DISSERTATION

GROWTH FACTORS, ONCOGENES, AND ANTIONCOGENES IN THE PATHOGENESIS OF OSTEOPETROSIS AND OSTEOSARCOMA

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 1991

COLORADO STATE UNIVERSITY

March 5, 1991

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ABDELAZIZ REBATCHI ENTITLED GROWTH FACTORS, ONCOGENES, AND ANTIONCOGENES IN THE PATHOGENESIS OF OSTEOPETROSIS AND OSTEOSARCOMA BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

GROWTH FACTORS, ONCOGENES, AND ANTIONCOGENES IN THE PATHOGENESIS OF OSTEOPETROSIS AND OSTEOSARCOMA

MAV-2(0) induced avian osteopetrosis is characterized by periosteal bone proliferation in long bones. Comprehensive cellular investigations to detect and understand factors involved in the proliferation of osteopetrosis cells have not been reported. In addition, osteopetrosis has never been shown to evolve to malignancy or neoplasia and this characteristic of the disease has not been studied. To investigate the evolution to neoplasia through the study of gene expression, canine osteosarcoma samples were also included in these investigations. cDNA probes specific for tumor suppressor genes, growth factors and oncogenes, were used to determine the expression of these genes in osteopetrotic, non-inoculated controls, 10 day-old inoculated chickens, and canine osteosarcoma samples. Prior to these investigations, a protocol for bone RNA extraction was developed. In these studies, mRNA specific for Wilms' tumor suppressor gene was detected in osteopetrotic samples. This gene was not expressed in non-infected

chicken controls, 10 day-old-inoculated chickens or in canine osteosarcoma samples. The expression of a potent mitogenic factor c-erb B, confirmed the proliferative nature of osteopetrosis. Since osteopetrotic cells display some level of differentiation as opposed to canine osteosarcoma cells which are not differentiated, it is concluded that Wilms' tumor gene acts as a differentiating factor preventing osteoblastic cells from entering the cycle of neoplasia, since the action of Wilms' tumor gene is not antioncogenic.

These results indicate that avian osteopetrosis appears to be the result of a concomitant expression of both an oncogene (c-erb B) feeding the proliferative phenotype, and a tumor suppressor gene (Wilms' tumor suppressor gene) that keeps these cells in check. Other supporting results were obtained; platelet-derived endothelial cell growth factor was significantly expressed in both osteopetrotic and osteosarcoma samples, suggesting a common pathogenic aspect of both clinical entities. Bone morphogenic protein-1 (BMP-1) was highly expressed in one sample only. Platelet-derived growth factor was weakly expressed in one osteopetrotic sample. These results suggest a sequel of a previous involvment in the pathogenesis of osteopetrosis and confirm that osteopetrotic cells are at higher stage of differentiation. BMP-2 and BMP-3 were not expressed in this system suggesting that they might be brought to the bone matrix by the circulation, or act at an earlier stage

of differentiation. Finally, the observation that 10 day-old inoculated chickens did not show any expression of any mitogenic factor appears to confirm that these chickens do not develop osteopetrosis because their bone cells are differentiated and therefore have a different set of gene regulatory proteins which makes them non-permissive to the proliferative action of the virus.

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AKNOWLEDGEMENT

I should like to thank my adviser, Dr Ralph Smith for the outstanding literature he gave us to read. It helped. I also would like to thank him for giving me the green light to develop every project I wanted to do.

I should also like to thank my co-adviser, Dr Robert Norrdin for doing everything he could to help. In addition of his introducing me to the bone world, he provided me with every support, advice, suggestion, to make this project more successful.

My other members of committee, Dr. Leonard Pearson, Dr. Peter Gasper, Dr. James Collins are to be thanked, for I was impressed by their willingness to help and by their fairness and understanding.

During my molecular work, I was continuously helped by Dr Jonathan Carlson and his laboratory people. The list is too long to enumerate it. I will never stress their help enough. This help came especially from Vicki McGrane who was kind enough to take the time to show me some molecular techniques before I was able to fly with my own wings.

I am also very indebted to Dick!, sorry Dr Richard Grant, who never hesitated to leave his work to provide

me with any assistance I needed. I am pleased to see this help continued even after he left. The frequent discussions we had on different matters.....were almost the only fun I had in the lab and I owe him that.

Dr Dahn Clemens' support and advices were of great help during his time as a graduate student and especially after he left. He was also fun to be with and his departure was regrettable. I am very grateful for his help.

I am greatly indebted to all the scientists listed in table 4.1, who were kind enough to send me the cDNA probes. Without their help, this work would certainly not have been possible.

I should like to thank Roxanne Curtis and Erin Haywood in Dr Smith's laboratory for their excellent care of the chickens.

I certainly wish to thank all other graduate students and research associates that helped one way or the other.

I wish to thank Steve Costigan, then in Dr Demartini's lab for the numerous advices concerning RNA. His help came at a point where I really needed help with my RNA work and he definitely was the right person to talk to.

I should like to thank Dr Carol Blair, our department head, for her help and understanding, as well as her staff especially Carmen Pando and Marlene Dunlap for their efficient handling of administrative or other matters.

I should like to thank the department of microbiology (C.S.U) for awarding me the Graduate Teaching Award. It was a very happy moment.

I would like to express my thanks for the kind and friendly help from Jo and Wayne in Dr. Cockerell's lab.

Thanking Dr Gordon Scott, my adviser at the University of Edinburgh as well as his staff, is not enough to express my gratitude, for he was the person who introduced me to the fascinating world of research and was ever since extremely helpful in every matter I seeked him for.

My former boss at the National Institute of Animal Health, Dr Toufik Bereksi was extremely understanding of my determination to carry on graduate studies. I would like to thank him for allowing me to pursue these studies I wanted so much. In addition, I would like to express my deep appreciation for the moral support he provided during difficult circumstances.

The care, concern and support I received from my family during these studies was extemely precious and boosted my moral when I needed it the most. These thanks are especially directed to my brother Ali who had the kindness of keeping my moral high during our frequent discussions.

DEDICATION

This dissertation is dedicated, with great pleasure, to my nieces Amina and Assia and to my nephew Abdel-Illah. They were my most fervent supporters. I hope this will encourage them to continue their scholastic success which I have no doubt will still be as remarkable as before.

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CHAPTER 1

INTRODUCTION

Ever since its first recognition and description (Pugh et al., 1927), chicken osteopetrosis was shown to have a viral etiology, and the virus classified within the avian leukosis complex (Holmes, 1959 ; Beard, 1963). Aurigemma et al., (1991), made and analyzed several viral recombinants, and demonstrated that the part of the genome responsible for the observed MAV-2(0) osteopetreosis was the env-LTR portion of the genome. While the mechanism by which ALV strains induce lymphoid leukosis was explained by Neel et al., (1981), the mechanism by which MAV-2(0) causes the proliferation of osteoblastic cells in osteopetrosis is not known. Given the low frequency of LL tumors observed compared to the otherwise high frequency in osteopetrosis induced by MAV-2(0), it becomes clear that their respective mechanisms has to be different. In contrast with lymphoid leukosis, osteopetrotic osteoblastic cells are not clonal (Robinson and Miles, 1985; Aurigemma et al., 1989); so far there has never been any proof linking the integration of the virus near one given oncogene as being at the origin of osteopetrosis. The probability of the virus integrating near one given oncogene is rather low and inconsistent with the observed high frequency of osteopetrosis.

In order for the frequency to be that high, one has to admit that the mechanism of osteopetrosis must be highly reproducible, otherwise the high frequency of the lesions would not be observed. Since the answer does not appear to be in the chicken genome, it has to be looked for elsewhere

An important feature of osteopetrosis is the persistent lack of progression to neoplasia despite the high proliferation rate of osteoblastic cells.
In acute myeloid leukemia (AML), cells from an important subset of AML patients differ in exhibiting autonomous proliferation in vitro based on an acquired capacity of the cells to synthesize one or more of the above regulators, and these cells seem to represent an example of genuine autocrine growth stimulation (Young et al., 1987; Oster et al., 1988). The remarkable colony stimulating factor dependency of myeloid leukemia populations indicate that CSFs are essential cofactors in the development of myeloid leukemia. Without the necessary proliferative stimulation by these CSFs, it would not be possible for a transformed myeloid leukemic cell to generate the expanding leukemic cell clone that is myeloid leukemia although this was not shown to be the case in all patients (Medcalf 1989).

Research in bone biology designed to develop drugs mimicking the action of specific growth factors to treat osteopenic diseases has lagged behind when compared to the known available treatment of anemia by the injection of erythropoietin (EPO) (Golde et al., 1988). This lack of

progress is attributed, in part, to the cumbersome and extremely complex way of extracting RNA from bone to study the sequential gene expression of the growth factors needed for the final development of the bone because of the texture of the latter (Jerome 1990).

The origin of the bone morphogenic proteins (BMPs), factors that specifically initiate the complex process leading to the formation of bone, is unknown (Wozney et al., 1988). There is also a controversy as to whether BMP-3 or "osteogenin" considered to be the same protein (Luyten et al., 1989) is involved in the proliferation of cartilage and ending up with fully differentiated bone cells (Sampath et al., 1987; Katz and Reddi, 1988; Vukicevik et al., 1989; Reddi et al., 1989) or induce cartilage only, when implanted sub-cutaneously together with demineralized bone matrix (Wozney et al., 1988).

The goal of this project was to develop a reliable protocol for the extraction of bone RNA, in order to study the pathogenesis of osteopetrosis as well as osteosarcoma through the expression of oncogenes, anti-oncogenes, and growth factors in osteopetrotic chickens and in dog osteosarcoma. A reliable and efficient protocol for the extraction of bone RNA was developed.

In addition, this work has provided insights into the pathogenesis of osteopetrosis and canine osteosarcoma at the cellular level, by demonstrating how MAV-2(0) causes bone cells to proliferate and why avian virus-induced

osteopetrosis does not evolve to neoplasia.

Furthermore it also showed that only bone morphogenic protein-1 (BMP-1) was expressed by bone cells. The non-expression of BMP-2 and BMP-3 appears to suggest that they are involved at an earlier stage of differentiation during chondrogenesis.

CHAPTER 2

LITERATURE REVIEW

Bone modeling and remodeling

Bone Modeling

Bone modeling is the process that results in the final shape. Modeling primarely affects the surface of the cortical bone, and is mainly observed during bone development and during bone repair following fracture.

Bone Remodeling

Although it is commonly assumed that bone is an inert tissue, it is continuously renewed in a process called remodeling. During bone formation, osteogenic cells (osteoblasts) deposit a matrix composed of collagen and a variety of proteins and carbohydrates. This matrix is further mineralized following deposition of calcium and phosphorous. This mechanism insures a continuous turnover and replacement of the bone matrix.

Bone resorbtion and deposition are continuous and are concomitant. While bone resorption is mediated by osteoclasts, bone formation is carried out by osteoblasts.

Origin of Bone Cells

Osteoblasts and osteoclasts were originally thought to be derived from a common precursor (osteoprogenitor) cell, which developed into either cell type depending on the local stimulis (Young, 1962; Owen, 1970). It is now believed that osteoclast and osteoblast are derived from the hemopoietic and stromal cell systems in marrow, respectively (Friedenstein, 1976; Owen, 1978).

Origin of Osteoblasts

The osteoblast is derived from a nonmigratory connective tissue cell (Friedenstein, 1976). Its immediate precursor is the preosteoblast, a fibroblastic cell capable of proliferation, located near osteoblasts and bone surfaces (Owen, 1971). On the basis of morphologic evidence, the soft connective tissue of periosteal and endosteal surfaces and haversian canals of bone (bone stroma) are thought to be continuous with marrow stroma (Bassett et al., 1961; McLean, 1968). From these studies a consensus emerged that marrow stroma and osteogenic soft connective tissue (periosteum) give rise to bone tissue and marrow stroma (Owen, 1978). The direct proof, associating fibroblasts as the origin of osteoblasts came with the demonstration that either marrow cells or fibroblasts grown *in vitro* from marrow cells form a viable calcified tissue within a few weeks (Friedenstein et al., 1970; Friedenstein, 1973). Fibroblasts cultured from the stromal system of other immunohemopoietic tissues (thymus,

spleen, lymph node), peripheral blood, and peritoneal fluid, were investigated with the same diffusion chamber method. In these cases, only a soft fibrous tissue which was not calcified was formed in the chambers (Friedenstein, 1973). However a calcified tissue was formed following the adding of an inducing agent (Friedenstein, 1968). This suggests that fibroblasts are different and their differentiation towards a given differentiated cell is conditioned by the action of a specific factor. Moreover, induction of cartilage and bone in skin and muscle by implantation of inducing agents such as transitional epithelium of the bladder, decalcified bone matrix, and bone morphogenic protein has been described (Reddi, 1975). These fibroblasts are different from the marrow stromal cells in that they require inducing agents in order to differentiate to bone cells. This suggests that these inducible agents contain a specific differentiating factor that specifically determines the future of these fibroblasts as being osteoblasts rather than any connective tissue cell. These cells could be compared to predetermined marrow stromal cells (before they are committed to the osteogenic lineage) (Friedenstein, 1976; Friedenstein, 1973).

Origin of Osteoclasts

Substantial research findings support the conclusion that the osteoclast is derived from the hemopoietic stem-cell via a blood-borne mononuclear cell (Fischman et al.,

1962; Gothlin et al., 1976). Experiments with beige mice were instrumental in associating the origin of osteoclasts with the mononuclear cell lineage. For example, beige mice have giant lysosomes in granular leukocytes, monocytes, and osteoclasts but not in fibroblasts and osteoblasts (Ash et al., 1980; Ash et al., 1980; Oliver et al., 1975). Furthermore both monocytes and macrophages demonstrate resorptive activity when incubated with bone _in vitro_ (Mundy et al., 1977; Teitelbaun et al., 1979; Kahn et al., 1978).

Generalities about fibroblasts

In order to understand bone cells' differentiation it is worth describing some concepts about fibroblasts. These cells seem to be the most versatile of connective tissue cells, displaying a remarkable capacity to differentiate into other members of the family such as adipocytes, smooth muscle cells, bone cells and cartilage cells. However there is good evidence that fibroblasts in different parts of the body are intrinsically different, and it is far from certain that all fibroblasts in a given region are equivalent (Conrad et al., 1977).

If a preparation of bone matrix, made by grinding bone into a fine powder and dissolving away the hard mineral component, is implanted in the dermal layer of the skin, some of the cells there, probably dermal fibroblasts, become transformed into cartilage cells and, a little later, others into bone cells, thereby creating a small

lump of bone, complete with a marrow cavity (Reddi et al., 1977). These experiments suggest that components in the extracellular matrix can dramatically influence connective tissue-cell differentiation. In fact, bone matrix has been found to contain, trapped within it, high concentrations of several growth factors that can affect the behavior of connective tissue cells (Hauschka et al., 1986; Schor et al., 1987). Furthermore, the extracellular matrix may influence the differentiated state of connective-tissue cells through physical and chemical effects. When chondrocytes are grown in suitable medium, cartilage cells proliferate and maintain their differentiated character. However under conditions where cartilage cells are kept at relatively low density and remain as a monolayer on the culture dish, a transformation occurs, in that the cells dedifferentiate to fibroblastic cells and stop producing type II collagen (type specific to cartilage) and start producing type I collagen (specific for fibroblasts). When chondrocytes are then transferred to a dish of agarose, and held suspended without any attachment to a substratum, they adopt a rounded shape and quickly revert to the chondrocyte phenotype and start making type II collagen (Benya and Schaffer, 1982). It appears therefore that chondrocytes can revert to their fibroblastic phenotype. Most importantly, it shows that the differentiation of fibroblasts into bone cells is not only consequent to the actions of growth factors but also to the

influence of the state of the surrounding matrix demonstrating therefore the complexity of fibroblast differentiation to bone cells.

Physiology of Bone Formation

Bone formation is a very complex phenomenon. Several components such as hormones, growth factors, vitamins as well as minerals and oligoelements contribute, in a complex interplay, to bone formation. Several components have been found to be directly involved, including hormones such as parathyroid hormone and calcitonin. Parathyroid hormone potentiates calcium levels in the blood by inducing the resorption of bone. Calcitonin is a hypocalcemic hormone and induces a decrease of calcium in the serum and contributes therefore to the stabilization of the calcium level in the bone.

Minerals

Many minerals contribute to the bone formation. However calcium and phosphorous are badly needed for such process to happen, since they mineralize the matrix.

Bone Matrix

Extracellular matrix accounts for about 90% of the total weight of compact bone and is composed of microcristalline calcium phosphate resembling hydroxyapatit (60%) and fibrillar type I collagen (27%). The remaining 3% consists of minor collagen types and other

proteins including osteocalcin, osteonectin, matrix 4-carboxyglutamic acid protein, phosphoproteins, sialoproteins and glycoproteins, as well as proteoglycans, glycosiminoglycans, and lipids (Boskey et al., 1984).

The role of growth factors in bone formation

Bone exibits the most complex spectrum of growth factors activities of any tissue yet described (Hauschka et al., 1986). Systemic and local growth factors interact in complex and as yet poorly understood ways to regulate the process of bone formation and remodeling (Redhead, 1990). Growth factors are divided into two categories: those factors that specifically induce new bone formation and local and systemic factors that regulate bone formation (Table 2.1 based on Centrella et al., 1990). Systemic and local factors regulating bone growth have mainly been studied in cell culture. Growth factors induce a membrane protein kinase that is intimately related or identical to the cellular receptor, has a high molecular weight (greater than 150,000), and enhances the phosphorylation of the receptor and of additional endogenous molecules. However it is not known whether these effects are related to the mitogenic activity of growth factors (Canalis, 1985). In addition, it is commonly thought that an overlap exist between growth factors and oncogene products. Many if not all, oncogenes are now perceived as functional components of a mitogenic cascade (Stiles, 1985).

Systemic growth factors

1. Platelet derived growth factor

PDGF involvement in bone formation is reported (Canalis 1985; Reddi et al., 1972). Sub-cutaneous implantation of demineralized bone matrix is followed by a transient inflammatory response (day 1), migration of mesenchymal stem cells by chemotaxis (Reddi et al., 1972; Somerman et al., 1983) into the area of the implant and proliferation on day 3 (Rath and Reddi 1979; Sampath et al., 1982). Proliferation of the cells is followed by their differentiation into chondrocytes (day 5-7), osteocytes (days 9-12) and hematopoietic marrow (day 21) (Reddi, 1981). Under some circumstances, soft tissue repair is not maximal (Grotendorst et al., 1984) and can be increased by various growth factors (Howes et al., 1988). It was of interest to determine whether PDGF could influence the rate of bone repair. To investigate this possibility, demineralized matrix powder was implanted in the presence of various doses of PDGF ranging from 20 to 100ng (Howes et al., 1988). However PDGF did not affect bone growth in young rats (70-100g), perhaps because of a high spontaneous growth rate (Howes et al., 1988). The results indicate a stimulatory action of PDGF on bone growth, since exogenous PDGF increased the alkaline phosphatase activity and the calcium content of the *de novo* induced bone plaque in older rats (250-350gr) (Wozney et al., 1988).

Table 2.1: Synoptic action of systemic and local factors on bone formation and resorption [Based on Centrella et al., (1990)

Effects of Local and Systemic Factors on
Bone Formation and Resorption

Osteoinductive factors isolated from bone matrix

Osteoinductive factor (OIF)

Bone morphogenetic factors (BMPs)
- BMP-1
- BMP-2A
- BMP-3 (osteogenin)

Factors regulating bone formation and resorption

	Bone formation (osteoblasts)	Bone resorption (osteoclasts)
Local growth factors		
Transforming growth factor-β (TGF-β)	+,0,-	+,-
Insulin-like growth factors (IGFs)	+	?
β_2 microglobulin (β_2m)	+	?
Platelet-derived growth factor (PDGF)	+	+
Colony-stimulating factors (CSFs)	?	+
Transforming growth factor-α (TGF-α)	-	+
Interleukins (ILs)	+,-	+
Tumor necrosis factors (TNFs)	+,-	+
Interferon gamma (INF-γ)	-	
Fibroblast growth factors (FGFs)	+	0
Systemic factors		
Vitamin D	+	+
Vitamin A	+	+
Parathyroid hormone (PTH)	+	+
Calcitonin (CT)	0	-
Glucocorticoids	-	+

This result was confirmed by histological studies. These authors think that PDGF may recruit cells and stimulate bone formation over resorption *in vivo* (Howes et al., 1988). These results complement previous observations on PDGF. Platelet-derived growth factor makes fibroblasts competent to replicate, confers to cells arrested in the GO/G1 phase of the cycle the ability to enter into S phase and is therefore considered to be a competence factor (Canalis et al., 1985). Progression of these competent cells from the S phase through the rest of the cell cycle requires the presence of additional plasma factors such as insulin, insulin growth factor and EGF, which are called progression factors (Canalis et al., 1985). Interest in studying PDGF originates from the reason that it is a mitogenic factor and would be of interest to test it in our system.

2. Endothelial Cell Growth Factor

Endothelial cell growth factor is a mitogen for human endothelial cells isolated from bovine brain and is a member of a family of polypeptides that includes acidic fibroblast growth factor (aFGF) and eye-derived growth factor II (Canalis, 1985; Thomas, 1987). Two forms of ECGF have been described: alpha-ECGF and beta-ECGF. The two forms have the same biological properties and significant amino-acid homology (Esch et al., 1985). Alpha-ECGF is identical to acidic FGF (Gimenez et al., 1985). When amino-acid ECGF was assayed for bone formation

it was shown that the administration of the protein at a dose 0.1 to 100 ng/ml stimulated fetal rat calvarial DNA synthesis, and resulted in a generalized increase in protein synthesis (Canalis and Raisz, 1987). This effect was shown to be time-dependent since this result was seen only after after 48-96 hours treatment. The stimulatory effect of ECGF was not specific for collagen, in fact, a larger effect was observed on non-collagen than on collagen protein synthesis, but the collagen synthesized was type I, indicating that ECGF stimulated the replication of cells which included those of the osteoblastic lineage (Canalis and Raisz, 1987). The stimulatory effect of ECGF on endothelial cell replication and neovascularization in correlation with those on bone cell replication may be important for bone repair, particularly after fractures (Canalis and Raisz, 1987). Moreover, the mitogenic effect of ECGF on endothelial and bone cells is enhanced by heparin, and the fracture callus is rich in heparin-containing mast-cells (Lindholm et al., 1969). Paradoxically ECGF decreased bone collagen synthesis in 24 hour cultures, which indicated a direct inhibition of osteoblastic function. ECGF had no affect on bone degradation or resorption. This action of aFGF (ECGF) was not observed by other authors because they were able to show that, in vivo, only PDGF induces cartilage and bone growth, whereas FGF just like insulin growth factor or EGF, did not induce any bone formation different from the

control non-inoculated animals (Howes et al., 1988).

3.Fibroblast Growth Factor

Fibroblast growth factor is a peptide with a Mr of 13,000 isolated from bovine pituitary glands, and stimulates cell replication in cultured fibroblasts, myoblasts, and chondrocytes, among other cells (Canalis, 1985; Thomas, 1987). The effects of pituitary FGF but not of the brain peptide (ECGF or aFGF), have been examined in bone, and they are similar to those of EGF, although the latter is more potent (Canalis, 1985). This factor stimulates cell replication in both cartilage and bone in culture systems. In bone, FGF inhibits the synthesis of type I collagen, and decreases alkaline phosphatase activity. Therefore FGF stimulates cell replication and inhibits osteoblastic differentiation (Canalis, 1985). FGF does not have major effects on bone resorption and its effects on bone mineralization are unknown.

3.Insulin-like Growth Factors

Insulin-like growth factors or somatomedins have been classified as growth hormone-dependent peptides that stimulate cartilage or linear growth. There are two major circulating somatomedins: IGF I and IGF II (Canalis, 1985). IGF I is produced in the liver and is GH dependent and considered to be the main circulating mediator of the growth-promoting effect of GH (Philips, 1980; Herington, 1983). Local administration of GH at the site of the epiphyseal growth plate stimulates unilateral bone growth

in hypophysectomysed rats (Isaksson et al., 1982; Russell, 1985). Chondrocytes from rabbit ear and epiphyseal growth plate have specific binding sites for GH (Eden, 1983). In order to determine the cellular expression of IGF I, a solution hybridization assay was used to detect specific IGF I mRNA in rat rib growth plate. Hypophysectomy resulted in a decrease in the number of IGF I mRNA copies compared to that in normal rats. Replacement treatment with GH restored the number of transcripts in a specific and dose dependent manner and give further support to the stimulatory effect of GH on longitudinal bone growth (Isgaard et al., 1988). In cartilage, IGF I stimulates DNA and proteoglycan synthesis whereas in bone it stimulates DNA, collagen and non-collagen protein synthesis (Canalis, 1980). Thus, and in contrast with the other systemic factors, IGF I induces cell proliferation and differentiation.

4. Platelet-derived endothelial cell growth factor (PD-ECGF).

Although this growth factor has not been shown to be involved in bone formation, the author thought it would be of interest to test it in this system because of its specific mitogenic action on endothelial cells. In addition Powers et al., (1987), suggested that thrombi generated by bone lesions, could induce the release of growth factors that will be responsible for the osteoblastic proliferation. This factor has a relative

molecular mass of ~45, 000 (45K) when purified to homogeneity from human platelets (Myazono et al., 1987; Myazono and Heldin, 1989). This protein, in contrast to the fibroblast growth factor family (Lobb et al., 1986; Gospodarowicz et al., 1986), does not bind heparin and does not stimulate the proliferation of fibroblasts (Myazono et al., 1987). This protein stimulates the specific cell growth of endothelial cells (Ishikawa et al., 1989). The deduced primary structure of PD-ECGF shows no similarity with other known proteins (Ishakawa et al., 1989). PD-ECGF is suggested to have a role in maintaining the integrity of blood vessels (Ishikawa et al., 1989). PD-ECGF has chemotactic action for endothelial cells in vitro (bovine aortic endothelial cells). In addition an angiogenic activity was shown in vivo; a strong angiogenic response on the developing vascular system of the chick chorioallantoic membrane was observed which was inhibited when specific antibodies to the factor were used (Ishikawa et al., 1989). These results demonstrate that the factor alone is responsible for the observed angiogenic response, eliminating therefore the possibility that an inflammation or any other factor could have induced this angiogenesis (Ishikawa et al., 1989). Angiogenic activity was also shown to greatly influence blood vessel development in tumors (Ishikawa et al., 1989) and justifies the examination of the role of this factor in our system because it might be a candidate for the pathogenesis of

osteosarcoma at least. Previous work on the pathogenesis of osteopetrosis has shown that MAV-2(0) induced vascular damage that resulted in the presence of thrombi (Powers et al., 1987). The authors hypothesized that this might be at the origin of the release of growth factors (BMPs), themselves responsible for the observed osteoblastic proliferation. However PD-ECGF could not be at the origin of bone proliferation because its action is limited to endothelial cells.

5. Prostate-Derived Growth Factors

Initial interest in the presence within prostatic tissue of growth factors for osteoblasts arose from the very high frequency with which prostatic adenocarcinomas are associated with osteogenic metastases (Jacobs et al., 1979). Two peptides with apparent molecular weights of 10,000 and 13,000 D were derived from hyperplastic tissue, whereas a single moiety of 10,000 D was obtained from malignant tissue. These entities increased cell numbers and alkaline phosphatase activity in osteoblastic cells consistent with effects on bone growth and differentiation (Koutsillieris et al., 1987).

6. Epidermal growth factor receptor.

This factor was not previously shown to be involved in bone formation. Therefore it was of interest to include it our system. Epidermal growth factor is an autophosphorylating tyrosine specific protein kinase (Ushiro and Cohen., 1980; Buhrow et al., 1982) and

stimulation of this activity by ligand is obligatory if the quiescent cell is to progress through the G1/S interphase of the cell cycle (Wells et al., 1990).

Activation of the EGF receptor initiates a cascade of cellular events mediated by triggering of the intrinsic tyrosine kinase (Carpenter et al., 1979). This is immediately followed by an increase in cytosolic free calcium concentration and receptor internalization before ending up with degradation of the EGF receptor. This triggered process leads to gene transcription within minutes. A few hours later, DNA synthesis and mitosis occur (Wells et al., 1990). Mutational studies showed that the tyrosine kinase activity is necessary for all subsequent receptor actions, including internalization (Wells et al., 1990). The identification of a mutant EGF receptor that does not undergo down regulation has provided a genetic probe to investigate the role of internalization in ligand induced mitogenesis (Wells et al., 1990). A transformed appearance in addition to anchorage independant growth were observed when these cells were subjected to ligand concentrations that previously failed to induce these responses (transformed appearance and anchorage independant) in cells expressing wild type receptors (Wells et al., 1990). According to the authors, these findings imply that activation of the protein tyrosine kinase at the cell membrane is sufficient for the growth induced (mitogenic) effect of EGF receptor. Consequently

downregulation could be an attenuating mechanism, without which, transformation would occur (Wells et al., 1990). The receptor mutant truncated at amino-acid 973 to 1022, was not followed by down regulation but had a competent kinase, with binding of ligand leading to increased transcription (Chen et al.,1989). An interesting approach to the factor is its downregulation by an adenovirus E3 gene product protein (10.4 K) (Carlin et al., 1989). This protein was shown to bind to EGF receptor and induces internalization and degradation of EGF receptor. The 10.4 K protein is not a growth factor that has some common sequences to EGF but rather has some common sequences to a region in EGF receptor at the cytoplasmic face of the transmembrane domain (Carlin et al., 1989). These authors conclude that down regulation of EGF receptor during adenovirus infection may occur by a new mechanism that necessitates the formation of heterodimers composed of 10.4 K protein and EGF receptor.

7. Immune factors

Other immune factors appear to have a mitogenic regulation role on osteoblasts, such as macrophage-derived growth factor (Rifas et al., 1984) and IL-I (Gowen et al., 1985).

Local growth factors

1. BMPs.

Urist first demonstrated that extracts of demineralized bone could induce new bone formation if implanted into ectopic sites in rodents (Urist, 1965). Further work with these extracts demonstrated that the whole process of bone formation complete with bone marrow formation, was shown to mimick the natural process of bone formation (Reddi et al, 1972). The next step was to purify the extracts and identify the biologically active proteins. Before knowing the specific protein in the bone extract that induces bone proliferation, the name BMP was used, because the active component in the bone extract was identified as being proteinaceous (Urist et al., 1979). Further studies in rats showed that the implantation of demineralized diaphyseal bone matrix in subcutaneous sites induced a sequence of events resulting in the local differentiation of endochondral bone (Reddi et al., 1972) and it is possible a specific bone inductive factor is associated with the matrix (Sampath et al., 1987). More recently a protein called "osteogenin" was isolated from bone matrix (Luyten et al., 1990). This protein stimulates the formation of new bone *in vivo* when implanted under the skin with insoluble matrix from collagen. However the purification of bovine bone extracts yielded three different polypeptides called BMP-1, BMP-2, and BMP-3 (Wozney et al., 1988). This lead to the isolation of the

three humans BMPs (Wozney et al., 1988). Sequencing studies showed that BMP-2 and 3 were members of the TGF Beta supergene family, while the third (BMP-1) appears to be a novel regulatory protein (Wozney et al., 1988). Each of the three appears to be independently capable of inducing the formation of cartilage in vivo (Wozney et al., 1988). This suggests that biological activities in original preparations were a mixture of different factors and that there is a complex interplay of factors in cartilage and bone formation (Wozney et al., 1988).

2. Transforming growth factor Beta

Bone represents the most abundant source of the peptide (Seyedin et al., 1985; Ellingsworth et al., 1986). In vitro studies have indicated that TGF Beta may be important in bone development, remodeling, or repair (Robey et al., 1987). However sequential studies of bone formation demonstrated that TGF Beta was expressed throughout the sequence of bone formation in implants (Carrington et al., 1988). However none of these aforementioned factors were shown to have a direct effect on bone formation when implanted sub-cutaneously with bone matrix (Wozney et al., 1988).

Antioncogenes

9. Wilms' tumor gene

The decision to investigate this gene in this system was not dictated by an interest in Wilms' tumor rather by

the observation that anti-oncogenes have a broad specificity. For example, the Wilms' tumor gene is involved in different clinical entities. In addition to its association with Wilms' tumor, this gene is involved in the genesis of aniridia (an absence of malformation of the iris), genitourinary (UG), and mental retardation (Francke et al., 1979; Riccardi et al., 1980; Turleau et al., 1984). Bladder carcinoma has also been correlated with the involvment of Wilms' tumor gene (Fearon et al., 1985). The Retinoblastoma gene was (in addition of its unequivocal involvment in retinoblasroma) shown to be involved in human osteosarcoma and in various other tumors (Bookstein et al.,1990; Hansen et al., 1985). It is now known that the <u>RB</u> gene has been shown to be the only gene implicated in the etiology of Retinoblastoma (Friend et al., 1986; Lee et al., 1987; Fung et al., 1984). The involvment of these gene products could be impairing oncogene expression or blocking the action of the encoded protein. The lack of the expression of this gene is shown to be at the origin of Wilms' tumor in 1 out of 15,000 children (Matsunaga, 1981). Wilms' tumor and retinoblastoma are considered to be genetically analogous in that both are explained in terms of a two-mutation model (Knudson, 1971; Knudson and Strong, 1972). Allelic mutations in germ line or postzygotic mutation must accumulate within a single gene (both alleles of genes need to be inactivated before a malignant transformation is initiated). Wilms' tumor gene is

situated on chromosome 11p13 in humans (Rose et al., 1990). Its location in the chicken chromosome is not known.

Pathophysiology of Osteopetrosis

Definition of Avian Osteopetrosis

Avian MAV-2(0)-induced osteopetrosis is a clinical entity characterized by an abnormal hyperplastic periosteal bone proliferation (Figure 2.1).

Morphometric studies were conducted in order to evaluate the relative number of osteoblasts and osteoclasts in MAV-2(0) induced avian osteopetrosis. There is no decrease in osteoclasts but an increase in osteoblasts (Schmidt et al., 1981). This shows that MAV-2(0) does not selectively destroy osteoclasts upon infection, since an increased number of these cells was observed. Therefore the enlargment of the bone is due to the unique increase of osteoblast numbers. This finding differentiates avian osteopetrosis from the mammalian form of the disease. Mammalian osteopetrosis has its origin in an osteoclastic defect rather than in an increase of osteoblasts and it occurs in humans, mice and rats (Bonucci et al.,1975; Handelman et al., 1958, Johnston et al., 1968, Schoefield et al., 1974; Shapiro et al., 1980; Walker, 1975a; Walker 1975b).

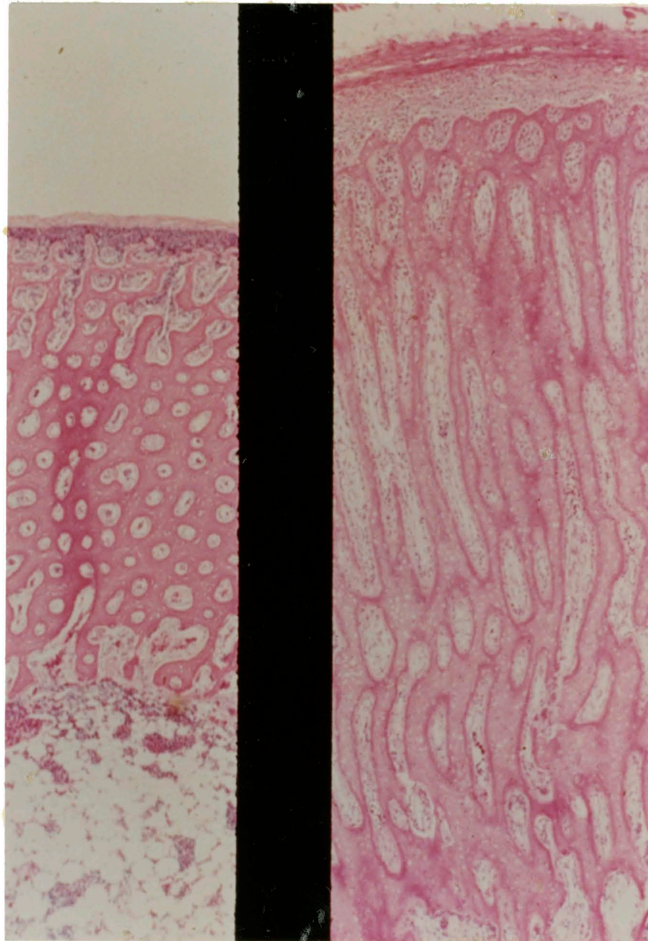


Fig.2.1: Cross section of a comparative 3 weeks old normal chicken bone (left side) and osteopetrotic chicken bone (right side). H.E stain. Same magnification (40 X). Decalcified. Courtesy, Dr Robert W. Norrdin. Pathl. Dept. C.S.U.

Recently, a more precise etiology for the genesis of osteopetrosis in mice was provided at the molecular level. The defect in op/op was thought to be associated with an intrinsic defect in haematopoietic progenitors (Yoshida et al., 1990). In order to investigate the origin of this deficiency, primary fibroblasts cell lines from op/op mice were established and their ability to stimulate the proliferation of macrophage progenitors was tested.

The results show that op/op fibroblasts are defective in production of functional macrophage colony stimulating factor (M-CSF), although its messenger RNA (Csfm mRNA) is present at normal levels.

The CSF mRNA was not effective because the sequence of the gene demonstrated a single base pair insertion in the coding region of the Csfms that generates a stop codon, 21 base pairs downstream from the initiating codon. This indicates that the generated protein is truncated and will not induce its specific biological action. Thus the op mutation is within the Csfm coding region of op/op fibroblasts, and the authors conclude that the pathological changes in this mutant resulted from the absence of effective M-CSF that will stimulate the proliferation of osteoclasts, themselves responsible for bone resorption (Yoshida et al., 1990).

Etiology of avian osteopetrosis

Retroviruses

The virus used for this study, MAV-2(0), is a member of the Retroviridae family. This family is subdivided into three 3 main subfamilies.

1. Spumaviruses

This is a group of foamy viruses found in cell culture and appear to have no importance in pathology.

2. Lentiviruses

This is an important subfamily because the viruses contained in this subfamily are characterized by latency, mutation, and induce immunosupression, and include Visna-Maedi and HIV.

3. Oncoviruses

These viruses are subdivided into two groups: (a) Oncogene-bearing viruses such as Rous sarcoma virus, avian myeloblastosis virus, and (b) oncogene- free viruses such as avian leukosis viruses, one strain of which, MAV-2(0), was used in these experiments.

General Considerations of RNA Tumor Viruses

On the basis of their appearance, RNA tumor viruses have been designated by the name of A,B,C particles by electron microscopists (Bernhard, 1958).

A type particles have toroidal nucleoids (Bernhard, 1958; Sarkar et al., 1972), B type particles such as the murine mammary tumor viruses, have a spherical nucleoid

eccentrically located in the virion. In addition these particles are characterized with distinct surface projections protruding from the outer membrane (Nowinski et al., 1971; Sarkar and Moore, 1972; Sarkar et al., 1969). C-type viruses contain a centrally located dense nucleoid and surface projections that are much less prononced than those of B particles (Bernhard, 1958; Sarkarand Moore, 1972). All avian and most mammalian RNA tumor viruses are classified as C-type particles (Tooze, 1973).

Within these C-types viruses there are differences related to three biological properties of the virus:interference, host range and neutralization. Because of the host range of the virus Vogt and Ishizaki (1966) classified avian RNA tumor viruses into two subgroups. Further work permitted these C-type viruses to be classified into seven subgroups. The resistance of certain chicken cells to various subgroups of virus has allowed the designation of chicken cells according to the virus to which they are resistant. If for instance a given chicken cell (C) is resistant (/) to a C subgroup, these cells will be designated (C/C), while C/O is used to designate cells that are suceptible to all known avian tumor viruses. Interference occurs when a cell infected with a given subgroup of viruses is resistant to further infection with viruses originating from the same sub-group. The RNA tumor viruses may be neutralized by antiserum prepared against members of the same subgroup, but not by antisera

prepared against different subgroups (Ishizaki and Vogt, 1966).

MAV-2(0)

Osteopetrosis in chickens is caused by viruses of the avian leukosis complex (Holmes, 1959) that belong to the Retroviridae family. Field strains of these viruses induce osteopetrosis and other disorders (Beard, 1963). However, chickens examined for osteopetrosis are usually old, 20 to 28 weeks of age (Blitz et al., 1965). Moreover, osteopetrosis is usually induced in a relatively low proportion of infected animals by most avian leukosis viruses since only 33% of infected birds developed osteopetrosis (Holmes, 1964).

Four strains of viruses have so far been shown to induce high frequency and a rapid onset of osteopetrosis.
1) A subgroup B virus, shown to be a contaminant of Schmidt Ruppin Rous sarcoma virus (SR-RSV), known as the ARC isolate, induces osteopetrosis in 100% of chickens in 1-2 weeks post-hatch (Dougherty et al., 1968).
2) NTRE, a subgroup E recombinant between a temperature defective Rous sarcoma virus (td- PrRSV-B) and the endogenous retrovirus RAV-0. This strain is responsible for the induction of approximately 30% osteopetrosis when inoculated into day-old K 28 strain of White Leghorn chickens after an incubation period of 2-6 months (Tsichlis and Coffin, 1980). Finally the two last strains, MAV-1(0)

and MAV-2(0), were isolated from the standard BA1 strain a stock of avian myeloblastosis virus (Smith and Moscovici, 1969).

MAV-1(0), a subgroup A virus, is responsible for the induction of osteopetrosis in 80% of chicks following a latency of approximately 3 weeks (Smith and Moscovici, 1969). The MAV-2(0) stocks were derived from a single clone of virus biologically purified by end point dilution Smith and Moscovici, 1969). The biologically purified virus induced an incidence of 80% osteopetrosis (Smith, 1982).

Storage of MAV-2(0) for several years diminished the pathogenicity of the virus and lesions were observed only after 3 months of latency (Smith, 1982). After four to five transfers over a period of 2 years, the rapid form induced osteopetrosis at an incidence of nearly 100% by two weeks post hatch (Banes and Smith, 1977). For a while, it was supposed that MAV-2(0) stocks were a mixture of a defective and helper viruses (Smith 1982). However plaque purification of 85 strains was instrumental in the identification of 3 isolates inducing massive bone growth by the time chicks were 3 weeks of age (plaque isolates 32,64, and 81). Three well-isolated plaque isolates of plaque No. 32 induced osteopetrosis with the same intensity and rapidity as their immediate ancestors (Smith and Morgan, 1982). In order to determine whether or not there was any difference between these slow and rapid onset

strain it was necessary to clone and sequence the different strains.

The first part of this work was recently completed (Aurigemma, 1990). A Hirt extraction (Hirt 1967) was performed during the early part of MAV-2(0) replication, when the viral genome is reverse transcribed into a double stranded DNA copy, to recover unintegrated MAV-2(0) DNA. After several restriction digest enzymes were tested, Sac I enzyme was identified as the only restriction enzyme which cleaved viral DNA at a single site and gave a linear DNA fragment of about 8Kb, while the uncut viral DNA was estimated to have a size of 10Kb (Aurigemma et al., 1990).

This MAV-2(0) linear DNA was then cloned into the arms of Sac I digested Lamda gt WES LamdaB arms.

To verify its ability to grow in culture just like the parent virus, cloned MAV-2(0) DNA was used to transfect CEF cultures, and the virus was propagated in cell culture.

Using the reverse transcriptase assay, it was shown that all the supernatants resulting from the transfection with different clones induced a reverse transcriptase activity. That clearly confirmed that cloned MAV-2(0) was viable and its genome was intact. Furthemore, in vivo experiments confirmed that the cloned virus retained its pathogenicity because 100% osteopetrosis was induced in hatched chickens (Aurigemma et al., 1990).

Pathologic Spectrum of MAV-2(O)

In contrast with ALV field isolates, the spectrum of neoplastic and non-neoplastic disorders induced by MAV-2(O) is rather narrow. The most important feature of this virus is its reproducible capability to induce an incidence of approximately 100% of osteopetrosis when inoculated into 10 day old embryos (Smith, 1982). Tumors are rarely found in MAV-2(O)-infected chickens. However other features have already been described by other investigators such as nephroblastoma, anemia, immunosuppression and stunting.

Nephroblastoma

Plaque purified MAV-2(O) induced an 80% incidence of osteopetrosis and a 20% incidence of nephroblastoma (Smith and Moscovici, 1969; Banes and Smith, 1977; Smith et al.,1976), while plaque purified MAV-2(N) induced greater than 80% incidence of nephroblastoma and less than 30% osteopetrosis in infected chickens (Watts and Smith, 1980; Watts et al., 1982). Nephroblastomas are embryonic kidney tumors consisting of mesenchymal and epithelial renal elements in different stages of differentiation (Watts and Smith 1980; Watts et al., 1982).

When DNA was extracted from tumors, the presence of virus /host junction fragments was demonstrated, indicating that the tumors arose from one of two clonal outgrowths of transformed cells (Boni-Schnetzer et al., 1985). Gene expression studies showed that c-Ha-Ras gene was provirally

activated within the tumors since chimaeric host-virus transcripts were cDNA cloned from a nephroblastoma (Westaway et al.,1986). Finally, studies conducted by Collart et al., (1990) showed an infrequent involvment of c-fos in 1 of the 16 clonal outgrowths.

Anemia and Immunosuppression

Anemia and immunosuppression is predominantly seen when the virus is inoculated at 8-10 days of age (Paterson and Smith,1978; Smith and Schmidt,1982; Cummins and Smith, 1988). The destruction of RBC precursors is responsible for the anemia rather than the diminished space due to invading osteopetrotic lesions (Smith 1982). Immunosuppression was shown to result from a macrophage dysfunction rather than a lack of a soluble factor (Cummins and Smith, 1988).

Stunting

The other feature observed in the examinations of this present study in all chickens when inoculated at 10 day-old embryos is stunting, in which infected birds are smaller than the non-inoculated ones (Banes and Smith, 1977).

PATHOGENESIS

MAV-2(0)- induced avian osteopetrosis is characterized by an abnormal proliferation of osteoblasts where the intrinsic number of osteoclasts is constant or slightly higher than in normal uninfected birds (Schmidt et al.,1981). However the studies leading to the

identification of the part of the genome responsible for the induction of the disease leading to the observed proliferation of osteoblastic cells has only recently been addressed (Schmidt et al., 1982). These latter studies of T1 ribonuclease fingerprints of MAV-2(0) isolates, showed that two oligonucleotides at the 3′ end of the genome were associated with osteopetrosis induction, especially when these nucleotides were not present in the genome of viruses which did not induce osteopetrosis. That was one of the first indications of the localization of the genome fragment responsible for osteopetrosis. Research undertaken with RAV-0, RAV-60, and NTRE-7 found that sequences in the 3′ end of the genome were responsible for the induction of fibrosarcoma, osteopetrosis, anemia, and adenocarcinoma (Robinson et al, 1982). In addition Robinson et al., (1985) studied virus recombinants of RAV-1 (inducing high frequency of lymphomas) and RAV-0 (non-oncogenic), and showed that sequences outside the LTR were responsible for inducing a particular tumor. It was postulated that the integration of the virus was near a given oncogene depending on the tumor induced (Robinson et al., 1985). Robinson et al., (1986) showed that sequences near the 5′ long terminal repeat of avian leukosis viruses determine the ability of the virus to induce osteopetrosis. Finally Aurigemma et al.,(1991) made and analyzed several viral recombinants and demonstrated that the part of the genome responsible for MAV-2(0) induced osteopetrosis was

the *env*-LTR portion of the genome. However, the cellular mechanism by which MAV-2(0) causes the proliferation of osteoblastic cells in osteopetrosis is not known. Powers et al., (1987), suggested, following the observation of thrombi in osteopetrotic lesions, that these latter could generate circulatory disturbances and subsequent cell necrosis may cause the release of bone factors such as bone morphogenic proteins (BMP) that stimulates periosteal proliferation. Looking at this pathogenesis from a different angle, the author thought that since osteopetrotic osteoblastic cells are not clonal (Robinson and Miles 1985; Aurigemma et al., 1990); there has never been any proof linking the integration of the virus near a given oncogene as being at the origin of osteopetrosis. Even if this virus happens to be integrated near different oncogenes at the same time, one could not affirm that osteopetrosis is consequent to these integrations since these integrations are random by essence and are different in every cell. This is inconsistent with the observed high frequency of osteopetrosis which has to rely on a highly reproducible mechanism. Thus the pathogenesis of osteopetrosis has to exclude virus integration as being responsible for the stimulation of the observed proliferation of osteoblasts leading to osteopetrosis. Furthermore the non-integrated viral DNA was also shown not to be responsible for the induction of osteopetrosis since osteopetrotic lesions were shown to appear before non-integrated DNA virus was

detected (Aurigemma et al., 1990). Therefore it becomes obvious that the pathogenesis of osteopetrosis has to involve another mechanism which has to be highly reproducible.

Hypothesis

Since the answer does not appear to involve clonal DNA integration near an oncogene, the explanation has to be elsewhere. The author proposes that the answer is at the cell membrane. The author thinks that part of the viral glycoprotein, gp85, during viral binding to the receptor or gp37 during the penetration or budding of the virus, may crossreact with a growth factor or a proto-oncogene receptor that is adjacent to a virus receptor. This crossreaction triggers the receptor and by cell signalling pathway induces the proliferation of the cells. How is this mechanism specified in detail?

The mechanism that the author proposes is related to the ways cells differentiate. Therefore one has to first discuss cellular differentiation.

Genes are expressed following the action of different repertoires of gene regulatory proteins (Garrels, 1979). Every cell has a different specific repertoire of gene regulatory proteins that specifically induce the expression of genes that characterize the differentiated specificity of the cell (Maniatis et al., 1987).

A particular differentiated cell expresses a specific particular set of genes (Figure 2.2). Of course, all cells express what are commonly known as the house keeping genes, which are necessary for the biochemical needs of every cell. Therefore the expression of these genes is the same in all cells.

The fact that every cell has a different gene regulatory protein repertoire is at the origin of the difference between differentiated cells (Garrer 1979).
This difference comes about during embryogenesis, when cells acquire different gene regulatory proteins along their differential pathway, because at every stage of differentiation, one or more new gene regulatory protein(s) is/are added (Maniatis et al., 1987). These gene regulatory proteins when binding the DNA can have a positive action (stimulate expression of a given gene) or a negative action (inhibit expression of a given gene) (Atchison et al., 1988). In addition the gene regulatory proteins can influence each other when binding to the gene regulatory sequences, therefore their respective specific actions may change following these interactions (De Combrugghe, et al., 1984)(figure 2.3).

An interesting illustration of how this model works is illustrated by the way interleukins work in immune cells. An example which is well known is the stimulation by IL-2 of different immune cells (T-Helper cells, B-cells, cytotoxic T-cells). However the response generated, although

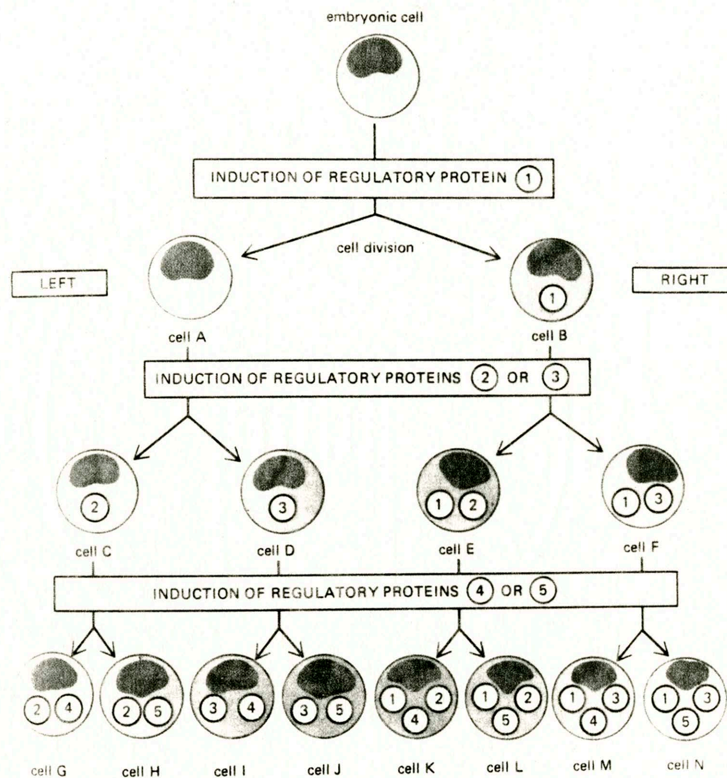
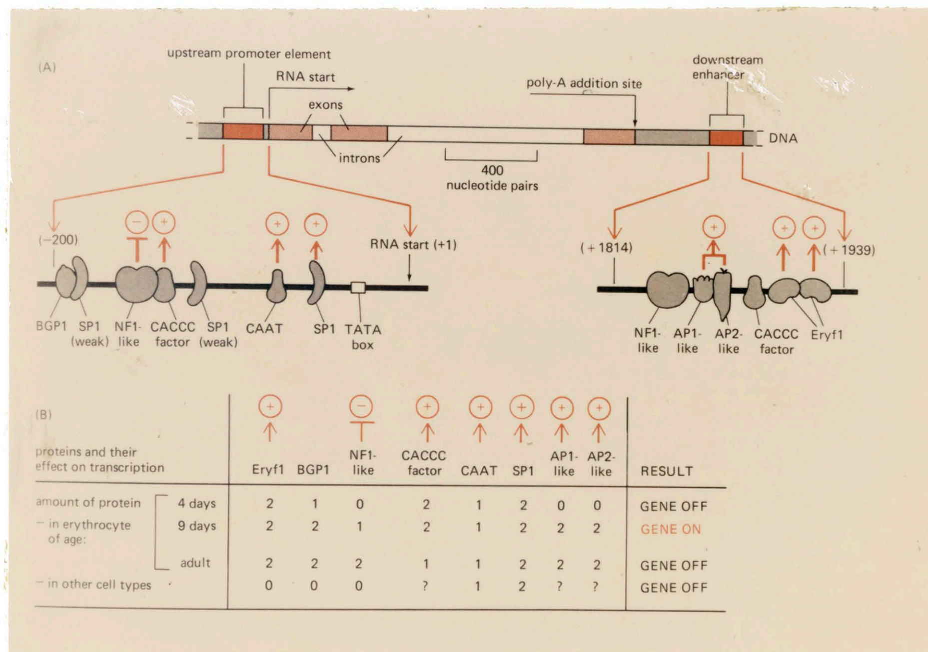


Fig.2.2: Schematic chart showing how the combination of a few gene regulatory proteins can generate many cell types in embryos. Reproduced from Molecular Biology of the Cell, Alberts, Bray, Lewis, Raff, Roberts and Watson, Garland, 1989. With kind permissin from the editor.



proteins and their effect on transcription		Eryf1	BGP1	NF1-like	CACCC factor	CAAT	SP1	AP1-like	AP2-like	RESULT
		+		−	+	+	+	+	+	
amount of protein in erythrocyte of age:	4 days	2	1	0	2	1	2	0	0	GENE OFF
	9 days	2	2	1	2	1	2	2	2	GENE ON
	adult	2	2	2	1	1	2	2	2	GENE OFF
in other cell types		0	0	0	?	1	2	?	?	GENE OFF

Fig. 2.3: Shows apattern of combination of the bonding of gene regulatory proteins for the expression of Beta globin. Reproduced from Molecular Biology of the Cell, Alberts, Bray, Lewis, Lewis, Raff, Roberts and Watson, Garland, 1989. With the kind permission of the Editor.

complementary (the response of the three cells is coordinated to induce a specific reaction towards the invading organism) is different since it induces autocrine stimulation of Helper T cells, maturation (differentiation) of B-cells and activation of cytotoxic T-cells. These cells have the same genome (Gurdon, 1962) at the exception of the differently rearrranged gene segments that will lead to the genesis of different B-cell (Dreyer et al., 1965), or T cell receptor specificities. These cells have the same receptor for the factor. And yet the same stimulation generates a different response in different cells. So where does the difference lie? The difference can lie only in between; a different set of gene regulatory proteins. This aforementioned example clearly demonstrates that every cell is endowed with a specific repertoire of gene regulatory proteins otherwise the same response would have been observed in the three different cells.

The author proposes that during embryogenesis, MAV-2(0) binds to cells from the osteoblastic lineage, thus infecting cells which have a specific gene regulatory protein repertoire permissive to this proliferation. Once a growth factor or an oncogene receptor is stimulated, it generates the synthesis of a given GRP by cell signaling pathway that will be added to the preexisting set of GRPS (Figure 2.2). The combination of a subset of these gene regulatory proteins, including the newly synthesized one, will specifically bind to gene regulatory sequences

(Atchison, 1988) responsible for the promotion of the transcription of a proliferative factor itself responsible for the observed proliferation. Another important aspect of osteopetrosis is the non-appeareance of osteopetrotic lesions following the viral inoculation of chicks at 10 day-old of age embryos (Smith, 1982).

When chickens are infected at 10 days of age, they have a different set of gene regulatory proteins that are being expressed. Therefore the stimulation of these cells generate a new different gene regulatory protein which when added to the pre-existing regulatory proteins does not induce the expression of the factor or oncogene responsible for the proliferation of these cells.

It is understood that these differentiated cells have had the time to acquire other GRPs along their differentiation pathway which clearly differentiate them from the early embryonic osteoblastic cells. Previous studies conducted by Smith and Morgan, (1984) showed that bursectomized chicks can develop osteopetrosis inoculated at six weeks of age. This clearly shows that without a a specific immune environment osteoblasts are infected and proliferate to produce osteopetrosis. These previous findings do not contradict this hypothesis because bursectomy induces a lack of production of B-cells that will result in the lack of antibodies as well as growth factors that are the consequences of a lack of a bursa.

Therefore one can postulate that the lack of these factors can generate different stimuli on the osteoblastic cells, influencing therefore the set of gene regulatory proteins existing in the osteoblastic cells. Since cells are continuously stimulated by different soluble factors, the cells have now a different set of gene regulatory proteins allowing them to be permissive to the mitogenic activity of the factor produced following viral stimulation. The immediate question is, can an immune factor interact with an osteoblastic cell? Cytokines were shown to influence bone formation (Simpson et al., 1984; Hauschka et al., 1986). Any stimulation can bend the balance towards the expression or repression of a given gene. The author beleives that this same gray story happens in the pathogenesis of AIDS.

Gary Nabel and David Baltimore described a factor which binds to the enhancer sequence of AIDS virus and induces its replication (Nabel et al.,1987).

Other studies found additional intervening factors. However, it is not possible to accept the fact this factor just by itself could generate the reactivation of the virus generating the observed disease. The answer is far from simple. If it were the case how come that the latency is different depending on the given individual one is dealing with? A plausible explanation is that every individual is subject to different stimuli, thereby generating different specific individual GRPs which either act on the

replication of the virus or contribute to its latency depending on the particular individual.

That's the reason the author thinks different people may develop AIDS at different times. This is also for the same reason the author thinks that some people never come down with the disease. It is of interest to emphasize that gene expression of both mammalian DNA viruses (McKnight and Tigan, 1986) and lower eukaryotes such as yeast (Struhl, 1987) uses the same mechanism. Thus, inducible and tissue specific expression of eukaryotic genes appear to operate by similar or identical rules regardless of the organism.

The enhancer elements are binding sites for specific DNA binding proteins which function as positive or negative regulators of gene transcription in specific cell types and/or in response to specific signals in the microenvironment. This involvment of the virus glycoprotein with the cell membrane is corroborated by recent findings, whereby a rapid increase in the RNA levels of the proto-oncogenes c-fos, c-jun, and c-myc followed the stimulation of cultured cells with human cytomegalovirus infection (Boldogh et al., 1990).

The same authors showed that neither inactivation of viral infectivity with U.V. irradiation, nor inhibition of translation with cycloheximide or anisomycin adversely affected the enhanced expression of proto-oncogenes. This result showed that the glycoprotein itself is involved in the stimulation of the cell.

One important remark relates to the fact that previous MAV-2(0) strains did not induce such a frequency of osteopetrosis. The author proposes the following explanation. MAV-2(0) envelope glycoproteins have been subject to mutations that differentiate them from the ALVs that induce a low frequency of osteopetrosis. This assumption is verified by the fact that MAV-2(0) before being passaged several times did not induce 100% osteopetrosis (Smith and Morgan 1982).

What might have happened is that the MAV2-(0) genome was subject to a critical mutation in a specific area of the glycoprotein that changed the three-dimentional spatial configuration of the glycoprotein and made it interact with this growth factor or oncogene receptor. This mutation seems to have been conserved since MAV-2(0) still induces approximately 100% osteopetrosis. The osteoblastic as well as the fibroblastic cells are then stimulated to grow. As a matter of fact, sequencing of the viral envelope by different authors (Kan et al., 1985) confirms the fact that mutations have occured. This is not a surprise, because the only way to explain this differing frequency of osteopetrosis is that there are mutations of the envelope gp85, for the simple reason that the mechanism of osteopetrotic induction is not going to change every time there is a mutation.

On the other hand, osteopetrosis has never been shown to evolve to neoplasia. The author suggests two

alternatives to explain this phenomenon.

1) The cells do not acquire the ability to synthesize their own growth factors and therefore do not evolve to neoplasia.

2) There is an antioncogene that blocks the action of an oncogene and therefore prevents the cells from evolving to neoplasia.

Canine Osteosarcoma

Introduction

Osteosarcoma is a neoplastic lesion affecting the osteoblastic cell lineage (Figure 2.4). This tumor has many similarities with human osteosarcoma. In the dog there is a male sex predilection, and most tumors appear in appendicular sites, primarily at the metaphysis. Less than 10% of patients have metastasis at presentation. Over 90% of the tumors have high grade histology. The metastatic rate was shown to be of 80% or more with amputation alone. The lung was shown to be the most common site of metastasis.

These aforementioned features are very similar to human osteosarcoma (Withrow et al., 1990).

Etiology

The exact cause of osteosarcoma in man and dogs is unknown. However, ionizing radiation has rarely been demonstrated to induce osteosarcoma in both men and dogs (Morgan and Pool, 1982; Rosenberg et al., 1982; Shives et al., 1986; Taylor et al., 1981; Wolff et al., 1980).

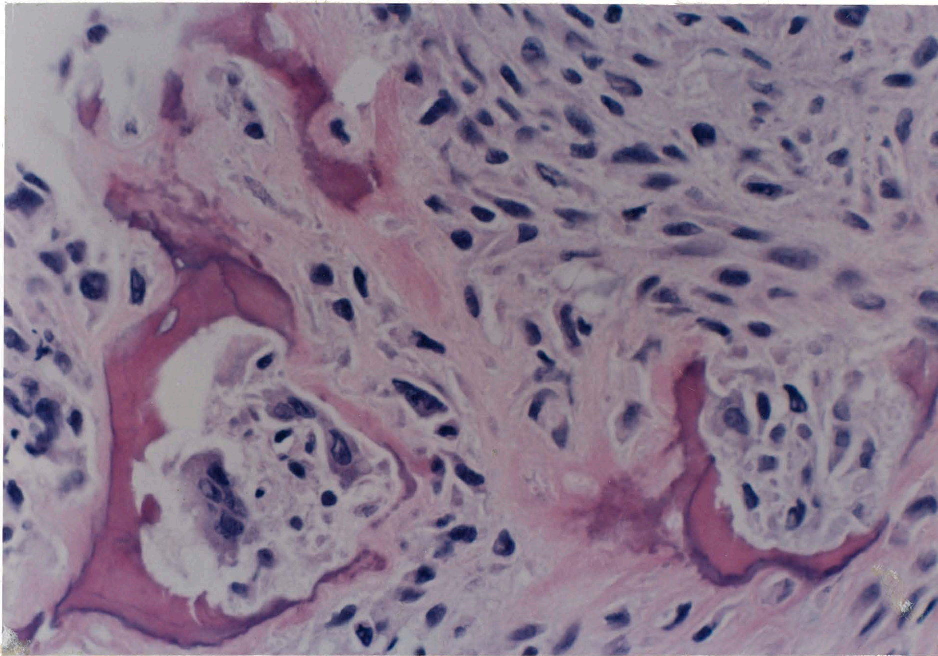


Fig.2.4: Histologic section of canine ostosarcoma 400x magnification. H. And E.stain. Bottom left= multinucleated osteoclast. Pink tissue = decalcified bone. Black cells= Osteoblasts. Sample kindly supplied by Dr Withrow (Veterinary Teaching Hospital, C.S.U).

Eigthy seven dogs representing 3.4% of the sample treated with radiation for soft tissue carcinomas developed osteosarcoma within the field of irradiation. All tumors developed 20 months or longer after the radiation therapy. Pre-existing bony defects such as multiple osteocartilagenous exostosis have undergone malignant transformation in both species (Banks et al., 1975; Cooper et al., 1983). In some cases, fracture repair followed by metallic implants induced the appearance of osteosarcoma (Withrow, personnal commununication, 1990).

These tumors display the same phenotype as the naturally occuring ones. Other proposed etiologies include viral and chemical carcinogens. Proof of their involvment is not well substantiated (Brostrom, 1980; Mankin, 1979; Sinkovicks et al., 1977; Storm et al., 1981). Nonetheless, there is now increasing evidence that a lack
or inactivation of a tumor suppressor gene (Retinoblastoma gene) is itself associated with osteosarcomas in humans.
In 1971, it was initially postulated that two "mutational" changes were sufficient for the development of retinoblastoma (Knudson, 1971). Hansen et al (1985) demonstrated molecular genetic evidence that the
development of retinoblastoma and osteosarcoma involves specific somatic loss of constitutional heterozygosity for the region of human chromosome 13, localized in the 13q14 region. Retinoblastoma cells invariably lack normal expression of the <u>RB</u>-encoded protein (RB) because of mutation of

both RB alleles, suggesting that loss of functional RB protein is an obligate event during retinoblastoma genesis (Lee et al., 1988). The RB gene has been successively called a "suppressor" or regulatory gene by some (Murphree et al., 1984) and "antioncogene" by others (Knudson, 1985). Individuals inheriting the susceptibility to develop Rb also have a high incidence of secondary malignancies, the most frequent of which are osteosarcomas and soft tissue carcinomas (Abramson et al., 1984). Moreover similar changes in the RB gene have been documented in such common tumors as adenocarcinoma of the breast (T'Ang et al., 1988). Furthermore, similar changes were also reported in small cell carcinoma of the lung (Harbour et al., 1988; Yokota et al., 1988). What's more this RB gene was also involved in human prostate carcinoma cells, since its transfection into these cells abolished their ability to form tumors in nude mice (Bookstein et
al., 1990).

While the loss of heterozygosity in many other tumors has been reported to be highly confined to a particular chromosome 13 (Fearon et al., 1985), other researchers reported a loss of heterozygosity on many chromosomes in more than 60% of tested tumors in osteosarcoma samples (Togushida et al., 1988). However such diversity of the loss of heterozygosity has also been reported in malignant melanomas (Dracopli, et al., 1985; Dracopli, et al., 1987), lung cancer (Yokota et al., 1987) and meningiomas

(Dumanski, et al., 1987). Of particular interest is that tumors which have a loss of heterozygosity at many loci on chromosome 13 also have a loss of heterozygosity at many loci on other chromosomes. These results contrast to the observations of Hansen et al., (1985) and Dryja et al., (1986) in that they were unable to find loss of heterozygosity at many loci on chromosomes other than chromosome 13. In malignant melanomas, the diversified allele loss occured during the progression of tumors as a result of some biological selection (Dracopli, et al., 1985; Dracopli, et al., 1987).

Such may be the case for osteosarcoma, especially in metastatic tumors. To make things more complex, one tumor studied, (KS-8), showed no loss in 4 of the loci studied and shown to be deleted in other tumors. On the other hand, another tumor lost heterozygosity for four of five informative loci at primary biopsy (KS 54). Chromosome 17 was also intimately related to chromosome 13 in term of loss of heterozygosity (Togushida, et al., 1988). Since p53 gene is also located in chromosome 17 as well and since the probe used was polymorphic, it is possible that it detected the altered p53 gene. Such concerted loss of heterozygosity observed in tumors is intriguing (Toguchida et al., 1988).

Therefore, and at this stage of study, the difference between observations can not be simply explained by the difference in the stage of tumor development. Moreover

other researchers used Southern blot mapping to find that 11 of 60 osteosarcoma samples had altered restriction patterns of the p53 gene and that six of these had loss of the other p53 allele. In contrast, no alteration of the p53 gene was detected in 50 samples from other types of sarcomas. Fifty per cent of osteosarcoma cell lines (4 of 8) also had gross rearrangements of one p53 allele with loss of the second allele, and these had no detectable p53 mRNA. These data show that human osteosarcomas can have rearrangements of the p53 gene which may cause loss of normal constraints on cellular growth (Miller et al., 1990). It therefore remains to be determined whether the changes reported within the RB gene in these other tumors are related to the initiation of malignancy or rather are involved in tumor progression. These examples show that the RB gene alteration has a broad role in the genesis of human tumors. However (with the exception of retinoblastoma), it is perhaps unlikely that functional loss of the RB gene is sufficient for tumor development (Benedict et al., 1990).

Objectives

One of the objectives of the current work is to provide a biological mechanism to explain the proliferation of osteoblastic cells in avian osteopetrosis and compare it to the non-appearance of osteopetrotic lesions in 10 day-old inoculated chicks.

The second objective is to find the reason why osteopetrotic cells never evolve to neoplasia.

CHAPTER 3

DEVELOPMENT OF AN *IN VIVO* MODEL FOR THE STUDY OF BONE FORMATION

Introduction

It has been shown that growth factors are of benefit in a pathologic condition such as anemia. These studies show that it is possible to induce an increase in red blood cells in patients suffering from mild anemia through the administration of eythropoietin (Golde et al. 1988). There is therefore an interest in factors stimulating bone formation as a possible treatment for osteoporosis (Reddi et al., 1988). The development of an animal model would be an important advance in studies of bone formation.

However, bone formation is a complex phenomenon, due to the highly regulated interplay of factors involved in bone growth (Wozney et al. 1988).

In addition to the role of systemic growth factors and local growth factors on bone formation (Centrella, 1985; Hauschka, 1986; Wozney et al., 1988) there is now a proven interaction between bone matrix factors and osteoblastic cells (Luyten et al., 1990; Hauschka et al., 1986). Therefore the influence of this interaction can best be monitored by gene expression studies to evaluate the

sequential difference in factors' expression. The development of an animal model would be an important advance in studies of bone formation.

Previous Models

Cell culture

Advantages. A cell culture system presents an opportunity for a controlled environment. In addition, it is much less difficult to obtain the desired amount of the needed sample. Moreover, RNA extraction is relatively easier than from animal tissues.

Disadvantages. The cells are at one stage of differentiation or development, therefore the phenotypic expression may not reflect the *in vivo* one. Moreover, this particular stage may not even reflect an homologous *in vivo* level of differentiation. This is because so many growth factors hormones, interaction between hormones and growth factors, take place in an *in vivo* situation. In cell culture, one has only fixed conditions, characterizing *in vitro* situation where the amount of serum added is fixed and the hormones and growth factors in it are not susceptible to change according to a given physiological condition.

In addition, the addition of the serum can generate a non-specific activation of c-*jun* (Boldogh et al.,1990).

These are the reasons that led to the author's adoption of an <u>in vivo</u> model for the present studies.

<u>In situ model or localized in vivo model</u>

This model consists of administring bone-matrix supplemented with the experimental growth factor subcutaneously, and followed by assessment of <u>de novo</u> bone formation (Reddi et al., 1977; Wozney et al., 1988).

<u>Advantage</u>. This model allows direct clinical studies.

<u>Disadvantages</u>. The amount of <u>de novo</u> bone is not high enough, to extract sufficient amount of RNA to be able to undertake large scale studies. Moreover, the undertaking of such experiments requires lengthy and tedious surgical procedures.

In addition, it is difficult to study the bone matrix and osteoblast relationship since the demineralized matrix used had previously been " washed" from any factor before its use in order to adequately assess the studied factor.

<u>Use of Osteopetrotic Chicken as a Model</u>

<u>Advantages</u>

1. MAV-2(0) stocks can be stored for long periods of time. In addition MAV-2(0) titer can be established by a simple plaque assay.

2. Avian osteopetrosis is easily induced by intraveinous inoculation of MAV-2(0).

3. Osteopetrosis is observed in approximately 100% of chicks inoculated, in a reproducible manner (Banes and Smith, 1977).

4. Because of the proliferative nature of avian-induced osteopetrosis, large numbers of cells are generated which facilitates the study of gene expression.

5. The virus is apathogenic for humans.

6. It is possible to monitor gene expression over a desired period of time.

Disadvantages. The only disadvantage encountered is the difficulty and exacting demands required for RNA extraction from the tissue. The present work has focused on the development of a reliable and efficent protocol for the extraction of RNA from bone.

Materials and Methods

Egg inoculation

Ten-day-old chicken-embryos, from the SC line of White Leghorn (Hyline, Dallas Center,Iowa) were infected by administration of virus via the intravenous route. Each embryo was given with 10^5 PFU of the cloned MAV-2(0),32/2/4 strain (Aurigemma et al., 1990). Uninfected control chickens were incubated and maintained under identical conditions. Hatched chicks were reared in isolation, in special cages where food and water were provided *ad libidum* at the Painter Center for Laboratory Animals, Colorado State University.

Protocol for Bone Sample Processing

Materials

1. Tris buffer solution (see Appendix). This solution was filtered and autoclaved.

2. Sleep away (Euthanasia solution, Fort Dodge Laboratories, Inc,Fort Dodge, Iowa 50501).

3. Rat-tooth and straight forceps.

4. Scalpel and blades.

5. Poultry shears.

6. Curved scissors.

7. Fine scissors.

8. Petri dishes (100 mm).

9. 10 ml syringes with 18-gauge needles.

All instruments, except the poultry shears were wrapped in aluminium foil and baked overnight at 120oC for ~ 10 hours.

First stage; bone sampling.

Birds were euthanized with 0.1 to 0.2 ml of Sleep Away using the intravenous route. Both femurs and both tibias were carefully removed. Muscles and periosteum were stripped off the bone.

The ends of the bones were removed at the epiphyses using the poultry shears and the remaining bones were flushed with Tris buffer to remove the bone marrow. The bones were then sectioned sagittaly, and the remaining

marrow was removed by scraping. These processes were undertaken in Petri dishes containing a Tris buffer solution pH 7.4.

The bones were transferred to a petri dish containing a guanidinium solution (see composition in the appendix) where further processing took place. Using a scalpel, the bones were shaved (Figure 3.3) and cut into very small pieces (~ 1mm cubed). The minced bone fragments were immediately frozen in liquid nitrogen until further processing.

<u>Second stage</u>; <u>bone processing</u>.

Frozen, minced bone samples were thawed at room temperature, and then further homogenized for 5 to 10 minutes (the length of this step depends on the quality of the previous processings) using a Tissumizer (Tekma, P.O Box 37202. Cincinnati, Ohio).

The material was further homogenized in a glass teflon homogenizer for 5-10 minutes, after which the homogenate was passed through an 18-gauge needle. It is essential that the bone particles are thoroughly ground before this step in the procedure. This is important to render the process easier and optimize the yield of RNA.
The following step depends on the technique of RNA extraction utilized.
<u>The single step technique</u> (Chomczynski et al. 1987) does not require that the homogenate be centrifuged beforehand since the application of the technique and centrifugation

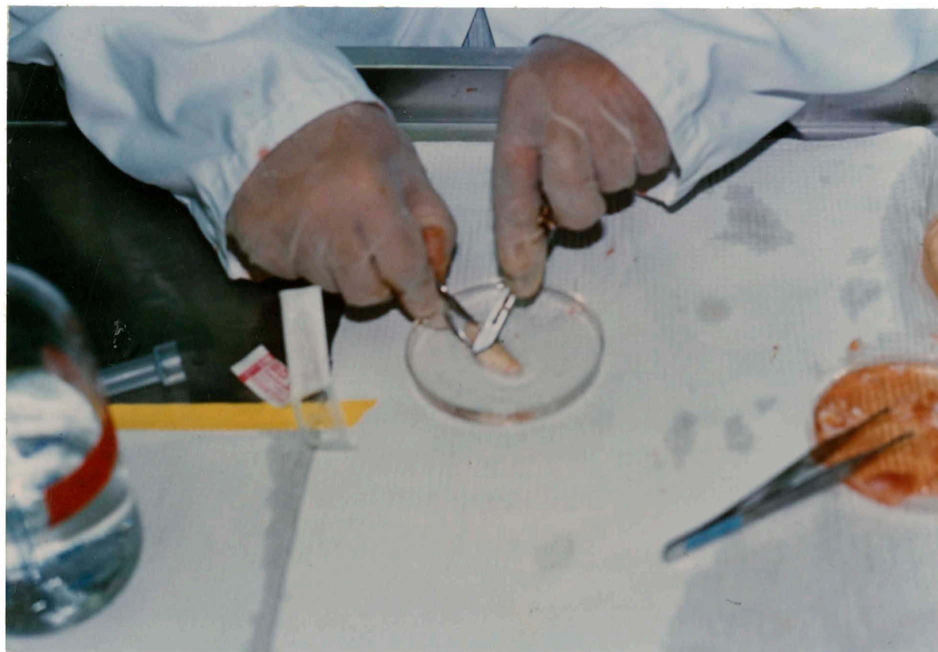


Fig.3.1: Shaving of a non-decalcified bone to be used for RNA extraction in a guanidinium solution.

of the samples will result in sedimentation of the bone debris to the bottom of the tubes while the RNA solution is in the aqueous phase. The aqueous phase was transferred to a fresh tube with 10 ml of isopropanol, and placed at -20° C for at least one hour to precipitate RNA. Sedimentation at 10,000g for 40 minutes was performed (an increase in the centrifugation time was found to further purify the sample from bone proteins). From this point onwards, the modification of the single step method described (Puissant et al., 1990), was used. The resulting pellet was resuspended by vigorous mixing in 2 ml of 4 M LiCl to solubilize polysaccharides.

The precipitate was pelleted at 10,.000 rpm for 5 min. and dissolved in 2 ml 0.5% SDS. Two ml chloroform was added and mixed to the water phase. After centrifugation for 10 min at 10,000g, the upper phase was collected and precipitated by the addition of 2 ml isopropanol in the presence of 0.2 M sodium acetate, pH 5.0. The insolubile was RNA of a purity of > 99%. Use of this procedure resulted in copious amounts of high quality of RNA (Figure 3.2). This technique is appropriate for bone RNA extraction because it allows the removal of all the proteins from bone RNA and the first stage of extraction, RNA is in a constant contact with the guanidinium solution. RNA extraction from bone is time-consuming and the procedure is very rapid.

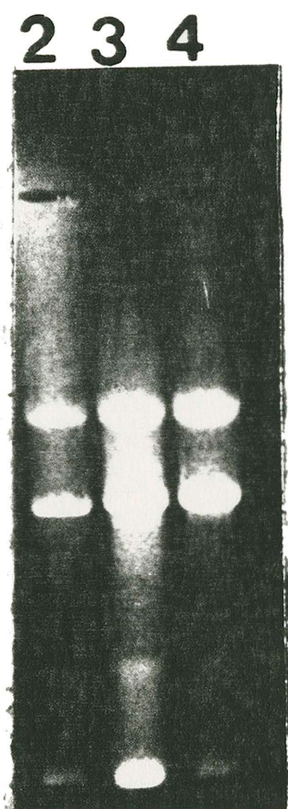


Fig.3.2: Electrophoretic gel of Hela cell RNA.Lanes 2, 3, and 4 represent separate samples from Hela cells RNA. This gel picture shows the four characteristic bands of total RNA. In addition the good quality of the RNA is demonstrated by the sharpness of the bands

<u>The Chirgwin method</u> (Chirgwin et al., 1979) was used to extract RNA for the Northern blots. The homogenate derived following bone processing is centrifuged at 10,000g for 10 min. to remove the bone debris before carefully layer it on a 5.7 M CsCl (BRL5507UB) in 4mM EDTA (pH 7.5) in a Beckman SW 40 or SW 41 tube. The CsCl solution and the SW tube were treated with 0.05% DEPC (Diethyl Pyrocarbonate, Sigma Chemical Co.) solution before use (Mc Donald et al., 1987). The tubes were then centrifuged in a Beckman L7-55 ultracentrifuge at 35,000g for 22 hours. The supernatant was removed carefully and when there was ~0.5 ml left, the tube was quickly turned upside down to prevent protease contamination of the RNA pellet. The pellet was left to air dry for 20 min at room temperature. To speed up the drying process the tube was cut down to 1 cm from the pellet. The walls of the tubes were then wiped with Kimwipes. The pellet was resuspended in DEPC treated water and precipitated by adding 0.1 volume of 2 M sodium acetate and 2.5 volumes of ethanol.

When RNA samples were suspected to be contaminated with RNAse, the pellets were resuspended in unbuffered 7.5 M guanidine-HCl neutralized to pH 7 with NAOH, supplemented with 10 mM dithiothreitol, and filtered (McDonald et al., 1987). The RNA was precipitated with 0.05 volume of 2 M potassium acetate (pH 5.5) and 0.5 volume of ethanol. The solution was then kept at -20oC overnight and the RNA recovered by centrifugation at

16,000g for 15 min. The pellet was resuspended in DEPC treated water and ready for use. The amount of water used to resuspend the RNA depended on the size of the pellet to insure a correct dilution of the RNA without the amount of RNA being too small to be detected by the spectrophotometer. The samples were then stored at -70°C .

The spectrophotometric analysis used 40 as the extinsion coefficient for the calculation of the RNA amount.

The formula was as follows:

A260 displayed data X 40 X dilution factor= ug/ul.

RNA samples were kept on ice during the determination of the amount of RNA and during all handling.

Northern blot To perform Northern blots, all solutions were DEPC treated (0.1% DEPC) (Colbert et al., 1990). The sodium phosphate solutions were prepared (as described in the index for solutions) the day before running the gel.

The electrophoretic tank and the comb were treated with 1 M sodium hydroxyde for at least one hour to remove any RNAse contamination. The gel buffer, reservoir buffer and transfer buffer solutions were then autoclaved.

1. The gel apparatus was neutralized from its alkalinity by using concentrated HCl solution. I found it convenient to pH the resultant solution to 6-7 before it

was discarded so that the added reservoir buffer did not have its pH diminished by the residual acidity of the tank. The gel apparatus was inverted and left to dry.

2. A water bath was set to 65oC.

3. The gel was then prepared with 1% highly purified molecular standard agarose (IBI Inc.,New Haven, Connecticut) in bi-distilled sterile water. The agarose was boiled with the aid of a hot plate and a stirrer bar. Once the agarose was melted completely, the gel solution was put in a water bath at 65°C to cool down for 15 min.

4. The ends of the gel tray were taped. The tray was put in the reservoir tank.

After cooling the gel buffer, formaldehyde was added to the gel buffer solution at a final concentration of 3%. After thorough mixing, the gel solution was laid on the tray before the gel began to set. The gel apparatus was covered with saran wrap for one hour while the gel hardened.

5. RNA samples were prepared as follows.

The desired amount of RNA sample was pipetted (different amounts from one sample were used here, 20ug, 10ug, 5ug, 2.5ug). The volume of the sample was adjusted to a total of 5 ul using FDA water (filtered, DEPC treated, and autoclaved). For the marker lane the author used 5 ul of RNA ladder (BRL Bethesda Research Laboratories, Gaithersburg, MD 20877).

The samples were kept on ice.

A 2X sample buffer was prepared immediately before use:

4ul   1M phosphate buffer, pH 6.8

10ul   10 mM EDTA, pH 7.5

16.5ul formaldehyde

19.5ul FDA water.

50 ul Formamide (deionized with analytical mixed bed resin AG 501X8(D).Bio.Rad.Lab., Richmond, California.). Five ul of this sample buffer was added to each microcentrifuge tube. In addition 1 ul of 400 ug/ml of ethidium bromide was added to the marker microcentrifuge tube. The samples were thoroughly mixed, briefly centrifuged and placed in a water bath at 65oC for 15min. RNA samples were quickly put on ice for 5 min.

After cooling, the samples were briefly centrifuged before being opened. Two ul of the loading buffer was added to every sample (50% v/v glycerol, 0.2% w/v bromophenol blue, 5 mM phosphate buffer (pH 6.8). The samples were mixed by tapping. The solution was a dark blue color if the ionic conditions and pH were correct. The loading buffer turned yellow if the pH changed. The tubes were briefly centrifuged again and ready to be loaded on the gel.

6. The tape was carefully removed from the ends of the gel tray. The reservoir buffer was added. The samples were loaded on the gel. During the run the reservoir

buffer was recirculated every 20 min. by turning off the apparatus and pipetting in and out the reservoir buffer for a minute. For adequate resolution of most species of mRNA, the gel was left to run for until the dye front was six centimeters from the wells, a process that usually took 3 hours.

7. While the gel was running, the transfer buffer was prepared (two liters of 25 mM phosphate buffer, pH 6.5. Buffer volume brought up to two liters after adjusting pH with phosphoric acid). The solution was poured into a baking dish with a glass plate bridge in place.

Two pieces of 3 MM paper (15cm x 27cm) were cut for a wick. In addition 4 pieces of 3MM paper were cut to the exact size of the gel after removing the marker lane. A piece of GeneScreenPlus™ nylon (Biotechnology Systems NEN® Research products, Boston, MA) was cut to the same size as the gel, after cutting the marker lane, and hydrated in DEPC treated distilled water for 3 min. Then the nylon was equilibrated for 15 minutes in transfer buffer.

8. Once the gel marker had migrated approximately 6 cm into the gel, the gel was rinsed with distilled DEPC treated water and a photograph was taken of the marker lane that was previously stained with ethidium bromide. A piece of parafilm was used to cover the gel limiting the marker lane in order to separate the marker lane from the other remaining RNA samples without infringing on the latter.

Two previously wet 3MM pieces of paper were laid over the wick that was previously wet in transfer buffer. To avoid that some bubbles slip between the wick and the 3MM paper, a pipette was rolled over the pieces of paper. The gel was placed upside down on these papers such that the smooth side was in contact with the nylon when it was laid on top of the gel. The two other pieces of 3MM paper were laid on top of the gel and a stack of blotting paper was laid onto these papers. Pieces of parafilm were cut and placed near the edges of the gel to prevent the upper layers of the blot from touching the lower layers after the gel had been compressed. A piece of glass was put on top of the stacked papers and a weight was placed on top of the glass.

An overnight blotting of the gel followed. The nylon and the compressed gel was removed and put on a light box and the wells were marked with a marking pen. The nylon was washed in transfer buffer for 15 min. Following which, the nylon was removed to dry for 15 min. Thereafter it was baked for 2 hours at 80° C (without vaccum) for two hours. The nylon was then stored in a seal-a-meal bag in a dark place.

<u>Preparation of the probe</u>. The plasmid containing the cDNA insert for B-actin was digested using Hind III restriction enzyme for one hour at 37°C and then it was run on a low melting agarose gel TAE buffer (see composition of the solution in the index) along with a marker lane. The

size of the insert was verified by comparing it to the marker lane and cut with a razor upon which the insert was purified using Geneclean kit (The Geneclean[R] Bio 101 Inc. La Jolla California).

After purification, the insert was dissolved in 14 ul of T.E. buffer. One ul was used to estimate the DNA concentration using a spectrophotometer and 25ng of the insert was labelled using the procedure described in the Amersham Labelling Kit.

Following two precipitations in sodium acetate and 100% ethanol, a labelled probe with a specific activity of 150,000 CPM/ng was obtained.

Prehybridization. The GeneScreenPlus recommended prehybridization solution (10 ml solution containing 5ml formimide, 2 ml of water, 2 ml of 50% dextran sulfate, 1 ml of 10% SDS, 0,58g of sodium chloride was added to the tube and mixed by inversion) was prepared.

The solution was placed at 37oC for 15 min to dissolve the salt. The amount of prehybridization solution was then adjusted to 8.4 mls because 0.1ml/square centimeter of the nylon was required.

The nylon was placed in a seal-a-meal bag and the prehybridization lasted for 4 hours at 42oC. To avoid background, 800,000 CPM of labeled probe were added to the prehybridizing solution and the blot was hybridized for 22 hours at 42oC. After hybridization, the blot was washed free of unbound radioactivity as follows:

1. Two washes with 100 ml of 2 x SSC at room temperature for 5 min. with constant agitation.

2. Two washes with 200 ml of a solution containing 2x SSC and 1.0% SDS at 60oC for 30 min with constant agitation.

3. Two washes with 100ml of 0.1 x SSC at room temperature for 30 minutes with constant agitation. The nylon was then covered with saranwrap and put in a cassette with an X-ray film and an amplifier at -70oC for 24 hours.

Results

The film was exposed for 24 hours upon which it was developed. The bands observed were rather large. In order to make sure that they were specific, another wash at high stringency was performed just like the third wash described above. Other films were put in the cassette and allowed to remain for different periods of time.

Two films, one after 6 hours and the other after 18 hours exposure were selected. The one shown (Figure 3.5) was exposed for 6 hours. Bands whose intensities were proportional to the amount of RNA were observed in every lane.

Discussion

This result shows that the RNA extracted was of high quality and the Beta-actin was detected in bone tissue of chickens. The implications of these results are simple. A reliable method has been developed for extracting RNA

from chicken bone tissue. This procedure therefore makes it possible to use osteopetrosis as a model for the study of bone growth. That would lead to the understanding of the physiologic process involved and consequently to the establishment of a therapeutic protocol that takes into consideration the biology of bone formation.

The development of a reliable method for bone RNA extraction, both in term of the quality of the RNA extracted and the yield of RNA, permits the use of avian osteopetrosis as an animal model for the study of bone biology.

Fig. 3.3. Autoradiograph of the northern blot detecting Beta-actin in bone cell RNA following a 6 hours exposure. Amounts of RNA shown originated from one sample.

CHAPTER 4

MATERIALS AND METHODS

Introduction

These experiments were designed to determine the differing gene expression in osteopetrotic chickens and in canine osteosarcoma samples.

Virus

The virus stock used was the MAV-2(0) 32/2/4 strain (Smith and Morgan, 1982) cloned by Aurigemma et al., (1990). The clone (clone 9) that mimicked best the parent virus was used (Aurigemma et al., 1990). This clone was grown and titered by plaque formation on 10-day-old chick embryo fibroblasts cells of the SC line of the White Leghorn chickens. The virus was stored at -70oC (Aurigemma et al., 1990). The virus was diluted in Tris buffer solution containing 10% FCS to a final concentration of 10^6 PFU/ml.

Inoculaion Technique

Eggs were candled at 10 days of incubation, and an appropriate chorioallantoic vein was delineated with a pencil. The pencil marks on the shells were drilled in

such a way that a small rectangular piece of egg shell was removed and the delineated vein appeared neatly (Dremel Motor Tool Model 260 series 55-3, Racine Wisconsin). A paraffin oil impregnated swab was used to improve the optical properties of the egg shell membrane above the vein. A one ml syringe with a 30 gauge needle 1/2 inch long was used to inject the virus to 10-day-old embryos. The 10 day-old chickens were inoculated using the jugular vein with 0.2 ml of the same concentration of virus.

Chickens Used

Chickens used were of the SC line of white leghorn chickens (Hyline, Dallas Center, Iowa) (Graf, 1972; Smith and Bernstein, 1973) are gsa negative (Hilgers et al., 1972) and chf negative (Hanafusa, 1970) and contain two unexpressed, endogenous retroviral loci, ev1 and ev4 (Humphries et al, 1981). Osteopetrotic chickens, resulting from inoculation of 10-day-old embryos, were sacrified at 11 weeks of age. Virus-infected, but non-osteopetrotic chickens, were obtained by infecting chickens at 10 days of age, and sacrified at 4 weeks post infection. Gene expression studies were performed using these two categories of chickens.

Dogs Used

In order to extend the comparison of gene expression to canine osteosarcoma two neoplastic osteosarcoma samples originated from 4 dogs were used. One big sample originating from one dog, the other was a pooled sample originating from three dogs.

mRNA Purification

Total RNA was extracted from chickens and dog osteosarcoma samples, as described in chapter two. In order to select for mRNA, the Fast Tract mRNA isolation Kit (Invitrogen, San-Diego, CA) was used. A part of the RNA extracted was run on a formaldehyde gel to test the integrity of the mRNA extracted. Electrophoresis of mRNA on formyldehyde gels revealed that mRNA extracted from both chicken osteopetrotic cells and dog osteosarcoma migrated in a fashion that shows that the mRNA was not degraded (Figure 4.1).

Plasmids Used

A list of plasmids obtained for this study from different scientists is shown in Tables 4.1a and 4.1b. The plasmids were introduced into E.coli strain HB101 by transformation in order to grow and isolate DNA for probes. Two techniques of transformation were used; the calcium chloride (Mandel and Higa, 1970) was used to transform Beta-actin and the one-step technique (Chung, et al., 1989) was used to transform the remaining plasmids.

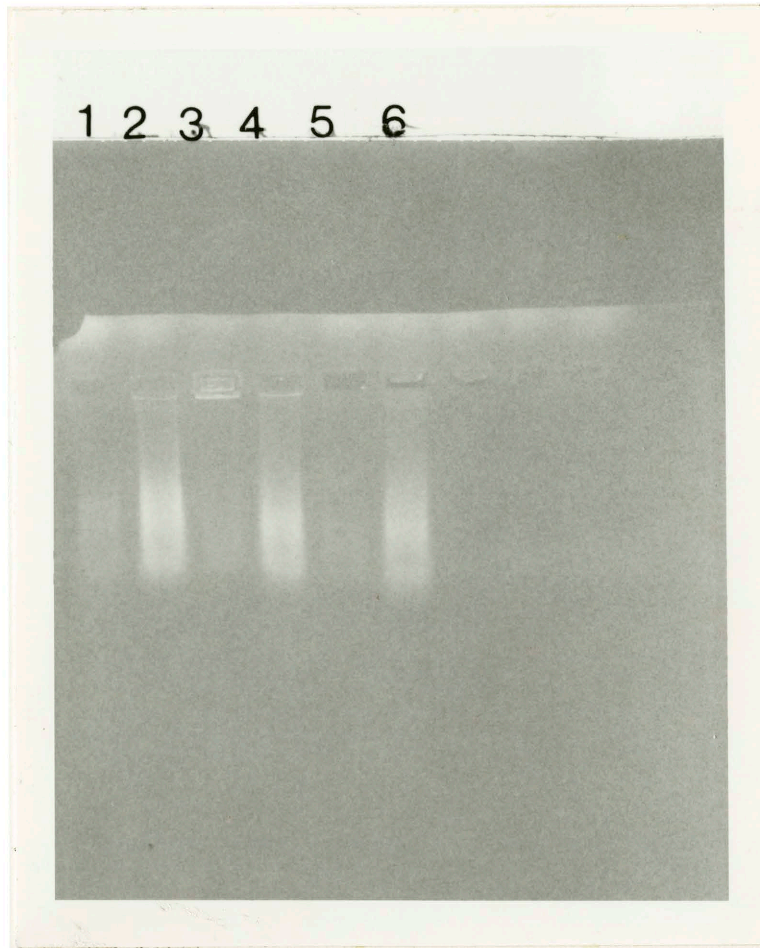


Fig. 4.1: Samples of mRNA from chicken and canine osteosarcoma: 1) 10 day-old inoculated control, 2)osteopetrotic, 3)uninoculated control, 4)osteopetrotic, 5)osteosarcoma, 6)osteosarcoma. Samples ran in a formaldehyde gel for 60 min.

The author should like to thank Dr Richard Grant, previous graduate student in Dr Carlson's laboratory for kindly supplying the already competent cells used to perform the quick method.

Large-Scale Isolation of Plasmid DNA

The transformed bacteria were grown in a 5 ml LB medium supplemented with ampicillin at a final concentration of 50 ug/ml. The bacterial suspension was grown overnight in an incubator shaker (Model G-25, New Brunswick Scientific CO.,Inc.,New Jersey) at 37oC. The following day, 500 ml of LB medium supplemented with 50 ug/ml ampicillin was prepared for every plasmid. Two ml of the overnight bacterial culture was used as an inoculum of the 500 ml LB medium. The OD_{600} was measured on a regular basis until it reached 0.5, at which time 2.5 ml of a ethanol-solution of chloramphenicol (37 mg/ml)was added at a final concentration of 170 ug/ml to the bacterial suspension. The flask was incubated overnight in a shaker incubator at 37°C, upon which time its contents were divided into 2 plastic flasks and centrifuged at 10,000 rpm for 5 min.

Lysis by Alkali (Ish-Horowicz and Burke 1981)

The pellet was resuspended in 4 ml of solution I (see composition in index) with 10 mg of lysozyme and left at room temperature for 10 min. To the bacterial lysate was

added 8 ml of solution II, mixed gently and left on ice for another 10 min. Six mls of solution III was added, mixed, and left on ice ice for another 10 min. The lysate was transferred to two corex tubes and centrifuged at 15,000 rpm for 20 min. The supernatant fluid was transferred to fresh sterile corex tubes and two volumes of 100% ethanol was added. The DNA was sedimented at 15,000 rpm for 10 min. in a refrigerated Sorval centrifuge. The DNA was then spun at 15.000 rpm for 10 min. in a refrigerated Sorval centrifuge (Sorvall RC-5B refrigerated superspeed centrifuge). The pellet was resuspended in 8 ml of T.E. buffer and 0.8 ml of a 10 mg/ml ethidium bromide solution and 8 g of cesium chloride (Var Lac Oid Chemical CO., INC., Bergenfield, New Jersey) was added.

Purification of Plasmid by CsCl Method (Maniatis et al., 1982).

The plasmid suspension was loaded into Quick-Seal centrifuge tubes (13 x 51 mm,Beckman Inst, Inc, Palo Alto, CA). The tubes were balanced, and centrifuged at 55,000 in a VTi 65.1 rotor at 18° C for 20 hours in an ultracentrifuge (Beckman L7-55 ultracentrifuge). The tubes were removed very carefully from the rotor and cut at the neck using poultry shears. A pasteur pipette was used to remove the plasmid band from the gradient. Previously this pipette had its tip darkened with a marking pencil to localize the tip inside the gradient (this suggestion came from Dr Dahn Clemens a previous graduate student in Dr

Carlson's laboratory). The plasmid band was removed and transferred to a Corex tube. The volume of the plasmid suspension was determined and an equal amount of 1-butanol saturated with water was added. The tubes were thoroughly mixed with the aid of a vortex mixer and the supernatant fluid was discarded in a liquid waste container reserved for ethidium bromide. The extraction was repeated until the supernatant was free of pink color. The extraction was performed twice more to make sure that there was no ethidium bromide left. One volume of T.E. buffer and 2 volumes of isopropanol were added to the plasmid suspension. The suspension was left overnight to precipitate, after which the suspension was centrifuged in a Sorval refrigerated centrifuge for 40 min. at 15,000 rpm. The pellet was left to dry for an hour and resuspended in 200 ul of T.E. buffer. The whole process took 5 days to complete.

Extraction of Inserts from their Respective Plasmid Vectors

Inserts were digested with appropriate restriction enzymes for an hour at 37oC. in buffers recommended for each enzymes (Figure 4.2).

A DNA marker was used to verify the size of the inserts. All samples were run in a low melting temperature agarose gel (see composition in Appendix for solution). After the inserts were adequately separated from their

plasmid vectors, they were excised from the gel using a razor blade for each insert. Using the Geneclean kit, inserts were purified and resuspended in 14 ul of T.E. buffer.

Slot Blot Technique

Before mounting the apparatus, the nylon membranes to be used were cut to the size of the slot blot apparatus which was 5.8/21.5 cm. The membranes were hydrated using distilled water for 3 min. They were equilibrated in transfer buffer for 15 min before being placed in the slot blot apparatus. Before loading the mRNA samples and the insert controls, the slot was hydrated another time with transfer buffer (750 ul was dispensed to every slot of the slot blot apparatus).

This buffer was further aspirated using a vaccum pump. The insert controls were used to check whether the labelling of the probe worked or not, in case there was no expression of the studied gene. Since a minimun of two inserts were loaded in every blot, this procedure allowed the author to have an idea of the specificity of the probe since a probe should specifically react with its homologous DNA and not with the DNA representing another probe.

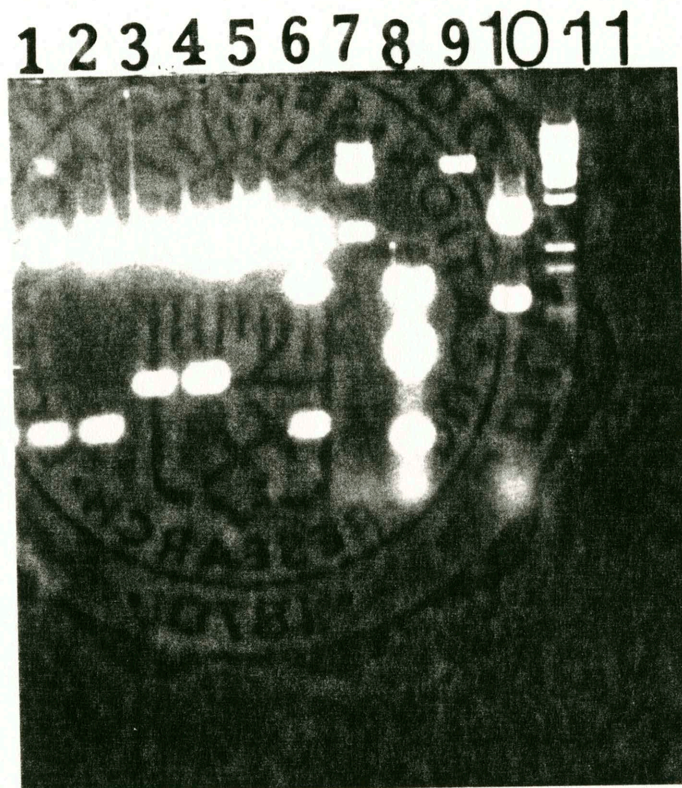


Fig.4.2: Restriction diagnostic digest. 1) BMP-3, 2) BMP-3, 3) BMP-1, 4) BMP-1, 5) not digested BMP-2, 6) Wilms'tumor gene, 7) incompletely digested plasmid, 8) c-myc.,9) linearized c-myc., 10) c-bic 11) Marker Lane, bands are located between lane 10 and lane 11.

Preparation of the Samples

mRNA was quantified using the spectrophotometer. Twelve identical blots were used in these experiments. Every slot of the slot blot contained the same amount of mRNA (0.5ug) from every sample. To every blot was added two different DNA inserts coding for two different genes, as a positive control. Some of the blots were hybrydized with a probe that did not have a specific insert control in the blot.

RNA samples and DNA insert controls were denatured at 65° C for 15 min. They were immediately chilled in ice, centrifuged for 5 seconds and replaced on ice. Samples were loaded on the nylon. Once the samples were loaded, the vaccum pump was used to fix the samples on the nylon. The slots were washed with 750 ul of transfer buffer each. The blot was allowed to dry on a piece of 3MM paper for 15 min. The blot was baked for two hours at 80°C and stored at room temperature until use. All the results obtained in these experiments were from these 12 blots.

Preparation of the Probes

All inserts extracted were carefully stored. Approximately 25 ng of each insert was labelled using the Amersham kit. Five ul containing 50uCi of ^{32}P dCTP having a specific activity of ~ 3000Ci/mmole, was added. Labelling was allowed to proceed overnight upon which the labelled inserts were precipitated twice with 0.5 volume of 7.5 M

ammonium acetate and 2.5 volumes of ethanol. This precipitation method was used because only labelled nucleotides are pelleted. By these method unlabeled nucleotides were removed and background labeling on blots was minimized (Wallace, 1987). Radioactivity was quantified using a scintillation counter (Beckman LS 7000 microprocessor controlled) and 1.2 millions counts per minute were used to hybridize the blots. The author did not want to add more than this amount of radioactivity to avoid having unnecessary background on blots.

Prehybridization and Hybridization Conditions

The expression of different genes was analyzed on different blots at the same time. The same conditions of hybridization were used in any set of experiment. The conditions used were either the ones described by the GeneScreenPlus manual or much lower ones. Exceptions will be noted when they were used.

High Stringency Conditions: Prehybridization was conducted for an average of 4 hours, as specified in the GeneScreenPlus manual just like for a homologous probe at 42 °C (2 ml of 50% dextran sulfate, 10% SDS, 5 ml formamide and 0,58 gr of sodium chloride per 10 ml solution). Hybridization was also conducted at 42° C and washings conditions were performed as follows:

1. Two washes with 100 ml of 2 x SSC at room temperature for 5 min. with constant agitation.

2. Two washes with 200 ml of a solution containing 2x SSC and 1.0% SDS at 60 oC for 30 min. with constant agitation.

Low stringency conditions

Another set of experiments was carried out with much lower stringency to verify the specificity of the previous responses as well as the degree of expression observed at high stringency. The conditions of these experiments were the following: 25% formamide, 1.16g of sodium chloride, 2 ml of 50% dextran sulfate, 1ml of 10% SDS /10ml of solution at 42° C. The washing conditions were also drastically different than the high stingency ones. The blots were first washed in 4x SSC twice for 5 minutes. The second wash was performed with 5x SSC and 1% SDS at 42 oC. The third wash was performed for three hours because it was noticed that the probe stuck to the nylon very strongly. The amount of background present on the blot was estimated by monitoring the blot with a Geiger counter.

Autoradiography

Upon high or low stringency prehybridization, hybridization and washings, the blots were wrapped in Saranwrap, put on X-Ray film, and inserted into film cassettes together with an amplifier. The cassettes were put at -70oC for different amounts of time as indicated for different blots.

Study of Gene Expression of the Different Genes

Wilms' Tumor Gene

A film was developed after 24 hours, and another film after 4 days following hybridization at high stringency. The hybridization was repeated using low stringency conditions. A film was developed after 24 hours, then the blot was washed at high stringency and another film was developed after 15 hours.

Platelet Derived- Endothelial Cell Growth Factor

Following high stringency hybridization, a film was developed after 24 hours, and another film after 4 days. The hybridization was repeated at low stringency and a film was developed 24 hours later.

Epidermal Growth Factor Receptor

This hybridization was performed was at high stringency. A film was processed after 24 hours of exposure, and another film developed after 4 days of exposure.

BMP-1

This hybridization was performed at low stringengy and a film was developed following 24 hours of exposure, and another film developed after 3 days of exposure.

BMP-3

This hybridization was performed at high stringency. A film was developed after 24 hours of exposure and after 4 days of exposure. This hybridization was repeated using low stringency conditions. A film was developed after 24 hours of exposure and another one after 4 days of exposure.

BMP-2

This experiment was also carried out using high stringency. A film was developed after 24 hours of exposure and another one after 3 days of exposure.

Retinoblastoma Gene

This hybridization was carried out twice. The first time hybridization was performed at high stringency, and the second time low stringency. A film was developed after 24 hours of exposure and the second one was developed after 4 days of exposure in both stringency conditions.

c-jun

Hybridization was carried out using high stringency conditions. A film was processed after 24 hours of exposure and another one was developed after 4 days of exposure.

Beta-actin

This experiment was carried on using high stringency conditions. A film was developed following 24 hours exposure.

MAV-2(O)

Hybridization conditions were low stringency. A film was developed after 24 hours exposure. The film showed a lot of background that did not allow the reading of the film. A film was then added to the cassette and exposed for 3 hours and upon which it was processed. The film was then washed at high stringency for 18 hours with distilled water. It was then left for 10 days unexposed. Thereafter another film was put in the cassette and processed 24 hours later.

Platelet Derived Growth Factor (PDGF)

This hybridization was also conducted at low stringency. A film was developed after 24 hours and 3 days post exposure.

PTPAse IB

This hybridization was carried out at high stringency A film was developed at 24 hours and a second after 3 days of exposure.

Table 4.1a. List of the different plasmids having cDNA inserts coding for different genes.

Plasmid	Insert size	Restric.enz.	Author. Address
pPDGF	960bp	Eco RI and Hind III	Dr D.Samols,. Dept of Bioch. Sch. of Med.Case West. Unv.Clvd. Ohio
pPDGF Receptor	2.65 Kb	Eco R1	ATCC, Dr D.Bowen-Pope. Dept of Path. Univ of Wash.
pBeta-actin	~1.9Kb	Hind III	Dr D.W Cleveland, Dept of Biol. Johns Hopkins .
pPD-ECGF	1.5 Kb	Eco RI	Dr C.H Heldin, Ludwig Cancer Inst.Uppsala Sweden.
pc-jun	~2.3 Kb	Bam HI	Dr I.M Verma, The Salk Inst.S.D,CA
pPJ3 RBC RB gene.	3.5 Kb	Bam HI	Dr Weinberg, Whitehead Inst. Boston, Mass.
pPTPase IB	2.7 Kb	Eco RI	Dr A Bruskin, Appd. Biotec. Cambridge.Mass.
pc-erb B	560 Kb	Bam HI	Dr N.J.Mahle. Dept of Bio. Mayo Clinic
pWilms' tumor gene	1.8 Kb	Eco RI	Dr D.E.Housman, M.I.T. Boston. Mass.
pc-bic	~1.9	Eco RI-Pst I	Dr W.Hayward.Sloan Kettering Inst.NY.
PBMP-1,2,3	849,350, 573.	EcoRI,HincII XbaI, XbaI-PstI	J.Wozney,Gen. Ins.Cambridge. Mass

Table 4.1b. List of plasmids containing inserts coding for different genes.

Plasmid	cDNA size	Restr. Enzyme	Author
TGF Beta	2.14	Eco RI	Dr H. Harris Dept of human Gen. Sch. of Med. Uni. of Pa.
CEF	350bp	Eco. RI	ATCC, Dr G. Bell H. Hughes Med Ins. Chicago
H-*ras*	6.6 Kb	Bam HI	Dr Waldren CSU, Ft Collins.

Table 4.2. Differing amounts of RNA in the different samples.
OP= Osteopetrotic birds.
Inoc= 10 day-old inoculated birds
Osteos= Osteosarcoma sample

Samples	A_{260}	A_{280}	A_{260}/A_{280}	Amount of RNA (ug)
Test 1 (OP) 47 ul	0.108	0.058	1.86	101.52
Test 2 (OP) 47 ul.	0.062	0.031	2.0	58.28
Ctl 1 (OP)30 ul.	0.063	0.033	1.90	37.8
Ctl 2 (OP) 27 ul.	0.035	0.018	1.94	18.9
Test I (inoc) 27 ul.	0.014	0.006	2.33	7.56
Test 2 (inoc) 27 ul.	0.017	0.008	2.12	9.18
Ctl 1 (inoc) 20 ul	0.025	0.01	2.5	10
Ctl 2 (inoc) 20 ul	0.02	0.00	---	8
Osteos. Test 1 45 ul	0.03	0.00	---	27.6
Osteos. Test 2 24 ul.	0.054	0.030	1.8	25.92

CHAPTER 5

RESULTS

Wilms' Tumor Gene

The use of this gene in these experiments was dictated by the need to verify whether this tumor suppressor gene was involved in osteopetrosis or osteosarcoma pathogenesis. Expression of the Wilms' tumor gene in both osteopetrotic samples was observed (Fig. 5.1). Neither the non-infected controls nor the 10 day-old inoculated birds showed any expression. More importantly, the canine osteosarcoma samples did not show any specific expression. Scanning densitometry showed that the two bands were expressed at almost the same intensity (Fig. 5.1).

This result was obtained after only 4 days of exposure. After 24 hours exposure, only the insert control lit up.

This hybridization was repeated at low stringency and was followed with a low stringency washing. There was a drastic difference in the exposure time since the film was exposed for only 24 hours and revealed a signal in both osteopetrotic samples and faint bands were observed in the two osteopetrotic controls. No hybridization was observed with 10 day-old infected birds or canine osteosarcoma

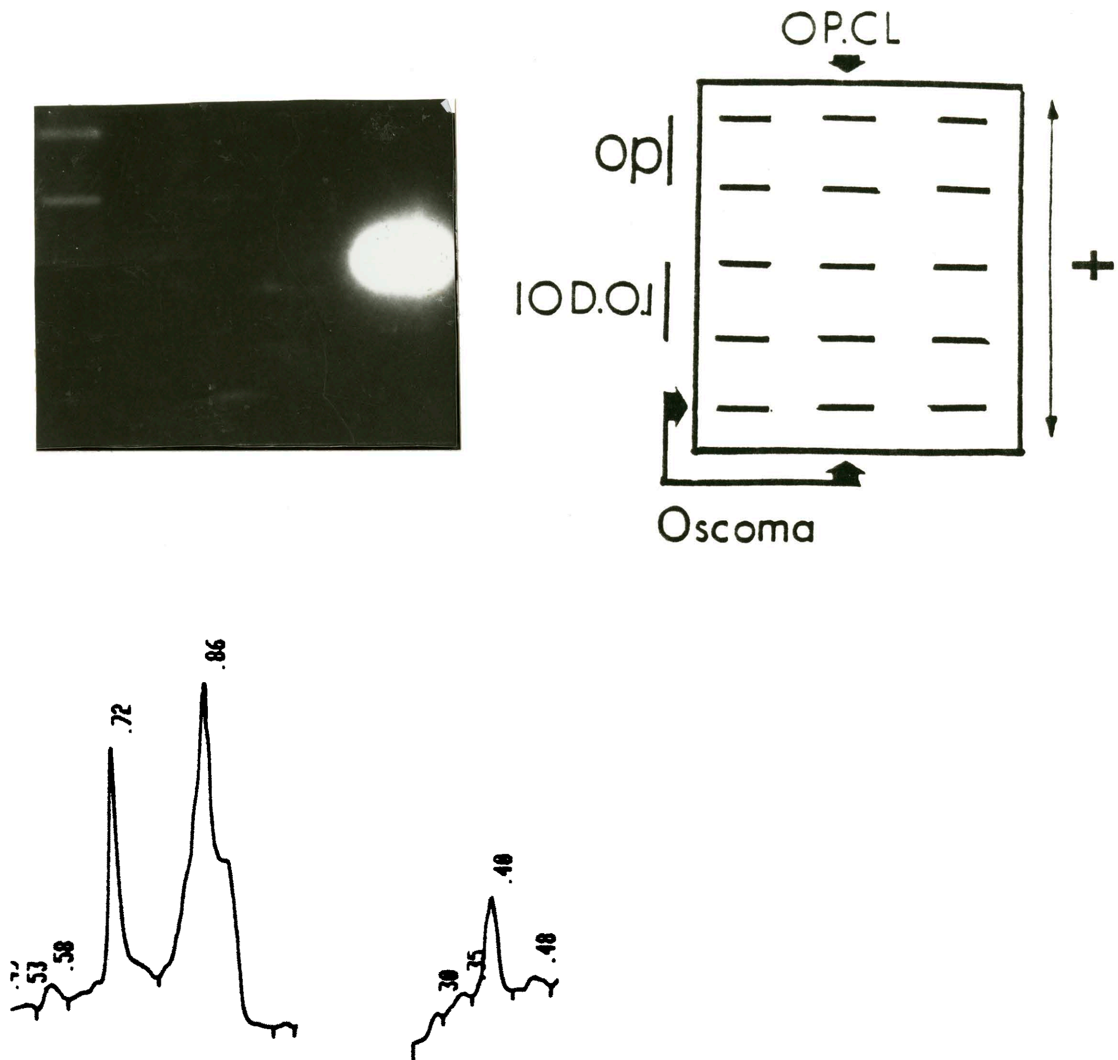


Fig.5.1: Expression of Wilms' tumor gene in the two osteopetrotic samples following high stringency hybridization and four days exposure. A non-specific signal appears in the osteosarcoma sample.
OP.Cl= Control non-inoculated birds.
OP= Osteopetrotic samples
10 D.O.I= 10 Day-old inoculated birds.
Oscoma= Osteosarcoma samples.

samples. In addition, there was a very strong background.

However the blot was washed at high stringency for three hours and exposed for 15 hours. The signal in the two controls disappeared demonstrating that the previously shown signal was non specific (Fig.5.2).

The second osteopetrotic sample showed a stronger band than in the first hybridization that was conducted at high stringency. It is likely that it was due to an extra background that persisted after the washing rather than greater expression. This, of course had had an increase in the density of the second band (Fig. 5.2).

c-erb B

According to the literature, there is no mention of any experiment that ever showed the action of c-erb B in bone formation. It was of interest to see whether or not this factor had any role in the pathogenesis of osteopetrosis. Indeed it had a role (Fig. 5.3)

A significant expression of c-erb B was observed after a 4 day exposure but not after 24 hours exposure. Expression of c-erb B was detected in both osteopetrotic samples and in one of the non-inoculated controls. This result was obtained following hybridization and washing at high stringency. Scanning densitometric studies do not indicate a significant difference between the two bands. On the other hand, the non-inoculated control shows a

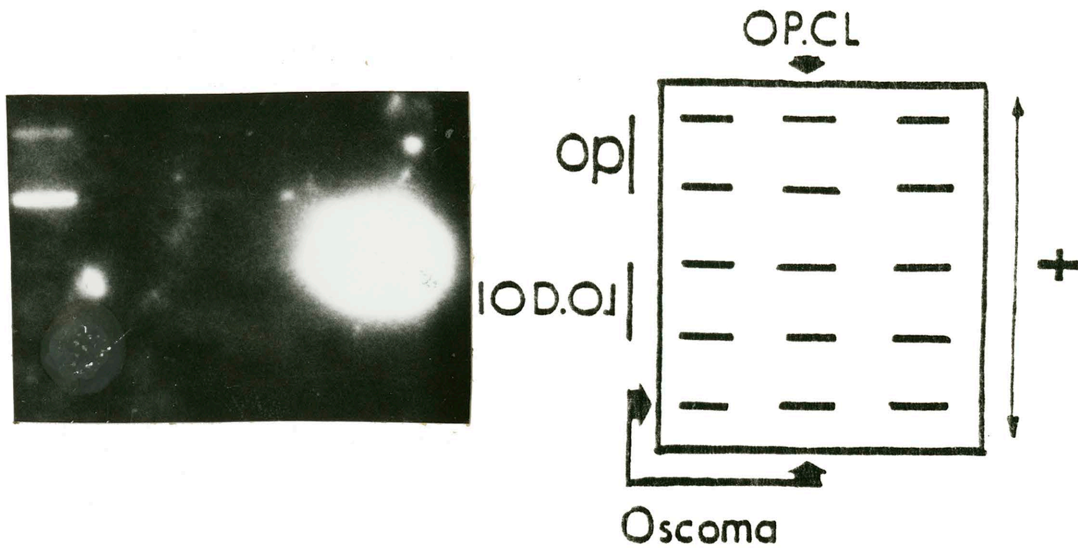
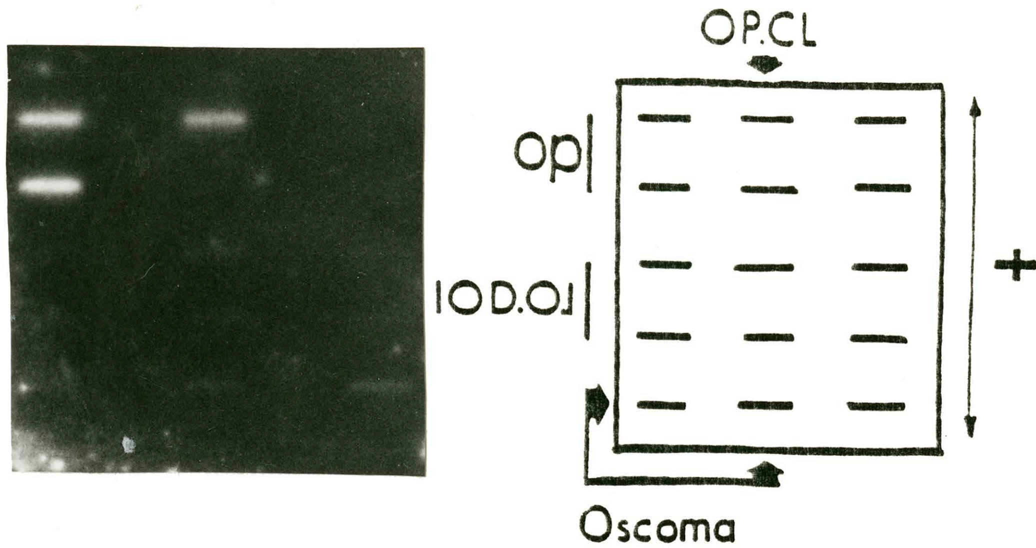


Fig.5.2: Expression of Wilms' tumor gene following low stringency hybridization. This blot was further washed at high stringengy and exposed for 15 hours.
OP.Cl= Control non-inoculated birds.
OP= Osteopetrotic samples
10 D.O.I= 10 Day-old inoculated birds.
Oscoma= Osteosarcoma samples.



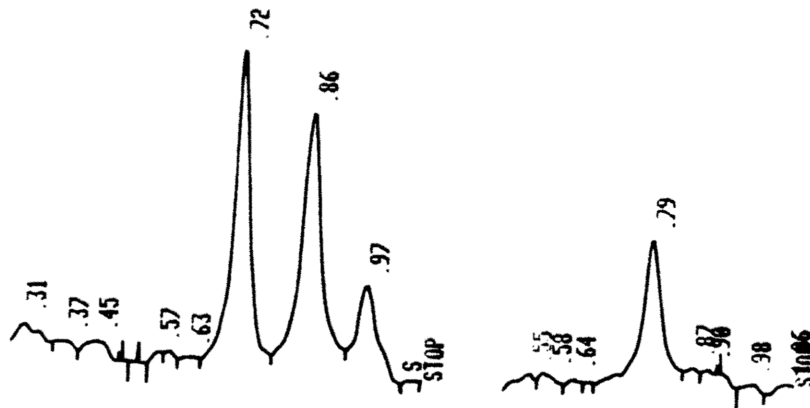


Fig.5.3: Expression of c-erb B receptor: Following high stringency hybridization and washing and four days exposure.
OP.Cl= Control non-inoculated birds.
OP= Osteopetrotic samples
10 D.O.I= 10 Day-old inoculated birds.
Oscoma= Osteosarcoma samples.

significantly lighter expression as shown by scanning densitometry.

BMP-1

Being a local-acting growth factor, it was of interest to test its expression in osteopetrotic cells. This hybridization was conducted at low stringency. An apparently weak signal appeared after 3 days of exposure. However the fact that the control positive insert had a weak detection might suggest that it was highly expressed (Figure 5.4).

BMP-3

Trying to detect this gene was an important experiment because the role of this gene is subject to controversy. This hybridization was conducted at high and low stringency. Films were examined following 1 day and 4 days of exposure. No expression was found at high or low stringency after either 1 day or 4 days of exposure (data not shown).

BMP-2

This factor was also tried because of its supposed action on bone growth. The experiment was conducted at high stringency and a film was examined three days of exposure. This factor was not shown to be expressed, neither in osteopetreosis nor in osteosarcoma samples (Data not shown).

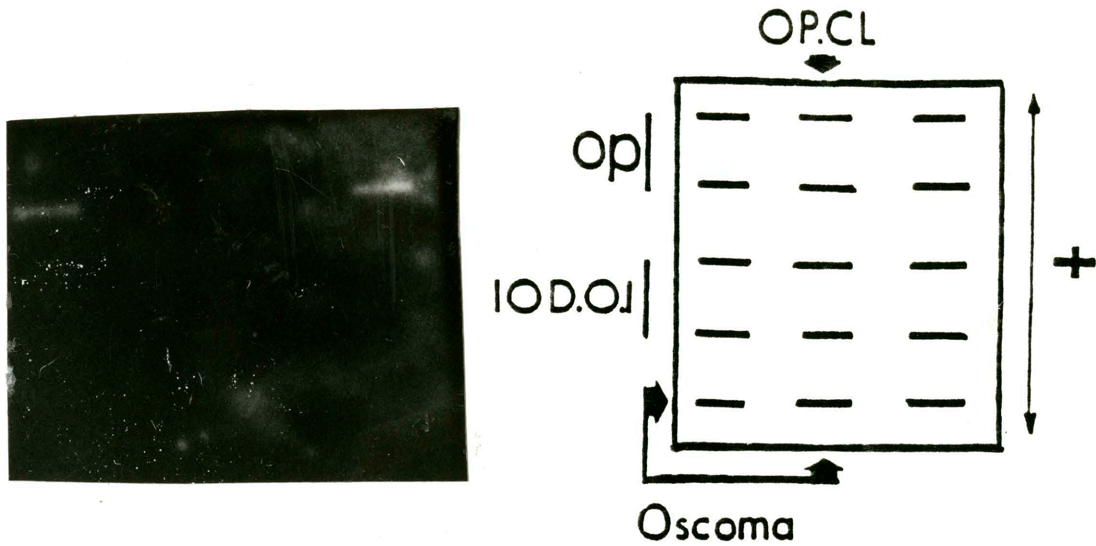


Fig.5.4: High expression of BMP-1 following high stringency hybridization and three days exposure.
OP.Cl= Control non-inoculated birds.
OP= Osteopetrotic samples
10 D.O.I= 10 Day-old inoculated birds.
Oscoma= Osteosarcoma samples.

Retinoblastoma Gene

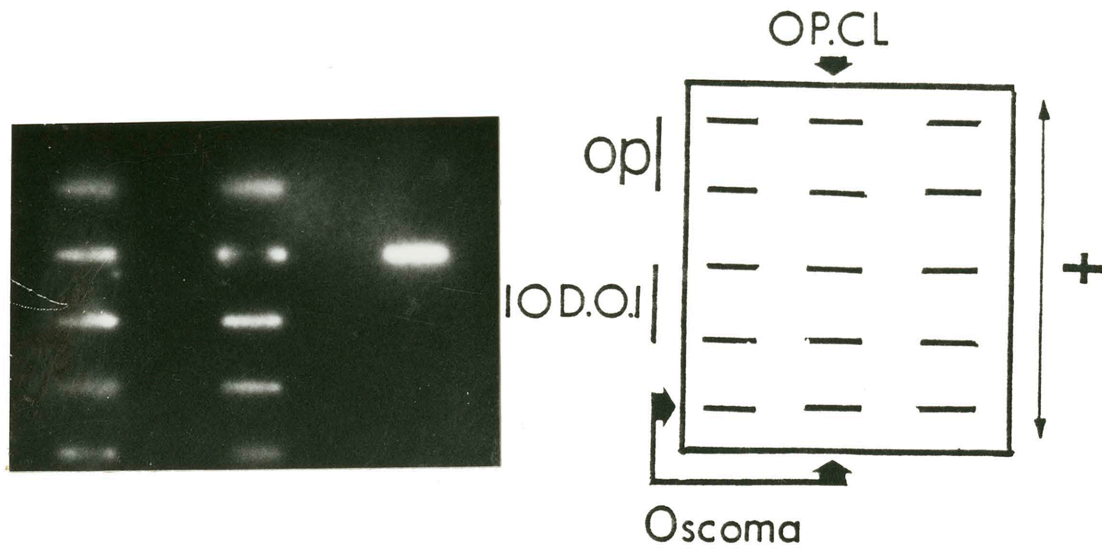
Given the involvment of the RB gene in human osteosarcoma, it was of interest to determine whether it was see whether or not it is involved in canine osteosarcoma or avian osteopetrosis. This hybridization was conducted at high and low stringency, no specific expression and films were developed after 1 day and 4 days of exposure. No expression of the RB gene was observed after 4 days of exposure at either high or low stringency. This blot showed a lot of background (data not shown).

c-*jun*

This oncogene is a DNA binding protein. Its expression is the result of mitogenic stimulation. This hybridization was conducted at high stringency and films were developed after 24 hours of exposure and after 4 days of exposure. They showed no expression at either exposure time.

Beta-actin

Beta-actin was used as an internal standard in order to verify that all samples had the same amount of RNA. This hybridization was conducted at high stringency and showed expression in all but one sample where expression was not appropriately shown. The film was examined following 24 hours exposure (Figure 5.5). It was then tested a second time but the probe did not work.



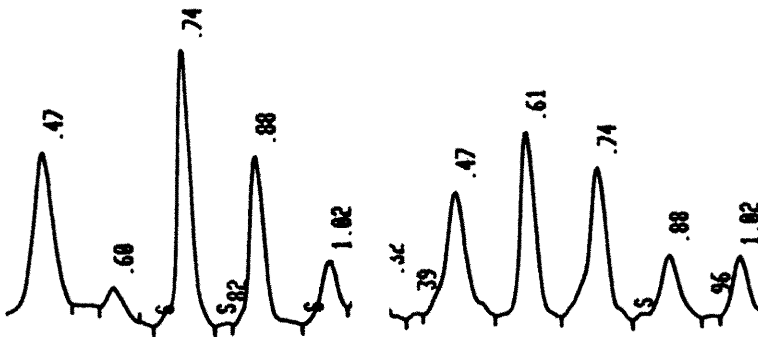


Fig.5.5: Beta-actin expression following high stringency hybridization and 24 hours exposure.
OP.Cl= Control non-inoculated birds.
OP= Osteopetrotic samples
10 D.O.I= 10 Day-old inoculated birds.
Oscoma= Osteosarcoma samples.

MAV-2(0)

This experiment was conducted to see whether the virus was detected in osteopetrotic chickens and in 10-day-old inoculated chickens. This hybridization was conducted at low stringency. Viral message was detected after 24 hours of exposure. The blot was then washed at high stringency. Ten days later another film was put into the cassette and was observed 24 hours later. The two osteopetreotic samples showed a signal. The first sample showed a much greater expression than the second one. As to the 10 day-old inoculated birds, no viral message was detected in the 10 day-old inoculated birds (Figure 5.6).

Platelet Derived Growth Factor (PDGF)

This factor is a mitogenic factor and induces bone cell proliferation. This hybridization was conducted at low stringency. A weak signal was observed in one of the osteospetrotic samples but in none of the other samples. This expression was detected only 3 days after exposure of the film (Fig. 5.7).

Platelet Derived- Endothelial Cell Growth Factor

This factor induces endothelial cell proliferation, but does not induce fibroblastic proliferation. Following high stringency hybridization, films were examined after 24 hours, and after 4 days of exposure. There was no detectable expression after 24 hours.

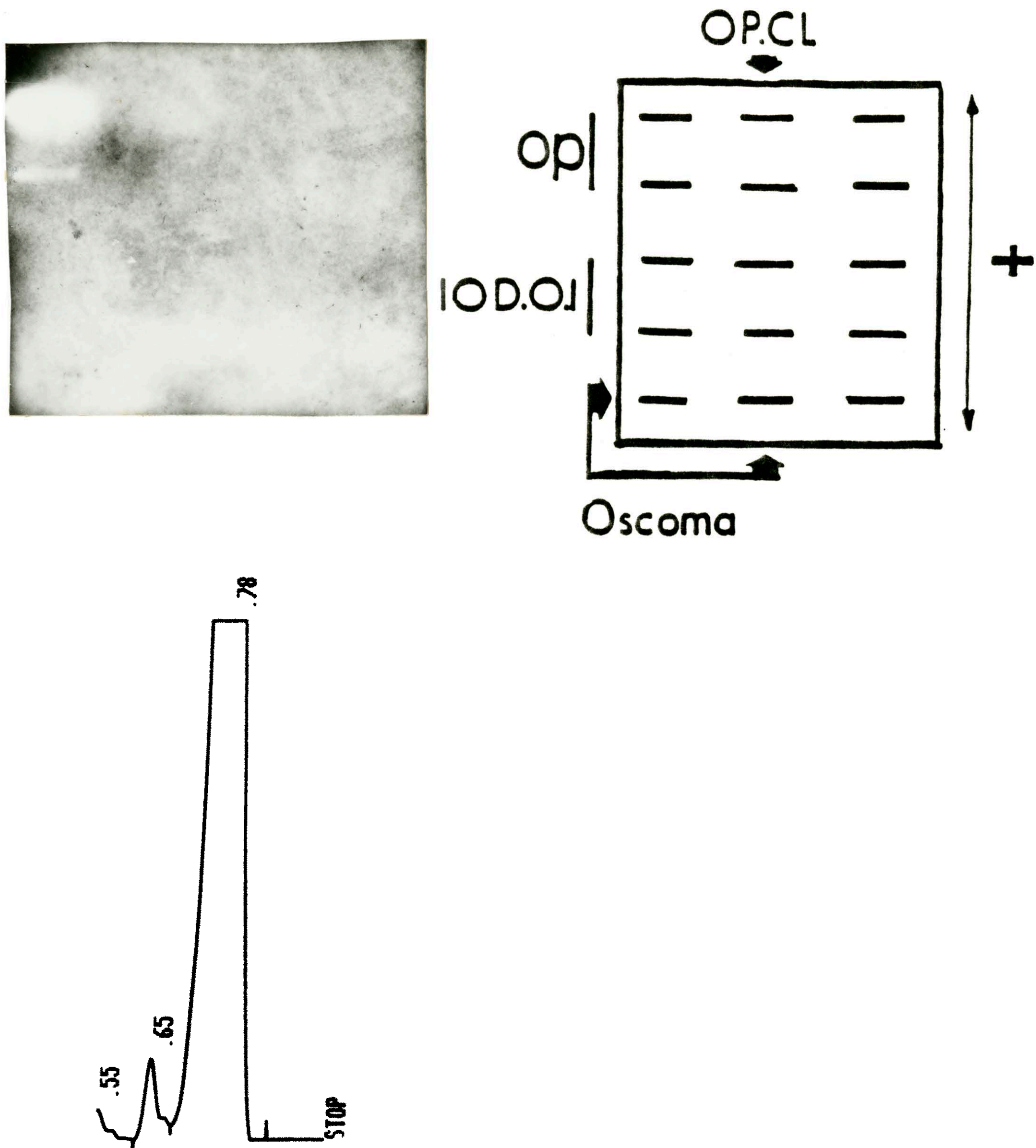


Fig.5.6: Detection of MAV-2(0) message. Hybridization was conducted at low stringency. Blot was washed at high stringency and stored ay -70 oc for 10 days. Film processed following 24 hours exposure.
OP.Cl= Control non-inoculated birds.
OP= Osteopetrotic samples
10 D.O.I= 10 Day-old inoculated birds.
Oscoma= Osteosarcoma samples.

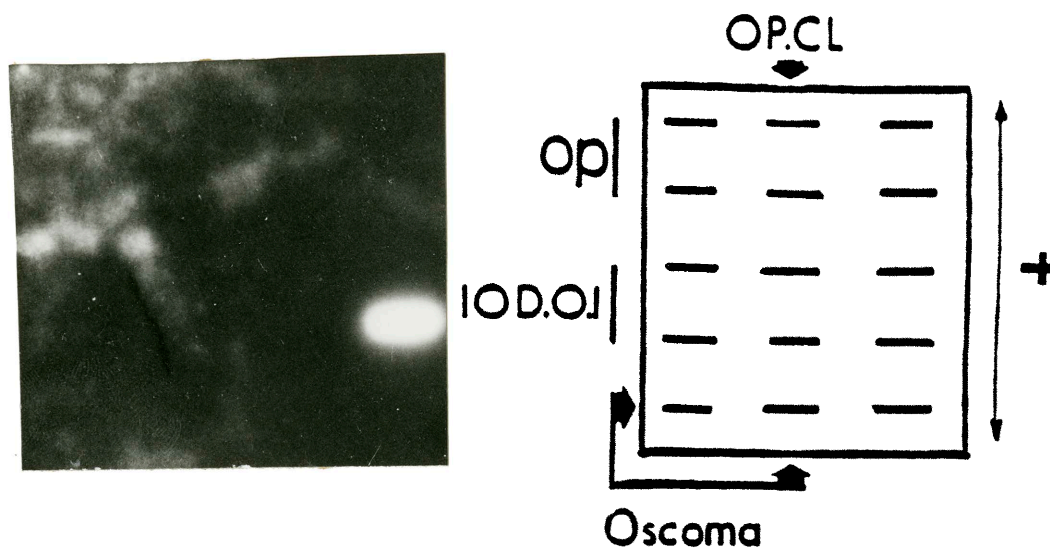


Fig. 5.7: Very weak expression of platelet-derived growth factor after 3 days exposure and low stringency hybridization.
OP.Cl= Control non-inoculated birds.
OP= Osteopetrotic samples
10 D.O.I= 10 Day-old inoculated birds.
Oscoma= Osteosarcoma samples.

After four days of exposure an expression was observed in one of the osteopetrotic and one of the canine osteosarcoma samples. Faint bands were observed in control non-inoculated chickens and in 10 day-old inoculated chickens (Figure 5.8).

PTPase IB

This is the only antioncogene whose mechanism is well known. It dephosphorylates the oncogene product and suppresses its action (Tonks et al., 1989). This experiment was conducted at high stringency.
Films were developed after 24 hours and 3 days of exposure. No expression of this gene was detected. Apparently this anti-oncogene is not expressed at all (data not shown).

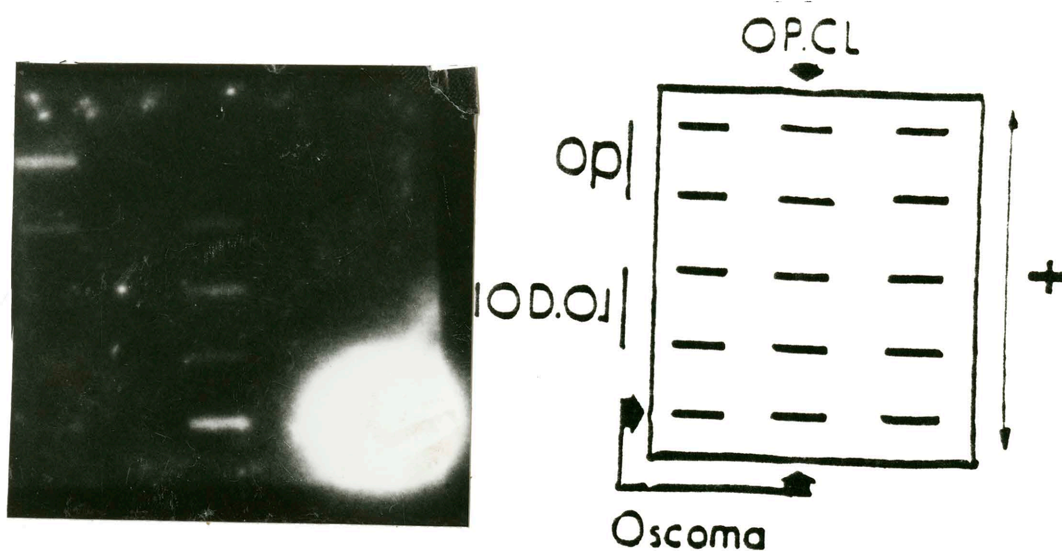


Fig.5.8: Expression of PD-ECGF following high stringency hybridization and four days exposure.
OP.Cl= Control non-inoculated birds.
OP= Osteopetrotic samples
10 D.O.I= 10 Day-old inoculated birds.
Oscoma= Osteosarcoma samples.

Table 5.1. Summary of results obtained in hybridization experiments. The criteria used to determine expression and non-expression were the observation or non observation of a signal.

Factors and controls used	Factors expressed	Factors not expressed
Wilms' tumor gene	Expressed in both osteopetrotic samples	
c-*erb* B	Expressed in both osteopetrotic samples	
PDGF	Expressed in one osteopetrotic sample.	
BMP-1	expressed in one sample.	
PD-ECGF	expressed in one osteopetrotic sample and one osteosarcoma.	
PTPase IB		Not expressed
Retinoblastoma		Not expressed
c-*jun*		Not expressed

Table 5.1b. Summary of results obtained in hybridization experiments.

Factors and controls	Expressed	Non-expressed
BMP-1	expressed in one osteopetrotic sample	
BMP-2		Not expressed in any sample
BMP-3		Not expressed in any sample
MAV-2(0)	Expressed in the two osteopetrotic samples	

CHAPTER 6

DISCUSSION AND CONCLUSION

Introduction

This examination of growth factors, oncogenes and antioncogenes in avian osteopetrosis has led to some expected findings, but most of the results were unexpected and original. These results have also demonstrated the high specificity of the slot blot technique used in these experiments to detect the expressed factors.

Expression of Wilms' Tumor Suppressor Gene

The expression of Wilms' tumor gene in osteopetrotic samples shows that there is involvment of Wilms' tumor suppressor gene in the pathogenesis of osteopetrosis. This expression was not observed in normal non-inoculated controls, 10-day-old inoculated chickens, or in canine osteosarcoma samples. The main difference between these 4 categories of samples is that the osteopetrotic cells harbor a proliferative phenotype while the non-inoculated controls and the 10-day-old inoculated chickens are in a quiescent stage. The canine osteosarcoma samples are at a high proliferation stage but they are not differentiated and do not induce bone deposition. This suggests that Wilms' tumor gene is only expressed in cells harboring a proliferative phenotype. The author speculates that this

gene is not expressed in normal controls because the cells are multiplying at a normal rate and their multiplication is subject to normal cellular control. Hybridization of the blots at a low stringency failed to show expression in any other sample.

A conclusion from these studies is that this factor is not expressed in quiescent differentiated cells. Because Wilms' tumor is a tumor suppressor gene and osteopetrotic cells are in a proliferative state, it seems appropriate to speculate that the expression of this gene is involved in the pathogenesis of osteopetrosis by controlling cell proliferation. Although the author does not consider the expression of Wilms' tumor gene in normal cells as likely, one cannot exclude the possibility it might be expressed at such a low level that it is difficult to detect. It is appropriate to speculate about how the expression of this gene influences the course of osteopetrosis. Expression of the Wilms' tumor suppressor gene certainly does not inhibit the proliferation of avian osteoblastic cells in avian osteopetrosis since the gene was expressed during continuous progression of clinical symptoms. Therefore this gene product does not appear to interfere with continuation of proliferation, otherwise the development of lesions would have stopped. Consequently, the product of this gene does not appear to counteract the expression of c-*erb* B gene. This c-*erb* B gene appears to

be involved in the proliferation of avian osteoblastic cells, since the expression of c-erb B is demonstrated in the two osteopetrotic samples. Therefore the action of Wilms' tumor gene appears to be indirect. Thus, this gene should be called a tumor suppressor rather than antioncogene because its action does not appear to impair oncogenic action. Given the observed bone deposition in osteopetrotic lesions as opposed to the osteosarcoma samples, it appears that osteopetrotic cells display some differentiation. This bone deposition would not have occurred had the cells been undifferentiated since bone deposition is an osteoblastic differentiated function. Therefore, it is possible that the Wilms' tumor gene acts as a differentiating factor, inducing the cells to differentiate, thereby preventing them from entering the cycle of malignancy, despite the continuous action of an oncogene (c-erb B). The non-expression of Wilms' tumor gene in canine osteosarcoma samples suggests that this gene is involved in the pathogenesis of osteosarcoma. Recent work shows that osteosarcoma is not solely induced by the RB gene but several other chromosomal loci on chromosome 13 and on diverse chromosomes including chromosome 11 are involved (Togushida et al., 1988). In addition, the RB gene is involved in a great number of tumors, such as adenocarcinoma of the breast (T'Ang et al., 1988), small cell carcinoma of the lung (Harbour et al., 1988; Yokota

et al., 1988) and in human prostate carcinoma cells, since transfection of this gene into these cells abolished their ability to form tumors in nude mice (Bookstein et al., 1975). However the concomittant action of an oncogene and a tumor supressor would appear to be a paradox had it not been shown that oncogenes act in a dominant manner in transformation assays (Frazier, 1989). On the other hand it also appears that tumor suppressor genes can act in a dominant manner to suppress the tumorigenic activity of dominant-acting oncogenes (Frazier, 1989). This apparent paradox is illuminated by examining the following experimental model.

When the EJ bladder carcinoma cell (containing an activated _ras_ oncogene), is fused with a normal cell, the hybrid cell is non-tumorigenic but grows in soft agar and, after repeated subcultures, eventually yields tumorigenic segregants. Analysis of gene expression in the non-tumorigenic hybrid cell shows that p21 (activated _ras_-coded protein) is expressed, therefore the _ras_ gene is indeed dominant (Stanbrisge, 1987). But the tumorigenicity of the cell is suppressed even with continued expression of an activated oncogene, indicating that a suppressor gene is also acting in a dominant manner. Therefore activation of a dominant acting oncogene is necessary but not sufficient for malignant transformation. Furthermore, the tumor-suppressing gene does not act directly to regulate synthesis of the activated oncogene, so it does not act as a typical

anti-oncogene. The author of this present work concludes that activated dominant oncogenes give the cells (in vivo) a limited proliferation advantage. During this time other changes must occur that move the cell into a cancerous state (perhaps by loss of tumor-suppressing genes) (Stanbridge, 1987). That neither the Wilms' tumor gene or the Retinoblastoma gene is expressed in canine osteosarcoma samples suggests the involvment of more than one tumor suppressor gene. These assertions are supported by the observation that several chromosomal deletions are responsible for a loss of heterozygosity in the same chromosome 13 and in other chromosomes including chromosome 11, in osteosarcoma tumor samples (Togushida et al., 1988). Therefore, the author suggests that the RB gene is a critical component of a tumor suppressor cascade. This deduction stems from the observation that cellular division involves several proto-oncogenes, some of which are DNA binding proteins or gene regulatory proteins. Thus, tumor suppressor genes may act as gene regulatory proteins. They may have a differentiating action to counteract the action of proto-oncogenes, and the end result may be full cell differentiation. In the case of virus-induced osteopetrosis, this proliferation may continue because cells are subjected to a proliferative stimulus generated by a continual stimulation of cells by the virus.

There are several implications of these results on osteopetrosis pathogenesis. There is evidence that

osteopetrosis is a highly proliferative disease, but does not evolve to malignancy because of the expression of a tumor suppressor gene. This gene may be a normal house keeping gene that is not involved in the basal metabolism of the cells but has the role of keeping the proliferation of cells in control. That is why the author believes there is a high probability this gene may be expressed in other cells during the same phenotypic conditions. The author suggests that the expression of this suppressor gene is subject to be stimulated by a cell signaling pathway, when the cell is at the threshold of malignancy. Therefore it is very likely that the expression of this tumor suppressor gene prevents osteopetrotic cells from evolving to malignancy since osteopetrosis has not been shown to evolve to neoplasia.

Epidermal Growth Factor Receptor

The expression of c-*erb* B may explain the high proliferation rate of these cells. C-*erb* B is known to be a highly mitotic factor. Its expression in both osteopetrotic samples confirms that it may be acting in such a manner in these cells. This conclusion is supported by the observation that c-*erb* B is the only factor expressed in both osteopetrotic samples at a significant level. This expression suggests active involvment in the proliferation process. However c-*erb* B was also expressed in one of the controls at low level. It is rather

difficult to explain why this factor is expressed in one of the controls. Normal differentiated osteoblastic cells do not need growth factors and therefore would not normally express proliferative factors. It is possible that this particular bird may have been subjected to an external stimulus that generated expression of this factor. This expression does not have an impact on differentiated cell proliferation since the bone was as normal as the other controls that did not show any expression. One possible explanation for this observation is that c-erb B is a highly mitotic factor and its expression does not correlate with the otherwise quiescent phenotype of differentiated bone cells. However the rapid proliferation of osteopetrotic cells suggests that a factor continuously exerts its action. The continual expression of the factor indicates that it is present to provide continual stimulation. This stimulation could be mediated by the virus that is released from the proliferating cell or from other adjacent cells. This possibility seems likely since MAV-2(0) was detected in these samples. The virus could stimulate cells by crossreacting with them as postulated in the hypothesis. Continuous stimulation could transform EGF receptor to an oncogeneic form, because the receptor appears not to be down regulated and this is sufficient to induce transformation, since downregulation is an attenuating mechanism ending the action of c-erb B (Wells

et al., 1990). According to this scheme, one can say that the cellular mechanisms are present and activated for osteopetrotic cells to be transformed, but this process is stopped by the counteracting expression of Wilms' tumor suppressor gene that prevented this transformation.

PD-ECGF

The background information in the literature review indicates that PD-ECGF's role is restricted to the stimulation of endothelial cells (Ishikawa et al., 1989). Experiments conducted as part of the present investigation demonstrate a significant expression of PD-ECGF in osteopetrotic and in osteosarcoma cells. To explain this expression, two reasons are proposed that are not mutually exclusive. It is established that MAV-2(0) induces anemia by destroying erythrocyte progenitor cells (Smith 1982). In addition, the proliferating lesions induce a drastic destruction of the bone marrow leading to diminished vascularization. The author wonders if PD-ECGF is secreted in order to replenish the damaged vascularisation, perhaps as a repair mechanism. Another explanation takes into account the observation that tumors at their inception secrete angiogenic factors. It is possible that PD-ECGF is expressed because cells may have undergone some early phase of oncogenic conversion, including a step calling for the expression of PD-ECGF. The lower expression in controls, may be attributable to the normal angiogenesis observed during remodeling.

PDGF

PDGF was expressed in one osteopetrotic sample and not in the other one. It was not expressed in controls or in the canine osteosarcoma samples. A low expression of PDGF in canine osteosarcoma samples has been found by others (Korchevar, 1990). This result is unexpected in that PDGF is expressed in vitro in tumor cells to facilitate the mitotic division of cells by reducing the adherence of cells on the glass bottle (Herman et al., 1985).

The interesting observation about this expression is that it is weak in avian osteopetrotic bone and present in only one sample. In the normal cycle of the cell, PDGF is a factor that leads to proliferation but its action is restricted to the beginning of the cell cycle (Canalis et al., 1985). PDGF makes cells competent to replicate, and the cells arrested at the GO/GI phase are stimulated to enter into S phase (Canalis et al ., 1985). This role for PDGF makes it likely that this factor is involved in bone formation but not at this level because the cells are at a more evolutive stage in their replication cycle and therefore do not need PDGF. Other factors need to take over in order to carry on the proliferative cycle in order to stimulate bone formation. Once a factor is expressed at a given time another will succeed it at another stage and so on. Despite its low expression in only one sample, it appears to represent the sequel of a previous involvment.

BMPs

The background information provided in Chapter 2 showed that BMPs induce the proliferation of cartilage cells (Wozney et al., 1988) and the process is continued for the proliferation of bone cells (Luyten et al., 1989). These factors are responsible for the initiation of bone formation. Of the three BMPs, only BMP-1 was significantly expressed in one osteopetrotic sample. The fact that this factor is expressed in one osteopetrotic sample appears to eliminate it's action as the origin of the prolififeration of cells in avian osteopetrotic cells. Although BMP-1 has biological characteristics that are similar to BMP-2 and BMP-3, BMP-1 is not a member of the TGF Beta super gene family as are the two others (Wozney et al., 1988). Important conclusions that may be drawn from these results are the following:

1) BMP-1 seems to act at an early stage of differentiation which may explain why it is only expressed in one osteopetrotic sample. It is suggested that BMP-1 like BMP-2 and BMP-3 is a differentiating factor commiting mesenchymal cells (fibroblasts) to the osteoblastic phenotype (Reddi, 1975). However the possibility that this factor is brought to the bone matrix by the blood circulation is not excluded, since the non-inoculated controls did not express the factor. One interpretation is that BMP-1 is either brought to the bone matrix by the

circulation or it is expressed at an early stage during chondrogenesis.

2) It appears that BMP-2 and BMP-3 are not expressed by bone cells, therefore one may speculate that they are brought to the bone matrix by the blood circulation and establish themselves in the bone matrix. Another possibility is that like BMP-1, they are involved at an early stage of differentiation. Another important consideration is that this factor was not expressed in canine osteosarcoma samples in contrast to its expression in an osteosarcoma cell line (Wozney et al., 1988). This might reflect the difference between cell culture and *in vivo* investigations, in that the cells *in vitro* may express a different phenotype since they have a different environment.

Retinoblastoma Gene

The experiment showed that this gene was not expressed in canine osteosarcoma samples which was expected given the association of this gene with human osteosarcoma (Hansen et al., 1985). However it was not expressed in osteopetrotic or in control chicken bone. This result was unexpected because this gene is associated with the induction of osteosarcoma and loses its heterozygosity in many but not all samples examined (Togushida et al., 1988). It is logical to think that the *RB* gene should be expressed in normal bone cells. It is unlikely that the gene was not

homologous enough for detection because the experiment was repeated at very low stringency and no expression was found.

PTPase IB

No expression to this gene was found in any of the chicken or dog osteosarcoma samples studied. This result may mean that the gene is not expressed at all in bone cells. It may also mean that the homology is so low that the homologous message was not detected. But this latter explanation does not appear to the author as likely because DNA sequences of antioncognes, oncogenes, growth factor and homeobox genes are highly conserved.

MAV-2(0)

The two osteopetrotic samples expressed a positive signal for viral message in both osteopetrotic samples but not for the 10 day-old inoculated birds. This confirms the presence of the virus in the osteopetrotic birds. However this result does not mean that the virus was absent in the 10-day-old inoculated birds. The author beleives that the amount of virus in these cells was not high enough to be detected when one considers the differing degree of expression in the two osteopetrotic samples used. There was a big difference of expression between the two samples and yet the two birds were of the same age and had

approximately the same degree of lesions. It is also possible that during extraction some of the message was lost in one of the samples.

Beta-actin

As expected, this constitutively expressed message was present in all samples with some degrees of variation due to the spectrophotometer inaccuracies in estimating the amount of RNA present. However, the result still indicates the presence of the message in all samples, with one exception that may be due to an abnormal deposition of the RNA in this particular slot.

Global Interpretation of These Results

The first hypothesis stated that the virus MAV-2(0) stimulates growth factors or oncogene receptors at the cell membrane. This in turn will generate the synthesis of a growth factor inducing osteoblastic proliferation. This proliferation was not shown when virus was inoculated into 10-day-old chickens because their cells are differentiated and have a different set of gene regulatory proteins.

How can these results be interpreted at the light of the first hypothesis formulated by the author ?

1) There is no one single result that contradict it.

2) A highly mitotic factor (c-*erb* B) was expressed demonstrating the proliferative phenotype of the disease. Most importantly, this factor was not expressed in 10-day-old inoculated birds that behaved just like the controls; although infected, they did not generate the expression of factors, oncogenes or antioncogenes with the exception of a slight expression of PD-ECGF known to have an action on endothelial cells, not on bone cells. In a way this is understandable because the birds do not show any lesion when they are inoculated at this stage therefore there is no proliferation of cells, since osteoblastic cells are differentiated and therefore at a quiescent stage, and do not need to express a growth factor.

Even if the cells are not proliferating, the virus could induce the expression of the factor (c-*erb* B) even if the factor is not going to induce a proliferative action since the cells are differentiated and are not likely to respond to a proliferating factor. So this supports an important point of the author's hypothesis, in that the cells are at a different stage of differentiation, and have a different set of gene regulatory proteins.

Since this set is different, the external stimulus will not have the same impact as it would have on embryos cells that have a different set. Proliferation of bone cells is accompanied by the expression of several growth factors, especially EGF receptor (c-*erb* B) which is a potent mitogenic factor known to generate DNA binding

proteins such as c-jun and induce the mitotic activity shown by the cell. This hypothesis is supported by the obervation that every proliferative stimulation of cells is initiated at the cell membrane (Freeman et al., 1989). Since MAV-2(0) is responsible for the proliferation observed in avian osteopetrosis, it follows that it is likely to act at the cell membrane. On the other hand, a perpetuation of the proliferative phenotype could only be explained by the continued action of the virus. This is not difficult to imagine, since these studies demonstrated viral activity in the 11 week-old chickens. A similar situation may exist in that herpes cytomegalovirus is implicated in the induction of oncogene expression although this virus was UV inactivated (Boldogh et al., 1990). However none of the authors provide an explanation as to how this mechanism works.

The fact that osteopetrotic chickens were different in age than the 10 day-old inoculated chicks is by no means a handicap for the interpretations of these results. The difference between the two is that the cells of the former are proliferating whereas the latter cells are differentiated and not proliferating. The difference between osteopetrotic birds and their respective controls, is greater than that between the 10 day-old-inoculated birds and the non-inoculated osteopetrotic controls that differ in age.

The other hypothesis formulated in this research concerned the non-evolution of osteopetrotic cells to neoplasia. This aspect has been debated in detail in the discussion section for the expression of Wilms' tumor gene, however it is worth mentioning that to the author's knowledge, no similar case has ever been reported _in vivo_. Only _in vitro_ has the antagonistic action of a concomittant oncogene and tumor suppresor gene been shown (Stanbridge, 1987). Because Wilms' tumor is a tumor suppressor gene, its expression in proliferative osteopetrotic cells is highly significant. The conclusion is that the only reason for the expression of the Wilms' tumor gene is to prevent cells from entering the malignant cycle by attempting to differentiate the osteoblastic cells.

Conclusion

This work has been extremely complex because it dealt with a very hard and a complex tissue. Intensive investigations were carried out and took a long time to complete since different techniques were tested to perfect every stage of bone sampling, processing and the RNA extraction itself, all in RNAse free conditions.

However a protocol of RNA extraction was developed which was instrumental in the success of this project. This is especially important given the fragility of RNA and the long time needed to process every sample.

The protocol developed gave consistent results and turned out to be quite reliable and efficient in both the quality and the yield of the mRNA obtained. Therefore it was a technical achievement to keep this RNA undegraded.

1. It is known that the study of gene expression in bone cells has been extremely difficult, because of its intrinsic complexity (Hauschka et al., 1986) and because of the painful and cumbersome work required to extract RNA. This was due to the fact that bone had to be ground without demineralization.

In the light of current bone research, this extraction protocol is expected to be used efficiently since it is a rapid one. Bearing in mind that any RNA work has to take into consideration the time factor, since an increase in the processing time can only increase the probability of RNA degradation, it has every chance to be successful.

Therefore it is important to have a rapid technique to use different samples at the same time to speed up the study of bone biology, to investigate the cascade of factors involved in the control of bone formation, using either normal or osteopetrotic bone. This knowledge is essential if one wants to establish a treatment for osteopenic and other related bone disorders.

2. Osteopetrotic chickens were a good animal model because one could reproduce the lesions easily. In addition, the proliferative status of this disease guarantees sufficiently high mRNA yields given the

selective proliferation of osteoblastic cells. Moreover, the fact that the virus used was shown to be apathogenic to humans adds to the importance of this model.

3. The author beleives that this the first large scale investigation undertaken in *in vivo* conditions that has involved such a large array of different probes to have provided a comprehensive view of avian osteopetrosis and canine osteosarcoma. Research in cell culture has provided conflicting results. Many researchers have advocated the role of some growth factors in bone induction, but none was shown to be directly linked to bone formation when tested in *in situ* (Wozney et al., 1988). This research has demonstrated that there is a significant difference between *in vitro* and *in vivo* work since BMP-1 was expressed in osteosarcoma cell lines (Wozney et al.,1988). The present investigations demonstrate that BMP-1 was not expressed at all, even at low stringency conditions, supporting the author's decision to work in *in vivo* conditions to have a real estimation of the events taking place in *in vivo* situation. The phenotypic status of cells living in physiologic conditions is different from cells grown in fixed growth conditions, therefore the response is different.

5. Another important finding was that BMP-2 and BMP-3 were not expressed in bone cells. These results eliminate bone cells as being at the origin of the secretion of these factors. BMP-1 was expressed in an apparently weak manner

but considering a low detection of the control, it is assumed that it is relatively highly expressed. However its expression in only one sample eliminates it de facto as having a significant importance in being directly involved for the observed bone proliferation or in the normal process of bone differentiation since this factor was not expressed in normal bone cells. Its expression in only one osteopetriotic bird may indicate an earlier involvment in the process of the observed high proliferation. This confirms the work of Wozney and his group (1988). BMPs were only able to initiate the proliferation of cartilage cells as opposed to another group (Luyten et al., 1988) that claimed that BMP-3 factor was by itself able to induce the proliferation of bone cells.

The present result demonstrates unequivocally that BMP-2 and BMP-3, are not expressed in bone cells, either in a proliferative (osteopetrosis) or in a quiescent stage (normal control birds).

However another significant point is that the origin of the BMPs is not known (Wozney et al., 1988). This work confirms that BMP-1, at least, could be expressed in bone cells and this may clarify the origin of this factor. This does not mean that it is secreted only in bone cells though.

4. The other positive result concerns the expression of a potent mitotic oncogene (c-erb B), confirming at the molecular level the proliferative nature of the disease.

The fact that 10 day-old inoculated birds did not express any factor, seems to confirm the author's hypothesis that cells at a different stage of differentiation will differ in their proliferative response to virus infection. The best way to unequivocally confirm this hypothesis is to use inactivated virus and inoculate it to 10-day-old embryos. MAV-2(0) virus was grown in cell culture and purified by sucrose gradient using the method of Smith (1979). This virus was further inactivated by U.V light for 40 min. The use of c-erb B terminology instead of EGF receptor is considered as appropriate because there is a strong feeling that this factor is expressed continuously in osteopetrotic cells, therefore this factor is not down regulated and in this circumstance is transformed to its oncogenic form. This transformation to the oncogenic form does not need a mutation of the oncogene. A perpetual stimulation is all that is needed.

Furthermore, the author believes that the study of the interaction of growth factors, oncogenes, and antioncogenes has revealed interesting aspects of osteopetrosis pathogenesis. The most important of all the results was finding the expression of Wilms' tumor antioncogene in osteopetrotic chickens. This could be regarded as a novel regulatory mechanism explaining why proliferating cells that accompany a pathologic situation are stopped from becoming neoplastic. Therefore its expression has an important implication in the pathogenesis of osteopetrosis in

particular, and in the biology of tumor induction in general. This conviction arises from the fact that this gene is not a growth factor or an oncogene, whereby its expression with other factors may make one wonder which one has a direct influence on the observed proliferation. Rather, its expression when compared to the other samples can only be justified by the need to keep the cell proliferation in control and prevent them from entering the cycle of neoplasia, since its expression does not stop the cells from proliferating (high expression of EGF receptor) confirming therefore the author's hypothesis.

BIBLIOGRAPHY

Abramson, D. H., Ellsworth, R. M., Kitchin, F. D. 1984. Second mononuclear tumors in Retinoblastoma survivors. Ophtalmology **91**:1351-1355.

Ash, P., Loutit, J.F., Townsend, and K. M. S. 1980. Giant lysosomes, a cytoplasmic marker in osteoclasts of beige mice. J. Pathology. **130**:237-245.

Ash, P., Loutit, J. F., Townsend, K. M. S. 1980. Osteoclasts derived from haemopoietic stem cells. Nature **283**:669-670.

Atchinson, M. L. 1988. Enhancers mechanisms of action and cell specificity. Annu. Rev. Cell Biol. **4**:127- 153, 1988.

Aurigemma, R. E., Comstock, R. D., and Smith, R. E. 1989. Persistent viral DNA synthesis associated with an avian osteopetrosis-inducing virus. Virology. **171**:626-629.

Aurigemma, R. E., Simon, C., Hayward, W. S., and Smith, R. E. 1990. Preparation of a detailed restriction map of the avian leukosis virus MAV-2(0). Avian diseases. **34**:99-105.

Banes, J., and Smith, R. E. 1977. Biological characterization of avian osteopetrosis. Infection and Immunity. **16**:876-884.

Banks, W. C., Morris, E., Herron, M. R., and Green, R. W. 1975. Osteogenic sarcoma associated with internal fracture fixation in two dogs. J. Am. Vet. Med. Assoc. **167**:166.

Bassett, C. A. L., Creighton, D. K., Stinchfield, F.E. 1961. Contribution of endosteum, cortex and soft tissues to osteogenesis. Surg. Gynecol. Obstet. **112**:145-152.

Beard, J. W. 1963. Viral tumors of chickens with particular reference to the leukosis complex. Ann. N. Y. Aca. Sci. **108**:1057-1085.

Benedict, W. F., Xu, H-J., and Takahashi, R. 1990. The retinoblastoma gene: Its role in human malignancies. Cancer Investigations. **8**:535-540.

Benya, P. D., Schaffer, J. D. 1982. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell. **30**:215-224.

Bernhard, W. 1958. Electron microscopy of tumor cells and tumor viruses. A review. Cancer Res. **18**:41-58.

Blidz, R. M., and Pellegrino, E. D. 1965. Avian osteopetrotic bone. J. Bone Jt. Sur. **47A**:1365-1377.

Boldogh I., AbuBAkar S., Albrecht T. 1990. Activation of Proto-oncogenes : an immediate early event in human cytomegalovirus infection. **247**:561-564.

Bolognesi, D. P., Huper, G. Green, R. W. and Graf, T. 1974. Biochemical properties of oncoviruses polypeptides. Biochim. Biophsys. Acta **355**:220-235.

Boni-Schnetzler, M., Boni, J., Ferdinand, F. J. and Franklin, R. M. 1985. Developmental and molecular aspects of nephroblastoma induced by avian myeloblastosis-associated virus 2-0. J. Virol. **55**:213-222.

Bonucci, E., Sartori, E., Spina, M. 1975. Osteopetrosis Fetalis. Report on a case, with special reference to ultrastructure. Virchows [Arch. Pathol]. **368**:10

Bookstein, R., Shew, J. Y., Phang-lang, C., Scully, P., and Lee, W. H. 1990. Suppression of tumorigenicity of human prostate carcinoma cells by replacing a mutated RB gene. Science. **247**:712-715.

Boskey, A. L., and Posner, A. J. 1984. Bone structure, composition , and mineralization. Orthop. Clin. North Am. **15**:597-612.

Broger, T. G., and Cavennee, W. K. 1990. several reference to ultrastructure. Virchows rch368 109

Brostrom, L. A. 1980. On the natural history of osteosarcomas: Aspects on diagnosis, prognosis and endocrinology. Acta. Orthop. Scand. **183**:9

Buhrow, C. A., Cohen, S. and Staros, J. V. 1982. Affinity labeling of the protein kinase associated with the epidermal growth factor receptor in membrane vesicles from A 431 cells. J. Biol. Chem. **257**:4019-4022.

Canalis, E. 1980. Effect of insulin growth factor I on DNA and protein synthesis in cultured rat calvaria. J. Clin. Invest. **66**:709.

Canalis, E. and Raisz L. G. 1979. Effect of epidermal growth factor on bone formation in-vitro. Endocrinology. 1979. **104**:862-869.

Canalis, E. 1987. Growth factors and bone formation. Calcif. Tiss. Int. **41**:89.

Canalis, E. 1985. Effect of growth factors on bone cell replication and differentiation. Clinical orthopedics and related research. **193**:246-263.

Canalis, E. 1985. Effects of endothelial cell growth factor on bone remodeling _in_ _vitro_. Journal of Clinical investigation. **79**:52-58.

Carlin, R. C., Tollefson, A. E., Brady, H. A., Hoffman, B. L., and Williams, S. M. 1989. Epidermal growth factor receptor is down regulated by a 10,400 MW protein encoded by the E_3 region of Adenovirus. Cell. **57**:135-144.

Carpenter, G. and Cohen, S. Epidermal growth factor. Annu. Rev. Biochem. 1979. **48**:193.

Carrington, J. L., Roberts, A. B., Flanders, K. C., Roche, N. S. and Reddi, H. 1988. Accumulation, localization, and compartmentation of transforming growth factor Beta during endochondral bone development. Journal Cell Biol. **107**:969-1975.

Centrella, M., McCarthy, T. L., and Canalis, E. 1990. Growth factors and cytokines in bone: A treatise, Vol. 6, B.K. Hall, Ed. (Telford Press, Caldwell, N.J.).

Chen, W. S., Lazar, C. S., Poinie, M., Tsien, R.Y., Gill, G.N., and Rosenfeld, M. G. 1987. Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. Nature. **328**:820-823.

Chirgwin, J. M., Prybyla, A. E., MacDonald, R. J. and Rutter, W. J. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochem. **18**:5294.

Chiu, I. M., Reddy, E. P. Givol D., Robins, K. C., Tronick, S. R. and Aaronson, S. A. 1984. Nucleotide sequence analysis identifies the human c-_sis_ proto-oncogene as a structural gene for platelet-derived growth factor. Cell **37**:123-129.

Chomczinski P. and Sacchi, N. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate phenol extraction. Anal. Biochem. **162**:156-159.

Chung, C. T., Niemela, S. L. and Miller, R. H. 1989. One-step preparation of competent Escherichia coli: Transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. **86**:2172-2175.

Colbert, J. T., Costigan, S. A. and Zhao, Z. 1990. Photoregulation of Beta-tubulin in RNA abundance in etiolatid oat and baby siblings. Plant physiology **93**:1196-1202

Collart, K. L., Aurigemma, R., Smith, R. E., Kawai, S. and Robinson, S. L. Infrequent involvment of c-fos in avian leukosis virus-induced nephroblastoma. Jounal of Virol. **64**:3541-3544.

Conrad, G. W., Hart, G. W., Chen, Y. 1977. Differences in-vitro between fibroblast-like cells from cornea, heart, and skin of embryonic chicks. J. Cell Sci. **26**:119-137.

Cooper, K. L., McLeod, R. A., and Beabout, J. W. 1983. Radiologic evaluation. In Sim, F. H. (ed.). Diagnosis Treatment of Bone Tumors: A Team Approach, Slack, Thorofare, N. J. P. 10.

Cummins, T. J. and Smith, R. E. 1988. Analysis of hematopoietic and lymphopoietic tissue during a regenerative aplastic crisis induced by avian retrovirus MAV-2(0). Virology **163**:452-461.

De-combrugghe, B., Busby, A., Buc, H. 1984. Cyclic Amp receptor: Role in transcription activation. Science **224**:831-838.

Dracopli, N. C., Aldahef, B., Houghton, A. N. and Old, L. J. 1987. Loss of heterozygosity at autosomal and X-linked loci during tumor progression in a patient with melanoma. Cancer Res., **47**:3995-4000.

Dracopli, N. C., Houghton, A. N. and Old, L. J. 1985. Loss of polymorphic restriction fragments in malignant melanoma: Implications for tumor heterogeneity. Proc. Natl. Acad. Sci. USA. **82**:1470-1474.

deThe, G., Becker, C. and Beard, J. W. 1964. Virus of avian myeloblastosis (BAI strain A). XXV. Ultrachemical study of virus myeloblast phosphatase activity. J. Natl. Cancer Inst. **32**:201-237.

Dougherty, R. M., Conklin, R. H., Whalen, J. P. and Distefano, H. S. 1968. Etiology of avian osteopetrosis. Fed. Proc. **27**:681.

Dreyer, W. J. and Benneth, J. C. 1965. The molecular basis of antibody formation : a paradox. Proc. Natl. Acad. Sci. USA. **54**:864-869.

Dumanski, J. P., Carlborm, E., Collins, V. P., and Nordenskjold, M. 1987. Deletion of a locus on human chromosome 22 involved in the oncogenesis of meningioma. Proc. Natl. Acad. Sci. USA. **84**:9257-9279.

Dryja, T., Papaport, J. M., Joyce, J. M., Petersen, R. A. 1986. Molecular detection of deletions involving band 14q of chromosome 13 in Retinoblastoma. Proc. Natl. Acad. Sci. USA. **83**:7391-94.

Eden, S. 1983. Specific binding of growth hormone to isolated chondrocytes from rabbit ear and epiphyseal plate. Endocrinology **112**:1127.

Ellingsworth, L. R., Brennan, J. E. Fok, F. Rosen, D. M. Bentz, Piez, K. A and Seyedin, S. M. 1986. Antibodies to the N-terminal portion of cartilage inducing factor Beta. J. Biol. Chem.**261**:12362-12367.

Emerson, B. M., Nickol, J.M., Jackson, P. D. and Felsenfel, G. 1988. Analysis of the tissue specific enhancer at the 3′ end of the chicken adult Beta-globin genes. Pro. Natl. Acad. Sci. USA **85**:5976-5980.

Esch, F., Uno, N., Baird, A., Hill, F., Denoroy, L., Ling, N., Gospodarowicz, D. and Guillemin, R. 1985. Primary structure of bovine brain acidic fibroblast growth factor (FGF). Biochem. Biophys. Res. Commun. **133**:554-562.

Fearon, E. R., Feinberg, A. P. Hamilton S. H., and Volgelstein, B. 1985. Loss of genes on the short arm of chromosome 11 in bladder cancer. Nature. **318**:377-380.

Ferguson, D. 1987. Bovine bone morphogenic protein (bBMP) fraction-induced repair of craniotomy defects in the rhesus monkey (Macaca speciosa). Clinical Orthopedics and Related Research **219**:251-258.

Fischman, D. A., and Hay E. D. 1962. Origin of osteoclasts from mononuclear leukocytes in regenerating newt limbs. Anat. Rec. **143**:329-333.

Francke, U., Holmes, L. B., Atkins, L. B., Atkins, L. B., and Riccardi, V. M. 1979. Aniridia-Wilms′ tumor association: evidence for specific deletion of 11p13. Cytogenet. Cell Genet. **24**:185-192.

Frazier, M. E. 1989. State of the art address oncogenes and tumor suppressing genes. Proc. 7th ACVIM FORUM. San-Diego.

Freeman, C. S., Kimes, B. W., Martin, M. R., and Marks, C. L. 1989. An overview of tumor biology. Cancer Investigation **7**:247-265.

Friedenstein, A. J. 1968. Induction of bone tissue by transitional epithelium. Clin. Orthop. Rel. Res. **59**: 21-35.

Friedenstein, A. J. 1976. Precursor cells of mechanocytes. Int. Rev. Cytol. **47**:327-355.

Friedenstein, A. J. 1973. Determined and inducible osteogenic precursor cells, hard tissue growth, repair and remineralization. Ciba Foundation Symposium II. New-York, Elsevier, pp 169-181.

Friedenstein, A. J., Chailakhjan, R. K., and Lalykina, K. S. 1970. The development of fibroblast colonies in monolayer cultures of guinea pigs bone marrow and spleen cells. Cell Tiss. Kinet. **3**:393-402.

Friend, S. H, Bernards, R., and Rogels, S. 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature **323**:643-646.

Fung, Y. K. T, Murphee A. L; T'ang, A. 1984. Structural evidence for the authenticity of the human retinoblastoma gene. Science **236**:1657-1355.

Garrels, J. I. 1979. Changes in protein synthesis during myogenesis in a clonal cell line. Deve. Biol. **73** :134-152.

Gimenez-Gallego, G., Rodky, J., Bennett, C., Rios-Candelore, M., Disalvo, J. and Thomas, K. 1985. Brain derived acidic fibroblast growth factor. Complete amino-acid sequence and homologies. Science. **230**:1385-1388.

Gold, D. W. and Casson, J. C. 1968. Hormones that stimulate the growth of blood cells. Scientific American. p62-70.

Gothlin G., Ericsson J. L. E. 1976. The osteoblast: review of ultrastructure, origin and structure function relationship. Clini. Orthop. Rel. Res **120**:201-228.

Gowen, M., Wood, D. D ., and Russel, R. G. 1985. Stimulation of the proliferation of human bone cells in vitro by human monocyte products with Interleukin-1. activity. J. Clin. Invest. **75**:1223-1229.

Graf, T. 1972. A plaque assay for avian RNA tumor viruses. Virology **50**:567-578.

Grotendorst, G. R., Pencev, D., Martin, G. R, Sodek, J. (1984). Molecular mediators of tissue repair. In: Hunt, TK, Heppenstall, R. B, Pines E., Rovee, D. (eds) Soft and hard tissue repair. Praeger, New-York,pp 20-40.

Gurdon, J. B. 1962. The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. J. Embrol. Exp. Morphol. **10**:622-640

Hanafusa, H., Miyomoto, T. and Hanafusa, T. 1970. A cell-associated factor essential for formation of an infectious form of Rous Sarcoma virus. PNAS **66**:314-321.

Handelman, C. S., Morse, A., Irving J. T. 1958. The enzyme histochemistry in the osteoclasr of normal and ia rats. Am. J. Anat. **115**:363.

Hansen, M. F., Koufos, A., Gallie, B. L., Phillips, R. A., Fodstad, O., Brogger, A., Gedde-Dahl T., and Cavenee, W. K. 1985. Osteosarcoma and retinoblastoma. A shared chromosomal mechanism revealing recessive predisposition. Proc. Natl. Acad. Sci. USA. **82**:6216-6220.

Harbour, J. W., Lai, S. L., Whang-Peng, J., Gazdor, A. F., Minna, J. D. and Kaye, F. J. 1988. Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. Science **241**:353.

Hauschka, P. V., Mavrakos, A. E., Iafrati, M. D., Doleman, S. E., and Klagsbrun. 1986. Growth factors in bone matrix : isolation of multiple types by affinity chromatography on heparin-sephadex. J. Biol. Chem. **261**: 12665-12674.

Heine, J., deThe, G., Ishiguro, H., and Beard, J. W. 1962. Morphologic aspects of Rous sarcoma virus elaboration. J. Natl. Cancer Inst. **29**:211-223.

Herington, A. C. 1983. Recent advances in the biochemistry and physiology of the Insulin-Like growth factor/Somatomedin family. Int. J. Biochm **15**:1201.

Herman, B., and Pledger, W. J. 1985. Platelet-Derived Growth Factor-Induced alterations in vinculin and actin distributions in Balb/c3T3 cells. J. Cell Biol. **100:** 1031-1040.

Hilgers, J., Nowinski, R. C., Geering, G., and Hardy, W. 1972. Detection of avian and mammalian oncogenic RNA viruses by immunofluorescence. Cancer Res. **32:**98-106.

Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Comp. Pat. **69:**385-389.

Holmes, J. R. 1959. Further studies on the experimental transmission of of avian osteopetrosis. J. Comp. Path. **69:**385-389.

Howes R, Bowness, J. M., Grotendorst, G. R, Martin G. R., and Reddi, A. H. 1988. Platelet derived growth factor enhances demineralized bone matrix-induced cartilage and bone formation. Calcif. Tissue Int. **42:**34-38.

Humphries, E. H., Allen, R. and Glover, C,. 1981. Clonal analysis of the integration and expression of endogenous avian retroviral DNA acquired by exogenous viral infection. J. Virology **39:**584-596.

Isaksson, O. G. P., Jansson, J. O., Gause, I. A. M. 1982. Growth hormone stimulates longitudinal bone growth directly. Science. **216:**1237.

Isgaard, J. C. Moller, O. G. P., Isaksson, A. Nilsson, Mathews, L. S., and G. Norsted. 1988. Regulation of Insulin-Like Growth Factor Messenger Ribonucleic Acid in Rat Growth Plate by growth hormone. Endocrinology **122:**1515-1520.

Ishikawa, F., Miyazono, K., Hellman, U., Drexler, H., Wernstedt, C., Hagiwara, K., Usuki, K., Takaku, F., Risau, W. and Heldin, C. H. 1989. Identification of an angiogenic activity and the cloning and expression of PD-ECGF. Nature. **338:**557-562.

Ishizaki, R. and Vogt P. K. 1966. Immunological relationships among envelope antigens of avian tumor viruses. Virology **30:**375-387.

Jacobs, S. C., Pikna, D., and Lawson, R. K. 1979. Prostatic osteoblastic factor. Inves. Urol. **17:**195-198.

Jerome, C. Sandoz Pharma AG, 360/405, CH-4002 Basel, Switzerland. Talk and handout prepared for bone Biology Workshop, Sun-Valley, Idaho, USA, August 1990.

Johnston, C. C., Lavy, N., Lord, T., Vellios, F., Merrit A. D and Dess, W. P. 1968. Osteopetrosis: a clinical, genetic , metabolic and morphologic study of the dominantly inherited, benign form. Medecine **47**:149-167.

Jones, P. A, Chandler, L. A., Ghazi, H. 1985. ras gene amplification and malignant transformation. Mol. Cell Bio. **15**:2836-2841.

Kahn, A. J., Stewart C. C., Teitelbaum S. L . 1978. Contact-mediated bone resorption by human monocytes in-vitro. Science. **199**:988-990.

Kan, N. C., Baluda, M. A. and Papas, T. S. 1985. Sites of recombination between the transforming gene of Avian Myeloblastosis Virus and its helper virus. Virology. **145**:323-329.

Kanter, M. R., Smith, R. E. and Hayward, W. S. 1988. Rapid induction of B-cell lymphomas: insertional activation of c-myb by avian leukosis virus. J. Virol. **62**:1423-1432.

Katz, R. W and Reddi, A. H. 1988. Dissociative extraction and partial purification of osteogenin, a bone inductive protein, from rat tooth matrix by heparin affinity chromatography. Biochemical and biophysical research communications. **157**:1253-1257.

Knudson, A. G. 1971. Mutation and Cancer. Statistical study of Retinoblastoma. Proc. Natl. Acad. Sci. USA. **68**:820-823.

Knudson, A. G., and Strong, L. G. 1972. Mutation and Cancer: a model for Wilms's tumor of the kidney. J. Natl. Cancer Inst. **48**:313-324.

Knudson, A. G. Jr. 1985. Hereditary cancer, oncogenes, and antioncogenes. Cancer Res. **45**:1437-43.

Koutsillieris, M. Rabbani, S. A., Bennett, P. J., and Goltzman, D. 1987. Characteristics of prostate-derived growth factors for cells of the osteoblast phenotype. Calc. Tiss. Intl. **80**:941-946.

Lee, E. Y. H. P., To, H., Shew, J. Y. 1988. Inactivation of the retinoblastoma susceptibility gene in human breast cancers. Science **241**:218-221.

Lee, W. H., Bookstein R., Hong, F., 1987. Human retinoblastoma suceptibility gene; cloning, identification and sequence. Science 235:1394-1399.

Lindholm, R., Lindholm, S., Liukkop, P., Paasimaki, J., Iskantaa, S., Rossi, R., Autio E., and Taminen, E. 1969. The mast cell as a component of callous in healing fractures. J. Bone J. Sur. Am V **51B**:148-155.

Luyten F. P. Cunningham, S. M. A , Muthukumaran, N., Hammonds, R. G., Wood, W. I., and Reddi, A. H. 1989. Purification and partial amino-acid sequence of osteogenin, a protein initiating bone differentiation "J. Biol. Chem. **264**:13377-13380.

Maihle, N. J., Raines, M. A, Flickinger, W., and Kung, H-J. 1988. Proviral Insertional Activation of c-erbB: Differential Processing of the Protein Products Arising from Two Alternate Transcripts. Molecular and cellular Biology. **8**:4868-4876.

Maniatis, T., Goudbourn, S., Fisher, J. A . 1987. Regulation of inducible and tissue specific specific gene expression. Science **236**:1237-1244.

Mankin, H. J. 1979. Current concepts in cancer: Advances in diagnosis and treatment of bone tumors. New Eng. J. Med. **300**:543.

Matsunada, E. 1981. Genetics of Wilms'tumor. Hum. Gent. **57**:231-246.

Massuda, H., Miller, C., Koeffler, H. P., Battifora, H. and Cline, M. J. 1987. Rearrangement of the p53 gene in human osteogenic sarcomas. Proc. Natl. Acad. Sci. USA. **84**:7716-7719.

McLean, F. C., and Urist, M. R: 1968. Bone: Fundamentals of the physiology of skeletal tissue. Third edition. Chicago, University of Chicago Press, p12.

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

Medcalf, D. 1989. The role of stem cell-renewal and autocrine growth factor production in the biology of myeloid leukemia. Cancer Research. **49**:2305-2311.

McDonald R. J., wift, G. H., Przbyla, A. L. and Chirgwin, J. M. 1987. Isolation of RNA using Guanidinium Salts. Methods in Enzymology. Guide to molecular cloning techniques. Edited by Shelby L. Berger **152**:219. Academic Press, INC. Harcourt Brace Jovanovich, Publishers.

Miller, C. W., Aslo, A., Tsat, C., Slamon, D., Ishizaki, K., Togushida, J., lampkin B., and Koeffler, P. 1990. Frequency and structure of p53 rearrangements in human osteosarcoma. Cancer Research. **50**:5950-5954.

Morgan, J. P., and Pool, R. R. 1982. Radium-226 induced bone lesions in beagles: Clinical, radiologic, and histologic observations. Vet. Radiolol. **23**:261.

Mundy, G. R., Altman, A. J., Gandek, M. D., Bandelin, J. G. 1977. Direct resorption of bone by human monocytes. Science. **196**:1109-1111.

Murphree, A. L., Benedict, W. F. 1984. Retinoblastoma: Clues for human oncogenesis. Science. **223**:1028-1033.

Myazono, K., and Heldin, C. H. 1989. High yield purification of PD-ECGF. Structural characterization and establishment of a specific antiserum. Bioch **28**:1,704-1,710.

Myazono, K., Okabe, T., Urabe, A., Takaku, F. and Heldin, C. H. 1987. Purification and properties of an endothelial cell growth factor from human platelets. J. Biol. Chem. **262**:103.

Nabel, G., Baltimore, D. 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. **326**:711-713.

Neel, B. J., Hayward, W. S., Robinson, H. l., Fang, J. and Astrin, S. M. 1981. Avian leukosis virus-induced tumors have common proviral integration sites and synthesize discrete new RNAs:oncogenesis by promoter insertion. Cell **23**:323-334.

Neiman, P., Payne, L. N. and Weiss, L. A. 1980. Viral DNA in bursal lymphomas induced by avian leukosis viruses. J. Virol. **34**:178-186.

Nowinski, R. C., Edynak, E., and Sarkar, N. H. 1971. Serological and structural properties of the Mason-Pfizermonkey virus isolated from the mammary tumor of a Rhesus monkey. Proc. Natl. Acad. Sci. USA **68**:1608-1612.

Oliver C., Essner E. 1975. Formation of anomalous lysosomes in monocytes, neutrophils and eosinophils from bone marrow of mice with Chediak-Higashi syndrome. Lab. Invest. **32**:17-27.

Oster, W., Lindemann, A., Ganser, A., Mertelsmann, R. and Herrmann, F. 1988. Constitutive expression of hematopoietic growth factor genes by acute myeloblastic leukemia cells. Behring Inst. Mitt. **83**:68-79.

Owen, M. 1970. The origin of bone cells. Int. Rev. Cytol **28**:213-238.

Owen, M. 1971. Cellular dynamics of bone, the biochemistry and physiology of bone. Vol III. Edited by G.H Bourne. New-York, Academic Press. pp 271-297.

Owen, M. 1978. Histogenesis of bone cells. Calcif. Tissue Int. **25**:205-207

Paterson, R. W. and Smith, R. E :Characterization of anemia-induced by avian osteopetrosis virus. Infection and Immunity. **22**:891-900.

Peterson, R. D., Purchase, H. G., Burmester, B. R., Cooper, M. D. and Good, R. A. 1966. Relationship among visceral lymphomatosis, bursa of Fabricius, and bursa-dependent lymphoid tissue of the chicken. JNCI. **36**:585-598.

Philips, L. S. 1980. Somatomedins. New-England Journal of Medecine. **302**:371.

Powers, B. E., Norrdin, R. W., Snyder, S. P. and Smith, R. E. 1987. A sequential study of bone lesions caused by isolates of an avian osteopetrosis virus, MAV-2(0). Bone. **8**:231-240.

Puh, L. P. 1927. Sporadic diffuse osteoperiotitis of fowls. Vet. Rec. **1**:189-190.

Puissant, C. and Houdebine, L. M. 1990. An improvement of the single-step method of RNA Isolation by acid guanidinium thicyanate-phenol-chloroform Extraction. Biotechnique **8**: 148-149.

Rath, N. C., Reddi, A. H. 1979. Collagenous bone matrix is a local mitogen. Nature. **278**:855-857.

Reddi, A. H. 1975. Collagenous bone matrix and gene expression in fibroblasts, extracellular matrix influences on gene expression. Edited by H.C Slavkin ,R.C Grenlich, New-York, Academic Press. pp 619-625.

Reddi, A. H., Gay, R., Gay, S., Miller, E. J. 1977. Transitions in collagen types during matrix-induced cartilage, bone, and bone marrow formation. Proc. Natl. Acad. Sci. USA. **74**:5589-5592.

Reddi, A. H., N. Muthukumaran, S. M. Jill, A., Carrington, L., Luyten, F. P., Paralkar, V. M., and Cunningham, N. S. 1989. Initiation of bone development by osteogenin and promotion by growth factors. Connective tissue Research. **20**:303-312.

Reddi, A. H. Huggins C. B. 1972. Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. Proc. Nat. Aca. Sci. USA. **69**:1601-1605.

Reddi, A. H . 1981. Cell biology and biochemistry of endochondral bone development. Collagen Res. 1:209-226.

Redhead, S. 1990. Bona fide osteoinductive factors. Osteogenin, a protein isolated from bone matrix, induces dramatic growth of new bone tissue in- vitro. Review article. The Journal of NIH Research. **2**:67-70.

Riccardi, V. M., Hittner, H. M., Francke, U., Yunis, J. J., Ledbetter, D., and Borges, W. 1980. The aniridia-Wilms' tumor association: the critical role of chromosome band 11p13. Cancer Genet. Cytogenet. **2**:131-137.

Rifas, L., Shen, V., Mitchell, K., and Peck, W. A. 1984. Macrophage-Derived growth factor for osteoblast-like cells and chondrocytes. Proc. Natl. Acad. Sci. USA. **81**: 4558-4562.

Robey, P. G., Young, M. F., Flanders, K. C., Roche, N. S., Kondaiah, P., Reddi, A. H., Termine, J. D., Sporn, M. B., and Roberts, A. B. 1987. Osteoblasts synthesize and respond to transforming growth factor type Beta (TGFBeta) in vitro. J. Cell Biol. **105**:457-463.

Robinson, H. L., and Miles, B. D. 1985. Avian leukosis virus-induced osteopetrosis is associated with the persistent synthesis of viral DNA. Virol. **141**:130-143.

Robinson, H. L., Reinsch, S. S. and Shank, P. R. 1986. Sequences near the 5' long terminal repeat of avian leukosis viruses determine the ability to induce osteopetrosis. J. Virol. **59**:45-49.

Robinson, H. L., Jensen, L. and Coffin, J. M. 1985. Sequences outside of the long terminal repeat determine the lymphomagenic potential of Rous-associated virus type 1. J. Virology **55**:752-759.

Robinson, H. L., Blais, B. M., Tsichlis, P. N. and Coffin, J. M. 1982. At least two regions of the viral genome determine the oncogenic potential of avian leukosis viruses. PNAS **79**:1225-1229.

Rose, E. A., Glaser, T., Jones, C., Smith, C. L. William, H., Lewis, K. M. Call, M., Minden, E. Champagne, L., Bonetta, H., Housman, Y. and D. E. Complete physical map of the WAGR Region of 11p13 localizes a candidate Wilms' tumor gene. 1990. Cell. **60**:495-508.

Rosenberg, S. A., Suit, H. D., Baker, L. H., and Rosen, G. 1982. Sarcomas of the soft tissue and bone. In DeVita, V.T., Hellman, S., Rosenberg, S.A.(eds.): Cancer: Principles and Practice of Oncology, J. B. Lippincott Co. p. 1067.

Russell, S. M. 1985. Local injections of human or rat growth hormone of purified human somatomedin-C stimulates unilateral tibial epiphyseal growth. Endocrinology **116**: 2563

Salomon, D. Perroteau, S. I. 1986. Growth factor in cancer and their relationship to oncogenes. Cancer invest.**4**:43-60.

Sampath, T. K., Muthukumaran N., and Reddi, A. H. 1987. Isolation of osteogenin, an extracellular matrix-associated, bone-inductive protein, by heparin affinity chromatography. Proc. Natl. Acad. Sci. **84**:7109-7113.

Sampath, T. K., DeSimone, D. P., Reddi, A. H. 1982. Extracellular bone matrix-derived growth factor. Exp. Cell. Res. **142**:460-464.

Sarkar, N. H., Charney, J. and Moore, D. H. 1969. Mammary-tumor virion sructure in mouse milk fractions. J. Natl. Cancer Inst. **43**:1275-1288.

Sarkar, N. H., Moore, D. H. and Nowinski, R. C. 1972. Symmetry of the nucleocapsid of the oncoviruses in RNA nucleocapsid of the oncoviruses, in RNA viruses and host genome in oncogenesis, eds,P. Emmelotand P. Bentvelzen (North-Holland, Amesterdam) ,pp.71-79.

Sarkar, N. H., Moore, D. H. and Nowinski, R. C. 1972. Electron microscopy in mammary cancer research. J. Natl. Cancer. Inst. **48**:1051-1058.

Schmidt, E. V., Crapo, J. D. Harrelson, J. M., and Smith, R. E. 1981. A quantitative histologic study of avian osteopetrotic bone demonstrating normal osteoclast numbers and increased osteoblastic activity. **44**:164173.

Schor, S. L., Schor, A. M. 1987. Clonal heterogeneity in fibroblast phenotype: implications for the control of epithelial-mesenchymal interactions. Bioessay **7**:200-204.

Seyedin, S. M., Thomas, T. C., Thomson, A. Y., Rosen D. M. and Piez, K. A. 1985. Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. PNAS. USA. **82**:2267-2271.

Shapiro, F. Glimcher, M. J., Holtrop M. E., Tashjian A. H., Brickley-Parsons, D., and Kenzona, J. F. 1980. Human osteopetrosis: a histological, ultrastructural and biochemichal study. J. Bone Joint Surg{ Am} **62**:384.

Shimokado, K., Raines, E.W., Madtes, D. K., Barrett, T. B, Benditt, E. P., Ross, R. 1985. A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. Cell **43**:277-228.

Shives, T. C., Dahlin, D. C., Sim, F. H., Pritchard, D. J., and Earle, J. D. 1986. Osteosarcoma of the spine. J. Bone Jt. Surg. **68**:660.

Shofield, B. H , Levin L. S , Doty, S. B. 1974. Ultrastructure and lysosomal histochemistry of ia rat osteoclasts. Calcified tiss. Inter. **14**:153

Simpson, E. 1987. Growth factors which afffect bone. Trends Biochem. Sci. **9**:527.

Sinkovics, J. G., Thota, H., Romero, J. J., and Waldinger, R. 1987. Bone sarcomas: Ethiology and Immunology. Can. J. Surg. **20**:494.

Smith, R. E. and Schmidt, E. V. 1982. Induction of Anemia by Avian Leukosis Viruses of Five Subgroups. Virology **117**:516

Smith, R. E. and Moscovici, C. 1969. The oncogenic effects of nontransforming viruses from avian myeloblastosis virus. Cancer Research. **29**:1356-1366.

Smith, R. E. and Bernstein, E. H. 1973. Production and purification of large amounts of Rous sarcoma virus-induced osteopetrosis in the chicken. J. Immunol. **125**: 523-530.

Smith, R. E. 1982. Avian osteopetrosis. **101**:75-94.

Smith and Morgan. 1982. Identification of plaque isolates of an avian retrovirus causing rapid and slow onset osteopetrosis. Virol. **119**:488-499.

Smith, R. E., Davids, L. J. and Neiman, P. E. 1976. Comparison of an avian osteopetrosis virus with an avian lymphomatosis virus by RNA-DNA hybridization. Journal of Virology **17**:160-167.

Smith, R. E. 1979. Large scale growth of Rous Sarcoma Virus. Methods in Enzymology. **LVIII**. Edited by Jacoby W. B. and Pastan, I. H. Academic Press.p 393

Somerman, M., Hevitt, A. T., Varner H. H., Schiffman, E, Termine, J., Reddi, A. H. 1983. Identification of a bone matrix-derived chemotactic factor. Calcified Tissue Int. **35**:481-485.

Stanbridge, E. J. 1987. Genetic regulation of tumorigenic expression in somatic cell hybrids. Adv. Viral Oncol. **6**:83-101.

Stiles, C. D. 1985. The biological role of oncogenes- Insights from PDGF. Cancer Research. **45**:5215-5218.

Storm, F. K., Morton, D. L., Eilber, F. R., and Saxton, R. E. 1981. Etiology and advances in therapy with immunotherapy, limb salvage surgery, and hyperthermia. Seminars in oncology **8**:229.

Struhl, K. 1987. Promoters, activator proteins, and the mechanism of transcriptional initiation in yeast. Cell **49**:295-297.

T'Ang, A., Varley, J. M., Chakraborty, S., Murphree, A. L., Fung, Y.-K.T., 1988. Structural rearrangement of the retinoblastoma gene in human breast carcinomas. Science **242**:263-266.

Taylor, G. N., Thurman, G. B., Mays, C. W., Shabestari, L. Angus, W., and Atherton, D. R. 1981. Plutonium-induced osteosarcomas in the St. Bernard. 1981. Rad. Res. **88**:180.

Teitelbaun, S. L., Stewart, C. C, Kahn, A. J. 1979. Rodent peritoneal macrophages as bone resorbing cells. Calcif. Tiss. Int. **27**:255-261.

Thomas, K. A. 1987. Fibroblast Growth factor. FASEB Journal **1**:434-440

Tooze, S. The Molecular Biology of Tumor viruses. 2nd Ed. (Weiss , Teich, Varmus and Coffin, Eds). Cold spring Harbor Laboratory. Cold Spring Harbor, N.Y. pp 502-584

Togushida, J. K., Ishizaki, M., Sasaki, S., Ikenaga, M., Sugimoto, M., Kotoura, Y. and Yamamuro, T. 1988. Chromosomal reorganization for the expression of recessive mutation of retinoblastoma susceptibility gene in the development of osteosarcoma. Cancer Research **48**:3939-3943

Tonks, T. K., Charbonneau, H., Diltz, C. D., Kumar, S. Cicirelli, M. F., Krebs, E. J. 1989. "Protein tyrosine phosphatases: structure, properties and role in signal transductions". Adv. Pro. phosphatases. **5**:149.

Tsichlis, P. N. and Coffin, J. M. 1980. Recombinants between endogenous and exogenous avian tumor viruses: role of the c-region and other portions of the genome in the control of replication and transformation. J. Virol. 33: 238-2491.

Turleau, C., de Grouchy, J., Nihoul-Fekete, C., Chavin-Colin, F., and Junien, C. 1984. Del 11p13/ nephroblastoma without aniridia. Hum. Genet. **67**:445-456.

Urist, M. R., Noganu H. 1978. Experimental myositis ossificans. Cartilage and bone formation in muscle in response to a diffusible bone matrix-derived morphogen. Arch. Pathol. Lab. Med. **102**:312-316.

Ushiro, H., and Cohen, S. 1980. Identification of phosphostyrosine as a product of epidermal growth factor-activated protin kinase in A431 cell membranes. J. Biol. Chem. **255**:8363-8365.

Urist, M., Mikulski, R. A., Lietze, A. 1979. Solubilized and insolubilized bone morphogenetic protein. Proc. Natl. Acad. Sci. USA. **76**:1828.

Urist, M. R. 1965. Bone formation by auto-induction. Science **150**:893-899.

Vogt, P. K. and Ishizaki, R. 1966. Patterns of viral interference in the avian leukosis and sarcoma complex. Virology **30**:368-374.

Vogt, P. K. and Ishizaki R. 1965. Reciprocal patterns of genetic resistance to two avian tumor viruses and in two lines of chickens. Virology **26**:664-672.

Vukicevic, S., Luyten, F. P.and Reddi, A. H. 1989. Stimulation of the expression of osteogenic and chondrogenic phenotypes <u>in vitro</u> by osteogenin. Proc. Natl. Acad. Sci. USA. **86**:8793-8797.

Walker D. G. 1975a: Bone resorption restored in osteopetrosis mice by transplants of normal bone marrow and spleen cells. Science **190**:784.

Walker D. G. 1975b. Spleen cells transmit osteopetrosis in mice. Science **190**:785.

Wallace, D. M. 1987. Precipitation of nucleic acids. Methods in Enzymology. Guide to molecular cloning techniques. Edited by Shelby L. Berger **152**:219. Academic Press, INC. Harcourt Brace Jovanovich, Publishers.

Watts, S. L, Smith, R. E, and Faras, A. J. 1982. Avian nephroblastoma virus MAV-2(0) are genetically distinct. J. Gen. Virol. **60**:185-189.

Watts, S. L., and Smith, R. E. 1980. Pathology of chickens infected with avian nephroblastoma virus MAV-2(N). Inf. Immun. **27**:501-512.

Weinberg, R. W. 1988. Finding the antioncogene. Scientific American. Sept 1988:44-51.

Wells A. Welsh, J. B, Lazar C. S, Steven Wiley, G. Gill, N., and Rosenfeld, M. G. 1990. Ligand-induced transformation by a noninternalizing epidermal growth factor receptor. **247**:962-964.

Westaway, D., Papkoff,J., Moscovici, C., and Varmus, H. E. 1986. Identification of a provirally activated c-Ha-Ras oncogene in an avian nephroblastoma via a novel procedure : cDNA cloning of a chimaeric viral-host transcript. EMBO J. **5**:301-309.

Withrow, S. J., Powers, B. E. Straw, R. C., Wilkins, R. 1990. Comparative aspects of osteosaecoma; dogs versus man. Unpublished Data.

Wolff, R. K., Merickel, B. S. Rebar, A. H., and Mewhinney, J. A. 1980. Comparison of bone scans and radiography for detecting bone neoplasms in dogs exposed to 238 PuO2. Am. J. Vet. Res. **4**:1804.

Wong, S. T., Winchell, L. F., Mc cune, B. K., Shelton Earp, H., Teixedo, J., Massague, J., Herman, B., and Lee, D. C. 1989. The TGF-alpha precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction. Cell **56**:495-506.

Wozney, J. M., Rosen V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Rodney, M., Hewick, E. Wang, A. 1988. Science. **242**:1528-1534.

Yokota, J., Akiyama, T., Fung, Y-K.T., Benedict, W. F., Namba, Y. 1988. Altered expression of the retinoblastoma (RB) gene in small-cell carcinoma of the lung. Oncogene **3**:471-475.

Yokota, J., Wada, M., Shimosoto, Y., Tenada, M., and Old, L. J. 1987. Loss of heterozygosity on chromosome 3, 13, and 17 in small cell carcinoma and on chromosome 3 in adenocarcinoma of the lung. PNAS. **84**:9252-9256.

Yoshida, H., Hayashi, S. I., Kunisada, T., Ogawa, M., Nishikawa, S. 1990. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature **345**:442-444.

Young, R. W. 1962. Cell proliferation and specialization during endochondral osteogenesis in young rats. J. Cell Biol. **14**:357-370.

Young, D. C., Wagner, K., and Griffin, J. D. 1987. Constitutive expression of the granulocyte-macrophage colony-stimulating factor gene in acute myeloblastic leukemia. J. Clin. Invest. **79**:100-106.

Yunis, J. J. 1983. The chromosomal basis of human neoplasia. Science **221**:227-236.

APPENDIX

INDEX FOR SOLUTIONS

INDEX FOR SOLUTIONS

1) Tris buffer
   4.5 liters

   36g NACl
   1.71g KCl
   0.45g NA_2HPO_4
   4.5 g Dextrose
   13.5 g Trizma
   4.5 ml Pen/strep

The pH is adjusted to 7.4 in 4 liters. The solution is added the remaining water to 4.5 liters. The antibiotic solution is added then it is filtered sterilized. In order to dilute the virus 10% of FCS is added.

2) Solution I used for "miniprep" and high scale plasmid preparation.

   50 mM Glucose
   25 mM Tris pH 8
   10 mM EDTA

3) Solution II

   0.2 N NaOH
   1% SDS

4) Solution III

   5 M potassium acetate(pH 4.8) prepared as follows.
   To 60 ml of 5 M potassium aceteate, add 11.5 of glacial acetic acid and 28.5 ml of H_2O. The resulting solution is 3M with respect to potassium and 5M with respect to acetate. Mix and let stand for 10 min.

5) LB Luria-Bertani medium

   Per liter:

Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	10g

   Adjust to pH 7.5 with sodium hydroxyde.

5) Sodium phosphate solution pH 6.8. One liter of 1 M sodium phosphate dibasic heptahydrate is prepared. Another solution of 1 M Sodium phosphate monobasic is prepared. This latter solution is added to the former until the pH is 6.8.

Solutions used for the Northern blot technique.

6) Gel buffer

20mM Sodium phosphate
3% Formaldehyde
The solution is adjusted to pH 6.8 using

7) Reservoir buffer

10mM buffered sodium phosphate pH 6.8.
3% formaldehyde.

8) Transfert buffer

25mM Sodium phosphate pH 6.5.

9)Prehybridization buffer

5ml formimide
2ml of 50% dextran sulfate. This solution is prepared the night before.
2ml H_2O.
10% SDS.
The whole solution is mixed by inversion and is left at ~42oC in an incubator. Then 0.58g of NaCl was added to the tube and mixed by inversion.

10) Salmon sperm DNA is prepared as described by Maniatis (1982).
100ug/ ml of hybridization solution was used.

11) Tris-Acetate (TAE).

Concentrated stock solution.

Tris base	242g
Glacial acetic acid	57.1ml
0.5 M EDTA	100 ml

9)Prehybridization buffer

5ml formimide
2ml of 50% dextran sulfate. This solution is prepared the night before.
2ml H_2O.
10% SDS.
The whole solution is mixed by inversion and is left at ~42oC in an incubator. Then 0.58g of NaCl was added to the tube and mixed by inversion.

10) Salmon sperm DNA is prepared as described by Maniatis (1982).
100ug/ ml of hybridization solution is used to prevent background.

11) Tris-Acetate (TAE).

Concentrated stock solution.

Tris base	242g
Glacial acetic acid	57.1ml
0.5 M EDTA	100 ml

12) Guanidinium solution (Chomczinsky and Nicoletta, 1987)

4 M guanidinium thiocyonate,
25 mM sodium citrate pH 7.
0.5% sarcosyl
0.1 M 2-mercaptoethanol.

To minimize handling of guanidinium thiocyanate (hazardous), a stock solution was prepared as follows. Two hundred and fifty grams of guanidinium thiocyanate was disolved in the manufacturer's bottle (without weighing) with 293 ml water, 17.6 ml of 0.75 M sodium citrate, pH 7, and 26.4 ml 10% sarcosyl at 65° (Chomczinski P. and N. Sacchi).