HUMAN GROWTH HORMONE REGULATION OF ONCOGENE EXPRESSION IN Nb2 LYMPHOMA CELLS

BY

WILLIAM HARVARD FLEMING

A Thesis
Presented to the Faculty of Graduate Studies
University of Manitoba
In Partial Fulfillment
of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

May, 1987

Winnipeg, Manitoba

ISBN 0-315-37446-2

HUMAN GROWTH HORMONE REGULATION OF ONCOGENE EXPRESSION IN NB2 LYMPHOMA CELLS

BY

WILLIAM HARVARD FLEMING

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Table of Contents

ACKNOWLEDGEMENTS

I would like to thank Drs. R.J. Matusik and H.G. Friesen for their supervision and support of this project.

The immunocytochemical studies were done in collaboration with Dr. Pettigrew, Dept. of Pathology, University of Manitoba. Drs. P.R. Murphy and L.J. Murphy collaborated in the animal studies and offered their assistance and advice with many aspects of this work.

Over the years many members of the Endocrine group, Department of Physiology provided advice on various aspects of this work. Drs. J.G. Dodd, M.L. Duckworth and I. Worsley were always available to help solve problems.

ABSTRACT

Although the growth response of Nb2 cells has been well documented, little is known about those events which occur immediately following the binding of a lactogenic hormone to these cells. Using scanning electron microscopy it has been possible to document the rapid development of ruffles and pseudopodia on an early response to lactogen stimulation.

The presence of c-oncogene transcripts is a common finding in rapidly growing normal tissue and their malignant counterparts. As it was originally thought that c-oncogene expression may be cell cycle regulated, the possibility that the activation of one or more of these genes may represent an early post-membrane event in the mitogenic activation of Nb2 cells was examined.

The c-abl gene and the ras gene family mRNA were found to increase between 6 hour and 9 hour post-lactogen treatment. This represents a relatively late event and occurs just prior to these cells entering S phase of the cell cycle. In contrast, an increase in c-myc transcripts was observed within 15 minutes of hormone addition. The c-myc transcript number peaked within 3 hours and then slowly declined as the cells progressed through G1 of the cycle. Removal of hGH after c-myc levels had peaked was associated with a rapid decrease in c-myc transcript levels and failure of the cells to proliferate. The dose response was identical to the dose response observed for Nb2 cell proliferation. These results indicate that lactogen induced Nb2 cell proliferation and c-myc expression are quite closely linked.

Recent studies implicate an increased expression of c-oncogenes in certain human malignancies. The screening of both benign and malignant

prostate tumor indicates that c-myc levels are significantly higher in the malignant tumor. As well, the largest and most slow growing benign tumors had the lowest levels of c-myc expression. These results indicate that the evaluation of c-myc expression may provide information about the growth characteristics of a particular tumor.

LIST OF FIGURES

LIST OF TABLES

List of Abbreviations

Units of Measure

g	gram
mg	milligram
g	microgram
ng	nanogram
pg	picogram
l	litre
ml	millilitre
l	microlitre
cm	centimetre
mm	millimetre
nm	nanometre
hr	hour
min	minute
sec	second
cpm	counts per minute
dpm	disintegrations per minute
Ci	microcuries
mCi	millicuries
M	molar
mM	Millimolar
M	micromolar
N	Normal
A_{260}	absorbance at 260 nanometres
O.D.	optical density
^{o}C	degrees Celsius
xg	times the force of gravity
uv	ultraviolet light

Biological Molecules

RNA	ribonucleic acid
poly (A^+) RNA	polyadenylated ribonucleic acid
mRNA	messenger ribonucleic acid
DNA	deoxyribonucleic acid
cDNA	complementary deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dTTP	deoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate
TdT	terminal deoxynucleotide transferase
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
oPRL	ovine prolactin

hGH	human growth hormone
TPA	12-0-tetradecanoyl-phorbol-13-acetate
SS	salmon sperm
BPH	benign prostate hyperplasia

Reagents

CO_2	carbon dioxide
Na_2EDTA	disodium ethylenediamine-tetra acetate
NaOH	sodium hydroxide
NaCl	sodium chloride
NaOAc	sodium acetate
KCl	potassium chloride
$CaCl_2$	calcium chloride
CsCl	cesium chloride
Na_2HPO_4	sodium phosphate dibasic
EtBr	ethidium bromide
oligo (dT)	oligodeoxythymidylate
NaHepes	sodium N-2-hydroxyethylpiper-azine-n'-2-ethanesulfonic acid
SSC	sodium chloride, sodium citrate
NBM	nitrobenzyloxymethyl paper
ABM	diazobenzyloxymethyl paper
NADPH	nicotinamide-adenine dinucleotide phosphate
^{32}P	^{32}phosphate
TRIS-HCL	Tris (hydroxymethyl) aminomethane hydrochloride
ETOH	ethanol
DNAase	deoxyribonucleotidase
RNAase	ribonucleotidase
BSA	bovine serum albumin
SV40	Simian Virus 40
ddH_2O	double distilled water
PBS	phosphate buffered saline

INTRODUCTION

THE ORIGIN OF ONCOGENES

In 1910, Peyton Rous demonstrated the transmissibility of sarcoma in fowl (1). This first experimental evidence for an oncogenic virus has become a prototype on which virologists have based their search for oncogenes. In the following 75 year, a viral etiology has been established for a variety of neoplasms in domestic animals and at least suggestive evidence exists for an etiologic role in human cancers. With the advent of molecular cloning and DNA sequencing techniques the fine structure of the retroviral genomes and their corresponding oncogenes have been deduced. These studies have resulted in the identification of more than two dozen different oncogenes whose role in both neoplastic transformation and normal development has just now begun to be studied (2).

The retroviruses are characterized by a genome consisting of two similar subunits of RNA ranging from 5 to 10 kilobases (Kb) in length. Expression of this genome requires the conversion of the genomic RNA into DNA. This feat is accomplished by the retroviral specific enzyme reverse transcriptase (3). The viral DNA thus produced (known as proviral DNA) becomes integrated into the host's genome where cellular enzymes transcribe the viral genes and translate the viral mRNAs into proteins. The retroviral

genome although exceedingly small specifies all components necessary for the assembly of new viral particles. Three genes constitute the retroviral genome, the gag gene which codes for the internal structural proteins; the pol gene which encodes the sequence of the enzyme reverse transcriptase and the env gene which specifies the protein component of the glycoprotein viral envelope. A stretch of repetitive nucleotide sequences referred to as an LTR (long terminal repeat) flanks both the 3' and the 5'end of the viral genome and is responsible for the promotion of the expression of these genes. The presence of such controlling elements enables the virus to direct the synthesis of large quantities of viral mRNA and thus permit rapid viral replication.

The life cycle of the retroviruses is unique in that viral replication requires the interaction of the viral genome and that of its host. The effect of the insertion of proviral DNA in the host genome has been proposed to subvert gene expression in a variety of ways that may produce neoplasia. Firstly, the insertion of proviral DNA may disrupt the integrity of a gene which is necessary for maintaining the differentiated function of a cell. Alternatively, the insertion of an active proviral genome into an otherwise transcriptionally inactive domain may lead to the inappropriate expression of cellular replicative genes which may then cause uncontrolled growth. A third

possibility is that through a series of recombination events a retrovirus may pick up and incorporate a gene with oncogenic potential and transfer this gene to another host.

The first example of the existence of a transforming gene carried by a retrovirus resulted from a series of experiments with the Rous sarcoma virus. Through the use of temperature sensitive mutants, Martin was able to demonstrate that the transforming property of the Rous sarcoma virus was attributable to a gene now known as src (4). At lower temperatures the mutated src gene is expressed and brings about the transformation of host cells (5). An increase in temperature inactivates the src gene and the transformed cells revert to their normal phenotype. With the more recent development of gene transfer experiments it has been demonstrated that the isolated src gene itself can be inserted into cells, expressed and translated into protein with the result that transfected cells undergo malignant transformation (6).

Using an entirely different approach to isolate genes with oncogenic potential, Weinberg's group (7) and Cooper's group (8) have developed a method of transfecting cells with DNA from primary tumors. The DNA from normal cells was unable to transform 3T3 cells whereas DNA from a variety of both human and animal tumors was found to demonstrate this capability. Subsequent studies revealed that the same oncogenes are active in similar tumors in a

variety of species . The most startling finding from these types of experiments was that the transforming gene of a human bladder carcinoma is homologous to the transforming gene of the Harvey rat sarcoma virus (Ha-ras), (9,10). This observation has been confirmed several times with a variety of primary tumors indicating that the number of active oncogenes is relatively small, perhaps numbering no more than several dozen.

The high incidence of retroviral infection among many species coupled with the oncogenic potential of many retroviruses led Todaro and colleagues to suggest that oncogenes may lie unexpressed in all normal tissues and that activation of these oncogenes may result in the development of neoplasia (11). DNA probes made from viral oncogenes were utilized to search for oncogene related sequences in the tissue of host animals. With one exception, the oncogene of the Focus Forming Virus in mice, it has been found that genes homologous to the retroviral oncogenes are present in their host genomes (12). Furthermore, these cellular homologs or cellular oncogenes are highly conserved through evolution from yeast to man (13).

Several lines of evidence indicate that the retroviral oncogenes originally arose from cellular genes. The viral oncogenes are not required for viral replication. The original Rous sarcoma virus is exceptional in that all

other retroviruses containing oncogenes are in fact replicative defective and require the presence of a helper virus in order to reproduce. This would indicate that the presence of an oncogene would carry with it a loss of selective advantage. The intron-exon structure of the cellular oncogenes more closely resembles the expected eucaryotic gene organization rather than the retroviral gene. Further support of this idea arose from the observation that sequence conservation amongst various species is higher than that observed between the viral and cellular counterparts.

The ubiquitous nature of these cellular oncogenes or proto- oncogenes underlines the importance of these genes as they have been shown to be expressed in a variety of normal tissues (12). The high degree of phylogenetic conservation of these genes coupled with their tissue specific patterns of expression during both cellular growth and differentiation suggest the retroviruses have now provided us with a unique set of tools with which to investigate the growth and development of both normal and malignant cells at the molecular level.

THE STRUCTURE AND FUNCTION OF CELLULAR ONCOGENES

The structure of most cellular oncogenes is reminiscent of the variety of primary structures found in most unique copy genes. Cellular oncogenes exhibit classical Mendelian

loci and maintain constant positions within the genome of a given species (14). They range in size from a few Kb to more than 20 Kb (15,16). The numbers of introns present ranges from none in the case of c-mos to more than 12 as found in a murine sarcoma virus (17).

While only minor variation in the structure of cellular oncogenes from species to species is the general rule, variation of gene copy number has been noted. The myc oncogene family is now known to comprise at least three members. The c-myc gene is known to be expressed in most dividing cells while the N-myc gene appears to be restricted to tissues derived from the neural ectoderm (18,19). A third member of this family, L-myc has been detected only in small cell carcinoma of the lung (20). In the case of the c-myc oncogene a single copy is found in the chicken while four loci have been described in man (21). While one of the human c-myc genes is quite similar in structure to chicken c-myc the other human loci appear to represent incomplete copies of the gene and are of no known functional significance (22). In the case of the chicken src gene, 2 loci have been described (23). Two distinct src proteins have been described in man, however it has not yet been determined whether these are the products of 2 distinct genes (24).

The ras family of oncogenes is thought to have evolved by the duplication of a single ancestral gene. To date 2

distinct cellular oncogenes have been identified by viral transduction from the Harvey strain of murine sarcoma virus (Ha-ras) and the Kirsten strain (Ki-ras) while a third cellular oncogene designated N-ras has been isolated from a neuroblastoma cell line (25). In the rat genome 2 loci for the Ha-ras have been identified (26). The c-rasH-2 is colinear with the viral ras gene while the other gene designated c-rasH-1 contains 3 introns. It has been demonstrated, using an SV-40 expression vector that the mRNA transcribed from the c-rasH-2 gene has a post-transcriptional defect which precludes translation into the p21 protein product (25).

The cellular gene homologous to the Kirsten sarcoma virus has been isolated and found to contain 2 loci (27). The locus designated c-rasK-2 contains 3 introns is more than 45Kb in length and is thought to be the functional gene. The intronless c-rasK-1 gene which contains several termination codons is unable to produce the p21 protein product.

Two members of the ras gene family occur in a substantially amplified form. The Ha-ras gene has a copy number of 10 in the rodent M.pohari while the Ki-ras gene is present in at least 8 copies in the Chinese hamster (28). Neither the mechanism of amplification nor the reason for this relatively late evolutionary divergence is known.

While the Harvey and Kirsten members of the ras gene

family appear to be only distantly related at the nucleotide level the p21 protein product encoded by these genes demonstrates 85% homology (29,30). Explanation of this duplication phenomena is further complicated by unexpected similarities between several gene families. Comparison of the cellular oncogenes src,yes and fps/fes by nucleic acid hybridization suggested that these oncogenes were not related . The amino acid sequence predicted from these individual genes however, demonstrates a striking similarity in structure particularly at the carboxy terminal (31). These regions of homology occur in the region of the src protein (pp60) which comprises the functional domain of the tyrosine-specific protein kinase activity associated with this protein (32). These findings indicate that several gene families may belong to a superfamily of genes which has arisen from a single ancient lineage (33).

The determination of the biochemical function of most cellular oncogene products has been a difficult task secondary to the relatively low level of these proteins. Initial studies utilizing antibodies produced against synthetic peptides have determined the sub-cellular location of several oncogene products. Functional correlations of these proteins are just now beginning to emerge. The product of the src gene has been located on the cytoplasmic side of the cell membrane where it appears to be concentrated in adhesion plaques that are responsible

for the attachment of cultured cells to their substratum (34,35). Following its synthesis the src protein becomes associated with two proteins in the cytoplasm as it travels to the cell membrane. One of these proteins has been identified as a major heat-shock protein while the other may itself be a substrate for the phosphorylating activity of src (36, 37). Upon insertion into the cell membrane the src protein the first time since its synthesis displays tyrosine- specific kinase activity (34). A 36 Kd protein associated with the cell membrane has been identified although the function of this phosphorylated protein remains obscure (38). While the src protein itself is phosphorylated, the significance of this observation is unclear. Point mutations introduced into the gene (resulting in the conversion of the tyrosine residue at position 415 to phenylalanine) prevented phosphorylation at this site but did not alter the transforming ability of the src protein (39).

The role of phosphotyrosine in normal cell function is obscured by the observation that the majority of phosphoproteins are phosphorylated at either serine or threonine residues and in fact phosphotyrosine has been reported to constitute only about 0.01% of all phosphoaminoacids in normal cells (40). In spite of this low abundance at least 7 tyrosine-specific kinases have been identified among the cellular oncogenes. It has been

suggested that phosphorylation of tyrosine residues may be an important biochemical step in several developmental pathways each of which is regulated by an independent enzyme system (12).

Another group of oncogenes has been identified based on the tendency of their protein products to accumulate in the nucleus. This group consists of myc and myb both of which are prominently expressed in hemopoietic tissue (41). A third oncogene fos has been found in a variety of embryological structures where it may play a role in development and differentiation (42). While the function of these gene products has not yet been determined, it seems likely that they may interact with the nucleoprotein matrix and thus be responsible for the regulation of certain functional domains in the genome.

The ras gene family encodes for a protein designated as p21 which has been found to bind the guanine nucleotides GTP and GDP (43). In addition, p21 has been shown to have autophosphorylating activity of a unique nature in that GTP is the phosphoryl donor (44). It has been demonstrated that the ras oncogene obtained from a bladder carcinoma is able to transform NIH 3T3 cells, while similar DNA fragments from normal cells do not possess this activity. Subsequent sequencing of these DNA fragments revealed that they are identical with the exception of a single nucleotide substitution at position 12 which results in the

substitution of valine for glycine (45). A series of additional experiments indicates that an alternate site of mutation exists at position 61 where leucine substitutes for the normal glutamine residue (46). Whether or not these substitutions result in a structural change as is observed in the single amino acid mutation found in the sickle cell anemia is currently under investigation.

The recent discovery of genes homologous to ras in yeast has permitted the well developed mutational tools of the yeast geneticists to be utilized in the investigation of the biochemical function of the p21 protein (47). Yeast has been found to contain two closely related genes designated as ras1 and ras2 which are about 90% homologous to the mammalian ras at the protein level (48). This homology however is found primarily at the amino terminus. Mutations which disrupt both the RAS1 and RAS2 genes are lethal while mutation of either gene alone is not, which suggests that one gene product may substitute for the other (49). The introduction of the human Ha-ras locus into yeast with a lethal mutation of both RAS genes was effective at restoring viability to about 40% of the yeast spores (50). Another study has demonstrated that like its mammalian counterpart the yeast RAS is capable of binding guanine nucleotides (51).

The CYR1 mutant in yeast is deficient in adenyl cyclase activity and resembles strains with disruptions of both ras1

and ras2. Both the CYR1 mutant and the strains with the disrupted ras genes will grow in the presence of a BCY1 mutation which is deficient in the regulatory sub-unit of a cyclic AMP dependent protein kinase (52). The absence of the ras genes has an effect on yeast which is similar to that observed when there is a reduction of adenyl cyclase activity. As other guanine binding proteins have been shown to influence the activity of adenyl cyclase it has been suggested that the ras genes may provide a source of GTP which may stimulate this enzyme.

Constructs of the ras gene to produce the valine to glycine substitution at position 19 are equivalent to those seen in the Ha-ras mammalian p21 at position 12. This mutation results in levels of cyclic AMP which are 4-fold higher than normal as well as increased GTP activity which in turn stimulates adenyl cyclase (52). The mutant ras2 protein which is analogous to the transforming p21 of the mammalian system stimulates adenyl cyclase resulting in increased levels of cyclic AMP.

The most promising new insights into the function of oncogenes in mammalian systems have arisen from the rapid accumulation of information linking oncogene products to growth factors and their receptors. Homology between the sis oncogene of the simian sarcoma virus and one of the two peptides of platelet- derived growth factor (PDGF) was the first evidence linking growth factors with oncogene

products (53). More recently, it has been demonstrated that glucocorticoids inhibit the growth of the androgen responsive cell line DDT1MF-2 and that these cells will re-enter the growth cycle upon addition of PDGF (54). Subsequently it has been demonstrated that the inhibition of cell growth by glucocorticoids correlates with an inhibition of the transcription of c-sis mRNA sequences (55). These results have led the authors to speculate that an autocrine mechanism of growth is important in this cell line. This would be the first example of autocrine regulated growth involving an oncogene product.

The next example of a growth factor related oncogene to emerge was that of erb B, the avian oncogene which causes erythroleukemia in chickens. Sequence data of this gene demonstrated some areas of homology with the src oncogene. However, with the sequencing of the epidermal growth factor (EGF) receptor it became apparent that 6 of 14 peptides associated with the EGF receptor are homologous to the erb B oncogene with 74 of 83 amino acids being identical (56). The authors conclude that the erb B represents only a portion of the EGF receptor gene and point out that it may represent a different albeit highly related gene. The erb A gene, the second oncogene associated with the avian erythroblastosis virus, produces a protein which is not oncogenic itself but which is known to increase the oncogenicity of erb B. Recent studies indicate that c-erb A

belongs to a multigene family several members of which share extensive homology with both the glucocorticoid receptor and the estrogen receptor (57, 58). This homology is most striking in a cysteine-rich region of the respective proteins which suggests the presence of a shared functional domain. More recently, the c-erb A has been demonstrated to have homology to the progesterone receptor (58), the vitamin D receptor (60), and the thyroid hormone receptor (59).

Regulation of the hemopoietic system is accomplished by a complex interaction between a variety of growth factors and hormones. One such growth factor referred to as colony stimulating factor 1 (CSF-1) stimulates hematopoietic precursor cells to form colonies of mononuclear phagocytes (61). Recent studies indicate that the receptor for CSF-1 is similar to if not identical to the product of the c-fms proto-oncogene (63). The distribution of c-fms, its associated tyrosine kinase activity and its ability to bind CSF-1 all indicate a close functional relationship between the CSF-1 receptor and c-fms. The gene for c-fms has been localized to the long arm of chromosome 5 in man (63). This segment of chromosome 5 is deleted in a hematologic disorder referred to as the "5q-syndrome" which often progresses to myelogenous leukemia (64). Perturbation of the structural gene, coding for a receptor or c-oncogene product, is associated with and

may be involved in malignant transformation.

Demonstration that the CSF-1 receptor and c-fms are identical awaits the complete sequencing of both molecules. One possibility is that this relationship may parallel that observed with the v-erb B gene and the EGF receptor. The v-fms gene may lack the CSF-1 binding domain of the receptor while encoding the intracellular tyrosine kinase domain . Alternatively the v-fms gene may encode the competent CSF-1 receptor and may deliver this to fibroblasts which normally produce CSF-1 but lack the receptor. This may produce sarcomas with an autocrine loop similar to that described with the product of the c-sis oncogene (55).

ONCOGENES IN HUMAN MALIGNANCY

The fact that most human malignancies do not seem to result from the infection of the host by an acutely transforming virus, as is so often the case in lower mammals, has made identification of transforming genes difficult. As previously mentioned, Weinberg and Cooper developed a transfection technique whereby the DNA from human tumors was used to transform murine 3T3 cells. This led to the discovery that a transforming gene homologous to the v-ras was present in these tumors.

The expression of a variety of c-oncogenes has been described in most of the more common human malignancies and often several different oncogenes are found in the same

individual tumor (65). Both quantitative and qualitative changes in the normal cellular oncogenes have been implicated in malignant transformation.

The most thoroughly studied oncogene is c-myc in human lymphoid neoplasms (66). In Burkitt's lymphoma the translocation of the c-myc oncogene is from the long arm of chromosome 8 to the immunoglobulin heavy chain locus on chromosome 14, and less commonly the light chain locus on chromosome 22 (67). This translocation results in the increased expression of c-myc in these active domains. The sites of translocation of c-myc into the immunoglobulin locus is imprecise and unfortunately has not yielded any clues as to which regions of either the c-myc gene or the immunoglobulin gene are involved in the enhancement of expression of these genes.

A study on the levels of c-myc expression in more than 100 fresh hematologic neoplasms indicates elevated levels of c-myc mRNA in approximately 20% of the cases examined (68). Elevated levels of c-myc expression have also been identified in a number of solid tumors including carcinoma of the stomach, colon ,breast and in small cell carcinoma of the lung (69-72). Although amplification of the c-myc gene is observed in some of these tumors, in the majority of cases elevated levels of c-myc mRNA are associated with a normal gene dosage.

A single point mutation within the coding region of the

c-Ha-ras gene in the T24 bladder carcinoma results in a single amino acid substitution which confers transforming activity to the gene product. Using antisera specific for the p21 protein product of the c-Ha-ras gene the level of the p21 protein has been quantitated in benign polyps and in adenocarcinoma of the colon (73). The levels of p21 were found to be higher in the adenocarcinoma and correlated with the depth of invasion of these tumors. It remains to be seen whether levels of p21 will correlate with the metastatic potential of a particular tumor.

Further evidence for the involvement of c-oncogenes in human malignancies comes from the study of chronic granulocytic leukemia. About 90% of patients with this disease exhibit the presence of the Philadelphia chromosome which is a chromosome 22 with the deletion of both long arms that are then translocated to chromosome 9. The formation of this chromosome also results in the translocation of the c-abl gene from chromosome 9 to 22 (74). Of particular interest is the existence of variable forms of this disease where the long arms of chromosome 22 are translocated to another autosome. In these cases the c-abl gene is still translocated from chromosome 9 to 22 (75). While the functional significance of these rearrangements remains to be determined it seems likely that the consistent involvement of the c-abl gene which is known to be expressed in a number of hematologic

malignancies is important in the etiology of this disorder.

THE Nb2 LYMPHOMA CELL LINE

The Nb2 cell line was originally derived from a lymph node of an estrogenized male Noble rat. Preliminary studies indicated that this lymphoid cell line was dependent on the presence of pituitary derived factors for growth (76). Further studies demonstrated that any known lactogenic hormone would support the growth of these cells (77). As the mitogenic effect of lactogenic hormones occurs at physiological concentrations the Nb2 cell line has been subsequently utilized as a sensitive bioassay for lactogens (78).

Studies on the receptor mediated mitogenic action of prolactin in Nb2 cells indicates that there are approximately 12,000 receptors per cell and that maximal growth stimulation occurs with only 35% occupancy of these sites (79). This same study also described the dissociation constant of prolactin receptors and found it to be 75 mmol or some 20-fold higher than the receptors in other tissue types. Additional experiments of action of prolactin in these cells indicate that internalization of the prolactin-receptor complex is not necessary for the mitogenic effects of prolactin.

The development of the Nb2 cell bioassay has permitted the quantitation of prolactin bioactivity in a variety of

normal and pathological states. With few exceptions the serum prolactin concentration as determined by radio-immunoassay correlates well with the value obtained by the Nb2 bioassay (80). While it appeared that the Nb2 cells had an absolute requirement for the presence of a lactogenic hormone for growth, several recent studies suggest that additional ligands may influence the proliferation of these cells. As a variety of phorbol-esters are known to influence the proliferation of lymphocytes, Gertler et al (81) investigated the effect of TPA on the proliferation of Nb2 cells. No effect on cell proliferation was observed when TPA alone was added to stationary cultures of Nb2 cells. A maximal enhancement of 25% in the proliferation rate was obtained when 20 nM TPA was added with various amounts of hGH. The addition of hGH to Nb2 cultures results in an increase in the rate of transition of cells from G1 to S phase of the cell cycle, and an increase in the number of cells entering this transition phase. The addition of TPA to this system enhanced all these effects although the most dramatic effects were seen at the lower end of the hGH dose response curve. The authors conclude that these effects were mediated at a post-receptor level as the binding of hGH to the Nb2 cells is not affected by the presence of TPA.

The observation that parallel dilutions of serum and prolactin standards was associated with a greater

enhancement of cell proliferation in the serum samples suggests the presence of additional factors in serum which may influence the bioactivity of prolactin in the Nb2 cell assay. Studies by McNeilly and colleagues indicate that a heat stable serum factor of greater than 8000 daltons may be responsible (82). This as yet unidentified factor has no intrinsic growth promoting activity itself and the authors suggest that it may act in a manner similar to insulin in this system.

Recently, the Nb2 cells which were originally thought to be dependent on the presence of lactogenic hormones exclusively for growth, have been shown to proliferate in the presence of the lymphocyte mitogen interleukin-2 (83). This proliferative response to interleukin-2 occurs over a similar concentration range as the proliferative response to lactogenic hormones (200pg-2.0ng/ml). While the mechanism of growth stimulation is not well understood, studies with growth hormone antagonists indicate that interleukin-2 does not act through the lactogenic receptor on Nb2 cells (84). In contrast to the enhancement of the proliferative response to prolactin observed with the addition of TPA an inhibition of interleukin-2 stimulated Nb2 cell growth results from the presence of TPA. These results indicate that a variety of growth factors may trigger the proliferative response in Nb2 cells by at least two separate and distinct pathways.

Studies on the mechanism of action of lactogen induced proliferation of Nb2 cells indicate that lactogen-deprived growth arrested Nb2 cells have very low levels of activity of a number of proliferation associated enzymes. Following the addition of a lactogen to quiescent Nb2 cultures, a rapid induction of both ornithine decarboxylase (ODC) and S-adenosyl methionine decarboxylase (SAM) occurs (85). Similar increases in ODC activity resulted from the transfer of high density cultures to fresh medium (87) indicating that the rapid induction of this enzyme system was not in itself sufficient to produce cell proliferation. The observation that specific inhibitors of polyamine synthesis ,such as difluoromethyl-ornithine, also inhibit cell growth suggests that polyamines are required but not sufficient for Nb2 cell proliferation.

The role of protein phosphorylation in the mitogenic response to lactogens has recently been explored. Two proteins (designated as pp33 and pp19) have been identified which become phosphorylated following prolactin stimulation of Nb2 cells (87). The pp33 is maximally phosphorylated 1 to 3 hours after hormone addition, while phosphorylation of the pp19 is maximal at 7 to 9 hours. The increased incorporation of labeled methionine into both the pp33 and the pp19 following oPrl treatment indicates that both increased synthesis of these proteins and their subsequent phosphorylation may be important steps in lactogen induced

cell proliferation.

Recent studies have implicated a role for the membrane bound Na/H^+ antiport system in the regulation of cell growth (88, 89). In the cases of both hGH stimulation (90) and TPA treatment (91), activation of the Na/H^+ antiport system in Nb2 cells is observed within 4 minutes. The TPA treatment caused a significant H^+ efflux but did not result in cell proliferation. These results indicate that activation of the Na/H^+ antiport system is not in itself sufficient to induce Nb2 cell proliferation.

The Nb2 lymphoma cell culture system is well suited to the study of the mechanism of hormone action. A consistent growth response to physiological concentrations of lactogenic hormones coupled with the large sample size obtainable using suspension culture techniques permits the systematic study of those factors which interact to produce cell proliferation.

OBJECTIVES

While a large number of tumor-associated genes have recently been described, little is known of the role of these gene products in cell proliferation. The Nb2 cell line provides an excellent system with which to examine the relationship between mitogen binding, gene expression and cell growth. Studies to further characterize the phenotype of the Nb2 lymphoma cells will provide a framework for

studying the biological response of these cells.

The prostate gland is influenced by a variety of hormones including the lactogenic hormone, prolactin. As prostate cancer is often a hormonally responsive tumour, the expression of the c-myc oncogene in a number of these tumors has been undertaken.

MATERIALS AND METHODS

CELL CULTURE

The Nb211c clone (81) of the Nb2 rat lymphoma cell line was utilized for all gene expression experiments. The originally described Nb2 cell line (77) was used for all cytochemical studies. Both cell lines were grown in Fischer's medium for leukemic cells of mice supplemented with 10% horse serum, 10% fetal calf serum, 0.1 mM 2-mercaptoethanol, penicillin (50 units/ml), and streptomycin (50 ug/ml). Cultures were maintained in 1-litre spinner flasks (Bellco Glass Co.) in a 5% CO_2-95% air atmosphere at 37°C. For all experiments involving hormonal stimulation, Nb2 cells were centrifuged and resuspended in the above described growth media minus the 10% fetal calf serum supplement. Experiments were carried out at cell concentrations of $5x10^5$ - $1x10^6$/ml unless otherwise indicated.

For experiments involving serum free culture conditions, Nb2 cells were growth arrested in Fischer's medium containing BSA Fraction V (0.8%) and transferrin (50ug/ml) for a period of 24 hours. Both the human growth hormone and the anti-sera to hGH were produced in the Protein and Polypeptide Hormone Laboratory, University of Manitoba.

DETECTION OF SURFACE IMMUNOGLOBULIN BY INDIRECT IMMUNOFLUORESCENCE.

Rabbit anti-rat IgG, goat anti-rat IgA, sheep anti-rat IgE and rabbit anti-rat IgM were purchased from Miles Laboratories. Fluorescein conjugated second antibodies were obtained from Cappel Laboratories and Cedar Lane Laboratories.

The Nb2 cells (original Nb2 cell line) growing in log phase were gently centrifuged at 1,500xg and prepared as described previously (92). These cells were rinsed 3 times in Hank's salt solution and then incubated with the primary anti-sera for 30 minutes at room temperature. Following 3 thorough rinses with Hank's salts, the fluorescein conjugated second antibody was added and allowed to incubate for another 30 minutes. Following 3 additional rinses, the cells were examined under a fluorescence microscope and photographed. As a positive control for all surface immunoglobulins, a suspension of cells prepared from normal rat spleen cells was used.

INTRACELLULAR ENZYME STUDIES

Pararosaniline hydrochloride and -naphthyl acetate were purchased from Sigma Chemical Co. Rabbit antiacp25 -human muramidase was obtained from Cedar Lane Laboratories and a terminal transferase immunofluorescence assay kit was purchased from Bethesda Research Laboratories.

Cells were examined for alpha-naphthyl acetate esterase activity by a method described previously (93). Briefly, cells were rinsed 2 times in Hank's salts and cytospin preparations were made. Cells were then fixed for 10 minutes in a formaldehyde/calcium buffer (70 mmol CaCl2 and 4.0% formaldehyde). The slides were then rinsed for 30 minutes in distilled water. A solution of a-naphthyl acetate (5mM), sodium nitrate (15mM), pararosaniline (4mM) and sodium fluoride(2 drops of a 0.1M solution) was then used to incubate the cells for 30 minutes. Following a rinse in distilled water the cells were stained with 2% aqueous green. Isolated rat spleen cells and thymocytes were used as a positive control.

Muramidase activity in Nb2 cells was examined after acetone fixation (92). The cells were washed in Hank's solution twice and cytospin preparations were made. Endogenous peroxide activity was quenched by incubating cells with 3% hydrogen peroxide for 10 minutes. Non-specific background binding was reduced by incubating cells with normal swine serum at room temperature for 30 minutes. Next, the cells were incubated with rabbit anti-human muramidase at room temperature for 30 minutes. Following a rinse in Tris HCl (pH 7.4) buffer the PAP complex was diluted 1:50 and applied to cells for 30 minutes. After rinsing the slides for 30 minutes in Tris HCl (pH 7.4) buffer, diaminobenzidine (2.3 mM) was added for a 10 minute

incubation period. Cells were placed in copper sulphate (0.5%) and then counter-stained in Harris's hemalum. Slides were dried and permanent mounts made.

The activity of TdT was determined in a manner similar to surface immunoglobulins using a Bethesda Research Laboratories TdT immuno-fluorescent kit (93). Cytospin preparations of Nb2 cells were fixed in methanol for 30 minutes. Slides were rinsed with PBS (pH 7.4) and then incubated with rabbit anti-bovine TdT for 30 minutes. Following 3 rinses with PBS, cells were incubated with fluorescein conjugated goat anti rabbit IgG for 30 minutes. Slides were again rinsed, a wet mount was prepared and viewed under a fluorescent microscope. Rat thymocytes were used as a positive control.

GUINEA PIG ERYTHROCYTE ROSETTE-FORMING TECHNIQUE

Trunk blood was obtained from normal guinea pigs and collected in heparinized tubes. Blood samples were centrifuged and the red cells separated using a ficoll gradient, washed twice in phosphate buffered saline and then diluted 1:200. The Nb2 cells were rinsed in a similar manner and resuspended in PBS and 10% fetal calf serum at a concentration of $5x10^6$/ml.

Next, 0.1 ml of Nb2 cells was added to 0.1 ml of guinea pig erythrocytes and incubated for 5 minutes at 37^o C. This mixture was then centrifuged at 1500xg for 3 minutes,

the majority of the supernatant was removed and the cells were resuspended in the remaining volume and a wet mount was prepared. At least 200 cells were counted and all cells binding 3 or more erythrocytes were considered positive (94). Thymocytes prepared from 4-week old Sprague-Dawley rats were used as a positive control.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

Log phase growing Nb2 cells were centrifuged at 1500xg and stained with osmic acid and then prepared according to the method of Spurr (95). Sections were examined and photographed using a Phillips Model 301 electron microscope. (The processing of these samples was done in the laboratory of Dr. Ian Adamson, Department of Pathology.)

SCANNING ELECTRON MICROSCOPY (SEM)

Nb2 cells at various times following stimulation by hGH were allowed to settle on poly-lysine coated cover slips. Samples were then fixed and processed for SEM (96). The cells were examined using a JSM-35c Scanning Electron Microscope. (Processing of samples was done in Dr. Bruni's Laboratory, Department of Anatomy)

RNA EXTRACTION

All tissue samples and cultured cells assayed for

specific mRNAs were quickly frozen on dry ice or in a dry ice/ethanol bath and then stored at -70°C prior to RNA extraction. Total RNA was extracted by modification of the method described by Rutter and colleagues (97).

The Nb2 cells, isolated thymocytes and isolated spleen cells were added to 10 volumes of 4M guanidinium isothiocyanate, 5 mM sodium citrate (pH 7.4), 100 mM 2-mercaptoethanol. This solution was mixed thoroughly using a 10ml pipette. The homogenate was layered on a cushion of 5.7M cesium chloride in 100mM EDTA (pH 7.4) in a polyallomer tube which was centrifuged using a Beckman SW50.1 rotor at 35,000 rpm for 12-16 hours. Following centrifugation the supernatant and the cesium cushion were carefully aspirated and the total RNA pellet adherent to the bottom of the tube was dissolved in 200-400 ul of dd H2O. Sodium chloride was added to a final concentration of 0.25M. Two volumes of 95% ethanol were added and the samples were allowed to precipitate overnight at -20°C. Prior to electrophoresis precipitated RNA was centrifuged at 10,000xg and redissolved in dd H2O. The concentration of RNA was determined using a UV spectrophotometer and the ratio of absorbance at 260 nm and at 280 nm was determined. A ratio of greater than 1.8:1 indicated the presence of nucleic acids free from protein contamination.

Total RNA was prepared from various animal tissues using the guanidinium solution described above with the

addition of 0.1% anti-foam A (Sigma). This mixture was homogenized using a Waring blender for about 90 seconds. The samples were centrifuged for 10 minutes at 3000xg in order to pellet insoluble connective tissue. The resulting supernatant was layered on a cesium cushion and processed as described above.

PURIFICATION OF POLY (A+) RNA

Total RNA, at a maximum concentration of 1.0 mg/ml was mixed with Tris HCl (pH 7.4) (final concentration 0.01 M) and EDTA (final concentration 1.0 mM). This solution was heated in a boiling water bath and quickly cooled on dry ice/ethanol. Potassium chloride was added to a final concentration of 0.5 M and this denatured total RNA solution was then applied to an oligo-dT cellulose column (Collaborative Research) as described previously (98). Following binding of the poly (A+) RNA in this high salt solution, it was eluted using the above mentioned buffer without the potassium chloride. These low salt conditions preclude the binding of poly (A+) sequences of the mRNA molecules to the oligo-dT cellulose. The poly (A+) RNA thus eluted was monitored using a UV absorbance monitor (ISCO) and precipitated using 2 volumes of 95% ethanol and 0.20 M sodium chloride.

NORTHERN BLOT ANALYSIS

Denaturing agarose gels (1%) were prepared by dissolving agarose in boiling dd H20 allowing this solution to cool to 60°C and then adding 5x gel buffer and 38% formaldehyde to produce a final concentration of 1x buffer and 2.2 M formaldehyde.

5x Gel Buffer	Sample Buffer	
0.2 M MOPS (pH 7.0)	RNA	4.5ul
50 mM sodium acetate	5x gel	2.0ul
5.0 mM EDTA	formaldehyde	3.5ul
	formamide	10.0ul

The RNA samples were prepared as indicated above, with a maximum of 20 ug/sample. This mixture was incubated at 55°C for 15 minutes. Next, 2 ul of a loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol) was added to the sample. These samples were loaded on a 1% agarose gel and electrophoresed at 20 mA for 14 to 16 hours.

Following electrophoresis the RNA was transferred to either nitrocellulose filters (99) or to DBM paper (100). The agarose gel was placed on several sheets of Whatman filter paper saturated with 20x SSC (3 M sodium chloride and 0.15 M sodium citrate). A sheet of nitrocellulose paper (Schleicher and Schuell) was placed over the gel and in turn covered by a layer of paper towels. The 20x SSC

moves through the gel by capillary action and transfers the RNA from the gel to the nitrocellulose paper. The transfer is complete in about 6 hours. During this time the sheets of Whatman filter paper is kept saturated with 20xSSC. Following the transfer, the nitro-cellulose is baked at 80°C for 3 hours in a vacuum oven. The baked filter is then stored dry at room temperature until use.

Alternatively, RNA samples were transferred to DBM paper to which they are covalently bound (100). The activation of ABM paper to its diazotized form (DBM) is accomplished by soaking a sheet in 125 ml of ice cold 1.2 M HCL containing 3.0 mg of sodium nitrate for 30 minutes. Following several washes in acetone the DBM paper was placed on top of the RNA gel as described above for the nitrocellulose transfer. A solution of sodium acetate (0.3 M, pH 4.0) was used as the buffer during the transfer. Following a transfer of at least 6 hours, the DBM paper was stored at 4°C until use.

For all RNA gels, outside lanes containing either RNA or DNA molecular weight markers were removed following electrophoresis, stained with ethidium bromide (0.5 ug/ml) and visualized on a UV transilluminator.

NICK TRANSLATION OF cDNA PROBES AND HYBRIDIZATION

Various cDNA probes (Table 3) were labeled with ^{32}P using a nick translation kit supplied by Amersham. For

each nick translation 0.1 ug of cDNA probe was labeled using 15 uCi of ^{32}p alpha-dCTP (specific activity 400 Ci/mMol) per reaction. This resulted in the labeling of a probe with a specific activity of approximately $1x10^{8}$dpm/ug. Following this reaction, the labeled cDNA was separated from the free nucleotides on a small G-100 Sephadex column. Before hybridization, the cDNA probe was heat denatured at 100°C and quickly cooled on dry ice/ethanol.

Prior to hybridization Northern blots were pre-hybridized for at least 2 hours in hybridization buffer at the appropriate temperatures.

Nitrocellulose

Hybridization	DBM Paper Hybridization
6xSSC, 5x Dendardt's	50% formamide, 0.1% SDS, 0.6M NaCl
0.1% SDS, 50ug/ml SS DNA	80mM Tris (pH 7.8), 4mM EDTA, 50 ug /ml SS DNA

After pre-hybridization in 3-5 ml of the appropriate buffer, $5x10^{6}$ DPM of ^{32}P-labeled cDNA was added to each ml of hybridization solution. The sealed plastic bags containing the Northern blots were then hybridized overnight at 42°C (DBM blots) or 65°C (nitrocellulose blots). Post hybridization, both the DBM and nitrocellulose blots were rinsed 3 times in 2xSSC and 0.1% SDS at room temperature

followed by 2 rinses in 0.2xSSC and 0.1% SDS each for 15 minutes at 65°C. The blots were then covered with Saran wrap and autoradiographs were exposed using an enhancing screen (Quanta lll) at -70°C.

QUANTITATION OF mRNA TRANSCRIPTS

Autoradiograms produced from Northern Gel analysis were scanned using a BioRad densitometer (100). The area under the curve of the densitometer tracing was determined using a digitizing tablet (Hewlett Packard). In certain experiments where the RNA samples to be compared were too numerous to be electrophoresed on a single gel, at least 2 internal standards were included for comparison on each gel. A ratio of signal strengths between these two standards allowed comparison of samples on different gels.

IN VIVO hGH INDUCED C-MYC GENE EXPRESSION

These studies utilized hypox rats (Sprague-Dawley) approximately 2-4 weeks after hypophysectomy. Only those animals demonstrating a weight gain of less than 3g/week were used. Groups of 5 rats were killed at various intervals following the injection of hGH (100ug/g) intraperitoneally. Control rats received an equivalent volume of vehicle. Various tissues were removed and quickly frozen on dry ice. RNA extraction was performed as described above.

HUMAN PROSTATE C-MYC GENE EXPRESSION

Samples of tissue obtained from patients with BPH or carcinoma of the prostate were obtained following either suprapubic prostatectomy or transurethral prostatectomy. Two normal prostates were obtained within 4 hours of death of otherwise healthy individuals. Tissue was rapidly frozen using dry ice then stored at -70^{o}C until RNA was extracted.

RESULTS

CELL SURFACE MARKERS ON Nb2 CELLS

Cultures of Nb2 cells whether stationary or in log phase growth, did not express surface immunoglobulin (Table 1). The "capping" phenomenon associated with aggregates of anti-immunoglobulin was absent from all preparations of cells examined. In addition cytoplasmic immunoglobulin was not detectable using the peroxidase staining technique .

A monoclonal antibody raised to rat thymocytes, W3/25-HLK, has been shown to bind specifically to an antigenic determinant on T-helper cells (102). Virtually 100% of the Nb2 cells bound this antibody (Figure 1). A second monoclonal anti-body 0X8-HL, which identifies rat nonhelper T-cells (103), similarly bound to 100% of the Nb2 cells. Both these anti-bodies produced patterns of capping which were indistinguishable from each other. The intensity of the binding of both antibodies was less than that observed in control cells from a normal rat spleen.

INTRACELLULAR ENZYMES

The lysosomal enzyme muramidase was not detectable in Nb2 cells (Table 2). Nonspecific esterase activity also was absent as was activity of the DNA polymerase enzyme terminal deoxynucleotide transferase. The presence of α-naphthyl acetate esterase activity in normal peripheral T

lymphocytes is indicated by a solitary red cytoplasmic nodule or several punctate red granules. Although Nb2 cells failed to exhibit this reaction product, about one third of the cells examined had a faint localized staining of variable intensity (Figure 2). This localized staining pattern (Thy-like) has previously been reported in a subpopulation of normal rat thymocytes (93). We observed a similar pattern in approximately 65% of the thymocytes of 4 week old Sprague-Dawley rats (data not shown).

ROSETTE FORMATION WITH GUINEA PIG ERYTHROCYTES

Guinea pig erythrocytes have been shown to spontaneously form rosettes with a subpopulation of cells in the normal rat thymus (94). The proportion of rosette forming cells in the rat thymus increases with age from the fetus to the adult. Approximately 25% of the thymic cells isolated from the young adult Sprague-Dawley rat (4 week old) formed rosettes in our study. No rosette forming cells were identified from spleen cells isolated from these animals. The Nb2 cells were found not to exhibit this rosette forming property.

TRANSMISSION ELECTRON MICROSCOPY

The Nb2 lymphoma cells were found to have an average diameter of 7.5 um under conditions of growth arrest while log phase growing cells had an average diameter of 9.0 um.

The cytoplasm of Nb2 cells was found to be typical of lymphoblasts with numerous polyribosomes and many mitochondria (Figure 3).

Table 1. EXPRESSION OF CELL SURFACE MARKERS

The Nb2 cells were incubated with the indicated fluorescein conjugated antibody. Specific binding was determined with UV light microscopy. Cells isolated from normal rat spleen cells were utilized as controls.

Antibody to	Result
IgG	–
IgA	–
IgE	–
IgM	–
T-cell nonhelper (0X8-HL)	+
T-cell helper (W3/25-HLK)	+

Figure 1. T-CELL SURFACE MARKERS ON Nb2 CELLS

Binding of the monoclonal antibody W3/25-HLK to Nb2 lymphoma cells. All Nb2 cells examined demonstrated binding (X500).

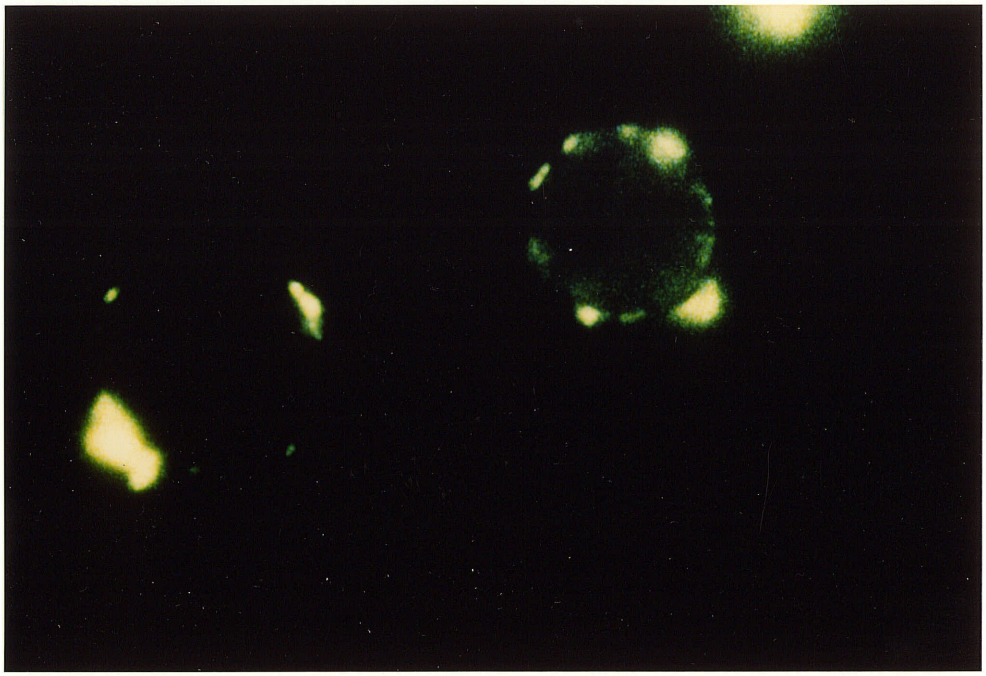


Table 2. INTRACELLULAR ENZYMES IN Nb2 CELLS

Nonspecific esterase activity and α-naphthyl acetate esterase activity were determined using α-naphthyl acetate as a substrate. Both terminal deoxynucleotide transferase activity and muramidase activity were determined using fluorescein conjugated antibodies. Isolated rat spleen cells were used as a control.

Enzyme	Result
Nonspecific esterase	−
α-Naphthyl acetate esterase	+ (Thy-like)
TdT	−
Muramidase	−

Figure 2. α-NAPHTHYL ACETATE ESTERASE ACTIVITY IN Nb2 CELLS.

The diffuse staining pattern indicated above in about 1/3 of the Nb2 cells is characteristic of the thy-like pattern observed in normal rat thymocytes (x200).

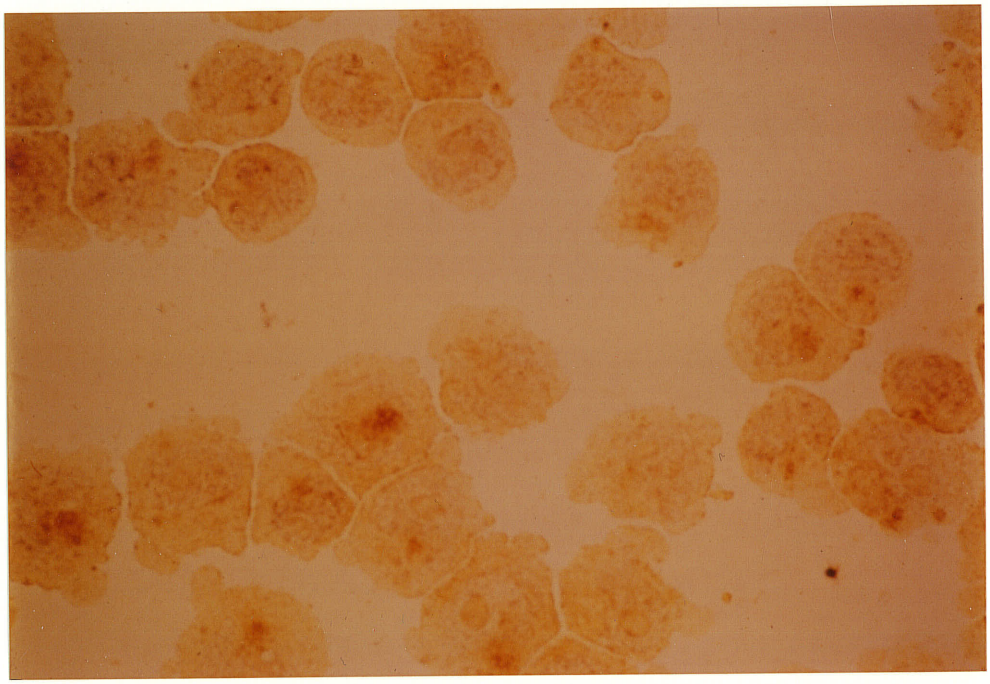
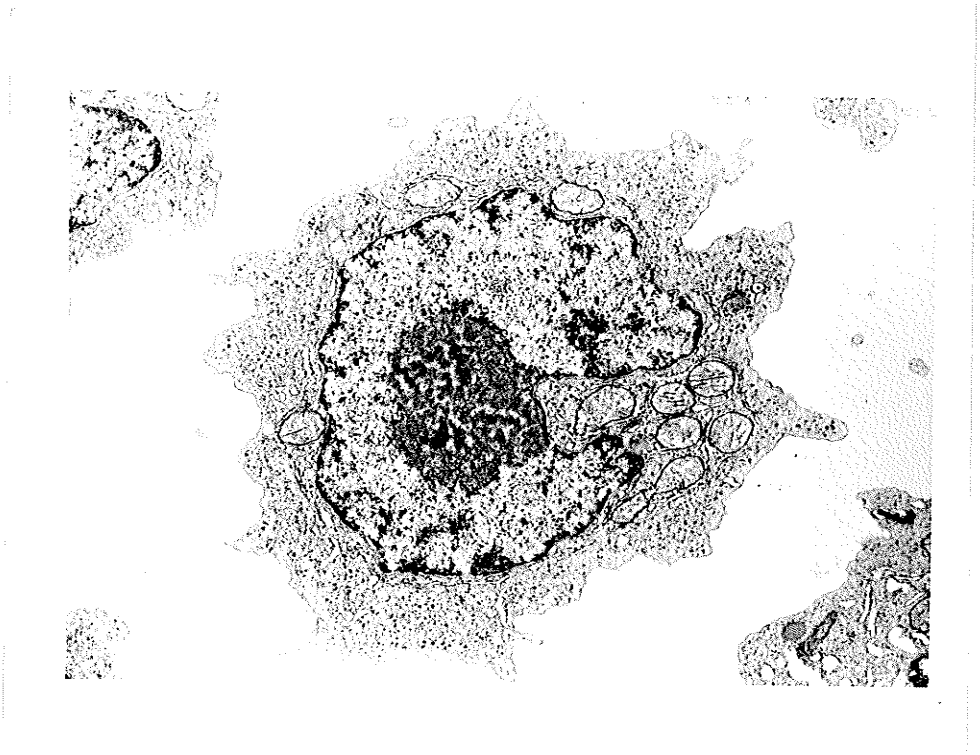


Figure 3. TRANSMISSION ELECTRON MICROSCOPY

A typical log phase growing Nb2 cell is presented. Rough endoplasmic reticulum, cytoplasmic vesicles and Golgi are sparse or absent. Heterochromatin is seen distributed along the nuclear membrane. A prominent nucleolus within a horse-shoe shaped nucleus is characteristic of Nb2 cells (x8300).



SCANNING ELECTRON MICROSCOPY

Growth arrested Nb2 cells were treated for variable periods of time with hPRL and allowed to settle on poly L-lysine coated microscope coverslips. These cells were fixed and then prepared for SEM.

As shown in Figure 4, unstimulated Nb2 cells have a relatively uniform surface with few undulations or projections. As early as 70 minutes after hormonal stimulation, there is an increase in cell volume associated with the appearance of numerous projections and pseudopodia-like structures. Within 2 hours, the highly ruffled Nb2 cell resembles the surface of a mitogen treated peripheral lymphocyte.

ONCOGENE EXPRESSION IN Nb2 CELLS

The following investigations of hormone stimulated growth utilized a clone of the original Nb2 lymphoma cell line. Designated Nb2 11c, this subline is representative of the most hormonally responsive cells in the original cell line. The cDNA probes used in this study are listed in Table 3. Also, this table lists the presence or absence of these oncogene transcripts in log phase growing Nb2 cells. The presence of c-abl, c-myc, and all three members of the ras gene family were detected in Nb2 cells. As shown in Figure 5 a single transcript of 6Kb characterized the H-ras and N-Ras transcripts while a doublet at 6Kb was detected

using the Ki-ras probe. The c-abl transcripts detected were 6Kb and 7Kb respectively. The c-myc transcript was 2.4 Kb.

Figure 4. SCANNING ELECTRON MICROSCOPY

Nb2 cells were treated for the time periods indicated with 1 ng/ml oPRL and then processed for SEM. (Time is indicated in minutes).

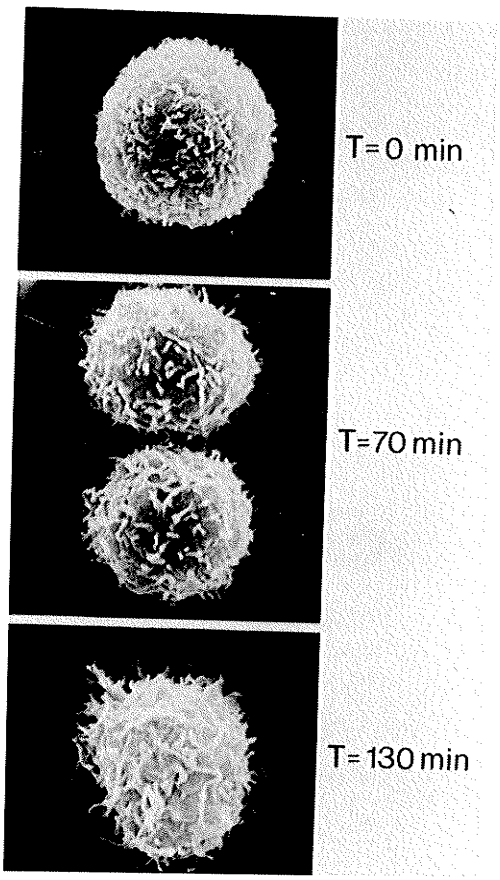

T=0 min
T=70 min
T=130 min

Table 3. ONCOGENE EXPRESSION IN LOG PHASE Nb2 CELLS.

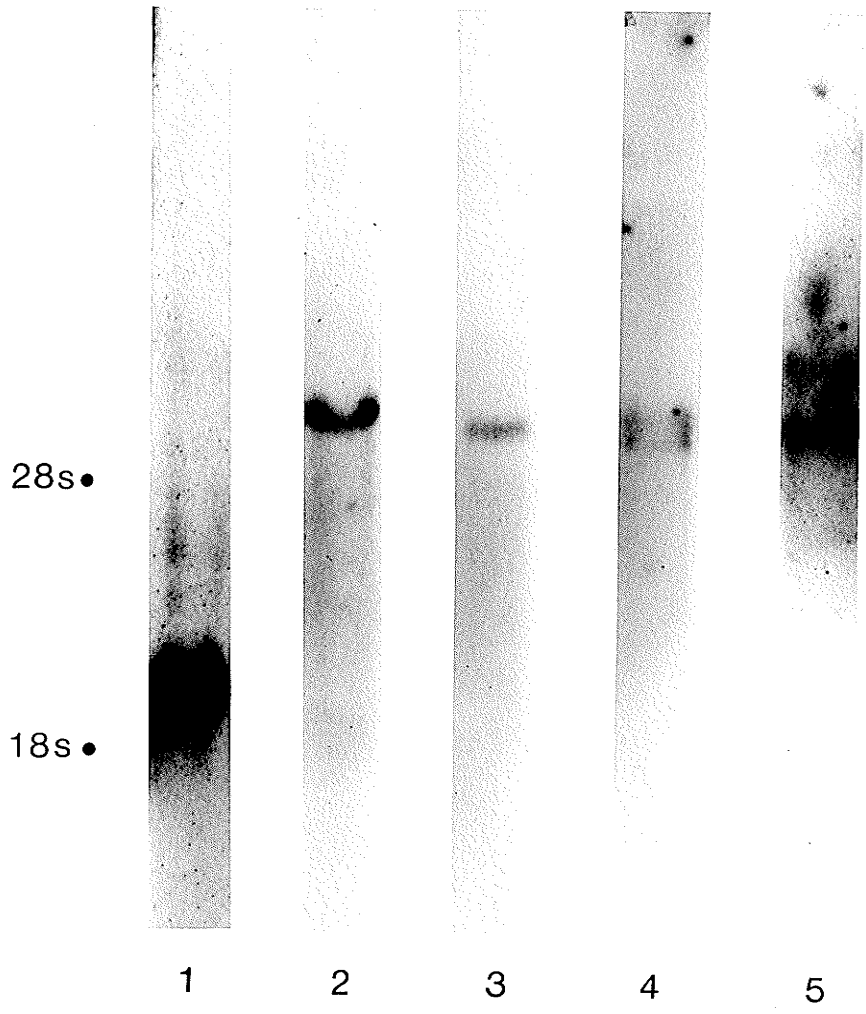
Expression of oncogene transcripts in log phase growing Nb2 cells is indicated as either +/- . The restriction fragment utilized as a probe along with a reference as to its origin is provided.

TABLE 3

ONCOGENE	SOURCE	DETECTION IN LOG PHASE NB2 CELLS	REFERENCE
abl	Ableson leukemia virus (7.8 kb, EcoR1)	+	16
myc	human (8.2 kb, EcoR1/HindIII)	+	17
Ha-ras	human (6.6 kb, BamH1)	+	18
Ki-ras	murine sarcoma virus (7.0 kb, EcoR1)	+	19
N-ras	human (1.0 kb, HindIII)	+	20
myb	avian myeloblastosis virus (7.2 kb, BamH1)	-	21
sis	human (1.7 kb, BamH1)	-	22
erbB	avian erythroblastosis virus (0.5 kb, BamH1/EcoR1)	-	23
src	Schmidt-kupplin a-2 genome (3.0 kb, EcoR1)	-	24
fms	SM Feline Sarcoma Virus (0.4 kb, Pst1)	-	25
fes	human (14.0 kb, EcoR1)	-	26
mos	Moloney sarcoma virus (2.5 kb, HindIII)	-	27

Figure 5. DETECTION OF C-ONCOGENE TRANSCRIPTS.

The RNA was examined from log phase cultures of Nb2 cells. Each lane contains 10 ug of poly (A+) RNA. Lane 1, c-myc; lane 2, N- ras; lane 3, H-ras; lane 4, Ki-ras; Lane 5, c-abl. Ribosomal RNA was utilized as size markers.


28s
18s
1
2
3
4
5

Further investigations were undertaken to determine both the kinetics of the hGH induced oncogene expression and the relationship between the activation of these genes and the Nb2 cell cycle. In order to quantitate changes in specific mRNA abundance following hormonal treatment, densitometeric analysis of the appropriate bands on the Northern gel autoradiograms were scanned and then digitalized. The areas under the corresponding peaks were calculated in square millimeters and then normalized to the largest peak. (An example of a digitalized scan of a gel containing human prostate samples hybridized with c-myc is shown in Figure 6). For all experiments involving Nb2 cells all samples from a particular experiment were electrophoresed on the same Northern gel. This approach eliminated any gel to gel variability.

The induction of c-myc transcripts following the treatment of Nb2 cells with hGH is shown in Figure 7. An increase in the abundance of c-myc transcripts is evident within 15 minutes of hormone addition. Peak abundance of c-myc transcripts occurs within 3 hours and then gradually declines as Nb2 cells proceed through G1 of the cell cycle. The induction of c-myc transcripts by 3 hours post hormone addition represents a 25 fold increase over the levels observed in growth arrested Nb2 cells.

The same time course following hGH induction was

examined using a probe for the β-actin gene. As shown in Figure 8 the β-actin transcripts increased about 5-fold during the first hour and then only increased slightly during the next few hours. These results indicate that factors other than a generalized increase in mRNA are responsible for the increase in c-myc transcripts.

Figure 6. DIGITIZATION OF DENSITOMETRIC SCANNING OF NORTHERN GELS

Lane numbers corresponding to lanes on the Northern gel are indicated in the row labeled L#. The absolute area of each curve is given in square millimeters in the row labeled mm. The third row labeled Nrm indicates the area under the curves normalized to largest peak which is automatically assigned the value of 100%. The signal ratio between lanes 7 and 1 is approximately 23 fold.

FILE: HPROSTATE GEL#: 1 GEL NAME: hprost myc

L#	1	2	3	4	5	6	7
mm	115	467	242	188	964	488	2664
Nrm	4.3	17.5	9.1	7.1	36.2	18.3	100.0

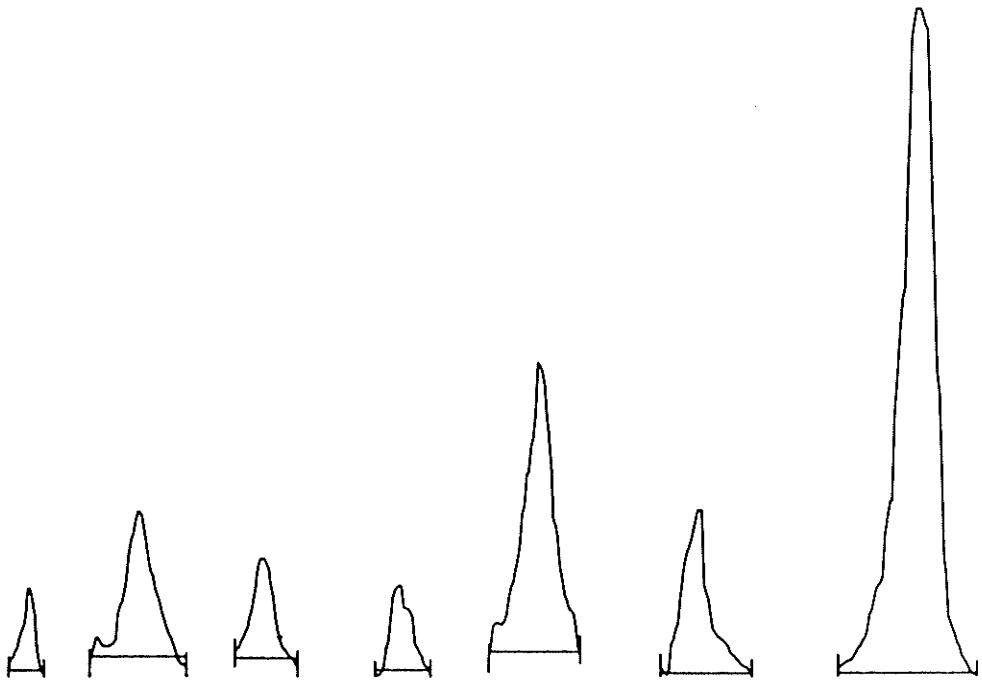


Figure 7. INDUCTION OF C-MYC IN Nb2 CELLS

The characteristic 2.4 Kb mRNA transcript is indicated above. The time following hGH administration is indicated in both minutes (') and hours (hr). Each lane contains 15 ug of Nb2 cell total RNA. Ribosomal RNA was used as size markers.

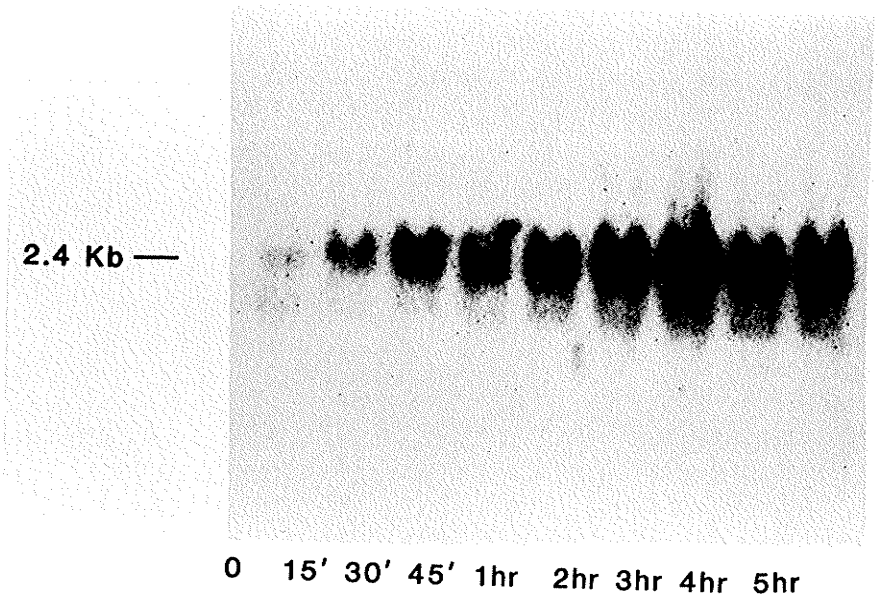
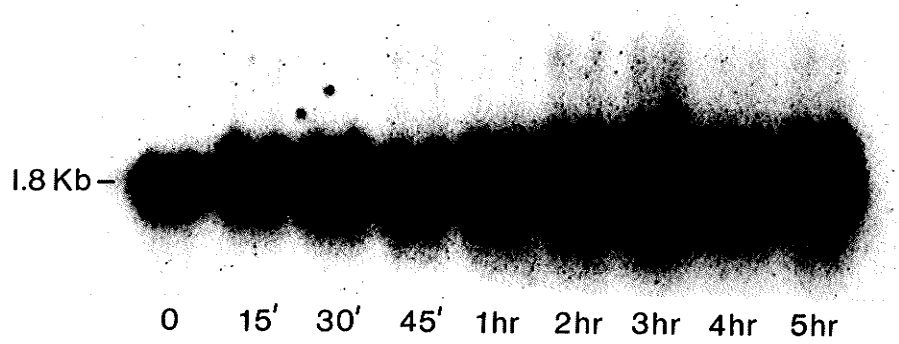

2.4 Kb
0 15' 30' 45' 1hr 2hr 3hr 4hr 5hr

Figure 8. INDUCTION OF β-ACTIN IN Nb2 CELLS

The 1.8 Kb β-actin transcript abundance is indicated following various times post hGH stimulation. Each lane contains 15 ug of total Nb2 cell RNA. Ribosomal RNA was used as size markers.


1.8 Kb
0
15′
30′
45′
1hr
2hr
3hr
4hr
5hr

The accumulation of H-ras transcripts following treatment with hGH is shown in Figure 9. In untreated cells no mRNA transcript is visible. A faint band at about 6 Kb is detected at 1 hour post hormone addition. The intensity of this sigmal increases gradually with the greatest accumulation occurring between 6 hr and 9 hr post hormone treatment. A prominent band persists at 12 hr at which time most cells are in S phase of the cell cycle (81). The same time course was examined using the c-abl probe. As seen in Figure 10 similar results were obtained with the maximal transcript accumulation occurring between 6 hr and 9 hr post hormone treatment.

In order to examine the relationship between cell growth and c-myc expression, dose response curves with respect to hGH was performed. As seen in Figure 11, the Nb2 cell growth and the accumulation of c-myc transcripts occur in a dose dependent manner. The dose response curves were fitted and the concentration of hGH required for half maximal stimulation (EC50) of both growth and c-myc accumulation were calculated using the ALLFIT program (116). The EC50 for the growth response was 193 ± 41 (SEM) pg/ml. The EC50 for c-myc accumulation was 299 ± 52 (SEM) pg/ml. The EC50 observed here is very similar to the EC50 described previously for prolactin stimulated proliferation of Nb2 cells (79). The EC50 for the growth response is not significantly different from the

EC50 for c-myc accumulation. This is consistent with the hypothesis that hGH induced c-myc induction is linked to Nb2 cell proliferation.

Figure 9. INDUCTION OF H-RAS TRANSCRIPTS IN Nb2 CELLS

Accumulation of H-ras transcripts following the addition of hGH (1 ng/ml) to growth arrested Nb2 cells is shown. Time post hormone addition is indicated in hours. The position of 28s ribosomal RNA and 6Kb H-ras mRNA is indicated.

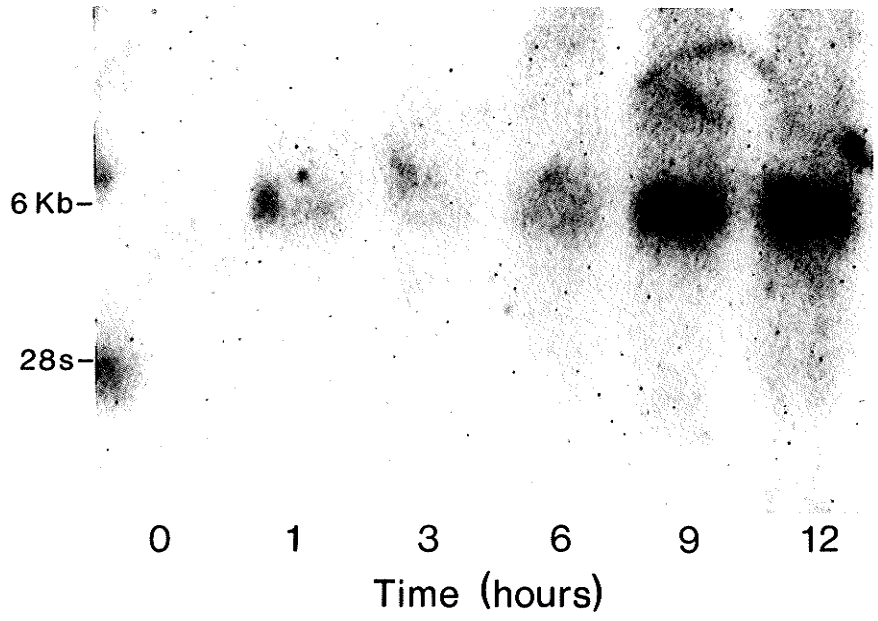

6 Kb–
28s–
0
1
3
6
9
12
Time (hours)

Figure 10. ACCUMULATION OF C-ABL TRANSCRIPTS IN Nb2 CELLS.

The induction of c-abl transcripts in growth arrested Nb2 cells following the addition of hGH (1 ng/ml) is shown. Time after hormone addition in hours is indicated. The position of 28s ribosomal RNA, the 7 Kb and the 6 Kb c-abl transcripts are indicated.

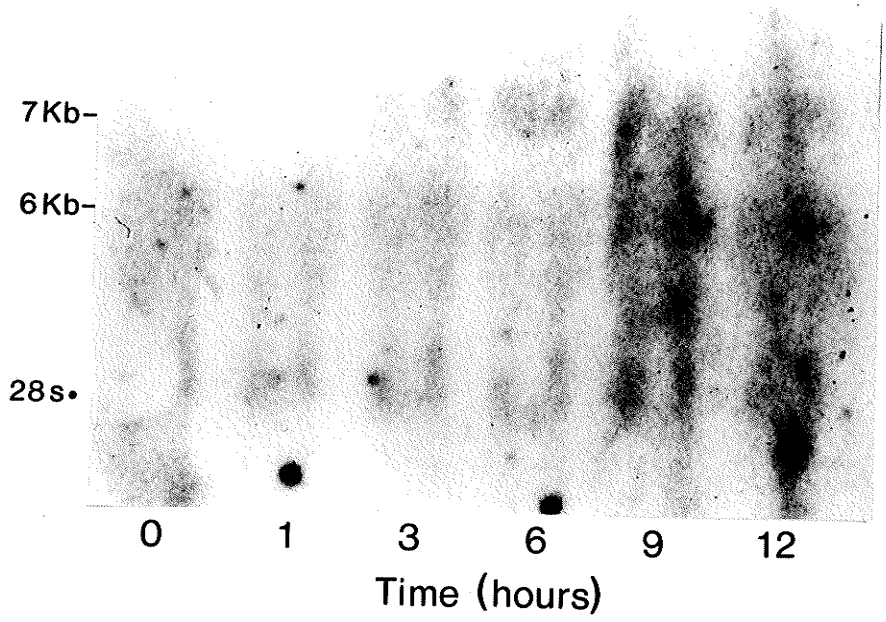
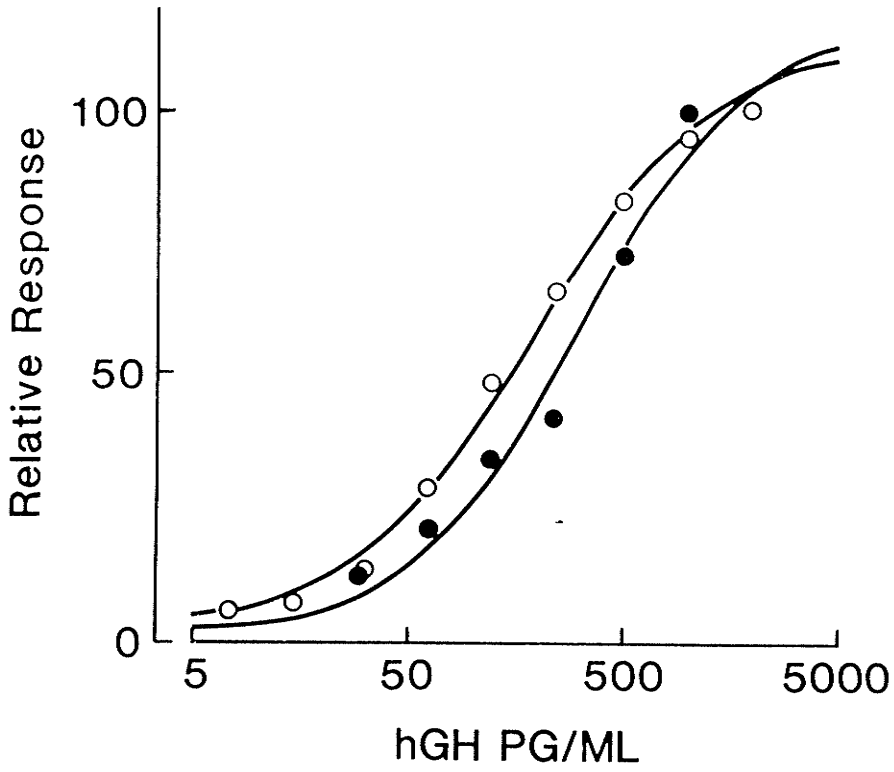

7Kb-
6Kb-
28s•
0
1
3
6
9
12
Time (hours)

Figure 11. DOSE RESPONSE FOR Nb2 CELL GROWTH AND C-MYC LEVELS.

Relative growth response (open circles) and relative c-myc accumulation (closed circles) are indicated. The growth response was measured after culturing Nb2 cells for 3 days as described previously. The c-myc accumulation was determined 3 hours after treatment with hGH. The EC50 for the growth response is 193 ± 41 (SEM). The EC50 for the c-myc response is 299 ± 52 (SEM).


Relative Response
100
50
0
5
50
500
5000
hGH PG/ML

In order to assess the effects of variable exposure times to hGH on cell proliferation, Nb2 cells were cultured for 1 to 4 hours in the presence of a maximally stimulating concentration of hGH and then washed and resuspended in medium containing 10% horse serum. As shown in Figure 12, this brief exposure to hGH was sufficient to elevate c-myc levels from 10 to 25 fold above control levels. After 40 hours of culture no significant cell proliferation was detected in those samples which were exposed to hGH for periods of between 1 and 4 hours. Those cells cultured in the continuous presence of hGH doubled during this time (Figure 13).

In order to examine the effect of hGH in maintaining c-myc levels in Nb2 cells, the pattern of c-myc accumulation following the removal of hGH from cultures was studied. As indicated in Figure 14, the levels of c-myc slowly decline after reaching their peak levels at about 3 hours. During the next 8 hours of culture there is a 3 fold decrease in c-myc abundance as the Nb2 cells proceed into S phase of the cell cycle. In contrast, removal of hGH from the culture system results in a much more rapid decline of c-myc transcript levels. At each time point examined the level of c-myc was 5-fold lower in those cultures in which the hGH had been removed than in those cultures maintained in the presence of hGH. This removal of hGH resulted in a 20-fold decrease in c-myc levels after 8 hours.

Figure 12. LEVELS OF C-MYC EXPRESSION AFTER hGH STIMULATION.

Duration of hGH treatment: Numbers on X axis indicate time in hours following hGH treatment. The relative abundance of c-myc transcripts was determined using densitometeric scanning. Aliquots of the cell cultures were taken and cell growth was determined (see Figure 13).

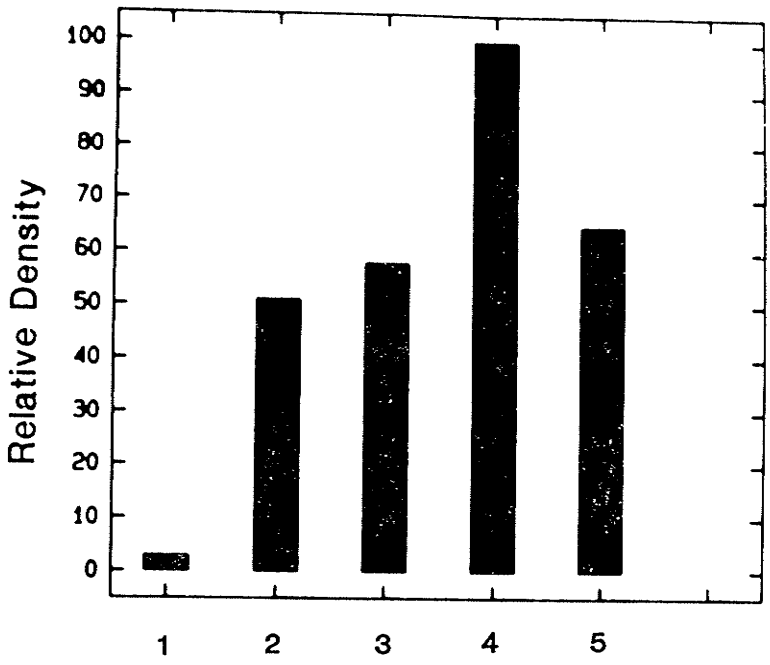

Relative Density
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5

Figure 13. Nb2 CELL PROLIFERATION AFTER hGH REMOVAL.

The duration of hGH treatment: A, 0 hr; B, 1 hr; C, 2 hr; D, 3 hr; E, 4 hr; F, Nb2 cells were washed and resuspended in medium containing hGH. Cell number was determined 40 hr after the initial hGH treatment.

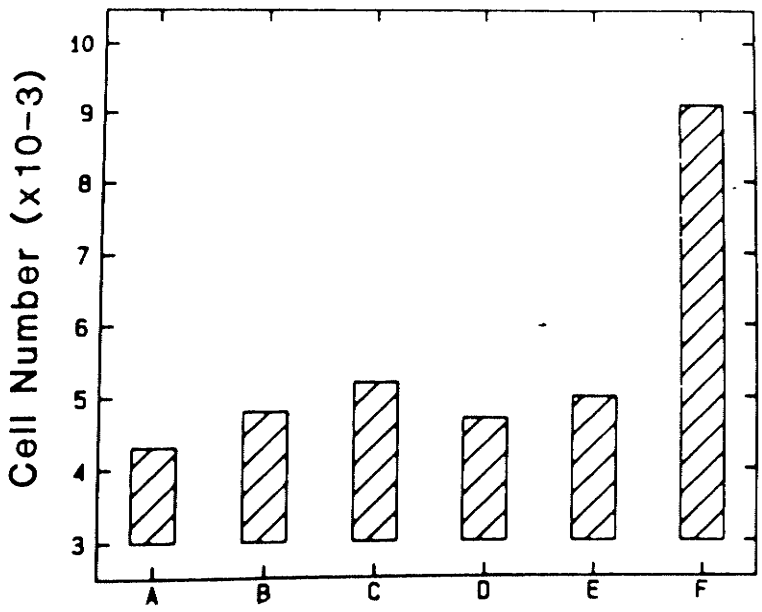
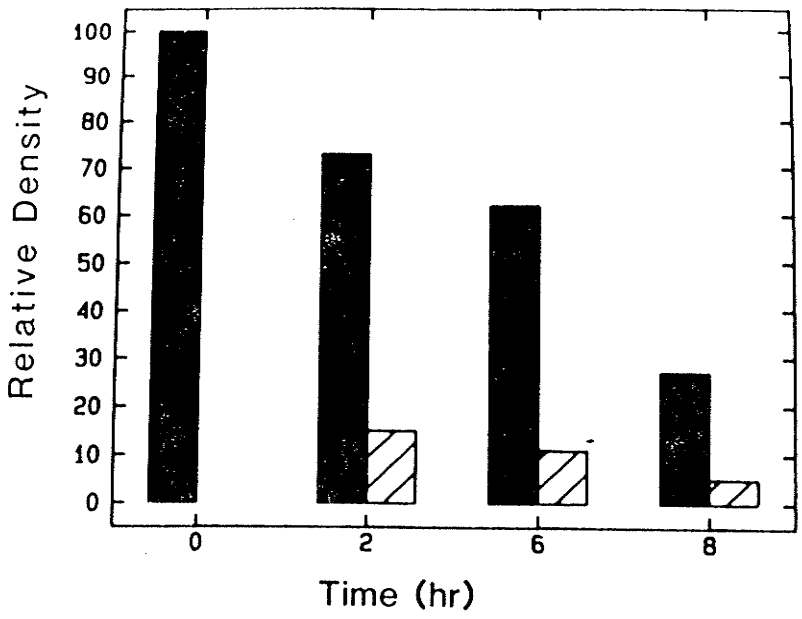

Cell Number (x10-3)
10
9
8
7
6
5
4
3
A
B
C
D
E
F

Figure 14. ABUNDANCE OF C-MYC TRANSCRIPTS AFTER hGH REMOVAL.

Following 4 hr stimulation of Nb2 cells with hGH, an aliquot of cells was removed for RNA analysis (time = 0) while the remaining cells were pelleted then resuspended in fresh medium with (solid bars) or without (hatched bars) hGH. At the indicated times, aliquots of cells were removed and the RNA was processed for Northern gel analysis. The relative abundance of c-myc transcripts was determined by densitometeric scanning.


Relative Density
100
90
80
70
60
50
40
30
20
10
0
0
2
6
8
Time (hr)

The cell proliferation observed when lactogenic hormones are added to stationary cultures of Nb2 cells may result from a direct effect of the hormone or alternatively lactogens may merely permit the action of various mitogens in the system. To investigate this further we examined whether hGH could induce c-myc accumulation and cell proliferation in a defined medium.

As shown in Figure 15 the hGH induction of c-myc transcripts in both the defined medium and the horse serum supplemented medium are similar. Cell growth during the first 48 hr was also parallel (data not shown).

Co-incubation of Nb2 cells with antisera to hGH has previously been shown to inhibit the growth response of these cells (78). Figure 16 shows that the accumulation of c-myc transcripts 3 hr post hGH addition is markedly reduced when the cells are pretreated with anti-hGH. The small increase in transcript number which is observed was not associated with detectable cell proliferation.

Earlier studies characterizing the kinetics of prolactin stimulated Nb2 cells indicate that the phorbol ester TPA enhanced the proportion of Nb2 cells which proliferated following lactogen stimulation (81). As indicated in Figure 17, TPA itself did not induce the accumulation of c-myc transcripts above those observed in untreated cells.

Figure 15. INDUCTION OF C-MYC TRANSCRIPTS IN SERUM-FREE MEDIUM.

Panel A: Nb2 cells growth arrested in Fischer's medium supplemented with 10% horse serum were then treated with hGH for the time periods indicated. Panel B: Nb2 cells growth arrested in defined serum free medium and then treated for the time periods indicated. Each lane contains 10 ug of total Nb2 cell RNA.

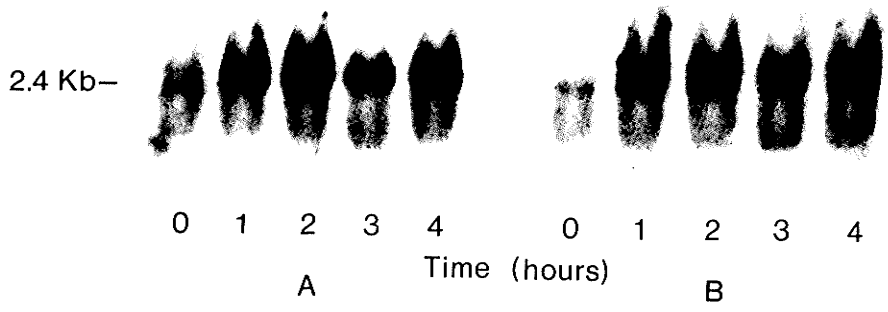

2.4 Kb–
0 1 2 3 4 0 1 2 3 4
Time (hours)
A
B

Figure 16. EFFECT OF ANTI-hGH ON C-MYC TRANSCRIPT ACCUMULATION.

Lane A: Nb2 cells 3 hr post hGH treatment. Lane B: Nb2 cells co-incubated with anti-hGH and hGH for 3 hr. Lane C: Nb2 cells treated with vehicle alone for 3 hr. Each lane contains 10 ug of total RNA.

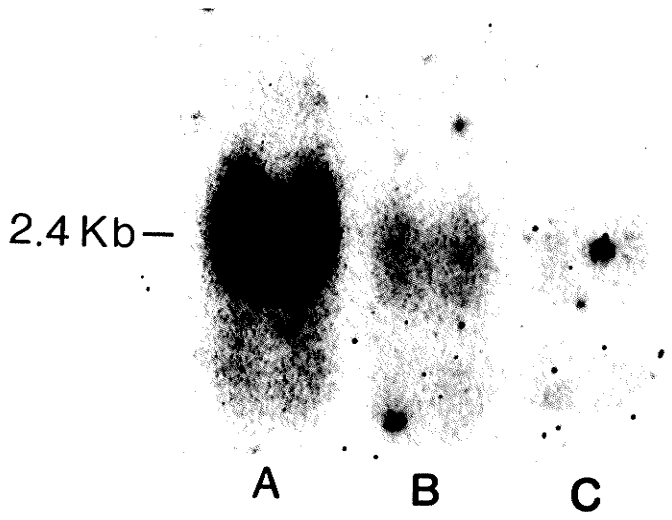
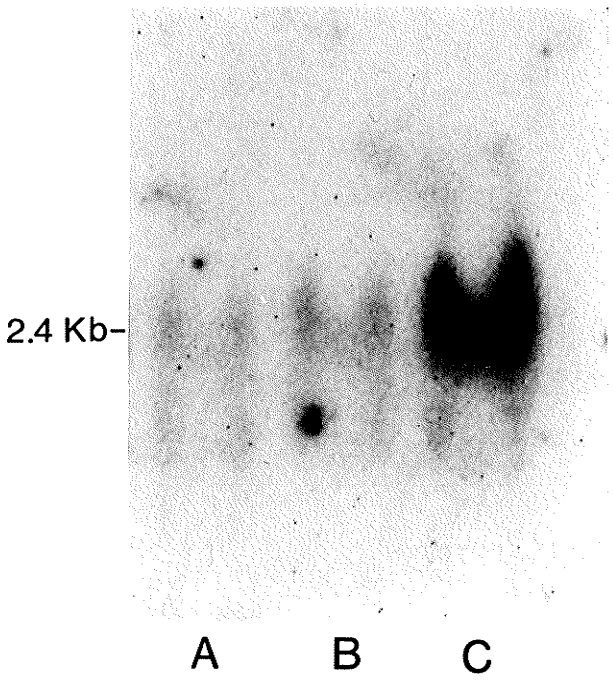

2.4 Kb
A
B
C

Figure 17. EFFECT OF TPA ON C-MYC TRANSCRIPT ACCUMULATION.

Lane A; vehicle treatment 3 hr. Lane B; TPA 20 nM for 3 hr. Lane C; hGH treatment for 3 hr. Each lane contains 10 ug of Total RNA.


2.4 Kb
A
B
C

In view of the preceding results which indicate that Nb2 cells have cytochemical markers associated with thymic lymphocytes, investigations were undertaken to determine whether lactogenic hormones could produce an *in vivo* induction of c-myc RNA transcripts in hypophysectomized (hypox) rats.

Following treatment of hypox rats with hGH for variable time periods, tissues were removed, quickly frozen then prepared for Northern blot analysis. As shown in Figure 18, treatment of hypox rats for up to 12 hours with hGH did not result in a detectable increase in the already abundant c-myc transcripts in the thymus. The relatively high levels of c-myc transcripts in the thymus may in fact obscure a significant increase in c-myc transcripts within a small sub-population of thymocytes. In contrast, a large increase in the abundance of c- myc transcripts was observed in the hypox rat liver within 1 hour of hGH treatment (139). These elevated levels persisted until 3 hours post hormone treatment and then declined to baseline by 6 hours post treatment. This induction of c-myc transcripts in rat liver was found to be secondary to the binding of hGH to the growth hormone receptor. Other lactogenic hormones such as oPRL did not produce this effect.

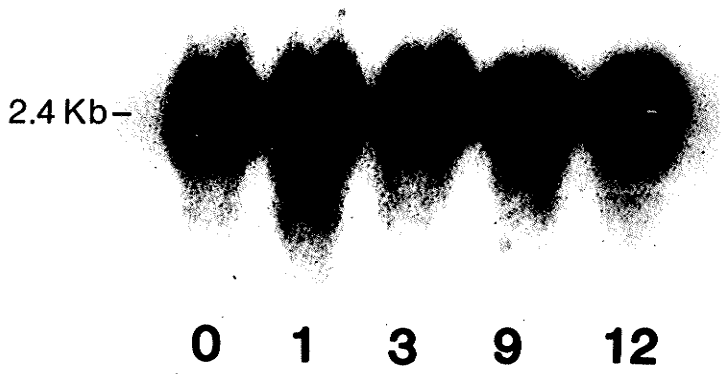

2.4 Kb–
0 1 3 9 12
Time (hours)

Figure 18. EXPRESSION OF C-MYC IN THE HYPOX RAT THYMUS.

Hypox rats were treated with ip injections of hGH for the time periods indicated. Each lane contains 10 ug of thymus gland total RNA. Ribosomal RNA was used as size markers.

EXPRESSION OF THE C-MYC ONCOGENE IN HUMAN PROSTATES

The expression of the c-myc gene has been associated with a large variety of human cancers. We have examined the expression of c-myc in both prostatic carcinoma and benign prostatic hyperplasia in order to determine whether the level of expression of this gene can help to distinguish between benign and malignant lesions or in predicting the presence of clinically aggressive disease.

The presence of c-myc transcripts in various prostate samples is shown in Figure 19. The level of c-myc expression in poly (A)+ RNA isolated from a total of 20 patients was quantitated by densitometeric analysis of Northern gels. One patient was assigned a level of 100 units and all other samples were expressed relative to this value (Figure 20). The levels of c-myc in 7 patients with carcinoma of the prostate (54 $\pm$ 40 SD) were significantly higher than that observed in 11 patients with BPH (26 $\pm$ 19) ($p<.05$). The normal prostate of 2 males aged 22 and 67 years demonstrated levels well below those observed in either the BPH samples or the adenocarcinoma samples. Subsequent hybridization of these samples with a probe for β-actin indicates that those samples with low c-myc levels contained intact RNA.

Levels of c-myc transcripts did not correlate with the mass of tissue removed (Table 4). The levels of c-myc in 2

patients over the age of 80 year were less than 35% of the mean value for the BPH group while the youngest patient in the group (58 yr) had c-myc levels more than 3 fold higher than the mean.

The levels of prostatic acid phosphatase at the time of diagnosis of prostate carcinoma did not correlate with c-myc levels (Table 5). Two patients (1 and 2) had high c-myc levels normal levels of acid phosphatase and no evidence of metastatic disease . Six months following diagnosis, patient 2 developed clinically detectable systemic disease with bone metastasis. Two other patients (3 and 5) presented with Stage D clinical disease the former having moderately elevated levels of c-myc while the latter showed a level consistent with BPH.

Histological evaluation of the adenocarcinoma samples indicated that the 2 patients with high levels of c-myc expression were M.D. Anderson grades 1 and 3 (Table 5). Patients (6 and 7) with grade 2 carcinoma and no evidence of metastatic disease had levels of c-myc comparable to those observed in patients with BPH.

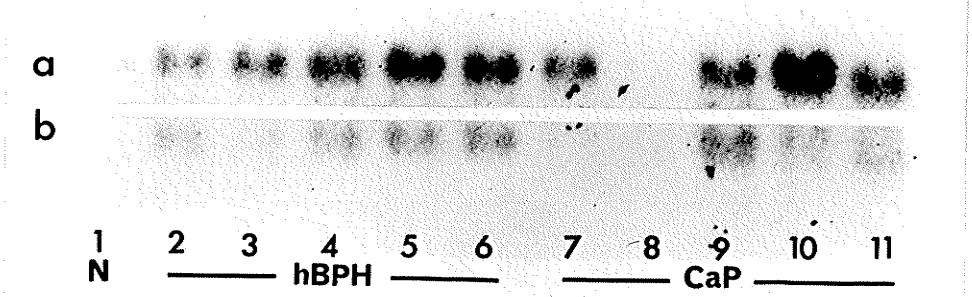

a
b
1
N
2
3
4
hBPH
5
6
7
8
9
CaP
10
11

Figure 19. EXPRESSION OF C-MYC IN PROSTATE TISSUE.

Lane 1: normal; Lanes 2-6: BPH; Lanes 7-11: prostate carcinoma. Each lane contains 6 ug of poly (A)+ RNA. Panel A: Northern blot probed with the human c-myc probe pKW3. Panel B: The same blot hybridized with the β-actin probe.

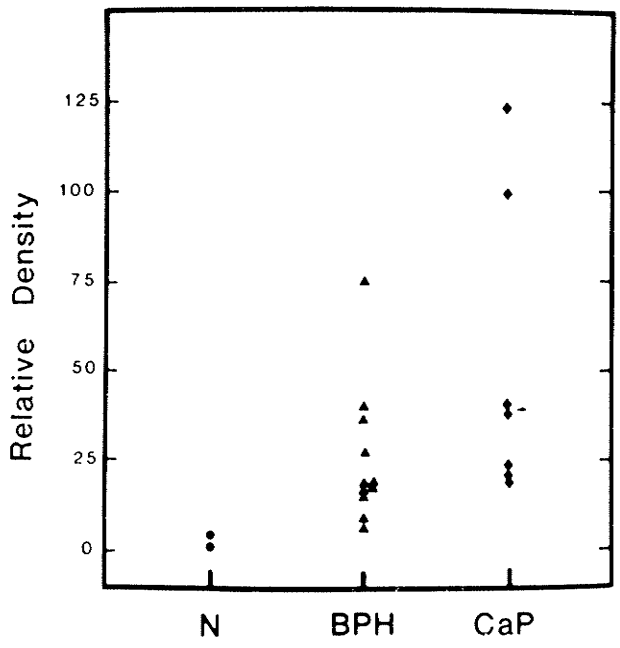

Relative Density
125
100
75
50
25
0
N
BPH
CaP

Figure 20. LEVELS OF C-MYC mRNA IN PROSTATE TISSUE.

Densitometeric scanning of the Northern gel analysis of prostate tissue from 20 patients expressed in relative units. The mean levels of c-myc in 11 cases of BPH is 26 $\pm$ 19 S.D. and in the 7 cases of carcinoma of the prostate (CaP) is 54 $\pm$ 40, ($p < .05.$); N, is normal prostate.

c-myc expression in BPH

Age of patient (yr)	Prostate wt (g)	c-*myc* expression
85	85	9.1
81	90	7.1
79	80	36.2
78	85	18.3
78	105	39.0
74	85	27.0
73	73	16.0
58	60	75.0

Table 4. C-MYC EXPRESSION IN BPH.

Three BPH samples were not included because the mass of tissue removed was not known. The value of c-myc expression is in relative units.

c-myc mRNA levels in prostatic carcinoma

Patient	Histological diagnosis			Prostatic acid phosphatase (μ/ml)	c-*myc* expression
	M. D. Anderson	Gleason	% tissue involved		
1	1	2/2	45	0.36	124.0
2	3	3/4	90	0.40	100.0
3[a]	2	2/3	100	0.60	41.0
4	3	5/3	100	0.60	38.0
5[a]	3	3/4	60	2.80	27.1
6	2	3/2	50	0.52	25.3
7	2	2/3	70	0.26	24.9

[a] Patients who presented with Stage D clinical disease.

Table 5. C-MYC LEVELS IN PROSTATIC CARCINOMA.

Histological evaluation was performed by 2 independent reviewers. The levels of prostatic acid phosphatase were determined at diagnosis using a Dupont Automatic Clinical Analyzer.

DISCUSSION

The absence of undetectable surface immunoglobulin indicates that Nb2 cells are not likely of B-lymphocyte origin. As well, the absence of cytoplasmic vacuoles and undetectable levels of the lysosomal enzyme muramidase suggests that this tumor did not originate from cells of the monocyte series.

The large solitary red nodule which characterizes the presence of α-naphthyl acetate esterase in mature T-lymphocytes was not present in Nb2 cells. Instead a faint, irregular reaction product previously described in normal thymocytes (93) was observed (Thy-like pattern). This pattern is thought to correlate with the early expression of this T-cell enzyme in a subpopulation of normal rat thymocytes (65%). This pattern of staining also is seen in a subset (1.8%) of rat bone marrow cells but is absent from cells isolated from both rat lymph nodes and spleen. While the function of the DNA polymerase enzyme TdT in lymphoid cells has not been clearly established, it is thought to play a role in the early growth and maturation of these cells. The absence of detectable TdT activity in Nb2 cells may indicate that these cells have completed an early phase of thymic development.

The mouse monoclonal antibody W3/25-HLK recognizes a subset of peripheral T-lymphocytes which mediate the helper

graft vs host reaction and the mixed lymphocytic response (102). The mouse monoclonal antibody OX8-HL will bind to all peripheral T-cells that are W3/25-HLK negative and which are responsible for functions such as the suppression of induction of antibody forming B-cells (103). Together these antibodies will bind to more than 90% of peripheral T-cells in the normal rat. No subset of peripheral T-cells that binds both antibodies has been identified.

All Nb2 cells examined from both stationary and log phase cultures bound both W3/25-HLK and OX8-HL. The intensity of fluorescence of both antibodies was greatest in stationary cultures. This is consistent with the observation that the volume of log phase growing Nb2 cells is twice that of stationary cells (data not shown). An increase in surface area without a corresponding increase in surface marker receptors would result in a decreased fluorescence intensity.

Thymocyte differentiation is often accompanied by a loss of membrane marker proteins. For example, murine thymocytes express the Thy 1.1 antigen while mature peripheral T-lymphocytes do not (117). The selective expression of either W3/25-HLK or OX8-HL antigens in peripheral T-cells is likely linked to the specialized function of these two cell types. The observation that both of these antibodies produce a much more intense pattern of fluorescence in their respective target cells in the normal

rat spleen is consistent with this idea.

The property of guinea pig erythrocyte rosette formation has been shown to be restricted to thymocytes in the rat (92). The increase in the number of rosette forming cells in the thymus as the rat matures (from birth to 4 weeks) suggests that a certain level of differentiation may be required before this rosette forming capacity is acquired. While we were able to demonstrate this rosette forming capacity in normal rat thymocytes, it was found that Nb2 cells did not exhibit this property.

In summary, these data suggest that the Nb2 lymphoma cell line may have arisen from a thymocyte at an intermediate stage of differentiation. While not clearly defined, this stage is likely to have followed an earlier stage of differentiation correlating with detectable TdT activity. Differentiation of these cells has not yet reached the point where either the W3/25-HLKB or the OX8-HL antigen is expressed exclusively. Both the absence of a T-cell α-naphthyl acetate esterase staining pattern and the presence of a Thy-like pattern confirm the thymic origin and the relative immaturity of these lymphoid cells.

The phenomena of lactogen regulated growth in Nb2 cells raises several questions as to the nature of the hormonal dependence of these cells. If in fact these cells are "frozen" at a particular stage of thymocyte development, the presence of functional lactogenic receptors suggests

the possibility that these hormones may be involved in normal thymocyte maturation. Alternatively, the lactogen dependence observed in these cells may be the result of malignant transformation and/or subsequent selection processes. Further investigation of the role of lactogenic hormones in thymocyte maturation and in the growth of leukemias and lymphomas may reveal a subset of lymphoid tumors whose growth is lactogen dependent.

As an increase in cell volume was observed to occur within a few hours following lactogen stimulation of Nb2 cells, studies were undertaken to examine whether significant morphologic changes accompanied the change in cell size. A relatively smooth surface characterizes quiescent Nb2 cells. The addition of physiological concentrations of oPRL produced numerous projections and pseudopodia-like structures within 70 minutes. By 2 hr post hormone addition the Nb2 cells highly ruffled appearance was similar to that of activated lymphocytes. At this time, this represented the earliest detected cellular response to a lactogenic hormone and indicated that a variety of biochemical changes were likely to precede these structural changes.

Recent studies have suggested a significant role for growth factor mediated oncogene expression in the proliferation of both normal and neoplastic cells. Increased levels of cellular oncogene expression in rapidly

growing embryonic tissues and the elevated levels of oncogene products in malignant vs benign tumors (118), suggest that these genes subserve vital cellular processes. The addition of mitogens to cultured cells results in the accumulation of oncogene transcripts, most notably that of c- myc (101). Conversely, both the depletion of growth factors and the treatment of tumor cells with differentiating agents results in a significant decline in the level of these gene products (41).

To further investigate early events involved in the response of Nb2 cells to mitogens, an asynchronous population of log phase growing cells was utilized. Of the dozen most common oncogenes recently identified, a total of five were found to be expressed. All three members of the ras family were detected as either a single 6Kb band on Northern gel analysis as in the case of H-ras and N-ras, while a doublet at 6Kb was detected with the K-ras probe. These transcripts are considerably larger than the 1.8 Kb transcript previously described in most cell types. Hematopoietic cells have previously been reported to contain this larger 6Kb transcript (41) and although the functional significance of the additional sequence remains obscure, this larger mRNA has been demonstrated to translate into the ubiquitous p21 ras protein (119). The two distinct 6Kb and 7Kb transcripts detected with the c-abl probe are similar to those reported previously. As well, the c-myc

probe hybridized to the familiar 2.4Kb transcript.

Expression of the c-myb gene has been described in a variety of hematopoietic cells with the highest levels occurring in cortical thymocytes. The level of c-myb transcripts were found to decline 10-fold as the immature cortical thymocytes matured and migrated to the lymph nodes (120). The absence of detectable c-myb transcripts in Nb2 cells is consistent with the previously described cytochemical data which suggest that these cells arose from a thymocyte at an intermediate or later stage of differentiation.

The erb B oncogene which is associated with the development of erythroleukemia in chickens has recently been shown to be identical to a truncated subunit of the epidermal growth factor (EGF) receptor (56). Transcripts of erb B were not detected in Nb2 cells and it is of interest to note that EGF is not an active growth factor for Nb2 cells (78).

The absence of c-sis transcripts in Nb2 cells is consistent with the observation that to date this gene product has only been detected in the circulating cells of chronic myelogenous leukemia in the accelerated phase (121). Similarly, the c-fms gene which encodes the CSF-1 receptor on macrophage precursors (62) is not detected in Nb2 cells. The absence of transcripts of c-src, c-fes and c-mos in Nb2 cells is consistent with the observation that

these genes have not been reported to be expressed in other hematopoietic cells.

When growth arrested Nb2 cells were stimulated with hGH, an increase in c-myc transcripts was detectable within 15 minutes, reached a maximum of 25-fold within 3 hours and then gradually declined as the cells proceeded through the G1 phase of the cell cycle. This pattern of expression is similar to that described following the addition of a variety of mitogens to both T and B lymphocytes (101). The pattern of induction of β-actin differs from c-myc in that it increases to a maximum of 10 fold within the first hour and then remains relatively constant. This result indicates that the increase in the c-myc signal after hGH stimulation is not simply due to variable amounts of mRNA being loaded on the gel nor due to a non-specific increase in the abundance of mRNA species in general.

The large 6Kb transcript associated with the hybridization of the various ras probes in Nb2 cells was shown to increase in late G1 of the cell cycle (between 6 and 9 hours post hGH addition). An increase in the more familiar 1.8 Kb ras transcripts has been demonstrated in late G1 phase fibroblasts and in regenerating liver (122). This phenomena has not yet been reported in hematopoietic cells in which the 6Kb transcripts have been described. As the 6 Kb and the 1.8 Kb transcripts have both been shown to translate into the p21 ras protein it is likely that the

major functional role of both mRNAs is conserved. While not yet experimentally demonstrated the functional significance of these gene products may involve modulation of either the S phase or the G2 phase of the cell cycle. The increased accumulation of c-abl transcripts follows a time course identical to that observed for the ras transcripts. This remains a novel finding to date, as the only study which discusses the level of c-abl transcripts in association with mitogen stimulated cells reports that this gene is not induced (123).

The demonstration of an increased accumulation of c-oncogene products in response to the mitogen treatment of a particular cell line does not demonstrate a causal relationship between c-oncogene expression and cellular proliferation. In an attempt to investigate the relationship between these events, an number of experiments were performed. The dose dependent growth of Nb2 cells observed following the treatment of growth arrested cells with increasing concentrations of a lactogenic hormone has previously been described (78). The observation that the accumulation of c-myc transcripts occurs in an identical dose dependent pattern indicates that the increase in c-myc transcripts closely parallels the growth response. Other studies have demonstrated that the rate of c-myc transcription correlates with the rate of cell division in a variety of tissues and that c-myc expression is higher in

fetal tissues than in adult tissues (42).

The experiments involving limited exposure of Nb2 cells to hGH indicate that while a 4 hour exposure was sufficient to maximally induce the c-myc transcripts, removal of the hormone at this time resulted in the failure of these cells to proliferate. This removal of hGH also was associated with a 5-fold reduction in the abundance of c-myc transcripts. In most cell proliferation model systems studied to date only a brief exposure to mitogens is required to render the cells competent to progress through the cell cycle (125). This induction of competence is thought to occur early and corresponds to the rapid increase in c-myc transcripts which then gradually decline as the cells progress through G1 of the cell cycle. These data have been interpreted to mean that c- myc is a member of a cell cycle regulated competence gene family which is expressed following PDGF stimulation of 3T3 cells (126). More recently, the level of both c-myc mRNA and protein have been shown to be constant throughout the cell cycle (127, 128). The initial induction of c-myc transcription following mitogen stimulation has now been attributed to the G0/G1 transition period.

Mitogen stimulated 3T3 cells require a 20 minute exposure to PDGF in order to be rendered competent to proceed through the cell cycle in the presence of progression factors such as insulin-like growth factor

(129). In contrast, Nb2 cells require prolonged exposure to hGH to proliferate . This requirement for the continued presence of a mitogen has previously been described in cultured hematopoietic cells (130).

In the Nb2 cell system hGH may act as both a competence factor and a progression factor. Alternatively, hGH may have a permissive effect, allowing other serum factors to induce proliferation. In either case it is evident that hGH is necessary for the rapid early induction of c-myc transcripts and for maintaining the level of these transcripts at later stages of the cell cycle. Although c-myc levels appear to closely correlate with the proliferative capacity of Nb2 cells, the peak of c-myc expression is not in itself sufficient to allow these cells to proliferate. A similar observation has recently been made by a group of investigators studying the growth of human B-lymphocytes in culture (131). They demonstrate that B-cell growth factor (BCGF) stimulated B-cells will proliferate only if they have been previously activated by either interaction with T-cells or by crosslinking of surface bound immunoglobulins (132). While this early activation of B-cells will result in the rapid accumulation of c-myc transcripts, these cells will not enter the S phase in the absence of BCGF. These results confirm our observation that c-myc transcript induction is not in itself sufficient for cell proliferation.

To investigate whether the presence of hGH was the major mitogenic stimulus to proliferation of the Nb2 cells these cells were cultured in a defined medium. The induction of c-myc transcripts in both serum supplemented and defined medium was identical as was the growth of these cells (data not shown) over the first 48 hours. The defined medium used in these experiments contained transferrin which has been shown to be a requirement for the growth of a variety of cell types. This is particularly relevant in the case of mature T-cells which are known to express transferrin receptors (133) following exposure to the growth factor interleukin-2 (IL-2). The subsequent binding of transferrin to its receptor is required to allow the cells to enter S phase of the cell cycle. Recent studies indicate that the Nb2 cells can be stimulated to proliferate in the presence of IL- 2 (83, 84). This observation supports previous experiments which suggest that Nb2 cells arose from a thymocyte at an intermediate stage of differentiation. The malignant transformation of a T-lymphocyte precursor has, in the case of Nb2 cells, been accompanied by the normal phenotypic state of IL-2 induced / transferrin dependent cell proliferation.

Previous studies have implicated a role for lactogenic hormones in the cell mediated immune response (134). A dramatic increase in thymus weight has been reported when both normal and hypophysectomized (hypox) rats have been

treated with oPRL. In view of these observations, studies were undertaken to determine if the treatment of hypox rats with hGH could produce an in vivo induction of c-myc transcripts. The absence of such a response may be due to a relatively small subpopulation of thymocytes responding to hGH, and any increase in myc transcripts then being undetectable against a high background level of c-myc expression. Alternatively, the growth promoting effects of hGH in thymocytes may have a latency period greater than that examined in our study. As well, the possibility that hGH induced thymocyte proliferation does not involve a G0 to G1 phase transition must also be considered.

EXPRESSION OF C-MYC ONCOGENE IN PROSTATIC TUMORS

The expression of the c-myc gene has been associated with a variety of human tumors (135). A number of mechanisms have been suggested to explain the transforming capacity of these genes and their associated high levels of expression in malignant tumors. Changes in the structural gene resulting in amino acid substitutions (9) or the deletion of exons (66) have been described. Increased levels of the mRNA or the protein coded for by the oncogene have also been proposed as important mechanisms (69-71). While these preliminary investigations suggest an aberrant pattern of oncogene expression to be associated with malignant transformation, studies on the diagnostic and

prognostic value of differential oncogene expression only recently have been undertaken.

The experiments described demonstrate the expression of c-myc transcripts in both benign and malignant prostatic tumors. Interpretation of the significance of c-myc expression was originally thought to be complicated by the fact that a large percentage of patients with benign prostatic hyperplasia are known to have concomitant prostatitis. An infiltrate of lymphocytes which are known to contain 20-fold higher levels of c-myc transcripts than resting fibroblasts may significantly increase c-myc levels (101). Histological evaluation of the BPH samples examined in this study indicate only a mild, diffuse prostatitis in all samples.

A recent review of the development of BPH indicates that the amount of hyperplastic tissue removed at surgery increases with advancing age (136). Calculation of the doubling time of these tumors shows a progressive increase with advancing age, ranging from a doubling time of 4.5 years in men between the ages of 31 and 50 years to a doubling time of 100 years in men over 70 years of age. In this study both subjects over 80 years of age had very low levels of c-myc transcripts. In contrast the youngest patient examined had levels of c-myc expression more than 3- times the mean value for the BPH group. These results are consistent with the hypothesis that c-myc levels are

proportional to the growth fraction of a particular tumor.

Two patients in this study with carcinoma of the prostate had levels of c-myc which were 2-fold higher than the mean value for the carcinoma group and 5-fold higher than the average for the BPH group. While neither of these patients demonstrated systemic disease at the time of diagnosis one patient (#2) has since developed bone metastasis following a 6 month interval. This is consistent with the outcome predicted by histological evaluation which indicated both a high Gleason and M D Anderson score. A similar correlation of histological diagnosis and oncogene expression recently has been reported in prostate carcinoma (137). This study showed a strong correlation between the amount of immunoreactive H-ras protein in prostate carcinoma cells and the histological grade of these tumors. A recent study of c-myc expression in carcinoma of the cervix also confirms the elevation of c-myc mRNA levels in those tumors which are clinically more agressive (138).

One major difficulty in evaluating the significance of elevated levels of c-myc transcripts is the variable infiltration of the prostate gland by tumor cells (Table 5). Additional studies using DNA probes for <u>in situ</u> hybridization and immunochemical techniques to quantitate the levels of the c-myc would permit direct evaluation of c-myc expression in individual tumor cells.

While histological grading correlates well with

survival in large populations of patients, difficulties arise in predicting the outcome in individual patients as significant mortality occurs among some of those patients assigned to favorable prognostic groups. The further evaluation of c-oncogene expression in these tumors may yield a new class of biologically and clinically significant tumor markers.

The following represents novel findings of this study:

1) The Nb2 lymphoma cells have those immunocytochemical characteristics associated with thymocytes at an intermediate stage of differentiation.

2) Lactogen stimulated mitogenesis in Nb2 cells is associated with the accumulation of the mRNA's of c-myc, c-abl and the c-ras gene family.

3) The early induction of c-myc is in itself not sufficient to induce cell proliferation.

4) The maintenance of c-myc transcript levels by the continuous presence of hGH is associated with cell proliferation.

5) Although there is great heterogenity, the level of c-myc transcripts is significantly higher in prostate carcinoma than in benign prostatic hyperplasia.

REFERENCES

1. Rous, P. A sarcoma of the fowl transmissible by an agent separable from the tumor cell. J. Exp. Med. 13: 397-410, 1911.
2. Bishop, J.M. Viral oncogenes. Cell 42: 23-36, 1985.
3. Baltimore, D. Viral RNA-dependent DNA polymerase. Nature 226: 1209-1211, 1970.
4. Martin, G.S. Rous sarcoma virus: a function required for the maintenance of the transformed state. Nature 227: 1021-1023, 1970.
5. Hanafusa, H. Cell transformation by RNA tumor virus. In Comprehensive Virology, Fraenkel Conrat H., Wagner R.R. eds., Plenum Press, New York, 1977, Vol. 9, p.341-355.
6. Czernilofsky, A.P., Levinson, A.D., Varmus, H.E., Bishop, J.M. Nucleotide sequence of an avian sarcoma virus oncogene (src) and proposed amino acid sequence for gene product. Nature 287: 198-203, 1980.
7. Shih, C., Shilo, B.-Z., Goldfarb, M.P., Dannenberg, A., Weinberg, R.A. Passage of phenotypes of chemically transformed cells via transfection of DNA and chromatin. Proc. Natl. Acad. Sci. USA 76: 5714-5718, 1979.
8. Krontiris, T.G., Cooper, G.M. Transforming activity of human tumour DNAs. Proc. Natl. Acad. Sci. USA 78: 1181-1184.

9. Premkumar, E., Reynolds, R.K., Santos, E., Barbacid, M. A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature 300: 149-152, 1982.

10. Tabin, C.J., Bradley, S.M., Bargmann, C.I., Weinberg, R.A. Mechanism of activation of a human oncogene. Nature 300: 143-149, 1982.

11. Huebner, R.J., Todaro, G.J. Oncogenes of RNA tumor viruses as determinants of cancer. Viral Carcinogenesis Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, September 16, 1969.

12. Bishop, J.M. Cellular oncogenes and retroviruses. Ann. Rev. Biochem. 52: 301-354, 1983.

13. Gallwitz, D., Donath, C., Sander, C. A yeast gene encoding a protein homologous to the human c-has/bas proto-oncogene product. Nature 306: 704-706, 1983.

14. Hughes, S.H., Payvar, F., Spector, D., Schimke, R.T., Robinson, H.L., Payne, G.S., Bishop, J.M., Varmus, H.E. Heterogeneity of genetic loci in chickens: analysis of endogenous viral and nonviral genes by cleavage of DNA with restriction enzymes. Cell 18: 347-359, 1979.

15. Goff, S.P., Gilboa, E., Witte, O.N., Baltimore, D. Structure of the Abelson murine leukemia virus genome and the homologous cellular gene: studies with cloned viral DNA. Cell 22: 777-785, 1980.

16. Van Beveren, C., van Straaten, F., Galleshaw, J.A., Verma, I.M. Nucleotide sequence of the genome of a murine sarcoma virus. Cell 27: 97-108, 1981.

17. Vennstrom, B., Bishop, J.M. Isolation and characterization of chicken DNA homologous to the two putative oncogenes of avian erythroblastosis virus. Cell 28: 135-143, 1982.

18. Kelly, K., Siebenlist, U. The role of c-myc in the proliferation of normal and neoplastic cells. J. Clinical Immunology 5: 65-77, 1985.

19. Nau, M.M., Brooks, B.J., Battey, J., Sausville, E., Gazdar, A.F., Kirsch, I.R., McBride, O.W., Bertness, V., Hollis, G.F., Minna, J.D. L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. Nature 318: 69-73, 1985.

20. Nau, M.M., Brooks, B.J., Battey, J., Sausville, E., Gazdar, A.F., Kirsch, I.R., McBride, O.W., Bertness, V., Hollis, G.F., Minna, J.D. L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. Nature 318: 69-73, 1985.

21. Vennstrom, B., Sheiness, D., Zabielski, J., Bishop, J.M. Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29. J. Virology 42: 773-779, 1982.

22. Favera, R.D., Gelmann, E.P., Martinotti, S., Franchini,

G., Papas, T.S., Gallo, R.C., Wong-Staal, F. Cloning and characterization of different human sequences related to the onc gene (v-myc) of avian myelocytomatosis virus (MC29). Proc. Natl. Acad. Sci. USA 79: 6497-6501, 1982.

23. Parker, R.C., Varmus, H.E., Bishop, J.M. Cellular homologue (c-src) of the transforming gene of Rous sarcoma virus: isolation, mapping, and transcriptional analysis of c-src and flanking regions. Proc. Natl. Acad. Sci. USA 78: 5842-5846, 1981.

24. Shealy, D.J., Erikson,R.L. Human cells contain two forms of $pp60^{c-src}$. Nature 293: 666-668, 1981.

25. Shih, T.Y., Weeks, M.O. Oncogenes and cancer: the p21 ras genes. Cancer Investigation 2(2): 109-123, 1984.

26. Defeo, D., Gonda, M.A., Young, H.A., Chang, E.H., Lowry, D.R., Scolnick, E.M., Ellis, R.W. Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. Proc. Natl. Acad. Sci. USA 78: 3328-3332, 1981.

27. Chang, E.H., Gonda, M.A., Ellis, R.W., Scolnick, E.M., Lowry, D.R. Human genome contains four genes homologous to transforming genes of Harvey and Kirsten murine sarcoma viruses. Proc. Natl. Acad. Sci. USA 79: 4848-4852, 1982.

28. Chattopadhyay, S.K., Chang, E.H., Lander, M.R., Ellis, R.W., Scolnick, E.M., Lowry, D.R. Amplification and

rearrangement of onc genes in mammalian species. Nature 296: 361-362, 1982.

29. Dhar, R., Ellis, R.W., Shish, T.Y., Oroszlan, S., Shapiro, B., Maizel, J., Lowy, D., Scolnick, E. Nucleotide Sequence of the p21 transforming protein of Harvey Murine Sarcoma Virus. Science 217: 934-936, 1982.

30. Tsuchida, N., Ryder, T., Ohtsubo, E. Nucleotide sequence of the onocogen encoding the p21 transforming protein of Kirsten murine sarcoma virus. Science 217: 937-938, 1982.

31. Levinson, A.D., Courtneidge, S.A., Bishop, J.M. Structural and functional domains of the Rous sarcoma virus transforming protein ($pp60^{src}$). Proc. Natl. Acad. Sci. USA 78: 1624-1628, 1981.

32. Barker, W.C., Dayhoff, M.O. Viral src gene products are related to the catalytic chain of mammalian cAMP-dependent kinase. Proc. Natl. Acad. Sci. USA 79: 2836-2839, 1982.

33. Doolittle, R.F. Similar amino acid sequences: chance or common ancestry? Science 214: 149-159, 1981.

34. Courtneidge, S.A., Levinson, A.D., Bishop, J.M. The protein encoded by the transforming gene of avian sarcoma virus ($pp60^{src}$) and a homologous protein in normal cells ($pp60^{proto\text{-}src}$) are associated with the plasma membrane. Proc. Natl. Acad. Sci. USA 77: 3783-

3787, 1980.

35. Rohrschneider, L.R. Adhesion plaques of Rous sarcoma virus-transformed cells contain the src gene product. Proc. Natl. Acad. Sci. USA 77: 3514-3518, 1980.

36. Oppermann, H., Levinson, W., Bishop, J.M. A cellular protein that associates with the transforming protein of Rous sarcoma virus is also a heat-shock protein. Proc. Natl. Acad. Sci. USA 78: 1067-1071, 1981.

37. Gilmore, T.D., Radke, K., Martin, G.S. Tyrosine phosphorylation of a 50K cellular polypeptide associated with Rous sarcoma virus transforming protein $pp60^{src}$. Mol. Cell. Biol. 2(2): 199-206, 1982.

38. Radke, K., Gilmore, T., Martin, G.S. Transformation by Rous sarcoma virus: a cellular substrate for transformation-specific protein phosphorylation contains phosphotyrosine. Cell 21: 821-828, 1980.

39. Snyder, M.A, Bishop, J.M., Colby, W.W., Levinson, A.D. Phosphorylation of Tyrosine-416 is not required for the transforming properties and kinase activity of $pp60^{v-src}$. Cell 32: 891-901, 1983.

40. Hunter, T., Sefton, B.M. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA 77: 1311-1315, 1980.

41. Westin, E.H., Wong-Stall, F., Gelmann, E.P., Dalla Favera, R., Papas, T.S., Lautenberger, J.A., Eva, A., Reddy, E.P., Tronick, S.R., Aaronson, S.A., Gallo, R.C.

Expression of cellular homologues of retroviral onc genes in human hematopoietic cells. Proc. Natl. Acad. Sci. USA 79: 2490-2494, 1982.

42. Muller, R., Slamon, D.J., Tremblay, J.M., Cline, M.J., Verma, I.M. Differential expression of cellular oncogenes during pre- and postnatal development of the mouse. Nature 299: 640-644, 1982.

43. Scolnick, E.M., Papageorge, A.G., Shih, T.Y. Guanine nucleotide-binding activity as an assay for the src protein of rat-derived murine sarcoma viruses. Proc. Natl. Acad. Sci. USA 76: 5355-5359, 1979.

44. Shih, T.Y., Papageorge, A.G., Stokes, P.E., Weeks, M.O., Scolnick, E.M. Guanine nucleotide-binding and autophosphorylation activities associated with $p21^{src}$ protein of Harvey murine sarcoma virus. Nature (London) 287: 686-691, 1980.

45. Tabin, C.J., Bradley, S.M., Baargmann, C.I., Weinberg, R.A., Papageorge, A.G., Scolnick, E.M., Dhar, R., Lowry, D.R., Chang, E.H. Mechanism of action of a human oncogene. Nature (London) 300: 143-149, 1982.

46. Yuasa, Y., Scrivastava, S.K., Dunn, C.Y., Rhim, J.S., Reddy, E.P., Aaronson, S.A. Acquisition of transforming properties by alternative point mutations within c-bas/has human proto-oncogene. Nature (London) 303: 775-779, 1983.

47. DeFeo-Jones, D., Tatchell, K., Robinson, L.C., Sigal,

I.S., Vass, W.C., Lowy, D.R., Scolnick, E.M. Mammalian and yeast ras gene products: biological function in their heterologous systems. Science 228: 179-184, 1985.

48. Powers, S., Kataoka, T., Fasano, O., Goldfarb, M., Strathern, J., Broach, J., Wigler, M. Genes in S. cerevisiae encoding proteins with domains homologous to the mammalian ras proteins. Cell 36: 607-612, 1984.

49. Tatchell, K., Chalfeff, D.T., DeFeo-Jones, D., Scolnick, E.M. Requirement of either of a pair of ras-related genes of saccharomyces cerevisiae for spore viability. Nature 309: 523-526, 1984.

50. Kataoka, T., Powers, S., Cameron, S., Fasano, O., Goldfarb, M., Broach, J., Wigler, M. Functional homology of mammalian and yeast ras genes. Cell 40: 19-26, 1985.

51. Temeles, G.L., Gibbs, J.B., D'Alonzo, J.S., Sigal, I.S., Scolnick, E.M. Yeast and mammalian ras proteins have conserved biochemical properties. Nature 313: 700-702, 1985.

52. Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Breck, D., Cameron, S., Broach, J., Matsumoto, K., Wigler, M. In yeast, RAS proteins are controlling elements of adenylate cyclase. Cell 40: 27-36, 1985.

53. Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C.,Aaronson, S.A., Antoniades,

H.N. Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221: 275-277, 1983.

54. Syms, A.J., Norris, J.S., Smith, R.G. Androgen stimulated elevation in androgen receptor levels is inhibited by the synthetic glucocorticoid triamcinolone acetonide. Biochem. Biophys. Res. Com. 116: 1020-1025, 1983.

55. Norris, J.S., Cornett, L.E., Hardin, J.W., Kohler, P.O., Macleod, S.L., Srivastava, A., Sym, A.J., Smith R.G. Autocrine regulation of growth: II. Glucocorticoids inhibit transcription of c-sis oncogene-specific RNA transcripts. Biochem. Biophys. Res. Com. 122: 124-128, 1984.

56. Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ulbrich, A., Schlessinger, J., Waterfield, M.D. Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature 307: 521-527, 1984.

57. Hollenberg, S.M., Weinberger, C., Ong, E.S., Cerelli, G., Oro, A., Lebo, R., Thompson, E.B., Rosenfeld, M.G., Evans, R.M. Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 318: 635-640, 1985.

58. Green, S., Walter, P., Kumar, V., Krust, A., Bornett, J.-M., Argos, P., Chambon, P. Human oestrogen

receptor cDNA: sequence, expression, and homology to v-erb-A. Nature 320: 134-139, 1986.

59. Weinberger, C., Thompson, C.C., Ong, E.S., Lebo, R., Gruol, D.J., Evans, R.M. The c-erb-A gene encodes a thyroid hormone receptor. Nature 324: 641-646, 1986.

60. McDonnell, D.P., Mangelsdorf, D.J., Pike, J.W., Haussler, M.R., O'Malley, B.W. Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. Science 235: 1214-1217, 1987.

61. Stanley, E.R., Guilbert, L.J. Regulation of macrophase production by a colony-stimulating factor. In Mononuclear Phagocytes - Functional Aspects, Part 1, R. van Furth, ed. (The Hague:Martinus Nijhoff), p. 417-433, 1980.

62. Sherr, C.J., Rettenmier, C.W., Sacca, R., Roussel, M.F., Look, A.T., Stanley, E.R. The c-fms proto-oncogene is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell 41: 665-676, 1985.

63. Rousel, M.F., Sherr, C.J., Barker, P.E., Ruddle, F.M. Molecular cloning of the c-fms locus and its assignment to human chromosome 5. J. Virol. 48: 770-773, 1983.

64. Wisniewski,L.P., Hirschhorn, K. Acquired partial deletions of the long arm of chromosome 5 in hematologic disorders. Am. J. Hemat. 15: 295-310, 1983.

65. Salmon, D.J., deKernion, J.B., Verma, I.M., Clin, M.J. Expression of cellular oncogenes in human malignancies. Science (Wash. DC) 224: 256-262, 1984.

66. Waldmann, T.A., Korsmeyer, S.J., Bakhshi, A., Arnold, A., Kirsch, I.R. Molecular genetic analysis of human lymphoid neoplasms. Annals of Internal Medicine 102: 497-510, 1985.

67. Taub, R., Kirsch, I., Morton, C. et al. Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc. Natl. Acad. Sci. USA 79: 7837-7841, 1982.

68. Rothberg, P.G., Erisman, M.D., Diehl, R.E., Rovigatti, U.G., Astrin, S. Structure and expression of the oncogene c-myc in fresh tumor material from patients with hematopoietic malignancies. Mol. Cell. Biol. 4: 1096-1103, 1984.

69. Shibuya, M., Yokota, J., Ueyama, Y. Amplification and expression of a cellular oncogene (c-myc) in human gastric carcinoma. Mol. Cell. Biol. 5: 414-418, 1985.

70. Alitalo, K., Schwab, M., Lin, C.C., Varmus, H.E., Bishop, J.M. Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-myc) in colon carcinoma. Proc. Natl. Acad. Sci. USA 80: 1707-1711, 1983.

71. Kozbor, D., Croce, C.M. Amplification of the c-myc oncogene in one of five human breast carcinoma cell

lines. Cancer Res. 44: 438-441, 1984.

72. Little, C.D., Nau, M.M., Carney, D.N., Gazdar, A.F., Minna, J.D. Amplification and expression of the c-myc oncogene in human lung cancer cell lines. Nature (London) 306: 194-196, 1983.

73. Thor, A., Horan Hand, P., Wunderlich, D., Caruso, A., Muraro, R., Scholom J. Monoclonal antibodies define differential ras gene expression in malignant and benign colonic disease. Nature (London) 311: 562-565, 1984.

74. de Klein, A., Van Kessel, A.G., Grosveld, G., Bartman, C.R., Hagemeijer, A., Bootsma, D., Spurr, N.K., Heisterkamp, N., Groffen, J., Stephenson, J.R. A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. Nature 300: 765-767, 1982.

75. Bartram, C.R., de Klein, A., Hagemeijer, A., van Agthoven, T., van Kessel, A.G., Bootsma, D., Grosveld, G., Ferguson-Smith, M.A., Davies, T., Stone, M., Heisterkamp, N., Stephenson, J.R., Groffen, J. Translocation of c-abl oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. Nature: 277,280, 1983.

76. Noble, R.L., Beer, C.T., Gout, P.W. Evidence in vivo and in vitro of a role for the pituitary in the growth of malignant lymphomas in Nb_2 rats. Cancer Res. 40:

2437-2440, 1980.

77. Gout, P.W., Beer, C.T., Noble, R.L. Prolactin-stimulated growth of cell cultures established from malignant Nb rat lymphomas. Cancer Res. 40: 2433-2436, 1980.

78. Tanaka, T., Shiu, R.P.C., Gout, P.W., Beer, C.T., Noble, R.L., Friesen, H.G. A new sensitive and specific bioassay for lactogenic hormones: measurement of prolactin and growth hormone in human serum. J. Clin. Endocrin. Metab. 51: 1058-1063, 1980.

79. Shiu, R.P.C., Elsholtz, H.P., Tanaka, T., Friesen, H.G., Gout, P.W., Beer, C.T. Noble, R.L., Receptor-Mediated mitogenic action of prolactin in a rat lymphoma cell line. Endocrinology 113: 159-165, 1983.

80. Rowe, R.C., Cowden, E.A., Faiman, C., Friesen, H.G. Correlation of Nb_2 bioassay and radioimmunoassay values for human serum prolactin. J. Clin. Endocrin. Metab. 57: 942-947, 1983.

81. Gertler, A., Walker, A., Friesen, H.G. Enhancement of human growth hormone-stimulated mitogenesis of Nb_2 node lymphoma cells by 12-0-Tetradecanoyl-Phorbol-13-Acetate. Endocrinology 116: 1636-1640, 1985.

82. McNeilly, A.S., Friesen, H.G. Presence of a nonlactogenic factor in human serum which synergistically enhances prolactin-stimulated growth of Nb_2 rat lymphoma cells in vitro. J. Clin. Endocrin.

Metab. 61: 408-411, 1985.

83. Rayhel, E.J., Albright, J.F., Shenk, B.A., Hughes, J.P. Stimulation of Nb_2 cell growth by interleukin-2 and human growth hormone. Program of the 68th Annual Meeting of the Endocrine Society, Anaheim, CA, p. 218 (Abstract), 1986.

84. Walker, A., and Friesen, H.G. Stimulation of growth of Nb_2 lymphoma cells in serum-free medium by human interleukin-2. (Submitted).

85. Richards, J.F., Beer, C.T., Bourgeault, C., Chen, K., Gout, P.W. Biochemical response of lymphoma cells to mitogenic stimulation by prolactin. Mol. Cell. Endocrinol. 26: 41, 1982.

86. Elsholtz, H.P., Shiu, R.P.C., Friesen, H.G. Role of ornithine decarboxylase in proliferation of prolactin-dependent lymphoma cells. Biochem. Cell Biol. 64: 381-386, 1985.

87. Elsholtz, H.P. Prolactin action and mechanism of action in Nb_2 lymphoma cells. Ph.D. Thesis, University of Manitoba, Winnipeg, Canada, 1984.

88. Moolenaar, H.W., Boonstra, J., van der Saag, P.T., de Laat, S.W. Sodium/proton exchange in mouse neuroblastoma cells. J. Biol. Chem 256: 12883-12888, 1981.

89. Moolenaar, W.H. Effects of growth factors on intracellular pH regulation. Ann. Rev. Physiol. 48:

363-367, 1986.

90. Too, C.K.L., Cragoe, E.J., Friesen, H.G. Amiloride sensitive Na^+/H^+ exchange in rat Nb_2 node lymphoma cells: II. Stimulation by prolactin and other mitogens. (Submitted).

91. Too, C.K.L., Walker, A., Murphy, P.R., Cragoe, E.J., Jacobs, H.K., Friesen, H.G. Identification of amiloride-sensitive Na^+/H^+ exchange in rat Nb_2 node lymphoma cells: I. Stimulation by 12-0-tetradecanoyl-phorbol-13-acetate. (Submitted).

92. Johnson, G.D., Dorling, J. Immunofluorescence and immunoperoxidase techniques. In: R.A. Thompson (ed.), Techniques in Clinical Immunology, pp. 85-116. Oxford: Blackwell Scientific Publications, 1979.

93. Kulenkampff, J., Janossy, G., Greaves, M.F. Acid esterase in human lymphoid cells and leukemic blasts: a marker for T lymphocytes. Br. J. Haematol. 36: 231-240, 1977.

94. Takeichi, N., Boone, C. Spontaneous rosette formation of rat thymus cells with guinea pig erythrocytes. Cell. Immunol. 27: 52-59, 1976.

95. Spurr, A.R. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26: 31-43, 1969.

96. Bruni, J.E., Clattenburg, R.E., Paterson, J.A. Ependymal cells of the rat fourth ventricle: response

to injury. Scanning Electron Microscopy, pp. 649-661, 1983.

97. Chirgwin, J.M., Przybyia, A.E., MacDonald, R.J., Rutter, W.J. Isolation of active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294-5298, 1979.

98. Aviv, H., Leder, P. Purification of biologically active globin messenger RNA by chromatography on oligo thymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA 69: 1408-1411, 1972.

99. Thomas, P.S., Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA 77: 520-525, 1980.

100. Alwine, J.C., Kemp, D.J., Parker, B.A., Reser, J., Renart, J., Stark, G.R., Wahl, G.M. Detection of specific RNA's or specific fragments of DNA by fractionation in gels and transfer to diazobenyl-oxymethyl paper. Meth. Enzymol. 68: 220-233, 1979.

101. Kelly, K., Cochran, B.H. Stiles, C.D., Leder P. Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet derived growth factor. Cell 35: 603-610, 1983.

102. Williams, A., Galfre, G., Milstein, C. Analysis of cell surface by xenogenic myeloma hybrid antibodies: differentiation antigens of rat lymphocytes. Cell 12: 663-673, 1977.

103. Brideau, R.J., Carter, P.B., McMaster, R.W., Mason, D.W., Williams, A.F. Two subsets of rat T lymphocytes defined with monoclonal antibodies. Eur. J. Immunol. 10: 609-615, 1980.

104. Srinivasan, A., Reddy, E.P., Aarson, S.A. Ableson murine leukemia virus: Molecular cloning of infectious integrated proviral DNA. Proc. Natl. Acad. Sci. USA 78: 2077-2081, 1981.

105. Dalla Favera, R., Gelmann, E.P., Martinotti, S., Franchini, G., Papas, T.S., Gallo, R.C., Wong-Staal, F. Cloning and characterization of different human sequences relatd to the onc gene (v-myc) of avian myelocytomatosis virus (MC29). Proc. Natl. Acad. Sci. USA 79: 6497-6501, 1982.

106. Shih, C., Weinberg, R.A. Isolation of a transforming sequence from a human bladder carcinoma. Cell 29: 161-169, 1982.

107. Tsuchida, N., Uesugi, S. Structure and functions of the Kirsten murine sarcoma virus genome: Molecular cloning of biologically active Kirsten murine sarcoma virus DNA. J. Virol. 38: 720-727, 1981.

108. Shimizu, K., Goldfarb, M., Perucho, M., Wigler, M. Isolation and preliminary characterization of the transforming gene of a human neuroblastoma cell line. Proc. Natl. Acad. Sci. USA 80: 383-387, 1983.

109. Klempnauer, K., Gonda, T.J., Bishop, J.M. Nucleotide

sequence of the retroviral leukemia gene v-myb and its cellular progenitor c-myb: The architecture of a transduced oncogene. Cell 31: 453-463, 1982.

110. Dalla Favera, R., Gelman, E.P., Gallo, R.C., Wong-Staal, F. A human onc gene homologous to the transforming gene (v-sis) of simian sarcoma virus. Nature (London) 292: 31-35, 1981.

111. Vennstrom, B., Fanshier, L., Moscovici, C., Bishop, J.M. Molecular cloning of the avian erythroblastosis virus genome and recovery of oncogenic virus by transfection of chicken cells. J. Virol. 36: 575-585, 1980.

112. DeLorbe, W.J., Luciw, P.A., Goodman, H.M., Varmus, H.E., Bishop, J.M. Molecular cloning and characterization of avian sarcoma virus circular DNA molecules. J. Virol. 36: 50-61, 1980.

113. Hampe, A., Gobet, M., Sherr, C.J., Galibert, F. Nucleotide sequence of the feline retroviral oncogene v-fms shows unexpected homology with oncogenes encoding tyrosine specific protein kinases. Proc. Natl. Acad. Sci. USA 81: 85-89, 1984.

114. Trus, M.D., Sodroski, J.G., Haseltine, W.A. Isolation and characterization of a human locus homologous to the transforming gene (v-fes) of feline sarcoma virus. J. Biol. Chem, 257: 2730-2733, 1982.

115. Blair, D.G., McClements, W.L., Oskarsson, M.K.,

Fishinger, P.J., Van de Woude, G.F. Biological activity of cloned Moloey sarcoma virus DNA: Terminally redundant sequences may enhance transformation efficiency. Proc. Natl. Acad. Sci. USA 77: 3504-3508, 1980.

116. Delean, A., Munson, P.J., Rodbard, D. Simultaneous analysis of families of sigmodial curves: application to bioassay, radioligand assay and physiological dose response curves. Am. J. Physiol. 235: E977-E102, 1978.

117. Acton, R.T., Morris, R.J., Williams, A.F. Estimation of the amount of tissue distribution of rat Thy 1.1 antigen. Eur. J. Immunol. 4: 598-602, 1974.

118. Thor, A., Horan Hand, P., Wunderlich, D., Caruso, A., Muraro, R., Schlom, J. Monoclonal antibodies define differential ras gene expression in malignant and benign colonic disease. Nature (London) 311: 562-565, 1984.

119. Ellis, R.W., DeFeo, D., Furth, M., Scolnick, E.M. Mouse cells contain two distinct ras gene mRNA species that can be translated into a p21 onc protein. Mol. Cell. Biol. 2: 1339-1345, 1982.

120. Sheiness, D., Gardinier, M. Expression of a proto-oncogene (proto-myb) in hemopoietic tissues of mice. Mol. Cell. Biol. 4: 1206-1212, 1984.

121. Romero, P., Blick, P., Talpaz, M., Murphy, E., Hester, J., Gutterman, J., C-sis and c-able expression in

chronic myelogenous leukemia and other hematologic malignancies. Blood 67: 839-841, 1986.

122. Goyette, M., Petropoulos, C.J., Shank, P.R., Fausto, N. Expression of a cellular oncogene during liver regeneration. Science 219: 510-512, 1983.

123. Greenberg, M.E., Ziff, E.B. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature (London) 311: 433-437, 1984.

124. Stewart, T.A., Bellve, A.R., Leder, P. Transcription and promoter usage of the myc gene in normal somatic and spermatogenic cells. Science 226: 707-709, 1984.

125. Stiles, C.D., Capone, G.T., Scher, C.D., Antoniades, H.N., Van Wyk, J.J., Pledger, W.J. Dual control of cell growth by somatomedins and platelet derived growth factor. Proc. Natl. Acad. Sci. USA

126. Armelin, H.A., Armelin, M.C.S., Kelly, K., Stewart, T., Leder, P., Cochran, B.H., Stiles, C.D. Functional role for c-myc in mitogenic response to platelet derived growth factor. Nature 310: 655, 1984.

127. Thompson, C.B., Challoner, P.B., Neiman, P.E., Groudine, M. Levels of the c-myc oncogene mRNA are invariant throughout the cell cycle. Nature (London) 314: 363-366, 1985.

128. Hann, S.R., Thompson, C.B., Eisenman, R.N. c-myc Oncogene protein synthesis is independent of the cell cycle in human and avian cells. Nature (London) 314:

366-369, 1985.

129. Leof, E.B., Wharton, W., Van Vyk, J.J., Pledger, W.J. Epidermal growth factor (EGF) and somatomedin C regulate G1 progression in competent Balb/c 3T3 cells. Expt. Cell Res. 141: 107, 1982.

130. Metcalf, D. The graulocyte-macrophage colony-stimulating factors. Science 229: 16-22, 1985.

131. Smeland, E., Godal, T., Ruud, E., Beiske, K., Funderud, S., Clark, E.A., Pfeifer-Ohlsson, S., Ohlsson, R. The specific induction of myc protooncogene expression in normal human B cells is not a sufficient event for acquistion of competence to proliferate. Proc. Natl. Acad. Sci. USA 82: 6255-6259, 1985.

132. Howard, M., Nakanishi, K., Paul, W.E. B cell growth and differentiation factors. Immunolog. Rev. 78: 185-210, 1984.

133. Neckers, L.M., Cossman, J. Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin 2. Proc. Natl. Acad. Sci. USA 80: 3494-3498, 1983.

134. Nagy, E., Berczi, I., Friesen, H.G. Regulation of immunity in rats by lactogenic and growth hormones. Acta Endocrinol. 102: 351-357, 1983.

135. Salmon, D.J., deKernion, J.B., Verma, I.M., Cline, M.J. Expression of cellular oncogenes in human malignancies.

Science (Wash., DC) 224: 256-262, 1984.

136. Berry, S.J., Coffey, D.S., Walsh, P.C., Ewing, L.L. The development of human benign prostatic hyperplasia with age. J. Urol. 132: 474-479, 1984.

137. Viola, M.V., Fromowitz, F., Oraves, S., Deb, S., Finkel, G., Lundy, J., Hand, P., Thor, A., Schlom, J. Expression of ras oncogene p21 prostate cancer. New Eng. J. Med. 314: 133-137, 1986.

138. Riou, G., Barrois, M., Le, M.G., George, M., Le Doussal, V., Haie, C. C-myc proto-oncogene expression and prognosis in early carcinoma of the uterine cervix. The Lancet : 761-763, 1987.

139. Murphy, L.J., Bell, G.I., Friesen, H.G. Growth hormone stimulates sequential induction of c-myc and insulin-like growth factor I expression in vivo. Endocrinology (In press) 1987.