the regulation of other genes involved in bone metabolism.

Perturbations in the sequential pattern of gene expression to form a functional cell will lead to developmental[23] defects. It is likely that this complex pattern of gene expression must be tightly controlled in order to permit the appropriate establishment of the normal osteoblastic phenotype and function and that perturbations in that ordered sequence of events may lead to various pathologies. For example, studies in transgenic mice have shown that continuous expression of the c-fos proto-oncogene, which is normally down-regulated after the proliferative phase, leads to aberrant bone development and induces lesions that

resemble those seen in patients with fibrous dysplasia. Inhibition by targeted mutations of the expression of another proto-oncogene, c-src, induces osteopetrosis in homozygous chimeric animals. Thus a better comprehension of the role and control of the expression of particular genes at each phase of the osteoblastic differentiation pathway is necessary and may permit to establish links between those genes and various pathologies that affect the cell.

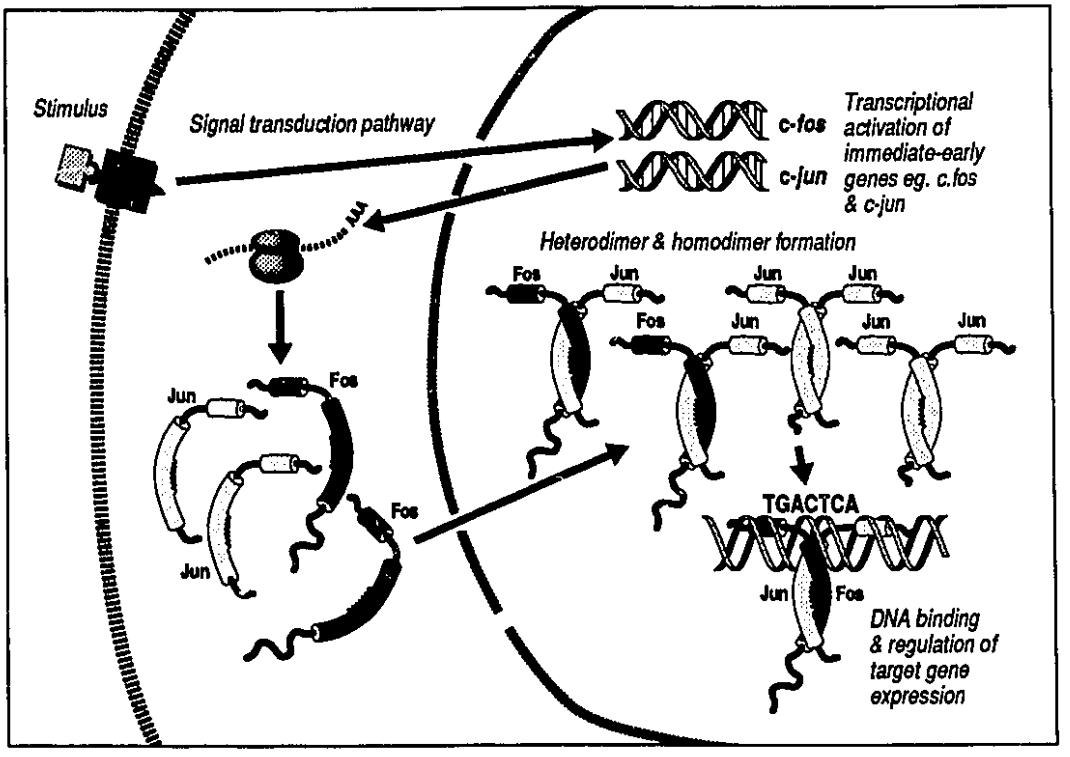

Figure 1: The AP-1 transcription factors (fos/jun). An extracellular stimulus induces the transcription of different members of the fos and jun family of proto-oncogenes via numerous signal transduction pathways. The Fos and Jun proteins are subsequently translated and translocated to the nucleus where they can form homo- and heterodimers. Members of the Fos family can only heterodimerize with Jun family members which can, on the other hand, homodimerize with themselves. The schematic cylinders represent protein alpha helices. The active transcription factor complex is an AP-1 dimer which binds to a specific AP-1 site on the promoter of AP-1 responsive genes as shown.

1. Mechanisms of c-fos Regulation

Regulation of the c-fos gene, like many other immediate-early response genes, is extremely tightly controlled. This is in contrast to a housekeeping gene, such as alpha tubulin, which is controlled much less rigorously. The cell has devised many complex mechanisms to ensure appropriate up- or down-regulation of immediate early response genes. From viruses, it is known that these genes are rapidly expressed to start a coordinated response cascade of gene expression toward a specific end result. For a virus, the result is an increase in the replication cycle by sequestering cellular proteins toward this goal.

Regulation of the initiation of transcription of c-fos is through the binding of specific transcription factors which recognize DNA elements on this complex promoter. Peptide growth factors indirectly activate the transcription of c-fos by multiple second messenger pathways from the cell surface that act on at least three distinct upstream regulatory domains on this promoter. All three regions contribute to basal and induced promoter activity of the human c-fos gene[167,168]. The distal region encompasses three elements which have been well characterized (see below). An uncharacterized medial region has also been described to resemble an NFI-like recognition sequence. A third proximal region is well charaterized and consists of two elements[169] (see below). Steroid hormones, on the other hand, directly activate the c-fos promoter via steroid

response elements. Prior to this work, only response elements for estrogen have been characterized[170]. Other steroids, such as retinoic acid, had been shown to influence c-fos transcription but response elements on the promoter had not been characterized.

Three characterized elements of the distal region are the AP1/FAP, SRE (serum response element), and the SIF-E elements which are implicated in both the repression and induction of transcription. The FAP site will bind AP-1 and ATF family members. A variety of signal transduction pathways converge on a ternary complex binding the SRE via protein kinase C dependent and independent pathways. One independent pathway involves c-fos activation by NFIL6 in response to the activation of the cAMP signal transduction pathway[171]. The SRE and the FAP site are constitutively occupied, activation of c-fos through these sites is probably via phosphorylation or replacement of ternary complex factors. Various proteins such as SRF, TCF, Elk-1[172], SAP-1[173], DBF/MAPF1, Phox1, NFIL6, SRE-ZBP, and E-12 have been shown to bind the SRE[174]. The SIF-E site, on the other hand, is not constitutively occupied in vivo and activation is through direct binding of the p91 transcription factor from the cytoplasm after interaction with the epidermal growth factor receptor[175].

In many cell types the steady state levels of c-fos are normally kept very low. Several mechanisms account for this repression. One mechanism involves the

SRE element located 307 bp upstream of the start site[176]. Transient c-fos induction via the SRE shuts itself off by a negative autoregulatory loop either through the binding of the adjacent AP-1 site by the c-Fos heterodimer[177] or by dephosphorylation of TCF[178]. Another mechanism involves the attenuation of transcription past sequences located within the first intron-exon junction[179]. A third mechanism leads to rapid translation-linked degradation via AU-rich sequences in the 3' nontranslated region of c-fos mRNA[180].

The well characterized proximal region contains a GC-rich repeated element and two partially overlapping sites for transcription factors ATF/CREB and MLTF/USF. The GC-rich repeated element is of importance in basal activity but is also the site shown to bind the retinoblastoma protein in the repression of c-fos induction[181]. In the human c-fos promoter it was found that the proximal cAMP response element (CRE) promoter domain is the major determinant of fos promoter activity[167]. Mutations in this element strongly reduce both basal and induced levels of expression. Transcriptional activation by cAMP or Ca^{2+} is via phosphorylation of a CREB family protein by either protein kinase A or by a Ca^{2+} calmodulin regulated kinase[182]. CREB proteins are constitutively bound and transcription is activated by the phosphorylation at a specific serine residue (position 133). In contrast to the SRE negative autoregulation loop c-Fos is unable to down-regulate CRE-mediated activation[183]. Heterodimerization with a CRE modulator (CREM) is used instead to block cAMP induction. Another

CRE was identified in the transcribed region of c-fos and was also shown to be important in cAMP activation[184]. Interestingly, it does not bind the same CREB complex that binds the proximal element.

Most studies on the transcriptional regulation of c-fos have documented regulatory regions in the c-fos promoter that initiate transcription. A strong block to transcriptional elongation occurs in the the 5' part of the mammalian c-fos proto-oncogene. A unique motif within the first intron has been characterized to be responsible for transcriptional arrest[185]. The mechanism of arrest seems either to be through pausing or premature termination of RNA polymerase II. Interestingly, it was shown that calcium second messenger signaling is responsible for the relief of the block to elongation independent of promoter activity[186,187]. In addition to an elongation block, the induction of full length c-fos is dependent on the linkage of RNA polymerase II with topoisomerase I to relieve torsional consequences of transcriptional elongation[188].

In practical terms the regulation of transcriptional activation involves the coordinated integration of all signal transduction pathways to the nucleus[189]. This is dependent on the cellular context and the coordinate response to simultaneous signals. Furthermore, any one signal can act to induce c-fos simultaneously through different mechanisms of regulation. For example, okadaic acid will regulate c-fos expression via transcriptional as well as post-

transcriptional mechanisms[190].

In addition to transcriptional activation mechanisms, various posttranscriptional mechanisms have also been described to regulate gene expression. Regulation of splicing and transport of the mRNA is possible[191]. However, many steroid hormones, such as estrogens, can modify gene expression through mechanisms that increase mRNA stability[192]. The c-fos message is extremely labile. One described mechanism shown to affect the posttranscriptional regulation of the c-fos protooncogene is the level of stability of the mRNA via two destabilizing regions[193]. Rapid degradation of c-fos mRNA is mediated by both a c-fos protein-coding region and an AU-rich element in the 3'-untranslated region. These domains function independently of each other by distinct mechanisms. The protein coding region is recognized as RNA by cellular proteins[194,195]. The deadenylation and decay process can be impeded by cyclohexymide, a protein synthesis inhibitor, indicating that this process might be coupled directly to its translation by ribosomes[196]. It has however been suggested that the decay mechanism for the 3' untranslated region may not be coupled to translation[197]. Moreover, within the 3' untranslated region, two structurally distinct but functionally interdependent RNA domains[198] are recognized by cellular proteins[199,200]. One RNA domain is responsible for deadenylation and the other for decay.

Many different post-translational mechanisms for regulating c-Fos half-life exist. The role of c-Fos as a transcription factor is complex and and can be regulated at several levels[201]. It is a phosphorylated protein which translocates to the nucleus, undergoes redox activation in the DNA-binding domain, associates with different dimerization partners, and interacts with other transcription factors.

In addition to control of mRNA stability, regulation of the stability of the c-Fos protein has been described[202]. The protein stability was found to decrease after dimerization with phosphorylated c-jun. Growth factors have been shown to regulate c-Fos by affecting its stability[203]. The degradation mechanism is by the classical ubiquitin system[204].

Although c-Fos is a nuclear protein, in the absence of serum the protein is located in the cytoplasm until serum stimulation when it translocates to the nucleus by an active mechanism[205]. This suggests that nuclear targeting sequences in the protein can also be a regulatory point for cellular localization[206].

Phosphorylation and dephosphorylation of AP-1 is critical in the regulation of the function of this complex. The phosphorylation of the AP-1 protein occurs by several different protein kinase whose activity is modulated by dimerization and binding to DNA of the AP-1 complex[207,208]. There is an enormous amount of cross-talk between different kinase cascades. Some of these kinases are new

tyrosine kinases[209,210] and others are MAP tyrosine kinases[211,212], glycogen synthase kinase, casein kinase II, and p34 cdc2 kinase. Furthermore, both the PKC and PKA second messanger pathways can phosphorylate c-Fos at multiple regulatory sites[213]. The cyclic AMP-dependent protein kinase A pathway seems to be important in the regulation of both the fos gene and the Fos protein. The PKA phosphorylation of the c-Fos protein at the serine 362 site augments its transforming potential[214]. Moreover, PKA phosphorylation may also stimulate nuclear translocation of c-Fos.

In addition to phosphorylation, an oxidation/reduction (redox) modification has also been suggested to regulate DNA-binding activity[215]. A conserved cysteine residue located in the basic DNA-binding domain of c-Fos, when reduced, allows the AP-1 dimer to bind DNA and enhances its transforming activity[216]. Reduction of this residue occurs in the cell via a cellular redox factor called Ref-1[217] which is also involved in DNA repair[218].

The activity of c-Fos also depends on dimerization with other transcription factor partners on or off of DNA elements. In addition to members of the Jun family, there are numerous new proteins that have been demonstrated to dimerize with c-Fos. The Fos/Jun and certain Fos/ATF heterodimers have been shown to also bind a variety of different CRE-like recognition sequences in addition to AP-1 sites[219]. Most recently, members of the Maf protooncogene family have been

shown to heterodimerize with Fos and Jun and bind AP-1 sites as well[220,221]. A novel T cell specific factor was also shown to interact with Fos/Jun[222,223]. New heterodimeric partners are continually being discovered[224,225]. Heterodimeric interactions on DNA allows for the possibility of having different binding specificities for various elements and having either activation or repression of transcriptional activity. This phenomena results in a varied and coordinated pattern of late gene expression that will vary depending on the cellular context.

Moreover, several interactions between Fos/Jun proteins and other nuclear receptor transcription factors have been reported. The actual molecular mechanisms for the multiple regulatory interactions described between the Fos/Jun family and nuclear receptors with cotransfections at different promoters and in different cell types remains contradictory[226,227,228]. Many models have been proposed[229]. For example, direct protein-protein interactions before the heterodimer binds DNA will mutually inhibit AP-1 and nuclear receptor regulated gene expression. In fact, c-fos was shown to be the preferential target of the glucocorticoid receptor inhibition of AP-1 activity by such a mechanism[230]. Other mechanisms, such as co-occupancy of a regulatory element or a competitive interaction with other proteins (tethering), have been proposed to explain some of these interactions[231]. It is conceivable that several of these mechanisms can co-exist; however, a mechanism of mutual inhibition is inconsistent with other mechanisms because c-Fos would not be available to interact with other proteins (tethering) or bind DNA

(co-occupancy).

2. Role of c-Fos in Bone

These studies on the molecular biology of fos and jun do not address the physiological role of these gene products on tissue development. Several lines of evidence suggest a putative role for c-Fos in skeletal tissue development[232]. The c-fos proto-oncogene was actually one of the first genes used to demonstrate that cellular genes homologous to viral oncogenes may have a role in pre- and postnatal animal development[233].

There are numerous reports that have localized the expression of c-fos and c-jun to regions of active skeletal tissue development. Moreover, the expression pattern of the c-fos proto-oncogene during skeletal development suggests that this gene plays a role in the differentiation of the cellular precursors of osteoblasts and chondrocytes (mesenchymal lineage) as well as precursors to the osteoclast lineage. In the mesenchymal lineage, c-fos mRNA was detected in perichondrial growth regions of the mouse[234]. Detailed studies which looked at c-Fos protein expression have identified it in regions with cells that are capable of becoming both osteoblasts and chondrocytes[235]. Furthermore, the expression of c-jun during mouse development was also associated with rapidly proliferating chondrocyte precursor cells[236]. In human development, the expression of c-fos was observed in areas with active endochondral[237] and intramembraneous bone formation[238]. Again, the mRNA was detected in precursors to osteoblasts and

chondrocytes. The preosteoblasts are found in both types of bone formation whereas prechondrocytes are only found in endochondral bone formation. Surprisingly, both these reports also observed a constitutive expression of c-fos in differentiating osteoclasts as well. Moreover, these results suggest that c-fos could have a role in both skeletal growth and remodeling during development.

The association between c-fos expression and areas of active skeletal development is supported by other observations. Interestingly, osteogenic differentiation of newborn mouse mandibular condyle is preceded by a burst of c-fos expression in vitro[239,240]. These cells then progressively continue to express the osteoblastic tissue-specific pattern of differentiation which is also preceded by a burst of c-fos. The transient expression of c-fos is also observed preceeding fracture healing[241]. Moreover, there are elevated levels of c-fos in both murine[242] and human[243] osteosarcomas and the viral form of c-fos, v-fos, induces chondro-osseous neoplasms in newborn mice[244].

To identify the putative in vivo role that c-Fos could have in skeletal development, both gain-of-function and loss-of-function experiments have been performed. Overexpression of an exogenous c-fos sequence in transgenic mice perturbs normal bone development[245]. This is displayed by striking disturbances in bone remodeling characterized by bone marrow fibrosis and enhanced formation of new bone. These lesions are not osteosarcomas; however, these transgenic mice have

a low frequency predisposition to form osteochondroid tumors[246]. Moreover, there was some evidence that osteoblasts were the target cells of c-fos overexpression[247]. A recent report, however, demonstrated conclusively that the osteoblast lineage is the principle target for transformation in c-fos transgenic mice and that this phenotype was specifically induced by c-fos, and not fosB or c-jun[248].

In contrast to the c-fos transgenic mouse phenotype, embryonic stem cell chimeras which express c-fos ectopically developed chondrogenic tumors[249]. These animals were generated to study the target cell specificity and consequence of c-fos overexpression during embryonic development. Transgenic animals, for reasons unknown, do not express a transgene during embryonic development and chimeric animals enable one to bypass this phenomena. It seems that the chondrocyte lineage is a novel target cell for c-fos overexpression during the embryonic development of the mouse[250]. This observation is not contradictory to previous transgenic mouse results since both the osteoblast and chondrocyte lineage originates from mesenchymal stromal cell precursors.

Perhaps the most striking evidence for the critical involvement of c-fos in bone development comes from c-fos null mutant mice. It was reported that mice lacking a functional c-Fos protein are growth retarded and develop an osteopetrotic phenotype with deficiencies in bone remodeling[251,252]. They also

displayed altered B-cell differentiation probably due to an extrinsic impaired bone marrow stromal cell microenvironment[253]. Furthermore, it was suggested that these mice could have an intrinsic deficiency in osteoclast differentiation (E.F. Wagner-personal communication). This suggests that c-fos may also play an essential role in osteoclast differentiation as well as an important role in osteoblast development. It was indeed surprising to find that c-fos was not essential in the fundamental processes of proliferation and differentiation of cells[254]. Prior to the fos-/- mouse, in vitro experiments suggested that c-fos may have been essential in the proliferation and differentiation of most, if not all, cell types[255,256]. It was also suggested that c-fos may have a role in cellular senescence[257]. Recent results however suggest that there are numerous overlapping pathways that control these fundamental developmental processes[258], including senescence[259]. The c-fos proto-oncogene may still have some role in these processes, but it is not essential in every cell type. These results unequivocally demonstrate that c-Fos is a critical transcription factor involved in the development of skeletal tissues. It has a biological role in both mesenchymal stromal cell precursors to osteoblasts and chondroytes as well as osteoclast precursors.

The c-Fos protein undoubtedly has an important role in osteoblastic growth and function as well as a significance in osteoblast pathology. It thus not only becomes important to understand the normal regulation of c-fos expression in osteogenic cells but it also becomes extremely important to understand what this

gene does in the regulation of osteoblast development. Furthermore, since the c-Fos protein interacts with the Jun family of proteins to regulate gene transcription, such studies should also account for jun family members in those cells. More specifically, the AP-1 transcription factor complex is fundamental in the regulation of bone specific gene products. In committed osteoblasts the transient nature of the expression of c-fos leads one to believe that it has a role in initiating a coordinated cascade of late gene expression leading to a more differentiated and functional osteoblast. In vitro osteoblastic differentiation involves the sequential expression of a number of structural genes such as type I collagen, alkaline phosphatase, osteopontin, osteocalcin, bone sialoprotein, and osteonectin[7]. Certain osteoblast related genes such as collagenase, type I collagen, osteopontin, alkaline phosphatase, and osteocalcin have putative AP-1 sites in their promoters. The regulation of these genes at the molecular level is complex, but AP-1 is thought to somehow be intricately involved in this process. In fact, in vitro experiments with cultured cells from transgenic lesions having constitutive overexpression of c-fos in osteoblast-like cells show perturbed cell differentiation and enhanced osteoblastic proliferation[248]. These cells express high levels of type I collagen, alkaline phosphatase, and osteopontin. They have very low osteocalcin expression partially consistent with a phenomena of phenotype suppression[260,261]. Furthermore, these cells do not have the normal ability to respond to calcitriol with increased levels of alkaline phosphatase and osteocalcin. An attempt to investigate this phenomena using transfected MC3T3-E1 cells revealed, in contrast

to cultured cells from transgenic lesions, an inhibition in type I collagen synthesis[262].

In addition to deregulated osteoblast lineage development, another consequence of overexpressed c-fos in the osteoblast seems to be the regulation of osteoclast maturation and activity in remodeling. This additional role may also be supported by the c-fos knockout mice which have little remodeling activity and by the c-fos transgenic mice which have an enormous amount of remodeling activity. The c-fos proto-oncogene may have a partial involvment in the initial activation step of the ARF series. It was observed that osteoclast cultures treated with the supernatant from MC3T3-E1 cells overexpressing c-fos caused a large increase in osteoclast maturation and bone resorption via the release of humeral factors[50]. In fact, it was shown that tumor necrosis factor α may act on MC3T3-E1 cells, via c-fos and c-jun, to secrete a soluble factor called JE which chemoattracts osteoclast lineage cells[165]. Interestingly, calcium homeostasis is extremely tightly regulated. The body needs a fine tuned immediate supply of calcium when in demand. The restoration of baseline plasma calcium is very rapid; it begins within minutes to hours[1]. Signaling via c-fos, an immediate early response gene, can be quickly transduced into an activation of resorption which would increase calcium to meet the body's demand. Such a mechanism is supported by the observation that the subcutaneous administration of PTH induces a transient and sequential in vivo expression of c-fos in cells of the osteoblast and chondrocyte lineage

followed by a transient expression of c-fos in the osteoclast and stromal cell lineage even though these cells do not have PTH/PTHrP receptors[159]. Therefore, PTH was shown to act indirectly on osteoclasts and stromal cells via soluble factors or cell to cell contacts mediated by the osteoblast.

D. Fibrous Dysplasia

Fibrous dysplasia is a sporadic developmental condition. It is characterized by monostotic or polyostotic preneoplastic expanding fibrous lesions of bone-forming mesenchyme. Bone formation is arrested at a disorganized immature woven stage. An enormous amount of osteoid is formed. There is, however, an extremely high bone turnover in these lesions which results in the inability of lamellar bone to develop. Instead, mesenchymal cells actively proliferate and secrete fibrous tissue which eventually crowds the marrow cavity. In fact, these proliferating cells can accumulate additional mutations that may transform the lesions into a neoplasm[263]. These lesions (figure 2) histologically closely resemble the ones described in the long bones of transgenic mice overexpressing an exogenous c-fos sequence. In addition, just as these mice are predisposed to osteosarcoma, so are children affected with fibrous dysplasia[264].

McCune-Albright syndrome is a clinical disorder generally characterized by polyostotic fibrous dysplasia accompanied by café-au-lait skin lesions and multiple endocrinopathies that can result in sexual precocity and/or growth retardation. Recently, activating mutations in the stimulatory G-protein subunit of the receptor /adenylate cyclase-coupling G protein were found in McCune-Albright's Syndrome[265]. There is no description of a case of fibrous dysplasia, without any of the other symptoms, that does not have this mutation. It is therefore difficult

to categorize these diseases as distinct. The various possible symptoms are thought to be due to embryonic somatic mutations within different tissues leading the different clinical manifestations of this disease. The mutation was indeed found in variable abundance in several affected tissues, consistent with the mosaic model[266]. Early reports had suggested that fibrous dysplasia patients had elevated levels of sex steroid hormone receptors[267,268]. This was offered as an explanation for certain endocrinopathies in this disease. These observations however, do not offer a satisfactory explanation for the complete clinical spectrum of manifested symptoms in this disease.

The proposed etiology for McCune-Albright syndrome stems from an activating mutation in $G_s\alpha$ which results in constitutively high levels of cyclic AMP[269]. An arginine in position 201 is substituted for either a histidine or cysteine in the α-subunit of the G protein complex. This mutation results in impaired GTPase activity of the α-subunit and causes constitutive activation of adenylate cyclase resulting in enormous amounts of cAMP second messenger. This is in contrast to the normal situation where persistent stimulation by agonists results in a rapid diminished responsiveness to activation called desensitization[270]. The various clinical characteristics of this disease are thought to lead to autonomous signaling in different tissues[271]. Elevated levels of cAMP in endocrine organs, for example, would result in specific endocrinopathies. The elevated levels in skin and bone would also manifest themselves in the specific clinical symptoms of the

disease, whereas no clinical manifestations would result in other unresponsive tissues. Thus, the specific cellular environment is important for the different clinical manifestations of this disease[272]. Moreover, within an individual tissue, cAMP activates a nuclear pattern of gene expression that causes specific clinical symptoms in that tissue[273].

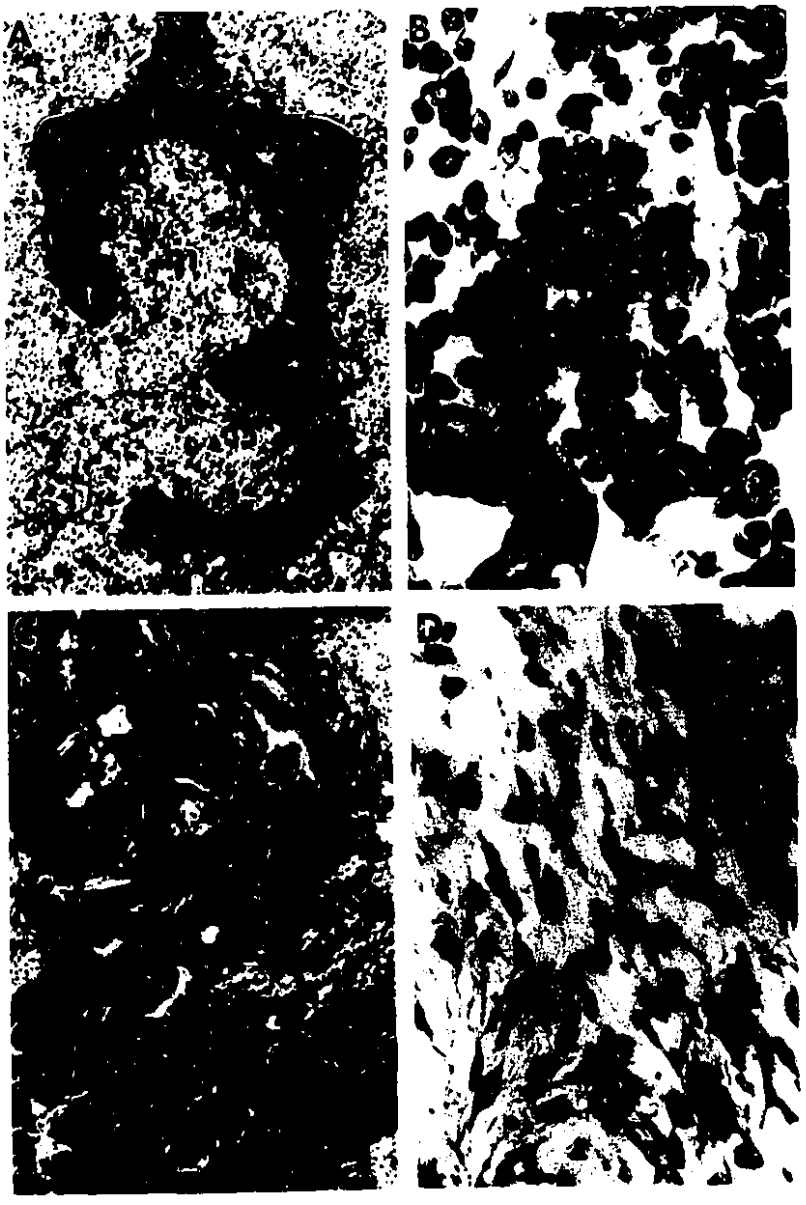

Figure 2: Histopathology of Fibrous Dysplasia. Low magnification (100X, panel A and C) brightfield microscopic histology of a biopsy specimen taken from a normal individual (panel A) and a fibrous dysplasia patient (panel C). Normal individuals have trabecular bone (t) that is well organized into lamellae (panel A). Bone from fibrous dysplasia patients is disorganized, woven and immature (i) bone undergoing excessive turnover (panel C). High magnification (1000X, panel B and C) of the bone marrow (m and f) between both populations is tremendously different. Normal individuals mostly have varied types of haematopoietic cells (panel B). Fibrous dysplasia patients have an enormous amount of fibrotic cells (panel D). Abbreviations used: Trabecular Bone (t), Immature Bone (i), Normal Marrow (m), Marrow Fibrosis (f).

IV. Rationale and Objectives of the Study

A. Control of Fos Expression in Bone Cells by Calcitriol

Since aberrant c-fos expression induces drastic phenotypes of bone development, it becomes extremely important to understand its normal regulation in a physiologically relevant target tissue. Furthermore, since the family of fos proteins interact with the jun family members to regulate gene transcription, such studies should also address the control of the expression of the jun family members in those cells. Calcitriol is a known modulator of osteoblast function. As a result, we studied the calcitriol (1α,25 dihydroxyvitamin D_3) regulated expression of fos/jun family members in bone cells[160]. The calcitriol-induced stimulation of c-fos expression was modulated at the level of transcription.

The most likely mechanism for this transcriptional regulation involves the interaction of the ligand-bound calcitriol receptor with a putative vitamin D response element (VDRE) to activate transcription. The c-fos promoter does not have a previously described VDRE; furthermore, there is no classical DR-3 sequence element in the promoter. Therefore, we wished to identify and characterize this novel VDRE in the c-fos promoter.

B. Fos Expression in the Etiology of Fibrous Dysplasia

Transgenic animals which overexpress c-fos in their tissues develop bone lesions identical to the bone lesions of patients with polyostotic fibrous dysplasia. We propose that since elevated levels of c-fos cause the transgenic mouse lesions then elevated levels of c-fos may also cause the human lesions. We used in situ hybridization on undecalcified bone sections to detect the presence of c-fos transcripts in the lesions of patients with polyostotic fibrous dysplasia and to determine the putative contribution of elevated levels of c-fos expression in the etiology of the disease.

V. Materials and Methods

A. Hormones and Reagents

Calcitriol and 24,25$(OH)_2$-D_3 were kind gifts of Dr. Milan Uskokovic from Hoffman LaRoche (Nutley, NJ). Actinomycin D was purchased from Gibco-BRL (Burlington, Ont). The primary monoclonal anti-c-Fos antibody (Ab-1) was obtained from Oncogene Science (Manhasset, NY) and the secondary goat anti-mouse antibody conjugated to fluorescein isothiocyanate (FITC) was from TAGO Immunologicals (Burlingame, CA). The anti-VDR was a rat IgG2b antibody specific for an epitope located C-terminally of the DNA binding domain purchased from Chemicon International Incorporated (Temecula, CA). Other chemicals were of the highest available grade and purchased from standard suppliers.

B. Plasmids and Probes

Plasmid pc-fos (mouse)-3[274] was obtained through the American Type Culture Collection (ATCC) (Rockville, MD). Probes derived from pc-fos (mouse)-3 include a 657 basepairs (bp) SphI-SacI fragment covering the fourth exon (used for Northern blot analysis), a 1.4 kilobasepairs (kb) HindIII-EcoRI fragment encompassing the first exon, part of the 5'-flanking region and a portion of the

first intron, as well as a 1.7 kb Eco RI-Nco I fragment covering from the first intron to the end of the fourth exon (these two last probes used in the *in vitro* nuclear run on assays). The fos B probe was a generous gift from Dr. Rodrigo Bravo (Squibb Institute for Medical Research, Princeton, NJ), and the fra-1 probe was provided by Dr. Tom Curran (Roche Institute for Molecular Biology, Nutley, NJ). All jun plasmids were kindly supplied by Dr. Daniel Nathans (Johns Hopkins University School of Medicine, Baltimore, MD). The c-jun probe was a 1.8 kb EcoRI fragment representing the complete cDNA, whereas the jun-B and jun-D probes were either the 5' untranslated regions described by Chiu et al.[275] (for Northern blots) or the complete cDNAs (for run-on assays). Other probes used include pMAT 1.1[276] to assess alpha-tubulin levels (obtained from Dr. Paul R. Dobner, University of Massachusetts Medical School, Worcester, MA) and the Pst I fragment of the rat glyceraldehyde-3-phosphate-dehydrogenase cDNA clone, pRGAPDH13[277]. All probes were labelled using [^{32}P]dCTP in an oligonucleotide-primed reaction according to the specifications of the manufacturer (Amersham Canada, Oakville, Ont).

The pc-fos (mouse)-3 plasmid was also used to generate the expression vectors featuring various portions of the c-fos promoter driving the luciferase reporter gene for transient transfection assays. The starting luciferase vector, pGL-2 Basic, was purchased from Promega Corporation (Madison, Wisconsin). The murine c-fos promoter region between Eag-1 (-276) and Acc-1 (+112) was subcloned

upstream of the luciferase reporter to generate the pEAfos plasmid. The pBAfos luciferase vector was derived in a similar manner using the c-fos promoter region between BssHII (-100) and AccI (+112). To generate the mutant pmEAfos plasmid, a point mutation was introduced by sequential PCR steps[278]. The pRSVCAT vector (ATCC #37152) was obtained from the American Type Culture Collection. The vector was used for expression of chloramphenicol acetyltransferase in eukaryotic cell lines under the control of Rous Sarcoma Virus long terminal repeats for normalization of transient transfections.

The hVDR and mRXRß plasmids were used for in vitro transcription and translation reactions. The hVDR[279] was a kind gift from Dr. J.W. Pike (Ligand Pharmaceuticals, San Diego, CA). It encompasses the complete human vitamin D receptor cDNA sequence subcloned in Promega's pGEM-7Zf(+) vector. The mRXRß[103] was a kind gift from Dr. V. Giguere (Hospital for Sick Children, Toronto, Canada).

In situ hybridization probes for c-fos were generated from the fourth exon of the human c-fos sequence. A riboprobe construct was made using Promega's pGEM-3Z plasmid with part of the human c-fos sequence as an insert. The c-fos sequence was from a unique NcoI site within the fourth exon to a unique ScaI site within the second intron of the gene. The riboprobe was generated by linearizing the plasmid at a unique BsgI site in the fourth exon of the c-fos gene and in vitro

transcribed using T7 polymerase. The actual riboprobe was 71 basepairs long with 12 labelled uridine residues. The non-specific probe was derived from Stratagene Corporation's pBluescript II SK- phagemid (Stratagene Corporation, LaJolla, CA). The probe was generated from the T7 promoter to the end of the SmaI linearized plasmid. The non-specific riboprobe was 73 basepairs long with 15 labelled uridine residues. All riboprobes were labelled with [^{35}S] UTP from Amersham. The DNA template was then digested away with DNase I and half the riboprobe reaction from unlabelled pGEM-3Z vector was added as a competitor. The remaining RNA probe was heat denatured and resuspended in hybridization mix (50% deionized formamide, 0.3M NaCl, 10mM Tris-HCl [pH 8.0], 1mM EDTA, 1x Denhardt's solution, 500 µg/ml yeast tRNA, 500 µg/ml poly(A) (Pharmacia, Baie d'Urfé, QC), 50 mM DTT, 10% polyethylene glycol (MW 8000; BDH, Pointe Claire, QC)). The riboprobe mix volume was then adjusted to $5x10^5$ cpm/µl and approximately 50 µl was added per section.

C. Cells and Tissue Culture Conditions

The MC3T3-E1 cells were obtained from Dr. Hiroaki Kodama (Ohu University School of Dentistry, Koriyama, Japan) and maintained as described[17] except that 10% fetal calf serum (FCS) was replaced by a mixture of 7.5% donor bovine serum and 2.5% FCS. For RNA isolation, nuclei extraction or nuclear protein extracts, the cells were grown to confluency, starved overnight in media

containing only 0.3% FCS, then treated with appropriate concentrations of calcitriol or 24, 25 $(OH)_2$-D_3 in ethanol or with a corresponding volume of ethanol alone (0 minute control time point).

The primary cultures of osteoblasts were obtained from newborn mouse calvaria as previously described[20], starved overnight in 2% FCS and treated with calcitriol in the same manner as the MC3T3-E1 cells.

ROS 17/2.8 cells were obtained from Dr. G. Rodan (Merck & Co., Westpoint, PA). Cells were maintained in Ham's F-12 modified to contain 28mM Hepes, 1.1mM Ca^{++} supplemented with 5% fetal bovine serum. The cells were passaged when confluent using 0.05% trypsin/0.53mM EDTA (Gibco/BRL, Burlington, Ont).

D. RNA Extraction and Analysis

Total RNA was isolated by the method of Chomczynski and Sacchi[280]. Northern blot hybridization was performed as described by St-Arnaud et al[281]. For mRNA half-life measurements, the cells were treated as described above except that 5 µg/ml actinomycin D was added immediately following incubation with calcitriol (10^{-9}M). The RNA was extracted at intervals following actinomycin D chase and analyzed by Northern hybridization. The blots were then exposed to pre-flashed

Kodak XAR-5 film (Picker Int. Canada, St-Laurent, Qc) and the autoradiograms were scanned on a Pharmacia-LKB UltroScan XL densitometer (Pharmacia Canada, Baie d'Urfée, Qc) using the GelScan 2000 software package.

E. Immunofluorescence

MC3T3-E1 cells were plated on gelatin-coated coverslips and starved in media containing no serum for 48 hours while still subconfluent. The cells were challenged with 10^{-9}M calcitriol or vehicle for various amounts of time, then fixed and stained as described by Adamkiewicz et al[282]. The antibody dilutions were 1:20 and 1:25 for the primary and secondary antibodies, respectively.

F. Nuclear Run-On Transcription Assays

Nuclei were isolated from calcitriol-treated MC3T3-E1 cells and nascent transcripts were elongated for 30 minute according to the procedure of Schibler et al.[283], as modified by Nepveu et al.[284,285]. In vitro synthesized RNAs were hybridized to immobilized double-stranded DNA probes as detailed in Nepveu and Marcu[286].

G. Transient-Transfection Assays

Ros 17/2.8 cells were plated at low density in 60-mm-diameter dishes. They were transfected the following morning according to a modification[287] of the calcium phosphate precipitation technique described by Graham and van der Eb[288]. Each dish was co-transfected with 4.5 μg of test plasmid and with 0.5 μg of RSVCAT plasmid as an internal control for the efficiency of transfection. Cells were glycerol shocked for two minutes after a four to six hour incubation with the DNA precipitate and allowed to recover overnight in complete media. The next morning, triplicate dishes were harvested to determine the average amount of luciferase activity prior to stimulation (basal activity). The remaining dishes were stimulated with either 1 μM calcitriol or 95% ethanol vehicle for 30 minutes prior to harvesting. Cells were harvested in lysis buffer (25mM Tris-phosphate [pH 7.8], 2mM dithiothreitol [DTT], 2mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100). Cell lysates were assayed for luciferase and chloramphenicol acetyltransferase (CAT) activity. Luciferase activity was measured directly in a luminometer using the luciferase assay system (Promega). CAT activity was measured by the phase-extraction assay of Neumann et al[289]. Luciferase values were then adjusted by variation in CAT activity.

The average luciferase results from the three test groups was analyzed. A first

average luciferase activity of three dishes prior to any stimulation was determined as the baseline activity. A second average luciferase activity of three calcitriol stimulated dishes was determined as the calcitriol stimulated activity. A final third average activity from three dishes that were treated with vehicle, instead of calcitriol, was determined as the activity in vehicle alone. Basal activity (baseline) was subtracted from vehicle- and hormone-treated luciferase activities. These values were a reflection of the transcriptional activity during the time the cells were exposed to calcitriol or vehicle. Results are reported as fold induction and represent the ratio between hormone-induced luciferase activity and the activity measured in vehicle treated dishes. Results are the mean fold induction $\pm$ S.E.M. of four independent experiments performed in triplicates.

H. Nuclear Extracts

MC3T3-E1 cells were treated with 10^{-8}M calcitriol or vehicle alone after overnight serum starvation in media containing 0.3% FCS. Nuclear extracts were then prepared essentially as described by Dignam et al.[290] with modifications[291]. In addition, to prevent any possible degradation of nuclear proteins, 10 µg/ml of both pepstatin A and leupeptins was added to all buffers. Once the extract was complete, the protein concentration was determined using a Bio-Rad Protein Assay (South Richmond, CA).

I. Gel Retardation and DNase I Footprinting Assays

For gel retardation assays[292], 5 μg of nuclear extract was incubated with 4 x 10^3 cpm of probe (approximately 2 ng) in gel retention mix (10mM Tris-HCl [pH 7.3], 50mM NaCl, 1mM DTT, 1mM EDTA, 2mM $MgCl_2$, 0.2% Nonidet P-40, 12% glycerol, 300 μg/ml BSA) in the presence of non-specific competitor DNA (poly(dIdC); 8 μg for promoter fragment probes or 2 μg for oligonucleotide probes). After incubation for 20 minutes at room temperature, bound probe was separated from free fragment or oligonucleotide on a 1x TBE (89mM Tris Borate [pH 8.3], 2mM Na_2EDTA) non-denaturing 4% or 8% 30:1 acrylamide:bisacrylamide gel, respectively. Antibodies were pre-incubated for 20 minutes prior to the addition of labelled probe. Receptors were translated in vitro using Promega corporation's TNT coupled reticulocyte lysate system following the instructions of the manufacturer. The binding reaction with in vitro translated receptors used the same gel retention mix except that 10^{-7}M calcitriol, 10 μg poly (dIdC) and 0.2 μg salmon sperm DNA were also added. The gel was migrated in a Bio-Rad Protean II apparatus at 150V for 3 hours with recirculating 1x TBE. It was then dried and autoradiographed on Kodak XAR-5 film.

DNase I footprinting assays were performed essentially as described by Jones et al.[293], except that the binding reaction was carried out in gel retention mix. Footprint reactions used 10 μg of nuclear extract. Binding reactions were for 15

minutes on ice, prior to the addition of the appropriate concentration of DNase I.

K. Patients' Clinical Descriptions

In total, 18 different biopsies were examined. There were 7 biopsies from lesions of polyostotic fibrous dysplasia patients. Another 2 fibrous dysplasia biopsies were from regions without any histological lesions. Nine other non-fibrous dysplasia biopsies were examined. Of these nine, two were from normal individuals, one from a hypocalcemic vitamin D resistant rickets (HVDRR) patient, one from a patient diagnosed with renal insufficiency, one from a person diagnosed with Paget's disease, one from a patient with Proteus Syndrome, and three were from patients diagnosed with Osteogenesis Imperfecta type IV.

L. Preparation of Undecalcified Bone Sections

Biopsies from the iliac crest or femur were prepared using standard bone histomorphometric techniques. Samples were fixed up to five days at room temperature with 12% formaldehyde solution in phosphate buffer prepared from bottled formalin. All fixed samples were dehydrated in increasing concentrations of ethanol for up to 15 days, then cleared in xylenes for a maximum of 4 days. They were infiltrated for up to 4 days with a 7:2.5 mixture of methylmetacrylate and Di-butyl phthalate containing 1% benzoyl peroxide. Infiltration was allowed

to continue at 4°C for another 4 days in the same resin but with 4.5% benzoyl peroxide. Polymerization was conducted in tightly sealed vials for at least 10 days at room temperature. Once polymerized, 6 μm serial sections were cut (Reichert-Jung, Polycut E microtome (Heidelberger, FRG)) and mounted on glass gelatin-coated slides. Once dry, they were incubated at 50°C for 20 hours under pressure to assure firm attachment. Sections were then stored at room temperature in a slide box until probed.

M. In Situ Hybridization

The in situ hybridization protocol was developed from modifications of standard techniques[294]. Sections were deplastified in four successive 10 minute baths of Ethylene Glycol Monoethyl Ether Acetate. Sections were then gradually rehydrated and prepared for pretreatment. Specimens were acid and heat denatured. Sections were incubated 20 minutes in 0.2N HCl. They were then incubated 15 minutes in 2x SSC (0.15M NaCl, 0.015M NaCitrate) at 70°C. No pronase digestion step was used. The prepared tissue was then mildly refixed prior to blocking of non-specific sulfur-binding sites. Specimens were post-fixed 5 minutes at room temperature in freshly prepared 4% paraformaldehyde. Non-specific sulfur binding sites were blocked using DTT, iodoacetamide, and N-ethylmaleimide. The tissue was also blocked with acetic anhydride and 2x SSC prior to dehydration. Hybridization was at 42°C for 30 minutes. Sections were

then washed at 45°C. A 1 hour prewash in 2x SSC with 5 solution changes was followed by a 0.2x SSC wash for 0.5 hours with one solution change. Sections were then dehydrated in ethanol and exposed to film overnight to estimate the exposure time for emulsion autoradiography with Kodak NTB-2 emulsion.

VI. Results

A. Control of Fos Expression in Bone Cells by Calcitriol

1. Differential Stimulation of fos and jun Family Members by Calcitriol in Osteoblasts

In order to assess the effect of calcitriol treatment on fos and jun expression in bone cells, cultures of the established osteogenic cell line MC3T3-E1 were grown to confluency, starved overnight in culture media containing low concentrations of fetal calf serum, and then challenged with relatively low doses of calcitriol for various periods of time. As shown in Figure 3, calcitriol transiently stimulated the steady-state mRNA levels of c-fos, c-jun and jun-B in MC3T3-E1 cells. The level of fos-B mRNA was also stimulated by calcitriol treatment in those cells (data not shown). Interestingly, calcitriol had no effect on the expression of jun-D (Figure 3) and fra-1 (data not shown). Similar expression patterns were observed in each of eight independent experiments.

Although c-fos, fos-B, c-jun and jun-B all showed a transient enhancement of their expression following calcitriol treatment, the actual kinetics of the stimulation varied somewhat for the individual genes. Both c-fos and fos-B showed maximum mRNA levels at around 20 minutes post-calcitriol addition (Figure 3

and data not shown). The c-jun transcripts reached peak levels more rapidly, whereas the jun-B response was maximal at slightly longer intervals (Figure 3). Densitometric analysis of the autoradiograms revealed an average 8 to 10-fold stimulation of both c-jun and jun-B transcripts levels by calcitriol (7.8 ± 0.3 [n=3] and 10.5 ± 1.5 [n=4] for c-jun and jun-B, respectively). The actual fold stimulation of c-fos expression by the steroid could not be estimated since the untreated levels of c-fos mRNA were too low to be quantified even in overexposed autoradiograms (data not shown).

We next determined if calcitriol could modulate fos/jun expression levels in primary cultures of osteoblasts isolated from newborn mouse calvaria. Figure 4 shows that two different doses of calcitriol transiently stimulated the levels of c-fos mRNA in primary osteoblasts. The magnitude of the stimulation induced by 10^{-9}M calcitriol appeared comparable in both the primary cultures and the established osteoblastic cell line (Figure 3 and 4A). However, treatment of the primary cultures of osteoblasts with 10^{-8}M calcitriol caused a much stronger stimulation of c-fos expression than the lower dose (Figure 4, panel B; see also below). Some degree of c-fos expression can be detected in the untreated primary cultures of bone cells (see Figure 4B, 0 minute lane) because the cells were starved in 2% serum instead of 0.3% in order to retain cell viability. Both c-jun and jun-B mRNA levels were also transiently stimulated by calcitriol in primary cultures of osteoblasts, whereas jun-D expression was not affected (data not

shown). Thus the differential stimulation of fos and jun family members by calcitriol in bone cells appears to be a true physiological response of that cell type and not an artefact or a peculiarity of the established osteogenic cell line.

The observed stimulation of fos and jun family members was also specific for the active metabolite of vitamin D, calcitriol. Figure 5 shows that 24, 25-dihydroxyvitamin D_3 (24, 25 $(OH)_2$-D_3), another dihydroxylated metabolite of the vitamin with no demonstrated effect on target tissues, did not stimulate c-fos expression in MC3T3-E1 cells. The expression of c-jun and jun-B also proved unaffected by high doses of 24, 25 $(OH)_2$-D_3 (data not shown).

Figure 6 shows that the stimulation of c-jun expression induced by calcitriol varied with the dose used, with half-maximal stimulation observed at around $5x10^{-9}$M. The calcitriol effect on c-fos and jun-B expression in osteoblasts was also dose-dependent (data not shown).

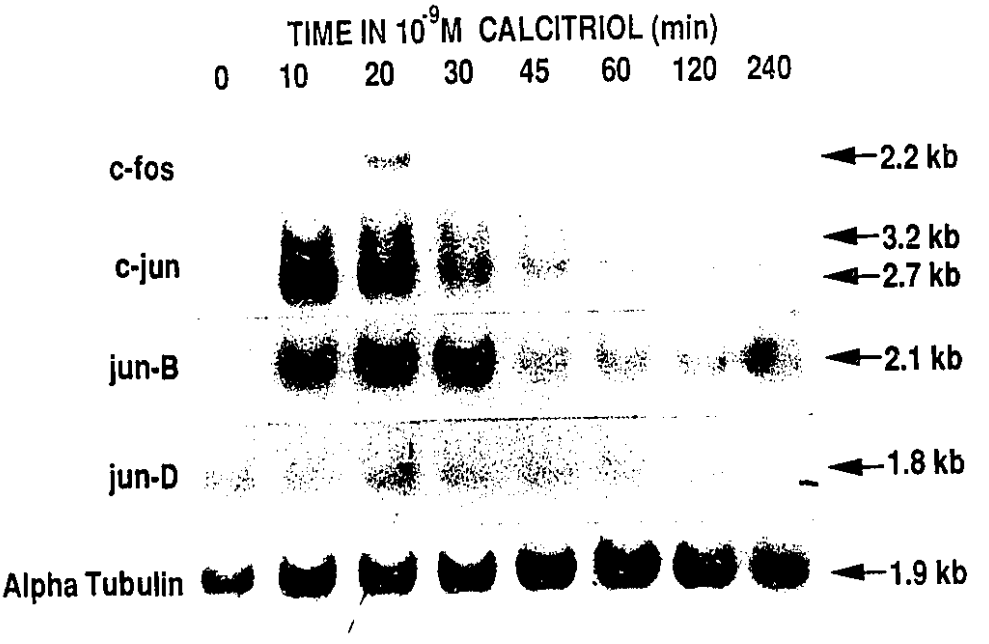

Figure 3: Transient stimulation of c-fos and jun family members by calcitriol in osteoblastic cells. MC3T3-E1 cells were grown to confluency, then starved for 24 hours in media containing only 0.3% serum prior to stimulation by 10^{-9}M calcitriol. Total RNA was harvested at the indicated times and analyzed by Northern blot assay using specific probes as described in Materials and Methods.

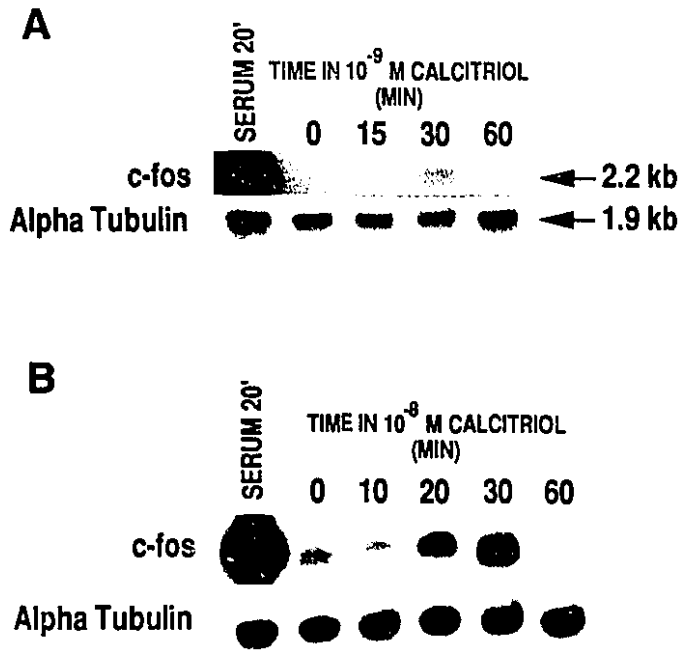

Figure 4: Calcitriol transiently induces c-fos expression in primary cultures of mouse osteoblasts. Primary cultures of mouse osteoblasts mechanically isolated from newborn mouse calvaria were starved for 24 hours in 2% serum prior to stimulation by calcitriol. Total RNA was extracted at the specified times and assayed for c-fos and tubulin expression by Northern blot analysis. A positive control of stimulation by serum for 20 minutes is shown in the left-hand lane. A. Cells were treated with 10^{-9}M calcitriol. B. Cells were treated with 10^{-8}M calcitriol.

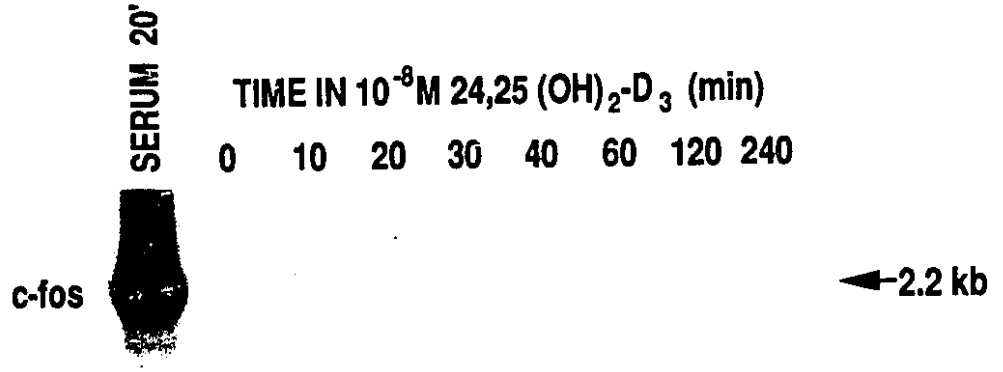

Figure 5: c-fos expression is not affected by treatment of osteoblastic cells with 24, 25 $(OH)_2$-D_3. Confluent cultures of osteoblastic MC3T3-E1 cells were starved in low serum for 24 hours prior to treatment with 10^{-8}M 24, 25 $(OH)_2$-D_3. Total RNA was harvested at intervals and c-fos mRNA levels were measured by Northern assay as described in Methods. Stimulation by serum treatment for 20 minutes is shown as a control on the left.

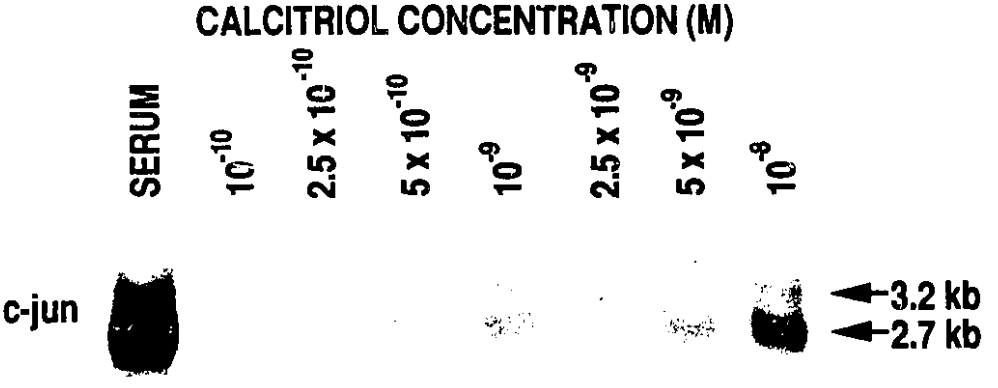

Figure 6: Calcitriol stimulates c-jun expression in a dose-dependent manner. Starved, confluent cultures of MC3T3-E1 cells were incubated for 20 minutes with increasing doses of calcitriol. Total RNA was extracted and assayed for c-jun expression by Northern blot analysis. Control stimulation by serum for the same length of time is shown in the left-most lane for comparison.

2. c-Fos Protein Levels

We used indirect immunofluorescence techniques with Fos-specific antibodies to determine if calcitriol treatment of osteoblasts stimulated c-fos protein (Fos) levels concomitantly with the levels of c-fos mRNA. As shown in Figure 7, calcitriol induced enhanced Fos levels in MC3T3-E1 cells. The effect was transient and showed a slight lag behind the stimulation of c-fos mRNA (compare Figure 3 and Figure 7), but overall Fos expression in calcitriol-treated bone cells mimicked c-fos gene expression.

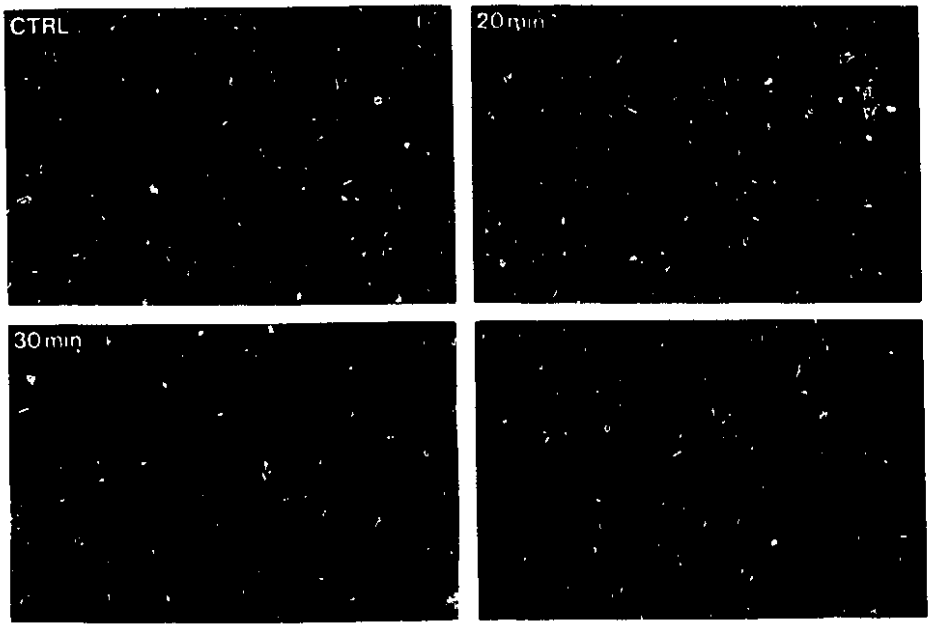

Figure 7: Calcitriol stimulates c-Fos protein levels in osteoblastic cells. Starved, subconfluent cultures of MC3T3-E1 cells were stimulated with 10^{-9}M calcitriol for the indicated times and then fixed. Fos protein was then detected by indirect immunofluorescence using a Fos-specific monoclonal antibody followed by a goat secondary antibody directed against mouse immunoglobulins and coupled to fluorescein isothiocyanate (FITC).

3. fos/jun Transcriptional Activity in Calcitriol-Treated Osteoblasts

We next addressed the molecular mechanisms implicated in the stimulation of the expression of c-fos, c-jun and jun-B by calcitriol in osteoblasts. The stimulation was observed even in the presence of protein synthesis inhibitors (data not shown), indicating that the effect was a primary response that did not require de novo protein synthesis. Calcitriol has been shown to act through a receptor that is a member of the steroid receptor superfamily. Since the members of this family of receptors act as transcription factors to regulate expression of their target genes, we used in vitro nuclear run-on transcription assays to determine if calcitriol regulates the expression of c-fos, c-jun and jun-B at the level of gene transcription.

Nuclei were isolated from MC3T3-E1 cells at various times following calcitriol exposure and the rates of transcription of the c-fos, c-jun and jun-B genes were measured. Figure 8 shows that a low level of c-fos transcriptional activity was detected in unstimulated cells. Interestingly, no transcription could be measured across the second, third and fourth exons of the c-fos gene in untreated cells (Figure 8), indicating a block to elongation of the nascent c-fos transcripts. In each of three separate experiments, calcitriol treatment of MC3T3-E1 cells induced a rapid stimulation of the initiation of c-fos transcription, as detected by a probe covering the first exon of the gene (Figure 8). Moreover, exposure to calcitriol

partially relieved the block to elongation (see Figure 8, 10 minute time point, c-fos exons 2-4 probe). This stimulation was transient and transcription rates returned to pre-treatment levels by 30 minutes following addition of the steroid (Figure 8). Although the stimulation of transcriptional activity induced by calcitriol was not comparable to the transcriptional enhancement achieved by stimulation with serum (Figure 8, left-hand lane), these data suggest that the augmentation of c-fos expression induced by calcitriol in osteoblasts is mediated, at least in part, at the transcriptional level.

Both c-jun and jun-B showed higher transcription rates than c-fos in untreated cells (Figure 8). However, calcitriol treatment had no effect on the rates of transcription for c-jun and jun-B in MC3T3-E1 cells (Figure 8; the slight enhancement of jun-B transcription between 20-30 minutes of calcitriol exposure was not reproducible in subsequent experiments). As a control, serum stimulation of the starved osteoblastic cells resulted in the strong stimulation of c-jun and jun-B transcription. Thus, the stimulation of c-jun and jun-B expression induced by calcitriol in bone cells appears modulated at the post-transcriptional level.

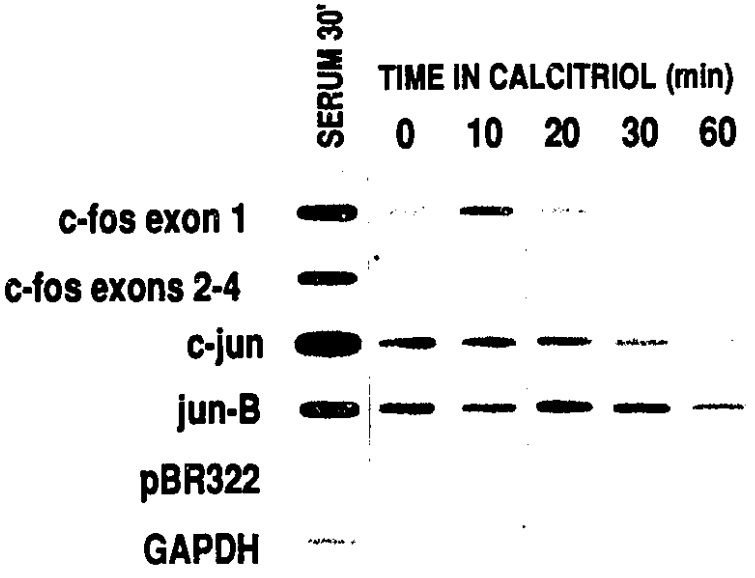

Figure 8: Transcriptional activity of c-fos, c-jun and jun-B in osteoblastic cells treated with calcitriol. Confluent, starved cultures of MC3T3-E1 cells were treated with either 10% serum or $5x10^{-9}$M calcitriol. Nuclei were harvested at the indicated times and transcriptional activity was assessed using *in vitro* nuclear run on transcription assays. Probes used are described in the Materials and Methods section. The pBR322 probe was the Eco RI-digested pBR322 plasmid DNA.

4. Stability of c-jun and jun-B Transcripts in Calcitriol-Treated Osteoblasts

To determine if calcitriol induced the expression of c-jun and jun-B in osteoblasts by modulating the stability of their mRNAs, we measured the half-lives of the c-jun and jun-B transcripts in MC3T3-E1 cells treated with or without calcitriol in the presence of actinomycin D, an inhibitor of transcriptional activity. The levels of c-jun messages decayed rapidly under those conditions whereas alpha-tubulin transcripts, used as an internal control for small variations in sample handling, displayed more stable kinetics (Figure 9, panel A). The rate of decay of the c-jun mRNAs was plotted (Figure 9B) and the half-life of the transcripts calculated for untreated cells and cells treated with calcitriol for 10, 20 or 30 minutes. Table I shows that both the 2.7 kb and the 3.2 kb c-jun transcripts exhibited short half-lives in osteoblastic cells (15.3 ± 2.0 min and 14.2 ± 2.1 min, respectively [n=6]). Moreover, calcitriol treatment had no significant effect on the stability of c-jun mRNAs. Similar results were obtained for jun-B mRNA (table I). Taken together, these data indicate that calcitriol stimulates c-jun and jun-B expression in bone cells by a post-transcriptional mechanism that does not involve mRNA stabilization.

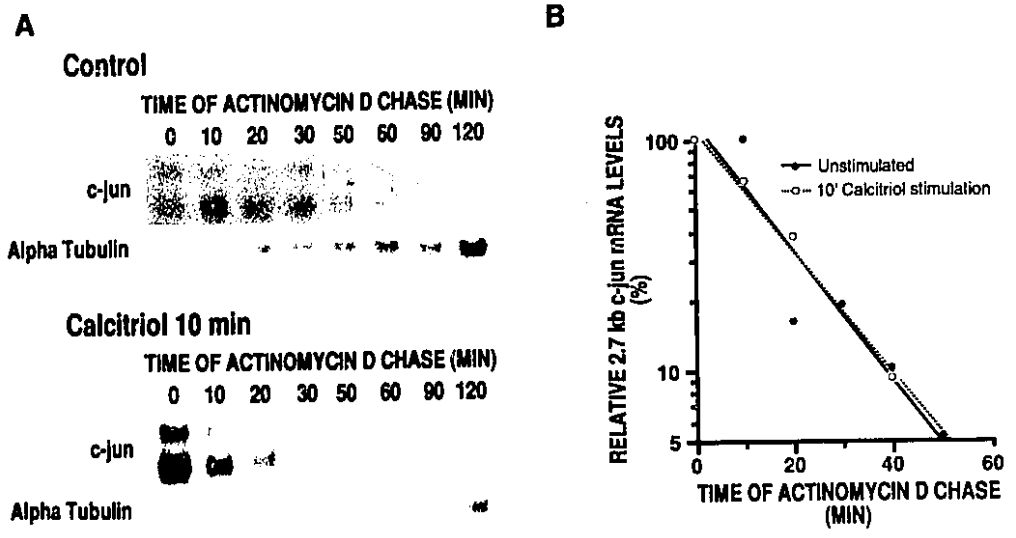

Figure 9: Stability of c-jun transcripts in calcitriol-treated osteoblastic cells. Total RNA was extracted from starved, confluent cultures of MC3T3-E1 cells treated with 10^{-9} M calcitriol or vehicle for 10 minutes, then chased for the indicated times with 5 µg/ml of actinomycin D. Northern blot analysis was then performed as described in Methods. A. Autoradiograms showing the decay of c-jun transcripts in untreated cells and cells treated with calcitriol for 10 minutes. The blots were stripped and re-probed with an alpha-tubulin probe to account for small variations in sample handling. B. The relative amount of c-jun mRNAs was assessed by densitometric scanning and plotted as a function of time of exposure to actinomycin D in order to calculate the half-lives of the transcripts.

Table I.
Half-Lives of c-jun and jun-B Transcripts in Calcitriol-Treated MC3T3-E1 Cells

Time in calcitriol (min)	Half-life (min) c-jun (2.7 kb)	c-jun (3.2 kb)	jun-B
0	15.3 ± 2.0 (6)	14.2 ± 2.1 (6)	28.0 ± 6.5 (3)
10	18.3 ± 3.6 (5)	19.6 ± 2.5 (5)	20.5 ± 6.5 (3)
20	13.8 ± 1.6 (5)	15.4 ± 1.9 (5)	14.8 ± 4.7 (3)
30	15.6 ± 2.7 (5)	17.8 ± 3.5 (5)	14.8 ± 4.8 (3)

Values are means ± S.E.M. Numbers in parenthesis represent numbers of individual determinations (n).

5. A Functional vitamin D response element (VDRE) in the c-fos Promoter

Since the vitamin D stimulation of the c-fos gene was shown to be controlled, at least in part, at the transcriptional level, we set out to characterize a vitamin D response element (VDRE) in the 5' promoter region of the c-fos gene. Based on computer analysis of the promoter sequence, two deletion constructs were generated using luciferase as a reporter enzyme (Figure 10). One of these vectors contained sequences up to position -276 of the promoter and encompassed a putative VDRE site (based on sequence similarity with previously characterized VDREs). In the other construct, stretching to position -100 of the promoter, the putative VDRE sequence was deleted. These plasmids were transfected into ROS 17/2.8 cells[18]. The cells were allowed to recover until the next morning when they were challenged with 1 μM calcitriol for 30 minutes. A short stimulation time was chosen because nuclear run-on assays (Figure 8) clearly demonstrated that maximal transcriptional stimulation peaked after 10 minutes of calcitriol stimulation. We thus compared the change in luciferase activity in a 30 minute period by stimulation with calcitriol over vehicle. This is a measure of the fold activation of transcription by calcitriol. This experiment was performed in triplicate for each sample concurrently and variation was compensated for by RSVCAT cotransfection. It was repeated four independent times.

The results shown in Figure 10 revealed a functional vitamin D responsive region

between -276 and -100 basepairs upstream of the murine c-fos transcription start site. Calcitriol activated transcription of the luciferase gene was nearly four times greater than basal levels of transcription with vehicle alone. When we removed the vitamin D responsive region there was no calcitriol stimulation of transcription above basal levels.

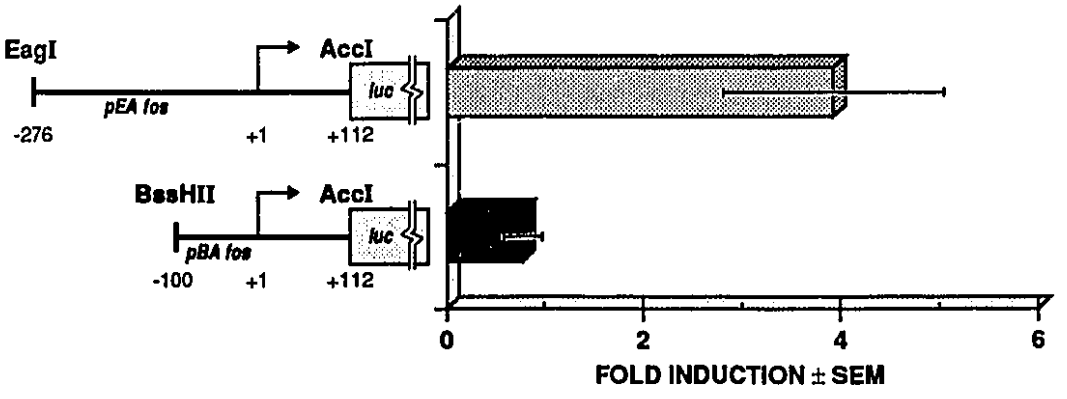

Figure 10: Activity of deleted c-fos promoter constructs in calcitriol-treated osteoblastic cells. Ros 17/2.8 cells were transfected with 4.5 μg of a promoter deletion test construct and 0.5 μg of RSVCAT to monitor for fluctuations in the efficiency of transformation. After an overnight recovery, the cells were challenged with 1μM calcitriol for 30 minutes. The fold induction of transcription by calcitriol is shown. Luciferase activity was measured and standardized for the activity of the internal control. Values shown are the mean fold induction ± S.E.M. of four independent experiments done in triplicate.

6. Nuclear Factors Binding the c-fos Promoter

The calcitriol-responsive region of the c-fos promoter identified in transient-transfection assays was used as a probe in gel retardation assays in order to identify the nuclear factors binding that promoter region. Figure 11 shows that this fragment contains a binding site for a factor that is not present in starved cells, but is rapidly induced upon calcitriol treatment of the cells. Results from one series of nuclear extracts are shown. Six independent series of nuclear extracts showed similar results. The binding of the induced factor followed kinetics that were similar to those of the transcriptional induction of c-fos expression by calcitriol, suggesting that the factor could be the ligand-bound vitamin D receptor binding its putative cognate response element.

Specificity of binding was assessed by attempting to compete the binding of the factor with a substantial molar excess (up to 1000-fold) of oligonucleotides encoding binding sites for other transcription factors. None of the competitors tested were able to compete for binding of the induced complex (Figure 12). Taken together, these results demonstrate that the promoter fragment contains a specific binding site for a nuclear factor that was induced upon calcitriol treatment of MC3T3-E1 cells.

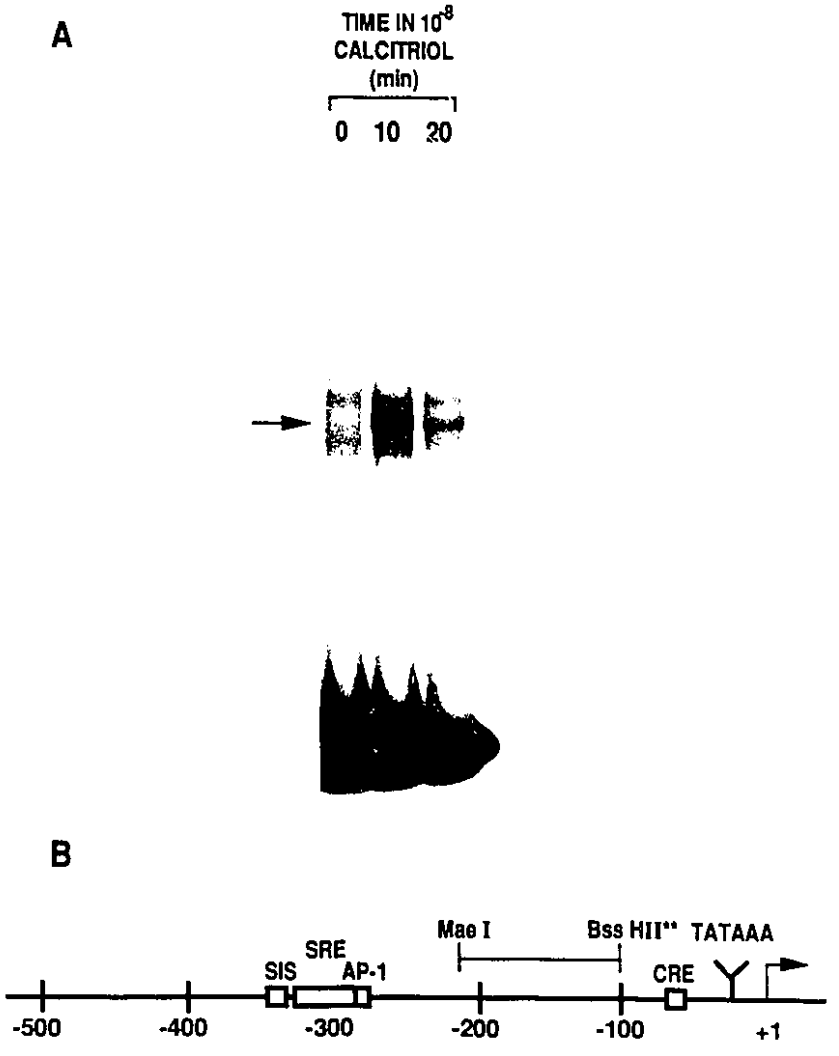

Figure 11: Calcitriol-induced nuclear factors bind to a region of the c-fos promoter. Nuclear extracts were prepared from MC3T3-E1 cells treated with 10^{-8}M calcitriol for 0, 10 or 20 minutes following overnight serum starvation. A. Gel retardation assay. The arrow points to the complex that is induced by calcitriol. B. Partial map of the c-fos promoter region. The MaeI to BssHII probe used in the gel retardation assay is depicted above the map. Abbreviations used: PDGF-response element (SIS), serum response element (SRE), fos/jun heterodimer binding site (AP-1), cyclic-AMP response element (CRE).

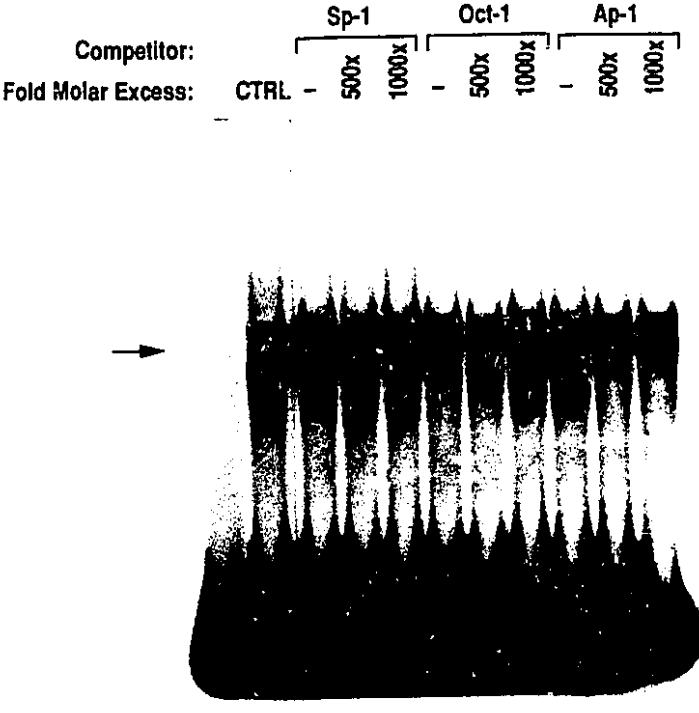

Figure 12: Binding specificity of the calcitriol-induced binding factor. Prior to migration of the gel retardation assay, extracts were incubated with increasing molar amounts of unlabelled competitor oligonucleotide. Binding sites for the Sp-1, Oct-1, and AP-1 transcription factors were used as competitors.

7. Binding Sequence Identification

The precise binding site of the factor was delineated using DNase I footprinting. As shown in Figure 13, crude extracts from MC3T3-E1 cells protected a region comprising positions -178 to -144 of the c-fos promoter. The protected region was observed both in uninduced and calcitriol treated cells (Figure 13). This is most likely due to the different binding conditions and sensitivity of the footprinting reaction compared to the gel retardation assay, where clear induction of the binding factor was observed (see Figure 11). Such differences have been observed before in different systems[295]. The protected sequence was as follows:

5' AGGTGAAAGATGTATGCCAAGACGGGGGTTGAAAG 3'

This region shall hereafter be referred to as the c-fos vitamin D response element (VDRE).

The binding site of the induced factor was confirmed by the fact that an oligonucleotide corresponding to this sequence was able to directly bind (Figure 14, panel A) and compete for binding (Figure 14, panel B) in a gel retardation assay. Furthermore, we have tried similar binding and competition experiments with different regions of the promoter (table II). None of the synthetic oligonucleotides covering partial regions of the protected sequence were able to bind the factor or compete for its binding to the promoter fragment (Table II and data not shown). These experiments suggest that the entire footprinted region is necessary for binding.

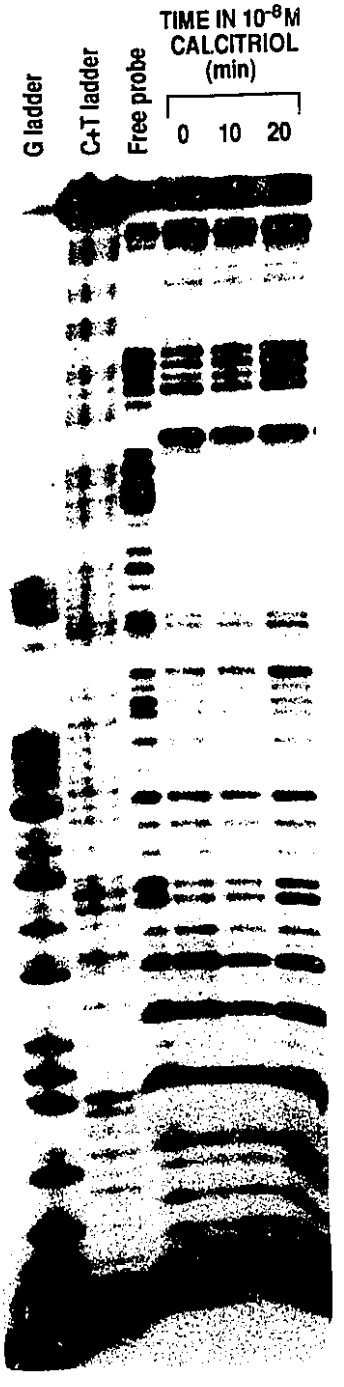

Figure 13: DNaseI footprint of the murine c-fos promoter. Nuclear extracts from serum-starved and calcitriol-stimulated MC3T3-E1 cells were analyzed by DNAse I footprinting using a DNA probe spanning residues -216 to -100 of the c-fos promoter. The probe was labelled at the BssHII site on the coding strand by Klenow fill-in.

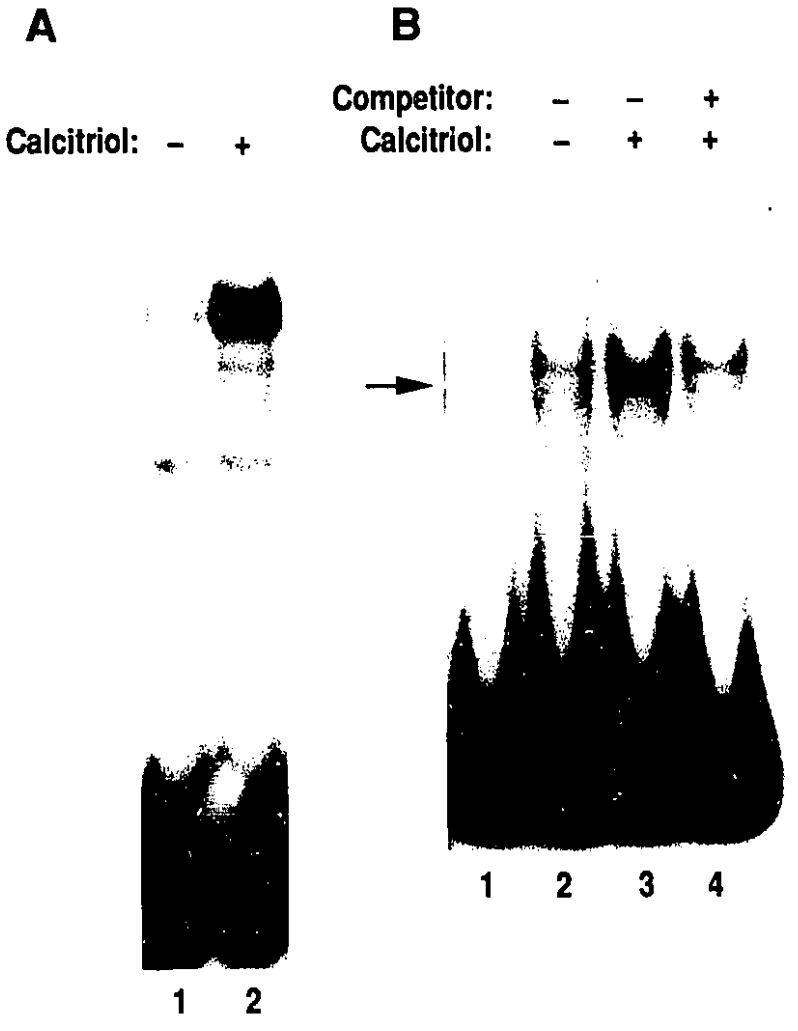

Figure 14: The footprinted c-fos VDRE sequence directly binds and competes the induced complex. A. An oligonucleotide sequence corresponding to the footprinted region of the c-fos promoter was used as a probe in a gel retardation assay. B. The calcitriol-treated MC3T3-E1 nuclear extract was pre-incubated with (+) or without (-) an excess (50-fold molar excess) of the unlabelled oligonucleotide prior to migrating the gel retardation assay. The probe used in panel B is described in figure 11, panel B.

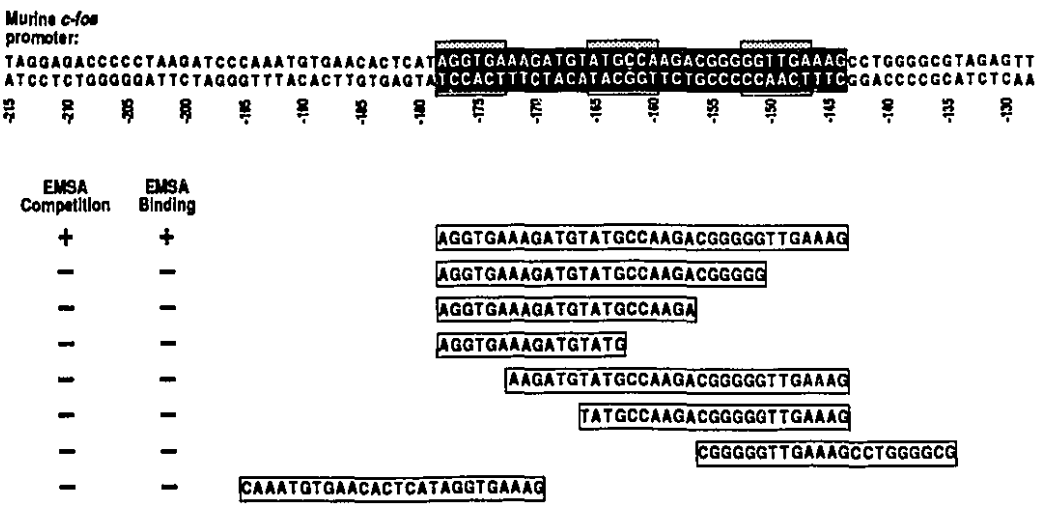

Table II: Deletion mutational analysis of binding to the c-fos VDRE. The partial sequence of the c-fos promoter region (residues -215 to -128) is indicated with the c-fos VDRE (protected area of the DNase I footprint) highlighted. The boxed residues represent putative hexameric half-sites (see text). Various synthetic oligonucleotides tested for binding and competition in electrophoretic mobility shift assays (EMSA) are outlined below.

8. VDR Binding

We then attempted to characterize the protein(s) binding the protected region. The most likely candidate was the ligand-bound vitamin D receptor. We have accumulated direct and circumstantial evidence suggesting that the VDR does indeed bind the c-fos promoter. First, an oligonucleotide encoding the mouse osteopontin (mOP) VDRE efficiently competed for the binding of the factor to the c-fos promoter (Figure 15). The mOP VDRE has been previously shown to bind the VDR[296].

Secondly, in vitro pre-incubation of nuclear extracts from calcitriol-starved cells with calcitriol induced the binding of the factor to the c-fos probe (Figure 16).

Finally, the VDR complex was directly recognized using antibodies directed against the vitamin D receptor. Specific antibodies can either disrupt or further retard protein DNA complexes in nondenaturing gels such as those used in gel retardation assays[295]. The antibody that was used recognizes an epitope on the DNA binding domain of the receptor and prevents its binding[297]. When nuclear extracts were pre-incubated with this antibody, it could specifically inhibit binding to the fragment (Figure 17, panel A). This result directly proves that the VDR is part of the protein complex that recognizes the fragment. Moreover, the antibody also prevented the binding of the complexes to synthetic oligonucleotides encoding

the osteopontin VDRE (Figure 17, panel B) or the c-fos VDRE (figure 17 panel B). These data confirm that the VDR is part of the complex that recognizes both the osteopontin VDRE and the c-fos VDRE.

It has been shown that the VDR heterodimerizes with members of the RXR family of steroid receptors to bind its cognate binding site with high affinity. We tested whether RXR family members could be partners involved in binding of the VDR to the c-fos VDRE. The in vitro translated RXRß and VDR were tested with the murine osteopontin and c-fos VDRE probes in gel retardation assays. We could effectively detect binding of the VDR homodimer and VDR-RXR heterodimer to the OP VDRE (Figure 18, lanes 1 to 3). Under the same conditions, we neither detected homomeric nor heterodimeric binding to the c-fos VDRE (Figure 18). The two retarded complexes observed with the c-fos VDRE probe represent non-specific binding to the probe, as they were also detected in the reticulocyte lysate control lane (Figure 18, lane 4). The factor recognizing the c-fos VDRE probe in nuclear extracts from MC3T3-E1 cells did not co-migrate to the same position as either of the in vitro translated complexes bound to the mOP VDRE (data not shown), further supporting the observation that neither VDR homodimers nor VDR-RXR heterodimers bound the c-fos VDRE.

It has recently been shown that the VDR can heterodimerize with RARs and T_3Rs to bind certain VDREs with high affinity[96,106]. We used nuclear extracts from P19

embryonal carcinoma cells to test whether VDR-RAR heterodimers bound the c-fos VDRE. P19 cells express all RAR isoforms[298]. Nuclear extracts from undifferentiated P19 embryonal carcinoma cells and from RA-treated P19 cells did not bind the c-fos VDRE (data not shown). In addition, co-incubation of the in vitro translated VDR with P19 nuclear extracts did not reconstitute the binding activity to the c-fos VDRE (not shown).

We also tested for the putative binding of VDR-T_3R heterodimers to the c-fos VDRE. We used three different polyclonal antisera directed against isoforms of T_3R in gel retardation assays with nuclear extracts from vitamin D-treated MC3T3-E1 cells. These antibodies have been shown to further retard the migration of the bound T_3Rs to their cognate response elements in gel retardation assays[299]. None of the antisera affected the binding of the vitamin D-induced complex from calcitriol-treated MC3T3-E1 cells to the c-fos VDRE probe (data not shown).

Taken together, we interpret these results to mean that the VDR heterodimerizes with a novel partner to bind the c-fos VDRE. This partner appears to have a restricted pattern of expression, since we could not detect binding using nuclear extracts from non-bone cells (data not shown). The identity of this binding partner remains elusive to date.

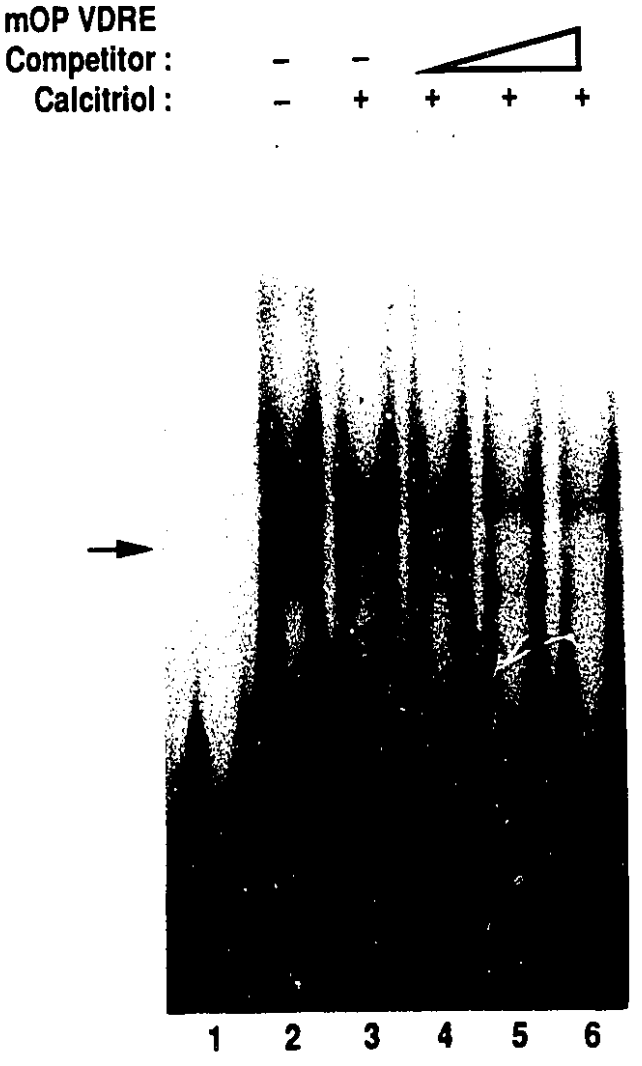

Figure 15: The calcitriol induced complex is specifically competed by a synthetic oligonucleotide encoding the murine osteopontin VDRE. Nuclear extracts from calcitriol (10^{-8}M) stimulated MC3T3-E1 cells were incubated with the MaeI to BssHII probe from the c-fos promoter. Increasing amounts of the murine osteopontin VDRE were simultaneously added as competitor to binding reactions for 20 minutes at room temperature. The bound complexes were analyzed by gel retardation assays. The arrow denotes the induced band.

Figure 16: Calcitriol induces the c-fos promoter-binding complex *in vitro*. Nuclear extracts from starved, untreated MC3T3-E1 cells were pre-incubated with 10^{-8}M calcitriol for 10 minutes prior to the gel retardation assay. Lane 1: extract from cells treated with 10^{-8}M calcitriol for 10 minutes. Lane 2: extract from untreated cells. Lane 3: extract from untreated cells pre-incubated with calcitriol *in vitro*. The arrow points to the calcitriol-induced binding complex.

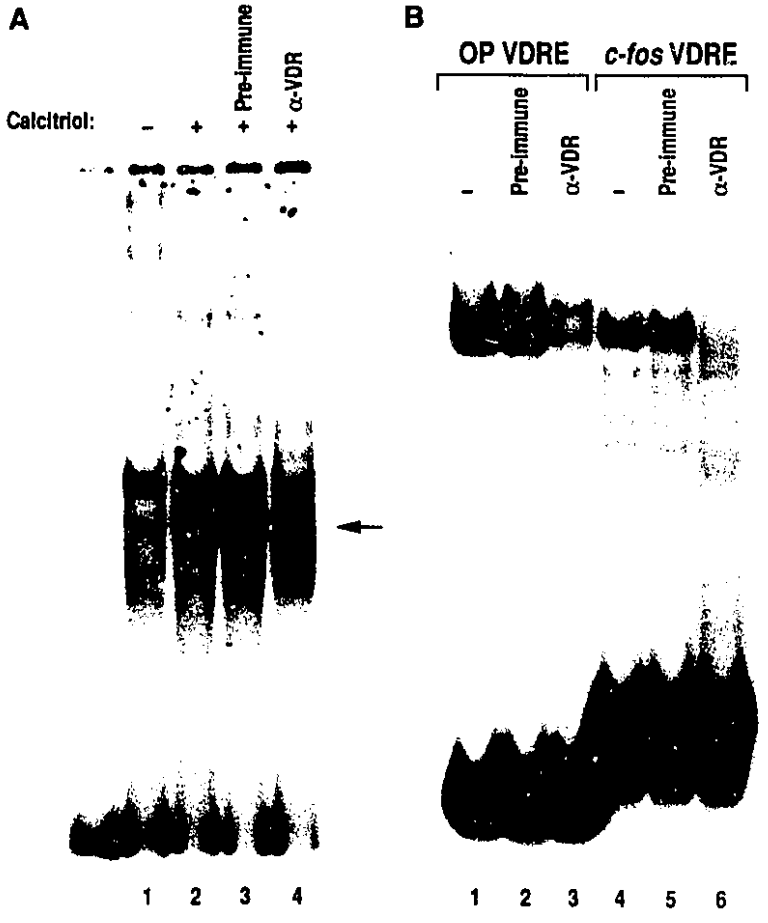

Figure 17: The VDR binds the c-fos VDRE. Pre-immune sera or specific anti-VDR antibodies were added to the binding reactions prior to the gel retardation assay. Panel A: Nuclear extracts from untreated or calcitriol-treated MC3T3-E1 cells were incubated with the c-fos promoter region from Mae I to BssH II. Bound complexes were separated from the free probe on a 4% gel. Panel B: An oligonucleotide corresponding to the mouse osteopontin VDRE (OP VDRE) was used as probe in lanes 1 - 3, whereas the c-fos VDRE oligonucleotide was the probe in lanes 4 - 6. Nuclear extracts from calcitriol-treated MC3T3-E1 cells were bound to the probes. Separation was on an 8% gel.

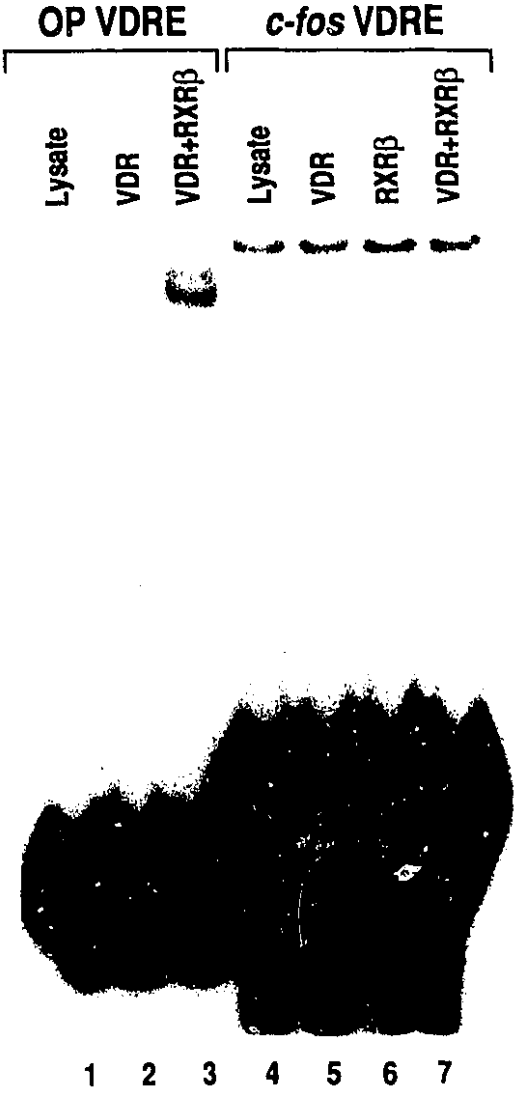

Figure 18: *In vitro* translated VDR and RXRß do not bind the c-fos VDRE. Gel retardation assays with *in vitro* translated nuclear receptors and the osteopontin (OP) VDRE (lanes 1 to 3) or the c-fos VDRE (lanes 4 to 7) used as probes. Reticulocyte lysate alone was used as a control (lanes 1 and 4).

9. VDRE Structure-Function Relationships

To assess the functional importance of the c-fos VDRE in the calcitriol-stimulated expression of c-fos, we mutated the VDRE binding site within the context of the partial c-fos promoter using site-directed mutagenesis. First, we mutated various residues of the VDRE (Figure 19, panel A) and tested the ability of the corresponding synthetic oligonucleotides to compete (Figure 19, panel B) or directly bind (Figure 19, panel C) the calcitriol-induced complex.

Previously characterized VDREs consist of pairs of hexameric consensus binding sites separated by a specific number of residues. Analysis of the wild type c-fos VDRE (panel A, wt) suggests three possible consensus binding sites separated by seven basepairs: an AGGTGA motif between positions -178 and -173, followed by a degenerate ATGCCA motif seven basepairs downstream, and another GGTTGA motif located at positions -152 to -147. We mutated various residues within and between the hexameric "half-sites". Mutations in the three basepairs directly upstream of the last two motifs (oligo m1) did not prevent this oligonucleotide to compete for binding. Mutating residues in the flanking regions of the hexameric imperfect repeats (oligo m2, panel A and B) marginally impaired the ability of the sequence to compete for binding. Surprisingly, mutating the first and last motif did not affect the binding competition (oligo m3, panel A and B). This suggests that the degenerate middle motif is crucial for binding. Indeed, this

motif must itself be mutated to effectively prevent competition (oligo m4, panel B) and binding (oligo m4, panel C) to the complex. In fact, it was sufficient to mutate the single middle G residue of the middle degenerate hexameric site (oligo m5) to affect both competition (panel B) and binding (panel C) to the complex.

We explored the effect of the m5 mutation in vivo using transient transfection assays of expression vectors featuring the wild-type c-fos promoter (pEAfos) or a corresponding promoter region in which the m5 mutation had been engineered by site-directed mutagenesis (pmEAfos). Figure 20 shows that mutating the G residue at position -163, which prevented binding of the VDR complex, effectively abolished the induction of the reporter gene by calcitriol. These results confirm that the c-fos VDRE is a functional response element. Further proof of the functionality of the element was obtained by subcloning synthetic oligonucleotides encoding the c-fos VDRE upstream of a minimal heterologous promoter fused to a reporter gene. The c-fos VDRE conferred specific copy-number dependent transcription of the reporter gene in calcitriol-treated Ros 17/2.8 cells (data not shown).

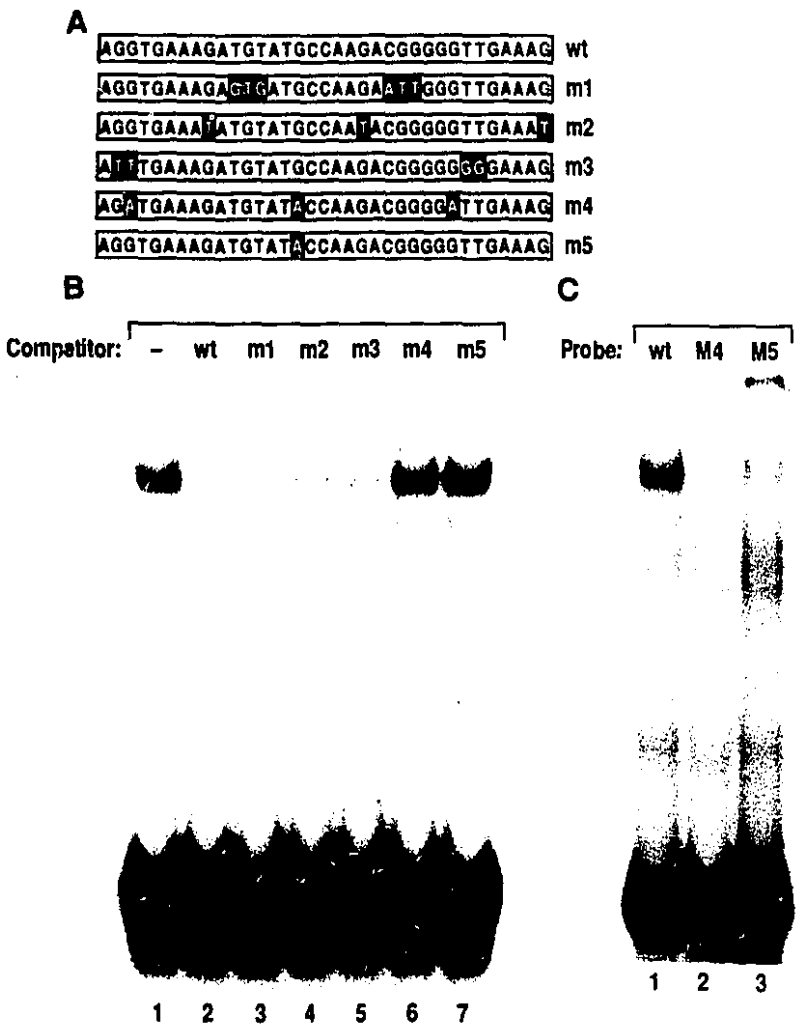

Figure 19: Site-directed mutational analysis of the c-fos VDRE. Panel A. Structure of the mutated oligonucleotides (wt, wild type). Panel B. An excess of the unlabelled oligonucleotides described in panel A was pre-incubated with calcitriol-treated MC3T3-E1 nuclear extracts. Binding was analyzed in a gel retardation assay on an 8% gel using the wt oligo as probe. Panel C. The wt, m4, and m5 oligonucleotides were end-labelled and used as probes in gel retardation assays with nuclear extract from calcitriol-treated MC3T3-E1 cells.

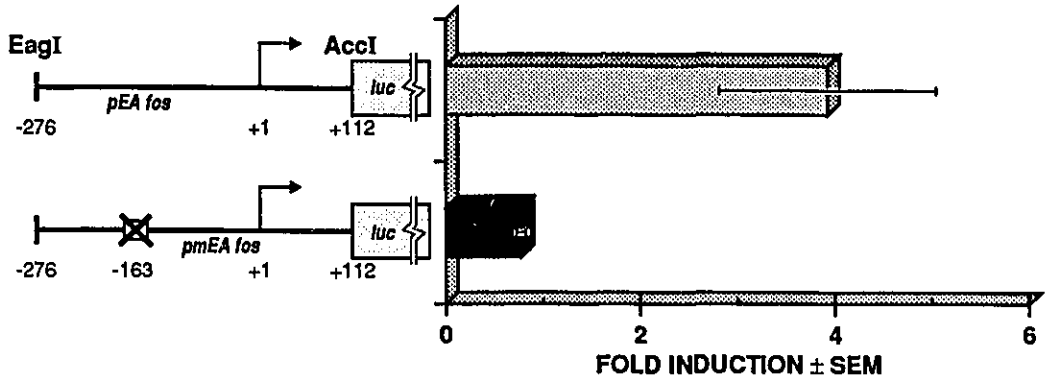

Figure 20: The c-fos VDRE is necessary for *in vivo* calcitriol induction. The c-fos VDRE was mutated by site-directed mutagenesis (m5 mutation, see figure 19A) in an expression vector fusing the c-fos promoter to the luciferase reporter gene (plasmid pmEAfos). The activities of the wild-type (pEAfos) and mutated expression vectors were analyzed by transient transfection assay with an internal control for efficiency of transfection. Transfected cells were challenged with 1μM calcitriol or vehicle for 30 minutes. Baseline luciferase values were subtracted from each sample. Values shown are mean fold induction ± S.E.M. for four independent experiments performed in triplicates.

10. Human c-fos VDRE

Our data showed that the murine VDRE was functional in a rat cell line. This was the first indication suggesting that the c-fos VDRE may be conserved across some species. To document this possibility we analyzed the human c-fos promoter for a conserved c-fos VDRE sequence. Sequence conservation between species is thought to imply evolutionary pressure due to functional importance. We aligned a putative VDRE sequence from the human c-fos promoter with the murine c-fos VDRE (Figure 21, panel A). The alignment showed some sequence conservation. Furthermore, key residues established in previous structure-function experiments appear conserved (Figure 21, panel A, highlighted residues). In particular, the critical G residue at position -163 of the murine VDRE was conserved in the putative human VDRE. Previous experiments have revealed that mutation of this single residue was sufficient to completely inhibit binding and function of the murine VDRE (see Figures 19 and 20).

We then tested the putative alignment using gel retardation assays. Interestingly, we detected binding to the putative human c-fos VDRE using nuclear extracts from calcitriol-treated MC3T3-E1 cells (Figure 21, panel B). Moreover, the data presented in Figure 21, panel B shows that the VDR was part of the bound complex, since the antibody that recognized the VDR abrogated binding (lane 3). Furthermore, the unlabelled wildtype murine c-fos VDRE effectively competed for

binding of the factor to the human sequence (lane 4). The mutants which could not compete for binding of the factor to the murine c-fos VDRE also could not compete for binding of this complex to the human c-fos VDRE (lanes 5 and 6). These results suggest that the VDR complex recognized both the murine and human VDRE. The functionality of the human sequence remains to be addressed.

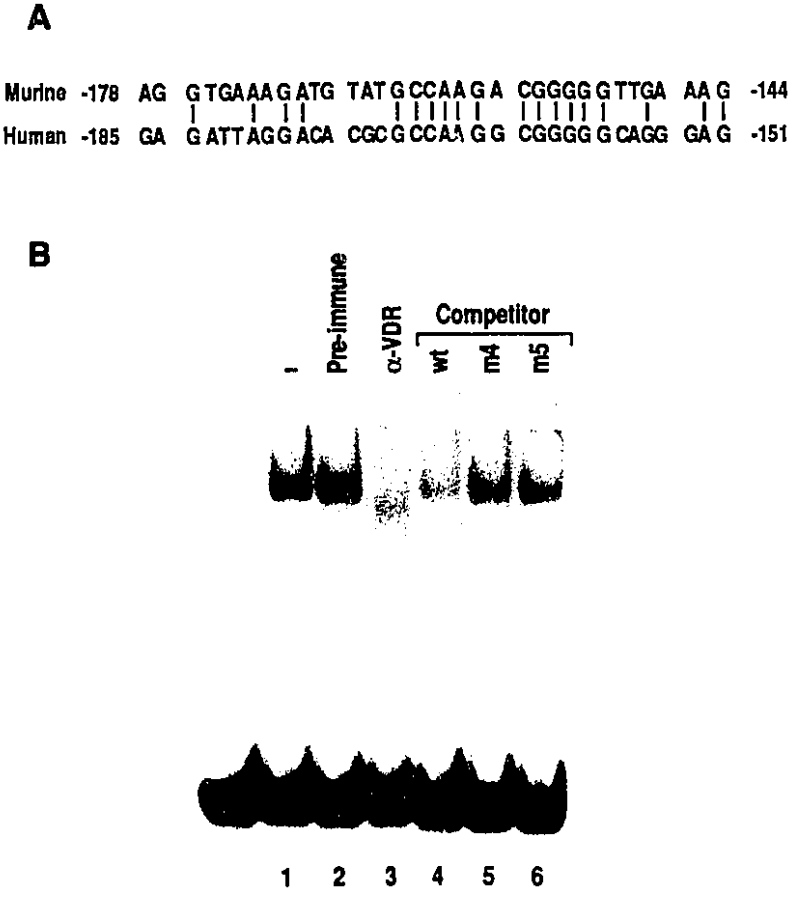

Figure 21: The murine VDR complex interacts with a human c-fos VDRE sequence. Panel A shows a putative human c-fos VDRE aligned with the murine c-fos VDRE sequence by computer analysis. Panel B shows the oligonucleotide corresponding to the putative human c-fos VDRE used as a probe in a gel retardation assay. The probe was incubated with MC3T3-E1 nuclear extracts (lane 1) in the presence of either pre-immune serum (lane 2), anti-VDR antibody (lane 3) or previously used unlabelled competitor VDREs (lanes 4 to 6).

B. Fos Expression in the Etiology of Fibrous Dysplasia

1. In Situ Detection of c-fos

The second aim of this thesis was to compare c-fos expression levels in bone biopsies from normal individuals and patients with fibrous dysplasia. Since transgenic mice overexpressing an exogenous c-fos sequence develop lesions that resemble those observed in patients with fibrous dysplasia, we hypothesized that elevated levels of c-fos expression might be implicated in the etiology of the disease.

We used in situ hybridization on undecalcified sections from bone biopsies to assess relative c-fos expression levels. Figure 22, panel A, shows the extensive hybridization signal (silver grains) observed throughout the fibrotic marrow space when a sample from a patient was probed with a specific c-fos riboprobe and examined under low magnification (100X) using bright phase optics. Higher magnification revealed that the signal was primarily localized to the fibrotic cells filling the marrow cavity (Figure 22, panel B).

The specificity of the observed signal was addressed. It was important to confirm that this signal (Figure 24, panel A) was due to probe binding to RNA inside the cell and not due to some nonspecific precipitate. In order to confirm that the

probe had bound to an RNA species inside the cell, the sections were pre-treated with RNase, which degraded all accessible RNA on the section. Subsequent probing of the sections with the labelled c-fos probe resulted in the virtual disappearance of any signal (Figure 24, panel C). This demonstrated that the c-fos probe did indeed recognize an RNA species. Pre-incubating the section with unlabelled c-fos probe prior to the addition of the corresponding labelled probe effectively competed away any signal from the section (Figure 24, panel B). This result demonstrates that the observed signal was due to specific hybridization of the c-fos riboprobe to its cognate target mRNA within the cells from the lesion.

Probing of the sections with an unrelated labelled riboprobe further confirmed the specificity of the hybridization signal observed with the c-fos probe. The c-fos fragment used spanned the fourth exon of the human sequence. This permitted the detection of complete mRNA transcripts. We engineered an unrelated probe of identical length. Figures 26B and 27B show that no signal was observed when sections were probed with an unrelated probe under identical conditions. This control experiment further established the specificity of the observed signal.

Close examination of particular sections featuring lesioned and non-lesioned tissue provided additional evidence that the c-fos signal was specific to particular cells in the lesions and not caused by artifactual precipitation of the probe or the emulsion over the entire area of the section. Figure 24 shows a particular section

containing both fibrotic cells (upper part of the photomicrograph) as well as normal haematopoietic cell types (bottom part of the photomicrograph). When probed with the specific c-fos probe, only the fibrous spindle-shaped cells characteristic of fibrous dysplasia lesions showed detectable levels of signal (Figure 24). Taken together, we interpret these results as demonstrating that our c-fos riboprobe allowed specific detection of c-fos mRNA. We then used in situ hybridization with this probe to compare c-fos expression levels in bone from patients with fibrous dysplasia and normal individuals or patients with unrelated pathologies.

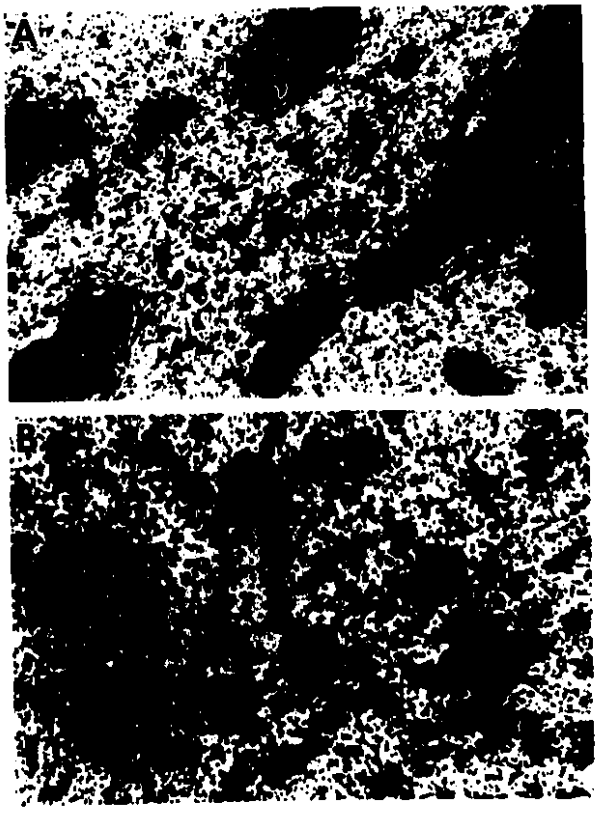

Figure 22: c-fos hybridization signal detected in fibrous dysplasia lesions. Brightfield microscopic appearance of the in situ hybridization signal obtained by assaying c-fos expression on an undecalcified bone biopsy section from a fibrous dysplasia patient. Panel A shows a low power magnification (100X) of the fibrotic tissue. Panel B is a high power magnification (1000X) showing that the silver grains localize to the fibrotic cells in the marrow cavity. Abbreviations used: Immature Bone (i), Marrow Fibrosis (f), Fibrotic Cells (c), Hybridization Signal (s).

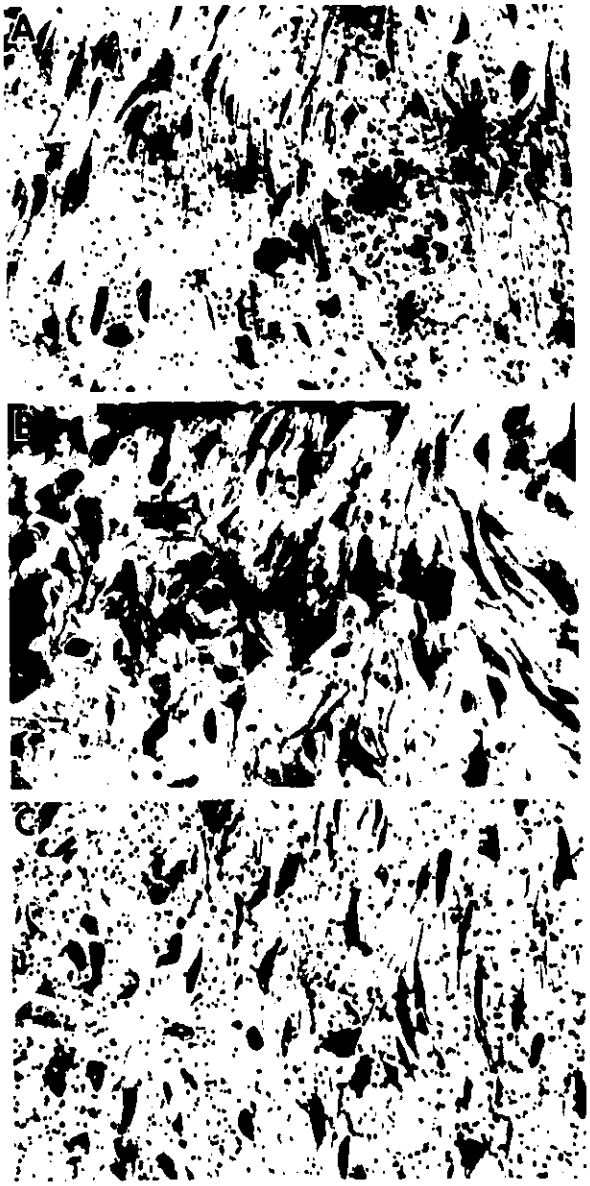

Figure 23: The c-fos probe binds specifically to intracellular RNA. Panel A, High power bright field micrograph of the c-fos signal detected in the fibrotic cells in the bone marrow of a fibrous dysplasia patient. Panel B, Pre-treatment with an unlabelled c-fos competitor probe. Panel C, Pre-treatment of the section with RNase prior to probing.

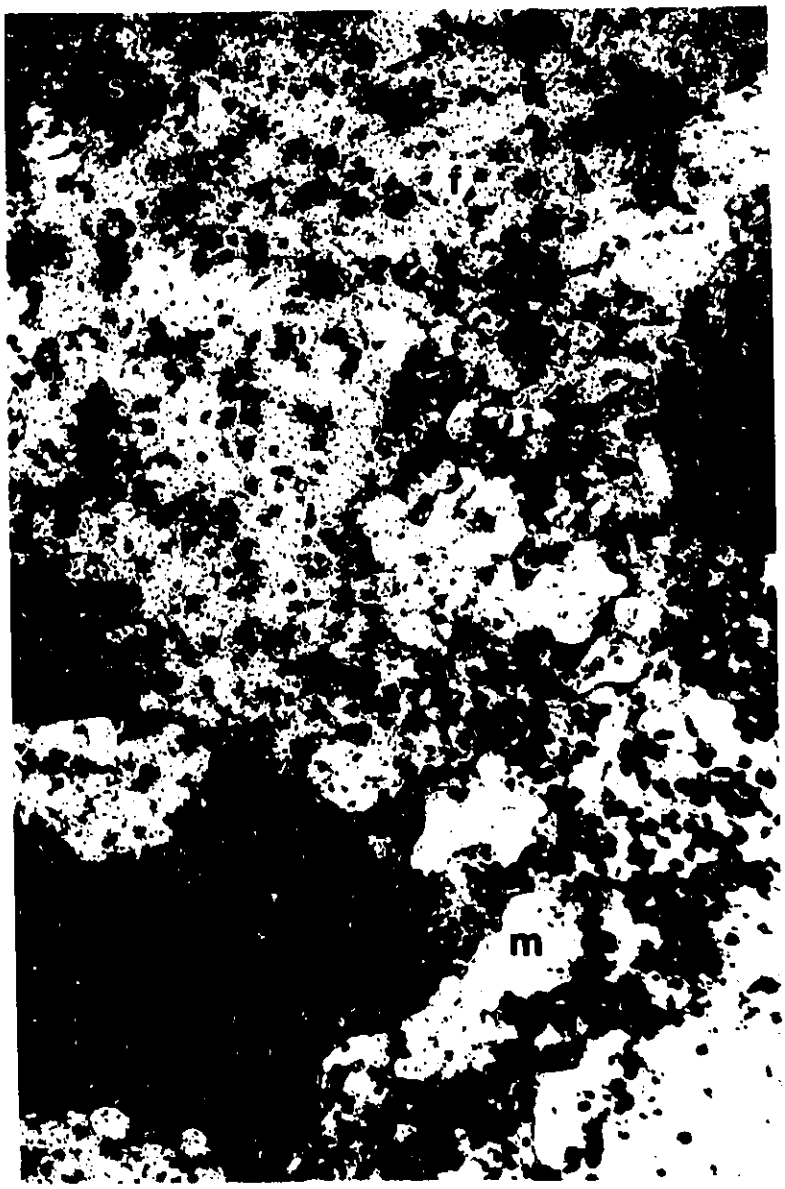

Figure 24: c-fos expression is localized to a specific cell type. Low power photomicrograph of an in situ hybridization assay with the specific c-fos probe. Only the fibrous marrow spindle-shaped cells have detectable levels of signal while normal bone marrow cells do not. Abbreviations used: Fibrous Marrow (f), Normal Marrow (m), Hybridization Signal (s), Trabeculae (t).

2. Elevated levels of c-fos in patients with Fibrous Dysplasia

We compared levels of c-fos expression between different biopsy specimens. Elevated c-fos levels were detected in bone biopsies from fibrous dysplasia lesions of seven different patients. Figure 25, panel A shows the result of probing patient #1 with a specific c-fos probe. The cells that expressed c-fos in the affected lesions were the fibroblastic cells that populate the bone marrow space. Although each biopsy did not express the same level of c-fos, all had significantly more signal than eleven other non-fibrous dysplasia lesion biopsies (see Figure 25, panel C, for example). Background grains were observed when the sections were probed with an unrelated probe under identical conditions (Figure 25 panels B and D). These results demonstrate that patients with fibrous dysplasia expressed more c-fos in the lesion than controls.

The extent of the variation in c-fos expression within the same patient was assayed. Fibrous dysplasia patients do not have their entire skeleton affected, despite the fact that they may have various fibro-dysplastic lesions throughout. Two patients, from which both lesion and non-lesion biopsies were available, demonstrated elevated levels of c-fos mRNA in the lesion. One of these patients had a monostotic lesion. Expression levels in two biopsy sections taken from patient #2, with polyostotic lesions, are shown in Figure 26. Panel A is a biopsy from the lesion probed for c-fos whereas panel C is a biopsy from an unaffected

bone area. Panel B and D show background levels of signal as detected with a non-specific probe. Interestingly, a biopsy taken from patient #3 had both normal and fibrotic cell types in the bone marrow (Figure 24). Only the region with fibrotic cells expressed elevated levels of c-fos as compared to the unaffected region. These results show that elevated levels of c-fos expression depend on the area from which the biopsy is taken. Increased expression of the c-fos proto-oncogene was observed only in the affected bone tissue.

To determine whether elevated c-fos expression in bone lesions is restricted and specific to patients with fibrous dysplasia, various different pathologies which exhibit extensive fibrous tissue were probed for c-fos expression. Only the fibrotic cells in biopsies taken from fibrous dysplasia patients showed elevated c-fos signal (see Figure 27, panel A and B). We did not detect increased c-fos mRNA levels in a bone biopsy sample from a patient with hypocalcemic vitamin D-resistant rickets (HVDRR) exhibiting very high bone turnover (Figure 27, panel C and D). One biopsy from a Pagetic patient (Figure 27, panel E and F) showed virtually no signal as did a biopsy from a patient diagnosed with Proteus Syndrome (Figure 27, panel G and H). Three biopsies from patients diagnosed with Osteogenesis Imperfecta type IV were also probed with c-fos. These samples had little signal above background (data not shown). All these diseases show extensive fibrosis, but only biopsies from patients with fibrous dysplasia showed elevated c-fos expression. Our findings that only bone biopsy samples obtained

from fibrous dysplasia patients show increased levels of c-fos expression support a pivotal role for this gene product in the etiology of the disease.

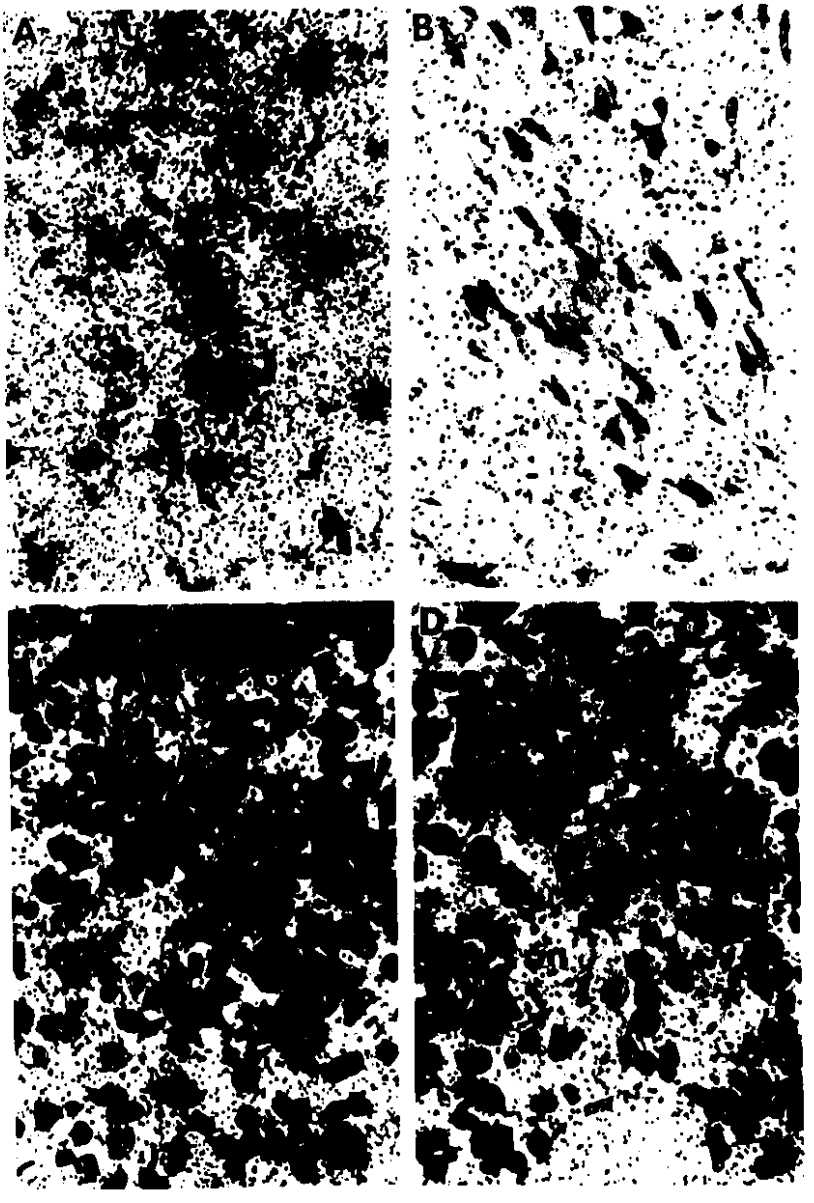

Figure 25: Elevated c-fos levels in biopsies from fibrous dysplasia patients. A biopsy specimen taken from a fibrous dysplasia lesion showed higher c-fos expression than a control biopsy. More fibrotic cells in the lesion (panel A) have detectable levels of c-fos message than the normal complement of bone marrow cells (panel C). The level of background in the lesion (panel B) and the control (panel D) was shown by probing the sections with a non-specific probe under identical conditions.

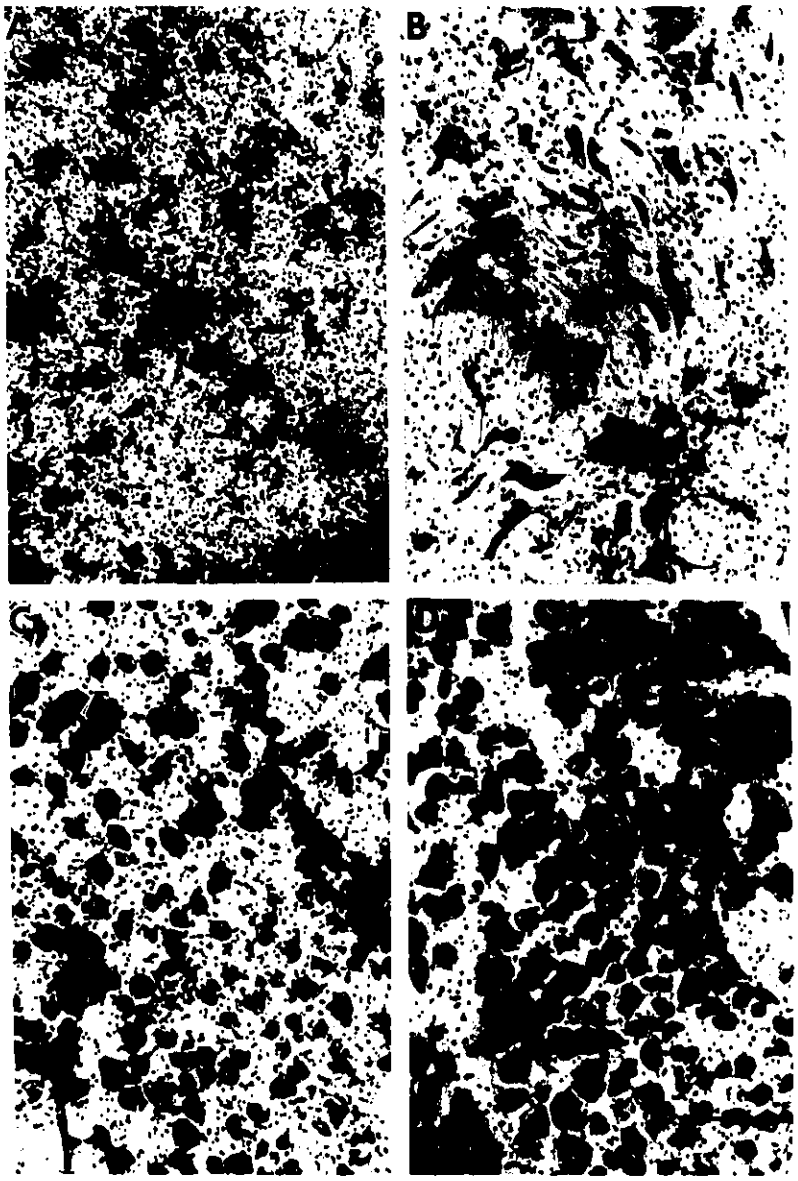

Figure 26: Elevated c-fos levels in fibrous dysplasia lesions relative to unaffected areas. Bone biopsy specimens taken from fibrous dysplasia patient #2. A specimen from the lesion probed with c-fos (panel A) was found to have elevated levels of signal compared to an unaffected area (panel C). Corresponding background signal is shown using a non-specific probe (panels B and D).

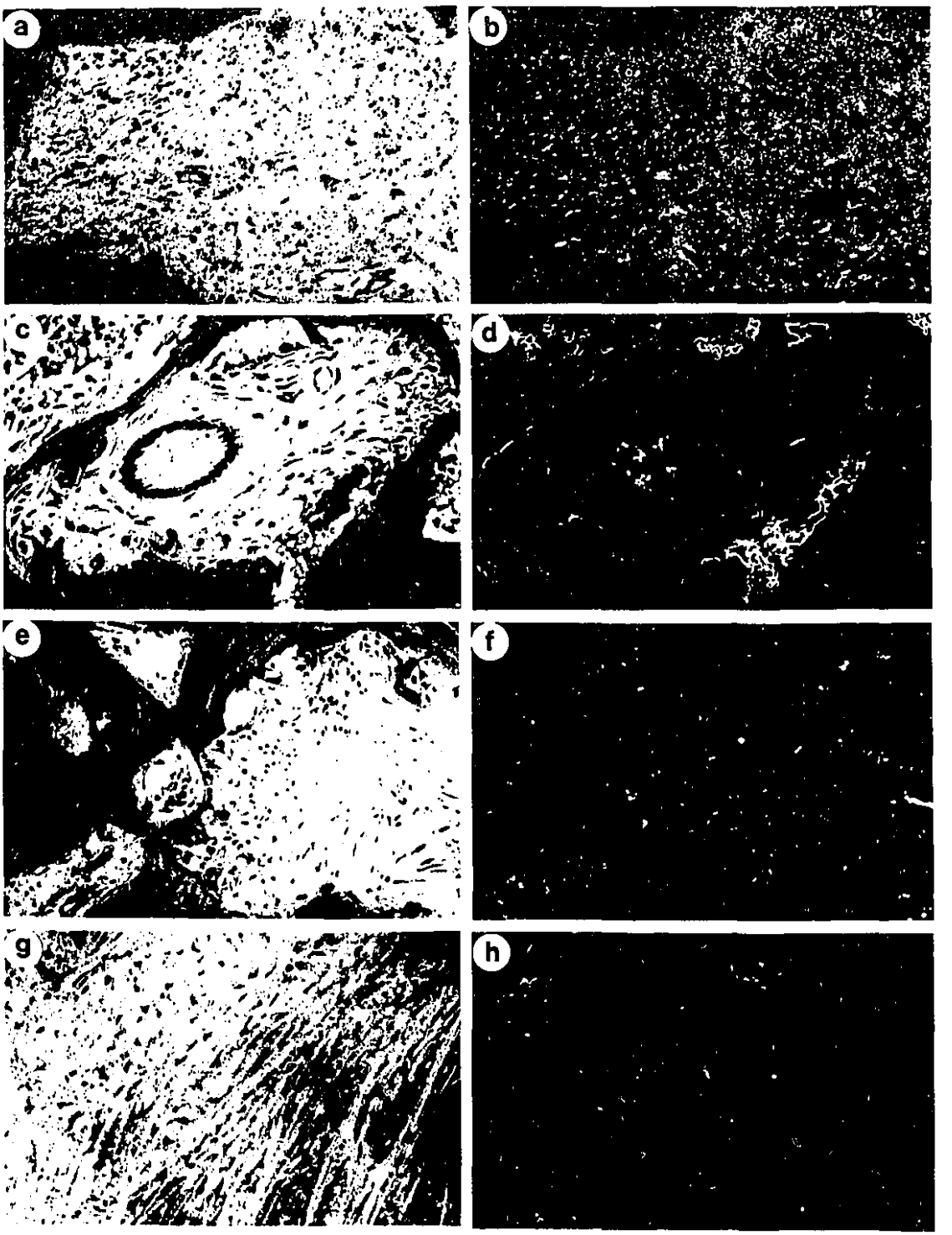

Figure 27: The increased levels of c-fos mRNA are specific for bone lesions of fibrous dysplasia patients. Brightfield (a,c,e,g) and darkfield (b,d,f,h) photomicrographs of sections from bone biopsies probed with the specific c-fos riboprobe. Biopsies were obtained from patients with fibrous dysplasia (a,b), hypocalcemic vitamin D-resistant rickets (c,d), Paget's disease (e,f) or Proteus syndrome (g,h). Magnification, 320X.

VII. Discussion

A. Differential Stimulation of Fos and Jun Family Members by Calcitriol

Our results show that calcitriol treatment of osteoblasts induces a rapid and transient stimulation of the expression of c-fos, fos B, c-jun and jun-B with varying kinetics. The expression of fra-1 and jun-D is not affected by calcitriol in those cells. The calcitriol effect is specific and dose-dependent. The expression of the c-Fos protein correlates with the expression of the mRNA in calcitriol-treated cells. The calcitriol-induced stimulation of c-fos expression is modulated, at least in part, at the level of the initiation and elongation of transcription, whereas its effects on c-jun and jun-B expression is controlled at the post-transcriptional level by a mechanism that does not implicate stabilization of their respective mRNAs.

Other modulators of osteoblastic growth and differentiation, such as growth hormone[161,162], insulin-like growth factors[163],,m and retinoic acid[164] have been also shown to induce c-fos expression in bone cells. Calcitriol, together with these factors, most likely contributes to the overall in vivo pattern of AP-1 response in osteoblasts.

The modulation of the expression of fos and jun family members by calcitriol may

not be restricted to osteoblasts, particularly in vitro. Indeed, the effect of vitamin D on c-fos expression was originally studied in the human promyelocytic leukemia cell line HL-60[300]. However, the elegant in vivo data from c-fos "knock-out" mice[251,252] and the in vivo data from c-fos overexpressing transgenic animals[245] demonstrate a role for the c-Fos protein in bone cell growth, differentiation and function. Since c-Fos functions as a heterodimer, our data showing differential modulation of the expression of fos and jun family members by calcitriol in established and primary cultures of osteoblasts further support this idea. Our data using primary cultures suggests that the modulation is a physiological response of the bone cells and not an artefact of the established cell line.

The effect of calcitriol on fos and jun expression in established or transformed cell lines from non-bone origins thus appears to bear less physiological relevance. Moreover, earlier studies of the c-fos expression pattern following calcitriol treatment were conducted with relatively high doses of the hormone. Treatment of HL-60 cells with doses of calcitriol at least one order of magnitude higher than the doses used in our work induced a gradual increase in c-fos expression that was detectable up to 24 hours after addition of the steroid, which is an unusual pattern of expression for this immediate-early gene. The molecular mechanism of action of calcitriol on c-fos expression was not addressed in that study. Other experiments, also using high doses of calcitriol, showed no effect of concomitant treatment with calcitriol on the serum-induced stimulation of c-fos expression in

bovine parathyroid cells[301]. It should be noted that the doses of calcitriol used in our study are equivalent to the doses used for studying transcriptional activation of responsive genes by calcitriol through its receptor.

Recently, our observation that calcitriol induces c-fos in osteoblasts was confirmed in human cells. Calcitriol has been shown to upregulate the expression of c-fos in human osteosarcoma cells[302] although with slightly deregulated kinetics. The expression of c-fos which is normally barely detectable in cell lines or primary cultures was quickly upregulated by calcitriol. In osteosarcoma cells, expression is detected more readily. Moreover this report suggests that the expression of c-fos by calcitriol is slowly upregulated and stays on for a longer period. This deregulated expression is not unexpected since the cells are transformed and may not tightly regulate the expression of a proto-oncogene.

The observation that the different members of the fos and jun families are differentially regulated by calcitriol is of particular interest. The pattern of expression of AP-1 responsive genes in a cell is most likely dependent upon which members of the two families are available for dimer formation at any given time. Indeed, c-Jun homodimers have been shown to have lower DNA-binding affinity[151] and trans-activating capability[303,304] than c-Fos/c-Jun heterodimers. Moreover, jun-B inhibits the transforming and trans-activating activities of c-jun[275,305]. The various heterodimers formed between other fos family members

(Fos-B and Fra-1) and jun family members have also been shown to have varying DNA-binding affinities[306]. Thus the expression of AP-1 responsive genes in osteoblasts following exposure to calcitriol will most likely vary over time as the different members of the fos and jun families are sequentially turned on and off. The influence and physiological significance of the low constitutive levels of jun-D (fig. 3) and fra-1 (not shown) expression in osteoblasts is currently unknown. Finally, the DNA-binding activity of Fos/Jun dimers has recently been shown to be inhibited in a phosphorylation-dependent manner by the protein IP-1[307]. The expression of IP-1 in resting and calcitriol-stimulated osteoblasts remains to be investigated to obtain a clear picture of AP-1 dependent transcription in bone cells.

The influence of calcitriol on fos/jun expression may also vary depending on the stage of differentiation of the osteoblast population. We used non-mineralizing cultures of MC3T3-E1 cells and primary osteoblasts and it would be interesting to measure the effects of calcitriol on the expression of these proto-oncogenes in longer-term, mineralizing cultures. In fact, we have accumulated some preliminary results which suggest that the stimulation of c-fos by calcitriol decreases with time of culture. This observation supports the hypothesis that transient c-fos expression has a role in osteoblast differentiation and a role in the stimulation of bone remodeling. The stimulation of c-fos by calcitriol is also in accord with the physiological roles of this hormone on osteoblasts. A burst of c-

fos in preosteoblasts could be involved in cellular differentiation[256] by activating a cascade of events which leads to a more differentiated cell type. The action of low dose calcitriol on c-fos could promote cellular differentiation. The stimulation of remodeling observed at higher doses could also be through the action of c-fos. Immature bone lining cells could respond to calcitriol by secreting a soluble factor which stimulates osteoclast maturation and resorption[165,50]. Both these responses are in accord with the responses that calcitriol elicits from osteoblasts.

Experiments which study the effects of other modulators of osteoblast function on the expression of fos and jun family members should yield interesting results. Glucocorticoids exert numerous effects on bone cells[308]. It should prove interesting to study the effects of glucocorticoids on the expression of fos and jun family members in osteoblasts.

The availability of functional Fos/Jun and Jun/Jun dimers may play a significant role in osteoblast physiology. The presence of functional AP-1 binding sites in the promoter region of genes preferentially expressed in bone cells is also suggestive of an important implication of fos and jun in the regulation of osteoblastic gene expression. Indeed, the Fos/Jun heterodimer has been shown to bind the AP-1 motif nested amidst the vitamin-D responsive element (VDRE) of the human osteocalcin promoter and to inhibit basal and calcitriol-stimulated osteocalcin expression in vitro[309]. A putative VDRE in the alkaline phosphatase

promoter that also contains an AP-1 site within its sequence binds Fos/Jun in vitro and these observations are thought to be pivotal in modulating the establishment of the fully differentiated osteoblastic phenotype[261]. Thus the stimulation of the expression of calcitriol-responsive genes in osteoblasts may be further modulated by the presence of functional Fos/Jun dimers; it should however be noted that maximal expression of vitamin-D responsive genes, such as osteocalcin, is observed at much later times following treatment with calcitriol[127] than the rapid response of fos and jun family members described herein. Several groups have also recently reported an interaction at the protein level between nuclear receptors and Fos and Jun[310], this would also affect the overall pattern of gene expression.

Furthermore, it is plausible that the specific role that c-fos has in bone physiology is due to its interaction with novel protein partners in this tissue. In fact, a basic helix-loop-helix-zipper protein termed Fos-interacting protein (FIP) was identified by its ability to form heterodimers with Fos[224]. It is conceivable that a novel helix-loop-helix-zipper protein called microphthalmia (mi) can also interact with c-fos[311]. This idea is especially interesting since mi was shown to be mutated in a mouse model for osteopetrosis similar to the osteopetrotic c-fos knockout phenotype.

The mechanism of action of calcitriol through its specific trans-activating receptor molecule has been well characterized[86]. Therefore, we hypothesized that the

calcitriol-enhanced expression of c-fos, c-jun and jun-B would be modulated at the transcriptional level. This turned out to be only partially true, as evidenced by the data shown in figure 8. Only c-fos showed increased transcriptional activity following calcitriol treatment of the osteoblasts. Moreover, it is unclear if all the increased steady-state level expression of c-fos following calcitriol exposure of the bone cells is due solely to the transcriptional effect. Since we cannot accurately quantify the fold-increase in mRNA levels (see results), we cannot rule out the contribution of post-transcriptional mechanisms in the overall calcitriol-stimulated expression of c-fos. At any rate, the reproducible increase in c-fos initiation and elongation of transcription support the importance of that molecular mechanism in mediating the calcitriol effect on c-fos gene expression. Our identification and characterization of a novel type of VDRE within the c-fos promoter (see below) confirms the transcriptional component of the calcitriol-induced stimulation of c-fos expression.

On the other hand, calcitriol does not appear to modulate c-jun and jun-B transcription in osteoblasts (see figure 8). As steroids have also been shown to modulate gene expression by affecting the half-life of certain transcripts[192], we have measured the stability of the c-jun and jun-B mRNAs in calcitriol-treated osteoblasts to determine if the steroid could increase the half-life of these transcripts. Our data indicate that calcitriol treatment has no significant effect on the stability of the c-jun and jun-B transcripts (figure 9 and table I). These results

suggest that post-transcriptional events such as nuclear stability of the RNA, processing (splicing) and/or transport to the cytoplasm may play important roles in the stimulation of c-jun and jun-B mRNA levels in response to calcitriol treatment of osteoblasts. Such post-transcriptional mechanisms have previously been inferred to play a role in the modulation of the expression of other nuclear proto-oncogenes in differentiating cells[284]. It is interesting to note that the enhanced expression of the liver/bone/kidney isozyme of alkaline phosphatase in osteoblasts is also controlled via a post-transcriptional mechanism that does not involve increased cytoplasmic mRNA stability[312]. It would be interesting to further the work presented in this study by studying the post-transcriptional mechanisms implicated in the stimulation of c-jun and jun-B expression by calcitriol. However, such mechanisms have proven difficult to clearly elucidate.

B. A Novel VDRE in the c-fos Promoter

The mechanism of action of calcitriol through its specific trans-activating receptor molecule has been well characterized[86]. Following our observation that calcitriol stimulates c-fos transcription in osteoblastic cells, we went on to identify and characterize a vitamin D response element in the c-fos promoter.

Our results show that a region of the c-fos promoter is functionally responsive to calcitriol in vivo. An induced binding complex in nuclear extracts from MC3T3-E1 cells stimulated with calcitriol specifically interacts with the responsive region of the c-fos promoter. Transient transfection assays in osteoblastic cells using wild-type and mutated versions of the VDRE have confirmed its functional relevance. This complex, binding a 36 basepairs sequence centered around residue -161, contains the vitamin D receptor (VDR) as expected. However, our results suggest that the RXR, RAR, and T_3R nuclear receptors are not involved as binding partners with the VDR to this novel VDRE sequence. Finally, this novel VDRE sequence appears conserved in the human c-fos promoter.

The c-fos VDRE that we have identified is a functional response element. A mutation that abolishes binding to the sequence concomitantly inhibits vitamin D-dependent transcriptional activation of c-fos in osteoblastic cells (see figure 20).

It should be noted that the selected mutation was engineered in the context of the partial c-fos promoter region that was previously shown to be responsive to vitamin D (figure 10). We chose to engineer the minimal mutation (m5; see figure 19A) that would completely abolish binding of the VDR complex to the site. We anticipate that the other mutation abolishing binding (mutation m4) would also abrogate vitamin D transcriptional responsiveness since the key mutated residue in the m5 mutant is also modified in the m4 mutation (see figure 19A).

Another line of evidence confirming the functionality of the c-fos VDRE comes from testing of expression vectors in which one or multiple copies of an oligonucleotide corresponding to the c-fos VDRE sequence were subcloned upstream of the minimal thymidine kinase promoter driving the luciferase reporter gene (not shown). In transient transfection assays in osteoblastic cells, the VDRE oligonucleotide specifically conferred vitamin D-transcriptional responsiveness to the reporter gene. The extent of the vitamin D-dependent induction was copy-number dependent.

A region showing sequence similarity to the murine c-fos VDRE can be identified in the human c-fos promoter. Preliminary results have shown that this sequence also binds the VDR and that mutations affecting binding to the murine VDRE also inhibit binding to the human sequence (figure 21). Although the functionality of

the human element remains to be established, these observations support the evolutionary importance of the element that we identified. Work done with the human c-fos promoter in HeLa cells suggest that this same region of the c-fos promoter is important in controlling c-fos transcription[169]. Moreover, our work on the murine element together with the work done on the human medial region can now be combined to understand what transcription factors are binding this site. It was suggested that members of the NF-1 family of transcription factors could bind the medial region[167] since the binding sequence was similar to the NF-1 binding site[313]. It remains to be tested, using specific antibodies and by in vitro translation, if NF-1 family members are part of the complex recognizing the murine c-fos VDRE.

The magnitude of the transcriptional stimulation induced by calcitriol through the c-fos VDRE (around 4-fold) is comparable to what has been reported for other vitamin D response elements[86,127,296]. To our knowledge, the strongest VDRE-mediated transcriptional activation (about 25-fold) has been reported in GC pituitary cells for the response element identified in the Pit-1 gene promoter[113]. Factors that could influence transcriptional responsiveness through a given VDRE in various cell types include the levels of expression of the VDR and its dimerizing partners (see below). Indeed, when the Pit-1 RDE response element was tested in a different cell type (CV-1), the observed response was reduced to about 10 fold. It would be interesting to test the c-fos VDRE in different cell

types to assess its efficiency of transcriptional activation. However, our preliminary results suggest that the element might show bone cell-specific response since vitamin D activation was observed only in MC3T3-E1 and Ros 17/2.8 osteoblastic cells. No vitamin D transcriptional activation through the c-fos VDRE could ever be measured in CV-1 cells, even when co-transfected with a VDR expression vector (data not shown). These observations suggest that the expression of the partner allowing binding of the VDR to the c-fos VDRE (see below) might be restricted to bone cells.

We used relatively high vitamin D doses in the transient transfection assays. Such doses have been used in the past by other investigators[96]. We elected to use that concentration of calcitriol for experimental convenience since the serum used in the culture of our osteoblastic cells was never treated to remove endogenous vitamin D or vitamin D binding proteins[128]. Thus the high dose used was most likely a huge excess that afforded experimental reproducibility. Indeed, we observed transcriptional response with calcitriol doses that were two orders of magnitude lower, however, that response proved less reproducible (data not shown).

This work does not constitute the first report of a steroid response element in the c-fos promoter. Two groups have previously reported the identification and partial characterization of estrogen response elements (ERE) in the human and murine

c-fos genes, respectively. One element was characterized far upstream of the transcription start site of the human c-fos promoter[314], whereas the murine c-fos ERE was identified in a 200 bp fragment located 1.5 kb downstream of the polyadenylation signal[170]. In both cases, the transcriptional activation was sustained longer than thirty minutes as we observed for the vitamin D-mediated stimulation of c-fos transcription in osteoblastic cells, suggesting that the calcitriol-dependent transcriptional response might be under tighter control (see below).

This is the first description of a functional VDRE in the promoter of an immediate early gene. The c-fos gene is very quickly transcriptionally activated by calcitriol and this activity is transiently maintained for up to thirty minutes (see figure 8). This phenomenon dictated that we use short time points of stimulation and highly sensitive methods of detection. We used ROS 17/2.8 cells which are easily transfectable and commonly used to characterize VDREs[18]. We used luciferase reporter constructs which are much more sensitive than chloramphenicol acetyltransferase reporter enzymes. In a short stimulation period, the c-fos VDRE showed the same fold-induction in transcription than other previously characterized VDREs which show maximal induction after much prolonged periods[95,296]. This result may be related to the unusual structure of the element and suggests that increases in c-fos transcriptional activity through the c-fos VDRE are rapidly turned off in vitamin D-treated osteoblasts.

Various mechanisms can be proposed for the transcriptional shut-off of the c-fos gene following calcitriol induction. Composite response elements acting both as VDREs and AP-1 binding sites have been described in the promoters of the human osteocalcin[309] and alkaline phosphatase[261] genes. It has been proposed that occupancy of these sites by one family of transcription factors would prevent binding by the other class of factors. Thus, the stimulation of fos and jun family members by calcitriol (figure 3) could lead to a negative feedback mechanism in which the induced AP-1 dimers could bind a composite VDRE-AP-1 site, displace the VDR and shut-off calcitriol activated transcription. However, no putative AP-1 site can be identified within or flanking the c-fos VDRE region, rendering this hypothesis less likely.

Cross-talk between different signalling pathways via direct protein-protein interactions, independent of response element binding, can prevent the VDR complex to bind DNA. The direct interaction between the VDR and a protein acting to inhibit binding to the c-fos VDRE could transcriptionally silence the gene. Functional antagonism between steroid hormone receptors and AP-1 family members has been described[310]. In fact, the glucocorticoid receptor and c-fos or c-jun were shown to antagonize each other's binding to DNA via direct protein-protein interactions[315]. The retinoic acid receptor and c-jun/c-fos can antagonize each other's activities by a mechanism that does not require DNA interaction, probably via direct protein-protein interactions[316]. A similar antagonism is seen

with the thyroid hormone receptor[317] and AP-1 as well as with the estrogen receptor[318] and AP-1. This suggests that the antagonism between AP-1 and members of the nuclear receptor family may be a general feature of nuclear receptors. Thus it could be hypothesized that c-Fos or c-Jun would interact with the VDR to prevent its binding to the c-fos VDRE and rapidly turn off the calcitriol induced transcriptional stimulation of c-fos expression. Interestingly, such a mechanism has been proposed for the transient stimulation of c-fos transcription by estrogens[319]. This interaction was suggested to be important for the transcriptional shut-off mechanism of the gene resulting in the transient nature of the expression. In any case, no direct interaction between the VDR and AP-1 family members has been described to date.

If it is not a direct negative feedback loop with AP-1 that is inhibiting further expression of c-fos, an indirect loop could be envisioned. Calreticulin is an endoplasmic reticulum calcium-binding protein that has recently been shown to also localize to the nucleus. The protein has been shown to specifically inhibit the binding and transcriptional activating functions of the androgen, glucocorticoid, and RA receptors[320,321]. Calreticulin can also interact with the vitamin D receptor to prevent binding to its cognate response element (S. Dedhar - personal communication). Interestingly, preliminary results suggest that calreticulin is also induced by calcitriol in MC3T3-E1 cells with shortly delayed kinetics as compared to c-fos induction. It is thus possible that calreticulin could therefore bind the

VDR to rapidly inhibit any further calcitriol dependent transcription of c-fos by the vitamin D receptor complex by preventing it from binding the VDRE. Experiments are in progress to study the putative contribution of calreticulin to the rapid transcriptional silencing of c-fos expression in calcitriol treated bone cells.

To see whether c-fos or calreticulin will negatively inhibit the transcription of c-fos by vitamin D a strategy of co-immunoprecipitating one protein with an antibody directed to the other should be appropriate. The co-immunoprecipitation of the VDR with either c-fos or calreticulin from nuclear extracts in late time points of induction will reveal any strong direct protein-protein interactions with the VDR.

We identified the precise region that the binding complex was recognizing. Under these conditions, no induction was detected with footprinting assays. This was probably due to the fact this is a different assay. Footprinting assays can detect the weaker interaction between the unliganded receptor and the VDRE because higher protein to DNA ratios are used in footprinting assays than mobility shift assays. Nonetheless, we know that the footprinted region recognizes the induced complex in a gel mobility shift assay because it competes for binding and directly binds the complex itself. There is a controversy in the literature about whether the VDR-RXR heterodimer can indeed be induced to bind a classical VDRE without the help of its ligand[295]. This report, however, shows that the addition of

calcitriol will result in increased affinity of the VDR for the VDRE. Binding of nuclear extracts to the murine c-fos VDRE in our hands can indeed be detected after calcitriol treatment of the cells or extracts. This difference can be explained by the different binding conditions used between the different groups. Our conditions were similar to those used in a report where increased binding could be observed[98].

The c-fos VDRE has an unusual structure. The c-fos VDRE element is not a classical direct repeat spaced by three basepairs. The protected sequence in footprinting assays does not look like a classical VDRE. The sequence itself is unusually long and the entire protected region is needed to interact with the complex. Any deletion at the 5' or 3' ends will abrogate binding. It should be noted however, that the most 3' site is a preferential VDR binding site[107]. When analyzed in the context of the DR-type structures, three putative hexameric core sites seperated by seven basepairs can be identified (table I). These would give the c-fos VDRE either a DR7-like conformation (between residues -178 and -160 or between residues -165 and -147) or a DR20-type configuration (between the two extreme hexameric sites, positions -178 to -147). However, mutational analysis (figure 19) of this site has revealed that these alignments are not valid. Oligonucleotides representing both DR7-type structures do not bind the calcitriol induced complex (table I). Mutations in both hexameric binding sites of the putative DR20-type element had no effect on binding (figure 5). Moreover, three

conserved G residues in the putative spacer region 3' of the three putative hexameric sites seem to have some functional importance (m2). Furthermore, it is sufficient to mutate a single central G residue (m5) within the least conserved direct repeat to essentially eliminate all binding and expression. Thus, the c-fos VDRE does not strictly conform to a known DR-like configurations. To precisely understand what residues are important for binding, we must do mutations scanning the c-fos VDRE to see the result on competition and binding and/or do methylation interference experiments.

The identification of this novel element is not completely unexpected since c-fos is induced with much more rapid kinetics than the other vitamin D responsive genes. The osteopontin VDRE is maximally induced after a long 48 hour calcitriol stimulation. The c-fos VDRE, in a thirty minute period, will accumulate a similar fold induction. It is thus not unexpected that the c-fos VDRE element does not contain a classical DR3 structure like the well characterized osteopontin, calbindin and osteocalcin VDREs.

The importance of this VDRE is sustained by the observation that binding is conserved in the human c-fos promoter. We have shown that the murine VDR binding complex can recognize the human element. This experiment (figure 21) points to some important structural determinants of the element. It is interesting to note that the conserved residues are also the strongly protected long 3' GC box

residues in the murine footprint (figure 13). More experiments are needed to precisely understand and show functionality in vivo of the human site. Footprinting studies and structure-function studies must be done to prove that the human element has physiological relevance. Similar kinds of studies should be done on the cloned chicken c-fos promoter. By analysing the element in different species we should be able to identify important residues of the c-fos VDRE element.

These observations support the hypothesis that calcitriol induced complex in osteoblasts on the c-fos VDRE is composed of novel factors of which the VDR is one component. These proteins come together and interact with each other and with this DNA element to form a novel complex. Attempts were made to identify the protein constituents of the transcription factor complex.

We were able to demonstrate that the VDR was part of this complex. Binding could be induced with calcitriol both in vivo by stimulating cells and in vitro by stimulating extracts. Furthermore, the well characterized murine osteopontin VDRE also recognized components of the c-fos VDRE complex. Ultimately, an anti-VDR antibody directly recognized the receptor and abolished its binding.

Since RXRβ is a known partner for nuclear receptors to bind their response elements[100], we tested whether it would heterodimerize with the VDR to bind the

c-fos VDRE. Our results show that the c-fos VDRE does not bind the VDR-RXRβ heterodimer (figure 18). Using in vitro translated receptors it was not possible to get heterodimeric binding with RXRß. It is unlikely that other RXR family members[103] would heterodimerize with the VDR to bind this element since in vitro translated RXRβ and RXRα are functionally interchangeable as dimerizing partners for the VDR in gel retardation assays[99,100].

We have not detected homodimeric binding of the VDR to the c-fos VDRE (figure 18). Under the conditions used, both the DR3-type VDRE of the mouse osteopontin gene promoter (figure 18, lane 2) and the DR6-type VDRE of the human osteocalcin promoter have been shown to bind in vitro translated VDR homodimers[97,106]. The P0 and IP12 synthetic VDREs are also thought to bind VDR homodimers. The c-fos VDRE does not exhibit any similarity to the configuration of these response elements.

Other putative binding partners were subsequently investigated. The thyroid hormone receptors have been shown to enhance binding of the VDR to a synthetic DR6[97]. This was an attractive hypothesis because thyroid hormone, like vitamin D, increases bone remodelling. It could have thus been possible that this effect was mediated by c-fos through an interaction of both receptors on the c-fos VDRE. The thyroid receptor, however, was not detected in the VDR complex on the c-fos VDRE with specific antibodies to three different isoforms of the

receptor. We looked for the TRα_1, TRα_2, and TRβ_1 isoforms of the receptors (data not shown).

Our data also revealed that VDR-RAR and VDR-T_3R heterodimers do not bind the c-fos VDRE (not shown). It has recently been shown that RA could transcriptionally activate c-fos expression in osteoblastic cells[164], although the putative RARE involved has not been identified. Interestingly, the human osteoclacin VDRE has been shown to also mediate transcriptional activation by RA and thus also function as a RARE[310]. This suggested that the murine c-fos VDRE could be involved in the RA-dependent transcriptional activation of c-fos in osteoblastic cells. However, we have never detected RA-dependent stimulation of transcription through the c-fos VDRE, even using various cell types (not shown). This observation further supports the notion that the c-fos VDRE is structurally different from the osteocalcin VDRE and that VDR-RAR heterodimers do not bind the c-fos element. Thus we interpret these results to mean that the VDR must heterodimerize with a novel partner to bind the c-fos VDRE.

There is evidence in the literature for novel protein-protein interactions allowing the binding of nuclear hormone receptors to their cognate response elements. The receptor binding factor (RBF-1) is a nuclear protein enhancing the binding of the progesterone receptor to DNA[322]. The murine homolog of the Drosophila homeotic regulator brahma (mbrm) has been shown to potentiate transcriptional

activation by the glucocorticoid and RA receptors via a mechanism thought to facilitate binding of the receptors to their target sequences[323]. The major RARE of the Pit-1 gene promoter is absolutely dependent on the Pit-1 protein for RAR transcriptional activation[113], suggesting a close association between tissue-specific transcription factors and steroid hormone receptors.

Thus, our work has identified a novel type of VDRE within the murine c-fos promoter. This element binds the VDR in conjunction with a novel dimerizing partner that is expressed in osteoblast-like cells. The identification and characterization of this cofactor should provide new insight into the molecular mechanism of action of calcitriol. Co-immunoprecipitation studies using anti-VDR antibodies could allow identification of this novel partner. Various strategies including VDR affinity chromatography or VDRE-DNA affinity chromatography could be envisaged to further characterize this novel cofactor.

C. Elevated c-fos Levels in Fibro-Dysplastic Bone

We used in situ hybridization to evaluate the contribution of c-fos overexpression to the etiology of fibrous dysplasia. The analysis of c-fos expression was performed on undecalcified bone tissue sections. Appropriate controls established the specificity of the assay. High levels of c-fos transcripts were detected in bone biopsies of lesions from six different patients with polyostotic fibrous dysplasia and one patient with monostotic disease. Virtually no signal was observed in eleven non-fibrous dysplasia bone sections. The population of cells that overexpressed c-fos appeared to be fibrotic cells that overpopulate the marrow space. Elevated signal was restricted to the lesions in patients. Moreover, increased c-fos expression was not observed in biopsies from patients with other bone diseases where high bone turnover and fibrotic tissue are present. These results support a pivotal role for the c-fos gene product in the etiology of fibrous dysplasia.

In situ hybridization conditions were developed using biopsy specimens that were prepared for bone histomorphometry. This is not the first report of in situ hybridization conditions developed for undecalcified adult bone[324]. It is, however, the first example where the technique is used on bone tissue sections normally prepared for bone histomorphometry. The fixation and embedding conditions used for histomorphometric analysis are not optimal for in situ

hybridization. Thus extensive controls which established the validity of the technique were performed. We have shown that the hybridization signal was due to specific binding of the riboprobe to the c-fos mRNA transcript.

In situ hybridization is a qualitative technique that cannot discriminate between minute variations in gene expression levels. However, the large differences in c-fos hybridization signal observed between bone biopsy samples from patients with fibrous dysplasia and normal individuals support our conclusion of increased c-fos expression in patients with the disease. Moreover, the diseased cell population that overexpresses c-fos in the lesions is not present in normal bone.

The expression of the c-Fos protein normally correlates with c-fos mRNA levels. Attempts were made to detect the c-Fos protein on methylmetacrylate-embedded sections at our disposal. However, this type of embedding has a tendency to destroy antigenic epitopes[325]. The limited availability of biopsy material has restricted this avenue of investigation.

The overexpression of c-fos is restricted to fibrous dysplasia lesions. Both polyostotic and monostotic lesions show elevated levels of the proto-oncogene (not shown). We did not detect increased c-fos mRNA levels in biopsy samples from patients with other bone diseases showing high bone turnover, such as HVDRR (hypocalcemic vitamin D-resistant rickets) (data not shown). The expression of

c-fos was also normal in fibrotic lesions from Paget's disease, Proteus syndrome, and Osteogenesis Imperfecta type IV (figure 27). Thus, the elevated c-fos expression appears specific to fibrous dysplasia lesions and is not linked to a high turnover phenotype nor to fibrous tissue in general. It is interesting to note that lesions from Paget's disease of bone do not exhibit increased c-fos expression, since fibrous dysplasia is sometimes considered a juvenile form of Paget's disease due to the similar appearance of the lesions.

Shenker et al.[271] have recently reported the presence of an activating mutation in the alpha subunit of the stimulatory G protein (Gsα) coupled to adenylate cyclase in bone cells from patients with monostotic fibrous dysplasia. Similar activating mutations have also been described in tissues from patients with the McCune-Albright syndrome[265], which exhibit polyostotic fibrous dysplasia as one symptom of the disease. Thus somatic mutations in the Gsα gene appear to underlie the clinical manifestation of both monostotic and polyostotic fibrous dysplasia. The G proteins are a family of guanine-nucleotide binding proteins involved in various transmembrane signaling systems, including the synthesis of cAMP through the adenylate cyclase pathway. Gsα activates and Gi inhibits adenylate cyclase in response to hormonal stimuli. The G proteins are heterotrimers composed of α, β and g subunits. Upon binding of a stimulatory ligand to its receptor, the activated receptor interacts with Gs and causes it to release guanosine diphosphate (GDP). This allows the guanine nucleotide binding

site of the α subunit to bind guanosine triphosphate (GTP) and triggers the dissociation of the α chain from the heterotrimer. The Gsα protein stimulates the activity of the adenylate cyclase enzyme and thus the synthesis of cAMP. Since the α subunit has intrinsic guanosine triphosphatase (GTPase) activity, the hydrolysis of GTP to GDP promotes the reassociation of the Gsα subunit with the beta-gamma complex, thus returning the Gsα to its resting state. The activating mutations that have been described in the Gsα gene cause substitutions of the arginine residue at position 201 by either histidine or cysteine[265], thus resulting in an abnormal Gsα protein with reduced GTPase activity that can continually stimulate adenylate cyclase in a constitutive fashion.

Accumulation of intracellular cAMP activates protein kinase A to phosphorylate specific protein substrates and leads to the regulation of gene transcription by modulating the phosphorylation state of members of the CREB (cAMP Response Element Binding proteins) family of transcription factors[326]. These nuclear proteins bind specific palindromic sequences termed CREs (cAMP Response Elements) in the promoters of target genes to modulate their transcription[327]. The c-fos promoter contains a well characterized CRE[328]. Thus we propose that the increased production of cAMP in bone cells harboring the Gsα mutation leads to elevated c-fos expression through the c-fos CRE. Indeed, we have detected the cysteine and histidine mutations at position 201 of the Gsα gene in patient number 1 and number 2, respectively (data not shown). Antibodies are available to

specifically detect the phosphorylated form of CREB[329]. Moreover, since the transgenic animal lesions developed as the sole consequence of c-fos overexpression, the similar human lesions may also develop solely due to the overexpression of c-fos alone. Despite the pleotropic effects that elevated levels of cAMP may have on cellular regulation, the overexpression of c-fos may be sufficient to cause the bone disease symptoms alone.

Not every bone is affected in patients with fibrous dysplasia. It has been documented that there is a variable distribution of cells containing the Gsα mutation in tissues, including bone[271], from patients with the McCune-Albright syndrome[265]. These results suggest that the sporadic occurrence of the bone lesions in McCune-Albright and fibrous dysplasia is due to a postzygotic somatic mutation that results in mosaicism. Our results showing normal c-fos expression in non-affected bone from patients with fibrous dysplasia (see figures 24 and 26) also support the notion of mosaicism in the expression of the activating mutation.

Overexpression of c-fos through the use of strong constitutive promoters and removal of the A/U-rich destabilizing sequence leads to oncogenic transformation in tissue culture[274]. Transgenic mice overexpressing c-fos, which initially develop bone lesions resembling those observed in patients with fibrous dysplasia[245], go on to develop osteosarcomas[246] and chondrosarcomas[249]. The c-Fos protein is also overexpressed in the majority of human osteosarcomas[243]. It is interesting to note

that a certain proportion of fibrous dysplasia lesions eventually degenerate into osteosarcomas[330]. It does however, seem to be more difficult for human lesions to degenerate into osteosarcomas than for murine lesions. This phenomena is however also observed when transforming human cultured cells with c-fos as compared to murine cells[331]. In any case, our results showing increased expression of c-fos in fibrous dysplasia lesions suggest that the overexpression of c-fos may represent the first hit in the multistep carcinogenesis of bone sarcomas.

The overexpression of c-fos in osteogenic cells appears to impair their normal function in remodeling and proliferation, but not differentiation. It has been suggested that constitutive c-fos expression would result in phenotype suppression in bone cells[260]. This would prevent differentiation along the osteoblastic lineage and inhibit the expression of genes normally found in the fully differentiated osteoblast. Our data partially supports this model. Bone is being formed and rapidly turned over in fibrous dysplasia lesions despite the abnormal morphological appearance of the osteoblasts. This suggests that differentiated, bone-forming osteoblasts are present in the context of constitutive high levels of c-fos expression and are not suppressed. However, this bone is not properly remodeled. It would appear that the overexpression of c-fos in the osteoblastic lineage actually interferes with the regulation of bone remodeling and osteoblast proliferation rather than with osteoblastic differentiation per se. The data obtained from the transgenic mice lines overexpressing c-fos[248] supports this hypothesis.

These mice exhibit improperly remodeled long bones with active preosteoblastic proliferation. The overexpression of c-fos affects the osteoblast lineage in these cells since osteosarcomas can develop. The remodeling hypothesis is supported by the fact c-fos overexpression in osteoblasts in vitro can cause the secretion of a soluble factor which promotes differentiation and resorbtive function of the osteoclast[50]. This role in remodeling hypothesis is further supported by the observation that homozygous mice for null alleles of c-fos show impaired bone remodeling leading to an osteopetrotic phenotype.

Our work suggests that the transgenic mice lines overexpressing c-fos under the control of a heterologous promoter could be used as an animal model of the disease to test the therapeutic value of treatments aimed at reducing the expression of c-fos in the affected tissue. More effective therapeutic strategies can also be developed. Pamidronate therapy will bypass the effects of high cAMP and block the effects of c-Fos on bone turnover. By more precisely understanding the exact role of c-Fos in bone remodeling and proliferation we can then devise even better and more effective therapeutic strategies to block these effects. By manipulating ways in which c-fos can be suppressed we may be able to utilize these mechanisms to inhibit the expression of the gene. Moreover, we now have a transgenic animal model to test these ideas.

The qualitative properties of in situ hybridization may make it difficult to use the

technique as a diagnostic tool. It will be difficult to establish how much c-fos overexpression is too much. Until it can be shown otherwise, only the molecular diagnosis of the Gsα mutation is definitive. It is more simple to directly isolate DNA from the blood and amplify the mutation by PCR. We can then identify it using a dot blot technique. The in situ hybridization technique can instead be applied to study aberrant gene expression in other pathologies.

Our results show increased c-fos expression in bone lesions from patients with fibrous dysplasia. This suggests that we have identified a nuclear event occurring downstream of the activating mutation in the alpha subunit of the G protein regulating adenylate cyclase activity. Since the overexpression of c-fos in the transgenics was sufficient to cause the disease phenotype, c-fos overexpression in the lesions may be sufficient to cause the human bone symptoms. Moreover, we have demonstrated that the high c-fos expression levels are specific to fibrous dysplasia and are not observed in other bone diseases exhibiting fibrous tissue or high bone turnover. This strongly supports a role for the increased expression of c-fos in the etiology of fibrous dysplasia. Therefore, fibrous dysplasia provides a unique situation whereby we can link a cell surface mutation to a nuclear event that may be sufficient to result in a disease phenotype.

VIII. Summary and Conclusion

The expression of the c-fos proto-oncogene during normal and pathological bone development was investigated. The calcitriol regulated expression of c-fos in osteoblast-like cells and the role of c-fos in the etiology of fibrous dysplasia was addressed.

Calcitriol is a known modulator of bone remodeling and cellular differentiation. The transient expression of the c-fos proto-oncogene has also been implicated in regulating bone remodelling and cellular differentiation. We have looked at the effects of calcitriol (1α,25-dihydroxyvitamin D_3) on the expression of the members of the fos and jun families of proto-oncogenes in an osteoblastic cell line and in primary cultures of osteoblasts. Calcitriol treatment of starved, confluent cultures of MC3T3-E1 cells induced a rapid and transient stimulation of the expression of c-fos, fos B, c-jun and jun-B with varying kinetics. The expression of fra-1 and jun-D was not affected by calcitriol in those cells. The selective stimulation of fos and jun family members by calcitriol was also observed in primary cultures of osteoblasts isolated from newborn mouse calvaria, suggesting that this modulation is a physiological response of the bone cells and not an artefact of the established cell line. The calcitriol effect was specific and dose-dependent. The expression of the c-Fos protein correlated with the expression of the mRNA in calcitriol-treated cells. The calcitriol-induced stimulation of c-fos expression was

modulated, at least in part, at the level of the initiation and elongation of transcription, whereas its effects on c-jun and jun-B expression was controlled at the post-transcriptional level by a mechanism that does not implicate stabilization of their respective mRNAs. The differential stimulation of the expression of certain members of the fos and jun families by calcitriol support a role for these oncoproteins in bone cell physiology.

The mechanism by which calcitriol transiently stimulated the transcription of the c-fos proto-oncogene in osteoblastic cells was further investigated. We have identified and characterized a vitamin D response element (VDRE) in the promoter of c-fos. The vitamin D responsive region was delineated between residues -178 to -144 upstream of the c-fos transcription start site. Mutations that inhibited binding to the sequence concomitantly abolished 1,25-(OH)2D3-induced transcriptional responsiveness, demonstrating that we have identified a functional response element. The structure of the c-fos VDRE was found to be unusual. Mutational analysis revealed that the c-fos VDRE does not conform to the direct repeat configuration where hexameric core binding sites are spaced by a few nucleotide residues. In contrast, the entire 36 bp sequence was essential for binding. As expected, we have identified the vitamin D receptor (VDR) as a component of the complex that bound the c-fos VDRE. However, our results showed that the previously characterized dimerizing partners of the VDR, retinoid X receptors, retinoic acid receptors, and the thyroid hormone receptors, were not

involved as binding partners to the c-fos VDRE in bone cells. These data suggest that the VDR heterodimerized with a novel partner to bind the c-fos VDRE and further hint at new molecular mechanisms of action for vitamin D.

We have used in situ hybridization to analyze c-fos expression levels in bone biopsies from normal individuals and patients with fibrous dysplasia to evaluate the putative contribution of c-fos overexpression to the etiology of the disease. The analysis of c-fos expression was performed on undecalcified bone tissue sections. Appropriate controls have established the specificity of the assay. High levels of c-fos expression were detected in bone biopsies from eight patients with the disease. Virtually no signal was observed in bone sections from four control individuals. The cells that express c-fos in the dysplastic lesions are the fibroblastic cells that populate the marrow space. No signal was detected in four biopsies from patients with other bone diseases where high turnover and fibrotic tissue are present (osteogenesis imperfecta, Paget, vitamin D pseudo-deficiency). These results suggest that high levels of c-fos expression are characteristic of fibrous dysplasia lesions and are not associated with other fibrotic bone tissue. Our results support the implication of c-fos in the etiology of fibrous dysplasia and suggest that we have identified the molecular mechanisms acting downstream of the activating Gsα mutation in this disease.

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