STRUCTURAL FEATURES ASSOCIATED WITH THE EXPRESSION OF THE PROLACTIN-INDUCIBLE PROTEIN(PIP) GENE IN HUMAN BREAST CANCER

by

Cindy Marie Bryan

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ABSTRACT

The role of the pituitary hormone prolactin in human breast cancer is poorly understood. An effective model with which to study the effect of prolactin was developed when Shiu and Iwasiow(1985) isolated and characterized a prolactin-inducible protein(PIP) from the human breast cancer cell line T-47D. Subsequently the PIP gene has been isolated and studies regarding its regulation have been performed. PIP is a 11K secreted protein with two glycosolated forms of 14K and 16K. The PIP cDNA is 577 bps long and the PIP mRNA is 800 bps in length. Several other human breast cancer cell lines express lower amounts of PIP than T-47D in response to treatment with prolactin and androgens whereas others show no detectable PIP expression. Thus, studies were performed to determine whether structural features of the PIP gene were contributing to the observed variation in its expression. Methylation of cytosine residues as detected by restriction enzyme assays and 5-Azacytidine manipulation does not appear to act as a gene silencing mechanism. Likewise, histone acetylation(as studied through sodium butyrate treatment) is not sufficient to induce PIP expression in a non-expressing cell line. In addition, the PIP gene is not rearranged between cell lines. The PIP gene has recently been assigned to a highly polymorphic region of chromosome 7(Myal et al, 1988). Studies were performed to establish whether the PIP gene was also polymorphic. Analysis of 52 samples of genomic DNA derived from human breast cancer cell lines, and lymphocytes from breast cancer patients as well as healthy individuals using a wide range of restriction enzymes revealed that the PIP gene was not polymorphic. Thus methylation, histone acetylation, and rearrangement or polymorphism do not appear to be contributing to PIP expression. However, this system represents an important model with which to study the molecular mechanism of prolactin in human breast cancer.

LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
BC	Breast Cancer
bps	Base Pairs
BSA	Bovine Serum Albumin
C	Cytosine
CM	Complete Media
cDNA	Complementary Deoxyribonucleic Acid
DHT	Dihydroxytestosterone
DMBA	Dimethylbenz(*a*)anthracene
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
E2	Estradiol
EGF	Epidermal Growth Factor
ER	Estrogen Receptor
ETOH	Ethanol
F	Hydrocortisone
FCS	Fetal Calf Serum
5-AzaC	5-Azacytidine
5mC	5-Methylcytosine
5mCMP	5-Methylcytosine Monophosphate
G	Guanine
GCDFP-15	Gross Cystic Disease Fluid Protein-15
GH	Growth Hormone
HBC	Human Breast Cancer
HMG	High Mobility Group

IGF-1	Insulin-like Growth Factor 1
IGF-11	Insulin-like Growth Factor 11
K	Kilodalton
kb	kilobase
MDBP	Methylated DNA Binding Protein
ml	Millilitre
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
MW	Molecular Weight
NaBut	Sodium Butyrate
NH2	Amino
PBS	Phosphate-Buffered Saline
PDGF	Platelet-Derived Growth Factor
PIP	Prolactin-Inducible Protein
Poly(A)	Poly Adenylated
PRL	Prolactin
PTH	Parathyroid Hormone
RFLP	Restriction Fragment Length Polymorphism
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
RT	Room Temperature
SAM	S-Adenosyl-Methionine
T3	Tri-iodothyronine
TGF-alpha	Transforming Growth Factor-alpha
TGF-beta	Transforming Growth Factor-beta

tRNA	Transfer Ribonucleic Acid
ul	Microlitre
uM	Micromolar

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INTRODUCTION

PART 1

HORMONES AND BREAST CANCER

A wealth of epidemiologic and experimental information points to the involvement of estrogens and other reproductive hormones in human breast cancer(HBC) (Siiteri et al, 1986). However, despite the many endocrine studies carried out over the past 30 years, the precise role of these hormones remains to be defined. Whether stated or not, the basic premise underlying these studies has been the notion that some abnormality of hormone production, transport, metabolism or action may be causally related to the development of breast cancer(Siiteri et al, 1986). Recent advances in our knowledge of the biology of breast cancer(BC), such as the potential role oncogenes, suggest that while hormones may be extremely important signals for the transformation and/or stimulation of cancer cell growth, they do not act alone in this regard(Siiteri et al, 1986).

The concept that BC is an endocrine-related disease was first demonstrated by Beatson in 1896 when he obtained regression of advanced metastatic disease following oopherectomy(Moore et al, 1986). In the 90 years that followed, a massive amount of evidence was obtained from studies in laboratory animals, and from epidemological research that implicated the estrogenic hormones directly or indirectly in the etiology of BC (Moore et al, 1986).

A. BREAST CANCER RISK FACTORS AND HORMONES

Before reviewing the role of hormones in the etiology of BC, several indirect lines of evidence linking HBC risk factors to hormones exist which warrant review. Endocrinologic studies of varying design have been conducted to attempt to identify specific hormonal aberrations of etiological

importance(Thomas,1986). The risk of BC is 100 times greater in women than in men. In women, risk increases remarkably with age from a few years after her menarche to menopause, and then increases less strongly with age after cessation of normal ovarian function. The earlier in life that a woman begins menstration, the greater her risk(Thomas, 1986).

Multiparity and a young age of first full-term pregnancy have been thought to have a protective effect against BC. Risk is directly related to the age at which a woman has her first full term pregnancy, and nulliparous woman have about the same risk a women who delay their first pregnancy until their mid-30's. An early age of first birth is only protective if the pregnancy goes to term, and some studies have provided evidence to suggest that risk is actually increased if the first pregnancy is aborted. These risks appear to have a hormonal mechanism(Thomas, 1986). Other risk factors for BC which could also have a hormonal mechanism of action include a family history of the disease, high socioeconomic status(which is associated with a high fat, high protein diet), obesity, and benign breast disease(Thomas, 1986). Nutrition is also a factor as geographical variations in BC risk are highly correlated with certain dietary factors. Consumption of animal fat or protein and refined sugar is positively correlated to an increased risk of BC(Moore et al, 1986).

Daughters of BC patients have been reported to have raised levels of prolactin(PRL) and estradiol(E2) (Henderson et al, 1975). Kwa and Wang(1977) found that women with a family history had significantly raised levels of PRL in the evening. This result was extended by Levin and Malarkey(1981) who reported increased levels of PRL throughout 24 hours in daughters of patients. These authors also noted a partial resistance to PRL secretion in response to dopamine suppression(Moore et al, 1986).

More recently, studies of hormone receptors in mammary tissue have also

been conducted to investigate the etiology of BC(Thomas, 1986). Indeed, estrogen receptor(ER) status of breast tumors has come to play a major role in the clinical care of breast cancer patients. This will be discussed in the next section in a more detailed manner.

In summary, the major risk factors associated with breast cancer are age of first full term pregnancy, age of first menarche, age of menopause, a diet high in fats and protein, and a family history of the disease. Most, if not all of these factors appear to be associated with endocrine activity.

B. STEROIDS

Much evidence has accumulated implicating E2 in the pathogenesis of human breast cancer. Observations dating back nearly a century ago saw that some breast tumors will regress following ovariectomy; however by the time it has reached the stage of overt metastatic disease, it retains the hormone-dependant phenotype of the normal breast only 1/3 of the time(Lippman et al, 1986a). In addition, the frequency of breast cancer in women who have never had functional ovaries is only 1% that in women with intact ovaries(Dickson et al, 1987). The role which E2 plays remains an important issue and is under extensive study.

Approximately 2/3 of human breast tumors are ER positive, 1/3 of the tumors responding to treatment directed towards removal of 17β-estradiol from the circulation, such as ovarioectomy and/or its action by antiestrogenic compounds such as tamoxifen (Dickson and Lippman, 1986). Antiestrogens have a high affinity for the ER and the most likely explanation of antiestrogen action *in vivo* appears to be simple antagonism of the growth promoting effects of E2 as mediated by the ER(Dickson and Lippman, 1986).

Studies of E2 metabolism through plasma and urinary E2 analysis have

revealed little abnormality(James et al, 1986). However, much of the information available today regarding the role of E2 in breast cancer was obtained from studies using continuous HBC cell lines in culture. Direct proliferative changes in clonal breast cancer cell lines culture have been demonstrated using physiologic doses of E2(Lippman et al, 1986b). In particular, the HBC cell line MCF-7 displays many of the properties of breast tumors *in vivo*, showing similar responses to hormones(Braunsberg et al, 1986). Cultured MCF-7 and other HBC cell lines serve as a model for examining the response to E2(Braunsberg et al, 1986). MCF-7 cells respond to E2 stimulation *in vitro* by increased secretion of growth factors and proliferation *in vivo* by tumor formation in the nude mouse(Dickson et al, 1987). Other cell lines which show a proliferative response to E2 include ZR-75-1, T47-D and CAMA 1(Lippman et al, 1986b). Thus, E2 directly stimulates HBC cells and gene expression at the level of mRNA concentration. This results in increased activity of many enzymes involved in cell growth(Lippman et al, 1986b).

In addition to stimulating the growth of MCF7 breast cancer cells in culture, E2 appears to induce at least two other functions thought to be critical for tumor invasiveness(Dickson and Lippman, 1986). E2 induces the secretion of plasminogen activator and other enzymes capable of proteolytic digestion of basement membrane(Dickson and Lippman, 1986). This membrane is largely comprised of the proteins collagen, laminin and fibronectin and may be a barrier to tumor spread(Dickson and Lippman, 1986). In addition E2 increases laminin receptor levels on the cell surface in MCF-7 cells. This binding protein is thought to be critical in cancer cell migration through a laminin-containing basement membrane. Thus E2 induces several functions required for various aspects of tumor development(Dickson and Lippman,1986).

Estrogens can directly regulate the activities of enzymes involved in nucleic acid synthesis(Lippman et al, 1986b). By means of independant probes for *de novo* and salvage DNA synthesis, it has been demonstrated that the two pathways for pyrimidine synthesis are concurrently stimulated by E2 and inhibited by antiestrogens(Lippman et al, 1986b). Specific enzyme activators, including those of thymidine kinase, thymidylate synthetase, aspartate transcarbamylase, carbamyl phosphate synthetase, dihydroorotase uridine kinase, and dihydrofolate reductase, are all regulated by E2 (Lippman et al, 1986b). Using a cDNA probe for thymidine kinase mRNA activity, it has been shown that E2 regulates thymidine kinase mRNA activity, as would be predicted from enzyme studies(Lippman et al, 1986b). Thus E2 directly stimulates HBC cells and later gene expression at the level of mRNA concentration.

The exact mechanism through which estrogens exert their effects upon BC cells is linked to the induction of growth factors. Growth inhibitors such as antiestrogens act by two mechanisms: first, down regulation of stimulatory growth factors, and second, increased production and/or secretion of substances with growth inhibitory activity(Lippman et al, 1986b).

The role of other steroid hormones in HBC is not as well understood as that of E2. Urinary secretion of testosterone has been seen to be dramatically increased in BC patients with atypical endometrial patterns(Moore et al, 1986). Similar urinary patterns were found in women with a family history of BC, breast hyperplasia or BC itself(Moore et al, 1986). The role of progesterone in the etiology of BC is far from clear and measurement of pregnandiol in the urine or progesterone in the blood or saliva has not helped to resolve the issue(Moore et al, 1986). Glucocorticoid hormones have been shown to have an inhibitory effect upon

HBC cells in culture mediated through the glucocorticoid receptor(Lippman, 1981). Glucocorticoid receptors are found in about 50% of all human biopsies however they do not appear to have a significant association with biological responses to classical endocrine therapies(Lippman, 1981). Other groups have shown similar results, i.e. dexamethasone is inibitory in general and progesterone and testosterone are without effect.

About one third of HBCs contain androgen receptors and androgen dependance has been shown in at least one rodent breast cancer cell line(Smith and King, 1972). Several HBC cell lines are also known to possess the androgen receptor(Lippman, 1981). In addition some HBCs will improve with androgen therapy but the basis of this response is not understood. In all the role of androgens in BC is not yet clear.

Although retinoic acid is not a traditional steroid hormone, its receptor has recently been identified as belonging to the steroid hormone receptor gene family(Petkovich et al, 1987). The appearance of DMBA-induced tumors in rats can be substantially decreased by the administration of vitamin A derivitives to rats. Retinoic acid induces several effects on cell growth, depending on the cell lines studied. These include a slower growth rate and cell death following a period of increasing growth inhibition(Lippman, 1981). Physiological concentrations of retinoic acid will also decrease the growth of several HBC cell lines, including MCF-7 and T-47D(Petkovich et al, 1987).

Vitamin D receptors have been described in human breast cancer tumor samples and the MCF-7 cell line(Lippman, 1981). Because bone is a major site of human breast cancer metastasis vitamin D may have a functional role in this respect.

In summary, the role of steroids in breast cancer may involve tumor

initiation in some malignancies and induction of critical events in the malignant progression of these cancers once they develop(Dickson and Lippman, 1986).

C. GROWTH FACTORS

In hormone-dependent cancers, estrogens regulate the secretion of specific proteins that possess autocrine and paracrine growth regulatory activities with multiple actions including the initiation of growth of quiescent cells and further growth stimulation of invasive potential(Lippman et al, 1986a) . These proteins are also thought to induce the secretion of factors capable of causing reversible aquisition of the malignant phenotype in non-transformed cells(Lippman et al, 1986a). Taken collectively, these growth factors are responsible for E2-mediated tumor progression(Lippman et al, 1986a). Indeed the fact that important endogenous mediators of tumor progression are secreted polypeptide growth factors is clearly of interest to endocrinologists(Dickson and Lippman, 1986). Both the growth factors and their receptors represent new targets for anticancer therapies(Dickson and Lippman, 1986).

MCF-7 cells and other ER-containing cell lines respond to 17*B*-estradiol treatment *in vitro* with an increased growth rate and with secretion of growth factor activities such as transforming growth factor-alpha(TGF-alpha), TGF-*B*, insulin-like growth factor 1(IGF-1) and a platelet-derived growth factor(PDGF)-related polypeptide(Dickson et al, 1987). The fact that epidermal growth factor(EGF) and IGF-1 are growth promoting for MCF-7 and some other HBC cell lines has led to the belief that TGF-alpha and IGF-1 might be autocrine growth factors(Dickson et al, 1987). TGF-**B**, found in platelets and usually secreted by cultured cells in an inactive form, is growth inhibitory for receptor-containing HBC cell lines,

while PDGF is not known to be mitogenic for BC, PDGF and TGF-*B* may stimulate stromal proliferation around the tumor *in vivo*, acting as supportive autocrine hormones(Dickson et al, 1987).

The first growth factor studied in conditioned medium from HBC cell lines was IGF-1(Somatomedin C). MCF-7, ZR-75-1, MDA-MB-231 and Hs578T produce IGF-1 and respond to exogenously added authentic IGF-1 with enhanced proliferation. IGF-1 receptors are demonstratable of the surfaces of all breast cancer cell lines tested(Lippman et al, 1986a). Myal et al(1984) have shown both IGF-1 and IGF-11 to be stimulatory to HBC cells in culture.

Growth factors may be involved as endocrine stimuli in very early stages of tumor initiation to autocrine stimuli in E2-regulated fully malignant cancer cells. EGF, a close relative of TGF-alpha, which also shares a common receptor with TGF-alpha, may have a role in initiation as well as progression of mouse mammary tumors. In many HBC cell lines EGF functions as a mitogen(Lippman, 1981). EGF stimulates the growth of both MCF-7 and T-47D cells, whereas pituitary-dervied fibroblast growth factor(FGF) stimulated the growth of T47D but not that of MCF7 cells(Shiu, 1981). *In vivo*, removal of the submandibular glands, a major source of EGF, dramatically reduces the incidence of spontaneous tumor formation and reduced the rate of tumor growth(Dickson and Lippman, 1986). EGF is known to have tumor promotional, growth promoting and immunosuppressive effects(Dickson and Lippman, 1986). EGF receptors are present in HBC and probably mediate the effects of EGF and the autocrine effects of TGF-alpha, produced by BC cells(Murphy et al, 1986).

Taken together, growth factors are capable of promoting *in vivo* tumorigenesis of hormone dependent BC cells in nude mice, though not to the same extent as E2 treatment of the mice. Hormone independant BC cells,

whether spontaneously arising in culture or constructed by DNA mediated gene transfer, appear to secrete such growth factors in increased amounts. Such a system of autocrine and paracrine cell stimulation and inhibition obviously suggests multiple interventions which may eventually have clinical applicability in the control of neoplastic breast cancer(Lippman et al, 1986a).

In addition, a possible mechanism through which breast tumors may become hormone independant is through loss of hormonal regulation of growth factor production(Lippman et al, 1986a). The mechanism by which hormone-responsive breast cancer is converted to the hormone-independant phenotype is by increased, nonhormonally regulated secretion of a similar or identical collection of autocrine and paracrine growth factors(Lippman et al, 1986a).

D. PITUITARY HORMONES

That E2 is the predominant hormone involved in HBC growth regulation is a well-established fact. The possible physiological role of other hormones, prolactin(PRL) in particular, is not well understood. However, a substantial body of evidence has accumulated supporting a role for polypeptide hormones, especially PRL in the etiology of BC.

As early as 1951 it was shown that anterior pituitary hormones are critical, perhaps essential for the genesis of spontaneous murine mammary tumors, for hypophysectomized rodents do not develop tumors of the mammary gland(Moon et al, 1951). In addition, grafting of multiple pituitary isografts to a variety of strains of female mice was shown to markedly increase the incidence of spontaneous mammary tumors(Muhlbock and Boot, 1959). Following this, work by Welsch and colleagues in the 1960's demonstrated a pivotal role for PRL in the development of 7,12 dimethylbenz(*a*)anthracene(DMBA) induced tumors in the rat(Welsch, 1981).

Drug-induced suppression of prolactin release decreases the growth of established tumors(Welsch, 1981). Since then, the role of PRL in the induction and progression of mammary tumors in experimental animals has become well established(Biswas and Vonderhaar, 1987).

The mammary gland of the mouse has also been studied extensively as an animal model of the human breast in terms of normal or neoplastic growth function. A primary role for PRL in normal and neoplatic mammary gland growth in mice has been established(Nagasawa et al, 1986). While PRL is essential for normal mammary gland growth and function, on one hand, it is also a key hormone for mammary tumorigenesis on the other. The causes of these different types of PRL action are poorly understood but may be due to to interaction with other hormones(Nagasawa et al, 1986). PRL exerts its influence directly to the glands and indirectly through its luteotropic effects by stimulation of ovarian progesterone secretion(Nagasawa et al, 1986). Furthermore, in mice, the action of PRL, whether in promoting normal growth and function or enhancing the progression of neoplastic foci, depends upon the circulating levels of other mammotropic hormones as well as the level of PRL(Nagasawa et al, 1986).

While there is ample evidence that PRL has an important role in the development and growth of rodent mammary carcinomas, its relationship to HBC is less clear(Love and Rose, 1985). Assays of PRL in plasma from breast cancer patients produces equivical results, some investigators have found no abnormality while others have observed increased levels in some patients(Love and Rose 1985). However, in women with advanced stages of the disease high blood concentrations of PRL have been reported associated with unfavorable prognosis(Wang et al, 1986).

In an attempt to determine the hormonal basis for the protective effect

of a young age of first pregnancy on BC risk, PRL levels in parous women were measured and found to be lower than that of nulliparous(Moore et al, 1986). Blood PRL levels have also been found to be increased in obese and postmenopausal women(Moore et al, 1986). Kwa et al(1974) were the first to demonstrate elevated PRL levels in family members of women with breast cancer. Henderson et al (1975) and Wang et al (1986), have found elevated plasma PRL levels in the daughters of women with BC. First degree relatives of women with bilateral BC diagnosed by age 50 have a 9-fold increased risk of the disease(Anderson, 1974). PRL levels in women with benign breast disease has been unremarkable(Moore et al, 1986). Attempts to induce tumor regression by the use of PRL suppressing drugs such as bromocryptine has generated conflicting and inconclusive results(Shiu, 1981). Overall, if PRL is playing a role in HBC it appears to be doing so at physiological concentrations.

A key problem when studying the relationship between PRL levels and BC risk may be that blood levels are generally determined by radioimmunoassay, which may not necessarily provide a true reflection of biologically active PRL(Rose, 1986). However, defining the effect of PRL on HBC growth is of major importance, since hyperprolactinemia is frequently encountered in women, both under physiological conditions , such as pregnancy and oral contraceptive administration and in pathological states such as the presence of pituitary tumors(Manni et al, 1986).

PRL receptors have been detected in human breast tumor biopies in as much as 70% of samples, although no clinical response is seen when serum PRL levels in patients with the disease are suppressed(Biswas and Vonderhaar, 1987). In 1980 Kevjer-Anderson and Beuhring reported that pharmacologic concentrations of ovine and human PRL can affect the growth rate of

malignant human mammary epithelial cells. Malarkey et al(1983) have shown that physiological levels of human PRL and hGH(not ovine PRL) increases the population doubling time of primary breast tumor cultures. Similar results were obtained using benign human breast tissue by Welsch and McManus(1977).

Manni et al(1986) have recently reported that under the conditions of the soft agar clonogenic assay, the vast majority of HBCs are PRL sensitive. Modest stimulation of colony formation is observed when PRL is added at the physiological dose of 20 ng/ml. When the dose is increased to 200 ng/ml, a superior colony-stimulating effect is seen which is virtually identical to that observed with the administration of E2(Manni et al, 1986). Although this is clearly a pharmacological dose, it nevertheless corresponds to the observed circulating levels of PRL in common hyperprolactinemic states(Manni et al, 1986).

A substantial body of evidence supporting a role for PRL in HBC has been accumulated using HBC cell lines maintained in long-term culture. Two of the most popular HBC cell lines studied are MCF-7 and T-47D. In 1979 Shiu identified prolactin receptors in a number of human mammary tumor cell lines, including MCF-7 and T-47D, which are also ER positive(Henderson and Pike, 1981). Conflicting results exist concerning the mitogenic role of PRL in these cells. Shafie and Brooks(1977) and Shiu(1981) have reported that PRL is not mitogenic to MCF-7 cells. However, Biswas and Vonderhaar(1987) presented data which suggests that MCF-7 cells are stimulated to grow in the presence of hGH and hPRL when added to the culture media of cells in the presence of charcoal-stripped serum. In addition, MCF-7 and T-47D cells have been shown to be stimulated by PRL when grown as solid tumors in nude mice in the presence of E2(Biswas and Vonderhaar, 1987), although others have failed to observe this.

Other physiological effects of PRL on HBC cells have been documented. Treatment of T-47D cells with hPRL or hGH in the presence of hydrocortisone(F) results in a change in shape of the cells from flat and polygonal to round and refractile(Shui and Paterson, 1984). In addition, a reduction in adhesiveness to the plastic substrate and and an increased lipid synthesis was observed(Shiu and Paterson, 1984). Interestingly, physiological changes of this nature can generally be correlated with the *in vivo* changes that correspond to increasing invasiveness of tumors(Shiu and Paterson, 1984). Thus the role of PRL in the etiology of HBC is not simple, but appears to be part of a complex, multi-response system(Biswas and Vonderhaar, 1987).

E. INSULIN AND T3

Insulin is a critical hormone in the normal differentiation of the mammary gland and is required for normal lactogenesis in rodents(Lippman, 1981). Major derangements in glucose homeostasis induced by surgical or chemical interference with pancreatic function have been associated with altered tumor growth rates in several murine systems(Lippman et al, 1986b). In addition high concentrations of insulin have been shown to be mitogenic for both MCF-7(Barnes and Sato, 1979) and ZR-75-1(Allegra and Lippman, 1978) possibly by acting through receptors for IGFs.

The thyroid hormone T3 is growth stimulatory for T-47D and MCF-7(Shiu, 1981). However *in vivo* studies of thryoid function and breast cancer are inconclusive(Bulbrook et al, 1981).

F. BREAST CANCER CELL LINES IN RESEARCH

There are many advantages in using cancer cell lines to study the physiological effects of hormones on human breast cancer. First, the problems that stem from studying mammary neoplasm in other species are

eliminated. Second, one can control the experimental variables so that an observed effect of a hormone on a particular cell type can be attributed to that hormone alone and not other unknown factors that may be present. Lastly, it is now possible to develop drug and hormone-appropriate biochemical and genetic complementation techniques to gain new insight into the mechanism of hormone dependancy of breast cancer cells(Lippman, 1981).

There are also disadvantages in using tissue culture methods in studying the effects of hormones on HBC. The influence of hormones on HBC cells *in vivo* is a complex phenomenon involving the interplay of many trophic factors derived from many organs. Thus the conditions of *in vitro* culture may not reflect the physiological milieu *in vivo*. As such, problems may be encountered when studying the effects of various hormones or agents on HBC cells. Cell culture conditions may lead to selection of cells capable of growth in the abscence of factors omitted from the *in vivo* condition. In addition, culture conditions invariably select against slower growing cell populations(Lippman, 1981). Because of this, many populations of cells that may express hormonal responses of interest may be overgrown by hormone-independant cell populations(Lippman,1981). Because most breast cancer cell lines have been established from malignant effusions, they represent a subset of the original tumor population which has metastatic ability and can grow in a local environment unlike that of the original tumor(Lippman, 1981). Thus, when a cell culture is started the cells have already undergone natural selection and a number of theraputic treatments such as irradiation, cytotoxic drugs and other hormonal manipulations(Lippman, 1981). These factors may affect the response of the cells to the hormones used in experiments.

Other drawbacks associated to the use of continuous HBC cell lines to

study the effects of hormones include the possibility of viral or mycoplasmic contamination which can alter the expression of various phenotypic parameters(Lippman, 1981). In addition, the cloning of cells may result in selection of a subtype which doesn't possess the same responses as the primary tumor did. Thus one must bear in mind potential problems that may affect experimental results when working with human cells in culture. Another factor to be considered is that a cell line developed from a pleural effusion of a patient with cancer of the breast and grown *in vitro* under artificial conditions for more than 10 years may not resemble the cell populations of a patient's tumor(Biswas and Vonderhaar, 1987).

THE PROLACTIN-INDUCIBLE PROTEIN

In 1985 Shiu and Iwasiow reported the isolation and characterization of a glycosylated PRL-inducible protein(PIP) in the HBC cell line T-47D. This important discovery marked the first time that hormonal induction of specific proteins via gene regulation in human target cells was seen. The induction of PIP and its mRNA represents a useful model in which to study the mechanism of PRL action in target cells. In addition the study of PIP provides a good basis for studying the role of PRL in HBC.

Treatment of T-47D cells with hPRL and hydrocortisone results in an increased synthesis of PIP and an increased accumulation of its mRNA. Both hormones are necessary to illicit PIP expression. The three PIP proteins have MWs of 11,000, 14,000 and 16,000. The 14K and 16K species represent glycosylated forms of the 11K protein(Shiu et al, 1987). *In vitro* translation of poly A+ mRNA from hormone-treated T-47D cells followed by immunoprecipitation using the PIP antiserum from rabbit resulted in the detection of a 12.5K protein. The 12.5K form is the precursor of the other PIP proteins(Shiu and Iwasiow, 1985). Thus the 11K species is formed due to cleavage of the signal peptide from the 12.5K form. The subsequent glycosylation of the 11K form generates the 14K and 16K forms.

Following the isolation of PIP, its cDNA clone was isolated by screening with antibodies a lambda gt11 expression library prepared from PRL and hydrocortisone treated T-47D mRNA(Murphy et al, 1987a). Isolated PIP clones were subcloned into pBR322(Shiu et al, 1987). The longest clone contained the entire coding region of the PIP gene of 577 bps. This was confirmed using hybrid-select translation followed by immunoprecipitation(Murphy et al 1987a). Subsequent Northern analysis showed a single mRNA species of about 900 bps. Assuming 200 nucleotides compose

the poly(A) tail in the mature mRNA the PIP cDNA of 577 nucleotides is probably missing about 120 bps in the 5' untranslated region(Murphy et al, 1987a).

Studies regarding the regulation of PIP expression by Murphy et al(1987b) showed that treatment of T-47D cells with hPRL and hGH increases PIP mRNA levels approximately 4.6 fold while a more pronounced increased(12.4 fold) was seen when cells were treated with both hGH and hydrocortisone or the androgen dihydroxytestosterone(DHT). DHT is the most potent steroid in increasing PIP expression. Nuclear run-off experiments show that DHT alone can increase the transcription of the PIP gene by 3.7 times(Murphy et al, 1987b). Thus it is thought that PRL(or hGH) plays a role in the post-transcriptional regulation of the PIP message. The PIP gene was sequenced and one open reading frame of 146 amino acids sufficient to encode the precursor form of the secreted PIP protein is evident(Murphy et al, 1987b).

The expression of the PIP gene is variable between HBC cell lines as well as tissues. PIP mRNA is detectable and hormonally-inducible in other HBC cell lines including ZR-75-1, BT-474, T-47D clone 8, clone 11 and a T-47D mutant line 5 at considerably lower levels(Murphy et al, 1987a). All of these cell lines are positive for the PRL and E2 receptors. Two PRL receptor positive cell lines, MCF-7 and EFM-19 contain no detectable PIP mRNA. T47-D contains approximately 25,000 PRL receptors per cell, MCF-7 has about one third of that amount(Shiu, 1979). Two other breast cancer cells lines(ER negative), BT-20 and MDA-MD-231 as well as a "normal" human mammary epithelial cell line HBL-100 also do not express detectable levels of PIP mRNA. The levels of PRL receptor are lower in HBL-100 than in MCF-7.

Protein homology studies show that the amino acid sequnce of PIP is

similar to the sequence of a protein encoded by a gene expressed in the mouse submaxillary gland. Subsequent Western blot analysis has confirmed the presence of PIP in human saliva(Murphy et al, 1987a) and human sweat and tears(Shiu, unpublished).

Further studies on the localization of PIP mRNA shows that *in vivo* expression of the PIP gene occurs in 61% of HBC tumor biopsies as well as in cystic and proliferative breast disease specimens(Shiu et al, 1987). Carcinomas of the prostate, pituitary, bladder, colon, bowel, kidney, benign prostatic hyperplasia and demotafibrosarcoma do not express PIP(Shiu et al, 1987). An RIA developed to detect PIP in the blood of breast cancer patients shows about 35% of samples analyzed show levels greater than 3 ng/ml.

In 1986 Haagensen reported the isolation and identification of a cystic breast fluid protein he called GCDFP-15 which was subsequently discovered to be the PIP isolated from T-47D cells as reported by Shiu and Iwasiow in 1985.

The molecular weight and other physical parameters regarding PIP are identical as reported by both groups. Haagensen has also developed a RIA which has been used to detect levels of GCDFP-15 ranging from 10 ng/ml in cycstic breast disease fluid to up to 70,000 ng/ml in the plasma of several patients with metastatic breast carcinomas. Haagensen reports GCDFP-15 is detectable in plasma of women with no past history of breast disease with a median blood level of 19 ng/ml, which is higher than that reported by Shiu et al(1987). Although the presence of gross cystic disease incurs increased plasma levels of GCDFP-15, the overall level of the protein in the blood of women with fibroadenomas is close to the level in normal adult women.

The plasma level of GCDFP-15 is believed to function as a marker of breast apocrine metaplastic activity and may also reflect an increased risk

for breast carcinoma development(Haagensen, 1986). Preliminary studies by Haagsensen's group suggests that women with blood levels GCDFP-15 above 50 ng/ml have an increased risk of breast cancer. Interestingly, Haagensen has also speculated that the hormonal milieu which induces breast cystic disease may also be a promoter stimulus to breast carcinoma development. This speculation is interesting given what is known about the hormonal dependance of PIP(GCDFP-15).

Both Haagensen and Shiu have reported that about 40% of women with breast cancer possess detectable levels of PIP in their blood. However, as metastatic breast carcinoma progresses, an increasing percentage of patients develop elevated levels(Haagensen, 1986). Treatment of patients with fluoxymesterone, a synthetic androgen used for pallative treatment of metastatic breast carcinoma caused an increase in plasma levles of GCDFP-15 by 200%. Again, these clinical results are in accordance with what is known from *in vitro* studies on the androgen-dependance of PIP expression in T-47D cells(Murphy et al, 1987b).

Other features regarding PIP as shown by Haagensen includes its localization to the apocrine epithelia of tissue from axilla, perineum, eyelid, external auditory canal and the breast. PIP(GCDFP-15) has also been located in areas of apocrine metaplasia such as intraductal papilloma, adenoma, and gynescomasita. The bronchial submucosa, sweat glands in skin and the serous cells of the submandibular glands and accessory lacrimal glands also possess PIP, again in accordance with Shiu's results(Shiu et al, 1987). The localization of PIP to these diverse areas is not surprising for the apocrine glands are specialized sweat glands under the influence of sex hormones and most of these tissues are phylogentically-related. Normal tissue and maligant tumors from the bladder, colon, espohagus, kidney ,

liver, thymus, pancreas, skin, stomach, thyroid, tonque, and uterus show no immunoreactivity for PIP.

The combined information obtained through the study of PIP by both groups suggest that PIP has valuable potential as a clinical marker for breast disease. However further study is still necessary to determine the physiological function of PIP in normal and disease states.

PART 11

GENERAL GENE REGULATION

The series of events associated with regulated expression of eukaryotic genes involves: 1) initiation of transcription, transcript elongation, processing and transport of mRNA into the cytoplasm, 2) translation of mRNA into protein and 3) posttranslational modifications of the protein(Levine et al, 1986). Although regulation of expression can take place at one or more of these steps, current evidence suggests that a major level for eukaryotic gene regulation involves initiation of transcription(Levine et al, 1986). It is widely accepted that gene regulation results from an interplay between DNA and chromosomal proteins, nucleosomal proteins, nonhistone proteins, high mobility group proteins and specific regulator proteins(such as repressors and activators(Razin and Riggs, 1980)). Histone modification by acetylation may also be one of the control mechanisms. DNA modification by methylation probably is also an important part of this complex hierarchy of controls(Razin and Riggs, 1980).

PART 111

METHYLATION

A. INTRODUCTION

The methylated base 5-methylcytosine (5mC) was discovered in calf thymus DNA about 38 years ago(Razin and Riggs, 1980). Since then the occurence of this minor base has been demonstrated in a wide variety of organisms, including all vertebrates and plants that have been studied. In vertebrates, 5mC is the only modified base yet found in DNA(Razin and Riggs, 1980). The presence of this abnormal base is the result of post-replicational methylation of C residues in the DNA and not of the incorporation of 5mCMP during DNA synthesis. After DNA replication, a

transmethylase enzyme transfers a methyl group from S-adenosyl-methionine(SAM) to the 5-carbon in the pyrimidine ring of cytosine(Riggs and Jones, 1983).

In mammalalian DNA, 2-7% of the cytosine residues are converted to 5mC shortly after DNA replication(Razin and Riggs, 1980). The majority(about 90%) of 5mC residues in eukaryotic DNA occur in the dinucleotide sequence CpG and between 50 and 70% of CG sites are methylated, depending on the species and the tissue(Compere and Palmiter, 1981). Although 5mC residues are found mainly at the 5' side of guanine residues, low but significant amounts are probably found next to C, T, and A residues(Van der Ploeg and Flavell, 1980). The dinucleotide sequence CpG in bulk vertebrate DNA occurs at about one-fifth of the expected frequency, its rarity thought to be due to its methylation(Bird 1986). The scarcity of CpG is most probably caused by the failure of DNA repair mechanisms to recognize deamination of 5mC to give thymine(Bird 1986).

Recent experiments indicate that several higher eukaryotic genes show differential patterns of DNA methylation that are cell specific, and which correlate with the tissue-specific expression of these genes. These observations suggest a role for DNA methylation in controlling transcriptional activity of genes and further indicate that tissue and gene-specific changes in DNA methylation may be one of the molecular mechanisms by which specific gene expression is regulated during development(Levine et al, 1986). As early as 1975 Riggs(1975) and Holliday and Pugh(1975) postulated that changes in DNA methylation would provide a means of controlling gene expression in an inheritable manner. Indeed, recent evidence indicates that there is an inverse correlation between the extent of DNA methylation and the expression of several genes(Riggs and Jones, 1983).

B. EXPERIMENTAL TECHNIQUES USED IN METHYLATION STUDIES

Rapid progress has been made in recent years in the development of highly sensitive and reliable methods for the identification and quantification of 5mC in DNA. A convenient and widely applicable approach to demonstrate the correlation between the extent of methylation in the nieghbourhood of a gene to its transcriptional activity became available with the Hpa 11/Msp 1 restriction endonuclease assay(Felsenfeld and McGhee, 1982). This useful assay was based on the observation that Msp 1 cleaves CCGG sites methylated at the internal cytosine residue while Hpa 11 was inhibited by methylation at the site(Bird and Southern, 1978). Thus with the use of gel electrophoresis of digested DNA followed by Southern transfer and hybridization with the appropriate labelled probe, the pattern of methylation at those CpG sites which are embedded in a CCGG sequence can be determined in some detail(Felsenfeld and McGhee, 1982). A comparison of the restriction fragments seen for Hpa 11 with those seen for Msp 1 shows the location of methylated CCGG sites(Razin and Riggs, 1980). Several other restriction endonucleases have been used to probe for methylation including Ava 1 and Hha 1, yet the most widely used remain HpaII and MspI.

Thus it is often possible to determine the presence or absence of 5mC at a given position in the genome when the position concerned is part of a restriction cleavage site. However, the use of restriction enzymes permits only an indirect measure of DNA methylation(Van der Ploeg and Flavell, 1980). For example, in the HpaII/MspI assay only methylation of CCGG sites is measured and such sites represent only about one sixteenth of the total methylated sites(Riggs and Jones 1983). Changes in critical sites could easily be missed. Sano and Sager(1982) have reported tissue specific differences in bovine satellite DNA that are readily seen at TCGA sites. In

this case it was possible to determine methylation patterns by direct DNA sequencing as the Hpa II/MspI assay would have missed the tissue-specific differences. Results obtained by the use of restriction endonucleases show that the pattern of DNA methylation at a given site in the DNA of higher organisms is highly specific(Van der Ploeg and Flavell, 1980).

Most experiments to be discussed which have used enzymes to detect the methylation status of repective genes have done so in light of the disadvantage that only 10-15% of total CpGs present in DNA can be detected. Indeed, DNA transfection studies indicate that only a small subset of CpGs are relevant for gene expression(Jaenisch and Jahner, 1984). It is therefore possible that all experiments where no correlation between expression and methylation was found have failed to detect the biologically relevant sites(Jaensich and Jahner, 1984).

C. 5-METHYLCYTOSINE AND CONTROL OF GENE EXPRESSION

Much experimental support has accumulated favoring the involvement of 5mC in gene control(Riggs and Jones, 1983). Many publications exist which indicate that CCGG sites in non-expressing tissues or cells are more highly methylated than in expressing tissue. The correlation is excellent in the 5'-flanking region and, in most cases, the coding region and even introns are also less methylated when the gene is being expressed(Razin and Riggs, 1980).

The notion that 5mC may be a significant factor in mammalian gene control rests on a theoretical framework built from established principles of protein-DNA interactions(Riggs and Jones, 1983). Experimentally verified predictions now include: 1) methylation affects protein-DNA interactions; 2) methylation is symmetrical in both strands; 3) methylation patterns exist; 4) methylation patterns are tissue-specific; 5) methylation patterns are

somatically heritible; and 6) maintenance methylases exist(Riggs and Jones, 1983). Methylation patterns of specific genes in cells and tissues is both species and tissue specific(Levine et al, 1986).

Many groups have used Hpall/Mspl anaylsis to show a correlation between undermethylation of CpG sites in the region of a gene and its expression. The pattern of variation of methylation between tissues is complex and usually does not involve complete demethylation of extensive regions of the DNA(Felsenfeld and McGhee, 1982).

In 1979 McGhee and Ginder first reported that several CCGG sites in the region of the chicken beta-globin genes are less methylated in erythrocytes and reticulocytes than in oviduct tissue. The same year Mandel and Chambon(1979) found a correlation between undermethylation and gene activity of the chicken ovalbumin gene. In their study 3 classes of Hpa II sites were observed, namely, nonmethylated in all tissues, fully methylated in all tissues and variably methylated in all tissues(Mandel and Chambon, 1979).

In 1980 Van der Ploeg and Flavell used Hpa II and Msp I to demonstrate that the DNA of tissues expressing the globin genes and the region surrounding and including the genes expressed showed low levels of methylation whereas the nieghbouring DNA regions have higher levels of methylation. This data suggests that hypomethylation may be a necessary but not sufficient condition for gene expression in higher eukaryotes(Van der Ploeg and Flavell, 1980).

Since then the Hpall/Mspl system has been used to show correlations between methylation and expression of many genes. Examples include: 1) The work of Levine et al(1986) who showed tissue specific hypomethylation of the parathyroid hormone(PTH) gene in the parathyroid gland as compared to non-expressing tissues such as leukocytes, anterior and posterior pituitary

and placenta. However, no correlation was seen between the degree of hypomethylation of the PTH gene and the level of parathyroid gland secretory activity. This data suggests that methylation of specific DNA sequences may exert a critical role in determining the tissue-specific distribution of PTH gene expression. 2) The methylation status of the rPRL and rGH genes in pituitary cell lines which produce either large or minute amounts of both hormones has been assessed. In the cell lines which produced the most rGH and rPRL the genes were found to be hypomethylated whereas in lines which expressed low amounts of the two hormones the genes were methylated(Laverriere et al, 1986). 3) Studies involving the human thyroglobulin gene indicate that the extent of methylation at the 5' end of the gene reflects the activity of the gene in adult somatic tissues(Libert et al, 1986). Out of four Hpa II sites, three were found to be nonmethylated in thyroid DNA while full methylation was observed in liver, salivary gland and sperm DNA. In placental DNA, all four sites were non-methylated regardless of the activity of the gene(Libert et al, 1986). In addition, methylation was low in extra-embryonic lineages regardless of gene activity. These are only some examples of a large amount of work that has shown a correlation between hypomethylation of specific genes and their expression.

Other lines of evidence suggest that DNA methylation may play a role in the regulation of gene activity in animal cells. The connection between DNA methylation and gene expression is supported by studies of mouse retroviruses. A number of workers had previously shown that the inactive, genetically acquired proviral sequences are heavily methylated whereas the active, somatically acquired copies are undermethylated. In a recent study, non-methylated proviral sequences were shown to be infectious in

transfection studies while the methylated sequences were not(Felsenfeld and McGhee, 1982). Furthermore a copy of the endogenous inactive provirus was cloned in bacteria and the cloned DNA (now unmethylated at CpG sites) was found to be highly infectious(Felsenfeld and McGhee, 1982).

DNA synthesized during chemically induced differentiation of Friend erythroleukemia cells is hypomethylated, as measured by its ability to accept methyl groups transferred by homologous DNA methyltransferases *in vitro*(Christman et al, 1980). This work suggests that a minor % of sites normally methylated in FL cell DNA are not methylated in DNA made during chemically induced differentiation(Christman et al, 1980).

The availability of cell lines that undergo differentiation in tissue culture has allowed direct examination of the hypothesis that methylation of the genome may be regulating gene expression during differentiation. Mouse erytholeukemia cells that undergo erythroid differentiation after exposure to chemical inducing agents the following correlations have been made: 1) Differentiation is induced in Friend erythroleukemia cells by exposure to L-ethionine at concentrations sufficient to inhibit *in vivo* methylation of DNA and tRNA. 2) DNA isolated from FL cells undergoing differentiation in response to other agents unrelated to ethionine is hypomethylated, as judged by its ability to act as a methyl acceptor *in vitro*(Christman et al, 1980).

In addition to a strong *in vivo* correlation between gene expression and DNA undermethylation in specific cell types, *in vitro* methylation of many gene sequences causes an inhibition of their expression when measured either in fibroblasts or Xenopus oocytes(Cedar et al, 1982). In 1983 Busslinger et al were able to methylate CpG sequences in the members of the *B*-globin gene family. Briefly, the globin gene is cloned in a single-stranded M13 vector and a second strand is synthesized in which all cytosine residues are

methylated(Busslinger et al, 1983). The DNA is then transfected into mouse cells where cellular methylase enzymes act upon the unmethylated strand only at CpG. Upon growth the methylation status of CpG sequences is maintained while methylation at other cytosine residues is lost. The mouse cells tranfected with both a methylated gamma-globin gene and an unmethylated B-globin gene were only able to express the beta-globin gene. This experiment provides additional proof of a role for methylation in the inhibition of transcription.

Subsequently the specific areas of the gene where methylation of CpG sequences were silencing gene expression were localized. To do this, DNA strands were synthesized where certain segments were protected from methylation(Busslinger et al, 1983). Protection of DNA regions upstream from the 5' region resulted in expression of the previously silent transfected gamma-globin gene. However, when upstream regions were methylated and the rest of the gene left unmethylated no transcription was seen. The conclusion of these experiments suggest that between 760 bps upstream and 100 bps downstream from the 5' end of the gene are one or more CpG sites whose methylation is sufficient to prevent transcription of the gamma-globin gene in mouse cells(Busslinger et al, 1983).

In conclusion, the experiments discussed provide strong support for the hypothesis that the function of methylation of cytosine residues in DNA is to silence gene expression and that hypomethylation is associated with active gene expression. However, further support of this theory comes from the results of experiments performed with the cytosine analog, 5-azacytidine(5-AzaC).

D. 5-AZACYTIDINE

If a specific pattern of cytosine methylation in DNA is associated with

gene expression, it follows that compounds which change these patterns would change the differentiated state of cells. In particular, an analogue of cytosine modified in the 5 position would be expected to be resistant to enzymatic methylation and hence affect gene expression(Jones and Taylor, 1980). 5-AzaC is a nucleotide analogue which differs from cytosine only in that a nitrogen is substituted for the 5-carbon in the pyrimidine ring. 5-azaC has been used extensively in methylation studies as its incorporation into DNA results in an inhibition of DNA methylation.

One of the first experimental uses of 5-azaC came when Jones and Taylor(1980) treated mouse 10T 1/2 cells for a short time with the analog and observed that after several divisions the cells had differentiated into muscle cells, chondorocytes and adipocytes. Subsequent studies proved the reason for the differentiation was that 5-azaC was causing undermethylation of DNA. In addition it was shown that the changes were clonally heritable(Jones and Taylor, 1980). Maximal response was observed at concentrations between 2 to 5 uM 5-azaC.

In 1981, Compere and Palmiter studied the expression of the metallothionein-1 gene in the W7 mouse thymoma cell line which does not express the gene in presence of glucococorticoids. Hpa 11 analysis showed that the gene was methylated in the W7 line as compared to expressing lines studied. W7 cells were then treated for several hours with 5-azaC and the gene became inducible by both cadmium and glucocorticoids. In addition all Hpa 11 sites that were previously methylated became unmethylated as a result of 5-azaC treatment(Compere and Palmiter, 1981).

Other studies have extended the use of 5-azaC to analyze the state of other individual genes after cell growth in 5-azaC. Groudine and Conklin(1985) have reported that 5-azaC induces expression of an endogenous

avian retrovirus, and that the expression is associated with both DNA demethylation and increased nuclease sensitivity of the viral sequences chromatin.

Several previously inactive genes on the X chromosome have been activated by 5-azaC(Razin and Riggs, 1980). The genes for *hprt*, *pgk*, and *g6pd* are located on the inactive X-chromosome. However, treatment of mouse-human hybrids with 5-azaC results in up to 1% of surviving cells expressing the *hprt* from the previously inactive human chromosome. Moreover about 10% of cells expresing human *hprt* also expressed *pgk* or *g6pd*. After treatment with 5-azaC, the *hprt* gene on the human X chromosome functions much more efficiently in gene transfer experiments, presumably due to undermethylation of DNA(Razin and Riggs, 1980). These results are consitent with the proposal that DNA methylation is the basis of X chromosome inactivation in female mammals.

Laverriere et al(1986), have used 5-azaC to study the differential expression of rPRL and rGH in several pituitary cell lines. As mentioned, parrallel digestion with Hpa II and Msp I showed an overall correlation between gene expression and methylation. The 5-azaC studies suggested a variable importance of gene methylation in the control of Prl and GH gene expression(Laverriere et al, 1986). DNA methylation is differentially involved in the rPRL and rGH gene's expression within the same cell population and for a given gene from one strain to another. Interestingly, an apparent identical state of methylation could be associated with different levels of hormone production.

From the known mode of 5-azaC action, it might be expected that the greatest biological effect would occur either immediately or after a short lag period after drug exposure, and that *de novo* methylation would result in

a gradual decrease of gene expression over time(Laverriere et al, 1986). Razin and Riggs(1980) have proposed that after one round of replication in the presence of 5-AzaC, 50% of the alleles will preserve the original methylation pattern, while after two rounds of replication in the presence of 5-AzaC, 25% of the alleles will preserve the parental pattern.

Incorporation of 5-azaC into DNA results in a daughter cell containing hemimethylated DNA that cannot be made fully methylated at sites of 5-azaC incorporation. A subsequent cell division would result in one daughter cell with an unmethylated allele at the site of the 5-azaC incorporation and one daughter cell with a normally methylated allele(Compere and Palmiter, 1981). 5-azaC incorporation into DNA therefore causes a permanent change in the methylation pattern of half the daughter cells produced. In most studies, the effects of 5-azaC appear to be stably inherited for many cell generations(Compere and Palmiter, 1981).

The mechanism by which 5-AzaC causes demethylation is unclear. The analogue apparently must be incorporated in DNA to inhibit methylation, but it cannot function merely by virtue of a lack of a methylatable atom in the 5 position, since the extent of demethylation far exceeds the amount of 5-azaC incorporation(Felsenfeld and McGhee, 1982). It has been suggested that the methylating enzymes walk along the DNA helix, so it is possible that the occurence of a 5-AzaC residue at a methylation site may severely impede the progress of the enzyme(Jones and Taylor, 1980). Indeed, the current model consistent with present data is that DNA containing 5-AzaC in place of cytosine functions as an irreversible inhibitor of the methylase enzyme(Friedman, 1981). Even low levels of 5-AzaC incorporation into DNA reduces methylase activity in cell extracts for one to two days after treatment(Felsenfeld and McGhee, 1982).

There is evidence that methylase can act as a processive enzyme, so that a single 5-AzaC in a daughter DNA strand could affect the methylation of cytosines downstream on the strand. If binding of methylase *in vivo* were coupled to replication, the effect of 5-azaC on gene expression could be a complicated function of the distance between the methylatable control region of the gene and the nearest replication origin(Felsenfeld and McGhee, 1982). 5-AzaC has other effects on treated cells including inhibition of protein, RNA and DNA synthesis at high drug concentrations(Cihak et al, 1974). The drug also interferes with processing of RNA and the methylation of tRNA(Lu et al, 1976).

In summary, it must be noted that not all genes can be activated by non-lethal concentrations of 5-AzaC, perhaps because activation of some genes requires more extensive demethylation than can be achieved with non-toxic levels of the drug(Felsenfeld and McGhee, 1982).

E. EXCEPTIONS

Although methylation levels are clearly correlated with expression in the cases discussed above, there are some genes, even in organisms with a high general level of methylation, that do not display such obvious correlations. For example, some organisms(notably insects), manage quite well without any extensive methylation. The mechanisms for marking expressed genes in such organisms, like the mechanisms that mark genes for demethylation, remains unknown(Felsenfeld and McGhee, 1982) In addition, many expressed structural genes are extensively methylated in sperm(Jahner and Jaenisch, 1984, Rossant et al, 1986).

Examples of specific genes in which no correlation between expression and methylation status exist include the alpha 2(1) collagen gene of chicken which has a pattern of Hpa II site methylation that is invariant(with some

sites completely unmethylated in five cell types that have been studied, whether or not the cell synthesizes detectable amounts of collagen(Felsefeld and McGhee, 1982)). Another anomaly has been reported in the case of the X chromosomes from normal human cells, which in contrast to X chromosomes from mouse-human hybrids reveal no simple correlation between chromosome inactivation and the pattern of methylation at Hpa II sites(McKeon et al, 1982).

During development of chicken or *Xenopus*, the hormone inducible vitellogenin genes(Perry,1983, Burch and Weintraub, 1983, Gerber-Huber et al, 1983) or the gamma crystalline gene(Grainger et al, 1983) are expressed despite being highly methylated. In addition the X-chromosome linked genes in extraembryonic tissues (Kratzer et al, 1983) also show no correlation between methylation status and expression. Sheffrey et al(1982) have observed no changes in globin gene methylation after induced differentiation of erythroleukemic cells. In addition, ribosomal DNA genes in *Xenopus laevis* are also expressed despite heavy methylation(Macleod and Bird, 1983). Although these may be examples of situations in which control of expression is not coupled to altered levels of methylation, it is possible that the crucial methylation sites are not Hpa II sites(Felsenfeld and McGhee, 1982).

Just as *in vitro* methylation has been used to show a relationship between methylation and expression, it has also been used to show the opposite. SV40 DNA *in vitro* methylated at all CpG sites by the rat liver methylase enzyme and microinjected into cells show active transcription of the early region (Graessmann et al, 1983). These observations suggest that expression of certain genes may be insensitive to inhibition by methylation.

Critics of the hypothesis that methylation can regulate gene expression

state that there is no obvious reason why methylation should critically affect transcription. For example, Razin and Riggs(1980) point out that the HpaII site in the rabbit globin gene is in an intron, *i.e.* it exists in an area thought to be relatively unimportant in gene regulation. In this case undermethylation could be just a trivial result of transcription inhibiting methylation(Razin and Riggs, 1980).

In summary, the presence of 5mC in specific CpG sequences appears to be one factor controlling the expression of many viral and cellular genes. As mentioned, the correlation between hypomethylation and gene expression does not always prove true. One factor that might explain apparent discrepancies concerning the role of specific DNA methylation is our limited ability to analyze the critical methylation sites of complex gene(Levine et al, 1986). Most experiments correlating DNA methylation and gene transcriptional activity have used HpaII and MspI therefore have measured methylation of only CCGG sequences. Thus failure to find a correlation between expression and methylation may arise simply because the endonucleases do not detect the relevant sites(Levine et al, 1986).

F. DNA METHYLATION IN DEVELOPMENT AND DIFFERENTIATION

The overall level of methylation remains constant during development but the methylation patterns of specific genes changes and hypomethylation correlates with activation of some genes(Jaenisch and Jahner, 1984). The pattern of DNA methylation is stably inherited to progeny cells presumably because the enzyme that brings about methylation acts much more efficiently on hemimethylated sites than on unmethylated ones, and therefore methylates the daughter strand of a newly replicated duplex wherever the parent strand is methylated(Felsenfeld and McGhee, 1982). Three points must be emphasized when considering gene control during cellular differentiation: 1)

differentiation rests on a foundation of specific protein-DNA interactions; 2) DNA modification by the formation of 5-methylcytosine profoundly affects specific protein-DNA interactions; and 3) information encoded in methylation patterns can be stably maintained through repeated DNA replication cycles by the maintenance methylase system(Riggs and Jones, 1983).

As mentioned, Riggs(1975) and Holliday and Pugh(1975) have postulated that changes in DNA methylation would provide a means of controlling gene expression in an inheritable manner(Razin and Riggs,1980). A methylation site(CG) can exist in three states- unmethylated, methylated in both strands, and methylated in one strand(hemimethylated). Hemimethylated sites are generated from methylated sites by DNA replication, because only deoxycytidine is incorporated by DNA polymerase. By definition, a maintenance methylase acts on hemimethylated sites, but has little activity on unmethylated sites. Methyl groups in a CG site are, in effect, semiconservatively replicated, and become a heritable entity. Thus symmetrical methylation of both strands coupled with a methylase acting only a half-methylated sites would lead to the maintenance through DNA replication of a methylated pattern on the DNA. Unmethylated sites would remain unmethylated(Riggs and Jones, 1983). Therefore there is clonal inheritance of a methylation pattern once established(Riggs and Jones, 1983).

Regarding the role of methylation in regulating gene expression during development and differentiation, it is known that the DNA in mammalian sperm and early embryoes is highly methylated and undermethylation is correlated with gene activity(Razin and Riggs, 1980). Several investigators have suggested that the specific event controlling the expression of embryonic genes must be demethylation. Once the specific demethylation events occur,

the differentiated methylation pattern would be inherited clonally as a result of the maintenance methylase system, provided that *de novo* methylation is slow(Razin and Riggs, 1980).

Indeed, numerous studies have shown that major changes in DNA methylation patterns occur during early embryonic development(Riggs and Jones, 1983, Rossant et al, 1986). It is likely that the paternal and maternal genomes are differentially methylated in the fertilized egg, and yet it is generally assumed that allelic gene copies present on autosomes in more mature tissues bear identical patterns of methylation. Groudine and Conklin(1985) have suggested that *de novo* methylation during spermatogenesis plays a role in the templating of genetic information in sperm DNA.

In 1987 Chandler et al performed a series of experiments using restriction fragment length polymorphisms(RFLPs) to detect an epigenetic difference in allelic loci demonstrating that individual alleles of the human c-Ha-ras-1 gene can be differentially methylated. This was the first time an allele-specific methylation pattern had been observed on an autosome, previously this phenomenenon was usually seen in X-linked genes(Chandler et al, 1987). In addition it was found that the c-Ha-*ras*-1 gene was extensively methylated at CCGG sites in sperm DNA, whereas the same sites were considerably undermethylated on both alleles in epithelial and mesenchymal cells cultured from 19 week old fetuses. This data indicates that paternal *ras* gene copies must become demethylated during early human development(Chandler et al, 1987).

Further evidence suggesting a role for methylation of DNA in controlling gene expression in a heritable manner came from White and Parker and their studies on the prostatic steroid binding protein. The expression of the genes which code for the subunits of the protein are stimulated by

testosterone. In rats that were less than 15-20 days old, the expression of the prostatic steroid binding protein is negligible and the genes in the prostate are methylated. Above this age, the pattern of DNA methylation around the genes for C1, C2 and C3 in a variety of rat tissues including the prostate supports previous studies whereby an inverse correlation between DNA methylation and transcriptional activity(White and Parker, 1983).

G. METHYLATION: MECHANISM OF ACTION

Several different mechanisms by which DNA methylation might interfere with gene expression have been proposed. One class of models postulates that 5mC directly affects the affinity of RNA polymerase or presumptive regulator proteins for their DNA-binding sites(Chandler et al, 1987). Indeed Razin and Riggs stated "It has long been our opinion that the essential function of 5mC is to modify protein-DNA interactions". The conversion of cytosine to 5mC introduces a methyl group into an exposed position in the major grove of the DNA helix and the binding to DNA of proteins such as the lac repressor, histones, and hormone receptors is known to be affected by changes in the major grove(Razin and Riggs, 1980). For example, it has been shown that changing the thymine residue at nucleotide position 13 in the lac operator to uracil or to cytosine greatly decreases the affinity of the repressor for operator. Changing position 13 to 5mC restores the affinity for repressor to normal. Therefore, the lac repressor only senses the presence or abscence of a methyl group at position 13(Razin and Riggs, 1980).

Additionally, the isolation of a methylated DNA-binding protein(MDBP) from human placenta that binds selectively in a sequence-specific manner to methylated DNA sequences has been reported(Zhang et al, 1986). Thus a substantial body of evidence exists which suggests that the effect of

methylation at critical sites on the DNA is to alter the way in which proteins interact with the region by modifying local interactions with the active sites of sequence-specific proteins.

Alternatively, methyl groups may control nucleosome phasing near important regulator sites, and proper phasing may be essential for gene expression. Another class of models postulates that DNA methylation may control higher order packing of chromosomes and may thus determine whether or not large domains of chromatin are exposed to regulator signals(Chandler et al, 1987).

The effect of methylation may be through longer range effects of DNA structure. In the synthetic polynucleotide poly(dCdG), poly(dC-dG), which consists entirely of CpG repeats, methylation of the cytosine residues causes the polymer to adopt the left handed Z DNA conformation under physiological salt conditions(Felsenfeld and McGhee, 1982). Furthermore the Z form has so far proven incapable of forming nucleosomes. Such results lead to the idea that methylation *in vivo* may have a major effect on DNA and chromatin structure(Felsenfeld and McGhee, 1982). Perhaps methylation is used primarily for stable memory of a differentiated state through DNA replication, a process that might otherwise disrupt delicate protein-DNA and protein-protein interactions. This possibility is suggested by the observation that some organisms in which differentiated cells do not continue replication may not utilize 5mC DNA modification(Razin and Riggs, 1980).

A hypothesis consistent with the available evidence which summarizes the above-mentioned ideas suggests that methylation or hypomethylation is a means of stablizing the structure of a gene in an active or inactive state. Thus, the hypomethylated state would be a prerequisite of transcriptional

competence. During one or a few critical cell cylces *trans* acting molecules may trigger a gene to switch from an inactive to a competent state which precedes gene activition(Jaensich and Jahner, 1984). This switch may involve alterations of chromatin structure as well as demethylation. Demethylation may be due to inhibition of the maintenance methylase at specific sites(Jaensich and Jahner, 1984). The altered pattern of methylation may be used to establish and maintain the gene in the new state of competence during subsequent cell replication. Depending of physiological stimuli, transcription may be initiated by interaction of specific *trans* acting molecules with regulatory sequences of the gene. "Enhancer" sequences, which control gene activity present in cellular genes (Gillies et al, 1983) are likely to represent targets for differentiation-specific regulatory signals. These signals are cell-specific. This model of gene control suggests that demethylation can be dissociated from actual gene transcription, as has been observed in several genes(Jaensich and Jahner, 1984).

In summary it can be concluded that methylation of cytsosine residues in mammalian DNA is probably an important component of a multilevel control system for gene expression(Chandler et al, 1987). Active genes are often hypomethylated, hypomethylation can activate genes, and it has recently been demonstrated that methylation can induce the formation of an inactive chromatin configuration(Chandler et al, 1987). A theory that is generally accepted by most investigators is that hypomethylation is but one of a series of necessary but not sufficient events associated with eukaryotic gene expression(Levine et al, 1986).

H. DNA METHYLATION AND CANCER

Because of the role DNA methylation is thought to play in normal cell

differentiation and gene expression, it is possible that derangements in DNA methylation may be responsible for some of the abberant gene expression seen in cancer(Riggs and Jones, 1983). It has been suggested that cancer represents an alteration in DNA, heritable by progeny cells, that leads to abnormally regulated expression of normal cellular genes; DNA alterations such as mutation, rearrangments and changes in methylation have been proposed to have such a role. Because of increasing evidence that DNA methylation is important in gene expression, several investigators have studied DNA methylation in animal tumours, transformed cells and leukemia cells in culture(Feinberg and Vogelstein, 1983).

Analysis of overall levels of cytosine methylation in transformed cells have been performed(Riggs and Jones, 1983). Several lines of cultured human tumor cells have been examined for total DNA methylation and found to have decreased levels relative to human fibroblast DNA(Diala and Hoffman, 1982, Ehrlich et al, 1982, Wilson and Jones, 1983). Therefore there is substantial evidence that the majority of human tumor cell lines contain lowered levels of DNA methylation.

In 1983 Feinberg and Vogelstein purified DNA from primary human cancers and from adjacent normal tissues of the same patients. The DNA was digested with Hpa II, transferred to nitrocellulose and hybridized with 32P-labelled probes made from the cDNA clones of the human growth hormone, gamma-globin and alpha-globin genes. These genes were chosen because they are all known to have Hpa II sites, they are widely scattered in the genome, and they are not normally expressed in the tissues from which the cancers were derived. In all cases studied using all of the mentioned probes, progressive hypomethylation of the genes were seen in the tumor tissue as compared to the normal adjacent tissue. No differences in the digestion patterns were

observed with Msp 1 digestion(Feinberg and Vogelstein, 1983).

A working model has been proposed by Riggs and Jones(1983) which attempts to explain the relationship between DNA methylation and cancer. The initial event or a later event could be a mutation, however inappopriate DNA methylation is an alternative possibility. Carcinogens may inhibit methylases or otherwise interfere with normal methylation(Riggs and Jones, 1983). The initial event could result in inappropriate activity of a cellular oncogene. Given the initial event and resulting replication pressure, there is now strong selective pressure favoring cells with abnormal gene expression. Since the inheritance of methylation patterns is not totally rigid(Shmookler-Reis and Goldstein, 1982), it is likely that aberrant cell division would increase the chances of altered methylation(Riggs and Jones, 1983).

In summary, a substantial body of evidence exists suggesting that abnormalities in methylation patterns may contribute to the aberant gene expression observed in cancer.

I. DNA METHYLATION AND HORMONES

In 1984 Jost, Seldran and Geiser performed a series of experiments showing that hypomethylation of the chicken vitellogenin II gene appears to be associated with the binding of the steroid receptor to cis elements upstream from the transcription initiation site of the gene. At the time of their study, it was generally accepted that steroid hormones exerted their effects upon specific genes via binding of the steroid receptor complex to the chromatin(Jost et al, 1984).

HpaII/MspI analysis of the 5' end of the chicken vitellogenin II gene revealed the appearance of a hypomethylated site concurrent with E2 treatment of the liver. DNA-cellulose competition binding assays were used

to measure the ability of cloned DNA fragments of the chicken vitellogenin II gene to displace the estrogen-receptor complex from total chicken DNA coupled to cellulose(Jost et al, 1984). The results showed that the nuclear ER complex has a preferential binding to a DNA fragment situated upstream from the 5' end of the gene containing the Msp I hypomethylation site(Jost et al, 1984).

In 1986 the studies of the hormonal regulation of hypomethylation of the chicken vitellogenin II gene continued when Saluz et al used genomic sequencing to study the extent of cytosine methylation at four CpG sites within the regulatory region of the gene. Before E2 treatment, three out of four of the sites were fully methylated, two of the sites lying within the ER binding site. Analysis of DNA isolated from hormonally-treated liver nuclei revealed that demethylation of the four sites had occurred and that it correlated well with the induction of vitellogenin mRNA synthesis(Saluz et al, 1986). All four sites remained demethylated even after cessation of gene transcription. Subsequent comparison of the methylation state of these four sites in DNA from different tissues demonstrated a clear dependence of the demethylation on estradiol. These results suggest that this hormone-dependent event occurs via an active pathway through excision repair and/or enzymatic dememthylation. An explanation of this effect would be that both steroid receptors and the hormone are present in all tissues, suggesting that the binding of complexes initiates the demethylation and helps to maintain it(Saluz et al, 1986). The hormone-receptor complex may have a higher affinity for binding to the site than the methylase enzyme, thus preventing enzymatic methylation. In all this study shows that hormones can alter the DNA methylation status of specific hormonally-responsive genes.

PART IV

HISTONE ACETYLATION AND GENE EXPRESSION

A. INTRODUCTION

The DNA of the somatic cells of higher organisms occurs in association with small basic proteins called histones. In most cell types, histones can be divided into five major classes differing in size, positive charge, and amino acid composition(Ruiz-Carrillo et al, 1975). In the nucleus of all eukaryotic cells, DNA is packaged basically the same way, two of each of the histones H2A, H2B, H3 and H4 form a specific aggregate which is surrounded by a stretch of DNA 146 base pairs long. This structure forms fundamental chromatin unit known as the nucleosome(Doenecke and Gallwitz, 1982). A major structural function of histones is to organize the long, fibrillar molecules of DNA into a more compact form(Ruiz-Carillo et al, 1975).

With the exception of rRNA, both active and inactive genes are packaged into nucleosomes forming the characteristic "beads on a string" structure. However, the organization of genes that are actively transcribed differs from that seen in the bulk of DNA. Nucleosome structure is subject to variation due to covalent modifications of histones. Histones may vary in their primary structure or they may be post-synthetically modified through events such as ADP-ribosylation, methylation, phosphorylation or acetylation(Leiter et al, 1984). Histone modification has been proposed as a possible mechanism for gene activation(Isenberg, 1979). Indeed, the modulation of chromatin and nucleosome structure must be considered as a prerequisite for the transcription of genetic information(Doenecke and Gallwitz, 1982).

After the discovery of histone acetylation(Allfrey et al, 1964), it was suggested that this post-synthetic modification of histone structure could

provide an enzymatic mechanism for modulating the interactions between histones and DNA in ways that affect the structure of chromatin(Vidali et al, 1978). Regulation of eukaryotic gene expression occurs at different levels of genome organization, one of which includes the arrangement of chromosomal proteins. In particular, histone acetylation at specific lysine residues in the NH2 region has been implicated in this respect.

The distribution of lysine and other positively charged amino acid residues is not random, indeed there is a clustering of lysine, arginine and histidine in the amino terminal portion, resulting in a high net positive charge(Vidali et al, 1978). Thus all histone classes in the octet comprising the nucleosome core are structured to favor DNA binding throughout their amino terminal regions(Vidali et al, 1978). These regions would be expected to interact electrostatically with the negatively-charged phosphate groups of the DNA helix which envelopes the nucleosome core(Vidali et al 1978). Acetylation of histones causes neutralization of positive charges which changes the internal structure of the nucleosomes and the ability of the nucleosomes to interact with each other(Doenecke and Gallwitz, 1982). Neutralization of the positive charges of histones also weakens their interaction with the phosphate groups of the DNA strand enveloping the nucleosome core(Vidali et al, 1978). Thus histone acetylation and deacetylation may be part of a system which regulates the state of higher order chromatin interactions in relation to chromosomal interactions(Doenecke and Gallwitz, 1982).

All four nucleosomal histones can be reversible enzymatically acetylated at specific lysyl residues within the NH2-terminal regions. The fact that these lysyl residues are conserved in histones from species as distant as yeast and man, emphasizes their structural importance for histone

function(Doenecke and Gallwitz, 1982). Histone acetylation is a ubiquitous phenomenon and has been observed in fungi, protists, plants and animals(Doenecke and Gallwitz, 1982).

When histone acetylation was first seen(Allfrey et al, 1964), the structural modifications of DNA it caused were speculated to affect its template function in RNA synthesis(Ruiz-Carrillo et al, 1975). Since the turnover of acetyl groups on the core histones is extremely rapid(half life of ten minutes), it has frequently been suggested that acetylation of histones may be involved in rapid alterations of genetic activity(McKnight et al, 1980). The effect on transcription would occur through a weakening of the histone-DNA contacts within the nucleosome and between nucleosomes themselves allowing access to, and transcription of, the appropriate regions of chromatin(Nelson et al, 1980).

A considerable body of indirect evidence exists supporting a role for histone acetylation in the regulation of gene expression (Reeves and Cserjesi, 1979). In 1964 Allfrey et al were the first to examine the possible role for histone acetylation in the regulation of RNA synthesis when it was shown that acetylation of histones lowered their effectiveness as inhibitors of RNA synthesis by the DNA-dependant RNA polymerases. In 1966 Pogo et al showed that human and equine lymphocytes increase their rates of RNA synthesis within minutes after exposure to phytomegglutinin(PHA). Histone acetylation was greatly increased in lymphocytes responding to PHA. The increase in histone acetylation appeared to precede the increase in nuclear RNA sysnthesis. This work represents one of the first time that histone acetylation was linked to a change in the fine structure of chromatin and in the capacity of DNA to serve as a template for RNA synthesis(Pogo et al, 1966).

Other correlations have been made between histone acetylation and RNA-synthetic capacity in cell types from different species and phyla(Ruiz-Carrillo et al, 1975). In the protozoan *Tetrahymena pyriformis*, RNA-synthesizing macromolecules occur in their acetylated forms, while histones in the inactive micronucleus are unacetylated(Gorovsky et al, 1973). There are also numerous examples of increased histone acetylation at very early stages in gene activation, for example in lymphocytes stimulated by mitogens(Pogo et al, 1966), in hepatocytes during regeneration of the liver(Pogo et al, 1968), and in different endocrine target cells following stimulation by the appropriate hormone(Jungmann and Schweppe, 1972). During embryogenesis, histones are extensively modified. In sea urchin eggs, H3 and H4 are most highly acetylated during the last blastula and the gastrula stage, when gene expression is highest(Doenecke and Gallwitz, 1982). In all these cases, increased acetylation of the core histones was observed(Ruiz-Carrillo et al, 1975). Based on this evidence it appears that highly acetylated core histones are playing a role as an important auxillary mechanism that facilitates the binding of RNA-polymerase(Leiter et al, 1984).

Another striking example of acetylation of histones being found in conjunction with an increased trancriptional activity occurs in the yeast, *Saccharomyces cerevisiae*. The relatively small genome of this lower eukaryote appears to be entirely derepressed, that is, it is being transcribed or in a persistant state of activation. As a consequence H3 and H4 occur predominantly in a hyperacetylated form(Doenecke and Gallwitz, 1982).

Conversely, correlations have been made suggesting that deacetylation of histones will cause gene inactivation. For example, in the mature sperm

cells of the echinoderm *Arbacia lixula*, in cells that are incapable of RNA synthesis, histones H3 and H4 occur entirely in their nonacetylated forms(Wangh et al, 1972). After fertilization the acetylated form of H3 and H4 reappear with the resumption of RNA synthesis at the blastula stage(Wangh et al, 1972). Similarly there is a progressive decrease in the proportion of acetylated histone molecules in avian erythrocyte nuclei as the cells mature and lose their capacity of RNA synthesis(Wangh et al, 1972).

Chromatin containing acetylated histones is more soluble in magnesium chloride-containing buffers after nuclease digestion than chromatin associated with unacetylated histones(Leiter et al, 1984). Hyperacetylation also provides nucleosomal particles with a lower electrophoretic mobility than those which are not acetylated (Leiter et al, 1984). In 1978 Davie and Candido examined the patterns of histone acetylation in a chromatin fraction from trout testis enriched in actively transcribed sequences. Chromatin was digested with DNase 11 and the digestion products separated into Mg2-soluble and insoluble components and examined using 2-D polyacrylamide gel electrophoresis. The results showed that the fraction enriched in actively transcribed seqeunces were also enriched in the multiacetylated form of H4.

Active and inactive chromatin differ in structure and organization. Active chromatin has the appearance of loosely wound filaments while inactive chromatin is found in dense clumps of compacted fibrils(Ruiz-Carrillo et al, 1975). Transcriptionally-active DNA sequences are preferentially degraded during limited digestions with DNase 1. DNase 1 will also readily attack chromatin containing hyperacetyled histones(Leiter et al, 1984). Mild treatment with micrococcal nuclease also yields chromatin fragments which are enriched in transcribed sequences(Doenecke and Gallwitz,

1982). Thus nucleases have been particularly useful in studying acetylation of histones as they provide a means to describe the protein moiety which is associated with active portions of the genome(Doenecke and Gallwitz, 1982).

Other evidence linking histone acetylation and the mechanism of hormone action includes the work of Jungmann who in 1972 observed increased rates of histone acetylation following gonadotropin injection into ovaries of immature rats, suggesting that gonadotropin may exert its effect on target tissues through a mechanism that involves histone acetylation at some stage(Jungmann and Schweppe, 1972). In 1986 Csordas et al studied the correlation between transcription of the hormonally-induced vitellogenin gene and histone acetylation. The induction of vitellogenin synthesis in the chicken liver was used in this study. After administration of E2 to immature animals there is a massive induction of vitellogenin mRNA synthesis. The appearance of hyperacetylated histones following E2 treatment was monitored and found to correlate with the appearance of DNase 1 hypersensensitive sites in the vitellogenin gene as reported by other groups. Thus histone acetylation appears to be playing a role in the induction of vitellogenin gene transcription by steroids.

B. BUTYRATE AND HYPERACETYLATION

Butyrate is a naturally occuring short-chain fatty acid that produces a wide range of effects on cells in culture(Oritz-Caro et al, 1986). Among the many documented effects of millimolar concentrations of butyrate on mammalian cells growing in tissue culture is the induction of hyperacetylation of most of the histones found in the nucleosome(Reeves and Cserjesi, 1979). Over 80% of H4 is converted to the mono-,di-,tri-,and tetra-acetylated forms in a number of differents types of cultured cells after sodium butyrate(NaBut) treatment. Furthermore in all of these cell

types, H3 also becomes hyperacetylated as do histones H2A and H2B(Reeves and Cserjesi, 1979). In addition to histone acetylation, the chromosomal proteins of NaBut-treated cells are modified by phosphorylation as well(Doenecke and Gallwitz, 1982). Other effects of butyrate on cultured cells include the induction of certain enzymes, induction of hemoglobin in Friend erythroleukemic cells and inhibition of cell division(Leiter et al, 1984). In addition NaBut influences cytoskeleton assembly and membrane composition and function(Oritz-Caro et al, 1986).

NaBut exerts its effect on histone hyperacetylation through inhibition of the histone deacetylase enzymes rather than by a stimulation of the histone acetyltransferase enzymes(Reeves and Cserjesi, 1979). There are two histone acetyl transferase enzymes with defined biochemical properties. Histone acetylase A is tightly bound to the chromatin, whereas acetyltransferase B ia highly enriched in the cytoplasm(Doenecke and Gallwitz, 1982). The two enzymes are further distinguished by their chromatographic behavior, molecular weight and substrate specificity(Doenecke and Gallwitz, 1982). The effects of Nabut on histone acetylation are completely reversible once it has been removed from cells after short term exposure(Vidali et al, 1978). The effect of NaBut on acetate uptake and removal shows that it has little or no effect on the rate of acetylation of nucleosomal histones. Therefore the increased level of histone modification seen in butyrate-treated cells cannot be attributed to stimulation of acetyl transferase activities(Vidali et al, 1978). In addition, NaBut has a marked inhibitory effect on the turnover of previously incorporated acetyl groups(Vidali et al, 1978).

Studies on histone acetylation have been greatly aided through the use of NaBut, allowing experimental alteration of the level of histone

acetylation in cultured cells and observation of the effects of such changes on gene expression. However, it has not been established whether or not other changes in morphology, enzyme levels, DNA synthesis and specific protein synthesis due to NaBut treatment are the result of a primary effect on histone acetylation(McKnight et al, 1980).

The following paragraphs represent a review of some of the experiments researchers have performed using NaBut to study histone acetylation. An important theme will become evident as one progresses through the discussion, i.e. the expression of different genes in the same and different cells and tissues can be increased, inhibited or not affected at all by butyrate treatment. The effect of NaBut on gene expression does not necessarily reflect its affect on histone acetylation. As mentioned, NaBut has other effects upon cell morphology and physiology besides that of histone acetylation. Indeed, other nuclear proteins such as those in the High Mobility Group are also acetylated by NaBut. Thus one must excercise caution before making generalizations regarding the effect of NaBut in different systems.

The enhanced DNase I sensitivity of transcriptionally active DNA sequences appears to depend upon their association with multiacetylated forms of the histones(Ruiz-Carrillo et al, 1975). The diffuse, extended regions of the chromatin, which are the predominant sites of chromosomal RNA synthesis, have the highest levels of histone acetylation and are digested more rapidly by DNase I than are the transcriptionally inert, condensed area of chromatin. The relaxation of the DNA helix also influences its accessibility to other enzymes and regulatory proteins involved in transcription. This would account for the many spatial and temporal correlations between histone acetylation and RNA synthesis(Vidali et al, 1978).

HeLa cells treated with 7mM NaBut accumulate multiacetylated forms of histones H3 and H4 which in turn causes a more rapid rate of degradation of the associated DNA sequences during limited digestion with DNase 1(Vidali, et al, 1978). In HeLa cells and in avian erythrocytes, the multiacetylated forms of histones H3 and H4 are preferentially released during limited DNase 1 digestions, further confirming their association with transcriptionally active regions of the chromatin(Vidali et al, 1978).

Exposure of Friend erythroleukemia cells to Nabut for 24 hours causes a complex but dramatic and characteristic series of changes in both *de novo*-synthesized proteins and unique sequence DNA transcripts in the cells(Reeves and Cserjesi, 1979). Here, histone hyperacetylation plays an important necessary, but not sufficient, role in the activation of new genomic expression in the erythroleukemic cells. In 1984 Leiter et al studied the effects of NaBut on the hyperacetylation of histones in relation to induction of erythroid differentiation. Using high resolution *in vivo* [3H]acetate labelling of histones isolated from inducible and non-inducible murine erythroleukemic cells, it was found that histones became acetylated yet the cells did not become committed to differentiation. All cell lines, regardless of their ability to differentiate, responded to NaBut treatment by exhibiting highly acetylated histones. Again these results suggests that histone hyperacetylation per se is insufficient to promote genes in an active state since relative acetylation reaches a maximum at 8 hours after NaBut addition when the expression of the erythroid program is still reversible(Leiter et al, 1984).

NaBut has also been used to study the activity of specific genes. In 1984 Ginder et al reported that *in vivo* treatment of adult Leghorn chickens with both NaBut and 5-azaC resulted in both the formation of a DNase 1

hypersensitive site on the 5' end of one of the globin genes and the demethylation of several CpG sites around the gene which resulted in increased levels of embryonic globin RNA in circulating reticulocytes(Ginder et al, 1984). This study points out the complexity of the interactions between the measurable features of higher eukaryotic gene activity. Neither demethylation nor NaBut treatment alone could induce DNase I hypersensitivity or high levels of globin RNA, and demethylation is a necessary prerequisite for expression(Ginder et al, 1984).

In the above-mentioned examples NaBut appears to have a stimulatory effect upon gene expression in the systems discussed. In several cases it has been shown to have either no effect or opposing effects upon different genes in the same system. In 1982 Covault et al treated HTC cells with NaBut and saw no immediate and direct effect on the transcription of chromatin in the intact cell as measured by total RNA synthesis. In 1984 Burgess et al studied the effect of butyrate upon the genes coding for the heat shock polypeptides in a trout cell line(Burgess et al, 1984). Treatment of cells with concentrations of NaBut ranging from 5mM to 50mM for 24 hours resulted in acetylation of histones but no increase in heat shock protein synthesis In this case it was concluded that the activation of the heat shock protein genes required a process that was susceptible to inhibition by sodium butyrate(Burgess et al, 1984).

In the Glial cell line C6, regulation of glycerol phosphate dehydrogenase and glutamine synthetase are inducible by hydrocortisone and lacatate dehydrogenase by catecholamines. Interestingly, NaBut treatment of C6 cells results in an inhibition of induction of glycerol phosphate dehydrogenase synthesis by hydrocortisone. In contrast, NaBut induces glutamine synthetase specific activity and has an additive effect in

combination with hydrocortisone. NaBut has no effect at all upon the induction of lactate dehydrogenase by catecholamines. Thus the histone acetylation caused by NaBut exerted different effects upon two genes normally inducible by hydrocortisone (Kumar et al, 1984).

Along a similar line, in 1984 Stanley and Samuels showed that treatment of GH1 cells with NaBut alone will not stimulate growth hormone production but will combine with L-T3 to increase L-T3 stimulation of growth hormone production 1.5 to 2 fold compared to L-T3 alone. In contrast with the growth hormone response, NaBut increases basal PRL production by 5-fold. Interestingly, whereas PRL production is normally inhibited by T3, it was stimulated between 20 and 70-fold by L-T3 and n-butyrate. PRL and growth hormone mRNA levels paralleled the changes in PRL and growth hormone production rates(Stanley and Samuels, 1984). These studies suggest a relationship between inhibition of chromatin-associated deacetylase activity and L-T3 stimulation of PRL and GH mRNA production, however the precise mechanisms surrounding these events remains unclear.

Several examples of an inhibitory effect of NaBut upon gene expression also exist. NaBut treatment of cultured myoblasts in the L6 cell line where the alpha actin and myosin heavy chain genes are normally switched on during muscle differntiation resulted in total inhibition of expression of the myosin heavy chain genes and partial inhibition of the switching on of the alpha-actin gene(Leibovitch et al, 1984). In 1983 Plesko et al(1983) reported that NaBut treatment of HTC cells will completely abolish the induction of tyrosine aminotransferase by dexamethasone and dibutyryl cAMP.

NaBut has also been used to study the affect on the inducibility of the casein genes by PRL. Induction of casein gene synthesis by PRL is accompanied by an accumulation of casein gene mRNA which results from

increased rates of trancription and mRNA stablization(Martel et al, 1983). Treatment of rabbit mammary explants with NaBut results in an inhibition of PRL action on the induction of casein synthesis and casein mRNA accumulation. However, it is generally accepted that the effects of NaBut in this respect are not due to histone acetylation alone. In this case, the inhibition of PRL's effect on casein gene expression is thought to occur at the level of the cell membrane itself, where, somehow, NaBut is disrupting the molecules involved in signal transmission(Martel et al, 1983). The binding of PRL to its receptors was shown to be not affected.

Several other examples where NaBut has been used to study the alteration of expression of specific genes warrant attention. McKnight et al(1980) have shown that the effect of NaBut upon the E2-inducible ovalbumin gene in chicken oviduct explants loses its E2-inducibility when treated with 2mM NaBut. Normally, estrogens cause a rapid accumulation of ovalbumin mRNA, while NaBut produces nearly complete inhibition of induction. Histone acetylation studies showed that the degree of histone acetylation correlated well with the effectiveness of NaBut in preventing egg white mRNA induction *in vitro*. It was also shown that inhibition observed was not due to an inhibition of protein synthesis or the prevention of estrogen receptor translocation to the nucleus. These results suggest that acetylation of histones blocks the productive interaction between the ER and chromatin which leds to increased transcription of egg white genes(McKnight et al, 1980).

Conflicting results have been obtained by different groups studying the effect of NaBut upon the thyroid hormone receptor. In 1986 Oritz-Caro's group studied the effect of NaBut on thyroid hormone receptor levels in glial C6 cells. Here thyroid hormone receptor levels appeared to increase

in a dose and time-dependant fashion. This increase in receptor levels was not accompanied by an increase in the level of multiacetylated forms of core histones as studied by gel electrophoresis. However Samuel's work in 1980 showed that that NaBut elicits a reduction in thyroid hormone nuclear receptor levels in GH1 cells.

Mitsuhaki's results also show that treatment of GH1 cells with NaBut negatively regulates thryoid hormone receptor levels. However in non-pituitary cell types tested, NaBut treatment increased thyroid hormone nuclear receptor levels(Mitsuhaki et al, 1987). In both cases changes in the electrophoretic mobilites of H3 and H4 resulted which were presumably caused by acetylation. This observation suggests that the acetylation of histones H3 and H4 is involved in the regulation of thyroid nuclear receptor levels and that there is a difference in the receptor regulation between pituitary and non-pituitary cells(Mitsuhashi et al, 1987).

In summary, it is important to understand that many factors have to co-operate when gene expression is regulated and butyrate-induced changes in cell function should not be solely linked to hyperacetylation of histones(Lieter et al, 1984). NaBut has manifold effects on nuclear function, which means tha NaBut treatment has far greater consequences than inhibition of histone deacetylases would suggest(Lieter et al, 1984).

Evidence is accumulating that gene activation is a multistep process in which the occurrence of DNase 1 hypersensitive sites play a role in addition to the association of the transcribed genes with the structure of the nuclear matrix(Csordas et al, 1986). Thus the degree of acetylation of histones in certain regions of the chromosome might also play a role in the complex, multistep process of gene expression of the eukaryotic cell(Csordas et al, 1986).

PART V

POLYMORPHISM

A. INTRODUCTION

DNA restriction enzymes recognize specific sequences in DNA and catalyze endonucleoytic cleavages, yielding fragments of defined lengths(Davies et al, 1983). DNA restriction fragment length polymorphisms(RFLPs) are detected as a result of DNA base changes or small insertions or deletions of DNA which remove, insert or rearrange restriction enzyme sites(Davies et al, 1983). The genotypic differences between individuals can be visualized by the altered mobility of restriction fragments on agarose gels followed by Southern transfer and hybridization to the appropriate DNA probe(Botstein et al, 1980).

The base pair changes, deletions, additions and other local rearrangements which give rise to RFLPs show Mendelian inheritance(Botstein et al, 1980), which makes them useful in conventional linkage studies as genetic markers(Davies et al, 1983). Recombinant DNA techniques provide a vehicle for dramatically expanding the numbers of markers available for genetic linkage study through the identification of RFLPs(Gusella et al, 1983).

Variations in restriction sites around the human β-globin genes have been estimated to occur once in every 100 base pairs(Murray et al, 1982). Polymorphisms are present in all regions of the genome(Gusella et al, 1983), and are sufficiently frequent to assume that one will be found adjacent to any random cloned DNA probe several kilobases in length(Murray et al, 1982). Unlike classical expressed markers, DNA polymorphisms can be found in regions of the genome irrespective of whether they encode a protein. No knowledge of the biochemical nature of a trait or of the nature of the

alterations in the DNA responsible for a trait is required(Botstein et al, 1980). Probes that detect RFLPs can therefore be derived from a known gene loci or from anonymous DNA segments(Gusella et al, 1983).

If a gene or DNA sequence is indeed polymorphic, RFLPs should be shown to be inherited as simple Mendelian co-dominant markers and verified through family studies(Botstein et al, 1980). Multigenerational pedigrees are more useful than nuclear pedigrees and large families give more information that smaller ones(Botstein et al, 1980). The pedigree analysis of the RFLP is then correlated to a qualitative trait such as cholesterol or weight for example.

RFLPs were first used as a tool for genetic analysis in 1974. Linkage of temperature-sensitive mutations of adenovirus to specific restriction fragment length differences was used to locate the mutation on a physical map of the restriction fragments(Davies et al, 1983). Since this time the study of RFLPs in families and individuals has led to the detection of the loci of a number of human genetic diseases(Donis-Keller et al, 1987). Identification of large numbers of polymorphic loci in many individuals is possible as a small volume of peripheral blood provides sufficient DNA for analysis(Botstein et al, 1980).

B. RFLPs AS MARKERS IN HUMAN DISEASE

A PstI RFLP found within the intervening sequence of the *gamma*-globin gene has been used to diagnose sickel-cell anemia in utero(Botstein et al, 1980). In this case the RFLP was recognized because of its relationship to the DNA seqeunce of the *gamma*-globin gene.

In 1982 Murray et al discovered two RFLPs on the short arm of the Human X chromosome that have been instrumental in characterizing the mutated DNA sequences that give rise to Duchenne Muscular Dystrophy(DMD). In 1983

Gusella et al identified anonymous DNA fragments from chromosome 4 that detect two different RFLPs in a Hind 11 digest of human genomic DNA that show close genetic linkage to the Huntington's chorea gene. Huntington's disease is a progressive neurodegnerative disorder with autosomal dominant inheritance, yet at present there is no reliable method of presymptomatic or prenatal diagnosis of the disease and no effective therapy(Gusella et al, 1983).

Adult polycystic kidney disease is a common and often lethal multi-organ disease with an autosomal mode of inheritance. The basic biochemical defect leading to the disease is unknown, however a linkage marker was identified on the short arm of chromosome 16 associated with the disease(Reeders et al, 1985). In 1985(Tsui et al, 1985) and 1986(Beaudet et al, 1986) found several polymorphic DNA markers that are genetically linked to the autosomal recessive gene that causes cystic fibrosis.

Use of RFLPS have allowed the chromosomal localization of the defective gene that causes Alzheimer's disease(St. George-Hyslop et al, 1987). The localization on chromosome 21 provides an explanation for the occurrence of Alzheimer's disease-like pathology in Down syndrome(St George-Hyslop et al, 1987).

Loss of RFLPs on chromosome 11 has been shown to occur with the development of breast cancer in 11 patients tested(Ali et al, 1987). The loss of heterozygosity of chromosome 11 loci has a significant association with tumors that lack estogen and progesterone receptors, grade 111 tumors, and distal metastasis(Ali et al, 1987).

Other structural genes shown to be polymorphic include the human liver alcohol and aldehyde dehydrogenase genes(Bosron and Li, 1987), the ceruloplasmin gene(Yang et al, 1986), the gene coding for plasma cell

membrane antigen PC-1(Van Driel et al, 1985) and the alphafetoprotein gene in cancerous rat liver(Petropoulus et al, 1985).

The importance of a DNA locus genetically linked to a disease locus has profound implications both for investigations of the basic gene defect and for clinical care(Gusella et al, 1983). Identification of a linked DNA polymorphism is the first step in the molecular analysis and characterization of a disease gene and its causitive role in a disease(Tsui et al, 1985).

For genetic counselling purposes, the number of markers needed and the degree of polymorphism desired are unlimited: more and higher are always better(Botstein et al, 1980). The more a probe is seen to be polymorphic, the easier it is to observe rare recombination events that are informative for linkage(Beaudet et al, 1986). Increased polymorphism at flanking loci also raises the proportion of families in which genotypic diagnosis can be proposed(Beaudet et al, 1985). Although loose linkage to a slightly polymorphic marker is better than no linkage, the tighter the linkage the more accurately one can predict genotypes(Botstein et al, 1980). Both cloned genes and anonymous DNA fragments closely linked to a disease locus showing RFLPs can be used to trace the incidence of a genetic disease through families(Reeders et al, 1985).

Thus the importance of studying RFLPs in human disease is based upon both their ability to lead to a better understanding of the basic genetic defect which causes a disease phenotype and their strong potential use in medical diagnosis. With linkage based on DNA markers, parents whose pedigrees might indicate the possibility of their carrying a deleterious allele could determine prior to pregnancy whether or not they actually carry the deleterious gene and consequently, whether amniocentesis might be

necessary(Botstein et al, 1980). Analogously, individuals potentially at risk for heritable disorders, including some forms of cancer, of which expression occurs later in life, ought to be able to determine in advance of obvious symptoms whether they are at risk for the disease(Botstein et al, 1980).

RESEARCH OBJECTIVE

The objective of this thesis is to provide answers to several important questions involving structural features of the PIP gene. The first question asks whether or not there are any structural features(such as DNA methylation, histone acetylation or DNA rearrangement) that can be correlated with the activity of the PIP gene in the various expressing and non-expressing HBC cell lines. As mentioned, methylation of cytosine residues in genes has often acted as a silencing mechanism. Therefore it is possible that T-47D(a high PIP expressor) is hypomethylated as compared to MCF-7(a non-expressor). Histone acetylation has been associated with both increases and decreases in the expression of various eukaryotic genes. Similarly histone acetylation may be contributing to PIP expression. Gross rearrangement of a gene due to occurrences such as chromosome breakage can affect expression of the gene in different tissues and cell types. Therefore the possibility that rearrangement of the PIP gene has occurred between the various HBC cell lines also warrants study.

Many genes have been discovered to be polymorphic and the derived restriction fragment length polymorphisms(RFLPs) have proven useful as markers for a variety of diseases(SEE POLYMORPHISM SECTION). The PIP gene has been localized to chromosome 7(Myal, unpublished), which has recently been shown to contain highly polymorphic sequences. Indeed, in 1987 Barker et al constructed a linkage map of chromosome 7 using 63 RFLPs as linkage markers. Because of the link between PIP and HBC and its localization to a chromosome which has been shown to contain genes showing a high degree of polymorphism, it is important to determine whether the PIP gene itself is

polymorphic. This is especially relevant considering that increased levels of PIP in the blood of women with cystic breast disease and breast cancer has considerable potential as a marker for the disease. To correlate the presence of the protein with a RFLP of the PIP gene itself would provide a powerful clinical tool in the detection of HBC.

METHODS AND MATERIALS

PART 1

MATERIALS

A. Tissue culture materials and media supplements

Culture dishes(100 x 20 mm), culture flasks(150 cm2), sterile pipettes, and 50 ml centrifuge tubes were obtained from Corning and Fisher Scientific Co. Fetal bovine serum(FBS), Dulbecco's Modified Eagles Medium(DMEM), penicillin-streptomycin, trypsin-EDTA, and L-Glutamine were supplied by FLOW and GIBCO. Bovine Serum Albumin(BSA) was obtained from Sigma.

B. Hormones and other chemicals

Human Growth Hormone was kindly supplied by Drs. Ian Worsley and Henry G. Friesen. Dihydroxy-testosterone and hydrocortisone were purchased from Sigma and insulin was obtained from GIBCO. Sodium butyrate(Sigma) was kindly provided by Dr. Peter Cattini. 5-Azacytidine(Sigma) was generously supplied by Dr. Bob Matusik as well as purchased from Calbiochem.

C. Cell lines and tumor

T-47D is a human breast cancer cell line derived from the pleural effusion of a 54-year old patient with disseminated carcinoma of the breast. It was obtained from E.G. and G. Mason Research Institute(Rockville, M.D.). The MCF-7 cell line was isolated from the pleural metastasis of a patient with adenocarcinoma of the breast was supplied from Dr. M. Rich, Michigan Cancer Foundation. BT-474 was a gift from Dr. E. Y. Lasfargues, Institute for Medical Research, Camden, N.J. HBL-100, MDA-MB-231, BT-20 were supplied through the Cell Culture Bank, EG & G/Mason Research Institute, National Cancer Institute, Rockville, Md. A subline of T47-D(clone 11) was kindly

provided by Dr. I. Keydar, University of Tel Aviv. T-47D mutant #5 was isolated by Dr. R. Sutherland, Ludwig Institute, Sidney, Australia. All of the above-mentioned cell lines are breast cancer cell lines with the exception of HBL-100 which is a nontumor human breast cell line generated from human breast milk.

One human breast carcinoma tumor was kindly supplied by Dr. Leigh Murphy(Dept. of Biochemistry, U. of M.).

D. DNA/RNA isolation material

The proteinase K and RNAse A used in the isolation of genomic DNA were obtained from Boehringer Mannheim. Materials used for isolation of plasmid DNA including ampicillin, tetracycline, and chloramphenicol were supplied by Sigma. Ethidium bromide used to visualize nucleic acid was purchased from Sigma. Cesium chloride and guanidine thiocyanate used in the isolation of total RNA were obtained from Cabot and Sigma respectively.

E. Nucleic acid probes

PIP cDNA had been previously isolated from a lambda gt11 expression library made from poly(A+) RNA isolated from T-47D cells and cloned into the plasmid PBR322 by Dr. Leigh Murphy. A 1.7 kb fragment of the genomic clone had been isolated from an EMBL human genomic DNA library and subcloned into the plasmid vector PUC 19 by Yvonne Myal. A polymorphic sequence from chromosome 8 was a gift of Carolyn Gregory of the Dept. of Human Genetics, U. of M. A human 28S ribosomal probe was kindly provided by Dr. Sonia DeToledo(Dept. of Physiology, U. of M.)

F. Southern and Northern analysis/hybridization materials

Agarose for gel electrophoresis was obtained from Bethesda Research Laboratories. Ammonium acetate used in the Southern transfer technique was purchased from Fisher Scientific. Nitrocellulose paper for use in all

nucleic acid transfer procedures was obtained from Biorad. N-lauryl sarcosine and salmon sperm DNA used in Southern prehybridization solutions were supplied by Sigma. SDS and formaldehyde used in Northern analysis came from Fisher. Formamide was obtained from Terochem. The nick translation kit and the 32P dCTP used to nick translate DNA probes came from Amersham. Kodak XAR-2 omat x-ray film was used in all autoradiographic analysis.

G. Restriction endonucleases and buffers

All restriction enzymes used in digestion of DNA were obtained from either Boehringer Mannheim, Pharmacia, or Bethesda Research Laboratories. Either the accompanying salt buffers, the manufacturer's suggested buffer or the digestion buffer suggested by Manniatis(1982) was used in all digestions. Spermidine was obtained from Sigma. .

PART II

METHODS

1. TISSUE CULTURE TECHNIQUES

i) Maintenance of cells in culture

All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with insulin(10 ug/ml), glutamine(4mM), glucose(4.5 g/l) streptomycin(50 ug/ml), penicillin(50 units/ml), and fetal bovine serum(10% v/v). This medium is referred to as complete media(CM). The cells were kept in an incubator at 37 degrees celcius within a humidified atmosphere of 95% air, 5% CO2. When confluence was reached the cells were split using a trypsin/EDTA solution. Existing medium was removed and the cells rinsed once with 2 ml of trypsin/EDTA. An incubation at 37 degrees in 3 ml of the trypsin solution was usually sufficient to dislodge the cells from the plastic substrate of the flask. 7 ml of CM was then added to the flask to stop the reaction and the cells were pipetted into new flasks containing fresh CM.

ii) Hormone treatment

Prior to the addition of hormones cells were collected and counted using a Coulter counter and plated at concentrations ranging from 650,000 to 1,000,000 cells per 100 x 20 mm culture dish. Cells were then grown in CM for 3 days before treatment. A low cell density has been shown to optimize conditions for the response of the cells to hormones.

After 3 days the cells were washed in DMEM(containing no insulin or FCS) twice and the media aspirated. DMEM(no insulin or FCS) containing 1% bovine serum albumin(BSA) was then added to the cells. One group of dishes then received 1 ug/ml human growth hormone(hGH) in addition to either 500 nM dihydroxytestosterone(DHT) or 1 ug/ml hydrocortisone(F). The control

group received the ethanol carrier only. Cells were then grown for 2 days in their respective media, a time period sufficient to induce substantial PIP expression. After the two day period the media was aspirated and cells were washed twice in phosphate-buffered saline(pH 7.0)(PBS). After the second wash the cells were gently collected using a rubber policeman, pipetted into a 50 ml centrifuge tube and spun at 4 degrees celsius at 2000 rpm in a swinging-bucket rotor. The supernatant was poured off and the cells immediately placed on ice. The pellet was then either used in the isolation of DNA or RNA or stored at minus 70 degrees celcius until further use.

iii) 5-Azacytidine experiments

Several forms of experiments were performed using the cytosine analog. MCF-7 cells were in all cases plated at approximately 1,000,000 cells per 100 x 20 mm dish and grown in CM for several days as indicated in section ii. After the 2-3 days of growth, filter-sterilized 5-Azacytidine(5-AzaC) was added in increasing concentrations ranging from 0 to 10 uM to 3 plates at each concentration. After 4 days the cells were collected for use in RNA and DNA analysis.

Another set of 5-AzaC experiments involved treating the cells as described above however after 2 days of growth in 5-AzaC(in CM) one group of cells were treated with 500 nM DHT and 1 ug/ml hGH along with the same concentrations of 5-AzaC. Thus the cells were treated as described in section ll however in addition to the hormones there were 1 um, 2 um, 4 um, 6 um, 8 um, and 10 um concentrations of 5-AzaC present. The control group was retreated with the described concentrations of 5-AzaC in DMEM (no insulin or FCS) in the presence of the ethanol. Both groups of cells were grown for an additional 3 days and then harvested. The cells were grown for three days as their growth was slowed in the presence of 5-AzaC and enough

cells were needed for RNA and DNA analysis.

The 5-AzaC experiments were repeated using concentrations of 6, 8, and 10 um in the presence or absence of hormones.

iv) Sodium butyrate experiments

MCF-7 and T-47D cells were plated at approximately 4,000,000 cells per dish and maintained for three days as described in section ii. After three days 4mM sodium butryate(NaBut) was added to all dishes and the cells allowed to grow for an additional 24 hours when they were washed and then plated in DMEM(no insulin or FCS). Within each cell type two groups were designated. One group was treated with hormones as described in section ii. The other group was treated with hormones and an additional 4 mM NaBut was added to the media. The cells were grown for an additional two days at which time they were harvested as described above. RNA was isolated from the cell pellets for analysis.

2. DNA PREPARATION AND ISOLATION

i) Genomic DNA derived from breast cancer cells

The genomic DNA from all cell pellets obtained from the human breast cancer cells lines was prepared as follows. Cell pellets were dissolved in a lysis solution containing 100 mM Tris/Cl(pH 8.0), 100 mM NaCl, 5mM EDTA, 1.0% SDS and 100 ug/ml proteinase K. The cells were incubated in the proteinase K solution overnight (O/N) at 37 degrees celcius. The viscous solution was then treated with RNAse A for 15 minutes at 65 degrees C. Following RNAse treatment the DNA solution was extracted twice with phenol(v/v), twice with phenol/chloroform(v/v), and once with chloroform(v/v). The DNA was then ethanol precipitated using 0.4 x vol of 5M NaCl and 2.2 vol of cold 95% ETOH at minus 20 degrees celcius O/N. The precipitated nucleic acid was centrifuged and the pellet dried under vacuum.

The dry DNA pellet was then dissolved in TE(10:1), pH 8.0. The amount of DNA was calculated by taking an O.D. 260 reading of 5 ul DNA diluted into 495 ul ddH2O on a u.v. spectrophotometer. Briefly, the reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50 ug/ml for double-stranded DNA(Maniatis et al, 1982).

The same procedure was used to isolate DNA from a breast tumor sample, with the exception that ground tumor tissue was used.

ii) Lymphocyte DNA derived from human blood samples

10 mls of blood from female and male volunteers were collected in heparinized vacutainer tubes. Dr. K. Tachibana graciously offered his assistance in taking the blood samples. Blood samples were treated with 5 volumes of an ammonium chloride:TRIS solution and incubated at 37 degrees C for five minutes(NH4Cl:TRIS solution= 900ml 0.155M NH4Cl added to 100 ml 0.170M TRIS pH 7.65). Following incubation the solution was centrifuged at 2000 rpm for 10 minutes in a swinging bucket centrifuge. The supernatant was removed and the resulting cell pellet was resuspended in 10 ml of 0.85% NaCl followed by centrifugation at 2000 rpm for 10 minutes. The saline wash was repeated, the sample recentrifuged and the cell pellet placed on ice. This technique was obtained from the Department of Human Genetics(U of M).

Once the pellet had been obtained, the same procedure followed to isolate the DNA from the lymphocytes as was used to isolate DNA from the breast cancer cells(see section i).

iii) Isolation of cloned DNA fragments

In order to generate enough cloned PIP fragments to use in experiments, the plasmids containing the PIP gene had to be amplified and isolated. The standard plasmid preparation technique as outlined in

Maniatis(1982) was used. Plasmids were transfected into *E. Coli* bacteria grown in an overnight culture in the presence of either 50 ug/ml ampicillin(for PUC) or 12.5 ug/ml tetracycline(for PBR322) which selected for bacteria containing the plasmid. Chloramphenicol(.34 mg/ml) was then added to amplify plasmid DNA for 16 hours. Bacterial cells were harvested via centrifugation and for each original 500 ml of culture 9.5 ml of lysis buffer(lysis buffer = 25mM TRIS(pH 8.0), 10mM EDTA and 20% glucose) was added to each pellet. Cells were lysed on ice for 30 minutes until the solution was clear. Ten ml of an NaOH/SDS solution was then added for a further ten minutes to denature protein and RNA. The preparation was then incubated for 30 minutes in an additional 10 ml of a sodium-acetate(NaAc) solution, followed by centrifugation at 18,000 rpm. The aqueous component was then combined with 0.6 volumes of isopropanol and incubated at room temperature(RT) to precipitate protein and bacterial DNA. This was followed by centrifugation at 15,000 rpm for 10 minutes(at RT).

The pellet was then dried and dissolved in TE(10:1) pH 8.0 and the DNA subjected to equilibrium centrifugation using a CsCl gradient in the presence of ethidium bromide. The DNA was dissolved in 5.7M CsCl and added to 13 ml quick seal polypropylene tubes overlayed with ethidium bromide and mineral oil and centrifuged in a Ti-75 ultracentrifuge rotor at 55,000 rpm overnight.

After centrifugation, the ethidium bromide-bound plasmid and bacterial DNA was visualized in the polypropylene tube under uv light. The plasmid DNA was removed and extracted 5 times with 3-4 volumes of isoamyl alcohol to remove the ethidium bromide. The DNA was then ethanol precipitated and the pellet dried and dissolved in TE(10:1) pH 8.0. To remove the human insert from the plasmid the DNA was restriction digested

with Eco R1(in the case of PIP cDNA) and Xba 1(in the case of the 1.7 kb fragment).

The human DNA sequences were isolated from the plasmid DNA using electrophoresis on a low melting point 1% agarose gel. The PIP cDNA and 1.7 kb piece were separated from the plasmid DNA as a result of differential mobilities in the gel. The gel segment containing the PIP DNA was cut out and the agarose removed by phenol extraction. The DNA was then dried, ethanol precipitated and dissolved in TE(10:1)(10 mM Tris, 1 mM EDTA) pH 8.0. The amount of DNA present was estimated by running a 1 ul sample of the insert next to a known amount of MW standard DNA. The inserts were then stored at 4 degrees C.

3. ISOLATION OF RNA

Total RNA from cells was isolated using the technique of Chirgwin et al(1979). Two ml of the denaturant guanidine thiocyante(GnSCN) was added to each cell pellet and vortexed thoroughly. The GnSCN solution was then added to 2 ml 5.7 M CsCl in 13 x 51 mm Beckman polyallomer centrifuge tubes and centrifuged in an SW.50.1 ultracentrifuge rotor at 25,000 rpm for 22 hours.

After centrifugation the supernatent was removed and the resulting translucent RNA pellet air dried and dissolved in TE(10:1) pH 8.0. This was followed by an ethanol precipitation using 0.2M NaAc and 2.2 vol ETOH at -20 degrees C. The precipitated RNA was centrifuged and the pellet dried and dissolved in TE(10:1) pH 8.0. The amount of RNA present in each sample was quantitated through spectrophotometric measurement(OD 260). An OD 260 of 1 corresponds to 40 ug/ml RNA(Maniatis et al, 1982). Samples were then stored at -70 degrees C.

4. DNA RESTRICTION DIGESTS

In all restriction digests 10 ug of genomic DNA was digested in total

volumes ranging from 20 ul to 30 ul. In each digestion 1/10th of the total volume consisted of the appropriate buffer and 1/10th of the volume consisted of enzyme. The buffers chosen were ones deemed optimal for each enzyme(through a trial and error process). In the majority of digests the low/med/high salt buffer recommendations of Maniatis(1982) were followed. However, several times the manufacturer's suggested buffer was utilized. All digestions were performed in the presence of 100 mM spermidine to increase the efficiency of cutting. In several instances, such as with Msp 1, it was necessary to boost the digestion using additional enzyme. With the exception of Taq 1, which was incubated at 65 degrees C, all digests were carried out at 37 degrees C.

5. GEL ELECTROPHORESIS AND SOUTHERN TRANSFER

Digested DNA fragments were size fractionated on 0.8% agarose gels in a 1 x TBE electrophoresis buffer(0.089M Tris-borate, 0.089M boric acid, 0.002M EDTA, pH 8.0) containing ethidium bromide(0.5 ug/ml). After electrophoresis DNA was visualized and photographed under uv light. Lambda Hind III standards were included on all gels for use in size determination.

A modification of the Southern transfer technique(Southern, 1975), developed by Smith and Summers (1980), was used to prepare and transfer the gels to nitrocellulose paper. The modified technique involves the use of alkaline solutions(Reed and Mann, 1985). Gels were denatured in a solution containing 1.5M NaCl and 0.5M NaOH at RT for one hour. Neutralization was carried out in 2 volumes of 1 M ammonium acetate, 0.02M NaOH for one hour at RT. Gels were then set up in a standard transferring apparatus O/N covered by nitrocellulose and Whatman paper using the neutralizing solution as a transfer buffer.

Nitrocellulose filters were then dried at 80 degrees C under vacuum for

a minimum of one hour. Filters were prepared for hybridization in a prehybridization solution containing SCP(20 x SCP = 2M NaCl, 0.6M Na2HPO4, 0.02 M Na2H EDTA, pH 6.2), N-lauryl sarcosine, sheared salmon sperm DNA, Denhardt's solution, 50% formamide and dd H2O. Prehybridizations were carried out at 42 degrees C for one to two hours.

6. NORTHERN ANALYSIS

The Northern transfer technique as developed by Alwine et al (1977) was used to evaluate levels of PIP RNA. 10 ug total RNA were denatured in 50%(v/v) formamide and 2.2M formaldehyde. Electrophoresis of the RNA samples was carried out on a 1.0% agarose gel containing 2.2M MOPS(pH 7.0), 50 mM NaAc, 5mM EDTA(pH 8.0) and H2O. The gel was blotted onto nitrocellulose(Thomas ,1980) using 20 x SSC as a transfer solution. Filters were baked for 2 hours at 80 degrees C under vacuum. The filters were then prehybridized in a prehybridization solution containing 50 % (v/v) deionized formamide, 5 x Denhardt's solution(1x Denhardt's = 0.02% w/v each of bovine serum albumin, Ficoll and polyvinylpyrrolidine), 5 x SSPE(1 x SSPE = 1.15M NaCL, 0.01M NaH2PO4, 1mM EDTA), 250 ug/ml denatured salmon sperm DNA and 0.1% SDS. Prehybridization occurred at 42 degrees celcius for a minimum of one hour.

7. HYBRIDIZATIONS AND AUTORADIOGRAPHY

i) Nick translations: All nick translation reactions were performed using 32P-labelled dCTP isotope purhased from Amersham. The amount of DNA nick translated in each reaction ranged from 500 to 600 ng. Standard reactions(including the DNA, nucleotide buffer, DNA polymerase 1 enzyme, ddH2O and isotope) using the Amersham Nick Translation kit were carried out for 90 minutes at 15 degrees C. The average specific activity of each probe ranged from 20,000,000 to 100,000,000 cpm/ug DNA. Probes were then run

through a Sephadex G-50 column to separate the nick tranlsated probe from the free nucleotide. The probes were then boiled for a minimum of 5 minutes and cooled in dry ice and ethanol to prevent renaturation before addition to the filters.

ii) Southern blot hybridizations: All hybridizations of DNA filters were carried out in the presence of 10% dextran sulphate in the described prehybridization solution. Dextran sulphate enchances the efficiency of the hybridization reaction(Wahl et al, 1979). Filters were hybridized from 18 to 36 hours. After hybridization, filters were washed twice in 6.6 x SCP, 1.0 % sarcosyl for 15 minutes, followed by two washings in 1 x SCP, 1.0% sarcosyl for 90 minutes each and a final washing of 0.1 x SCP, 1.0 % sarcosyl for 15 minutes. All washings occurred at 65 degrees celcius. After washing, filters were covered in plastic and set up in autoradiographic cassettes and exposed onto Kodak XAR film using an intensifying screen at -70 degrees celcius.

iii) Northern blot hybridizations: RNA filter hybridizations were performed in the prehybridization solution as described in part 6, in the abscence of dextran. All hybridizations were carried out for a minimum of 24 hours. After hybridization blots were washed twice in 2 x SSC, 0.1% SDS (1 x SSC = 0.15M NaCl, 0.015M sodium citrate) for 15 minutes at RT. This was followed by one wash in o.1 x SSC, 0.1% SDS for 45-60 minutes at 65 degrees C. Blots were then set up in autoradiographic cassettes and using Kodak XAR x-ray film were exposed using an intensifying screen at -70 degrees celcius.

RESULTS

1. METHYLATION STUDIES

A. Restriction enzyme digests:

i) HpaII/MspI

As mentioned, the HpaII/MspI enzyme system is particularly useful in determining the methylation status of a gene. The isoschizomers both recognize the sequence CCGG yet HpaII will not cleave if the internal cytosine residue is methylated. Figures 1 and 2 show the HpaII/MspI digestion patterns of a variety of breast cancer cell lines(T-47D, MCF-7 and ZR-75-1), a "normal" breast cell line(HBL-100) and lymphocyte genomic DNA. ZR-75-1 is a breast cancer cell line which expresses low levels of PIP. Hybridization with the 577 bp PIP cDNA probe shows a wide variation in the methylation patterns between cell lines. MCF-7, the cell line which does not express PIP, appears to be the most hypomethylated of the cell lines tested. Eight bands of MWs 12.0, 4.6, 3.8, 2.2, 1.2, 0.6, and 0.5 kb were detected in the HpaII analysis of DNA from T-47D, a cell line exhibiting a high level of PIP expression. The other cell lines analyzed possessed some bands in common with T-47D as well as a unique band in the case of ZR-75-1.

The MspI pattern is the same between cell lines suggesting that that variation observed with HpaII is due to methylation effects alone. Only 5 of the 8 bands(the 4.6, 3.8, 1.2, 0.6, and 0.5 kb) produced with HpaII cleavage of T-47D were observed in the MspI digestion. This indicates that the 12.0, 2.2, and the 2.0 kb bands represent fully or partially methylated CCGG sequences. Lymphocyte DNA is heavily methylated and is resistant to cleavage with the methylation-sensitive HpaII.

Figures 3 and 4 show the HpaII/MspI methylation pattern of a range

FIGURE 1. Hybridization of PIP cDNA to DNA from human breast cell lines and normal lymphocytes. DNA was digested with HpaII and MspI. Human breast cancer(HBC) cell lines T-47D(T), MCF-7(M), non-cancerous human breast cell line HBL-100(H), and peripheral blood lymphocytes from a normal individual(L). Sizes of restriction fragments are given in kilobases. Open arrows indicate bands due to incomplete digestion.

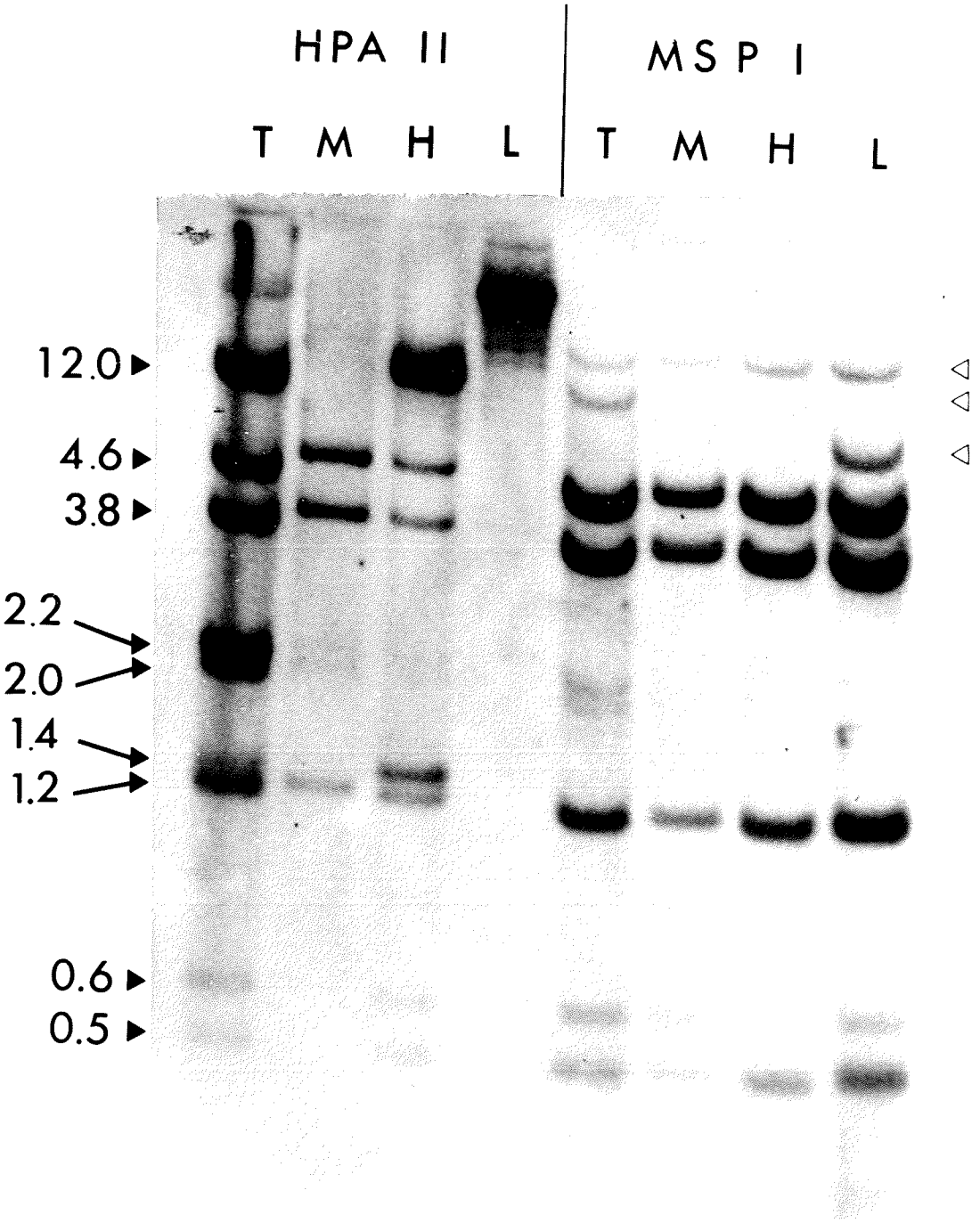

HPA II
MSP I
T M H L
T M H L
12.0
4.6
3.8
2.2
2.0
1.4
1.2
0.6
0.5

FIGURE 2. Hybridization of PIP cDNA to DNA from HBC cell lines and a non-cancerous human breast cell line. DNA was digested with Hpall and Mspl. HBC cell lines T-47D(T), MCF-7(M), ZR-75-1(Z), and a non-cancerous human breast cell line HBL-100(H).

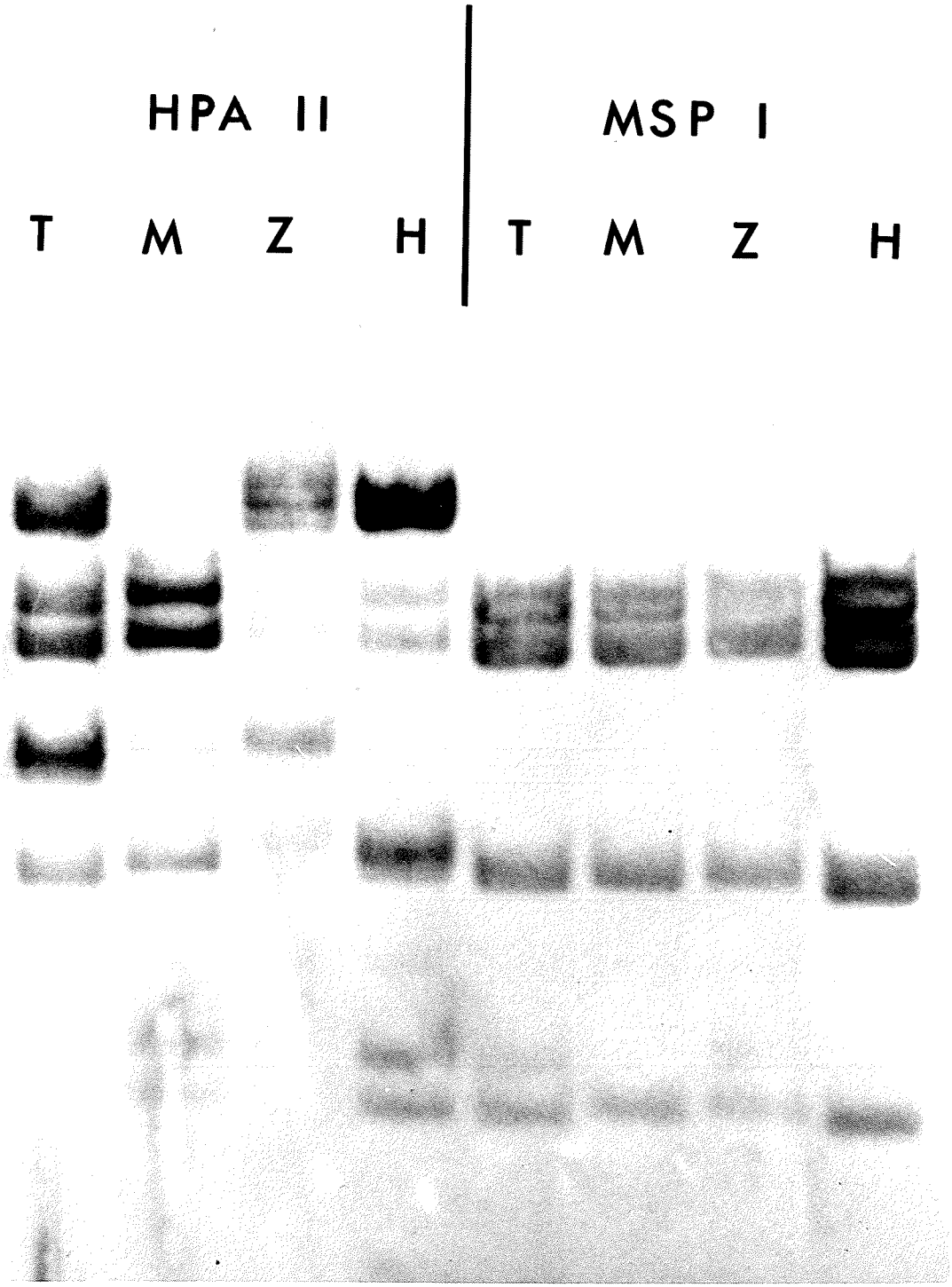

HPA II
MSP I
T
M
Z
H
T
M
Z
H

FIGURE 3. Hybridization of 1.7 Kb fragment(a genomic PIP clone) to DNA from HBC cell lines and a non-cancerous human breast cell line. DNA was digested with Hpall. HBC cell lines T-47D(T), T-47D mutant # 5(T5), BT-474(B4), MDA-MB-157(M1), ZR-75-1(Z), MCF-7(M), BT-20(B2), MDA-MB-231(M2), non-cancerous breast cell line HBL-100(H). pl indicates plasmid contamination.

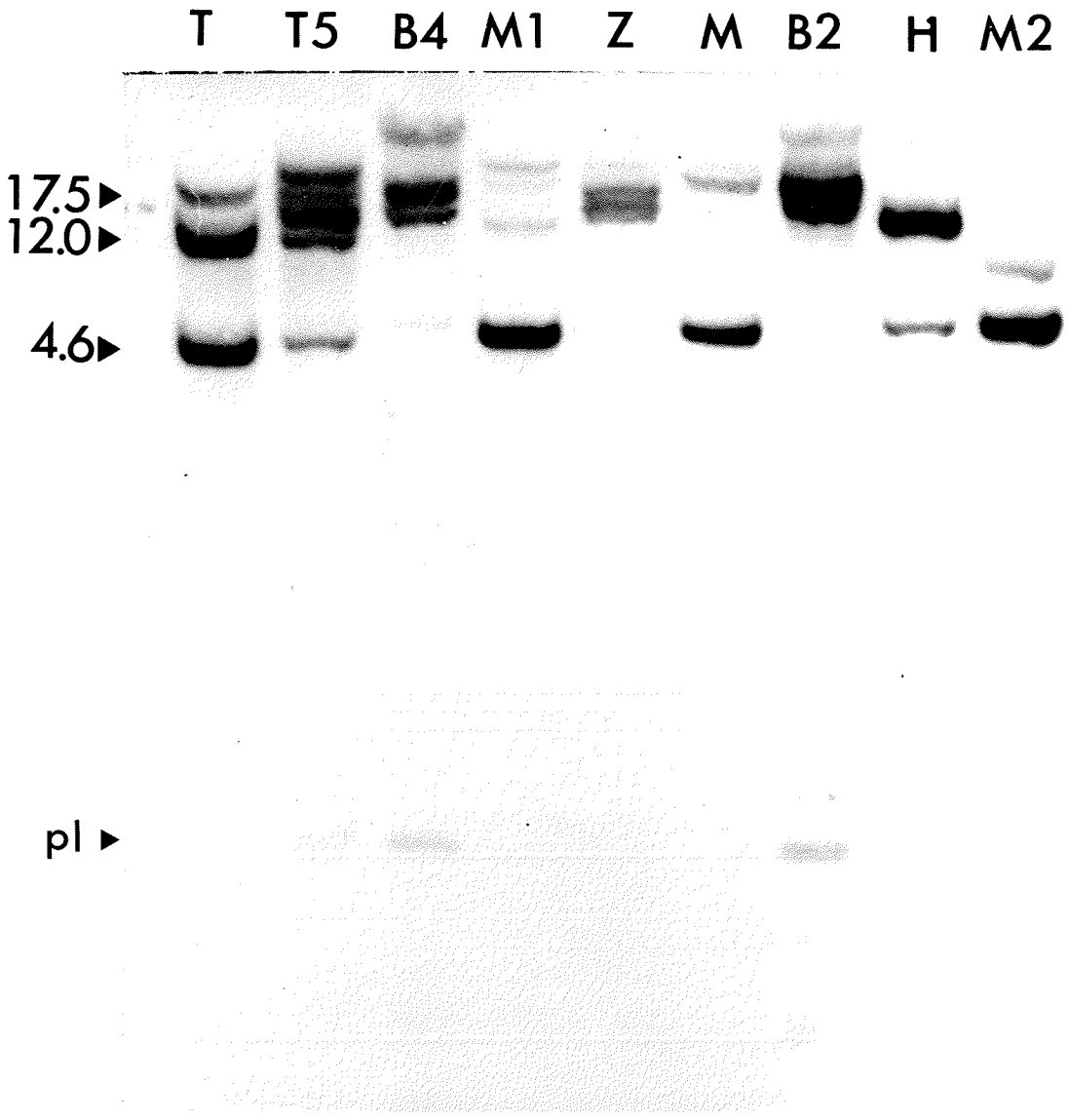
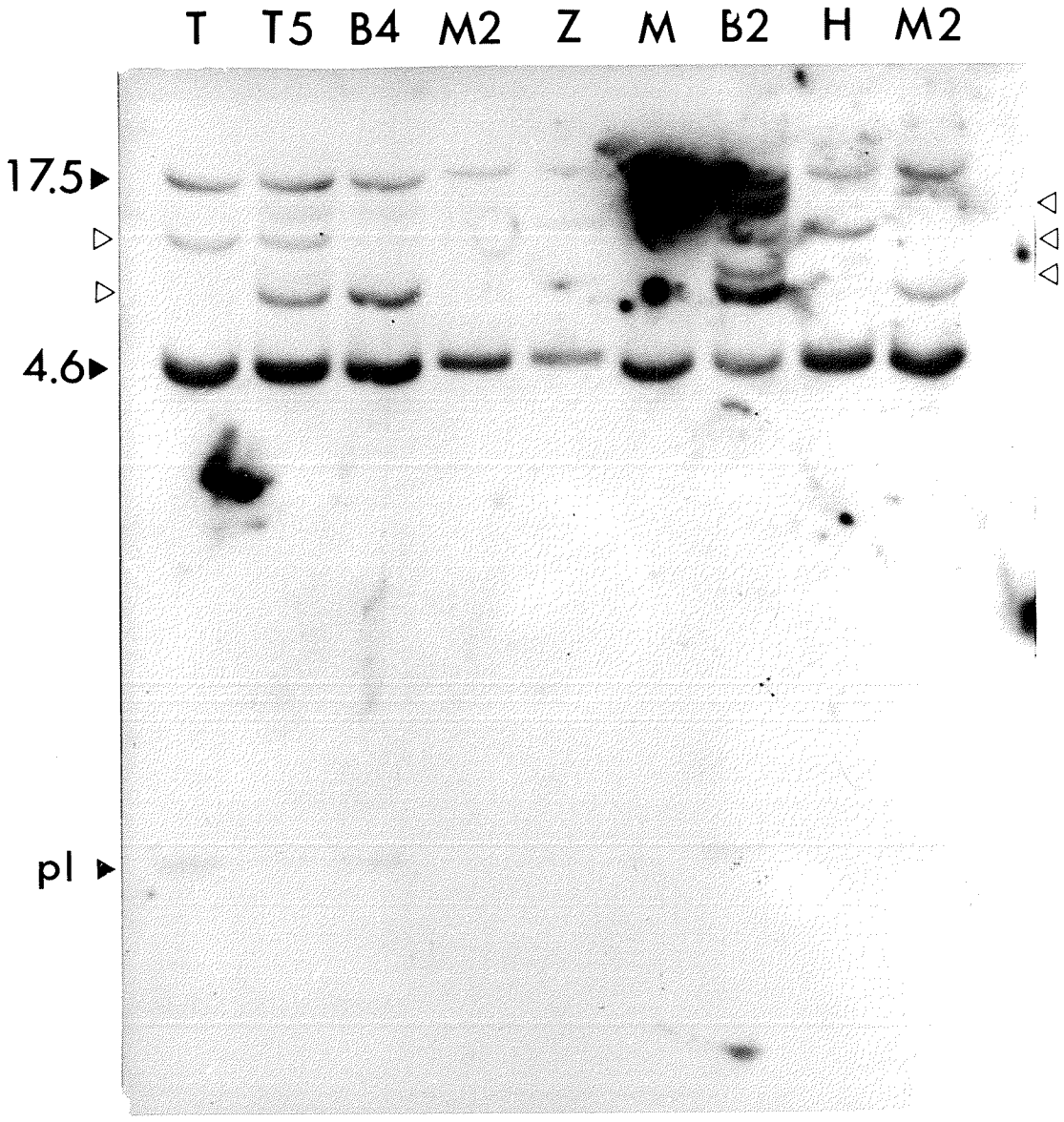

T
T5
B4
M1
Z
M
B2
H
M2
17.5
12.0
4.6
pl

FIGURE 4. Hybridization of the 1.7 kilobase fragment to DNA from HBC cell lines and a non-cancerous breast cell line. DNA was digested with Mspl. HBC cell lines T47-D(T), T-47D mutant #5(T5), BT-474(B4), MDA-MB-157(M2), ZR-75-1(Z), MCF-7(M), BT-20(B), MDA-MB-231(M2) and a non-cancerous breast cell line HBL-100(H). Open arrows indicate artifacts due to incomplete digestion(also see figure 22).


T
T5
B4
M2
Z
M
B2
H
M2
17.5
4.6
pl

of HBC cell lines using a 1.7 kb genomic DNA fragment as a probe. The 1.7 kb fragment of the PIP gene consists of 330 bps of 5' exon sequences and an undetermined amount of 5'-flanking region and intronic sequence making up the remaining 1.4 kb. Therefore it represents the probe with the most 5' information. A variety of methylation patterns is observable between cell lines. HpaII digestion of T-47D yields three bands of MW 17.5, 12.0 and 4.6 kb. The 12.0 and 4.6 kb bands are shared with the cDNA hybridization(figures 1 and 2). MCF-7 yields the 17.5 and the 4.6 kb bands plus an additional band of approximately 20 kb. Because MspI digestion yields only the 17.5 and 4.6 kb bands(figures 4 and 22), it appears that the 12.0 kb band in T-47D and the 20.0 kb band in MCF-7 represent different methylated CCGG sequences specific to each cell line. The other cell lines share bands in common with MCF-7 and T-47D as well as possessing their own unique bands. Interestingly, T-47D mutant #5, a low expressor of PIP(which maintains its hormone-inducibility) appears to be more methylated than T-47D. The most heavily methylated cell lines are ZR-75-1, a low expressor of the PIP gene, and BT-20 and BT-474, which do not express the PIP gene at all. The low molecular weight bands(pl) observable in both figures 3 and 4 are due to plasmid contamination(as determined by rehybridization of the blots with a plasmid probe).

MspI is a particularly difficult restriction enzyme to work with in terms of achieving complete cutting at all CCGG sites. The bands marked with an open arrow(e.g. in figures 1 and 4), have been determined to be due to incomplete cutting by comparison to other genomic Southern hybridizations of the same cell lines.

ii) Aval analysis

Aval is a methylation-sensitive restriction endonuclease which

recognizes the sequence GPyCGPuG and will not cleave the site if the internal cytosine residue is methylated. Figures 5 and 6a represent a wide range of HBC cell lines digested with AvaI and hybridized with the PIP cDNA probe. Six bands of MW 25.0, 9.6, 7.0, 4.9, 3.1, and 2.9 kilobases are produced when T-47D is digested with AvaI. MCF-7 shares the 9.6, 3.1 and 2.9 kilobase bands with T-47D, however an 8.0 kilobase band is also produced which is not present in T-47D. The difference in banding patterns between the two cell lines suggests that MCF-7 is relatively hypomethylated as compared to the T-47D and the other cell lines tested, which is consistent with HpaII analysis. A variety of methylation patterns are evident in the other cell lines. Lymphocyte DNA was not susceptable to cleavage by AvaI because of its high level of methylation.

Hybridization with the 1.7 kb probe(figure 6b) also shows a diversity of restriction patterns in these cell lines. The restriction fragments detected by the PIP cDNA probe and the 1.7 kb genomic probe are similar. This indicates that the majority of the sites recognizable by AvaI fall in an area of overlap between the 1.7 kb genomic fragment and the PIP cDNA.

In summary, the information revealed in figures 1 to 6 indicate that there is a wide range of methylation patterns between the various cell lines tested. In addition there appears to be no clear-cut correlation between the methylation pattern and PIP gene expression.

iii) Effect of hormones on PIP gene methylation

Because the PIP gene is hormonally-inducible, experiments were performed to determine if hormones could alter the methylation status of the gene in several HBC cell lines. Total genomic DNA was isolated from cells

FIGURE 5. Hybridization of PIP cDNA to DNA from human breast cell lines and normal lymphocytes. DNA was digested with AvaI. HBC cell lines T-47D(T), ZR-75-1(Z), T-47D clone #8(T8), MCF-7(M), T-47D Clone #11(T11), non-cancerous human breast cell line HBL-100(H), peripheral lymphocyte(L). Open arrows indicate bands due to incomplete digestion.

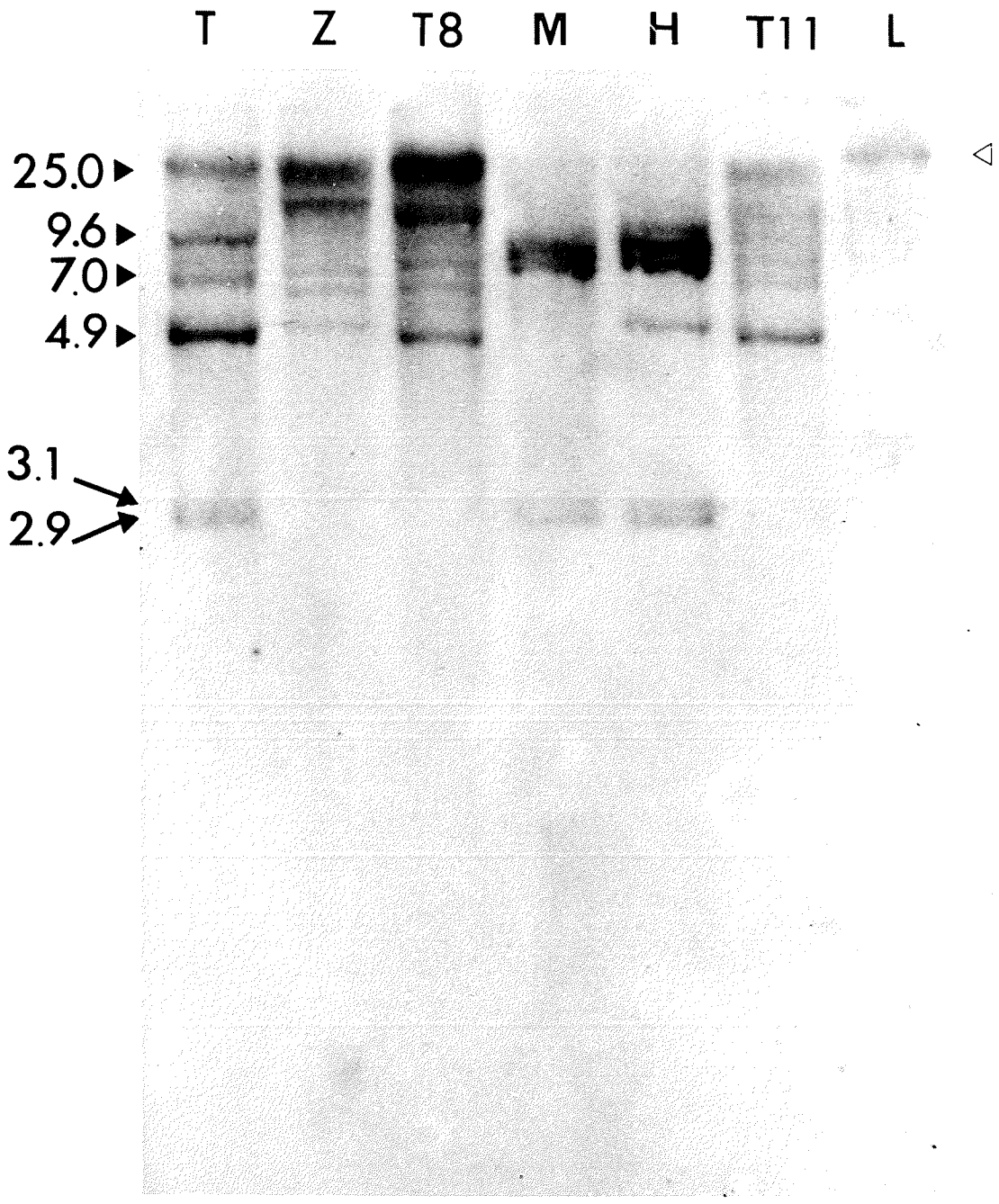
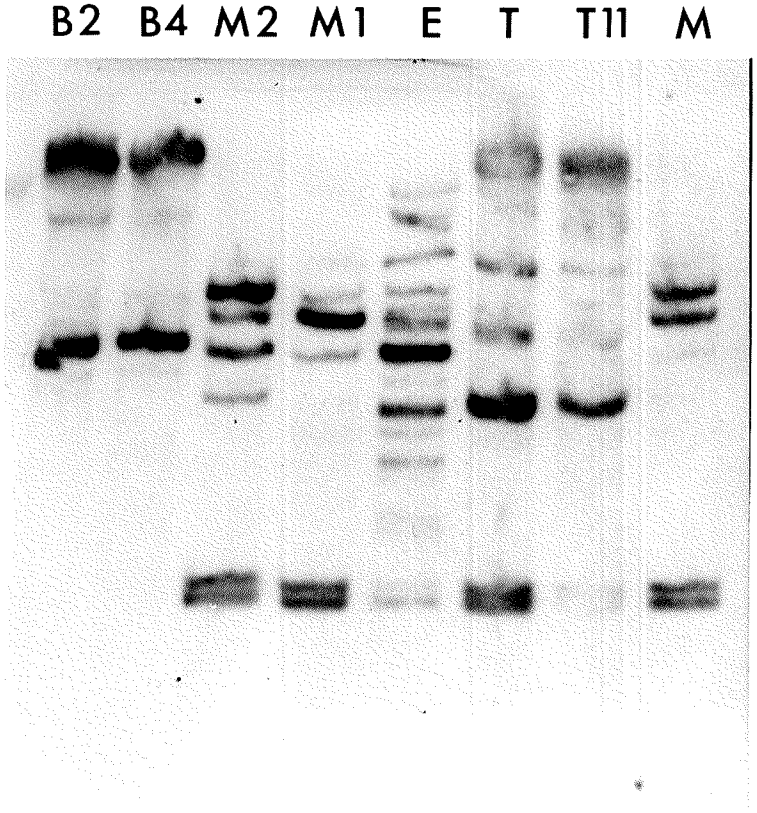
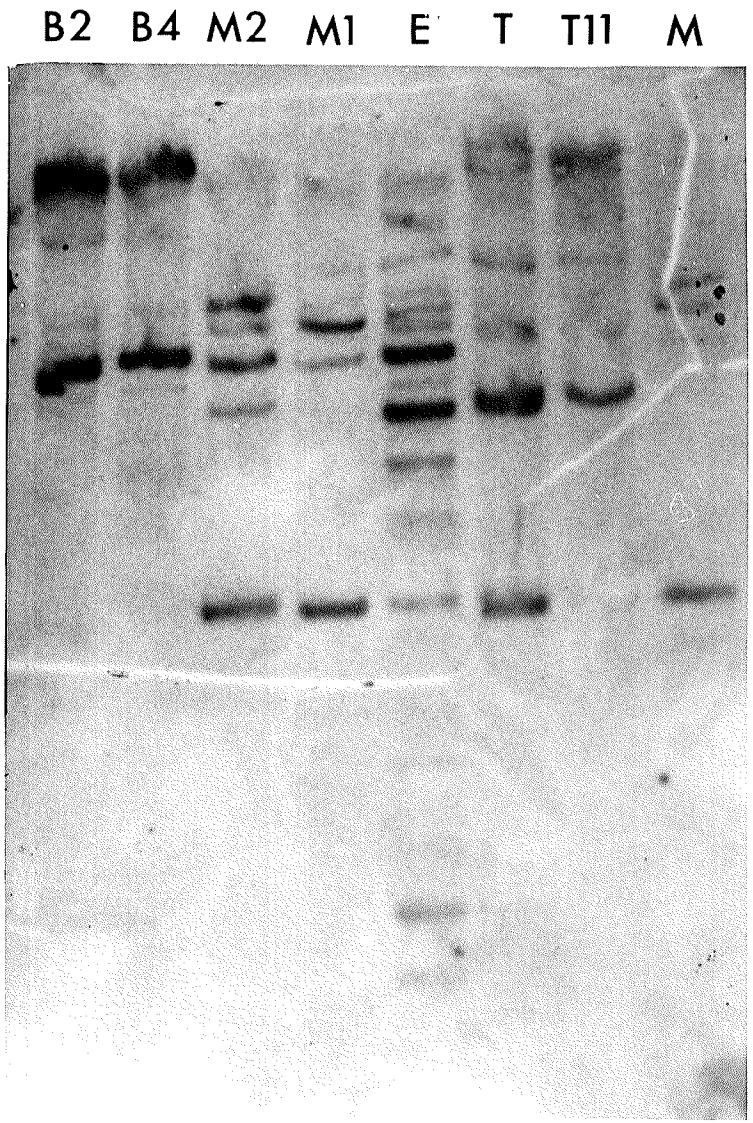

T
Z
T8
M
H
T11
L
25.0
9.6
7.0
4.9
3.1
2.9

FIGURE 6a. Hybridization of PIP cDNA to DNA from HBC cell lines. BT-20(B2), BT-474(B4), MDA-MB-231(M2), MDA-MB-157(M1), EFM-19(E), T47-D(T), T-47D clone #11(T11), and MCF-7(M).

FIGURE 6b. Hybridization of 1.7 kilobase fragment to DNA from HBC cell lines. See legend of Fig. 6a for cell lines.


B2
B4
M2
M1
E
T
T11
M


B2
B4
M2
M1
E
T
T11
M

treated with hGH and DHT and digested with Hpall, Mspl,and Aval. hGH was used in place of hPRL because it competes with equal efficiency for the PRL receptor and and been shown to elicit the same bioligical effect in inducing PIP expression; hGH is also more readily available.

The results of the Southern analysis are shown in figures 7 to 10. Figures 7 and 8 show Hpall/Mspl analysis of hormone-treated T-47D and ZR-75-1 cells using PIP cDNA and the 1.7 kb fragment as probes respectively. No difference attributable to hormone treatment is seen in either case. Figures 9a and 9b show the effect of hormones on the methylation status of the PIP gene in MCF-7 cells again no difference is seen. Aval digestion(figure 10) of the three cell lines representing a range from high to non-PIP-expressors also shows that hormone treatment of the HBC cell lines does not alter the methylation pattern of the PIP gene.

B. 5-AZACYTIDINE EXPERIMENTS

i) Northern analysis

If DNA methylation is playing a role in PIP expression it is conceivable that a compound that alters methylation of cytosine residues can alter the expression of the PIP gene. As mentioned, 5-azacytidine(5-AzaC) is a nucleotide analog that has been used extensively to activate previously inactive genes in cell culture. Based on this premise, MCF-7 cells(which do not express PIP in response to hormone treatment)were treated with 5-AzaC in an effort to induce PIP expression.

Northern analysis of total RNA isolated from MCF-7 cells treated with 5-AzaC is shown in figure 11. A range of concentrations of 5-AzaC were used ranging from 0um to 10um. MCF-7 cells have a cell doubling time of approximately 36 hours. Cells were treated in the analog for four days before harvesting, an adequate time to ensure its incorporation into DNA.

FIGURE 7. Hybridization of PIP cDNA to DNA from hormone-treated and control T-47D and ZR-75-1 HBC cells. Hormone manipulated cells were treated with 1 ug/ml human growth hormone(hGH) and 500 nM DHT(dihydroxy-testosterone) for 2 days. Control(C), Hormone-treated(H). Open arrows indicate fragments generated due to incomplete digestion of DNA. DNA was digested with Hpall and Msp1.

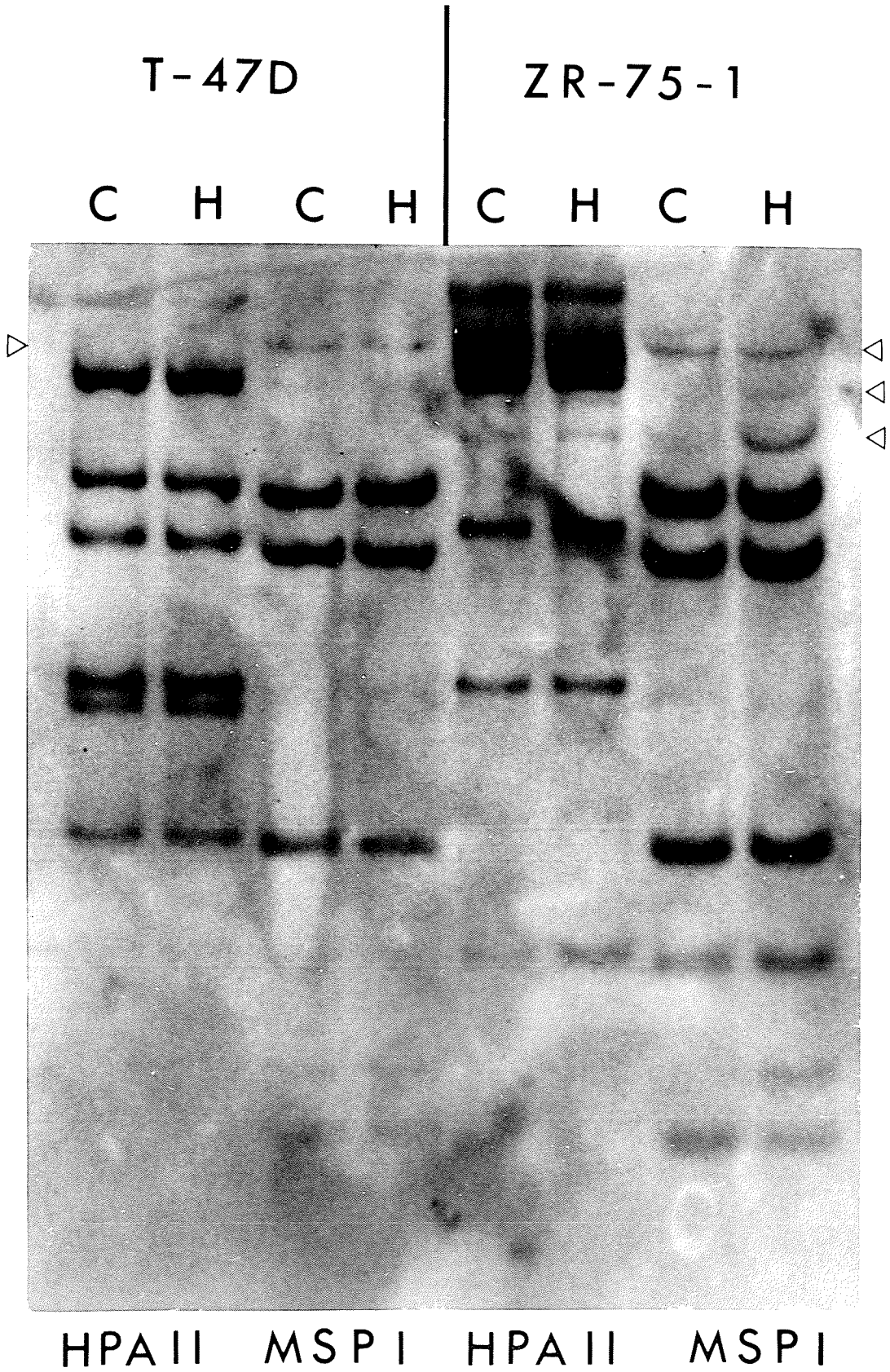

T-47D
ZR-75-1
C H C H C H C H
HPA II MSP I HPA II MSP I

FIGURE 8. Hybridization of 1.7 kilobase fragment to DNA from control and hormone-treated HBC cell lines T-47D and ZR-75-1. DNA was digested with HpaII and MspI. Control(C), Hormone-treated(H). Open arrows indicate bands due to incomplete digestion of DNA.

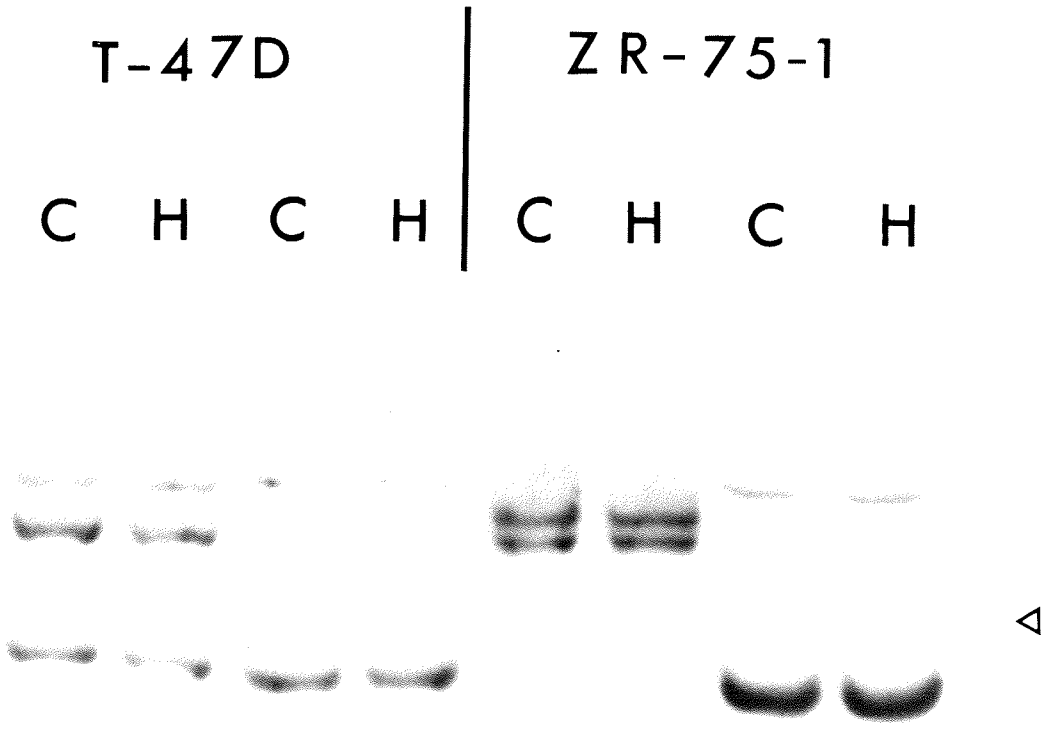
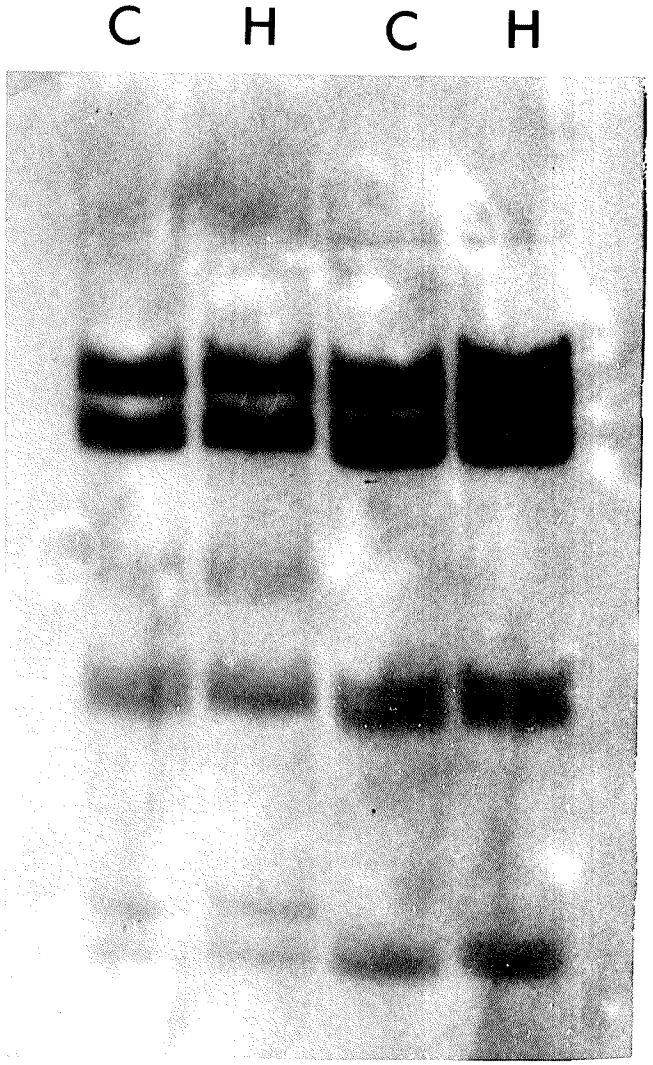

T-47D
ZR-75-1
C H C H C H C H
HPA II MSP I HPA II MSP I

FIGURE 9a. Hybridization of PIP cDNA to DNA from hormone-treated and control MCF-7 HBC cells. Control(C), Hormone-treated(H). DNA was digested with Hpall and Mspl.

FIGURE 9b. Hybridization of the 1.7 kilobase fragment to DNA from hormone-treated and control MCF-7 HBC cells. Control(C), Hormone-treated(H). DNA was digested with Hpall and Mspl.


C H C H
HPA II MSP I

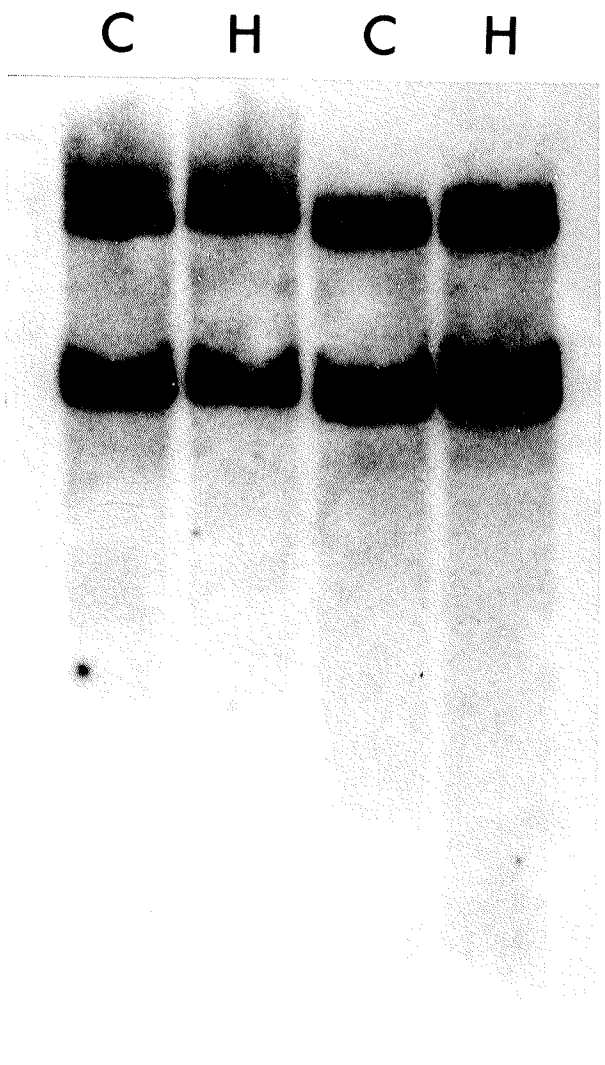

C H C H
HPA II MSP I

FIGURE 10. Hybridization of PIP cDNA to DNA from hormone-treated and control HBC cell lines. T-47D(T), MCF-7(M), and ZR-75-1(Z). DNA was digested with AvaI. Control(C), Hormone-treated(H). Higher intensity of MCF-7 bands is due to more DNA loaded as compared to the other cell lines.

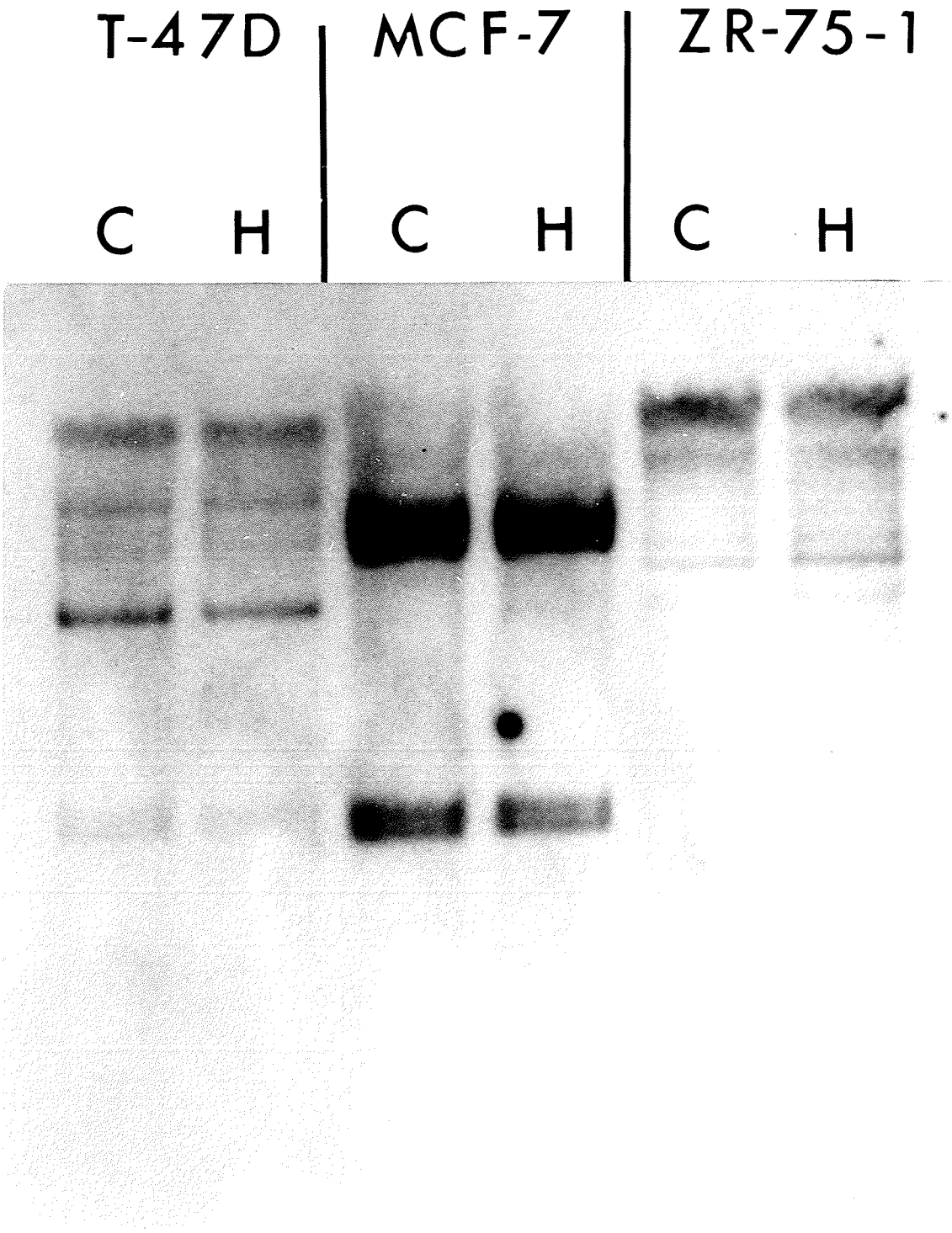
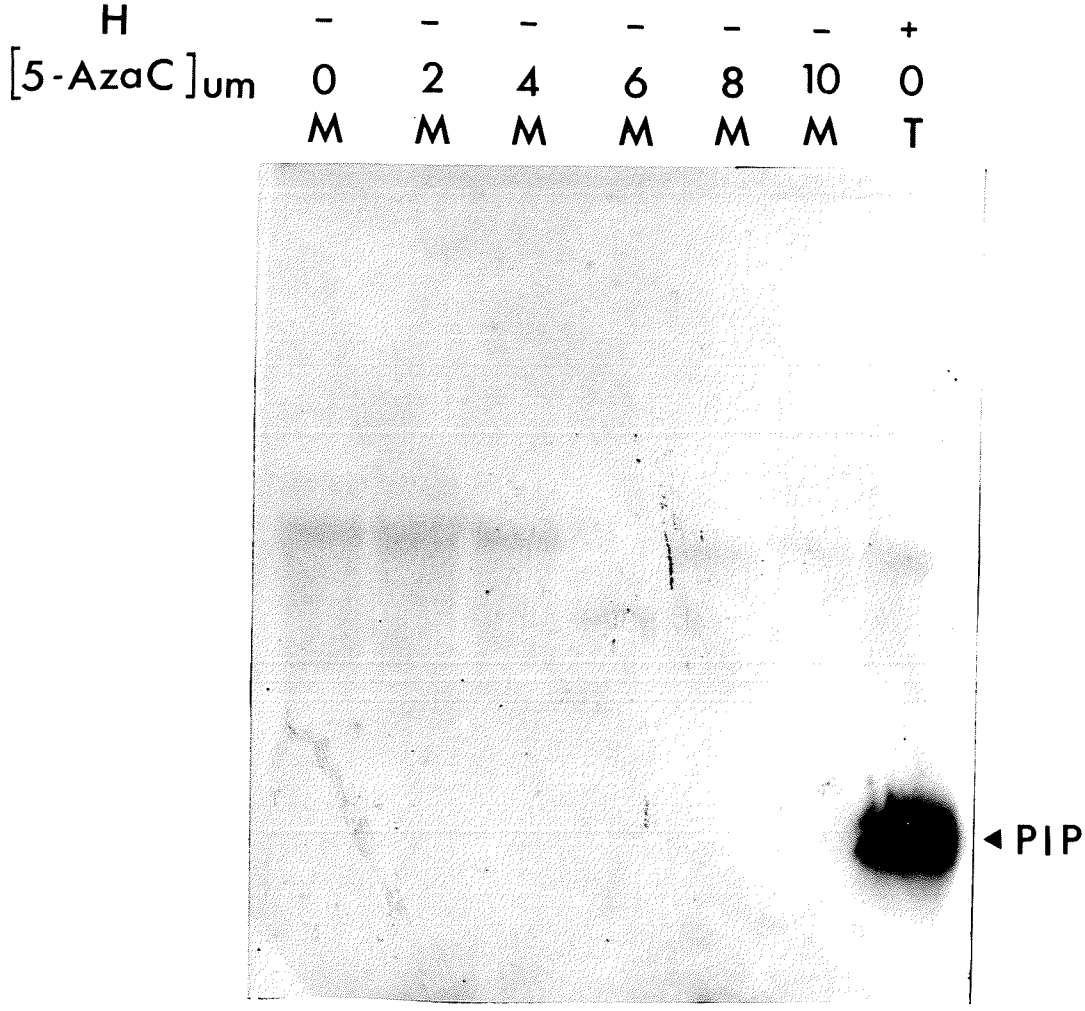

T-47D
MCF-7
ZR-75-1
C
H
C
H
C
H

FIGURE 11. Northern analysis of RNA from MCF-7 cells treated with 5-Azacytidine. MCF-7(M), T-47D(T). + or - indicates the presence or absence of hormone-treatment. Hormone-treated T-47D RNA was included as a positive control to indicate the position of the PIP mRNA. Faint bands in all lanes is due minor nonspecific hybridization to 28S ribosomal RNA. Northern blots were hybridized with PIP cDNA.


H - - - - - - +
[5-AzaC]um 0 2 4 6 8 10 0
M M M M M M T
PIP

Total RNA was also isolated from hormone-treated T47D cells and included in the Northern analysis for use as a positive control. No PIP RNA was detectable in MCF-7 cells regardless of the concentration of 5-AzaC used. Morphological changes including the change of shape and the development of cellular extensions were observed. In addition cells appeared to become localized into discrete clumps and increasing cell death was seen at higher concentrations of 5-AzaC. The 800 bp PIP message is present in the positive control(hormone-treated T47-D cells).

ii) Effect of hormones/Northern analysis

Because the PIP gene is normally hormonally-inducible, MCF-7 cells treated with 5-AzaC were simultaneously treated with hGH and DHT. Figure 12 represents Northern blot showing that a 10uM concentration of 5-AzaC in the presence of hormones does not result in PIP expression. Hormone-treated T-47D cells produce the expected 800 bp PIP message. This experiment was also conducted using lower concentrations of 5-AzaC in the presence of hormones however the Northern blot is not shown because the results were negative and no positive T47-D control was included. Thus the blot is blank and relatively uninformative. The combined results indicate that neither 5-AzaC or 5-AzaC in the presence of hormones can induce PIP expression in MCF-7 cells.

iii) Southern analysis

Genomic DNA was isolated from 5-AzaC-treated MCF-7 cells and analyzed using restriction analysis to determine if the analog was affecting the methylation status of the gene. Figures 13a and 13b show the results of digestion with HpaII, MspI and AvaI. The blot was hybridized with the 1.7 kb genomic probe.

No change in the methylation pattern as detected by HpaII and MspI

FIGURE 12. Northern analysis of RNA from hormone-treated and control MCF-7 cells. H (+ or -) indicates hormone-treatment status. MCF-7(M), T47-D(T). Hormone-treated T-47D RNA was included as a positive control. Northern blots were hybridized with PIP cDNA.

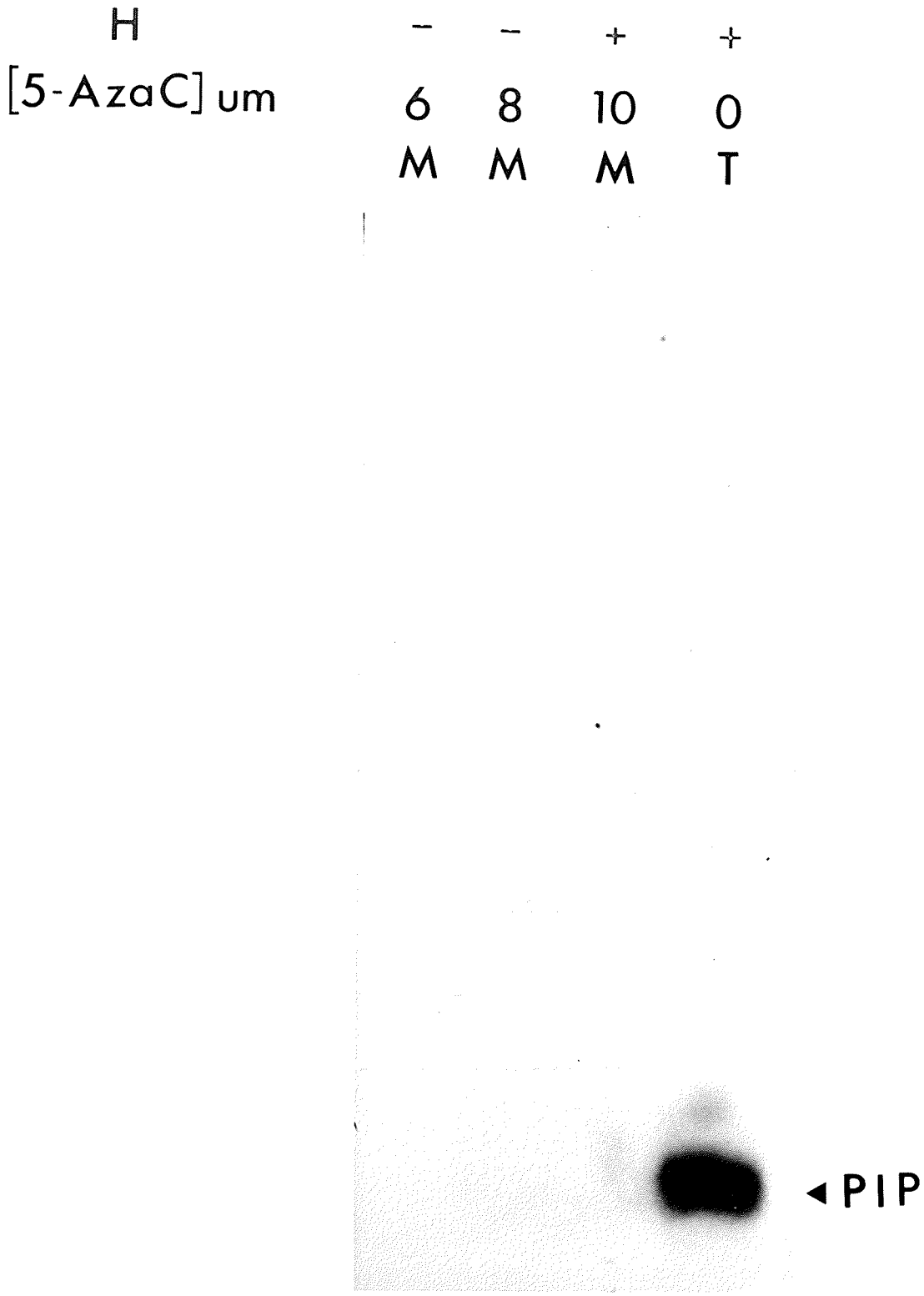
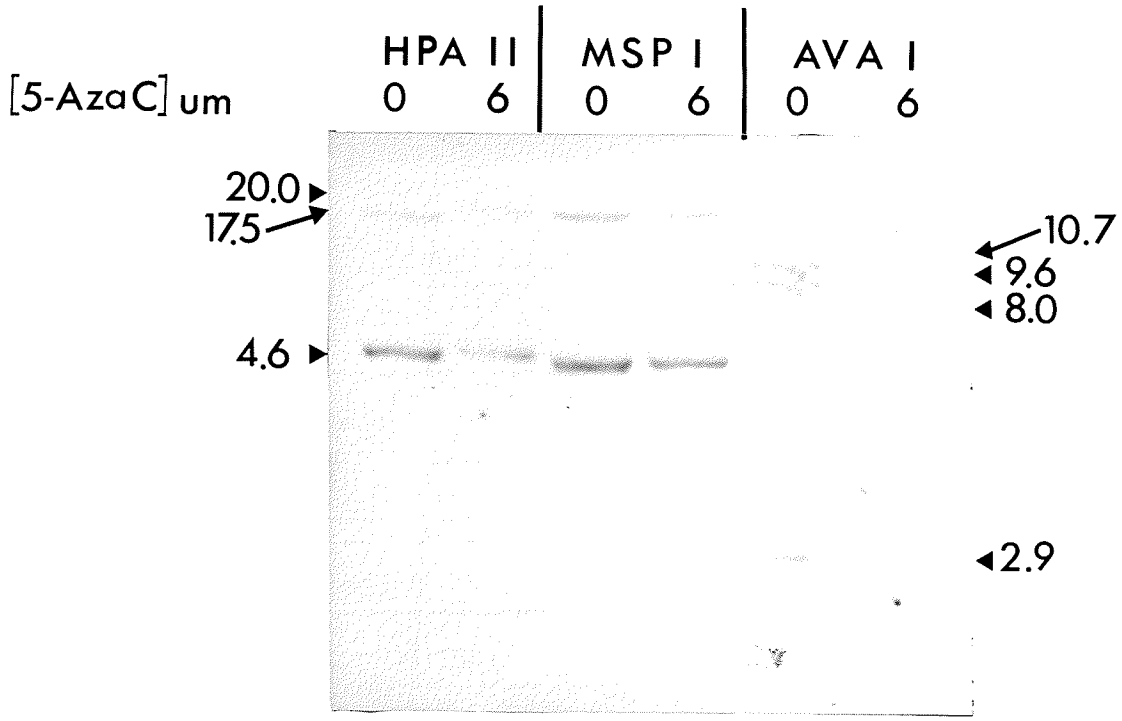
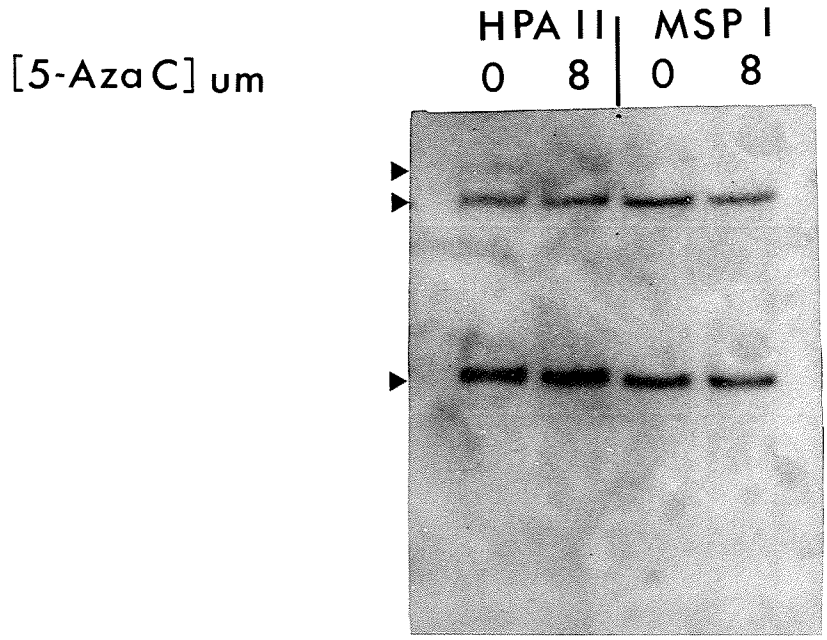

H
-
-
+
+
[5-AzaC] um
6
8
10
0
M
M
M
T
PIP

FIGURE 13a. Hybridization of 1.7 kilobase fragment to DNA isolated from 5-Azacytidine-treated MCF-7 HBC cells. DNA was digested with Hpa11, Msp1 and Ava1.

FIGURE 13b. Hybridization of 1.7 kilobase fragment to DNA isolated from 5-Azacytidine treated MCF-7 HBC cells. DNA was digested with Hpa11 and Msp1.


[5-AzaC] um
HPA II
MSP I
AVA I
0
6
0
6
0
6
20.0
17.5
4.6
10.7
9.6
8.0
2.9


[5-AzaC] um
HPA II
MSP I
0
8
0
8

was observed due to treatment with 5-AzaC. The expected bands of 20.0, 17.5 and 4.6 kb were observed with HpaII digestion regardless of whether or not the cells had been treated with 5-AzaC. MspI cleavage again showed the disappearance of the 20.0 kb band. AvaI cleavage produces four bands of MWs 10.7, 9.6, 8.0, anb 2.9 kb in both treated and untreated samples. These results correspond with the previously-discussed restriction enzyme methylation studies as well as the absence of the PIP message due to treatment with 5-AzaC. These results suggest that methylation of cytosine residues in the PIP gene is not playing a role in its expression.

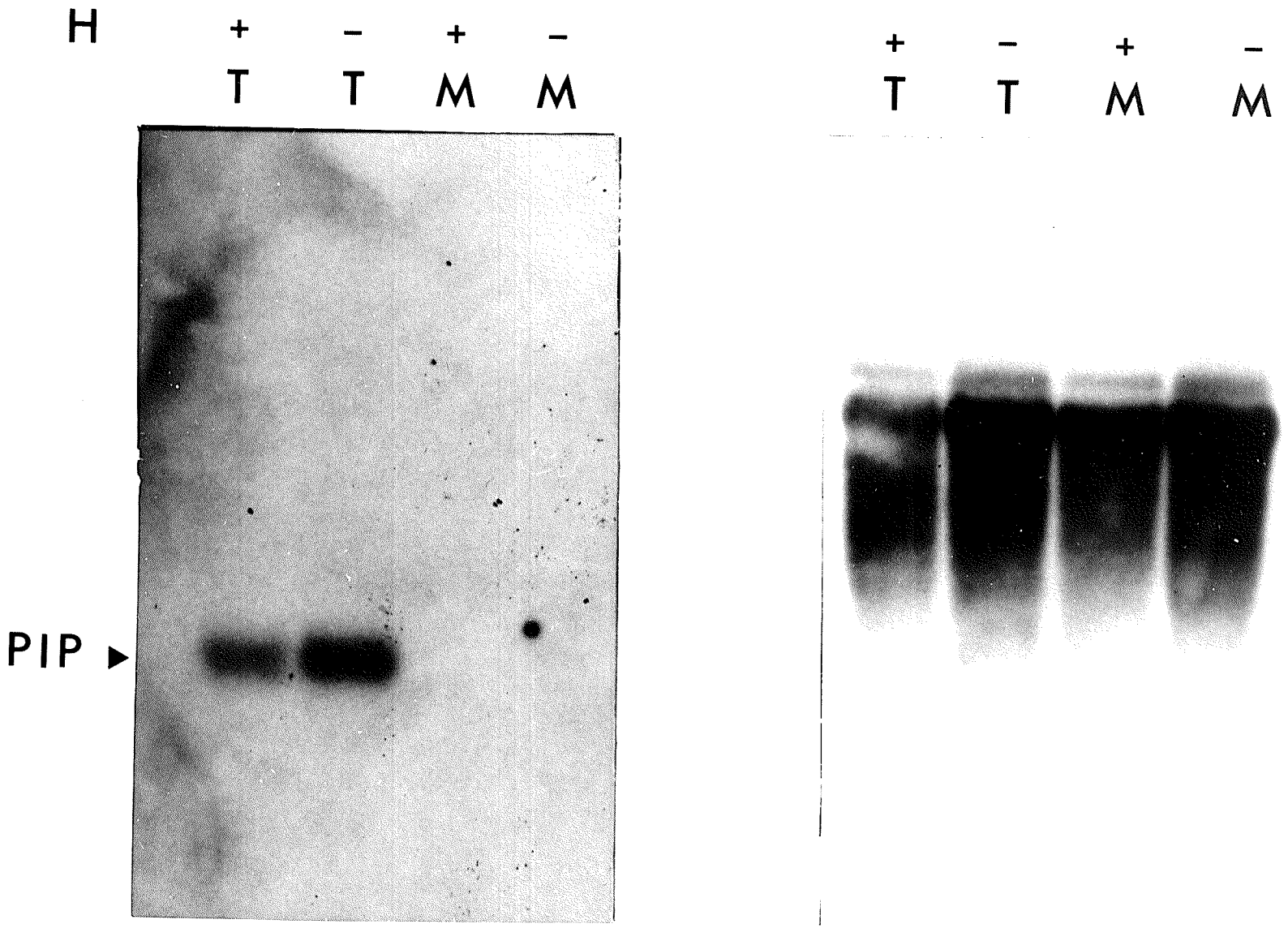
2. SODIUM BUTYRATE EXPERIMENTS

In order to determine if sodium butyrate treatment could affect PIP expression in HBC cell lines, T47-D and MCF-7 cells were treated with sodium butyrate(4 mM) and DHT and hGH. This experiment was originally carried out using 7 mM NaBUT which proved to be cytotoxic. Most other groups who have used NaBut in tissue culture experiments have found success using concentrations ranging from 1mM to 10 mM.

Figure 14a shows a Northern analysis of total RNA isolated from both MCF-7 and T-47D cells treated with NaBut. One group of cells within each type was treated with hormones (hGH and DHT) in addition to NaBut to determine if NaBut had any effect upon the hormonal induction of the PIP gene. Both hormone and NaBut/hormone-treated T47-D cells possess the 800 kb PIP mRNA. A weaker message from the NaBut/hormone-treated T47-D cells appears to be due to unequal loading of RNA samples. This is confirmed both through observation of the photograph of the gel and by figure 14b which shows the same Northern blot stripped and rehybridized with a 28S ribosomal

FIGURE 14a. Northern analysis of total RNA from sodium butyrate-treated HBC cells hybridized with PIP cDNA. H indicates presence(+) or absence(-) of hormone-treatment. T-47D(T) and MCF-7(M) cells were treated with 4 mM sodium butyrate for 24 hours. Northern blot was hybridized with PIP cDNA.

FIGURE 14b. Northern analysis of total RNA from sodium butyrate-treated HBC cells hybridized with 28S ribosomal probe. T-47D(T), MCF-7(M).


H
+
T
−
T
+
M
−
M
PIP

+
T
−
T
+
M
−
M

probe. It is evident that less RNA is present in the first lane containing hormone-treated T-47D RNA. Thus NaBut does not appear to effect the hormonal induction of the PIP gene in T-47D cells.

NaBut treatment of MCF-7 cells does not increase the expression of the PIP gene as no detectable message is present(figure 14a). Thus in both cell lines acetylation of histones caused by NaBut treatment does not appear to have an effect upon the expression or hormone-inducibility of the PIP gene.

3. POLYMORPHISM/REARRANGEMENT

Because the chromosomal location of the PIP gene is in a highly polymorphic region and it is differentially expressed in HBC cell lines and *in vivo*, experiments were performed to determine if the gene is polymorphic or rearranged. These studies essentially involved extensive restriction enzyme analysis of DNA obtained from HBC cell lines and lymphocytes of normal subjects and breast cancer patients.

Figures 15 to 26 show the result of Southern analysis of genomic DNA digested with a wide range of restriction enzymes. Several of the genomic DNA samples were inadvertantly contaminated with plasmid from an undetermined source in the lab during the isolation of DNA. The bands present on the Southern blots that are due plasmid DNA contamination were determined by stripping and rehybridizing blots with a plasmid probe. Plasmid bands are indicated on the figures with a dark arrow and the symbol "pl". In addition, because of the large number of restriction digests performed not all samples were thoroughly digested. Bands due to incomplete digestion were determined by comparison of the same samples between different blots as well as examination of the picture of the gel stained with ethidium bromide. Undigested DNA usually shows ethidium bromide-stained

strands extending from the wells in the gel. The bands that are present due incomplete digestion have been marked by an open arrow.

Figures 15a and 15b show the results of HindIII digestion using the 1.7 kb DNA fragment and the cDNA as probes respectively. Fig 16a compares the restriction pattern of a series of DNA samples hybridized with the 1.7 kb genomic probe. Figure 16b represents the same Southern blot stripped and rehybridized with a polymorphic probe(PSW50.5) derived from chromosome eight. The expected frequencies of two bands of 3.4 and 2.6 kb are observed. This indicates that HindIII enzyme is cutting the DNA to completion and yielding the correct fragments with another probe which detects polymorphism.

Figure 17 represents a HindIII digest of a variety of HBC cell line DNA samples using the 1.7 kb probe. The HindIII digestions hybridized with the 1.7 kb probe yields two bands of MW 10.0 and 6.5. Only the 10.0 is present with hybridization using the cDNA probe. Thus the results of figures 16 to 18 show that HindIII digestion shows no detectable polymorphism or rearrangement of the PIP gene in any DNA samples.

Figures 18a and 18b show a PstI digestion human breast cell line, patient and control lymphocyte DNA using the 1.7 and cDNA probes respectively. Three bands of MW 9.5, 3.1 and 2.8 kd are detected with the 1.7 kb probe. Only the 3.1 kb and 2.8 kb MW bands are in common between the cDNA and the 1.7 fragment. The cDNA probe also detects two additional bands of MWs 4.2 and 1.0. Excluding plasmid bands and uncut DNA no differences are observed between the various DNA samples.

Figures 19a and 19b show the restriction pattern obtained with an EcoRI digest using the 1.7 and cDNA probes. DNA from patient #6 and control #13 were incompletely cut. The 1.7 kb probe detects only one 6.0 kb band and

FIGURE 15a. HindIII digestion of DNA from HBC cell lines, and lymphocytes of breast cancer patients and normal individuals(controls), hybridized with 1.7 kb genomic probe. T47-D(T), MCF-7 (M). Sizes of restriction fragments are given in kilobases. pl indicates plasmid DNA. Numbers identify seperate individuals in control and patient DNA samples.

FIGURE 15b. HindIII digestion of DNA: Rehybridization of Southern blot from figure 16a with PIP cDNA probe.

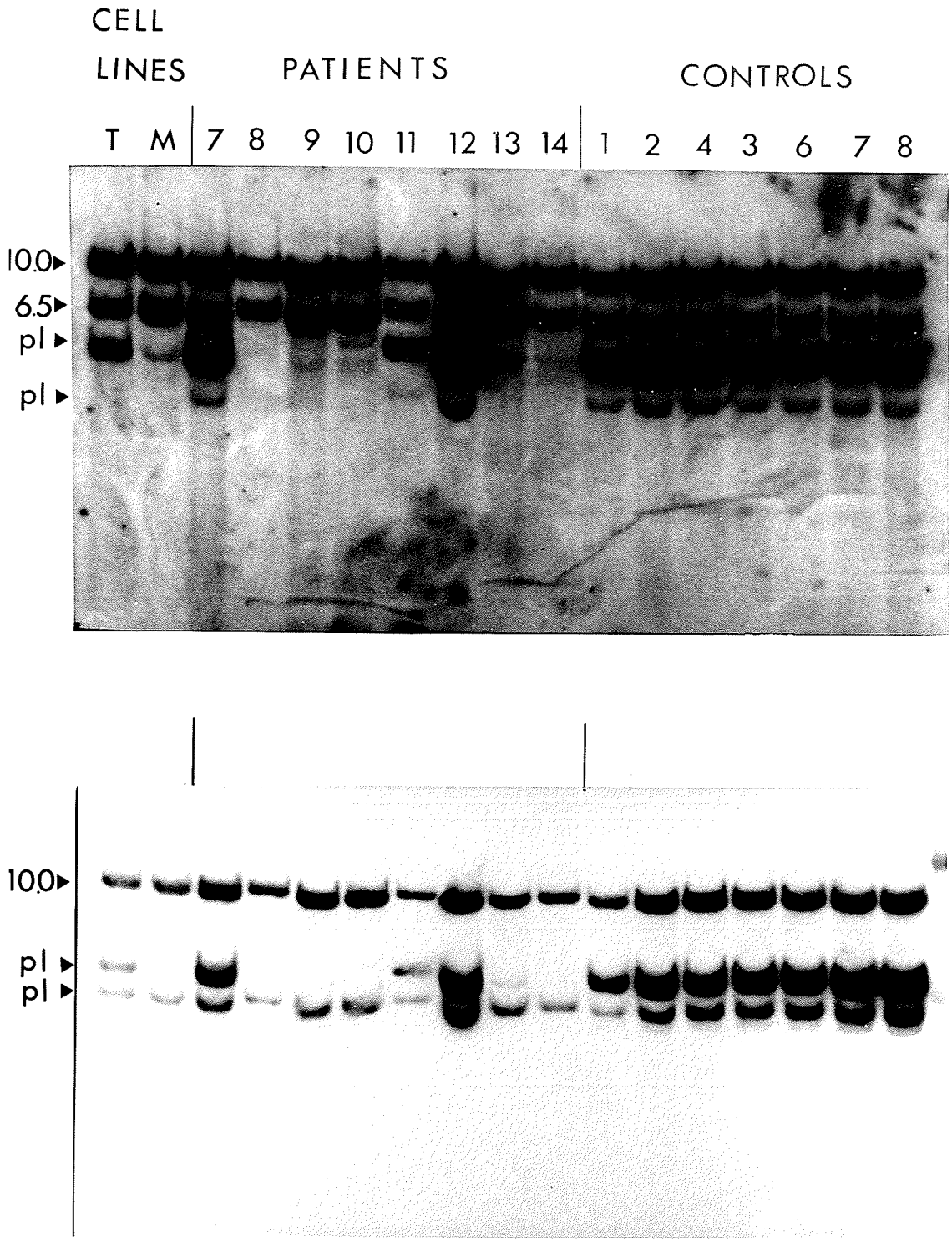

CELL
LINES
PATIENTS
CONTROLS
T M 7 8 9 10 11 12 13 14 1 2 4 3 6 7 8
10.0
6.5
pl
pl
10.0
pl
pl

FIGURE 16a. HindIII digestion of DNA from HBC cell lines, lymphocytes of breast cancer patients and normal individuals hybridized with the 1.7 kilobase fragment. T47-D(T), MCF-7(M). pl indicates plasmid DNA.

FIGURE 16b. HindIII digestion of DNA : rehybridization of Southern blot from Fig. 16a with a polymorphic probe (SW50.1) from chromosome 8. Faint upper bands is due to residual radioactivity from 1.7 kb probe that was not adequately stripped before rehybridization.

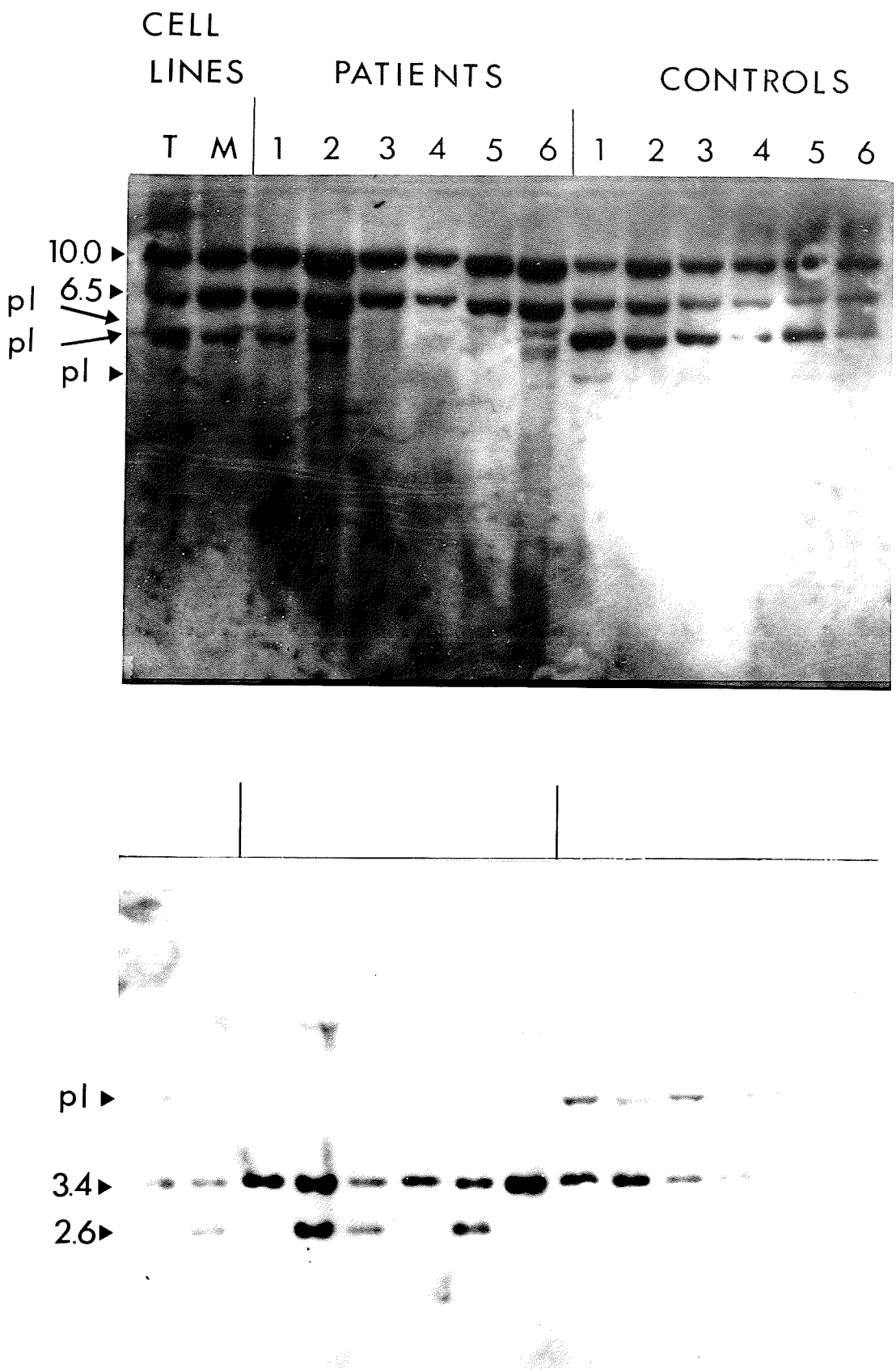

CELL LINES
PATIENTS
CONTROLS
T M 1 2 3 4 5 6 1 2 3 4 5 6
10.0
6.5
pI
pI
pI
pI
3.4
2.6

FIGURE 17. HindIII digestion of DNA from HBC cell lines, a non-cancerous human breast cell line and placenta hybridized with the 1.7 kilobase fragment. HBC cell lines T47-D(T), T47-D mutant #5(T5), T47-D clone #11(T11), MCF-7(M), MDA-Mb-231(M2), ZR-75-1(Z), non-cancerous human breast cell line HBL-100(H) and normal placenta(P). pl indicates plasmid DNA.

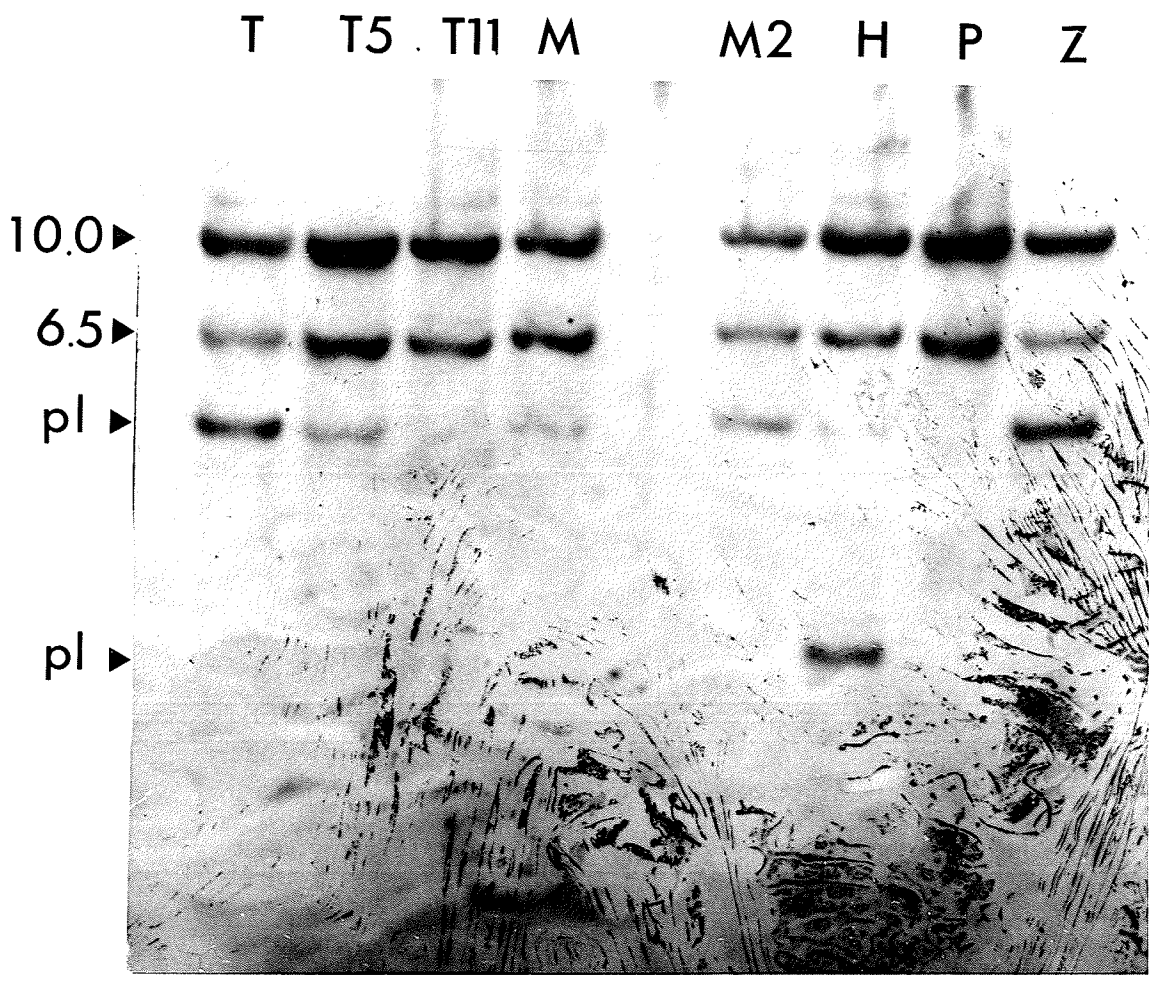

T
T5
T11
M
M2
H
P
Z
10.0
6.5
pl
pl

FIGURE 18a. Pst1 digestion of DNA from human breast cell lines, lymphocytes of breast cancer patients and normal individuals(control) hybridized with the 1.7 kilobase fragment. HBC cell lines T47-D(T), T47-D mutant #5(T5), MCF-7(M), T47D clone #11(T11), a non-cancerous breast cell line HBL-100(H). Open arrows indicate fragments due to incomplete digestion of DNA, pl indicates plasmid DNA.

FIGURE 18b. Pst1 digestion of DNA; rehybridization of Southern blot in Fig 18a with PIP cDNA probe.

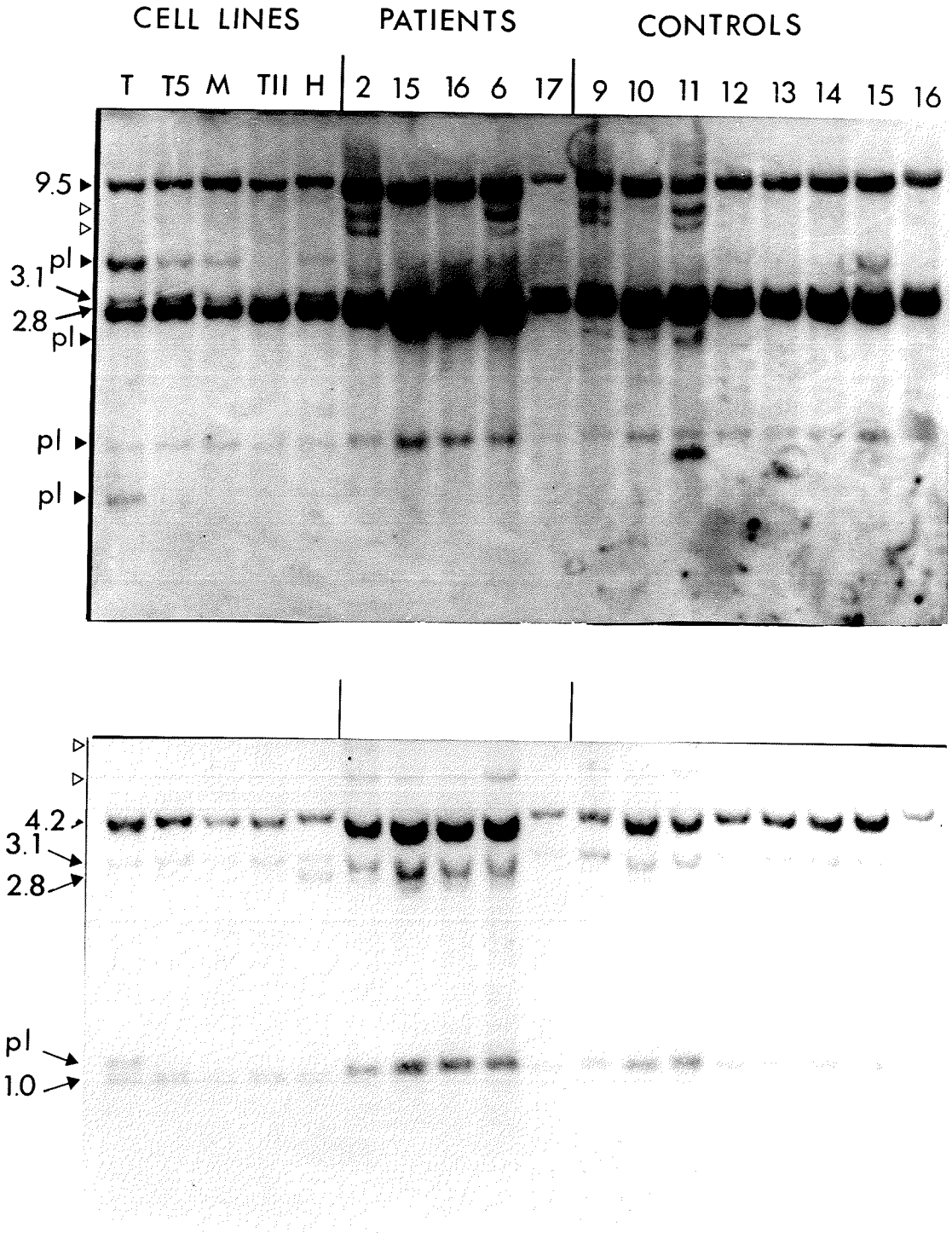
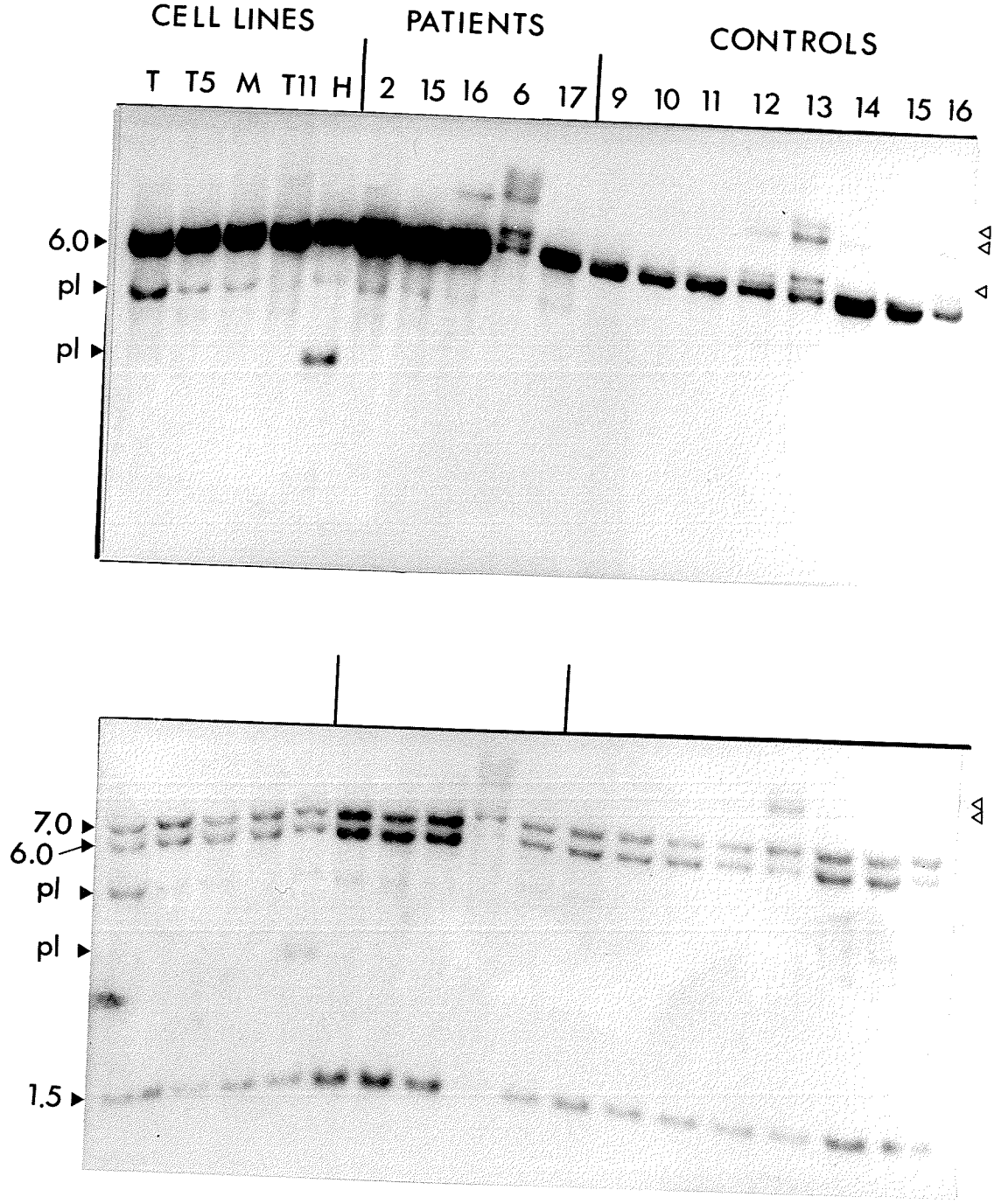

CELL LINES
PATIENTS
CONTROLS
T T5 M TII H 2 15 16 6 17 9 10 11 12 13 14 15 16
9.5
pl
3.1
2.8
pl
pl
pl
4.2
3.1
2.8
pl
1.0

FIGURE 19a. EcoRl digestion of DNA from human breast cell lines, lymphocytes of breast cancer patients and normal subjects(controls) tes hybridized with the 1.7 kilobase fragment. HBC cell lines T-47D(T), T-47D mutant #5(T5), MCF-7(M), T-47D clone #11(T11), a non-cancerous human breast cell line HBL-100(H). Open arrows indicate fragments due to incomplete digestion, pl indicates plasmid DNA.

FIGURE 19b. EcoRI digestion of DNA; rehybridization of Southern blot in Fig. 19a hybridized with PIP cDNA probe.


CELL LINES
PATIENTS
CONTROLS
T T5 M T11 H 2 15 16 6 17 9 10 11 12 13 14 15 16
6.0
pl
pl
7.0
6.0
pl
pl
1.5

hybridization with the cDNA shows three bands of MWs 7.0, 6.0 and 1.5 kbs. No structural differences are apparent in these 18 DNA samples tested using either probe.

Xba1 digestion shown in figures 20a and 20b show one 5.0 kb band using the 1.7 kb fragment as a probe and three bands of MWs 5.0, 3.0 and 1.8 as detected by the cDNA. Only one band of MW 23.2 is detected with the 1.7 kb probe in a BamHI digestion(figure 21a) while the cDNA probe detects both the 23.2 MW and a 10.0 MW band(figure 21b). No rearrangement or polymorphism is seen with either enzyme.

Figures 22 to 25 represent the results of digestion with Msp1, Xmn1, Taq1, and Pvull, four enzymes used extensively in polymorphism studies. All four Southern blots were hybridized with the 1.7 kb probe. Msp1 digestion(figure 22) yields two bands of MWs 17.5 and 4.6. Four bands of MWs 6.6, 3.0, 1.6, and 0.7 are observed with Xmn1 cleavage(figure 23). Taq1 digestion(figure 24) shows two bands of MWs 3.7 and 2.2 while Pvull(figure 25) cleavage yields two bands 23.2 and 4.9 kb in size. The restriction patterns of the PIP gene obtained using these enzymes indicate that no rearrangement or polymorphism has occurred.

Figure 26 represents the results of digestion of DNA from several cell lines with Alu1 and Bgl1 hybridized with the PIP cDNA probe. Alu 1 digestion yields two bands of MW 1.3 and 0.8 kb. There appears to be no sites for Bgl1 in the PIP gene detectable by the cDNA probe. Neither enzyme detects any rearrangement or polymorphism.

In summary, digestion of genomic DNA derived from the HBC cell lines and lymphocytes of normal subjects and breast cancer patients with 11 common restriction enzymes show no difference in the structure of the PIP gene.

FIGURE 20a. Xbal digestion of DNA from HBC cell lines, lymphocytes of breast cancer patients and normal subjects(control), hybridized with 1.7 kilobase fragment. T-47D(T), T-47D clone #11(T11), T-47D mutant #5(T5), MDA-MB-231(M2), MCF-7(M). Open arrows indicate fragments due to incomplete digestion of DNA, pl indicates plasmid DNA.

FIGURE 20b. Xba 1 digestion of DNA; rehybridization of Southern blot from Fig. 20a hybridized with PIP cDNA probe.

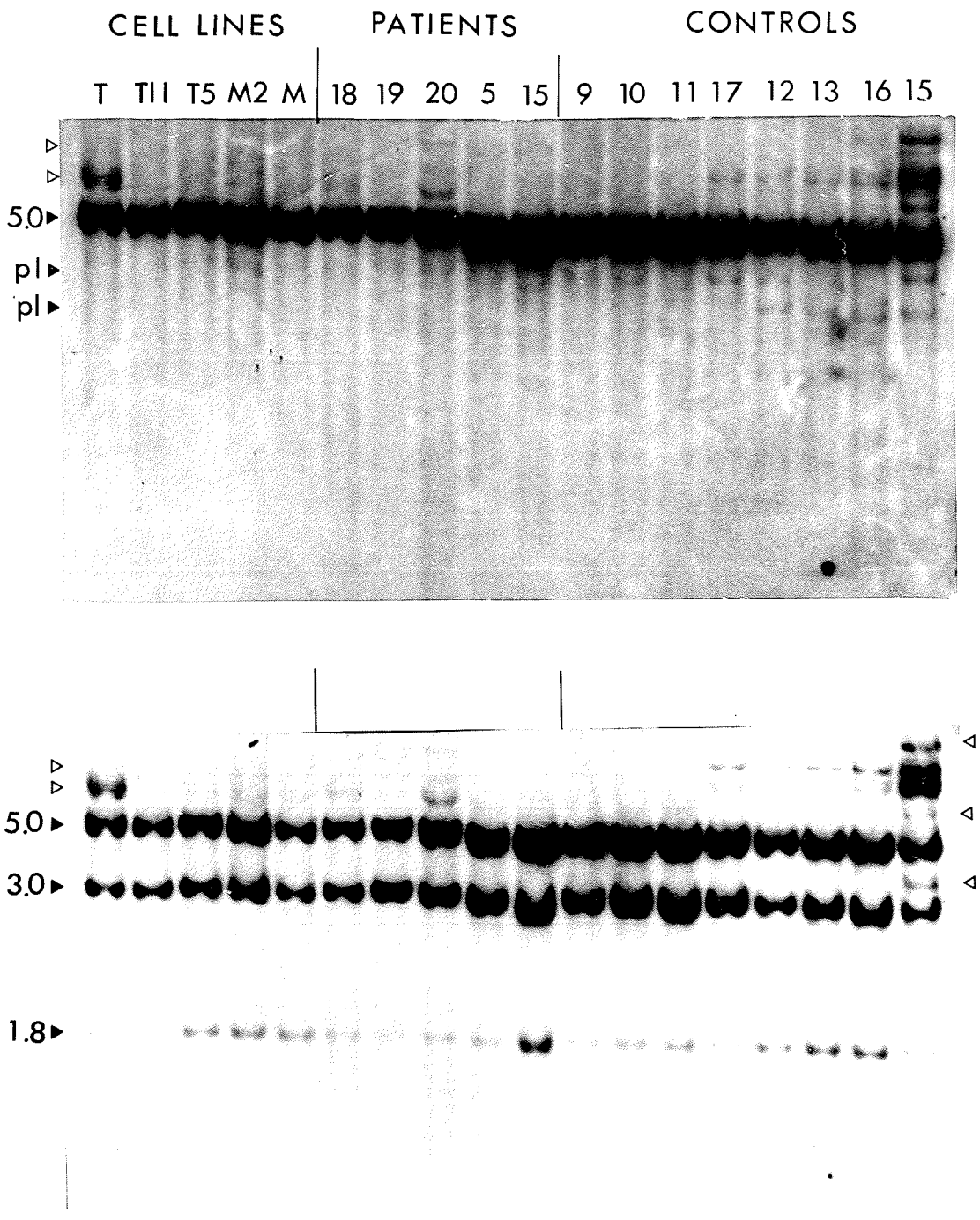

CELL LINES
PATIENTS
CONTROLS
T TI1 T5 M2 M 18 19 20 5 15 9 10 11 17 12 13 16 15
5.0
pl
pl
5.0
3.0
1.8

FIGURE 21a. BamHI digestion of DNA from HBC cell lines, lymphocytes of breast cancer patients and normal(control) subjects hybridized with 1.7 kilobase fragment. HBC cell lines T47-D(T), T-47D clone #11(T11), T-47D mutant #5(T5), MDA-MB-231(M2), MCF-7(M). pl indicates bands due to plasmid DNA.

FIGURE 21b. BamHI digestion of DNA; rehybridization of Southern blot in Fig. 21a with PIP cDNA probe.

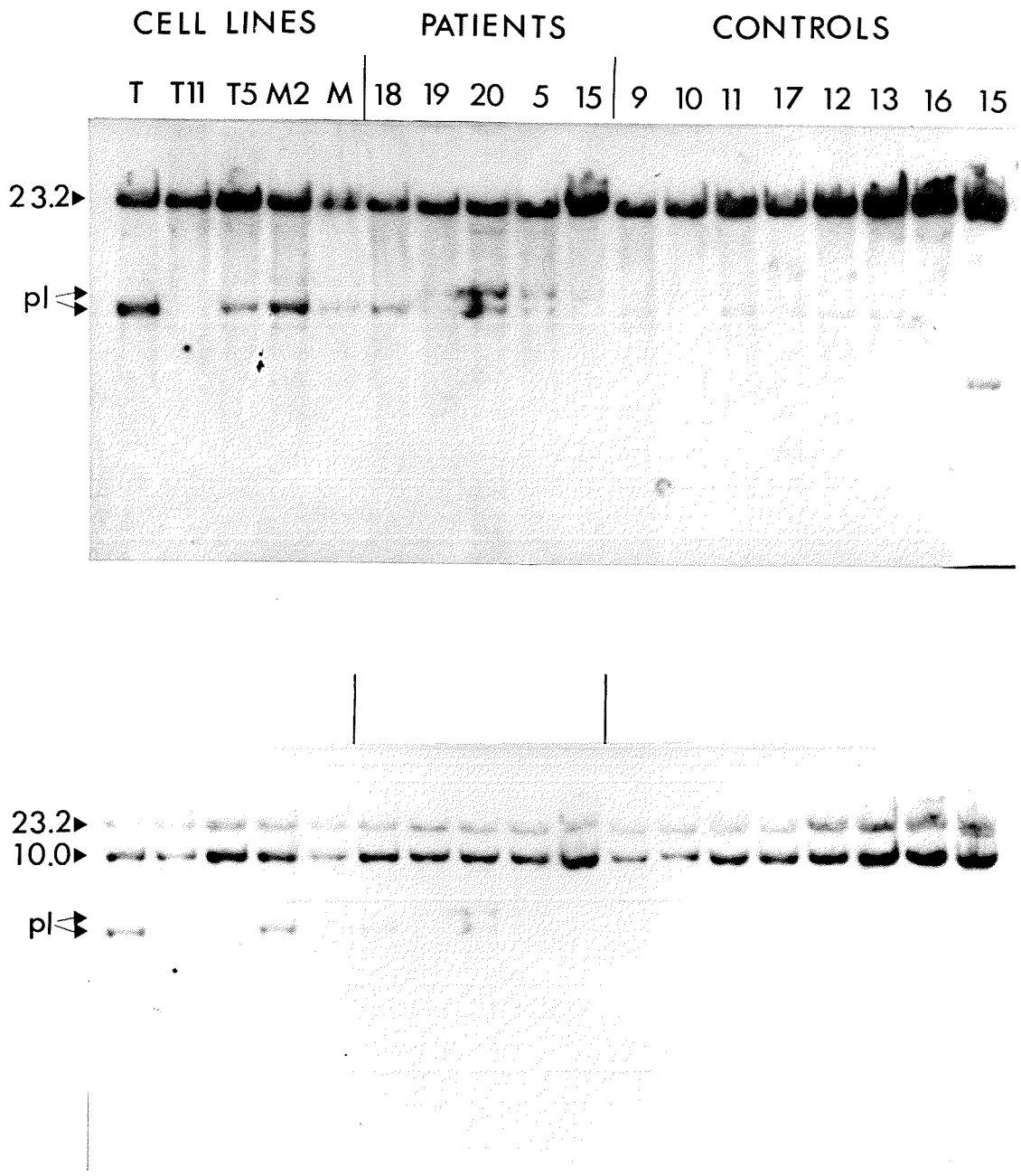

CELL LINES
PATIENTS
CONTROLS
T T11 T5 M2 M 18 19 20 5 15 9 10 11 17 12 13 16 15
23.2
pl
23.2
10.0
pl

FIGURE 22. MspI digestion of DNA from lymphocytes of normal and lymphocytes of breast cancer patients and from breast cell lines hybridized with 1.7 kilobase fragment. Human breast cancer cell lines T-47D(T), T-47D mutant #5(T5), MCF-7(M), a non-cancerous breast cell line HBL-100(H). pl indicates plasmid DNA, open arrow indicates fragments due to incomplete digestion.

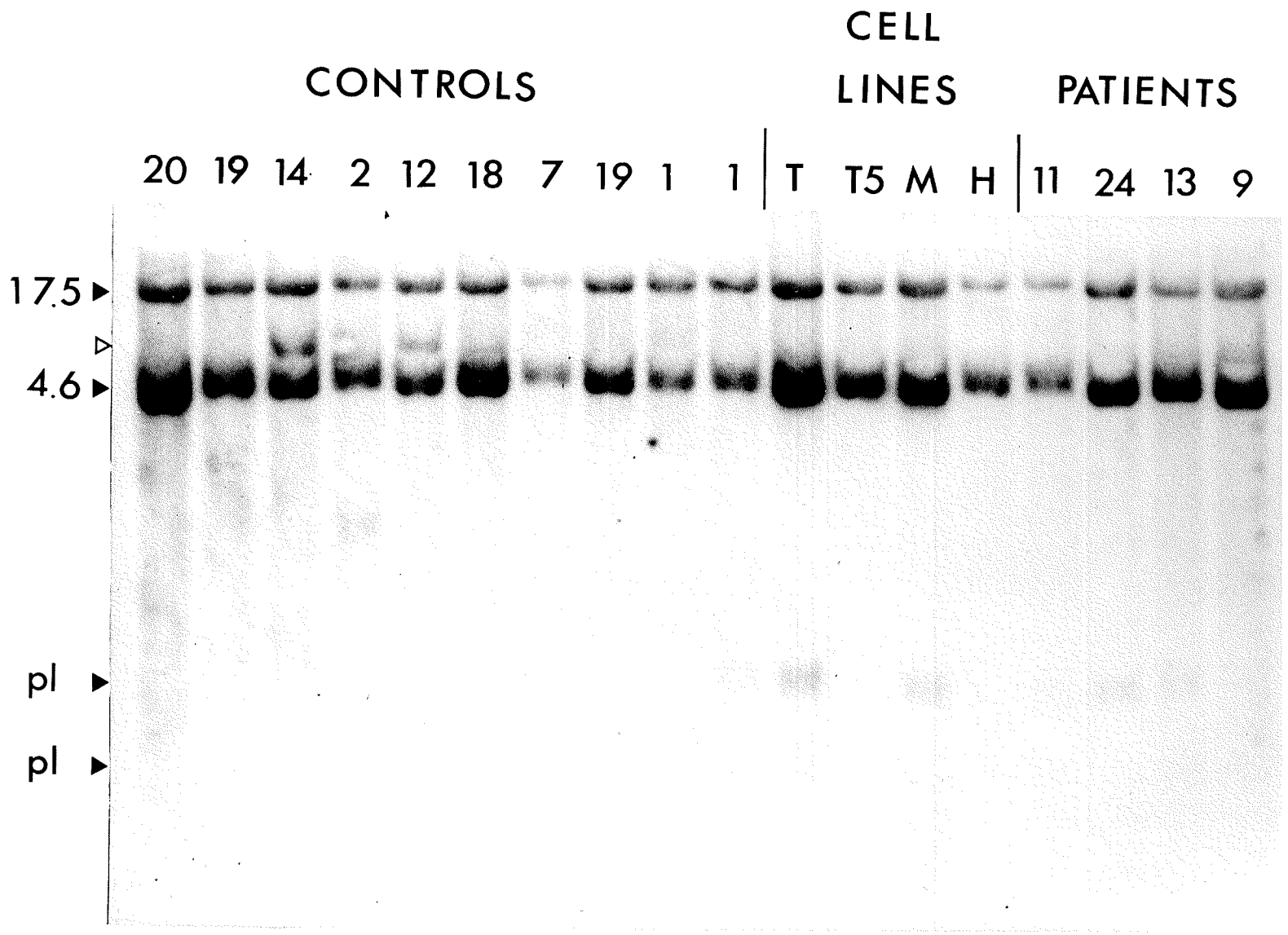

CONTROLS
CELL LINES
PATIENTS
20 19 14 2 12 18 7 19 1 1
T T5 M H
11 24 13 9
17.5
4.6
pl
pl

FIGURE 23. Xmnl digestion of DNA from HBC cell lines, and lymphocytes of breast cancer patients and normal(control) subjects hybridized with 1.7 kilobase probe. T-47D(T), MCF-7(M), MDA-MB-157(M1), MDA-MB-231(M2). pl indicates bands due to plasmid DNA, open arrows indicate fragments due to incomplete digestion of DNA.

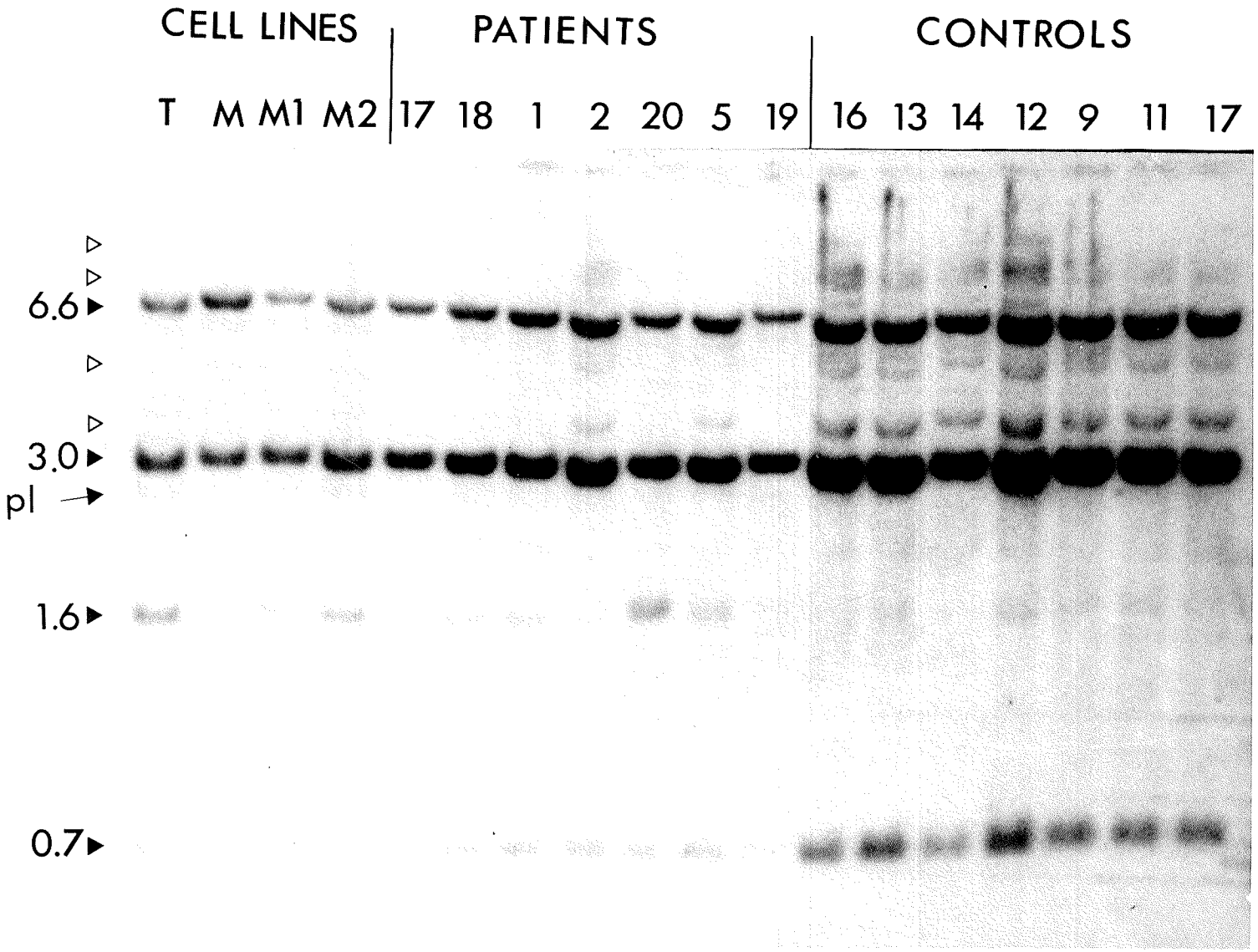

CELL LINES
PATIENTS
CONTROLS
T M M1 M2 17 18 1 2 20 5 19 16 13 14 12 9 11 17
6.6
3.0
pI
1.6
0.7

FIGURE 24. TaqI digestion of DNA from T-47D, and from lymphocyes of breast cancer patients and normal control subjects hybridized with 1.7 kilobase probe. pl indicates plasmid DNA, open arrows indicate fragments due to incomplete digestion of DNA.

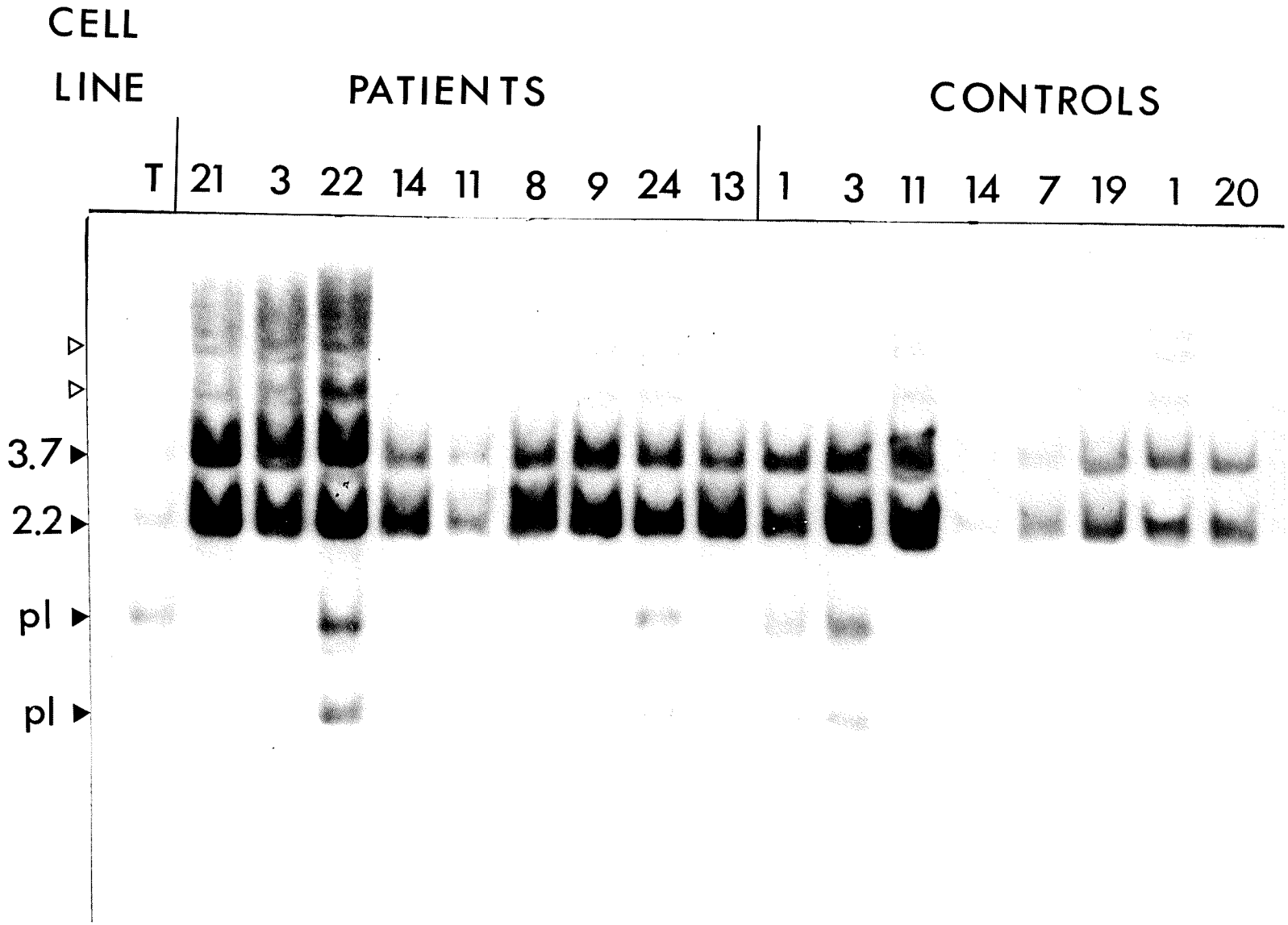

CELL
LINE
PATIENTS
CONTROLS
T
21
3
22
14
11
8
9
24
13
1
3
11
14
7
19
1
20
3.7
2.2
pl
pl

FIGURE 25. PvuII digestion of DNA from lymphocytes of breast cancer patients and normal subjects hybridized with 1.7 kilobase fragment. pl indicates plasmid DNA.

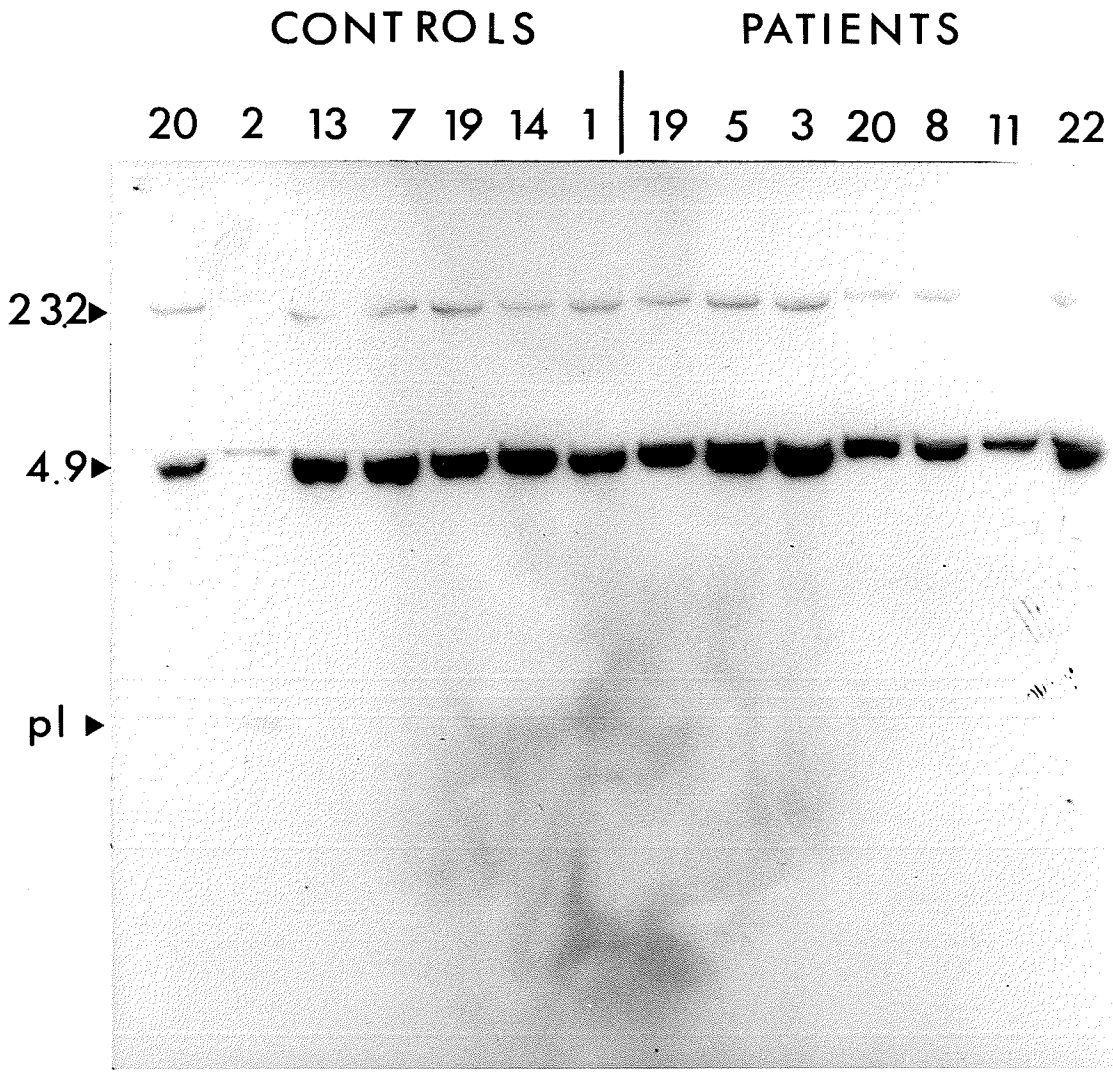
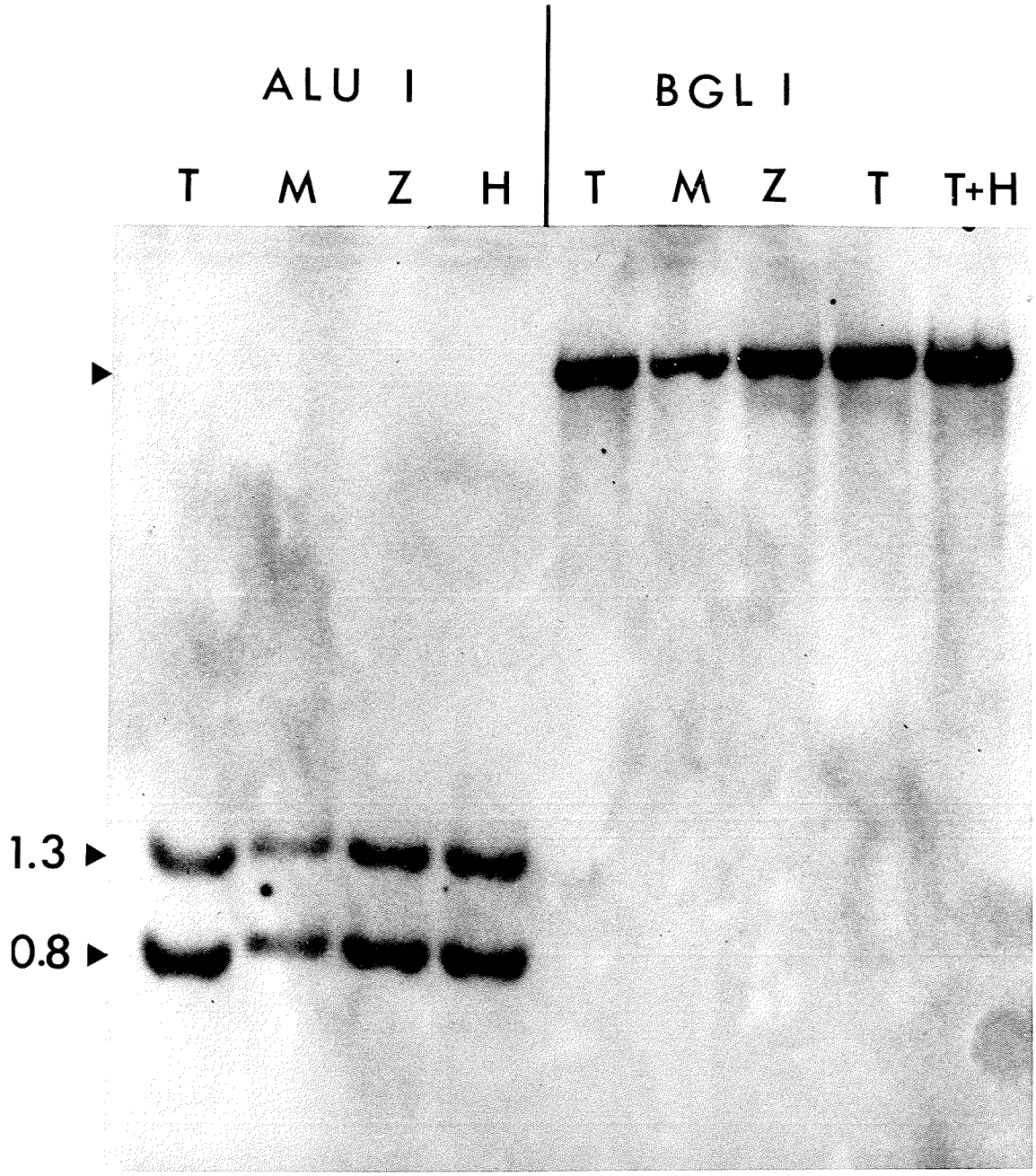

CONTROLS
PATIENTS
20 2 13 7 19 14 1 19 5 3 20 8 11 22
23.2
4.9
pI

FIGURE 26. Alu1 and Bgl1 digestion of human breast cell lines hybridized with PIP cDNA. HBC cell lines T-47D(T), MCF-7(M), ZR-75-1(Z), a non-cancerous human breast cell line HBL-100(H).


ALU I
BGL I
T
M
Z
H
T
M
Z
T
T+H
1.3
0.8

DISCUSSION

1. METHYLATION STUDIES

A large body of evidence exists which suggests that methylation of cytosine residues in DNA acts as a gene silencing mechanism. As mentioned, the PIP gene is expressed in some HBC cell lines but not others. Based on these factors, a possibility existed that methylation was contributing to the lack of PIP expression in HBC cell lines such as MCF-7. Two methods were employed to study the methylation status of the PIP gene in this thesis. The first involved the use of methylation-sensitive restriction enzymes, which have been used with success by other groups to detect the methylation status of specific genes. In many instances a strong correlation between gene expression and the methylation pattern detected by the restriction enzymes has been made. The second approach was one of chemical manipulation, i.e. an attempt to artificially demethylate the PIP gene in MCF-7 cells using 5-Azacytidine to see if the PIP gene would become actively expressed in the absence of the silencing effects of methylation.

i) Restriction enzyme analysis

The most widely used enzymes used to study the methylation status of genes are HpaII and MspI. As mentioned, both recognize the sequence CCGG, yet HpaII will not cleave if the internal cytosine residue is methylated. Thus a comparison of methylation patterns can be made within and between different cell lines. Any difference between the restriction patterns generated with HpaII and MspI indicates the presence of methylated cytosine residues. A similar restriction fragment pattern generated by both enzymes suggests that all CCGG sites being cleaved are not methylated. In addition, comparison of the HpaII pattern between cell lines detects methylation

differences that are cell line specific.

Studies of the PIP gene using HpaII and MspI digestion and the PIP cDNA as a probe, suggest that T-47D, the highest PIP expressor, is also one of the more highy methylated HBC cell lines tested. Indeed, the contrast appears to be greatest between T-47D and MCF-7(the poorest PIP expressor). T-47D possesses four detectable methylated sites(or partially methylated sites) that are absent in MCF-7 as seen in HpaII analysis(Figure 1). HBL-100, a "normal" human breast cell line in the sense that it is non-cancerous, also does not express PIP yet possesses a HpaII pattern more similar to that of T-47D than MCF-7. ZR-75-1, a HBC cell line which expresses PIP at negligible levels, also possesses a methylation pattern more closely related to T47-D than MCF-7. ZR-75-1 also has unique bands due to additional methylated sites.

Comparison of the HpaII pattern to the MspI pattern generated within each cell line shows that all of the observed differences using HpaII are due to methylation effects alone as no differences are visible with MspI digestion. Interestingly, MCF-7 possesses the same HpaII and MspI patterns(Figures 1 and 2) indicating that all CCGG sites detected by the PIP cDNA probe are nonmethylated in this non-expressing cell line. In contrast, T47-D is more methylated compared to MCF-7 as detected by HpaII/MspI analysis using the PIP cDNA probe. These results suggest that there is no correlation between hypomethylation and the presence of PIP expression in the HBC cell lines tested. Indeed, if any could be made it would be the opposite, i.e. a correlation between hypomethylation and a lack of active gene expression.

Studies performed involving HpaII/MspI analysis of a variety of HBC cell lines using the 1.7 kilobase fragment as a probe show a wide variation

in the methylation status of the PIP gene(Figure 3). Again, comparison with MspI digestion(Figure 4) shows that the differences in the restriction fragments observed with HpaII digestion are due to methylation effects alone. Comparison a T-47D to MCF-7 reveals that each cell line possesses a unique fragment(Figure 3) generated due to the presence of distinct methylated sites. Thus there is no apparent correlation between methylation and expression in this case either unless one speculates that the 20 kb band present in MCF-7 that is absent in T47-D represents a methylated CCGG site that is crucial to PIP expression. The other cell lines analyzed all appear to possess distinct methylation patterns yet no overall correlation can be made suggesting a link between PIP expression and the presence or absence of methylated cytosine residues.

AvaI, another methylation-sensitive enzyme, also detects a range of methylation patterns between the various HBC cell lines(Figures 5 and 6a and b). Because no isoshizomer is available with which to analyze methylation within a specific cell line, one cannot rule out the possibility that the observed differences are due to other factors such as polymorphism. However, in light of the evidence presented in a following section, this is unlikely to be the case. Again a striking difference in methylation is observed between T-47D and MCF-7. MCF-7 lacks the 25.0, 7.0 and 4.9 kilobase bands present in T47-D, suggesting the presence of methylated sites in T47-D that are absent in MCF-7. MCF-7 also possess a band of approximately 8.0 kilobases that is absent in T47-D. However, if a correlation is to be made again it is one between increased methylation and expression of PIP. Thus evidence is presented which suggests that in the case of the PIP gene, methylation is not acting as silencing mechanism.

Before coming to any conclusion regarding methylation and PIP gene

expression based on the restriction enzyme studies, it is necessary to point out the limitations involved using this approach. As mentioned in the introduction section, although the majority of genes studied do show a correlation between expression and methylation as detected by the HpaII/MspI system, this is not true for all genes. Exceptions have been documented where no correlation has been observed(see sec III, pt 3, INTRO.). Indeed, one of the major reasons behind these exceptions as well as the results with PIP may be that the enzymes simply aren't detecting the crucial methylated or unmethylated sequences involved in regulating gene expression. HpaII and MspI only detect one sixteenth of total methylated cytosine residues and the amount detected by AvaI does not significantly increase this number.

Another factor that must also be considered is the possibility that the nucleic acid probe used in the Southern analysis is not detecting the relevant CCGG sites. The PIP cDNA probe only detects coding regions of the gene. The 1.7 kilobase fragment contains both intronic and exonic information and so far is the most 5' genomic DNA probe available in the laboratory with which to conduct such studies. It is possible that the crucial area involved in regulating PIP expression is more 5' than can be detected with the available probes. Indeed, it is well-established that the 5' flanking area of a gene plays the most important role in regulating expression yet the 5' region of the PIP gene has not yet been fully characterized. Thus one must be aware that methylation may be playing a role in governing PIP expression, however because of the limitations involved it is simply not detected using the above approach.

The studies of Jost et al(1984) and Saluz et al(1986) on the hormonally-inducible chicken vitellogenin II gene revealed demethylation induced by hormone treatment. In addition the demethylation was detectable

using Hpall/Mspl analysis. In the studies involving the vitellogenin II gene the hormone involved was the steroid estradiol. The PIP gene is inducible by both steroid hormones(dihydroxy-testosterone and hydrocortisone), as well as prolactin(or growth hormone). Thus there existed the possibility that one of the possible mechanisms through which the hormones exerted their effect on PIP gene expression involved demethylation.

The approach used to study the effect of hormones on PIP gene methylation involved the use of methylation-sensitive restriction enzymes. Keeping the limitations when using this approach in mind, no hormone-induced change in the methylation status of the PIP gene in any of the HBC cell lines was observed(figures 7 to 10). There are several possible explanations for these results. First, the molecular mechanism through which steroids and prolactin exert their effect on PIP expression may not involve demethylation. As mentioned, gene regulation occurs at several different levels and within each level exists a variety of factors which may or may not be affected by hormones. This argument is supported by the restriction enzyme studies discussed above whereby methylation does not appear to be governing PIP expression. Second, it is again possible that the restriction enzymes used are simply not detecting the relevant methylated sites. In this case it would be possible that hormones are indeed affecting methylation but in sites that are not detectable using Hpall and Aval. Third, it is also possible that the sites affected by hormones are in the 5' region of the gene to which there is no corresponding molecular probe yet isolated or characterized.

Thus again no definitive conclusion can be made because of the limitations affecting this type of study. However, the results obtained

suggest that methylation of the PIP gene is not acting as a silencing mechanism in the non-expressing HBC cell lines tested. In addition demethylation does not appear to be one of the mechanisms through which hormones induce PIP gene expression.

In data not shown, DNA was isolated from a breast cancer tumor and digested with both Hpall and Mspl. Hpall digestion revealed a restriction pattern that differed from those of the cell lines. This indicates that in the tumor the PIP gene possessed a unique methylation pattern. This result is not unexpected given the variation in methylation patterns between cell lines. The tumor studies were not pursued for several reasons. First, the heterogeneity of cell types in a tumor makes analysis of the data difficult. For example, a typical breast cancer tumor consists not only of cancer cells but of other cell types such as fibroblasts and adipocytes. Thus one would not be able to rule out the possibility that the difference in cell type would be the cause of a certain result. In addition, no correlation could be made between the methylation status of the PIP gene and its expression as no information as to the presence of the PIP protein in the blood of the pateint was available. Thus minimal emphasis was placed upon tumor analysis.

As mentioned, 5-Azacytidine(5-AzaC) has been used extensively in methylation studies as its incorporation into DNA results in an inhibition of DNA methylation. The 5-AzaC experiments were performed to provide more information regarding the role of methylation in PIP expression in light of the limitations in interpreting results that are present when using restriction enzyme data alone. Theoretically, if methylation was playing a role in silencing PIP expression in a non-expressing cell line such as MCF-7, then by chemically inhibiting methylation one should be able to

induce PIP expression in MCF-7 cells. Normally no PIP RNA is detectable in Northern analysis of RNA derived from either control or hormone-treated MCF-7 cells. Thus experiments were performed whereby MCF-7 cells were treated with 5-AzaC in the presence and absence of hormones and RNA isolated for Northern analysis in an effort to detect the PIP message. No detectable PIP mRNA was present at any of the tested concentrations of 5-AzaC, in the presence or absence of hormone treatment(figures 11 and 12).

Southern analysis of DNA isolated from 5-AzaC-treated MCF-7 cells was also performed to see if the methylation pattern of MCF-7 had been altered(Figures 13a and 13b). Because hybridization using the PIP cDNA probe detects no differences in the HpaII/MspI pattern in MCF-7, the 1.7 kilobase fragment was used as a probe. One methylated site is present in MCF-7(as detected with the 1.7 kilobase piece), while no sites are detected with the PIP cDNA probe. Both HpaII and AvaI anaylsis of the 5-AzaC-treated MCF-7 DNA show no difference from that of DNA from untreated cells. This suggests that 5-AzaC was not incorporated into the one methylated CCGG sequence as detected by HpaII, or any of the methylated sites detected by AvaI. This result is not unusual as many CCGG sequences exist in the genome and only one or two critical methylated sites exist in a specific gene that may be governing their regulation.

The results of the 5-AzaC experiments support those obtained with restriction analysis, i.e. methylation does not appear to be contributing to the lack of PIP expression in MCF-7 cells. One can argue that the 5-AzaC was not incorporated into the methylated sequences in the PIP gene, indeed, this is supported by Southern analysis. However, as mentioned, certain limitations exist with Southern analysis, one being the nature of the nucleic acid probe used. The 1.7 kilobase probe is the most 5' probe

available, however it may not include regions of the gene that are essential in governing its expression. Perhaps if another probe was available a change in the HpaII or AvaI patterns would have been detected indicating that the 5-AzaC had been incorporated into the PIP gene. One cannot rule out these possibilities. In addition, 5-AzaC cannot activate all genes, as the extent of demethylation required to activate some genes may be too extensive and unattainable at non-lethal concentrations.

The combined results of both approaches used to study PIP gene methylation suggest that other mechanisms are governing its expression that do not involve methylation. It is possible that the appropriate cis-acting DNA elements are not present in the 5' end of the gene in non-expressing cell lines. Likewise the necessary trans-acting transcription factors may be absent in non-expressing lines. Once the gene has been fully characterized further studies can be performed in an effort to determine what other structural features are contributing to its expression.

2. HISTONE ACETYLATION

A substantial body of evidence exists in support of a role for histone acetylation and gene expression. However, as with methylation, the relationship between histone acetylation and gene expression is not always consistent, i.e. acetylation of histones is not always associated with active gene expression. One method that has been used in the past by other researchers to test the effects of histone acetylation on gene expression is *in vitro* treatment of cells with Sodium Butyrate(NaBut), a short chain fatty acid known to cause hyperacetylation of histones. Theoretically, if histone acetylation does increase PIP expression, then artificial acetylation of histones in the non-expressing cell line MCF-7 should result in

transcription of the PIP gene. Thus Northern analysis using the PIP gene cDNA as a probe should yield a detectable PIP message in MCF-7 cells after treatment with NaBut. In addition, an experiment was performed whereby T-47D, a cell line which expresses the PIP gene, were treated with NaBut. Thus the effects of NaBut on the hormone-inducibility of the PIP gene in an expressing cell line could be observed.

Figure 14a shows the results of NaBut treatment of T47-D and MCF-7 cells. No detectable PIP message is present in MCF-7 cells treated with butyrate in the presence or absence of hormones. These results suggest that histone acetylation is not a structural feature which contributes to PIP expression. In other words, the absence of histone acetylation is probably not responsible for the lack of PIP expression in MCF-7 cells.

Because histone acetylation has been implicated as a possible mechanism through which hormones exert their effect upon target genes(Csordas et al, 1986), T47-D cells were treated with NaBut in the presence and absence of hormones in an effort to detect any effect of histone acetylation on PIP induction. No substantial difference in PIP expression is caused by histone acetylation in T-47D cells. These results again suggest that acetylation of histones is not a structural feature involved in PIP gene expression. However, the exact role of histone acetylation as it is involved in regulation of gene expression remains controversial. As mentioned, in several cases NaBut treatment has been shown to have no effect or opposing effects upon different genes in the same cell type. This is well demonstrated by the work of Kumar et al(1984) who demonstrated that the induction of different enzymes by hormones in the same cell type were differentially altered by NaBut treatment. In addition, the fact that histone acetylation alone does not affect PIP expression is not surprizing

given that structural modifications of histone proteins are probably only one in a series of regulatory mechanisms affecting transcription.

A major limitation in this experiment that must be considered is the possibility that NaBut treatment was not successful in inducing hyperacetylation of histones in the appropriate region of the PIP gene involved in regulating its expression. Although other studies have shown that the majority of histones(especially H3 and H4, both of which are directly involved in gene regulation) are rapidly acetylated in the presence of NaBut, there is no direct method available to determine whether or not the critical sites involved in regulation of PIP expression were acetylated. This factor must be acknowledged when one considers the results of the NaBut experiment.

3. POLYMORPHISM STUDIES

The final feature that was studied with regard to PIP expression and structure was the possibility that the gene was either rearranged or polymorphic between cell lines and/or individuals. In terms of rearrangment, it is well established that in some cancers genes become rearranged. The rearrangement then contributes to the abberant gene expression involved in malignancy. Thus it was possible that the PIP gene had been rearranged or perhaps mutated in the different cell lines tested and this change was contributing to its variable expression.

In addition, a collaborative effort with the Department of Human Genetics, has revealed that the PIP gene is located in a highly polymorphic region of the long arm of chromosome 7(Myal, 1988). This provides additional evidence that the PIP gene may be polymorphic. As mentioned, polymorphism may be the result of point mutation, deletion or insertion of base pairs or rearrangment of a sequence of DNA.

The method employed to search for polymorphism involved Southern analysis of genomic DNA isolated from a wide variety of HBC cell lines, lymphocytes from breast cancer pateints and from healthy blood donors. A total of 52 different sources of DNA were used in this analysis. In addition, a wide range of restriction enzymes were used including HindIII, PstI, EcoRI, XbaI, BamHI, MspI, XmnI, TaqI, PvuII, AluI and BglI. If the PIP gene is polymorphic it should be detected by several of these enzymes. If a gene is to be useful as a RFLP marker, the polymorphism should be apparent in one out of every six samples tested (Carolyn Gregory, personal communication).

In the majority of experiments, both the 1.7 kilobase fragment as well as the PIP cDNA were used to probe the Southern blots. The results of the polymorphism studies indicate that the structure of the PIP gene is the same in both the expressing and non-expressing HBC cell lines as well as in both healthy individuals and breast cancer patients(figures 15 to 26). No gross rearrangement or changes in the gene can be detected using either probe or any of the restriction enzymes mentioned. This suggests that the structure of the PIP gene has not been altered and is not contributing to the variable expression of the gene between different breast cancer cell lines. Ideally one would expect to see a heritable RFLP which could be correlated to the presence of the PIP protein(thus the transcriptional activity of the gene) both *in vivo* and *in vitro*. No such RFLP has been detected which indicates that unlike other genes located in the same vicinity of chromosome 7, the PIP gene is not polymorphic.

The presence of plasmid DNA in the genomic DNA preparations provided misleading preliminary indications that the PIP gene was polymorphic. The source of the plasmid contamination could be several places, including

glassware or pipettes as well as solutions(such as phenol and chloroform) that are used in common in the laboratory. Once the presence of plasmid contamination was suspected, all blots were rehybridized with a plasmid probe and the bands on the genomic Southerns that were due to plasmid contamination were determined.

4. SUMMARY

As mentioned, the purpose of this thesis was to determine whether or not several structural features that have been associated with the regulation of gene expression in other systems were contributing to the expression of the PIP gene. The answers to several questions were obtained in this respect. The results of this thesis suggest that methylation, histone acetylation and rearrangement are not involved in the induction of PIP gene expression in the HBC cell lines tested. These results generate a further question, i.e. what factors are involved in the regulation of PIP gene expression? Other possiblities have been discussed including the presence or absence of the appropriate cis-acting DNA sequences and trans-acting transcription factors necessary for PIP gene expression in the cell lines tested. As mentioned, because the gene is not yet fully characterized studies have not yet been conducted in this respect. Future studies will determine the answers to these questions.

It is also possible that factors like methylation and histone acetylation are playing necessary but not sufficient roles in regulating PIP expression. It is well known that regulation of eukaryotic gene expression is a complex, multistep process. Thus altering the methylation status of the PIP gene with 5-Azacytidine may not be sufficient in itself to allow expression of the gene. The same arguement can apply to the sodium

butyrate experiments, i.e. histone acetylation alone is not sufficient to affect PIP expression.

Another structural feature that has been associated to gene expression is the presence and/or absence of hypersensitive sites in a gene. Hypersensitive sites are areas of a gene that are preferentially susceptible to cleavage by DNase1. The increased susceptibility of these areas to DNase1 cleavage is due to the unwinding or relaxation of the DNA double helix. Thus the structure of the gene in this area allows the binding of transcription factors to DNA which ultimately results in transcription of the gene. Hypersensitive sites are generally located in the 5' untranslated or 5' flanking areas of a gene. There is a possibility that cell lines such as MCF-7 which no not express PIP do not possess hypersensitive sites or such sites cannot be formed by hormone-treatment of the cells. In this respect T-47D would possess hypersensitive sites, which could explain why this cell line expresses the PIP gene.

Studies designed to detect DNase1 hypersensivity were not carried out because the 5' area of the PIP gene has not yet been fully characterized. As mentioned, most hypersensitive sites in DNA lie in the 5' untranslated or 5' flanking regions. Thus the probability of detecting hypersensitive sites with the genomic probes available were low. However, these studies will be performed in the future when the PIP gene has been fully characterized.

Another objective of this study was to determine if the PIP gene was polymorphic. The importance of determining genetic polymorphism lies in the ability of RFLPs to act as powerful clinical tools in the detection of certain disease states. In this study the disease in question was human breast cancer.

The polymorphism studies yielded important information, regardless of

the fact that no RFLPs were detected. The PIP gene now stands unique amongst the other genes in the same area as chromosome 7 in the sense that it is not polymorphic. It cannot be used as a genetic marker for breast cancer in this respect. However, this does not mean that the physiological role of the secreted PIP protein is not in some way linked to the presence of breast cancer. Thus PIP itself still has the potential to act as a marker for human breast cancer. Again studies have yet to be done to determine the function of the protein. Combined with the information obtained regarding the regulation of the PIP gene, an important system with which to study the physiology of breast cancer, the role of hormones in breast cancer, and the regulation of gene expression in breast cancer has been developed.

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