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EXPRESSION OF THE LIVER/BONE/KIDNEY-TYPE ALKALINE PHOSPHATASE GENE AND PROTO-ONCOGENES IN OSTEOSARCOMA CELL LINES

BY

JAMES DEAN THACKER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

IN

EXPERIMENTAL PATHOLOGY

DEPARTMENT OF PATHOLOGY

EDMONTON, ALBERTA

FALL, 1988

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ISBN 0-315-45629-9

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NAME OF AUTHOR: James Dean Thacker

TITLE OF THESIS: "Expression of the Liver/Bone/Kidney-type Alkaline Phosphatase Gene and Proto-oncogenes in Osteosarcoma Cell Lines"

DEGREE: Master of Science

YEAR THIS DEGREE GRANTED: Fall 1988

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **EXPRESSION OF THE LIVER/BONE/KIDNEY-TYPE ALKALINE PHOSPHATASE GENE AND PROTO-ONCOGENES IN OSTEOSARCOMA CELL LINES** submitted by **James Dean Thacker** in partial fulfilment of the requirements for the degree of **Master of Science.**

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ABSTRACT

The expression of the liver/bone/kidney-type (L/B/K) alkaline phosphatase gene and 10 proto-oncogenes was studied in six osteosarcoma cell lines. The amount of L/B/K alkaline phosphatase transcripts varied markedly among the osteosarcoma cell lines and correlated with alkaline phosphatase activities. Southern blot analysis failed to detect rearrangement of the L/B/K alkaline phosphatase gene and DNA slot blotting found no difference in the gene copy number in any of the cell lines investigated. Thus, the high level of alkaline phosphatase in some of the osteosarcoma cell lines appears to result from increased expression of the L/B/K alkaline phosphatase gene. The increased expression appears to occur by means other than gene rearrangement or amplification.

The proto-oncogenes c-sis and c-Ha-ras were expressed very weakly and transcripts of the proto-oncogenes c-mos, N-ras, c-ros, c-met were not observed in any of the osteosarcoma cell lines. Transcripts from the proto-oncogenes c-myc, c-fos, N-myc, and c-raf-1 were found in all of the osteosarcoma cell lines. Southern blot analysis did not detect rearrangement of any of these four proto-oncogenes in the osteosarcoma cell lines. The c-myc gene was found to be amplified in two of the osteosarcoma cell lines.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATCC	American Tissue Culture Collection
bcr	break point cluster region
cDNA	complementary DNA
c-onc	cellular oncogene
EDTA	Ethylenediaminetetraacetic acid, disodium salt
Hepes	4-(2 hydroxyethyl)-1 piperazineethane-sulfonic acid, sodium salt
High TE	100 mM Tris-HCl, 40 mM EDTA, pH 8.0
L/B/K	Liver/Bone/Kidney-type
Low TE	10 mM Tris-HCl, 1 mM EDTA, pH 8.0
LTR	Long Terminal Repeat
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
pNP	para-nitrophenylphosphate
RSV	Rous Sarcoma Virus
SDS	Sodium Dodecyl Sulphate
v-onc	viral oncogene

CHAPTER ONE

INTRODUCTION

It is the objective of this thesis to examine the expression of the liver/bone/kidney-type (L/B/K) alkaline phosphatase gene and several proto-oncogenes in osteosarcoma cell lines. In addition, the genes will be examined for changes in structure and copy number.

Human alkaline phosphatase exists in several isoenzyme forms. One form, the L/B/K isoenzyme, is often elevated in the serum of patients with osteosarcoma. The tumor cells produce the enzyme, and this provides the opportunity to study the tumor cells for alkaline phosphatase expression. Six osteosarcoma cell lines were examined for alkaline phosphatase activity. Any variations in alkaline phosphatase activity may be due to a number of mechanisms. One of these may be the amount of mRNA from the L/B/K alkaline phosphatase gene. RNA was isolated from the cell lines, bound to a nylon membrane, and hybridized with a cDNA probe corresponding to L/B/K alkaline phosphatase mRNA. From this any variation in the amount of L/B/K alkaline phosphatase mRNA among the cell lines was determined.

Structural alteration of the L/B/K alkaline phosphatase gene which affect its expression may account for differences in the level of L/B/K alkaline phosphatase mRNA. Deletions, translocations, and an increase in gene copy number may be detected by Southern blot analysis.

In addition to an increase in the mRNA from the L/B/K alkaline phosphatase gene, other genes may also have increased mRNA levels. This may include the recently discovered proto-oncogenes. Studies of cell lines and tumor material have revealed that these genes often undergo

mutations which alter their transcription rate or protein product. This is thought to be involved in the oncogenic process. Proto-oncogenes may be activated by several mechanisms. Southern blot analysis can be used to detect proto-oncogene activation by translocation, deletion, and gene amplification. RNA isolated from the osteosarcoma cell lines was bound to a nylon membrane and hybridized with probes specific for several proto-oncogenes. Those proto-oncogenes having detectable levels of RNA were then examined by Southern blot analysis for activation by rearrangement or amplification.

CHAPTER TWO

LITERATURE REVIEW

2.1 ALKALINE PHOSPHATASE

The wide distribution of alkaline phosphatase in nature suggests its involvement in an essential biological process. Alkaline phosphatase can be found in such diverse organisms as bacteria and mammals. Despite the vast amount of investigation involving alkaline phosphatase since its discovery early in the 20th century, no physiologic role has as yet been proven. Alkaline phosphatase in vitro is capable of catalyzing the hydrolysis of various phosphate esters at alkaline pH. Several physiologic roles for alkaline phosphatase have been suggested. This includes phosphotransferase action, mineralization of bone, and solute transport across membranes (McComb et al., 1979).

Human alkaline phosphatase is a multimeric glycoprotein localized mainly in the plasma membrane. Some of the highest amounts of enzyme are observed in tissues possessing an absorbtive or transport function (Butterworth, 1983). This includes the intestine, kidney, liver, and placenta. Histochemical studies demonstrate that the enzyme is concentrated in distinct cell regions. For example, in the liver, alkaline phosphatase is observed in cells making up the sinusoids, surface of the bile canaliculi, central veins, and portal veins (Uchida et al., 1981). Considerable alkaline phosphatase activity is also found in bone, with osteoblastic cells being rich in the enzyme and osteoclasts displaying little enzymatic activity (McComb et al., 1979). In human neutrophils alkaline phosphatase is localized within a cytoplasmic granule known as a phosphasome (Wilson et al., 1981).

Different forms of alkaline phosphatase are found in different tissues. Stability to heat, sensitivity to various inhibitors, antigenicity, and electrophoretic mobility have been used to distinguish three classes of human alkaline phosphatase. The three forms are placental, intestinal, and liver/bone/kidney (L/B/K). In addition to inter-tissue heterogeneity, alkaline phosphatase exhibits intra-tissue heterogeneity. Evidence suggests intra-tissue heterogeneity results from post-translational modification such as addition of sialic residues and variation in carbohydrate side chains (Butterworth and Moss, 1966). However, inter-tissue heterogeneity results from at least three gene loci corresponding to the different isoenzyme types (McKenna et al., 1979; Seargeant and Stinson, 1979). Amino acid sequencing has revealed that placental alkaline phosphatase and intestinal alkaline phosphatase are 86% homologous in protein sequence (Henthorn et.al., 1987). Whereas, the L/B/K alkaline phosphatase is 52% homologous with placental alkaline phosphatase (Weiss et al., 1986) and 56% homologous with intestinal alkaline phosphatase (Henthorn et al., 1987). Recently, complementary DNAs encoding the three human alkaline phosphatase isoenzymes have been cloned and characterized (Berger et al., 1987; Weiss et al., 1986; Henthorn et al., 1987). Comparison of the coding regions of the intestinal and placental alkaline phosphatase cDNAs reveals a DNA sequence homology of almost 90% (Henthorn et al., 1987). It was found that there is 60% homology between L/B/K and placental cDNAs (Weiss et al., 1986) The more closely related placental and intestinal alkaline phosphatase genes have both been mapped to chromosome region 2q34-37 (Griffin et al., 1987), while the L/B/K alkaline phosphatase has been mapped to chromosome region 1p34-36.1 (Weiss et al., 1987).

The determination of alkaline phosphatase activity in serum or plasma has been described as one of the most commonly performed tests in clinical biochemistry (Moss, 1983). Its measurement in serum has been used for over 50 years and thought to mark the beginning of clinical enzymology (Fishman, 1987). Elevated serum alkaline phosphatase activity is often indicative of bone or liver disease (McComb et al., 1979). The form of the enzyme that is increased is dependent upon the location of the disorder (McComb et al., 1979). In hepatobiliary diseases the highest serum alkaline phosphatase levels are observed in patients with bile stasis (Moss, 1983). Kaplan and Righetti (1970) have shown that obstruction of the bile duct stimulates alkaline phosphatase synthesis resulting in its serum elevation. Either intrahepatic or extrahepatic biliary obstruction can result in an increase in the enzyme (Moss, 1983). Primary carcinoma of the liver and other cancers metastatic to the liver may also result in elevated serum alkaline phosphatase (McComb et al., 1979). Monitoring serum alkaline phosphatase levels have been used to follow patients with other cancers for possible liver or bone metastasis (Mayne et al., 1987).

Hypophosphatasia is a rare genetic disorder in which low serum alkaline phosphatase activity is observed. Based on age of onset, the metabolic disease has been classified into infantile, childhood, and adult forms. Infantile hypophosphatasia is inherited as an autosomal recessive trait (McKusick, 1978). However, childhood hypophosphatasia is thought to exhibit either autosomal recessive or autosomal dominant transmission (McKusick, 1978) and adult hypophosphatasia is autosomal dominant with variable penetrance (McKusick, 1978; Whyte et al., 1982).

Isoenzyme studies revealed the presence of alkaline phosphatase identical to placental alkaline phosphatase in the serum of a patient having bronchogenic carcinoma (Fishman et al., 1968). This form of the enzyme, called the Regan isoenzyme, has subsequently been observed in malignancies of the testis, ovary, pancreas, colon, lymph tissue, kidney, stomach, and bladder (Fishman, 1987). Following the discovery of the Regan enzyme, the Nagao isoenzyme was detected in a patient with pleuritis carcinomatosa (Nakayama et al., 1970). The Nagao enzyme is similar to but distinct from placental alkaline phosphatase and has been called placental-like alkaline phosphatase (Fishman, 1987). It is found mainly in germ cell tumors and ovarian cancers (Fishman, 1987). The serum levels of placental and placental-like alkaline phosphatase are normally very low (Millan, 1981), and therefore are useful tumor markers for tumors expressing them (Wahren et al., 1979; Van De Voorde et al., 1981).

Patients with Paget's disease of the bone have some of the highest recorded serum alkaline phosphatase activities (Woodward, 1959). As with other bone disorders displaying an increase in osteoblastic activity, there is an elevation of circulating alkaline phosphatase (McComb et al., 1979). Osteosarcoma is a malignant tumor of bone. It is the most common primary malignancy of bone and usually appears in the second decade of life (Katznelson and Nerubay, 1982). When an osteosarcoma is of the osteogenic type, serum alkaline phosphatase activity is generally increased (Moss, 1983). In contrast, when an osteosarcoma is of the osteolytic type, serum alkaline phosphatase activity is normal or only slightly increased (Moss, 1983).

The expression of alkaline phosphatase has been determined in a number of cell lines from various malignancies (Neuwald et al., 1980; Benham et al., 1981). These studies have used assays for enzyme activity and antibody assays to determine the amount and type of alkaline phosphatase protei present. As yet, no studies have been performed to examine the level of alkaline phosphatase gene transcripts in tumor cell lines. With the cloning of cDNAs corresponding to the three gene loci for the alkaline phosphatase isoenzymes, it is now possible to determine the level of transcription of these genes in tumor material and cell lines.

2.2. PROTO-ONCOGENES

Much effort has been spent on a search for a cause of cancer. This has included studying the mechanism of action of carcinogens. It became evident that most carcinogens initiate cancer by their action on DNA. That is, they cause mutations which lead to tumor formation. Carcinogens include certain chemicals and radiation as well as viruses which affect cellular gene expression. The first tumor virus to be discovered was the Rous Sarcoma Virus (RSV) (Rous, 1911). In the years following its discovery few viruses having the ability to induce animal tumors were observed, and the notion of viruses as a major cause of cancer received little attention. The lack of viral particles in most tumor cells prevented investigators from accepting viruses as a major cause of cancer. However, it has been found that viral genetic material may be inserted into the host genome without the production of viral particles (Sambrook et al., 1968). Therefore, the absence of viral particles cannot be taken as evidence against a viral origin of cancer.

Today two groups of cancer causing viruses are known. One group with its DNA genome includes the papovavirus, adenovirus, and herpes virus. The other group consists of the retroviruses with their RNA genome. Investigations of retroviruses found that the viral genome becomes integrated into the genome of the host cell. The viral genes are then expressed causing the cell to become neoplastic (Oda and Dulbecco, 1968). Further studies revealed that the change was caused by a viral transforming gene (Vogt, 1977). This gene being foreign, was thought to be detrimental to the normal functioning of the host cell and when expressed causes tumor formation.

Using radioactive probes homologous to the viral transforming genes it was found that the genes are not viral genes, but rather are cellular genes (Stehelin et al., 1976). The genes were also shown to exist in uninfected animals (Spector et al., 1978). The retroviral transforming genes, known as viral oncogenes (v-onc), under the direction of viral transcriptional control elements lead to neoplastic transformation. The DNA sequences homologous to the v-onc genes and found in uninfected normal cells are termed proto-oncogenes or cellular oncogenes (c-onc). Proto-oncogenes that are mutated and act in a dominant manner to transform cells are known as oncogenes. Proto-oncogenes are believed to be acquired by retroviruses through recombination events between the genome of the infecting virus and that of the host cell (Bishop and Varmus, 1982).

The v-onc present in the RSV is called src. The RSV belongs to a group of oncogenic viruses known as acute transforming viruses. These viruses are capable of inducing tumors after a short latent period. The isolation of RSV mutants that are temperature sensitive (ts) for

transformation provided evidence that RSV produces a protein which leads to tranformation (Martin, 1970). The RSV ts mutants are capable of replication at 35°C and 41°C. When cells are infected at 41°C they are not transformed, however, when placed at 35°C they become transformed (Martin, 1970). Furthermore, when returned to 41°C they assume a normal morphology (Martin, 1970). This demonstrates that the src gene product is necessary for transformation and maintenance.

About 40 different proto-oncogenes and oncogenes have been discovered (Bishop, 1987). Some of these are related and form groups. They are found in a variety of species, from yeast to man. Studies on Drosophilia have shown the presence of such proto-oncogenes as c-ras (Neumann-Silverberg et al., 1984), c-erbB (Lineh et al., 1985), and c-myb (Katzen et al., 1985). That proto-oncogenes are highly conserved in metazoan evolution suggests they are involved in essential cellular functions.

The proto-oncogenes and there products fall into four general categories based on their subcellular localization, biological and biochemical properties. The four categories are protein kinases, nuclear proteins, guanosine triphosphate (GTP) binding and hydrolyzing proteins, and growth factors and their receptors.

One of the first oncogene products to be identified as a protein kinase was the src gene product. It was found to have the ability to transfer phosphate from adenosine triphosphate (ATP) to tyrosine residues in proteins (Hunter and Sefton, 1980). Most members of the protein kinase family of oncogenes have phosphotyrosine kinase activity, while a few (mos and raf) phosphorylate serine or threonine residues. Phosphorylation of tyrosine residues normally represents only

0.1% of the phosphorylation of serine or threonine residues (Hunter, 1984). The v-src gene product can increase tyrosine phosphorylation by 10 fold (Cooper et al., 1986). It is possible that the abnormal level of phosphorylation may disturb normal cellular processes resulting in transformation.

Some proteins encoded by oncogenes are located within the nucleus. This suggests they may be involved in the control of gene expression by affecting transcription and may also have an influence on DNA replication. One oncogene, c-myc, which has been studied extensively may be involved in both regulation of gene expression (Kingston et al., 1984) and DNA synthesis (Studzinski et al., 1986). Other oncogenes such as c-fos have been implicated as having a transcriptional trans-activation function (Setoyama et al., 1986).

The ras gene family makes up another category of proto-oncogenes. To date three c-ras oncogenes have been described; c-Ki-ras, c-Ha-ras, and N-ras. The ras genes encode proteins which bind and hydrolyze GTP (Shih et al., 1980). The ras proteins are located on the inner surface of the plasma membrane and have structural features in common with other GTP-binding proteins (Litosch, 1987). It appears that these genes are involved in the transduction of exogenous signals (Gordon, 1985).

The fourth category of proto-oncongenes consists of those which encode for proteins that are growth factors or growth factor receptors. Analysis of the v-sis oncogene product revealed its close similarity with the platelet-derived growth factor (PDGF) and was found to be derived from the gene or genes encoding for PDGF (Doolittle et al., 1983). The PDGF is released from storage following blood vessel injury and then plays a role in tissue repair by stimulating the proliferation

of fibroblasts (Gordon et al., 1985). The PDGF binds to a growth factor receptor which propogates the signal to within the cell. The receptor to which a growth factor binds may also be encoded by a proto-oncogene. One example is the epidermal growth factor (EGF) receptor. It is believed the EGF receptor is the product of the erbB proto-oncogene (Downward et al., 1984).

If normal cells contain proto-oncogenes how is it that these genes become involved in oncogenesis? With recent advances in molecular biology it has become possible to identify molecular events involved in tumor development. Proto-oncogenes can be activated to an oncogenic form by a number of means. This includes the transduction of proto-oncogenes into the genome of retroviruses, insertional mutagenesis, chromosomal translocation, deletions, point mutations, and oncogene amplification.

Oncogenic retroviruses belong to one of two types, the acute transforming viruses and the slow transforming viruses. The former, which includes the RSV, are capable of transducing proto-oncogenes into their genome. This often results in alteration of the proto-oncogene, and which may then be reinserted into the host genome in proviral form (Varmus, 1982). The v-onc gene activity is increased since it is now under the control of the viral regulatory sequences in the long terminal repeat (LTR) (Bishop, 1987). Both the increased level of transcription and structural mutation of the onocogene may be necessary for its transformation ability (Shalloway et al., 1984). The slow transforming retroviruses do not possess oncogenes and act by means of insertional mutagenesis (Bishop, 1987). In the host cells they replicate and reinsert their genomes in proviral form into the host genome. After a long latent period transformation may occur if the provirus is

integrated in the vicinity of a proto-oncogene. The proto-oncogene is activated through the action of the proviral regulatory elements within the LTR (Hayward et al., 1981).

Most human cancer cells have chromosome abnormalities. In certain cases specific chromosomal alterations are associated with specific types of cancer. One such case is the reciprocal translocation between chromosomes 9 and 22 to form the Philadelphia chromosome present in most chronic myelogenous leukemias (Nowell and Hungerford, 1960; Rowley, 1973). The c-abl proto-oncogene is located on the end of the long arm of chromosome 9 and becomes translocated to the break point cluster region (bcr) of chromosome 22 (De Klein, et al., 1982). Translocation of the c-abl gene to its new location results in its activation. Other chromosome abnormalities may also result in activation of proto-oncogenes. Some cancer cells have an increase in the number of copies of oncogenes through amplification. When amplifications are found within a chromosome they appear as homogeneously staining regions (HSRs) (Beidler and Spengler, 1976). However, the amplified DNA sequences may be found in small chromosomes called double minutes (DMs) (Beidler and Speigler, 1976). Amplification of specific DNA sequences is a phenomenon common in eukaryotic cells. It may be a purposeful event involved in normal development. For example, the ribosomal genes are amplified in Xenopus oocytes at a time when a large number of ribosomes are needed for rapid protein synthesis following fertilization (Brown and Dawid, 1968). Amplification of proto-oncogenes is thought to be an important mechanism of some proto-oncogenes. The N-myc and c-myc genes are the most frequently amplified oncogenes among the cell lines and tumors studied thus far (Gordon, 1985). A recent study has indicated that gene

amplification may be an important mechanism for tumor cell invasion (Bevacqua et al., 1988).

Somatic structural mutations, such as point mutations, may also result in the activation of proto-oncogenes. This is exemplified by the ras gene family. Using DNA transfection studies this family of genes was the first to be identified as being activated by a single nucleotide change (Bishop, 1987). In human tumors the ras genes have been found to be activated by point mutations at codon 12 or codon 61 (Reddy et al., 1982). Point mutations in the c-Ha-ras gene provide it with transforming ability, whereas, the normal c-Ha-ras proto-oncogene is not capable of transformation (Reddy et al., 1982). To obtain the ability to transform other oncogenes may require further mutations such as deletions or rearrangements (Hunter, 1987) Other oncogenes may require other mutations in addition the point mutations to obtain the ability to transform. This may include deletions or rearrangements (Hunter, 1987).

CHAPTER THREE

MATERIALS AND METHODS

3.1 TISSUE CULTURE

The osteosarcoma cell lines Saos-2, HOS (TE85), MG63, U2OS, and G-292 were obtained from the American Tissue Culture Collection (ATCC), and the KT005 osteosarcoma cell line was provided by Dr. Takashi Nakamura. The cell lines were received on dry ice, thawed at 37°C, and resuspended in the appropriate cultue medium. Cell lines KT005 and U2OS were maintained in minimum essential medium, alpha modification supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. Saos-2, MG63, and HOS (TE85) cell lines were grown in minimum essential medium, Dulbecco's modification supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. G-292 was cultured in McCoy's 5a medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. The cell lines were grown at 37°C in a moist atmosphere (95% relative humidity) of 5% carbon dioxide. Once the cells reached near confluency the medium was changed and the cells removed from the tissue culture flasks with trypsin-EDTA two days later. The cells were pelleted by centrifugation and used immediately or stored at -70°C.

3.2 ALKALINE PHOSPHATASE ACTIVITY

Alkaline phosphatase activity was determined as described by Stinson (1984). Cells pelleted from tissue culture were washed twice in 10 ml of rinse solution (10 mM Hepes, pH 7.6; 10% glycerol, v/v; 1 mM

magnesium chloride; 0.1 mM zinc chloride). The cells were centrifuged, the supernatant removed, and resuspended in 1 ml of fresh rinse solution containing 1% Triton X-100 (v/v). The cell suspension was centrifuged and the supernatant removed. Alkaline phosphatase activities were determined by spectrophotometrically (Varian 2200, 404 nm) measuring the release of para-nitrophenol from para-nitrophenylphosphate at 30°C in a solution of 10 mM para-nitrophenylphosphate, 1 M ethylaminoethanol (pH 10.3), and 1.5 mM magnesium chloride.

3.3 NUCLEIC ACID EXTRACTION

3.3 a) RNA

RNA was isolated by the guanidinium isothiocyanate method as described by Maniatis et al. (1982). To the cell pellet 5 volumes of lysis solution (4 M guanidinium isothiocyanate; 5mM sodium citrate, pH 7.0; 0.1 M 2-mercaptoethanol; 0.5% sarkosyl) was added and followed by vigorous mixing. One gram of CsCl was added to each 2.5 ml of homogenate and layered onto a 1.2 ml CsCl cushion. The RNA was sedimented using a TLA 100.3 rotor (Beckman) for 4 hours at 90,000 rpm. The supernatant was discarded and the RNA pellet dissolved in 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 1% SDS. The RNA was then precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2), 2.2 volumes of cold 95% ethanol, and overnight storage at -20°C. The RNA was recovered by centrifugation, washed with 70% ethanol, and stored in low TE (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA, pH 8.0) at -70°C.

3.3 b) DNA

High molecular weight DNA was prepared by adding 2 ml of high TE to the cell pellet, mixing, and adding 2 ml of lysis solution (0.2% SDS, 200 ug/ml of proteinase K in high TE). This solution was incubated overnight at 37°C and extracted three times with phenol and three times with chloroform;isoamaylalcohol (24:1). DNA was precipiatated with 0.1 volume of 3 M sodium acetate (pH 5.2), 2.5 volumes of cold 95% ethanol, and overnight storage at -20°C. The DNA was pelleted by centrifugation, dried under vacuum, and stored in low TE at -20°C.

3.4 DIGESTION WITH RESTRICTION ENDONUCLEASE

Restriction endonucleases were used as suggested by the manufacturer. Typically 1 ug of DNA was digested in a 20 ul volume with 3 units of enzyme at 37°C for 5 to 8 hours. Reactions were terminated by the addition of EDTA (pH 8.0) to a final concentration of 10 mM.

3.5 GEL ELECTROPHORESIS

3.5 a) RNA

RNA was electrophoresed through a denaturing agarose gel as described by Maniatis et al (1982). Fifteen micrograms of total RNA was dissolved in 50% formamide, 6% formaldehyde and 1 X gel running buffer (0.2 M morpholinopropanesulfonic acid, 50 mM sodium acetate, 5mM EDTA). The RNA samples were incubated at 60°C for 20 minutes and cooled on ice. Tracking dye (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, and 0.4% xylene cyanol) was added and the RNA samples loaded onto a horizontal slab gel apparatus containing 1% agarose (BRL, ultrapure), 1 X gel

running buffer, 6% formaldehyde, and 0.5 ug/ml ethidium bromide. The gel was run for 6 to 8 hours at 60 volts in 1 X gel running buffer containing 0.5 ug/ml ethidium bromide. The electrophoresed RNA was visualized with a long wave UV transilluminator and photographed with a Polaroid camera (Fotodyne mounted) using Polaroid 667 film.

3.5 b) DNA

Restricted genomic DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2), 2.5 volumes of cold 95% ethanol, and overnight storage at -20°C. The DNA was pelleted by centrifugation and resuspended in water with tracking dye (0.1% xylene cyanol, 0.1% bromophenol blue, 50 mM EDTA, and 50% sucrose). The DNA was electrophoresed on a horizontal submerged agarose gel. The gel contained 1% agarose (BRL, ultrapure) 1 X TAE (40 mM Tris-base, 5 mM sodium acetate, 1 mM EDTA, pH 7.9), and 0.5 ug/ml ethidium bromide. The gel was run for 12 to 14 hours at 45 volts. The separated DNA fragments were visualized with a long wave UV transilluminator and photographed with a Polaroid camera (Fotodyne mounted) using Polaroid 667 film.

3.6 TRANSFER TO NYLON MEMBRANES

3.6 a) NORTHERN TRANSFER

After electrophoresis of the RNA samples, they were transferred to GeneScreenPlus (Dupont NEN) following the method described by Maniatis et al. (1982) with modifications. The gel was soaked in several changes of water for 5 minutes. The gel was then soaked in an excess of 50 mM NaOH and 10 mM NaCl for 45 minutes. This was followed by neutralization

by soaking the gel in 0.1 M Tris-HCl (pH 7.5) for 45 minutes. After neutralization, the gel was placed on a wick consisting of 2 sheets of Whatman 3 MM paper saturated with transfer buffer (1.2 M NaCl, 2.4 M Tris-base, 80 mM EDTA, 1 M ammonium acetate) and overlaid with GeneScreenPlus transfer membrane. Six pieces of Whatman 3 MM paper were placed on the membrane and a 7 cm stack of paper towels topped with a 1 Kg weight. The transfer was allowed to proceed for 16 to 24 hours, after which the membrane was soaked in 2 x SSC (0.3 M sodium chloride, 40 mM EDTA) for 5 minutes, air dried, and baked at 80^{o}C in a vacuum oven.

3.6 b) SOUTHERN TRANSFER

DNA fragments electrophoretically separated on an agarose gel were transferred to GeneScreenPlus (Dupont NEN) according to the method described by Mason and Williams (1985) with modifications. Following electrophoresis the gel was soaked in an excess of 0.25 N HCl and gently shaken for 30 minutes with one solution change. The HCl solution was removed and the gel immersed in an excess of denaturing solution (0.6 M NaCl, 0.2 M NaOH) for 30 minutes with gentle shaking. The denaturing solution was then replaced with a neutralization solution (1.5 M NaCl; 0.5 M Tris-HCl, pH 7.5) and gently shaken for 30 minutes with one solution change. After neutralization, the gel was placed on a wick of Whatman 3 MM paper saturated with transfer buffer and the transfer completed as above using GeneScreenPlus as the membrane.

3.7 PROBE PREPARATION

3.7 a) PLASMID ISOLATION

Oncogenes were obtained from ATCC. They were received as lyophilized preparations of host bacteria harboring plasmids into which the genes had been inserted. The L/B/K alkaline phosphatase cDNA clone was obtained from Dr. Harry Harris as an agar stab of host bacteria containing a plasmid into which the gene had been inserted.

The freeze dried material was rehydrated in 0.3 ml of Luria broth (LB, Gibco) media and then transferred to a 250 ml erlynmeyer flask containing 100 ml of LB media with the appropriate antibiotic (either 100 ug/ml ampicillin or 25 ug/ul tetracycline). An inoculant from the agar stab was spread on an agar plate containing antibiotic and incubated overnight at 37°C. A single colony was removed and placed in a 250 erlynmeyer flask containing 100 ml of LB media with the appropriate antibiotic. The cultures were incubated overnight in a shaker incubator at 37°C. The following day 10 ml of overnight culture was innoculated into 1 litre of M9CA media (42 mM disodium hydrogen orthophosphate mM potassium dihydrogen orthophosphate, 86 mM sodium chloride, 19 mM ammonium chloride, 2 mM magnesium chloride, 0.2% w/v glucose, 0.1 mM calcium chloride, 0.2% w/v casamin acids) and incubated at 37° C in a shaker incubator. When the optical density of the M9CA media at 550 nm reached 0.6 to 0.8, 200 mg of choramphenicol was added. Incubation was continued for 18 to 20 hours at 37°C in a shaker incubator. Next the M9CA culture was chilled on ice and the cells pelleted at 8000 rpm using a Sorvall GS-3 rotor with 400 ml centrifuge bottles. The pellet was resuspended in a 5 ml of low TE and transferred to a 15 ml corex tube.

The cell suspension was centrifuged in a Sorval S-34 rotor at 6000 rpm for 7 mintues. The cells were then resuspended in 2 ml of cold 25% sucrose, 50 mM Tris-HCl, and 40 mM EDTA and mixed vigorously. To the tubes, 0.5 ml of 10 mg/ml lysozyme solution was added with gentle mixing and kept on ice for 5 minutes. This was followed by the addition of 1 ml of 0.5 M EDTA with chilling on ice for 10 minutes and then the addition of 0.2 ml of 20 mg/ml pronase also kept on ice for 10 minutes. To each tube 8.5 ml of lysis solution (0.3% Triton X-100, 6 mM EDTA, 0.5 mM Tris-HCl) was added and kept on ice for 15 to 20 minutes with occasional inversion of the tube. A Sorvall S-34 rotor was used to centrifuge the lysate for 60 minutes at 17,000 rpm. The supernatant was decanted into another tube with the addition of CsCl (0.92 g/ml) and ethidium bromide (300 ug/ml). The solution was transferred to a Beckman Quick Seal (16 x 76 mm) ultracentrifuge tube and centrifuged for 36 hours at 55,000 rpm in a Beckman 70TI rotor. The circular band of plasmid DNA was removed by dripping with a 22 gauge needle. Ethidium bromide was removed by the addition of 4 ml of isopropanol saturated with CsCl and water. This was repeated 5 times which was followed by overnight dialysis of the plasmid DNA against low TE. The DNA was phenol extracted 3 times and precipitated with 0.1 volume of sodium acetate (pH 5.2), 2.5 volumes of cold 95% ethanol, and overnight storage at -20^{o}C. The DNA was pelleted by centrifugatation, dried under vacuum, and dissolved in low TE.

3.7 b) ISOLATION OF HYBRIDIZATION PROBES

The plasmid DNA was digested with the appropriate restriction endonuclease(s) (Table I) using conditions prescribed by the manufacturer. The restriction reactions were stopped by the addition of

TABLE I. DNA FRAGMENTS USED AS HYBRIDIZATION PROBES

Gene	Fragment	Size	Reference
L/B/K alkaline phosphatase	EcoRI-EcoRI	2.5	Weiss et al., 1986
γ-actin (human)	BamHI-BamHI	2.1	provided by Dr. R. Sasi
c-Ha-*ras*-1	BamHI-BamHI	6.6	Santos et al., 1982
c-*fos*	NcoI-XhoI	3.1	Miller et al., 1984
c-*met*	EcoRI-EcoRI	1.1	Cooper et al., 1984
c-*mos*	EcoRI-EcoRI	2.7	Watson et al., 1982
c-*myc*	EcoRI-ClaI	1.3	Modjtahedi et al., 1987
N-*ras*	XbaI-XbaI	0.9	Murray et al., 1983
N-*myc*	EcoRI-BamHI	1.0	Schwab et al., 1983
c-*raf*-1	EcoRI-EcoRI	1.8	Bonner et al., 1985
v-*ros*	EcoRI-PvuII	0.75	Neckameyer and Wang, 1984
v-*sis*	PstI-PstI	1.2	Robbins et al., 1981

EDTA (pH 8.0) to a final concentration of 10 mM and tracking dye was added to the reaction solution which was then layered onto a 1% low melting point agarose gel (BRL, ultrapure, low melting point). The gel was run in cold room (8°C) for 6-8 hours at 60 volts. The gel and running buffer (1 X TAE) contained 0.5 ug/ml ethidium bromide which permitted visualization of the restriction fragments using a long wave UV transilluminator. The appropriate fragment to be used as a probe was excised with a scalpal and placed in a 1.5 ml microcentrifuge tube. Water was added at a ratio of 3 ml/g of gel. The tube was heated for 7 minutes at 100°C and then stored at -20°C.

3.7 c) PROBE LABELING

Radioactive probes were prepared according to the oligo-labeling method described by Feinberg and Vogelstein (1983, 1984). The reaction mixture consisted of 10 ul of water, 10 ul of oligo-labeling buffer (OLB: 100 ul each of the following 1.25 M Tris-Cl, pH 7.5; 125 mM MgCl, pH 8.0; 100 mM of 2-mercaptoethanol, dATP, dGTP, dTTP; 250 ul 2 M HEPES, pH 6.6; and 150 ul of hexadeoxyribonucleotides all suspended in low TE), 2 ul bovine serum albumin (10 mg/ml), 22 ul of DNA probe solution, 5 ul dCTP (α-P, 3000 Ci/mmole, 10 uCi/ul), and 2 units of DNA polymerase I large fragment. The reaction was terminated by the addition of EDTA (pH 8.0) to a final concentration of 10 mM. Tracking dye was added and the reaction mixture loaded into a Sephadex G-50 (medium grain) column equilibrated with low TE. Following separation of the radioactive DNA from unincorporated radioactive nucleotides, 1 ul of probe solution was blotted onto a glass fiber filter and washed twice with 15 ml of cold (4°C) trichloroacetic acid and twice with 15 ml of cold (-20°C) 95%

ethanol. The filter was dried and placed in a scintillation vial containing 15 ml of scintillation fluid. The specific activity of the labeled probe was determined by counting with a Beckman liquid scintillation spectrophotometer (model LS3100).

3.8 SLOT BLOTS

3.8 a) RNA SLOT BLOTS

The total RNA samples were first denatured by the addition of 50% formamide, 6% formaldehyde, and incubating for 1 hour at 50°C. Denatured total RNA samples were serially diluted and applied to a slot blot apparatus (BIORAD, Bio-Dot SF) using GeneScreenPlus as the transfer membrane. The top slot for each sample contained 8 ug of total RNA with each successive slot containing one-half that of the previous slot. Following application of the RNA samples the membrane was air dried and baked at 80°C for 2 hours.

3.8 b) DNA SLOT BLOTS

DNA slot blots were prepared by denaturing the DNA samples in 0.25 N NaOH for 10 minutes, serially diluting the samples in 0.125 N NaOH, and applying the samples to the slot blot apparatus. The transfer membrane was GeneScreenPlus. The top slot of each sample had 10 ug of DNA with each successive slot having half as much DNA as the previous slot.

3.9 HYBRIDIZATION

Oligo-labeled DNA probes were hybridized to the membranes as described by Anderson and Young (1985) with minor modifications. The membranes were prehybridized in 10 to 20 ml of 2 X set (300 mM NaCl, 600 mM Tris-base, 20 mM EDTA), 10 X Denhardt's (0.2% Ficoll, 0.2% polyvinyl pyrrolidone, 0.2% bovine serum albumin), 0.2% SDS, 10 ug/ml sonicated and denatured salmon sperm DNA, 100 ug/ml yeast tRNA, and 10 ug/ml poly (A) for 12 to 14 hours at 64°C. The prehybridization solution was removed and 12 to 20 ml of hybridization solution containing 2 X set, 10 X Dehnardt's, 0.2% SDS, 10% dextran sulphate, 100 ug/ml sonicated and denatured salmon sperm DNA, 100 ug/ml yeast tRNA, 10 ug/ml poly (A), and denatured probe (300,000 cpm) was added. The membranes were incubated at 68°C for 20 to 24 hours. The hybridization solution was removed and the filters washed in 2 X set with 0.1% SDS for 30 minutes at 68°C. This was followed by washing in 1 X set (150 mM NaCl, 300 mM Tris-base, 10 mM EDTA) with 0.1% SDS for 30 minutes at 68°C. The final washes were for 15 minutes each at 68°C in 0.5 X set (75 mM NaCl, 150 mM Tris-base, 5 mM EDTA), 0.25 X set (37.5 mM NaCl, 75 mM Tris-base, 2.5 mM EDTA), and 0.1 X set (15 mM NaCl, 30 mM Tris-base, 1 mM EDTA) each with 0.1% SDS. The membranes were covered in saran wrap and exposed to Kodak XAR-5 film for varying periods.

Membranes were stripped of probes for rehybridization with other probes following the method described by the manufacturer (New England Nuclear). Membranes to which DNA had been transferred were soaked in 0.4 M NaOH at 42°C for 30 minutes with gentle shaking followed by soaking under the same conditions in 0.2 M Tris-HCl (pH 7.5), 0.1 X SSC (15 mM

sodium chloride, 1.5 mM sodium citrate) with 0.1% SDS. Membranes to which RNA had been transferred were soaked for 3-5 minutes in several changes of boiling 0.01% SDS and 0.01 X SSC (1.5 mM sodium chloride, 0.15 mM sodium citrate) with gentle agitation. The filters were prehybridized and hybridized as described above.

3.10 DENSITOMETRY

A densitometer (Joyce Loebl, Chromoscan 3) was used to evaluate the copy number of the L/B/K alkaline phosphatase gene and proto-oncogenes in the DNA samples. Densitometric scans of the DNA slot blot autoradiograms were obtained. To estimate the relative amounts of the L/B/K alkaline phosphatase and proto-oncogene transcripts, densitometric scans of the RNA slot blot autoradiograms were obtained.

CHAPTER FOUR

RESULTS

4.1 ALKALINE PHOSPHATASE

4.1 a) Alkaline Phosphatase Activity

A portion of the cells pelleted following tissue culture were given to Dr. R.A. Stinson for determination of alkaline phosphatase activity. The alkaline phosphatase activities in the osteosarcoma cell lines are presented in Table II. The highest level of alkaline phosphatase activity was found in Saos-2 and the lowest in U20S. The remaining cells were used for the isolation of nucleic acids.

4.2 b) RNA Slot Blot and Northern Blot Analysis

Once RNA was isolated, 15 ug was denatured and run through a denaturing gel as described in Materials and Methods. A photograph of the gel was taken (Figure 1) and a Northern transfer performed. To assess the integrity of the RNA the 28S:18S ribosomal RNA ratio was examined. The ratio was found to be 2:1 or greater indicating no or little degradation (Figure 1). This helped to ensure that a negative result would not be due to RNA degradation, but rather an absence of transcripts in the sample. Following assessment for RNA integrity, 8 ug of total RNA was denatured and applied to a transfer membrane through the slot blot apparatus using the procedure in Materials and Methods. The membrane was prehybridized and then hybridized with ^{32}P-labeled L/B/K alkaline phosphatase probe. After washing an autoradiograph was made (Figure 2).

TABLE II. Alkaline Phosphatase Activity in Osteosarcoma Cell Lines

Cell Line	U/mg Protein	Relative Alkaline Phosphatase Activity
HOS(TE85)	0.34	85
MG63	0.04	10
Saos-2	27.3	6825
G-292	5.7	1425
KT005	1.7	250
U2OS	0.004	1

1 U = 1 umole pNP produced/min

Figure 1. Fractionation of Total RNA from Osteosarcoma Cell Lines. Lane 1, Saos-2; 2, KT005; 3, U20S; 4, MG63; 5, HOS(TE85); 6, G-292. For each cell line 15 ug of total RNA was denatured and then fractionated through a 1% agarose denaturing gel. The 28S and 18s ribosomal RNA bands with molecular weights of 5.8 and 2.0 respectively are shown. The 28S and 18s rRNA bands are well defined with the amount of 28S being roughly twice that of 18S, thereby indicating no or little degradation of the RNA samples.

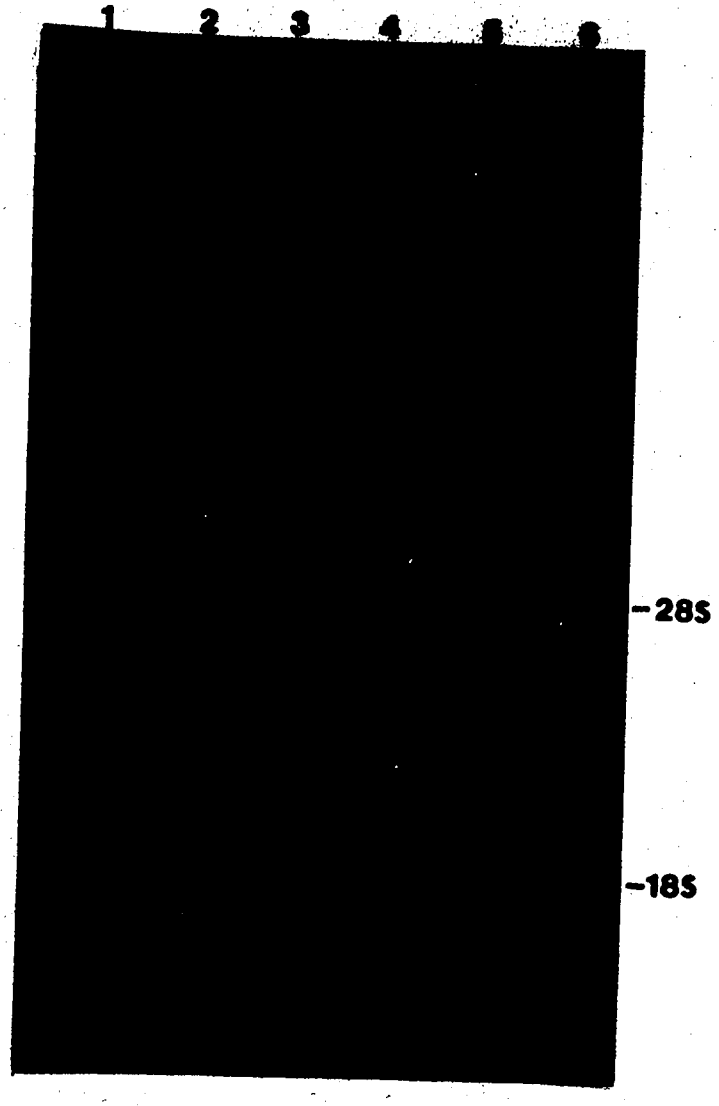
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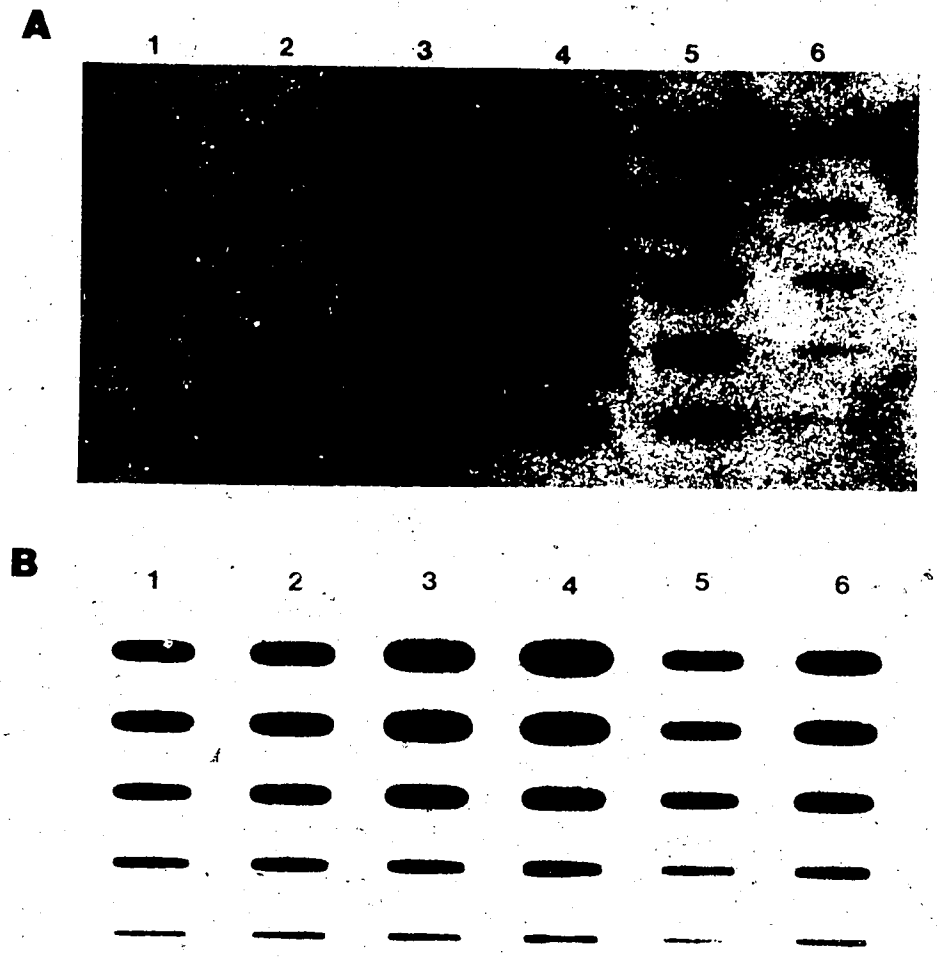
Figure 2. RNA Slot Blot Analysis of L/B/K Alkaline Phosphatase Transcripts in Osteosarcoma Cell Lines. Lane 1, HOS (TE85); 2, U2OS; 3, Saos-2; 4, G-292; 5, KT005; 6, MG63.

A. Total RNA was denatured, serially diluted, and bound to GeneScreenPlus. The top slot contained 8 ug of total RNA with each sucessive slot having half as much as the previous slot. The membrane was hybridized with ^{32}P labeled L/B/K alkaline phosphatase probe. After washing an autoradiogram was made. High levels of L/B/K alkaline phosphatase transcripts were found in Saos-2, G-292, and KT005.

B. The same membrane was stripped of the L/B/K alkaline phosphatase probe and rehybridized with ^{32}P labeled γ-actin probe. Simlar amounts of total RNA was found bound to the membrane for each of the cell lines. Densitometric scans of the autoradiograms were obtained. (Table III).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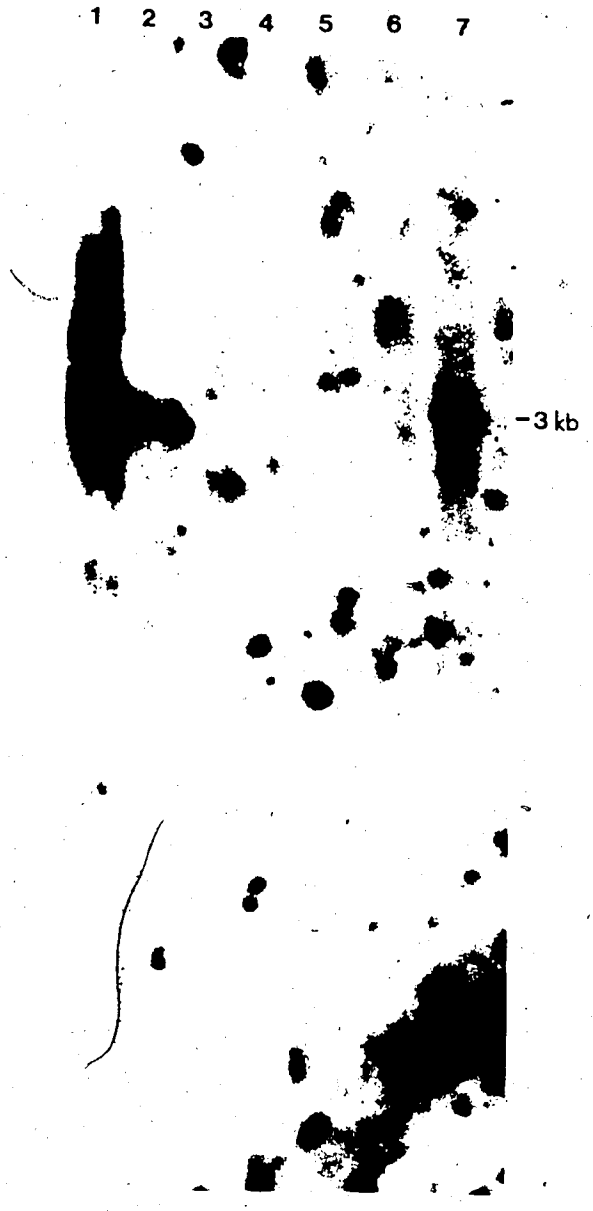
The difference in sample intensities on the autoradiograph may be due to variation in the number of L/B/K alkaline phosphatase gene transcripts or the amount of total RNA applied for each sample. To confirm similar total RNA amounts for each sample an "internal control" was used. For this a γ-actin probe for the γ-actin gene was used. The membrane was stripped of the L/B/K alkaline phosphatase probe and hybridized with ^{32}P-labeled γ-actin probe. The autoradiograph for the same membrane using the γ-actin probe is shown in Figure 2. A densitometer was used to scan the intensity of hybridization for both the L/B/K alkaline phosphatase probe and the γ-actin probe when hybridized to the membrane (Table III). The amount of total RNA for each sample varied somewhat as indicated by the actin scans. Variation in the amount of RNA to the membrane was corrected by determining the amount a sample's optical density had to be increased to equal the highest sample optical density. The optical densities for the corresponding samples on the L/B/K alkaline phosphatase hybridized membrane were then increased by the appropriate amount. In Table III the corrected densitometric scans are seen. The largest amount of L/B/K alkaline phosphatase gene transcripts is seen in the osteosarcoma cell line Saos-2 and the lowest is U2OS.

A Northern blot was prepared as outlined in Materials and Methods. Included in the Northern blot was total RNA from the osteosarcoma cell lines as well as from normal kidney. The Northern blot was prehybridized and then hybridized with ^{32}P-labeled L/B/K alkaline phosphatase probe. The membrane was washed and an autoradiogram made (Figure 3). Northern blot analysis of transcripts from the L/B/K alkaline phosphatase gene detected as single RNA species of 3 Kb. Only those osteosarcoma cell

TABLE III. Densitometric Scans of a RNA Slot Blot Hybridized with L/B/K Alkaline Phosphatase and γ-actin Probes.

Cell Line	Optical Density			Relative Level of L/B/K AP Transcripts
	L/B/K AP probe	γ-actin probe	corrected L/B/K AP probe	
HOS(TE85)	12.2	104	19	3
MG63	7.0	130	8.7	1.4
Saos-2	2880	101	4619	768
G-292	180	162	180	28
KT005	54	68	129	20
U20S	4	101	6.4	1

Figure 3. Northern Blot Analysis of L/B/K Alkaline Phosphatase Transcripts in Osteosarcoma Cell Lines. Lane 1, Saos-2; 2, G-292; 3, KT005; 4, U20S; 5, MG63; 6, HOS(TE85); 7, normal kidney. Fifteen micrograms of total RNA was denatured, fractionated through a 1% agarose denaturing gel, and transferred to GeneScreenPlus. The membrane was hybridized with ^{32}P labeled L/B/K alkaline phosphatase probe. The L/B/K alkaline phosphatase transcripts mirgrated as a 3 Kb band in the Saos-2 and G-292 osteosarcoma cell lines as well as in normal kidney. Transcripts were not detected for the other osteosarcoma cell lines.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~3 kb

lines having the highest amount of L/B/K alkaline phosphatase mRNA (Saos-2 and G-292, demonstrated by RNA slot blot analysis) and normal kidney exhibited detectable transcripts by Northern blot analysis. A single RNA species of the same size was detected in the osteosarcoma cell lines and normal kidney.

4.1 c) Southern Blot Analysis and DNA Slot Blot Analysis.

DNA samples from the osteosarcoma cell lines and control DNA (peripheral blood leukocytes) were digested with a restriction endonuclease (EcoRI or HindIII) and fractionated by electrophoresis in 1% agarose gels (Figure 4). The fractionated DNA samples were transferred to a membrane which was prehybridized and then hybridized with ^{32}P-labeled L/B/K alkaline phosphatase probe. The membranes were washed and autoradiograms prepared.

Gene rearrangement may be detected by Southern blot analysis. If a gene is rearranged it may have a restriction pattern different from that of the control. In Figure 5 the DNA bands for the EcoRI digested DNA samples are found. Here 5 restriction fragments are seen with molecular weights of 13 Kb, 10.5 Kb, 2.6 Kb, 2.5 Kb, and 1.6 Kb. The restriction pattern for the L/B/K alkaline phosphatase gene is identical for the control and the osteosarcoma cell lines. The DNA bands for the Hind III digested samples are seen in Figure 6. Again the restriction pattern with 4 bands having molecular weights of 22 Kb, 9.3 Kb, 4.6 Kb, and 1.5 Kb is the same for the control and osteosarcoma cell lines. Southern blot analysis, therefore, did not detect rearrangement of the L/B/K alkaline phosphatase gene.

Figure 4. Fractionation of EcoRI Digested Control and Osteosarcoma Cell Line DNA. Lane 1, Hind III digested lambda DNA; 2, Hind II/III digested lambda DNA; 3, Control; 4, Saos-2; 5, KTOO5; 6, U2OS; 7, MG63; 8, HOS (TE85); 9, G-292. Twelve micrograms of EcoRI digested DNA was fractionated through a 1% agarose gel. Included in the gel was Hind III and Hind II/III digested lambda DNA as molecular weight standards. Molecular weights of Hind III digested lambda DNA bands are indicated.

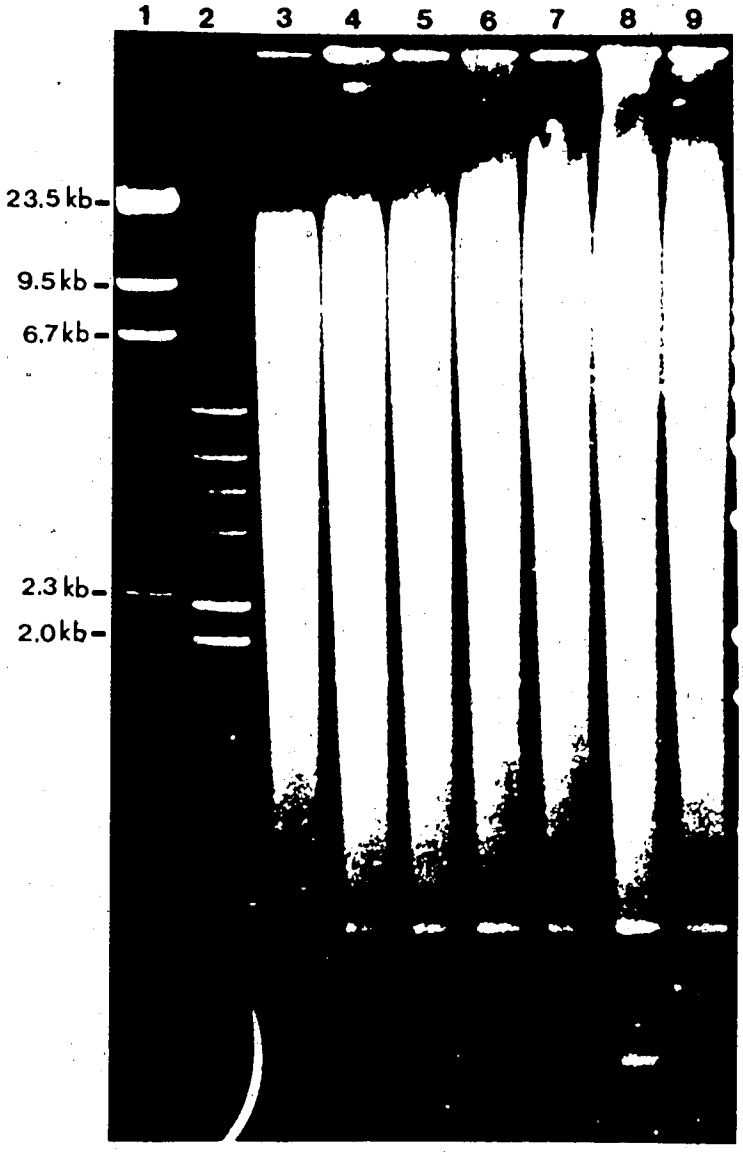
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Figure 5. Southern Blot Analysis of the L/B/K Alkaline Phosphatase Gene in EcoRI Digested Osteosarcoma Cell Line DNA. Lane 1, G-292; 2, HOS (TE85); 3, MG63; 4, U2OS; 5, KTOO5; 6, Saos-2; 7, Control; 8, Hind II/III digested lambda DNA; 9, Hind III digested lambda DNA. Twelve micrograms of EcoRI digested DNA was fractionated in a 1% agarose gel and transferred to GeneScreenPlus. The membrane was hybridized with ^{32}P labeled L/B/K alkaline phosphatase probe and ^{32}P labeled Hind II/III digested lambda DNA. After washing an autoradiogram was made. The restriction pattern of 5 bands with the molecular weights indicated was the same for control and osteosarcoma DNA samples. No rearrangement of the L/B/K alkaline phosphatase gene was found. Molecular weights of the Hind III digested lambda DNA bands are indicated.

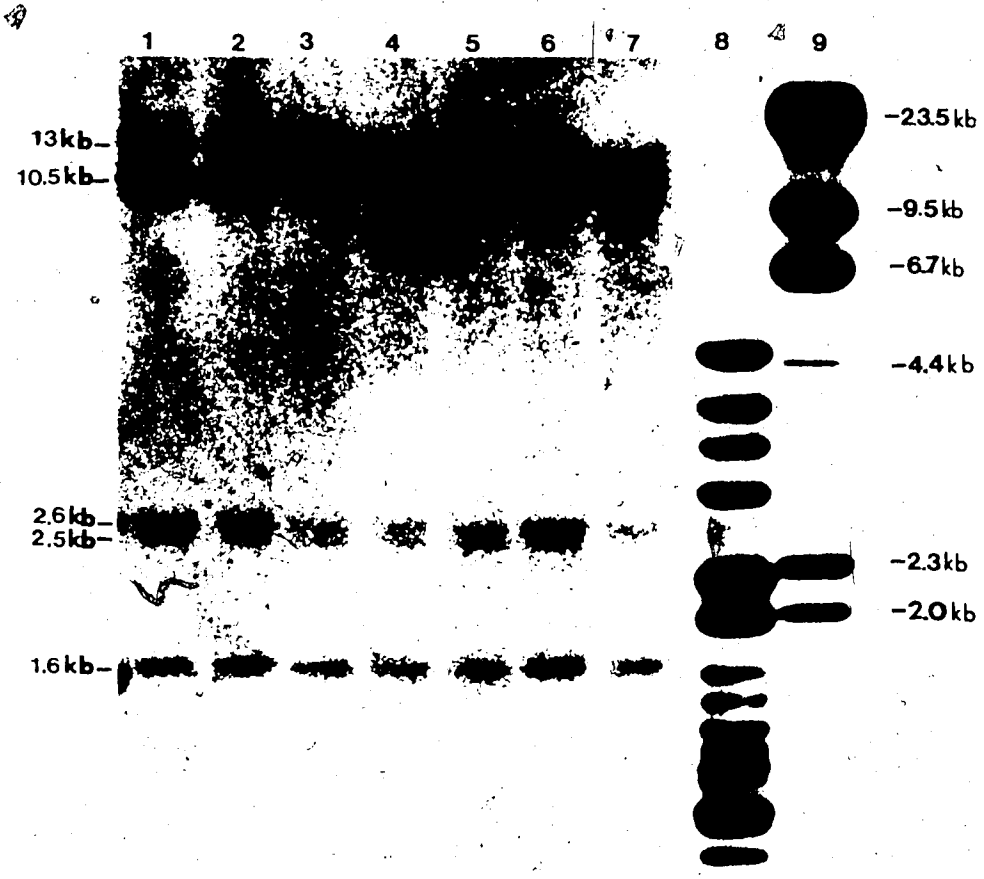
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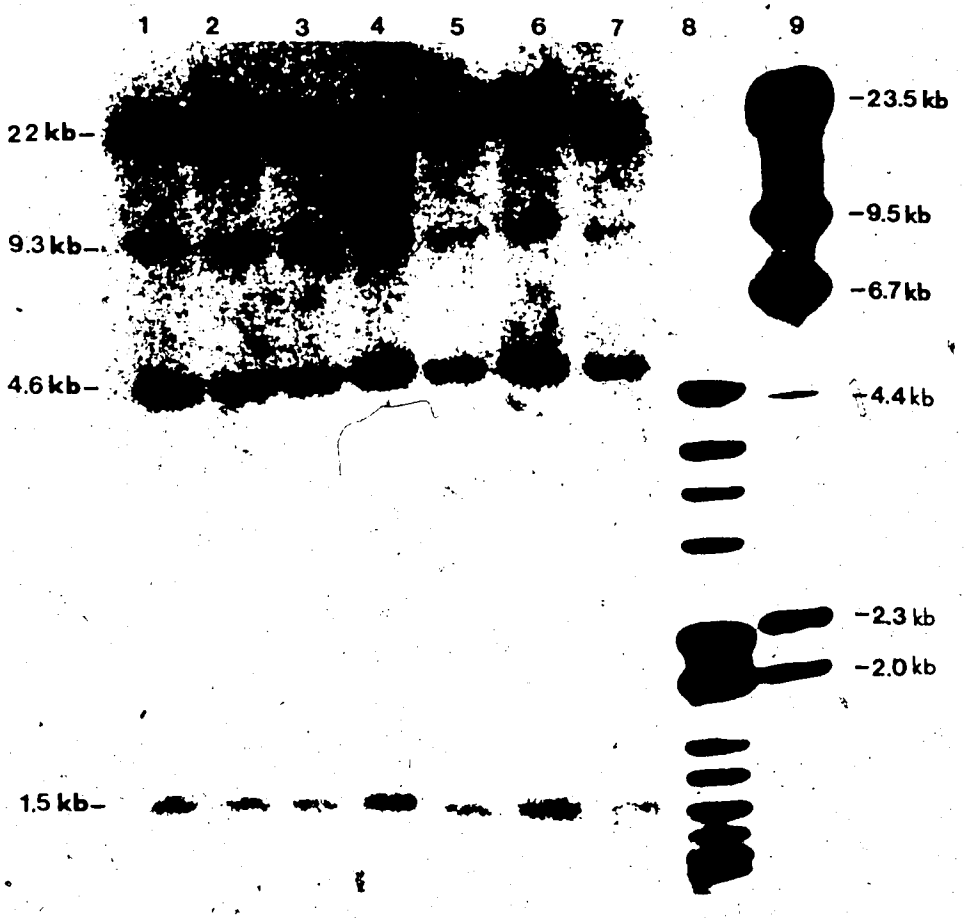
Figure 6. Southern Blot Analysis of the L/B/K Alkaline Phosphatase Gene in Hind III Digested Osteosarcoma Cell Line DNA Samples. Lane 1, G-292; 2, HOS (TE85); 3, MG63; 4, U20S; 5, KT005; 6, Saos-2; 7, Control; 8 Hind II/III digested lambda DNA; 9, Hind III digested lambda DNA. Twelve micrograms of Hind III digested sample DNA was fractionated in a 1% agarose gel and transferred to GeneScreenPlus. The membrane was hybridized with ^{32}P labeled L/B/K alkaline phosphatase probe and ^{32}P-labeled Hind II/III digested lambda DNA. The same restriction pattern of 4 bands with molecular weights indicated was found to be the same for the control and osteosarcoma cell lines. Molecular weights of the Hind III digested lambda DNA bands are found on the right.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The copy number of the L/B/K alkaline phosphatase gene was estimated by DNA slot blot analysis and densitometric scanning. Osteosarcoma cell line DNA and control DNA was bound to the transfer membrane using the slot blot apparatus. The membrane was prehybridized and then hybridized with ^{32}P-labeled L/B/K alkaline phosphatase probe. Following washing an autoradiogram was made (Figure 7). The membrane was then stripped of the L/B/K alkaline phosphatase probe and hybridized with ^{32}P-labeled γ-actin probe. The autoradiogram prepared of this hybridization is seen in Figure 7. Densitometric scans of the L/B/K alkaline phosphatase and γ-actin hybridized membrane were obtained. The optical densities are presented in Table IV.

For comparison purposes, ideally each of the samples should have the same amount of DNA bound to the membrane. However, the γ-actin internal control detected differences in the amount of DNA bound to the membrane. Some of the samples (Saos-2, KT005, HOS) had more DNA bound to the membrane while one had less DNA (MG63) than the control. To correct for the DNA differences the optical densities of the γ-actin control DNA was compared to that of the cell line samples. The optical densities of the cell lines were increased or decreased to equal that of the control DNA. The optical densities of the corresponding samples were increased or decreased the appropriate amount for the L/B/K alkaline phosphatase probe. The corrected optical densities and the L/B/K alkaline phosphatase gene copy number are found in table IV. Each of the osteosarcoma cell lines appears to have the same gene copy number as the control.

Figure 7. DNA Slot Blot Analysis of the L/B/K Alkaline Phosphatase Gene in Osteosarcoma Cell Lines. Lane 1, Control; 2, Saos-2; 3, KT005; 4, U20S; 5, MG63; 6, HOS (TE85); 7, G-292.

A. DNA samples were denatured, serially diluted and bound to GeneScreenPlus. The top slot had 10 ug of DNA and each successive slot had half as much as the previous slot. The membrane was hybridized with ^{32}P-labeled L/B/K alkaline phosphatase probe and after washing an autoradiogram made.

B. The L/B/K alkaline phosphatase probe was removed and the membrane rehybridized with P labeled γ-actin probe. The γ-actin probe acted as an internal control and indicated that the differences in the L/B/K alkaline phosphatase hybridization signals was due to the amount of sample DNA bound to the membrane. Densitometric scans of the autoradiograms were obtained (Table IV).


A
1 2 3 4 5 6 7
B
1 2 3 4 5 6 7

TABLE IV. **Densitometric Scans of a DNA Slot Blot Hybridized with L/B/K Alkaline Phosphatase (AP) and γ-actin Probes**

Sample	Optical Density			Estimated Gene Copy Number
	L/B/K AP probe	γ-actin probe	corrected L/B/K AP probe	
Control	121	41		
Saos-2	246	80	126	1
KT005	200	69	118	1
U20S	177	59	123	1
MG63	95	21	185	1-2
HOS(TE85)	212	60	145	1
G-292	104	37	115	1

4.2 PROTO-ONCOGENES

4.2 a) RNA Slot Blot Analysis

Total RNA isolated from the osteosarcoma cell lines was denatured and bound to transfer membranes using the slot blot apparatus. The membranes were prehybridized and then hybridized with radiolabeled proto-oncogene probes (Table I). A total of 10 proto-oncogene probes were used. After the filters were washed autoradiograms were prepared. No transcripts were detected for the c-met, c-mos, N-ras, and c-ros proto-oncogenes. Weak hybridiztion signals, barely above background, were detected for the c-sis and c-Ha-ras-1 genes (data not shown). Transcripts were detected for the c-myc, c-fos, c-raf-1, and N-myc proto-oncogenes. Autoradiograms of the RNA slot blots for these genes are presented in Figures 8, 9, 10 and 11.

All membranes hybridized with proto-oncogene probes were stripped and hybridized with ^{32}P-labeled γ-actin probe. Those membranes on which no (c-met, c-mos, N-ras, and c-ros) transcripts were observed, had detectable levels of -actin transcripts (data not shown). This ensured the negative signal was the result of lack of proto-oncogene transcripts and not the absence of RNA bound to the membrane.

Densitometric scans of the autoradiograms for those proto-oncogenes having easily detectable transcripts were obtained (c-myc, c-fos, c-raf-1, N-myc). In addition, scans of the same membranes when probed with γ-actin were made. The optical densities of these scans are presented in Tables V and VI. The autoradiograms and densitometric scans of the -actin probed membranes detected differences in the amount of RNA bound to the membranes. This was corrected for as with the L/B/K alkaline phosphatase RNA slot blots. The corrected

Figure 8. RNA Slot Blot Analysis of c-myc Proto-oncogene Expression in Osteosarcoma Cell Lines. Lane 1, Saos-2; 2, KT005; 3, U20S; 4, MG63; 5, HOS (TE85); 6, G-292.

A. Total RNA from the osteosarcoma cell lines was denatured and serilly diluted. The top slot containeds 8 ug of RNA and successive slots contained half as much as the previous slot. The membrane was hybridized with ^{32}P labeled c-myc probe and after washing an autoradiogram made. c-myc transcripts were detected in all of the osteosarcoma cell lines.

B. The c-myc probe was removed and the membrane rehybridized with ^{32}P labeled γ-actin probe. The γ-actin probe has shown near equal amounts of total RNA bound to the membrane for each of the osteosarcoma cell lines. Densitometric scans to the autoradiograms were obtained (Table V).

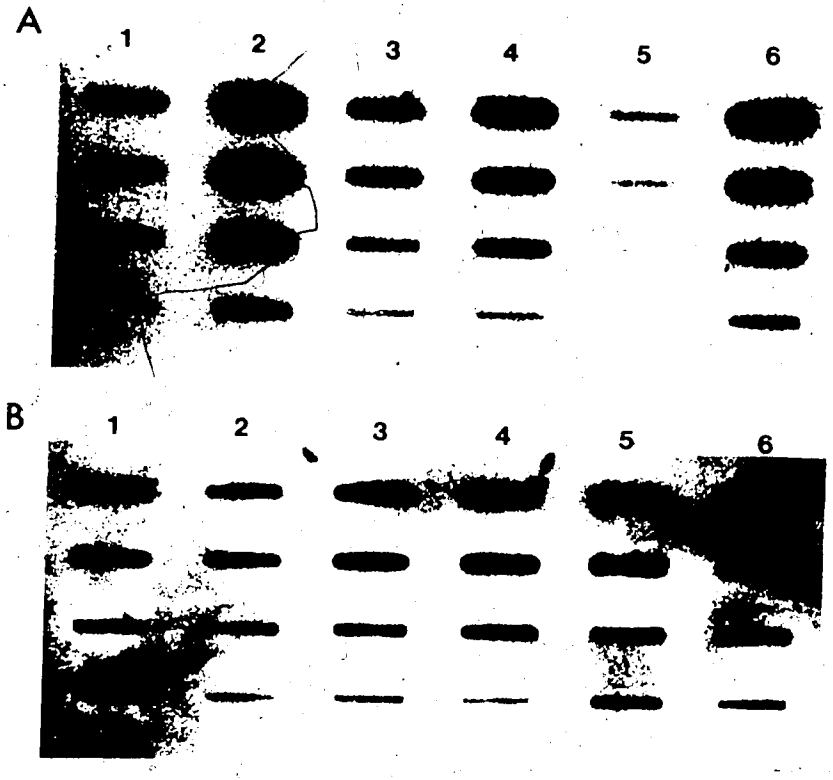
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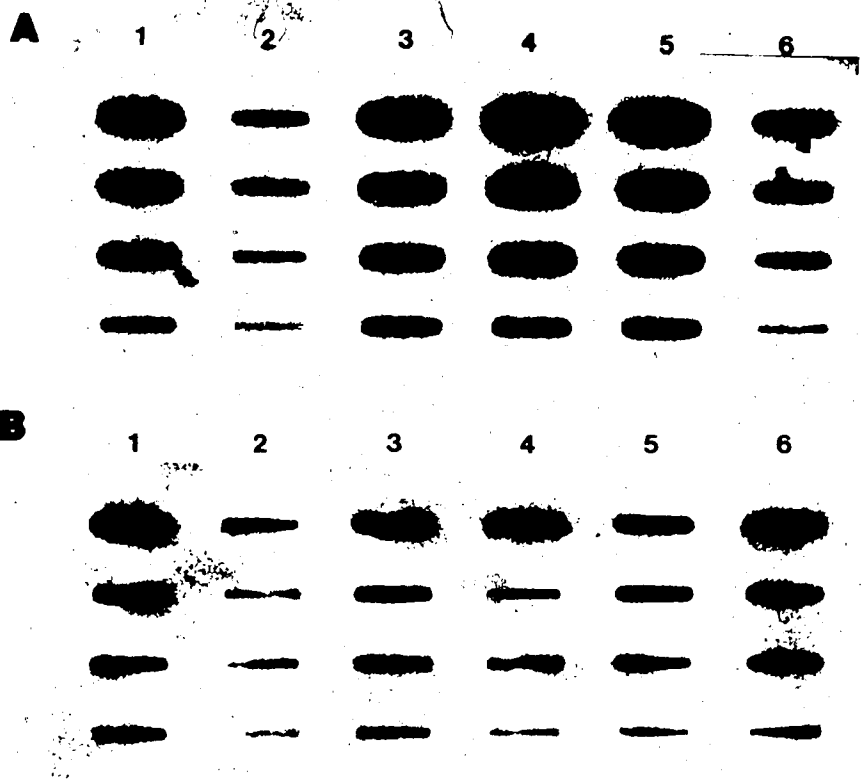
Figure 9. RNA Slot Blot Analysis of c-fos Proto-oncogene Expression in Osteosarcoma Cell Lines. Lane 1, Saos-2; 2, KT005; 3, U20S; 4, MG63; 5, HOS (TE85); 6, G-292.

A. Total RNA from the osteosarcoma cell lines was denatured, serially diluted, and bound to GeneScreenPlus. The top slot contained 8 ug of RNA and successive slots had half as much as the previous slot. The membrane was hybridized with ^{32}P labeled c-fos probe and after washing an autoradiogram made. c-fos transcripts were detected in all of the osteosarcoma cell lines.

B. The c-fos probe was removed and the membrane rehybridized with ^{32}P labeled γ-actin probe. The γ-actin probe has shown near equal amounts of total RNA bound to the membrane for each of the osteosarcoma cell lines. Therefore, the level of c-fos expression varied between the cell lines. Densitometric scans of the autoradiograms were obtained (Table V).


A
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2
3
4
5
6
B
1
2
3
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6

Figure 10. RNA Slot Blot Analysis of N-myc Proto-oncogene Expression in Osteosarcoma Cell Lines. Lane 1, Saos-2; 2, KT005; 3, U20S; 4, MG63; 5, HOS (TE85); 6, G-292.

A. Total RNA from the osteosarcoma cell lines was denatured, serially diluted, and bound to GeneScreenPlus. The top slot contained 8 ug of RNA and successive slots had half as much as the previous slot. The membrane was hybridized with ^{32}P labeled N-myc probe and after washing an autoradiogram made. N-myc transcripts were detected in all of the osteosarcoma cell lines.

B. The N-myc probe was removed and the membrane rehybridized with ^{32}P labeled γ-actin probe. The γ-actin probe has shown near equal amounts of total RNA bound to the membrane for each of the osteosarcoma cell lines. Therefore, the level of N-myc expression varied between the cell lines. Densitometric scans of the autoradiograms were obtained (Table VI).

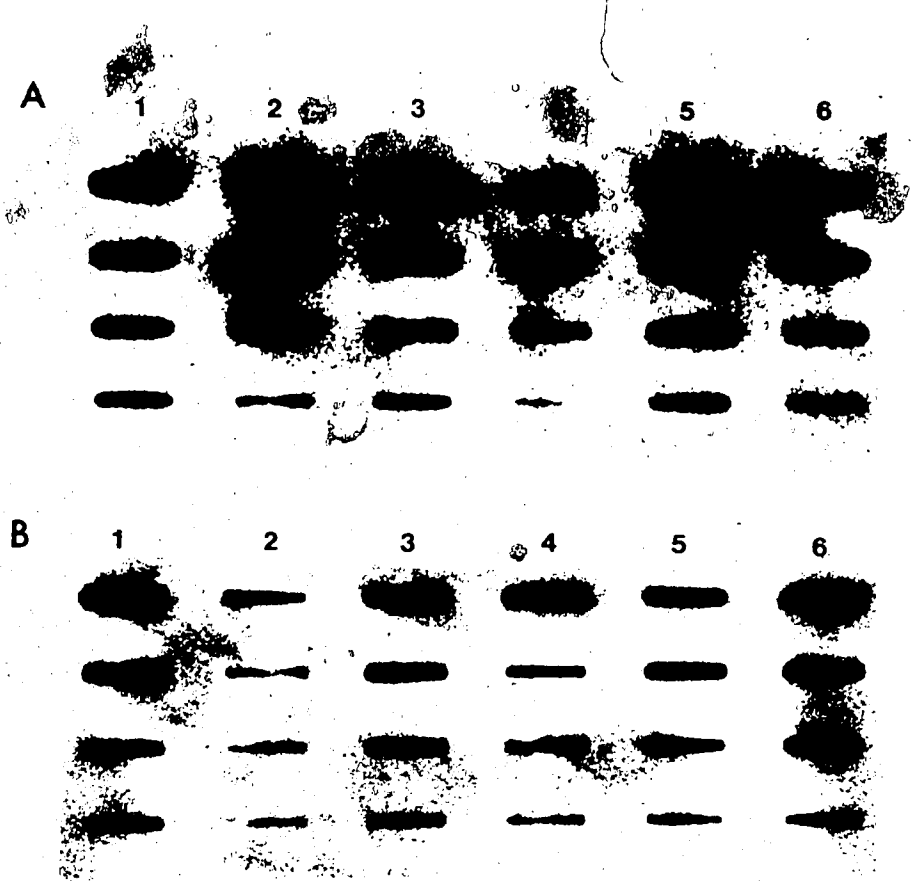
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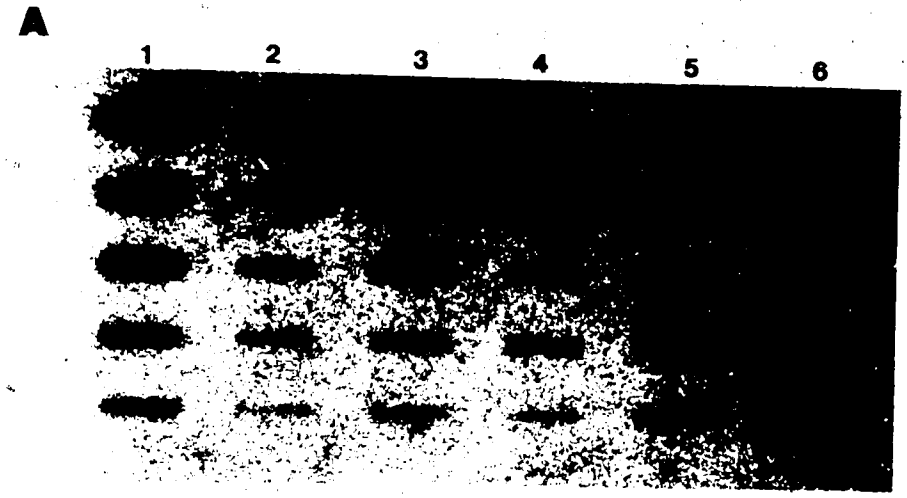
Figure 11. RNA Slot Blot Analysis of c-raf-1 Proto-oncogene Expression in Osteosarcoma Cell Lines. Lane 1, Saos-2; 2, KT005; 3, U20S; 4, MG63; 5, HOS (TE85); 6, G-292.

A. Total RNA from the osteosarcoma cell lines was denatured, serially diluted, and bound to GeneScreenPlus. The top slot contained 8 ug of RNA and successive slots had half as much as the previous slot. The membrane was hybridized with ^{32}P labeled c-raf-1 probe and after washing an autoradiogram made. c-raf-1 transcripts were detected in all of the osteosarcoma cell lines.

B. The c-raf-1 probe was removed and the membrane rehybridized with ^{32}P labeled γ-actin probe. The γ-actin probe has shown near equal amounts of total RNA bound to the membrane for each of the osteosarcoma cell lines. Therefore, the level of c-raf-1 expression varied between the cell lines. Densitometric scans of the autoradiograms were obtained (Table VI).


A
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2
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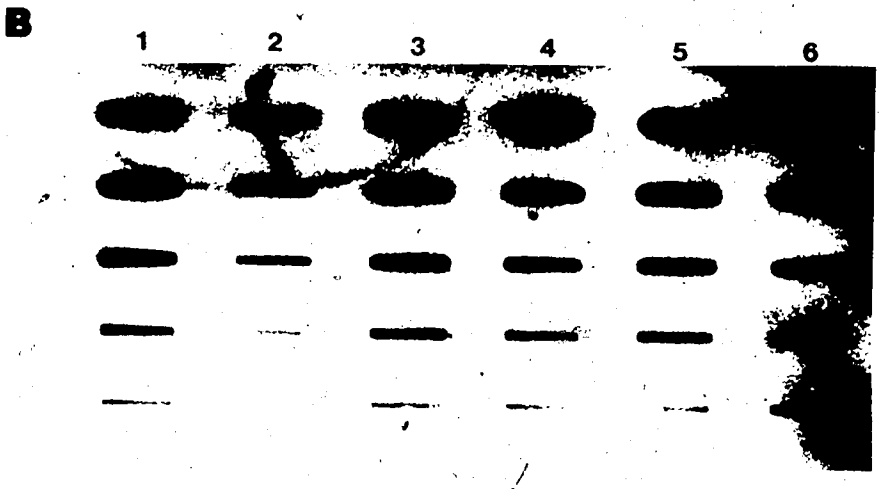

B
1
2
3
4
5
6

TABLE V. Densitometric Scans of RNA Slot Blots Hybridized with c-myc and c-fos probes.

Oncogene Probe and Cell Line	Optical Density			Relative Oncogene Transcript Level
	oncogene probed	γ-actin probed	corrected oncogene probed	
c-myc				
Saos-2	39	121	47	3.4
KT005	172	79	316	23
U20S	43	126	49	3.5
MG63	81	145	81	5.8
HOS(TE85)	13	136	14	1
G-292	130	139	136	9.7
c-fos				
Saos-2	110	142	121	1.2
KT005	24	37	101	1
U20S	101	111	142	1.4
MG63	155	66	366	3.6
HOS(TE85)	154	147	162	1.6
G-292	43	156	156	1.5

TABLE VI. Densitometric Scans of RNA Slot Blots Hybridized c-*raf*-1 and N-*myc* Probes

Oncogene Probe and Cell Line	Optical Density			Relative Oncogene Transcript Level
	oncogene probed	γ-actin probed	corrected oncogene probed	
c-*raf*-1				
Saos-2	180	52	249	3.8
KT005	53	22	173	2.6
U20S	93	51	131	2
MG63	51	56	66	1
HOS(TE85)	157	46	245	3.7
G-292	175	72	175	2.7
N-*myc*				
Saos-2	100	142	116	1.6
KT005	93	37	414	5.8
U20S	110	111	164	2.3
MG63	52	53	162	2.3
HOS(TE85)	123	150	135	1.9
G-292	71	165	71	1

optical densities and relative amounts of the transcripts are found in Tables V and VI.

4.2 b) Southern and DNA Slot Blot Analysis

The proto-oncogenes c-myc, c-fos, c-raf-1, and N-myc had detectable levels of transcripts which may be the result of activation by either gene rearrangement or amplification. The osteosarcoma cell lines were examined for possible rearrangement and amplification of these genes by Southern and DNA slot blot analysis.

Osteosarcoma cell line DNA and control DNA were digested with the restriction endonucleases EcoRI and Hind III. The digests were electrophoretically separated and transferred to a membrane. The membranes were prehybridized and then hybridized with one of the four radiolabled proto-oncogene probes.

Hybridization of the c-myc probe to the EcoRI digested DNA samples revealed a single 12.5 Kb DNA band for each of the cell lines and the control (Figure 12). A single restriction fragment of molecular weight 11 Kb was observed when the Hind III digested DNA samples were hybridized with the c-myc probe (Figure 12). Again the restriction pattern for the control and cell lines was identical. No rearrangement of the c-myc proto-oncogene was detected. However, the much darker bands for the osteosarcoma cell lines MG63 and G-292 indicated there may be an increase in the copy number of the c-myc gene in these cell lines. This was further pursued by DNA slot blot analysis (see below).

The c-fos probe revealed 9 Kb and 6.8 Kb restriction fragments when the DNA samples were digested with EcoRI and Hind III respectively (Figure 13). The restriction pattern for the control and cell lines was

Figure 12. Southern Blot Analysis of the c-myc Proto-oncogene in Osteosarcoma Cell Lines. Lane 1, G-292; 2, HOS (TE85); 3, MG63; 4, U20S; 5, KT005; 6, Saos-2; 7, Control. DNA from the control and osteosarcoma cell lines was digested with EcoRI (A) or Hind III (B), fractionated in a 1% agarose gel, and transferred to GeneScreenPlus. The membrane was hybridized with ^{32}P labeled c-myc probe.

A. The restriction pattern for the EcoRI digested samples consisted of a single 12.5 Kb restriction fragment in the control and cell lines. No rearrangement of the c-myc gene was detected. However, the greater hybridization intensity for the MG63 (lane 3) and G-292 (lane 1) cell lines indicated their possibly having an increse in the copy number of the c-myc gene. This was further pursued by DNA slot blot analysis.

B. The restriction pattern for the Hind III digested control and osteosarcoma cell line DNA samples consists of a single 11 Kb band. No rearrangement of the c-myc gene was detected. However, as above the hybridization intensities indicated a possible increase in copy number of c-myc in the MG63 and G-292 cell lines.

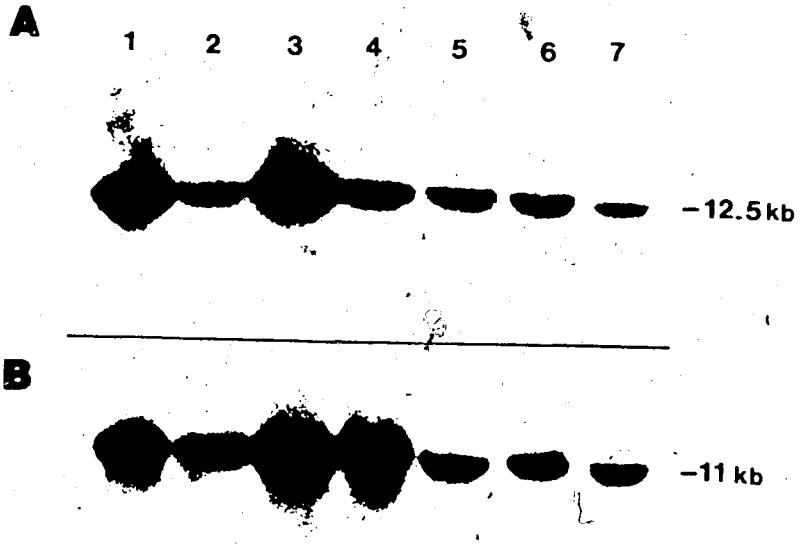
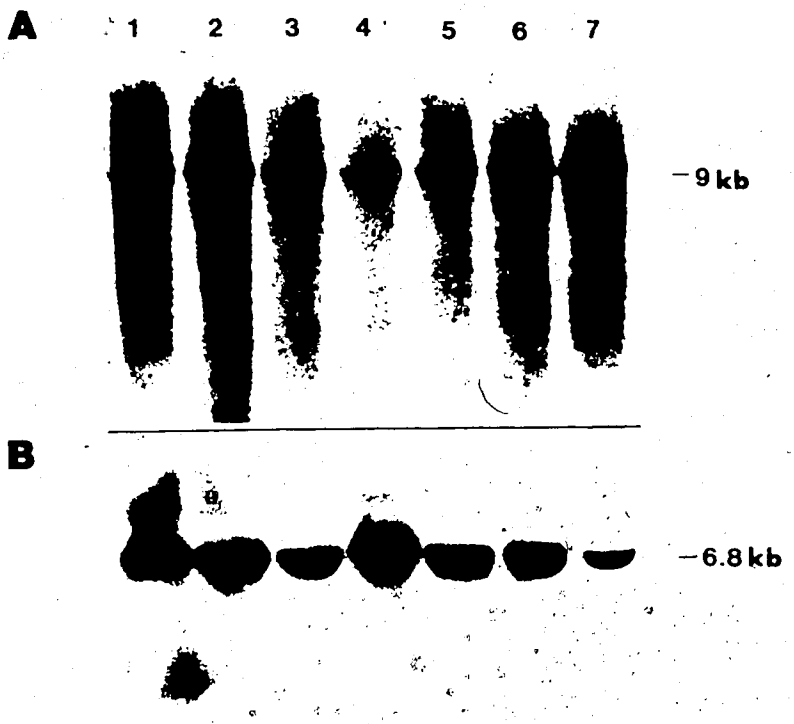

A
1
2
3
4
5
6
7
~12.5kb
B
~11 kb

Figure 13. Southern Analysis of the c-fos Proto-oncogene in Osteosarcoma Cell Lines. Lane 1, G-292; 2, HOS (TE85); 3, MG63; 4, U20S; 5, KT005; 6, Saos-2; 7, Control. DNA samples were digested with EcoRI (A) or Hind III (B), fractionated in a 1% agarose gel, and transferred to GeneScreenPlus. The membrane was hybridized with ^{32}P labeled c-fos probe.

A. The restriction pattern for the EcoRI digested control and osteosarcoma cell line DNA samples consisted of a single 9 Kb band. No rearrangement of the c-fos gene was detected.

B. The restriction pattern for the Hind III digested control and osteosarcoma DNA samples consisted of a single 6.8 Kb band. No rearrangement of the c-fos gene was detected. However, the greater hybridization intensity for the U20S cell line (lane 4) indicated possible amplification of the c-fos gene in this cell line. This was further pursued by DNA slot blot analysis.


A
1
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4
5
6
7
9kb
B
6.8kb

identical when digested with either EcoRI or Hind III. Therefore, no rearrangement of the c-fos proto-oncogene was found. The more intense band for the U2OS cell line (Figure 13, lane 4) indicated the possibility of c-fos amplification. DNA slot blot anlaysis was performed to examine this possibility (see below).

Hybridization with N-myc and c-raf-1 probes to the EcoRI and Hind III digested DNA samples did not uncover any rearrangement of these proto-oncogenes. The EcoRI digested DNA samples when hybridized with the N-myc probe revealed a single band of molecular weight 2 Kb (Figure 14). A single 6 Kb band was observed when the Hind III digested DNA samples were hybridized with the N-myc probe (Figure 14). The restriction pattern was the same for the control and cell lines. When the EcoRI digested DNA samples were hybridized with the c-raf-1 probe, the restriction pattern was the same for the control and cell lines with only a single 2.9 Kb band present (Figure 15). A single 7 Kb band was observed when the Hind III digested DNA samples were hybridized with the c-raf-1 probe. Furthermore, the intensity of the hybridization signals was much the same for the control and cell lines. No rearrangement or amplification of the N-myc and c-raf-1 proto-oncogenes was detected.

Southern analysis of the c-myc and c-fos proto-oncogenes revealed their possible amplification in some of the osteosarcoma cell lines. To determine the copy number of these genes DNA slot blots were prepared. Osteosarcoma cell line DNA and control DNA was denatured and bound to transfer membranes using the slot blot apparatus. The membranes were prehybridized and then hybridized with radiolabeled c-myc or c-fos probes. After washing autoradiograms were prepared (Figures 16, 17) and densitometric scans of the autoradiograms obtained (Table VII). To

TABLE VII. Densitometric Scans of DNA Slot Blots Hybridized with c-myc and c-fos Probes.

Oncogene Probe and sample	Optical Density			Estimated Gene Copy Number
	oncogene probed	γ-actin probed	corrected oncogene probed	
c-myc				
Control	70	73		
Saos-2	125	126	72	1
KT005	98	88	81	1
U20S	93	70	89	1
MG63	177	43	300	4-5
HOS(TE85)	129	97	97	1
G-292	160	62	188	2-3
c-fos				
Control	129	106		
Saos-2	180	185	103	1
KT005	181	156	122	1
U20S	139	107	137	1
MG63	77	49	166	1
HOS(TE85)	204	155	139	1
G-292	106	72	156	1

Figure 14. Southern Blot Analysis of the N-myc Proto-oncogene in Osteosarcoma Cell Lines. Lane 1, G-292; 2, HOS (TE85); 3, MG63; 4, U20S; 5, KT005; 6, Saos-2; 7, Control. DNA samples were digested with Hind III (A) or EcoRI (B), fractionated on a 1% agarose gel, and transferred to GeneScreenPlus. The membrane was hybridized with ^{32}P labeled N-myc probe.

A. The restriction pattern for the Hind III digested control and osteosarcoma cell line DNA samples consisted of a single 16 Kb band. No rearrangement of the N-myc gene was detected and the near equal hybridization intensity for the restriction fragments indicated no N-myc amplification.

B. The restriction pattern for the EcoRI digested control and osteosarcoma cell line DNA samples consisted of a single 2 Kb band. No rearrangement of the N-myc gene was detected.

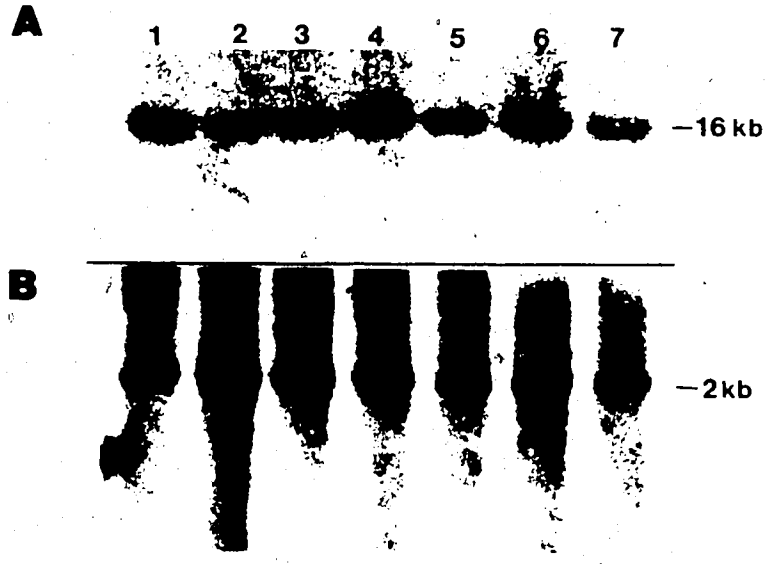
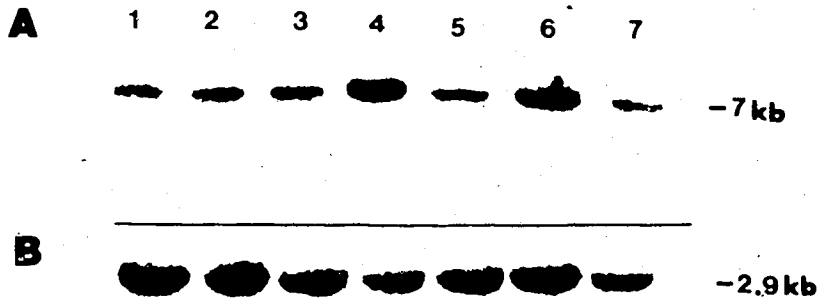

A
1
2
3
4
5
6
7
—16 kb
B
—2 kb

Figure 15. Southern Blot Analysis of the c-raf-1 Proto-oncogene in Osteosarcoma Cell Lines. Lane 1, G-292; 2, HOS (TE85); 3, MG63; 4, U20S; 5, KT005; 6, Saos-2; 7, control. DNA samples were digested with Hind III (A) or EcoRI (B), fractionated in a 1% agarose gel, and transferred to GeneScreenPlus. The membrane was hybridized with ^{32}P labeled c-raf-1 probe.

A. The restriction pattern for the Hind III digested control and osteosarcoma cell line DNA samples consisted of a single 7 Kb band. No rearrangement of the c-raf-1 gene was detected and the near equal hybridization intensity of the restriction fragments indicated no amplification of the gene.

B. The restriction pattern for the EcoRI digested control and osteosarcoma cell line DNA samples consisted of a single 2.9 Kb band. No rearrangement of the c-raf-1 gene was detected.


A
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2
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4
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6
7
~7kb
B
~2.9kb

Figure 16. DNA Slot Blot Analysis of the c-myc Proto-oncogene in Osteosarcoma Cell Lines. Lane 1, G-292; 2, HOS (TE85); 3, MG63; 4, U20S; 5, KT005; 6, Saos-2; 7, Control.

A. Control and osteosarcoma cell line DNA samples were denatured, serially diluted and bound to GeneScreenPlus. The top slot contained 10 ug of DNA with each successive slot containing half as much DNA as the previous slot. The membrane was hybridized with ^{32}P labeled c-myc probe and an autoradiogram made.

B. The membrane was stripped of the c-myc probe and rehybridized with ^{32}P labeled γ-actin probe. Densitometric scans of the autoradiograms were obtained. These scans showed variation in the amount of sample DNA bound to the membrane. However, when the densitometric scans of the c-myc and γ-actin probes were compared, the c-myc gene copy number was found to be increased in the MG63 and G-292 osteosarcoma cell lines (Table VII).

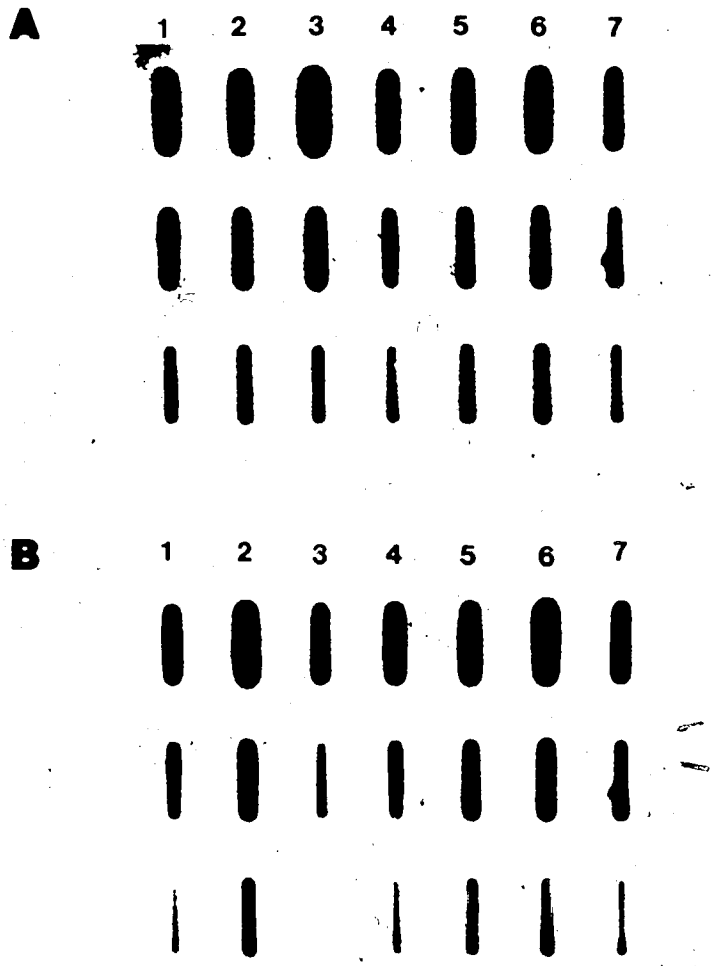
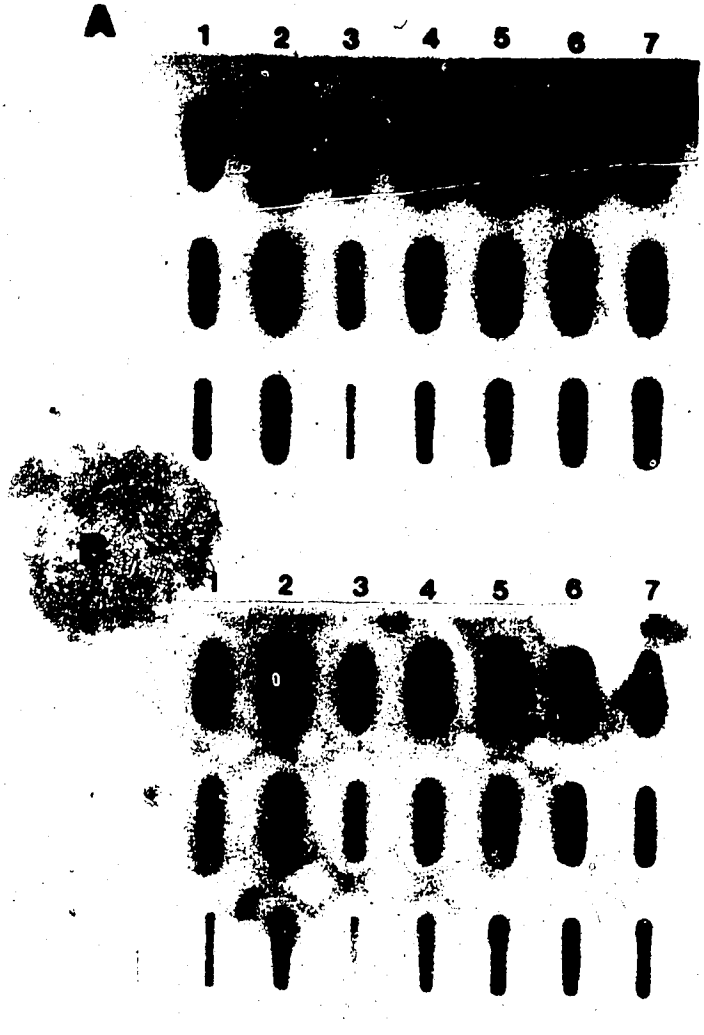

A
1 2 3 4 5 6 7
B
1 2 3 4 5 6 7

Figure 17. DNA Slot Blot Analysis of the c-fos Proto-oncogene in Osteosarcoma Cell Lines. Lane 1, G-292; 2, HOS (TE85); 3, MG63; 4, U2OS; 5, KT005; 6, Saos-2; 7, control.

A. Control and osteosarcoma cell line DNA samples were denatured, serially diluted, and bound to GeneScreenPlus. The top slot contained 10 ug of DNA with each successive slot containing half as much as the previous slot. The membrane was hybridized with ^{32}P labeled c-fos probe and an autoradiogram made.

B. The membrane was stripped of the c-fos probe and rehybridized with ^{32}P labeled γ-actin probe. Densitometric scans of the autoradiograms were obtained. The scans showed variation in the amount of sample DNA bound to the membrane. Comparison of the densitometric scans of the c-fos and γ-actin probes showed no amplification of the c-fos proto-oncogene (Table VII).


A
1 2 3 4 5 6 7
1 2 3 4 5 6 7

determine if the amount of DNA for each sample was the same the membranes were stripped of the proto-oncogene probes and hybridized with ^{32}P-labeled γ-actin probe. The autoradiograms of the γ-actin hybridized membranes are seen in Figures 16 and 17. The densitometric optical densities for these membranes are found in Table VII.

The γ-actin probe revealed differences in the amount of sample DNA bound to the membranes. This was corrected as was done with the L/B/K alkaline phosphatase DNA slot blot. The corrected optical densities for the c-myc slot and the estimated gene copy number for the cell lines are found in Table VII. A four or fivefold amplification of the c-myc gene was found in the osteosarcoma cell line MG63. Whereas a two to threefold amplification was found in the G-292 osteosarcoma cell line. In the remaining cell lines no amplification of the c-myc gene was found. The c-fos corrected optical densities values and estimated gene copy number are found in Table VII. The c-fos proto-oncogene appears not to be amplified in any of the osteosarcoma cell lines.

CHAPTER FIVE

DISCUSSION

5.1 ALKALINE PHOSPHATASE

The osteogenic form of osteosarcoma is a malignant neoplasm of osteoblasts (Moss, 1983). It is one of the most common malignant tumors of the skeletal system and in the young the most lethal of bone tumors (Bubis, 1982). Often the serum alkaline phosphatase level is elevated in patients harboring osteogenic sarcoma. Cell lines have been established from osteosarcoma tumors and the alkaline phosphatase activity in some of these cell lines studied (Neuwald et al.,1980; Benham et al., 1981). Furthermore, in some instances which alkaline phosphatase isoenzyme is present has been determined by immunoprecipitation (Murray et al., 1987). Some of the osteosarcoma cell lines are reported as having a high level of alkaline phosphatase activity while others are much lower (Benham et al, 1981; Murray et al., 1987).

Variation in alkaline phosphatase activity may be due to a number of mechanisms. The amount of alkaline phosphatase present may not differ much, but it's catalytic activity may be increased a great deal. For example, this occurs when the c-abl proto-oncogene on chromosome 9 is translocated to a gene referred to as bcr on chromosome 22 forming a fusion gene (De Klein et al., 1982). The product of the chimeric gene like that of the normal gene is a tyrosine kinase but with increased catalytic activity (Konopka et al., 1985). Differences in the amount of alkaline phosphatase protein may also account for variation in alkaline phosphatase activities. An increase in alkaline phosphatase protein may result in altered transcription of the alkaline phosphatase gene(s),

altered translation of the message, processing, and stabilization of the transcript and/or protein.

In the present investigation the alkaline phosphatase activity of six osteosarcoma cell lines has been determined. This was followed by examining the level of L/B/K alkaline phosphatase mRNA in the cell lines. In addition, possible activation of the L/B/K alkaline phosphatase gene by rearrangement or amplification was studied.

Alkaline phosphatase activity was found to vary considerably from one cell line to the next. The highest level of alkaline phosphatase activity was found in the Saos-2 osteosarcoma cell line (Table II). High alkaline phosphatase activity was previously reported for this cell line (Benham et al., 1981). The present study found the alkaline phosphatase activity in the Saos-2 cell line was over 6000 fold higher than that of the cell line having the lowest activity (U2OS). The alkaline phosphatase isoenzyme in the Saos-2 cell line has recently been demonstrated to be the L/B/K form (Murray et al., 1987).

RNA slot blot analysis using the cDNA probe corresponding to L/B/K mRNA (Weiss et al., 1986) in the present study revealed marked variation in the amount of L/B/K alkaline phosphatase mRNA in the osteosarcoma cell lines. The largest amount was found in the Saos-2 cell line. In comparing alkaline phosphatase activities in Table II to the amount of L/B/K alkaline phosphatase transcripts in Table III it can be seen that the level of alkaline phosphatase activity corresponds with the amount of L/B/K mRNA. That is, the cell line with the highest alkaline phosphatase activity has the largest amount of L/B/K alkaline phosphatase transcripts and so on. It, therefore, appears the alkaline phosphatase activity differences result from variation in the amount of

protein which in turn is due to differences in the amount of L/B/K alkaline phosphatase transcripts present. That is not to say that other mechanisms are not involved, but the amount of L/B/K alkaline phosphatase mRNA present appears to be the major mechanism.

The level of mRNA within a cell is dependent upon the rates of transcription, processing of the primary transcript and it's export, and the rate of cytoplasmic mRNA degradation. Gene expression may be affected by a change in any one of these processes. A change in any one or more of these processes may reflect a change in the level of L/B/K alkaline phosphatase within the osteosarcoma cell lines. In general, the most important control of gene expression is found at the level of transcription. This is substantiated by the observation that the steady state levels of an mRNA species correlates well with the transcription rate of the gene (Raghow, 1987). Changes in the rate of gene transcription may arise from mutation. Gene rearrangement or amplification are known to alter the transcription rate of genes thereby altering the amount of mRNA present. Rearrangement may release the gene from its usual controls and the level of expression increased. Gene amplification may also raise the mRNA level by providing an increase in the amount of template for transcription.

Southern blot analysis in the current study did not detect rearrangement of the L/B/K alkaline phosphatase gene in any of the osteosarcoma cell lines. The restriction pattern for the control and the cell lines was identical. Furthermore, Northern blot analysis showed a single RNA species of about 3 Kb in the osteosarcoma cell lines (Saos-2 and G-292) and normal kidney. Amplification of the L/B/K alkaline phosphatase gene was not detected by DNA slot blot analysis. This

indicates that a mechanism other than gene rearrangement or amplification is likely responsible for the large differences of the L/B/K alkaline phosphatase mRNA observed in the osteosarcoma cell lines.

Changes within cis-acting regulatory elements of the L/B/K alkaline phosphatase gene may result in altered expression of a gene. Mutations within the regulatory regions of the L/B/K alkaline phosphatase gene may be the cause of the high level of the enzyme in some of the osteosarcoma cell lines. Once the normal L/B/K alkaline phosphatase gene has been cloned and characterized the gene may be isolated from osteosarcoma cell lines and compared to that from normal cells. This may detect changes within regulatory sequences. Techniques that may prove useful in identifying mutations within L/B/K alkaline phosphatase gene regulatory genes are the polymerase chain reaction (PCR) and hybridization with oligonucleotide probes in slot blot format. This has been useful in identifying point mutations within the c-Ki-ras gene (Almoguera et al., 1988). PCR consists of repetitive cylcles of denaturation, hybridization, and polymerase extension using the reciprocal interaction of two oligonucleotides (Mullis et al., 1986). Once the specific DNA sequence is amplified it can be hybridized with oligonucleotide probes under varying stringencies to detect DNA sequence changes. PCR would also be useful to reduce the number of cloned DNA fragments to be screened if the L/B/K alkaline phosphatase gene from the osteosarcoma cell lines was to be cloned.

It is thought that the aberrant expression of developmentally regulated proteins in cancerous cells results from derepression of the normal gene (Fishman, 1974). For example, trans-acting factors may no longer be present or are incapable of exerting their influence on a

gene. Derepression of the L/B/K alkaline phosphatase gene may be the cause of the high level of its transcripts in some of the osteosarcoma cell lines. This could be the consequence of the loss of a trans-acting factor(s) which depresses the expression of the L/B/K alkaline phosphatase gene.

The loss of a trans-acting factor which exerts an influence on the L/B/K alkaline phosphatase gene could result from inactivation of the gene encoding for the factor. The loss of genetic information in some human tumors has been observed. Some pediatric cancers have a rare hereditary form in which specific chromosome deletions have been found. This includes the deletion in chromosome 13q14 observed in retinoblastoma (Knudson, 1978) and the deletion in chromosome 11p13 found in Wilm's tumor (Reeve et al., 1978). Cell fusion studies have also supported the idea of the loss of genetic information in the development of human cancer (Harris, 1988). Cell fusion has been used to investigate the abnormal expression of genes in tumor cells (Geiser et al., 1986). The use of somatic cell hybrids could prove useful in studying the expression of the L/B/K alkaline phosphatase gene in ostoesarcoma cell lines. If the high level of L/B/K alkaline phosphatase gene expression in some osteosarcoma cell lines results from loss of genetic information, then cell fusion studies may detect this. Cells from ostoesarcoma cell lines could be fused with normal human cells and the level of the L/B/K alkaline phosphatase gene expression examined.

In summary, alkaline phosphatase activity in the osteosarcoma cell lines varied markedly. The level of L/B/K alkaline phosphatase mRNA also varied considerably. The amount of L/B/K alkaline phosphatase mRNA in the osteosarcoma cell lines corresponds with their level of alkaline

phosphatase activity. Therefore, the differences in the alkaline phosphatase activities results from variation in the amount of L/B/K alkaline phosphatase mRNA in the osteosarcoma cell lines. This is likely the result of increased rate of transcription of the L/B/K alkaline phosphatase gene in some of the osteosarcoma cell lines. The current study found the gene not to be activated by rearrangement nor was the gene found to be activated by amplification. Further studies will be required to elucidate the mechanism responsible for the observed differences in the amounts of the L/B/K alkaline phosphatase mRNA.

5.2 PROTO-ONCOGENES

It has previously been shown that the v-src oncogene in the Rous sarcoma virus originated form a normal cellular gene termed a proto-oncogene (Stehelin et al., 1976). This finding helped to explain the transforming ability of retroviruses as well as suggest the origins of viral negative cancers. The finding of src and other proto-oncogenes indicated that the human genome has genes that if altered may be involved in transformation to the malignant state. Today a number of proto-oncogenes have been identified through their association with retroviruses or by gene transfer (Bishop, 1987). The three main ways in which proto-oncogenes are activated in humans are: point mutations, rearrangement, and amplification (Bishop, 1987).

The current study included investigation of osteosarcoma cell lines for the expression of 10 proto-oncogenes. Included in the 10 proto-oncogenes studied was at least one from the four major groups of proto-oncogenes (Table 1). Of the 10 proto-oncogenes investigated 6 were found to be transcriptionally active. This included the c-sis,

c-Ha-ras, c-fos, c-myc, N-myc, and c-raf-1 proto-oncogenes. Very weak hybridization signals, barely above background, were detected for c-sis and c-Ha-ras. The c-fos, c-myc, N-myc, and c-raf-1 genes had much stronger hybridization signals and were investigated for activation by rearrangement or amplification.

The N-myc gene was found to be expressed in all the osteosarcoma cell lines with the level of expression varying between the cell lines (Table VI). Expression of the N-myc gene has been found in other cell lines and tumors such as neuroblastomas, retinoblastomas, and small cell lung cancers (Kohl et al., 1984; Nau et al., 1986). Amplification of the N-myc gene appears to be a common mechanism of activation in these types of cancers. N-myc is amplified in most neuroblastoma cell lines and in about 40% of neuroblastoma tumors (Schwab et al., 1983; Seeger et al., 1985). Seeger et al., (1985) studied patients with neuroblastomas to determine if the number of copies of the N-myc gene was related to the outcome of the disease. They found that N-myc amplification was associated with poor prognosis, and furthermore, tumor aggressiveness was related to the degree of amplification. In the present study Southern blot analysis did not detect rearrangement of N-myc in any of the osteosarcoma cell lines. Moreover, the hybridization intensity of the restriction fragments was much the same indicating the absence of N-myc amplification (Figure 14). If the N-myc gene is involved in the development of osteosarcoma, its activation may occur by means other than rearrangement or amplification.

The product of the c-raf-1 gene is a serine/threonine kinase (Moelling et al. 1984). Its expression has been reported in other cell lines and thought to be involved in carcinogenesis (Bonner et al., 1985;

Fukui et al., 1987). In this study the c-raf-1 gene was found to be expressed in all of the osteosarcoma cell lines. However, rearrangement of the gene was not detected nor was there any indication of its amplification in any of the osteosarcoma cell lines. Another member of the raf family, B-raf, has recently been found to be activated by rearrangement (Ikawa et al., 1988). The c-raf-1 gene if involved in the genesis of osteosarcoma may be activated by a mechanism other than rearrangement or amplification.

The c-fos proto-oncogene has been studied extensively and much has been learned about its structure and expression. Its expression has been found to be closely regulated (Miller et al., 1984). Important components for regulation of c-fos include regulatory elements within the promotor region as well as both negative and positive trans-acting factors (Verma, 1987). The c-fos gene has been described as a "Master Switch" and thought to be involved in signal transduction (Marx, 1987). It has been found to be expressed in a number of tumors and cell lines (Slamon et al., 1984). In this study the c-fos gene was expressed in all of the osteosarcoma cell lines examined. Southern and DNA slot analysis did not detect rearrangement or amplification of the c-fos gene in any of the osteosarcoma cell lines. Activation of c-fos if involved in the formation of osteosarcoma, may occur by changes within its regulatory components or by other means.

As with N-myc, c-raf-1, and c-fos, the current study found the c-myc gene to be expressed in all of the osteosarcoma cell lines. The degree of expression of c-myc varied between the cell lines with it's expression in the KT005 cell line being over 20 times that of the cell line displaying the lowest expression (Table V). Southern blot analysis

did not detect rearrangement of the c-myc gene. However, amplification of the gene was found in the MG63 and G-292 osteosarcoma cell lines. Previously, the c-myc gene has been found to be activated by amplification in a number of cell lines including an osteosarcoma cell line different from those studied here (Masuda et al., 1987; Boegenmann et al., 1987). The c-myc gene encodes for a nuclear protein which is thought to play a part in cell proliferation (Hunter, 1984) and its activation by amplification may be an important step leading to osteosarcoma. It is interesting to note that the cell line KT005 was found to have the highest level of c-myc expression, yet the c-myc gene was found not to be amplified. Other means of activation of the c-myc gene may be important in the development of osteosarcoma.

Recently a group of genes were discovered that when absent cause tumor formation (Hansen and Cavanee, 1988). They have been termed tumor suppressors or antioncogenes. Antioncogenes act in a way opposite to that of oncogenes which act dominantly. This is, they are recessive to the wild type alleles (Hansen and Canvanee, 1988). The existance of antioncogenes was initially postulated in rare tumors such as retinoblastoma (Knudson, 1971). It was found that retinoblastoma tumors developed when there was a loss of genetic material mapping to the q14 band on chromosome 13 (Knudson et al., 1976). Retinoblastoma patients have a high risk of developing secondary cancers, especially osteosarcoma (Dryja et al., 1984.). A DNA segment thought to represent the "Rb" gene has been cloned (Friend et al., 1986). Using this DNA segment as a probe, the Rb locus was found to be deleted in most retinoblastomas and osteosarcomas (Friend et al., 1986). Further the Rb gene was found to be expressed in several tumors, but not in

retinoblastomas and osteosarcomas (Friend et al., 1986). Some human osteosarcomas have been found to become homozygous for chromosome 13q (Dryja et al., 1986). These observations suggest the loss of antioncogene function an important step in the formation of osteosarcoma.

It is possible that antioncogenes and proto-oncogenes might interact with one another (Bishop, 1987). This could involve antioncogenes exerting a regulatory function over proto-oncogenes. In this light, the loss of antioncogene function might release proto-oncogenes from their normal control. With the cloning of the DNA segment corresponding to the Rb locus it should be possible to undertake DNA transfection studies to see if introduction of the DNA segment will revert malignant characteristics. The osteosarcoma cell lines could be examined for expression of the Rb locus, proto-oncogene expression, and loss of tumorigenicity subsequent to introduction of the segment into cells.

5.3 EPILOGUE

The development of cancer is thought to be a multi-step process (Klein and Klein, 1986). The evolution of a cell clone from the normal to the malignant state involves progression through a number of intermediate steps (Klein and Klein, 1986). Statistical analysis of age-incidence curves has been used to estimate that at least four sequential mutational changes are necessary for the development of leukemias and at least 6 for carcinomas (Faber and Cameron, 1980). Included in these changes may be the activation of proto-oncogenes. In some cases it has been found that the activation of two proto-oncogenes

is required (Land et al., 1983). It is therefore not surprising that the current study found more than one proto-oncogene expressed in the osteosarcoma cell lines. The relevance of their expression in the osteosarcoma cell lines to the development of osteosarcoma is questionable. What has been found may have arisen from changes that occurred during the establishment of the cell lines or their maintenance in culture. Examination of osteosarcoma tumor material would help clarify this. Some of the changes that occur during progression to the malignant state may be independent, but necessary for transformation. For example, activation of the L/B/K alkaline phosphatase gene and proto-oncogene(s) in the osteosarcoma cell lines investigated here, may have arisen from quite separate events. Despite being independent events they may be necessary for the development of osteosarcoma. On the other hand, activation of the L/B/K alkaline phosphatase gene and proto-oncogene(s) may have been due to the same event. This could involve the functional loss of a common regulatory gene(s). An antioncogene exerting direct or indirect control over these genes may have been rendered inactive. However, given that carcinogenesis often proceeds through multiple stages of initiation, promotion, and progression it is more likely that expression of the L/B/K alkaline phosphatase gene arose from an event separate from that leading to proto-oncogene(s) expression.

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