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RNA METABOLISM IN BOVINE PITUITARY CELLS

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ABSTRACT

RNA METABOLISM IN
BOVINE PITUITARY CELLS

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A system for the culture of bovine pituitary cells was developed. Substiutution of D-valine for L-valine in the growth medium resulted in undiminished prolactin synthesis over a 30-day culture period. In serum-free medium or in medium containing charcoal treated serum addition of hydrocortisone stimulated growth hormone synthesis 3-5 fold. In either medium prolactin synthesis was diminished 50% by hydrocortisone addition. In serum-free medium bromoergocriptine reduced prolactin secretion 80-90% and prolactin synthesis by 40-50%. No stimulation of prolactin protein synthesis was observed using either estrogen or thyroid releasing hormone.

The primary bovine pituitary cell cultures were used in studies of the half-life of prolactin, growth hormone and tubulin mRNAs. Under conditions in which total poly A+ RNA decayed 50%, prolactin, growth hormone and tubulin showed no turnover. This result, coupled with the slow response of protein synthesis levels to hormonal effects, suggested a very long half-life for prolactin and growth hormone mRNA. It is not known how prolactin and growth hormone mRNA maintain their unusual stability. Studies to compare metabolism of these stable mRNAs to less stable mRNAs in the same cells were initiated. A transformed bovine pituitary cell line was characterized and a cDNA library prepared from these cells was screened for clones containing mRNAs of varying abundance.

The cloned DNA was characterized. Southern analysis showed that most cDNA clones represented single copy genes. The mRNAs produced by these genes varied in size from <700 bp to >2000 bp. Kinetic analysis of the cloned DNAs representing more abundant mRNAs revealed a 45 min to 8 hr range of half-lives.

One cDNA clone which hybridized to RNA with a half-life of 45 min was further analyzed. This cloned sequence contained a bovine short interspersed repetitive element. Sequence analysis of this element indicated 97% homology with the right monomer of the dimeric bovine A element. Transcription of this bovine repetitive element was measured in transformed pituitary cells and in normal kidney cells. High molecular weight RNA containing this element was observed. No evidence for small transcription products was found.

To Dan,

whose unwavering encouragement

and faith made this possible

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PART I

PART II

PART I

PRIMARY PITUITARY CELL CULTURE AND KINETIC ANALYSIS OF PROLACTIN AND GROWTH HORMONE mRNA

INTRODUCTION

My research interests are directed toward studying mRNA turnover and how mRNA stability might contribute to control of gene expression. A system in which a specific, high abundance mRNA could be studied, similar to casein production in mammary tissue or ovalbumin production in chickens, would be ideal. In this system, the levels of a particular mRNA could be increased or decreased by appropriate hormones and the relative contribution of transcription and turnover to specific mRNA levels could be measured. The research effort in our laboratory uses the bovine pituitary as a model system for studying control of expression of prolactin and growth hormone. Initially, the majority of my effort was directed toward establishing a bovine primary pituitary cell culture system that maintained proper hormone production and responded appropriately to other hormones.

After a functioning cell culture system was established, methods and conditions to label and analyze mRNA turnover were determined. The results of these studies combined with data from other laboratories indicated that both PRL and GH mRNAs probably have an unusually long half-life. How this extreme stability is maintained is not known. As a first step toward understanding why PRL and GH mRNA are so stable, a series of other bovine mRNAs were prepared for comparison with PRL and GH mRNA. These mRNAs were characterized as to size, abundance and turnover.

LITERATURE REVIEW

mRNA Stability

The proteins produced in a cell are determined primarily by the composition of its mRNA population. The molecular mechanisms responsible for controlling mRNA composition operate at a variety of levels. Initial control must be at the level of gene transcription, but this event is followed by a number of steps which are all potential control points. Nuclear RNA processing (capping, polyadenylation, methylation, splicing), transport to the cytoplasm, and cytoplasmic stability all contribute to the eventual mRNA content. The role of mRNA stability in regulation of gene expression has recently received considerable attention. It has been shown that stability of individual mRNAs is important in controlling the expression of certain genes.

Early estimates of mRNA half-life suggested that all mRNA in the cell turned over at equal, but relatively slow, rates (Greenberg, 1972, Singer and Penman, 1973). It was later found by methodologies described below that not all the Poly A+ RNA in a cell decayed at equal rates (Berger and Cooper, 1975, Puckett et al.,1975). Cloning technology has allowed us to study the half-life of individual mRNA species using a number of methods. Each has some drawbacks and in a given situation some methods are not appropriate; all are relatively tedious.

The most direct measurement of mRNA half-life is pulse-chase analysis. In this technique, cells are exposed to a pulse of ^{3}H-uridine for several hours. The chase is usually performed by adding fresh medium containing unlabeled uridine and cytidine. RNA is prepared at time intervals during the chase and the amount of radioactivity specifically hybridizing to an excess of a filter-bound DNA probe is determined. The time required for the cpm to be reduced to one half the initial cpm is the half-life. This method is easiest to use with an mRNA that has a high rate of transcription and a relatively long half-life. Since nucleotide chases are not efficient, the specific activity of the UTP pools should be monitored and factored into the calculations (Shapiro and Brock, 1985).

In an approach to equilibrium assay, cells are labeled continously for a long period of time (Greenberg,1972). RNA is prepared at time points throughout the labeling period and cpm hybridizing to a specific probe are determined. An equation is then applied which takes into account the initial accumulation rate, the length of time required to reach equilibrium and the doubling time of the cells. For this method to be valid, ^{3}H-uridine incorporation into total cellular RNA must be linear over the entire labeling period. This method is less direct than the previous one and assumes constant rates of synthesis and decay.

In a third method transcription is inhibited, usually by a drug such as actinomycin D. The reduction in mass of the mRNA may then be followed by northern or S_1 anaylsis using labeled complementary DNA probes. This method works well for species of low abundance or with low transcription rates since probes can be labeled

to very high specific activity. It has all the disadvantages associated with drug treatments which is particularly a problem for mRNAs that have long half-lives. There are some systems where withdrawal of a hormone or changes in cell physiology result in complete shut-off of transcription. In these cases northern analysis of decay can also be used without the necessity of labeling cells (Shapiro and Brock, 1985).

Until recently, studies on the half-life of specific mRNAs have focused on viral genes or cellular genes that are tissue-specific and produce a highly abundant mRNA. Vitellogenin, ovalbumin and conalbumin are egg proteins produced at high levels in the liver or oviduct. Their expression is positively controlled by the steroid estrogen. In all cases, estrogen increases the rate of transcription and also produces large increases in mRNA stability (Brock and Shapiro, 1983, McKnight and Palmiter, 1979). Vitellogenin mRNA in Xenopus liver has a half-life of 3 wk when induced and decays with a 17 hr half-life when estrogen is withdrawn. It has been postulated that the increased stability may be mediated by binding of estrogen to a cytoplasmic estrogen receptor (Shapiro and Brock, 1985).

In the mammary gland, casein production is stimulated to high levels by prolactin, a polypeptide hormone. Similar to estrogen, prolactin exerts its effect by increasing transcription and concomitantly stabilizing casein mRNA (Guyette et al., 1979). How prolactin increases stability of casein mRNA is not known.

Another gene product which needs to acheive high levels in committed cells is globin. When murine erythroleukemia cells are induced to differentiate, globin remains very stable with a half-life

of 60 hr (Vollach and Housman, 1981) while other mRNAs become less stable (Darnell, 1982). It has also been found that not all of the globins have the same stability. Human delta-globin which is only 1 - 1.5 % of total cell globin, has a half-life of 4.5 hrs as compared to 16.5 hrs for human beta-globin (Ross and Pizarro, 1983).

These are examples of genes whose products are controlled, to a large extent, by posttranscriptional mechanisms. Whether these genes were exceptional was not known.

Several attempts have been made to study larger numbers of genes expressed in the same cell. Darnell and coworkers (Harpold et al., 1979) isolated nine clones from CHO cells and studied their transcription rates and steady state levels. They found that differences in transcription rates between individual clones did not necessarily predict the steady state levels they would attain. The conclusion, therefore, was that posttranscriptional events were in part controlling the final mRNA concentration. Derman et al.(1981), studying a set of liver-specific mouse mRNAs, suggested that control of these sequences was exclusively at the transcriptional level. Using mouse L-cells, Carneiro and Schibler (1984) isolated mRNAs corresponding to housekeeping genes of either low or moderate abundance. They concluded that, in contrast to the accumulation of liver specific mRNAs, the accumulation of ubiquitous mRNAs is mainly posttranscriptionally controlled. It appears, therefore, that selective stabilization or destabilization may apply to many types of mRNAs, not only those which reach high levels in specilized cells.

How can stabilization or destabilization of a specific mRNA be accomplished? Within the last few years some information regarding

this question has been accumulated in a number of systems. So far it seems that there are a number of signals a cell can use to modulate mRNA levels. I will describe three reasonably well documented systems, tubulin, histone, and growth regulated mRNAs, which indicate the diversity of these control mechanisms.

The principal subunits of microtubules are α- and β-tubulin which belong to a small evolutionarily conserved multigene family. Each family contains approximately six genes which are expressed in specific, complicated programs during differentiation (Cleveland. 1987). Microtubules are formed by the polymerizations of heterodimers consisting of α- and β-tubulin. Tubulin protein synthesis is self regulated by the apparent intracellular concentration of heterodimers, so that an approximately 2-fold elevation in the concentration of unpolymerized tubulin is accompanied by a specific 5-10 fold repression of new tubulin synthesis. Reduction of unpolymerized subunit levels stimulates new tubulin protein synthesis 3-4 fold.

Anaylsis of tubulin mRNA levels showed that the altered rates of tubulin protein synthesis could be quantitatively accounted for by corresponding changes in tubulin mRNA levels. Transcription run-off experiments (Cleveland and Havercroft, 1983) and experiments with enucleated cells (Pittenger and Cleveland, 1985) have demonstrated that changes in tubulin mRNA transcription rates are not responsible for regulation of mRNA levels. Autoregulation is instead a result of cytoplasmic events which modulate tubulin mRNA stability. Control of β-tubulin mRNA stability apparently resides in the first 16 codons of the mRNA (Gay et al., 1987). Whether autoregulation is due to tubulin subunits interacting directly with β-tubulin mRNA or with nascent

tubulin protein is not yet known.

A number of mRNAs whose level is controlled during the cell cycle have also been studied. Messenger RNAs for the replication-dependent histone proteins required throughout S phase are selectively stabilized during DNA synthesis. These mRNAs do not have a 3′ poly A tail but are terminated instead by a highly conserved stem-loop structure (Schumperli, 1986). When DNA synthesis ceases, the half-life of these mRNAs decreases from 40 min to 8 min in Hela cells (Heintz et al., 1983). Accelerated mRNA turnover does not occur if protein synthesis is inhibited (Graves and Marzluff, 1984). It has now been shown that the terminal 30 nucleotides containing the stem-loop structure are all that is required for regulation of histone mRNA stability (Pandey and Marzluff, 1987) provided that translation of the mRNA terminates within 300 bp of this structure (Graves et al., 1987). These results suggest that the degradation system may recognize the free 3′ end of histone mRNA only after translation has proceded to a normal termination codon.

Another group of genes whose transcription and stability have been examined are those which are activated almost immediately after resting cells are stimulated to divide. Two oncogenes, c-fos and c-myc, are activated in this way (Greenberg et al., 1986, Piechaczyk et al., 1985) and ten clones identified by Lau and Nathans (1987) have been added to this group. In all cases these genes are regulated both transcriptionally and posttranscriptionally. When shut-off is required not only does transcription cease but the mRNA is destabilized to allow an extremely rapid response in gene expression.

Shaw and Kamen (1986) found that an evolutionarily conserved

AT rich 58 nucleotide sequence located in the 3' end of the lymphokine, granulocyte-monocyte colony stimulating factor (GM-CSF), could confer instability to globin mRNA. These authors propose that mRNAs containing a repeated AUUU motif are generally involved in the selective degradation of transiently expressed messengers. Control of c-myc is not so simple, however, since sequences in exon 1 or the first intron have been associated with mRNA instability (Piechaczyk et al., 1985) and at least some of the AUUU motif can remain at the 3' end in more stable varients of c-myc (Jones and Cole, 1987).

To date there is no clear understanding of how individual mRNAs may be stabilized or destabilized. We are just beginning to determine which sequences are important for mRNA stability or instability, but the mechanisms involved are not known. Endonucleolytic cleavage at a specific site, followed by exonucleolytic degradation is the most frequently suggested method for mRNA degradation. Results from studies using histone mRNA in a cell-free system support this speculation (Ross and Kobs, 1986). It will be of interest to see if polyadenylated mRNAs are also specifically controlled in this system. It seems likely that there will be more than one system for mRNA degradation.

Prolactin and Growth Hormone Expression

The system we have chosen for studying regulation of gene expression is the bovine anterior pituitary. In particular, we are interested in the aspects which control production of two major products of the pituitary gland, prolactin and growth hormone. One important tool for these studies was a cell culture system could be

used understand which factors control growth hormone and prolactin production.

The pituitary gland lies at the base of the brain and is connected to the hypothalamus by the hypophysial stalk. Physiologically the pituitary is divisible into two distinct portions, the anterior and posterior pituitary, separated by a zone called the pars intermedia. Embryologically the anterior pituitary originates from Rathke's pouch which is an embryonic invagination of the epithelium of the throat. The posterior pituitary is neural in origin and is an outgrowth of the hypothalamus.

Six hormones which play major roles in control of metabolic functions are secreted by the anterior pituitary. There are at least five types of secretory cells in this gland, each producing a different hormone. These cells can be distinguished by immunostaining techniques or on the basis of morphology using electron microscopy. The cell types and the hormones they produce are: 1) Somatotropes--growth hormone (GH), 2) Lactotropes--prolactin (PRL), 3) Corticotropes--Corticotropin (ACTH), 4) Thyrotropes--thyroid stimulating hormone (TSH), 5) Gonadotropes--luteinizing hormone (LH) and folicule stimulating hormone (FSH).

Almost all secretion by the pituitary is controlled by either hormonal or nervous signals from the hypothalamus. This control is complex and involves a number of factors for each secreted hormone. Some of these factors interact to influence the effect of other hormones on the secretion from the cell. The primary mode of regulation for most of the pituitary hormones is stimulation of secretion. Control of prolactin appears relatively unique among the

hormones secreted by the mammalian pituitary in that the major regulatory mechanism involves inhibition of PRL production (Meites and Clemens, 1972). This inhibition is demonstrated by in vivo experiments where removal of the pituitary from direct control of the hypothalumus results in an increase in PRL secretion.

Lactotrophs are distinctive in that they contain a mixture of small and very large secretory granules. PRL is synthesized on membranes of the rough endoplasmic reticulum as a precursor which is rapidly processed and transported into the golgi (reviewed by Maurer, 1982d, Dannies and Tam, 1982). Within one hour PRL is found in small secretory granules and at later times in the large granules. These large granules appear only when the cells are not secreting at a rapid rate. PRL is tightly packaged in the mature forms of the secretory granules and once packaged is very stable. Secretion occurs by fusion of the granule membrane with the plasma membrane and release of the contents (exocytosis). PRL in granules can be degraded within the cells by digestion in lysosomes (Farquhar, 1977).

A large number of hormones or factors have been found to influence secretion of PRL in vivo. For purposes of this review I will focus only on those that have been shown to work directly on the PRL secreting cell (Table 1). In most cases the effect on release is immediate, followed by much slower changes in levels of protein synthesis. Although it is possible that a particular hormone can affect secretion without affecting synthesis of PRL, factors which alter secretion have also been shown to change the synthesis of PRL (Laverriere et al., 1983). Most of the studies on PRL and GH release and synthesis have been performed using rat pituitary systems.

TABLE 1

Hormone effects on PRL/GH transcription

	Prolactin	Growth Hormone
Dopamine	Decrease	No effect
Estrogen	Increase	Increase(slight)
Epidermal Growth Factor	Increase	
Thyrotropin Releasing hormone	Increase	Decrease
Triiodothyronine(T_3)	Decrease	Increase
Vasoactive Intestinal Peptide (VIP)	Increase	
Somatostatin	Decrease	Decrease
Glucocorticoids	Decrease	Increase
Growth Hormone Releasing Factor	No effect	Increase
Ca^{++}	Increase	Increase(slight)

Hemipituitary or primary cell cultures have been used but cell lines derived from rat pituitary tumors (designated collectively as GH cells) have been most extensively studied. The original cell lines: GH_3 and GH_1, can secrete both PRL and GH. Clonal varients can secrete varying levels of one or the other of these hormones (Bancroft, 1981). They resemble normal pituitary cells in that they have retained their differentiated function since they respond to appropriate stimuli by secreting and synthesizing PRL or GH. It should be remembered that they differ from normal cells because they are transformed, do not have storage granules, and sometimes require concentrations of factors to affect release which are different from primary cells.

Regulation of Prolactin mRNA Levels

Dopamine, secreted by the hypothalamus, is thought to be the major hormone responsible for the inhibition of PRL production (MacLeod et al., 1970). Receptors for dopamine and dopamine agonists (primarily the catecholamines) are found on the plasma membrane of pituitary cells (Caron et al., 1978, Cronin et al., 1978). Dopamine has a limited stability in culture so most studies have been performed using the more stable ergocryptine agonists of dopamine. When these compounds interact with receptors on the lactotrophs they cause inhibition of PRL release within minutes, after which PRL accumulates within the cell for at least 8-16 hrs. Initially, bromocryptine inhibits release without affecting synthesis and stability of the PRL protein. Later PRL is degraded in the cell and PRL synthesis decreases (Dannies and Tam, 1982).

In primary rat pituitary cultures the dopamine agonist,

ergocryptine, was shown to decrease PRL synthesis and to also decrease mRNA levels (Maurer, 1980). Analysis of the time course of ergocryptine effects showed that PRL synthesis decreased rather slowly, so that 4-6 days were required for maximal inhibition. The effect on PRL gene transcription was more immediate with 70-80% inhibition of transcription within 45 min after addition of ergocryptine to cultured cells (Maurer, 1981). The rapid decrease in transcription but delayed effects on PRL mRNA levels and PRL protein synthesis suggest that PRL mRNA is very stable with an estimated half life of 48 hours.

The in vivo stimulation of PRL by estrogen has been recognized for many years. Estrogen affects most aspects of prolactin production, and the mechanism of its action has been extensively investigated (Dannies, 1985). One result of short term estrogen treatment is an increase in the number of PRL producing cells either in the gland (Gersten and Baker, 1980) or in primary cultures (Lieberman et al., 1982). In certain strains of rats long term exposure to estrogen can result in pituitary tumors. All of the increase in PRL production in estrogen treated cells is not simply a result of an increased number of secreting cells. In primary cell culture the increase in PRL synthesis is greater than the increase in cell number (Lieberman et al., 1982). Further analysis reveals that estrogen stimulates transcription from the PRL gene. Cells from 6 week old male rats treated with estrogen showed a 6-fold increase in PRL mRNA transcription at 24 hr after addition of estradiol to culture medium. The effect of estradiol on cells from female breeder rats was less. There was no effect on growth hormone transcription (Shull and

Gorski, 1986).

With the large number of regulators affecting PRL production their potential interaction can be very complicated. In the animal, estradiol greatly decreases the ability of dopamine to inhibit PRL secretion (Raymond et al., 1978). This antagonism extends to PRL synthesis in cultured primary cells (Maurer, 1982b). The mechanism of estrogen reduction of dopamine inhibition remains unknown but it has been shown that estrogen is not acting at the level of the dopamine receptor (DiPaolo et al., 1979). In contrast, estradiol has been shown to increase the number of receptors of thyrotopin releasing hormone, another hormone which stimulates PRL synthesis and release.

L-triiodothyronine (T_3) inhibits PRL synthesis in rat primary pituitary cell cultures. A 75% reduction in PRL synthesis with 6 days of treatment by 10 nM T_3 was paralleled by a decrease in PRL mRNA content (Maurer, 1982a). This inhibition was not blocked by estradiol, unlike the effect of estrogen on ergocryptine inhibition. Contrasting effects were seen in GH_3 cells where 50 nM T_3 caused a two fold increase in PRL production (Perrone et al., 1980) while 0.5 nM T_3 reduced PRL synthesis (Stanley and Samuels, 1984).

The peptide hormones, thyrotropin releasing hormone (TRH) and epidermal growth factor (EGF), have been shown to stimulate PRL synthesis in rat GC cells (Dannies and Tashjian 1973, Murdoch et al., 1982a). Transcription of PRL is stimulated within minutes after addition of either hormone to the culture medium with maximal stimulation occuring at 30-60 min (Murdoch et al., 1985). This stimulation of PRL transcription is interesting in that it is transitory. The initial burst of PRL gene transcription produces a

doubling of PRL mRNA synthesis within hours but then falls back to a new level that is still higher than the uninduced mRNA synthesis. Under this condition cytoplasmic PRL mRNA levels do not reach their maximum level until 16-20 hours after addition of the hormone to the cultures (Murdoch et al., 1985). Using these results the authors estimate that the half life of PRL mRNA in GH cells is approximately 8 hr. Using pulse-chase analysis Laverriere et al. (1983) report that TRH increases the half life of PRL mRNA from 17 hr in untreated cells to 27 hr in treated cells.

Murdoch et al., (1985) have identified a basic nuclear protein whose phosphorylation parallels the increases in PRL transcription. TRH and EGF stimulate phosphorylation on 3-5 sites on this protein, all on serine residues. It is possible that one mechanism by which peptide hormones affect transmission of their signal from the plasma membrane receptor to the 5′ regulatory DNA sequence is through phosphorylation of a specific nuclear protein.

A number of other factors have been found to regulate prolactin synthesis. Vasoactive intestinal peptide (VIP) is found in nerve termini in the hypothalamus and it has been shown to stimulate PRL secretion in vivo and in vitro. In GH_3 cells VIP increased PRL mRNA and mRNA precursor levels 25 hours after treatment (Carrillo et al., 1985). The increase in PRL mRNA levels was not blocked by concurrent addition of the glucocorticoid, dexamethasone, to the cultures. The glucocorticoids have been shown to reduce PRL mRNA synthesis in the GH and GC cell lines. (Potter et al, 1981, Evans et al., 1982). However, in primary cultures of rat and ovine pituitary cells no glucocorticoid effect on PRL synthesis was demonstrated

(Lieberman et al., 1978, Vician et al., 1979) leading Shull and Gorski (1986) to suggest that the glucocorticoid effect could be unique to pituitary tumor cell lines.

Most of the hormones which affect PRL synthesis and PRL mRNA transcription are factors which bind to plasma membrane receptors. Transduction of the signal generated by receptor hormone complexes at the cell surface to yield a nuclear transcription response is still not understood. Several cellular processes have been postulated to mediate the intracellular effects of the hormones. These include elevation of cytosolic calcium (Ca^{2+}), a stimulation of cAMP levels and activation of the phosphatidylinositol cycle (Bancroft et al., 1985, Murdoch et al., 1985).

In serum-free medium, calcium alone has been shown to specifically stimulate PRL mRNA levels (White and Bancroft, 1983). In GH cells either TRH or EGF require extracellular calcium to achieve maximal stimulation of PRL mRNA levels. When calcium is completely removed from the medium by chelation with EGTA, no response to TRH and a very small response to EGF is observed (Bancroft et al., 1985). That these cells were still hormone-responsive in the absence of calcium was shown by stimulation of GH mRNA by dexamethasone. Qualitatively similar results have been found using primary rat cultures, although these cells seem to regulate their intracellular calcium in such a way that the calcium in the medium must be much lower to demonstrate the response (Gick and Bancroft, 1985).

Adenosine 3',5'-monophosphate (cAMP) is often considered an intracellular mediator of hormone action. cAMP may be involved in regulation of PRL gene expression by certain hormones. Maurer (1982c)

found that cAMP could reverse the inhibition of egrocryptine on PRL mRNA levels. In GH_4 cells cAMP stimulated PRL transcription (Murdoch et al, 1982b) as did forskolin, an activator of adenylate cyclase. Forskolin also increased phosphorylation of the basic chromatin associated protein that was also phosphorylated in the presence of TRH (Murdoch et al., 1982b). In other studies PRL synthesis was not affected by cAMP. GH_1 cells showed no effect on PRL synthesis in the presence of forskolin even though it stimulated cAMP levels 400 fold (Stanley and Samuels, 1984).

In summary, PRL protein syntheis and mRNA levels are controlled by a wide variety of hormones (Table 1). In the animal the primary regulation is inhibition of PRL secretion, but interactions of all the hormones which affect PRL synthesis are undoubtedly important in the physiological regulation of circulating PRL levels. The nuclear effects of these hormones may be mediated by intracellular levels of calcium and/or cAMP.

The half-life of PRL mRNA has been very difficult to determine. In most of these studies it appears to a very stable mRNA. In inhibition of PRL by ergocryptine, days are required to reach a new steady state mRNA level. This implies that PRL mRNA is not rapidly turned over in treated cells. During induction by estrogen or the peptide hormones TRH or EGF, transcription rates change within minutes, but new steady state mRNA levels are not acheived for hours or days. It is possible that changes in PRL mRNA stability are occurring during these treatments but probably are not large and are superimposed on the kinetics of an mRNA which under all circumstances is relatively stable.

Regulation of Growth Hormone Transcription

Most of the hormones which affect PRL synthesis also influence GH synthesis, frequently in an inverse relationship (Table 1). GH synthesis and secretion are stimulated primarily by glucorticoid and thyroid (T_3) hormones. The interrelation of these two hormones in mediating expression of growth hormone is unclear. Studies using various GH cell lines have shown that glucocorticoid and thyroid hormone stimulate growth hormone synthesis (reviewed by Bancroft et al., 1985). Reports by two groups (Martial et al., 1977, and Samuels et al., 1979) suggested that glucocorticoids cannot induce significant stimulation of GH synthesis in the absence of thyroid hormone. In contrast, Dobner et al. (1981) using GH_3 cells reported significant glucocorticoid stimulation of mRNA levels in the absence of T_3. Evans et al. (1982) also showed that either dexamethasone (a synthetic glucocorticoid) or T_3 could increase mRNA transcription and steady state levels about 2-5 fold in G/C cells. These latter studies were performed in a serum-substitute induction medium where no hormones were added and 1% serum supplement was throughly treated to remove T_3 and hydrocortisone. Bancroft et al. (1985) found no synergism between dexamethasone and T_3 in GH_3 cells in the induction of mRNA levels. They consistently found a larger stimulation of GH synthesis by dexamethasone alone than by T_3 alone. Yaffee and Samuels (1984) and Nyborg et al. (1984) found that GC cells grown in 10% serum depleted of thyroid and steroid hormones showed a low and variable response to dexamethasone but always a synergistic response with T_3 plus dexamethasone. These authors feel that T_3 can act as the primary

regulator of the GH gene and glucocorticoids further amplify the T_3 response. Using GH_3 or GC cells in serum-substituted medium Diamond and Goodman (1985) found that dexamethasone alone produced no stimulation in GC cells and a slight induction of transcription in GH_3 cells. In their studies dexamethasone in combination with T_3 induced higher and faster transcription responses than T_3 alone.

Glucocorticoids and T_3 are probably the primary physiological factors which control GH production. Several groups feel that glucocorticoids potentiate the effects of T_3 while others feel that the two hormones function independently. The GH response to glucocorticoids has been variable, ranging from no response at all to production of high levels of GH. These differences may be the result of varying cell lines used for the studies, growth conditions (suspension vs. monolayer), or treatment of serum and medium components. The polypeptides, somatostatin and growth hormone releasing factor (GRF) have been shown respectively to inhibit and stimulate GH secretion (Vale et al., 1977, Rivier et al., 1982). GRF can also stimulate transcription of the GH gene as well as stimulating GH release (Barinaga et al., 1983).

Turnover of Growth Hormone mRNA

Estimates of the half life of GH mRNA and whether the half-life in influenced by hormones are also open questions. Evans et al., (1984) proposed that regulation of GH gene transcription can entirely account for the effects of dexamethasone and T_3 on GH synthesis. They did not, however, completely exclude the possibilty of additional modulation. Diamond and Goodman (1985) measured transcription rates

and changes in GH mRNA levels in cells induced with T_3 plus dexamethasone. They found a five-fold increase in transcription by nuclear run-on assay and a 50- fold increase in mRNA levels at 24 hr. using S_1 analysis. They concluded that the large accumulation of cytoplasmic GH mRNA contrasting with relatively small changes in transcription was not consistent with a purely transcriptional mechanism of hormone induction. They estimate 20 hr for the half life of GH mRNA in the presence of hormones and infer a two hour half life without hormones present.

Yaffe and Samuels (1984), on the other hand, determined a half life of 50 hr for GH mRNA in either induced or uninduced cells using pulse-chase analysis. The chase employed was unusual in that the initial time point measured was 27 hr after the start of chase and measurements were at 15-25 hr intervals after that. When transcriptional rates were measured they found that immediately after addition of T_3 and dexamethasone to the medium there was a 10-fold increase in the rate of transcription which then leveled to a new rate of about 3-4 times the uninduced level. This would result in a relatively greater increase in GH mRNA accumulation at early times as compared to later times during the hormone treatment. Therefore, the time required to achieve half maximal GH mRNA production would be shorter than the half life of the mRNA. In other words, approach to steady state would not be a valid method for measuring mRNA half life. Nyborg et al. (1984) also found a burst of transcription, followed by a decrease to a new level of transcription. The effect using T_3 was immediate while the effect with dexamethasone was slower, on the order of 30-60 min. When GC cells were removed from T_3 and dexamethasone-

containing medium, GH mRNA levels were reduced 92% in 24 hrs. They concluded that the half-life of GH mRNA in uninduced cells is 8 hrs.

Paek and Axel 1987, have reported that dexamethasone can affect the half-life of GH mRNA in a biphasic manner. This study involved transfection of a human GH cDNA under control of the HSV tk promoter into mouse fibroblasts. Since the HSV tk promoter is not glucocorticoid responsive, any changes in mRNA levels in the presence of dexamethasone must be due to stabilization of the mRNA. Significantly higher levels of mRNA accumulation were found in the presence of hormone while transcription rate measurements and approach to steady state showed no alteration due to hormone exposure. Pulse-chase analysis, using very low cpm, revealed that while 2/3 of the mRNA turned over at the same rate in induced and noninduced cells, about 1/3 did not seem to decay at all.

There is, at present, no consensus on the half-life of GH mRNA. Some of the variability between experiments may result from differences in culture conditions which produce different responses to hormone induction of GH protein synthesis. It seems possible that the hormones which affect GH synthesis may affect GH mRNA turnover in different ways.

MATERIALS AND METHODS

Primary pituitary cell culture.

Fresh bovine pituitary glands were obtained at a slaughterhouse and transported to the laboratory on ice. The intact glands could be stored on ice at least six hours without affecting the viability and yield of the resulting cell cultures. After dissection of the tissue the cell culture preparation must continue uninterrupted until the cells can be plated. Working under sterile conditions in a hood, the encapsulated gland was dipped in 70% ethanol, placed on a sterile cutting board, cut in half sagittally and the anterior pituitary was dissected free of surrounding connective tissue. Two to three hemipituitaries were placed on the platform of a Stadie-Riggs slicer and the tissue was sliced 1 mm thick. The slices were washed in cell culture medium (without serum), drained briefly and minced into 1 mm cubes using a double-edged razor blade. All blades used for tissue preparation were replaced frequently since dull blades compress tissue and damage cells adjacent to the cut. The pieces were placed in a beaker and washed with three to four changes of cell culture medium. The tissue was transferred to an Erlenmeyer flask and dissociated with 3 mg/ml collagenase (Worthington CLS) in culture medium with gentle stirring for two hours at 37^{o}C. Generally five to six glands were dissociated in 150 ml collagenase solution. After incubation the cell suspension was filtered thru several layers of cheese cloth,

centrifuged at low speed at room temperature for 5 min. to pellet the cells. The cells were resuspended in culture medium and repelleted two more times. The cells were suspended in trypsin solution [0.25% pancreatin (GIBCO), 50 ml/6 glands] and incubated with gentle stirring for 15-30 min at 37°C. The cells were centrifuged again, washed three to four times with very low speed centrifugation to separate red blood cells from the cell mixture. Cells from five to six glands were resuspended in 20 ml medium and counted. Generally 2-3 x 10^8 cells could be recovered from six glands. Cells were diluted in cell culture medium containing 10% calf serum (GIBCO) and distributed to dishes at 1 x 10^5 cells/cm^2. Cultures were maintained at 37° in a 5% CO_2 atmosphere.

To optimize maintainence of lactotrophs and somatotrophs in the cell culture a variety of media were tested. The specific types of media and additions to the medium are described in the text and figure legends. Initial experiments used primarily Swim's S-77 medium which had been especially formulated without methionine and valine (GIBCO). Unless otherwise stated all media contained D-valine in place of L-valine to inhibit overgrowth of the primary cultures with cells which were not hormone secreting cells (Padmanabhan et al., 1982).

Several types of serum (GIBCO) were tested in our experiments. We found that serum required dialysis to realize the full effects of D-valine substitution. We either purchased dialyzed serum or dialyzed normal serum for 2 days with 3 changes against 0.9 M NaCl in PB. After dialysis the serum was sterilized through 0.2u filters. In some experiments serum was treated with activated charcoal (Norite) to remove steroid hormones. For this 10 g/ml norite was added to

dialyzed serum and the suspension was stirred for 60 min at room temperature. The charcoal was removed by centrifugation at 10,000 x g rpm for 20 min and then filter sterilized through a 0.45 micro filter and then through a 0.2 micron filter to remove all residual charcoal.

Serum-free cell culture conditions were also developed. Several basic media along with a variety of additions were tested as described in the text and figure legends. The best medium was MEM D-Val(GIBCO) containing 10 ug/ml insulin and 5 ug/ml transferrin, buffered with 15 mM Hepes pH 7.2. For maintainence of fetal pituitary cultures serum-free medium was superior to medium containing serum but these cultures showed an absolute requirement for 40 ng/ml hydrocortisone. For all serum-free cultures the initial cell attachment required serum. Cell suspensions were plated in medium containing 10% calf serum, allowed to attach overnight, after which the medium was replaced with serum-free medium. A variety of cell attachment factors were tested in the course of these experiments. Inclusion of fetuin in the medium or pretreatment of the culture dishes with poly-D-lysine, gelatin or serum did not significantly enhance attachment.

Protein synthesis assay.

Cells were plated at 3 x 10^6 cells/60 mm culture dish. At the times indicated the cell culture medium was aspirated and 1 ml of labeling medium was added. Labeling medium generally contained 50 uCi/ml ^{35}S-methionine in Swim's medium containing 10% calf serum and 3 ug/ml methionine (20% of normal). At the end of labeling, medium was removed and cells were washed three times with 0.9% NaCl. Cells were lysed in 0.3 ml of 0.5% SDS, 2.5% 2-mercaptoethanol, and 10 mM TrisCl

pH 7.5. Since these samples are very viscous, the DNA was sheared by aspirating several times with a 1 ml syringe through a 22 gauge needle. Aliquots for measurement of total incorporation into protein were removed, and the remainder of the sample was boiled for 5 min. and stored in the freezer until ready for PAGE-gel analysis.

To determine total protein synthesis 25 ul of cell lysate was spotted in duplicate on 2.5 cm Whatman 3MM discs which had been saturated with methionine. The discs were air dried, transferred to a beaker containing 10 ml/disc of cold 10% TCA and incubated in ice 15 min. The discs were then transferred to hot 10% TCA and boiled for 10 min. The samples were rinsed briefly in 5% TCA, several times in EtOH for 1 min each, dried with a heat lamp and counted (Roberts and Patterson, 1973).

To quantitate prolactin and growth hormone protein synthesis labeled cell lysates were analyzed by discontinuous polyacrylamide gel electrophoresis (Laemmli, 1970) in tube gels. Gel tubes were cleaned in chromic acid and silanized using Siliclad or dichlorodimethyl silane in $CHCl_3$. The 10 cm separating gel was 12.5% acrylamide (30:0.8 acrylamide:bisacrylamide). The concentration of bisacrylamide in the separating gel was crucial for resolution of the small PRL and GH proteins. Bisacrylamide seemed to of variable purity and needed either recrystallization or new lots had to be screened. The stacking gel was 3% acrylamide (0.5 cm). Frozen samples were thawed, 10 ul dye was added to 40 ul of lysate and 50 ul total was applied per tube. Electrophoresis was at 2 mA/tube for about 8 hr. The gels were removed from the tubes using a tube eliminator (BioRad) and stained overnight in 0.05% Coomassie Blue in 25% isopropanol, 10% acetic acid.

The gels were destained in 10% acetic acid for several hours and sliced into 2 mm pieces. One to two slices were digested with 1 ml of 30% H_2O_2 at 60°C for 5-15 hours and counted in 10 ml Triton/toluene based counting fluid. NIH prolactin standard was included to verify the position of PRL cpm. Slab gels were similar to tube gels, except that 15-20 ul of lysate was applied per well. These gels were 1.5 mm thick, were run at 50 V until the dye was thru the stacking gel and then 100V for 5 hr. Staining and destaining were as described above. The gels were imbedded with PPO in DMSO or treated with Enhance (New England Nuclear) before autoradiography.

DNA Synthesis Assay.

Cell division in primary cultures could not be determined simply by counting cells. In the mixed cell population some cells were very refractory to trypsin while others lysed with little provocation. Cell division was determined, instead, by uptake of ^{3}H-thymidine (Steck et al., 1979). For each assay point, 1 x 10^6 cells in 35 mm dishes were labelled for 3 hr at 37°C with 0.5 ml of culture medium containing 0.5 uCi of ^{3}H-thymidine. At the end of the labelling period the medium was removed and the plates were rinsed three times with PBS. The cells were detached with 0.25% trypsin in PBS-EDTA for 10 min at 37°C. Cells were collected directly onto GF/A filters using a filtering apparatus, washed six times with PBS and lysed with two washes of cold 10% TCA, followed by two washes with methanol. Filters were dried and counted in a Triton-toluene scintillation fluid.

Measurement of RNA Turnover.

Cytoplasmic RNA was prepared by rinsing the cell monolayers two to three times with cold PBS. The cells were scraped in PBS, centrifuged at low speed and resuspended in 1 ml of buffer (25 mM NaCl, 25 mM TrisCl pH 7.5, 5 mM EDTA, 0.5% Triton) per 10^8 cells. Cells were broken using 20-30 strokes with a B pestle in the dounce homogenizer. Nuclei and cell debris were pelleted at 10,000 x g, 5 min. The supernatant was adjusted to 250 ug/ml Proteinase K, 100 ug/ml tRNA, 0.5% SDS and 0.11 M NaOAc and incubated for 25 min at 37°C. The RNA was extracted with two volumes of phenol/$CHCl_3$ followed by two extractions with two volumes of $CHCl_3$ and precipitated in two volumes of EtOH overnight.

Incorporation of ^{3}H-uridine into <u>total</u> RNA was measured by labeling 5 x 10^5 cells in 35 mm culture dishes with 0.3 ml medium containing 80 uCi/^{3}H-uridine. The cells were rinsed with PBS and lysed in 0.5% SDS, 2.5% 2-mercaptoethanol, and 10 mM TrisCl pH 7.5. The DNA was sheared with a syringe and 250 ul aliquots were precipitated with 5 ml of cold 10% TCA. The precipitate was collected on Whatman GFC filter discs.

<u>Preparation of filter-bound plasmid for RNA hybridization.</u>

Nitrocellulose (S & S) circles, 7 mm in diameter, were produced using a paperpunch. These were numbered with black ink to identify the plasmid they would contain, soaked in 2X SSC and arranged on blotting paper saturated with 2X SSC. Plasmid for binding was linearized with EcoRI, extracted with phenol/$CHCl_3$, and ethanol precipitated. The plasmid was resuspended in 2 M NH_4OH and denatured and nicked by boiling 1.5 min. The solution was adjusted to 2 M NaCl

and the final concentration of the plasmid was 50 ug/ml. Each filter was spotted with 4 ul of plasmid solution. The filters were air dried and baked at 80°C for 2 hrs, prewashed and air dried (McKnight and Palmiter, 1979) Prehybridization was for 10-18 hr in 1.2X hybridization buffer (McKnight and Palmiter, 1979) containing 0.4 mg/ml Poly(A) and 0.4 mg/ml tRNA. Labeled RNA was added at 10% of the final volume and hybridization was allowed to proceed for more than 48 hrs. Generally 10 ul of hybridization reaction was allowed for each filter. Reactions were performed in 10 x 75 mm polypropylene tubes (Sarstadt) tube with a tight fitting cap in a 45°C oven. Hybridization was performed in an oven instead of a waterbath so that it was not necessary to overlay the reaction with mineral oil to prevent evaporation and condensation in the tube. Filters were washed 6 times in 1X SSC containing 0.5% SDS at 68°C for 10 min each wash. To determine specifically bound RNA each filter was placed in a scintillation vial and the RNA was eluted by heating to 60°C for 1 hr in 0.5 ml of water.

End-labeling of RNA

Poly A+ RNA was nicked and end-labeled using polynucleotide kinase (Spradling et al., 1980). RNA (1.5 ug) was nicked by heating in 0.05 M Tris pH 9.5 for 10 min. at 90°C (2 ul total volume in sealed capillary tube). This reaction was added to 8 ul of ATP/Kinase (25 uCi gamma-^{32}P-ATP, 2.5 units T_4-kinase in 62.5 mM Tris pH 7.5, 12.5 mM $MgCl_2$, 2.5 mM dithiothreitol) and incubated at 37°C for 45 min. The reaction was terminated by addition of 10 ul 4M NH_4Ac. Carrier RNA (100 ug) and H_2O to make 200 ul total volume were added and extracted

with phenol/$CHCl_3$. The labeled RNA was precipitated 2 times from 0.2 M NaAc with 2 volumes of ethanol. The yield was 10^7 cpm/ug RNA.

CLONING OF cDNA

Preparation of mRNA

An SV40 transformed pituitary cell line, 5A5, was used as a source of mRNA for cDNA cloning. Confluent cell cultures were diluted 1:5 and plated at 5 x 10^6 cells/100 mm dish 36 hr before harvest for RNA. Monolayers from 40 dishes (3 x 10^8 cells) were washed three at a time in cold PBS and scraped into cold PBS. After collection the cells were pelleted at low speed in a desk top centrifuge and resuspended in 6 ml of lysis buffer [25 mM NaCl, 25 mM TrisCl pH 7.5, 5 mM EDTA, 0.5% Triton and 10 mM vanadyl nucleotide complex] Cells were broken in a dounce homogenizer with 40 passes of the B pestle. Nuclei were pelleted at 8000 rpm for 10 min in an HB4 roter. The supernatant was adjusted to 600 ug/ml Proteinase K, 0.075 M NaOAc pH 5.5, 0.5% SDS, and digested for 10 min at 37°C. The digestion mixture was extracted once with phenol, twice with $CHCl_3$, and ethanol precipitated overnight and RNA was collected at 8000 RPM for 30 min in an HB4 roter. The pellet was dissolved in 1 ml of water and the RNA concentration determined by absorbance at A_{260}. The RNA yield in this case was 1 mg. Yields of cytoplasmic RNA using the above procedure varied widely (1-5 mg/3 x 10^8 cells) probably because cell breakage using the dounce homogenizer was not always complete. Intactness of total RNA was judged by electrophoresis on a minigel of 1% agarose in TBE stained with ethidium bromide.

Poly A+ RNA was prepared using oligo dT cellulose fines (PL Biochemicals) in a batch elution procedure. For 1 mg of cytoplasmic RNA 25 mg of fines were swelled overnight in water, treated with 0.1 N NaOH for 30 min, and washed three times with HSB (0.12 M NaCl, 0.01 M TrisCl pH 7.6, 0.001 M EDTA, 0.2% SDS). RNA was denatured by heating at 65°C for 2 min, and an equal volume (1 ml) of 2X HSB was added. To this solution 25 mg of oligo dT cellulose fines in 0.5 ml HSB were added and the suspension was mixed gently for 15 min. The cellulose was pelleted and washed with 2 ml HSB 6 times with 5 min of mixing each time to remove Poly A- RNA. Poly A+ RNA was collected using 3 elutions with LSB (0.01 M Tris Cl, pH 7.6, 0.001 M EDTA, 0.2% SDS) of 0.2 ml each. The LSB fractions were pooled, adjusted to 0.2 M NaOAc pH 5.5 and precipitated with two volumes of ethanol.

Preparation of cDNA Library.

To prepare the cDNA library Poly A+ RNA was used as a substrate for oligo dT primed cDNA synthesis using reverse transcriptase. Double stranded DNA tailed with oligo dC was annealed to pBR322 which had oligo dG residues added to the PstI site (Maniatis et al., 1982). The general cloning scheme and important aspects of the reactions are detailed in Table 2.

Screening cDNA Library

Colonies from the initial transformation were picked to gridded agar plates, and onto gridded nitrocellulose disks which were layered on LB agar (master filter). The master filter was grown for 6 hr at 37°C, and replicated according to Maniatis et al., (1982). The

TABLE 2

CLONING SCHEME

Poly A+ RNA (10 ug RNA)

↓ Reverse transcriptase (Dr. J.W. Beard) 210 units
100 uCi ^{32}P-dNTP, 100 units RNasin (Promega)
20 ug Oligo dT primer
225 ul Rxn, 1.25 hr, 42°C

cDNA (1.25 ug, 4.5 x 10^5cpm)

↓ Phenol/Chloroform extract
Spun column, ethanol ppt.
0.16 N NaOH, 68°C, 10 min, neutralize, ethanol ppt.
Klenow polymerase (Boehringer Mannheim) 35 units/0.6 ug cDNA
330 ul Rxn, 13 hr, 16°C

dsDNA (470 ng)

↓ Phenol/chloroform, ethanol ppt.
(35K rpm, 45 min, SW 50.1)
S_1 nuclease 800 units/375 ng dsDNA
0.88 ml rxn, 45 min, 37°C

Blunt end dsDNA

↓ Phenol/chloroform, ethanol ppt.
(35K rpm, 45 min, SW 50.1)
Resuspend and precipitate from 0.15 M NaOAc/EtOH
Resuspend and precipitate from 0.12 M NaCl/EtOH
Resuspend in tailing buffer
Terminal transferase (NEN) 20 units/40 ng dsDNA
5 uCi ^{3}H-dCTP, 2515 pmole dCTP
37°C, 45 sec

dsDNA with 30 residue dC tail

↓ Phenol/chloroform, spun column in annealing buffer
PstI cut, dG tailed pBR322 (NEN)
4.2 ng tailed dsDNA
100 ul rxn, 10 min 68°C, 2 hr 45°C

Annealed vector and plasmid

↓ transform frozen competent HB101

5 x 10^6 transformants/ug insert DNA (97% Amp^S)

replica filters were grown 4 hr and each was replicated to two more filters which were grown for 11 hr. Filters were prepared for hybridization to ^{32}P labeled probe by the method of Grunstein-Hogness (Maniatis et al., 1982)

Probes for screening the cDNA library for abundance classes were prepared from Poly A+ RNA by two methods. One used reverse transcriptase primed with random primers prepared from calf thymus DNA according to the procedure described in Maniatis et al. (1982). The other used nicked RNA end-labeled with $AT^{32}P$ and polynucleotide kinase.

ANALYSIS OF CELLULAR SEQUENCES

Northern Analysis

Poly A+ RNA was prepared for analysis on 0.9% Agarose gels by heating 1 ug of poly A+ RNA in 50% formamide, 6% formaldehyde, 10% glycerol in 50 ul at 65°C for 5 min. The gel buffer and running buffer were 0.02 M boric acid, 0.2 mM Na_3EDTA pH 8.3, 6% formaldehyde. A typical 16 x 16 cm gel used 200 ml agarose and was run at 200 V for 2 hr.

Samples used as controls were stained in 1 ug/ml ethidium bromide in 0.1 M NH_4OAc for 1 hr. The rest of the gel was prepared for transfer by rinsing 3 times with H_2O, 5 min each time, then 30 min in 0.1M Tris pH 7.5, and 20 min with 1X SSC. Transfer was with 1X SSC to GeneScreen (NEN) which had been soaked in 1X SSC. Blots were prehybridized overnight at 45°C in 50% formamide, 5X SSC, 5X Denhardts, 100 ug/ml salmon sperm DNA, 20 ug/ml Poly A, 25 mM $NaPO_4$,

1% SDS. For hybridization, 0.25 ml/cm^2 of a solution containing 50% formamide, 5X SSC, 0.5% SDS, 100 ug/ml salmon sperm DNA, 20 ug/ml Poly A was used to replace the prehybridization solution. This was incubated at 45°C for two hours and then 10^6 cpm/ml (2 x 10^8 cpm/ug) cDNA containing probe was added and the hybridization continued for 24 hr.

Blots were washed 2 times with 2X SSC at room temperature for 10 min with shaking. Followed by two changes of 2X SSC/0.1% SDS at 68°C for a total of 90 min.

Southern Analysis

Total genomic DNA was prepared as described (Friderici, et al., 1984). Briefly, 5A5 cells were lysed in buffer containing 10 mM Tris pH 7.6, 10 mM EDTA, 0.5% SDS and 100 ug/ml Proteinase K (3 ml/5 x 10^7 cells). The lysate was digested overnight at 37°C, extracted once with phenol and twice with $CHCl_3$. The lysate was ethanol precipated, resuspended in 0.5ml 10 mM Tris pH 7.6, 1 mM EDTA (TE) and dialyzed against several changes of TE buffer for 1-2 days. Southern blotting was performed according to the procedures described in Maniatis et al. (1982).

Turnover of Selected mRNA Sequences

5A5 cells were plated at 6 x 10^6 cells/150 cm^2 flask in DME medium the day before initiation of labeling. Cells were labeled for 2 hr with 0.4 mCi/10 ml medium/flask. At indicated times one flask was rinsed with PBS-EDTA, trypsinized, suspended in PBS and pelleted. Cells were rinsed once with 5 ml PBS containing 4 mM vanadyl complexed

nucleotides. Cells were swollen on ice in 1 ml RSB + 10 mM vanadyl nucleotides. Cells were dounce homogenized with 20 strokes of the B pestle, 0.5% Triton X100 was added and cells were rehomogenized. Nuclei were removed at 5000 x g, 5 min and the supernatant was adjusted to 100 ug/ml Proteinase K, 0.15 M NaOAc and 0.5% SDS, and incubated 10 min at 37°C. The cytoplasmic extract was extracted with phenol and chloroform and ethanol precipitated. The RNA pellet was resuspended in 50 ul of water.

For hybridization analysis, filters were prehybridized overnight as described. The prehybridization mixture was removed and replaced with 90 ul hybridization solution containing 10 ul ^{3}H-uridine labeled total RNA for 9 filters. Hybridization reactions were performed in triplicate at 42°C for 3 days. Filters were processed as described under preparation of filter-bound plasmid.

RESULTS

When primary cell cultures were prepared from pituitary glands using standard techniques (Vale et al., 1977), a rapid decrease in PRL and GH production resulted, and the cells exhibited little response to added hormones. To circumvent this problem we substituted D-valine for L-valine in the growth medium. Certain cells (primarily fibroblasts) do not contain the enzymes necessary for conversion of D- to L-valine and cannot grow and divide in medium without L-valine (Gilbert and Migeon, 1975). In the pituitary, cells which produce PRL and GH are of epithelial origin and generally epithelial cells contain the enzymes necessary for this isomerization.

When primary cell cultures derived from adult animals were established in L-valine, a mixture of cell types was seen (Figure 1A). After cells were allowed to grow for 8 days in L-valine containing medium they produced a confluent monolayer (Figure 1B) dominated by one or two types of cells. Cells established in D-valine showed less spreading and cell division 2 days after plating (Figure 1C) and by 8 days, little cell division had occurred as measured by microscopy (Figure 1D). ^{3}H-thymidine incorporation into TCA precipitable material (Figure 2A) was very low in cells maintained in D-valine, whereas cells grown in L-valine showed high levels of incorporation during the first 7 days of culture and division ceased as the cultures reached confluence.

Figure 1. Primary bovine pituitary cell cultures maintained in D- or L-valine.

Cells were prepared from bovine pituitary glands as described in Materials and Methods and plated on cover slips in 35 mm culture dishes. Growth medium was Swims medium containing 10% dialyzed calf serum and either L-valine (A and B) or D-valine (C and D). The cells were stained with trichrome stain at 2 days (A and C) or 8 days (B and D) after initial plating.

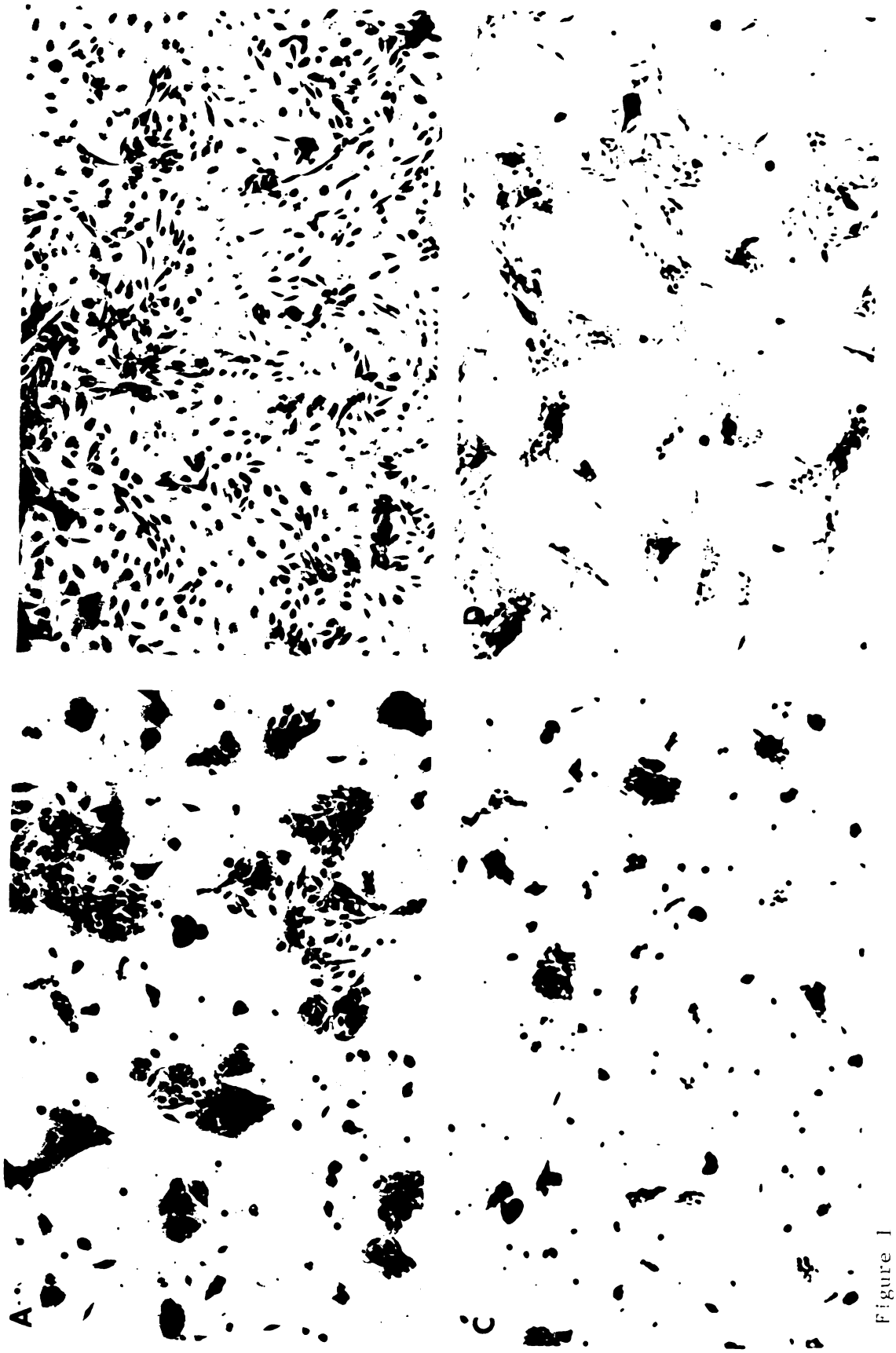


Figure 1

Figure 2. Effect of D-Valine on DNA and Protein synthesis.

Cells were maintained in Swims medium containing 10 % dialyzed calf serum and either D- or L- valine.

A. DNA synthesis measured by ^{3}H-thymidine incorporation into TCA precipitable cpm after 3 hr of labeling.

B. Total protein synthesis after 3 hrs of ^{35}S-methionine incorporation. Hot TCA precipitable cpm were determined for a portion of the cell lysate.

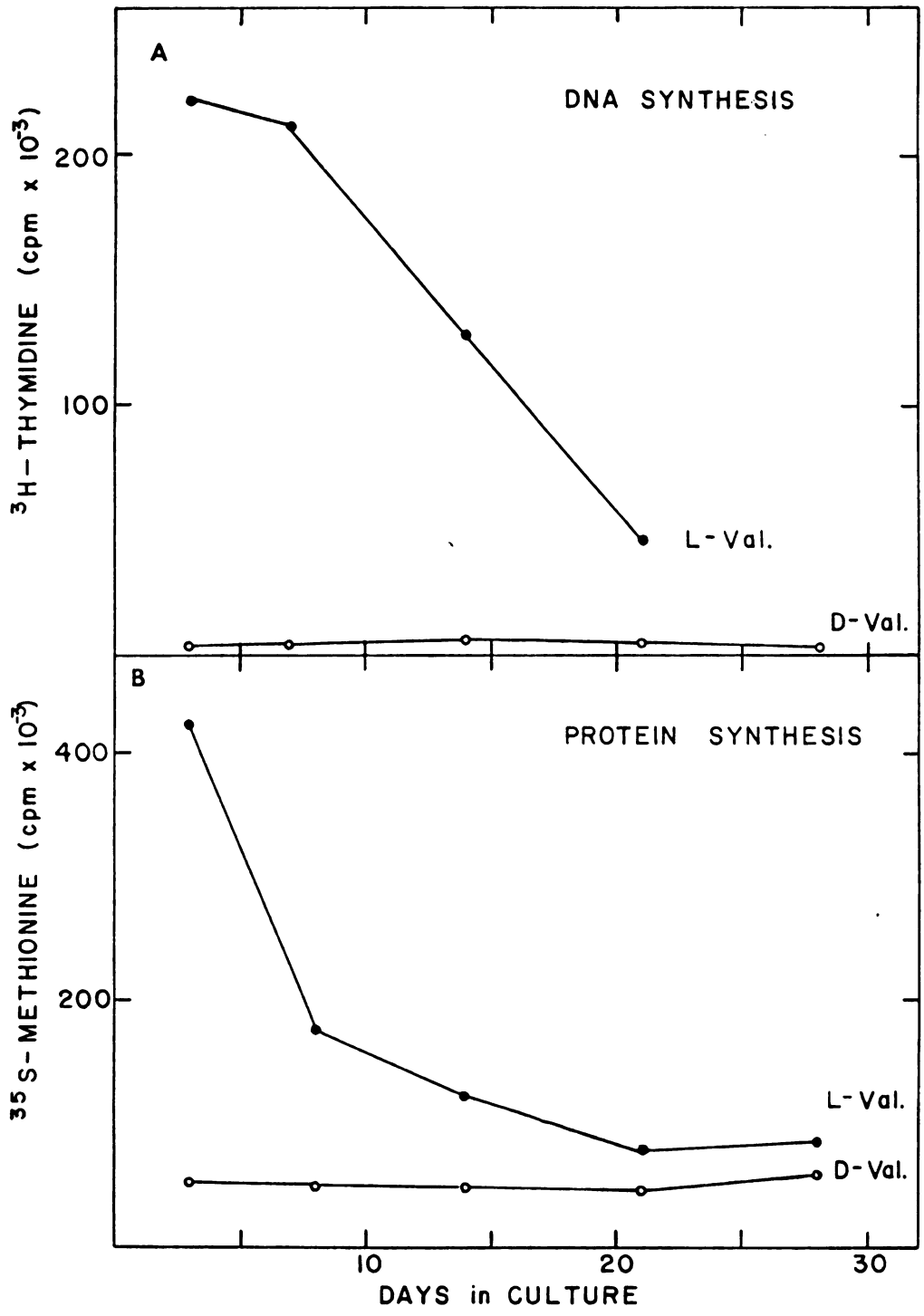

A
DNA SYNTHESIS
^{3}H-THYMIDINE (cpm x 10^{-3})
200
100
L-Val.
D-Val.
B
PROTEIN SYNTHESIS
^{35}S-METHIONINE (cpm x 10^{-3})
400
200
L-Val.
D-Val.
10
20
30
DAYS in CULTURE

Figure 2

Protein synthesis in D-valine medium remained at a constant low level. Cells in L-valine showed very high protein synthesis initially but incorporation decreased as cell division ceased and the cells became less active metabolically (Figure 2B). Epithelial cells from adult animals would not necessarily divide but would be expected to maintain a certain level of protein synthesis.

As a measure of maintenance of differentiated cellular function, PRL secretion into the medium was measured by radioimmunoassay (Padmanabhan et al, 1982). In L-valine medium, PRL secretion decreased steadily and by day 20 very little secretion occurred (data not shown). In D-valine medium, however, PRL secretion begins at the same level but stays high throughout the 30 days these cells were maintained in primary culture. Cells grown in D-valine also retained their ability to respond to thyrotropin releasing hormone by rapidly secreting increased amounts of PRL into the medium. Higher levels of PRL secretion were observed when cultures were maintained in 5 ng/ml estradiol. Initially the estrogen response is about 1.5 fold over controls but decreases with time to D-valine control levels.

Synthesis of PRL or GH can be used as a measure of the mRNA level of that protein since there is no translational control of PRL or GH protein production (Shull and Gorski, 1986). We, therefore, developed a system for determining the level of <u>de novo</u> protein synthesis so that optimization of culture conditions and hormone responsiveness could be evaluated. Cells were labeled with ^{35}S-methionine and lysed and the cell lysate was subjected to disc SDS polyacrylamide gel eletrophoresis (Figure 3). Even after 28 days in

Figure 3. Gel profile of newly synthesized protein.

Cells were maintained in either D- or L-Valine Swims medium for 28 days. Cell lysates prepared from cells labeled 3 hr with ^{35}S-methionine were subjected to SDS polyacrylamide gel electrophoresis. Gels were sliced 2 mm wide and radioactivity in each slice was determined.

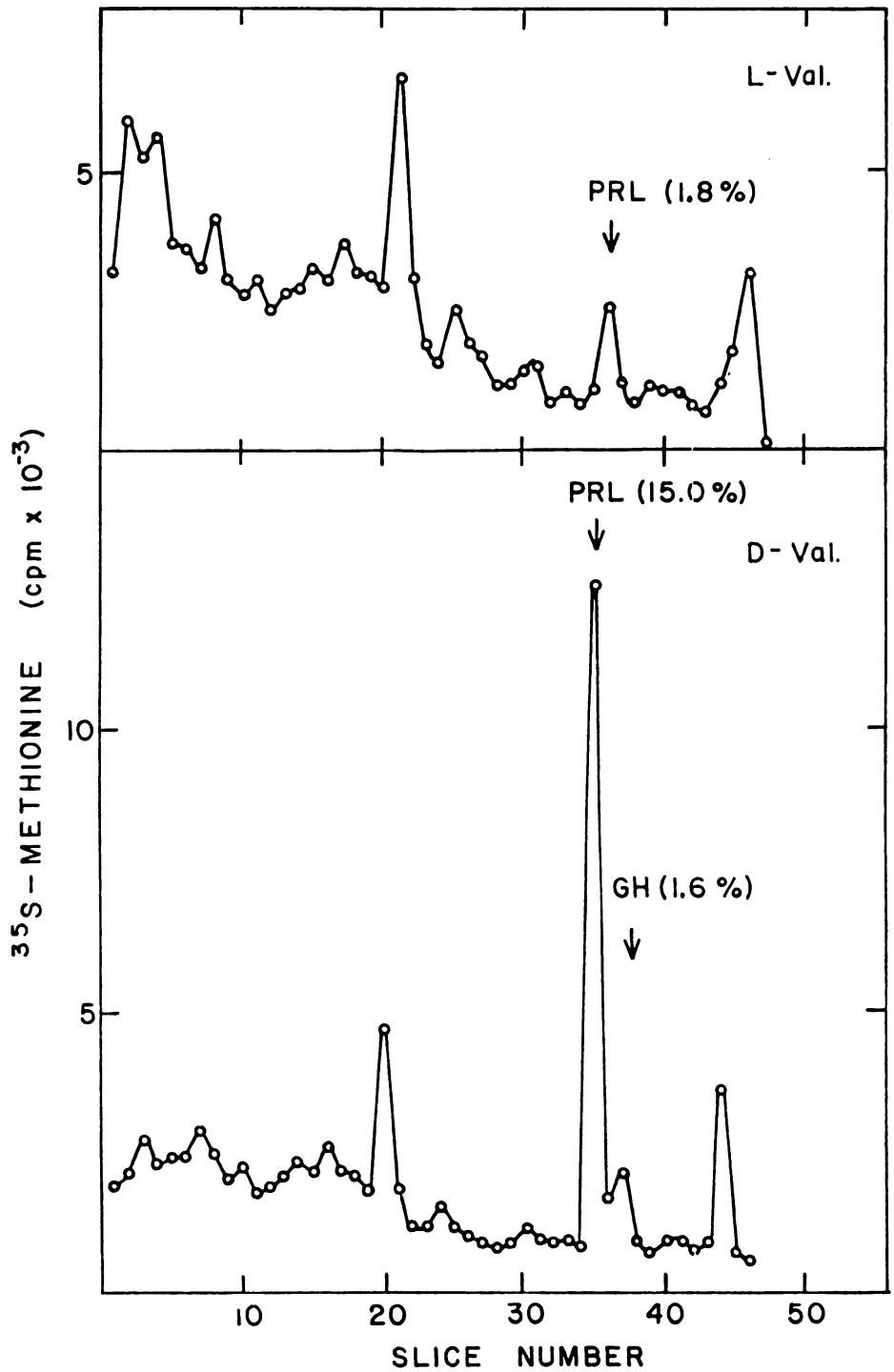

L-Val.
PRL (1.8%)
PRL (15.0%)
D-Val.
GH (1.6%)
5
10
5
^{35}S-METHIONINE (cpm x 10^{-3})
10
20
30
40
50
SLICE NUMBER

Figure 3

culture, PRL was the major protein product of cultures maintained in D-valine medium (Figure 3B). Only a low levels of PRL synthesis was detected in cultures maintained in L-valine medium. Prolactin accounted for 10-15% of the total protein synthesis throughout the culture period (Figure 4A). Growth hormone synthesis also remained relatively constant in this experiment (Figure 4B). That these major protein products were, in fact, PRL and GH was verified by comigration with authentic NIH standard bovine PRL and GH on these gels. Quantitation of PRL labeling was further analyzed in some lysates by immune precipitation. It was found that all of the material in the presumed PRL band could be precipitated with PRL antiserum (data not shown).

The results obtained for GH synthesis and secretion tended to be variable. We found that GH production could be maintained only with certain batches of serum. Serum which originally maintained GH synthesis lost that ability after charcoal treatment to remove steroids. Addition of 40 ng/ml hydrocortisone restored GH production (Figure 5B). PRL synthesis in these cultures showed very different results (Figure 5A). Charcoal treatment of serum increased PRL synthesis, while addition of hydrocotisone depressed PRL synthesis below control levels. These results can be explained if one considers endocrine control of the pituitary. PRL production is apparently primarily under negative control by the hypothalmus. Its primary inhibitor is dopamine, but hydrocortisone is also known to decrease PRL production. Hydrocortisone is responsible for increases in GH synthesis and secretion. Apparently serum from different groups of animals contained varying levels of hydrocortisone which was removed

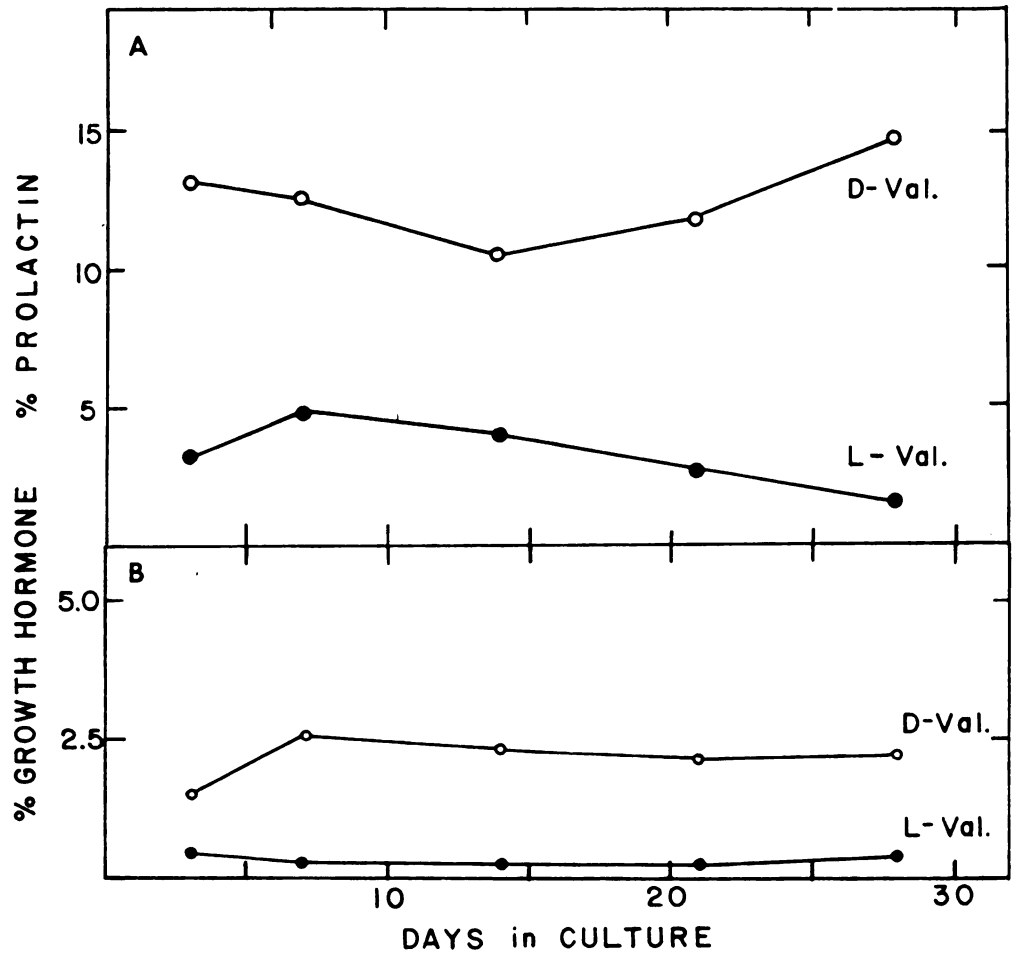


Figure 4. Prolactin and growth hormone as a percent of total protein synthesis.

Cell lysates were analyzed as in Figure 3. Percentage is the cpm migrating with NIH standard PRL or GH divided by total cpm recovered from each gel.

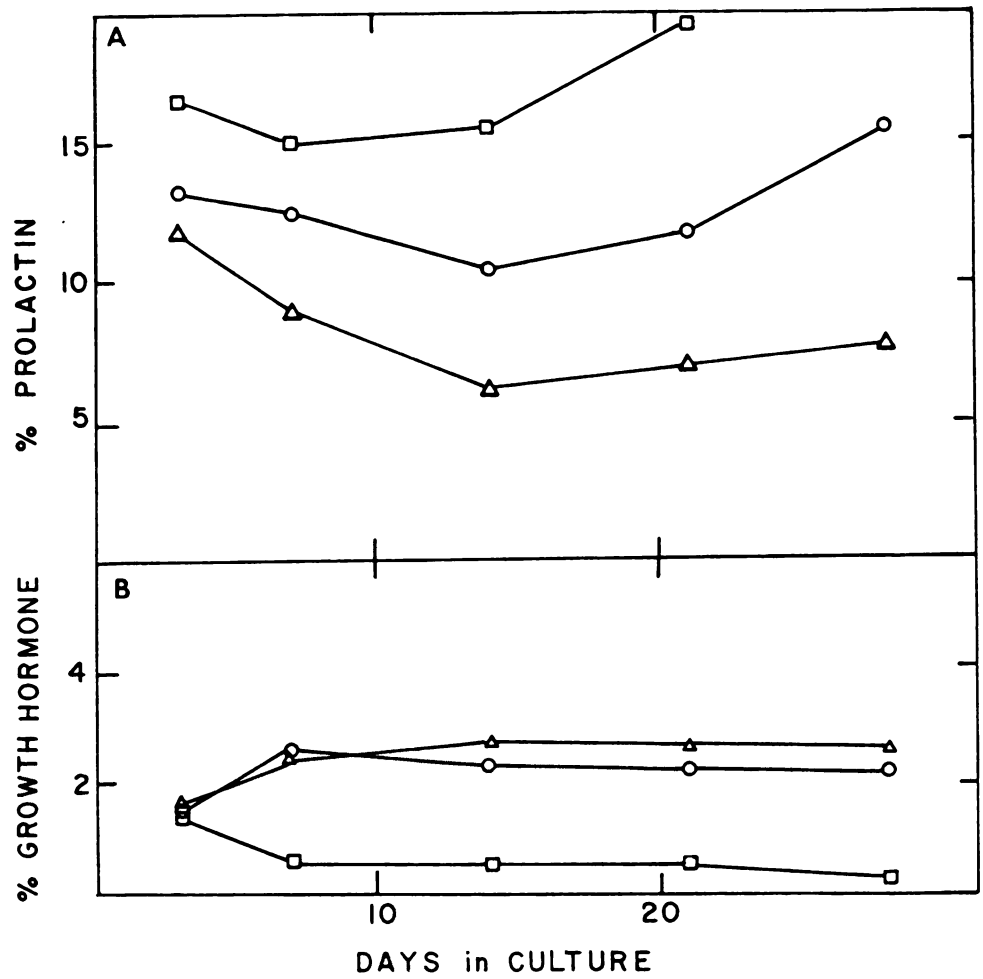


Figure 5. Effect of charcoal treatment of serum on GH and PRL synthesis.

Cells were maintained in D-valine Swims medium containing 10% of the indicated serum: (—○—) control, untreated serum; (—□—) charcoal treated serum; (—△—) charcoal treated serum plus 40 ng/ml hydrocortisone. Conditions for labeling and quantitation of PRL and GH synthesis were the same as in Figure 3.

by the charcoal treatment. This allowed PRL production to rise due to lack of repression by hydrocortisone or dopamine, while without these necessary stimulants GH production decreased.

At this point we had a primary tissue culture system that could be maintained for three to four weeks with continued PRL and GH production. However, when I attempted to change the levels of PRL and GH protein synthesis by adding hormones to the cultures the magnitude of the responses was small. The largest effects were seen with hydrocortisone which increased GH synthesis about five-fold and decreased PRL synthesis 50% (Figure 5). Results were still somewhat variable and this was not too surprising when one considers the number of factors in serum which can affect the synthesis and release of PRL and GH.

We felt that using a medium that was completely serum-free might increase the magnitude and reliabibity of these responses. Serum-free medium has been shown to allow increases in hormonal response of cells in culture (Barnes and Sato, 1980). A determined effort was made to establish pituitary cells in serum-free medium. Several types of commercial media were tested; plates coated with gelatin, serum, poly-D-lysine, or fibronectin were used to enhance cell attachment; and fetuin, insulin, transferrin and Hepes were added to the medium to replace serum components. Since fibroblasts do not proliferate in serum-free medium, the necessity of using L-Valine was reexamined. These media, with L-valine, generally supported considerable cell division for 1-2 weeks followed by a variable decline in total protein synthesis and a reduced number of cells per dish. There was a concomitant loss of PRL and GH synthesis. Hams's

F12 medium was used under conditions designed to maintain cell viability in epithelial cells from thyroid tissue (Ambesi-Impiombata et al., 1980). Using this medium, cell division occurred readily and viabiliy was maintained but no hormone response was demonstrated and PRL and GH production declined after 8 days (data not shown).

Serum-free MEM medium containing D-valine (GIBCO) and supplemented with only transferrin, insulin and HEPES did maintain a constant level of protein synthyses for more than two weeks. In serum-free medium, PRL synthesis tends to increase with time in culture for about five to six days (Figure 6). This probably reflects release of these cells from the hypothalmic repression that occured in the intact animal. Dexamethasone, an agonist of hydrocortisone, depresses PRL synthesis. Growth hormone synthesis showed a more complete reduction in these cultures than in those maintained in 10% charcoal treated calf serum (data not shown).

Using this serum-free medium a number of other attempts were made to produce larger changes in prolactin or growth hormone synthesis. Bromoergocryptine is a ergot alkaloid that is an agonist of dopamine. Dopamine is unstable in culture medium but bromoergocryptine is not. In rat pituitary cell cultures these compounds greatly reduce the amount of PRL secreted into the medium and the amount of PRL protein synthesized. When secretion of PRL into the medium was analyzed (Figure 7) it was found that bromoergocryptine reduced PRL secretion more than 80%. Hydrocortisone also reduced secretion of PRL to a lesser extent. While the effect of bromoergocryptine on PRL secretion is dramatic, the effect on PRL protein synthesis is less pronounced (Figure 6).

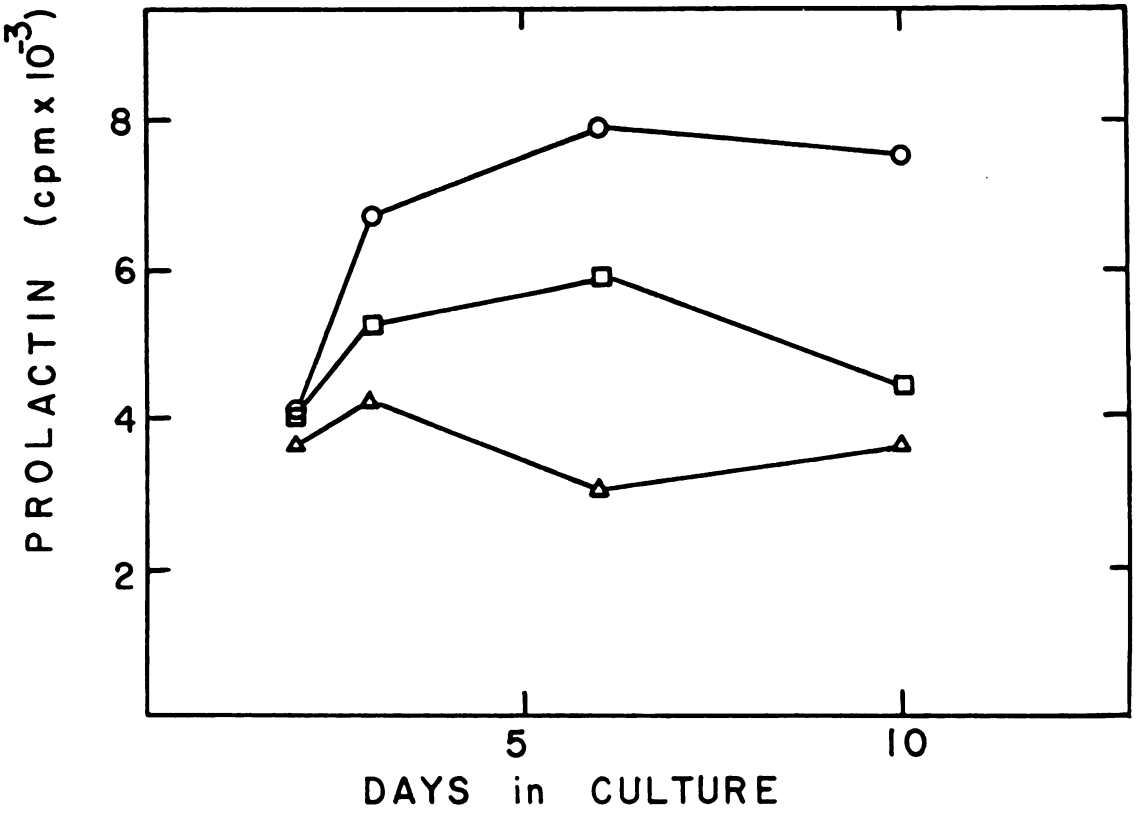


Figure 6. Prolactin protein synthesis in serum-free medium.

Primary pituitary cultures maintained in serum-free medium consisting of D-val MEM (GIBCO) were treated with 10 nM bromoergocryptine (△—△), 5 uM dexamethasone (□——□), or untreated (○——○), beginning one day after establishing the cultures and continuing throughout the culture period. Cell cultures were labeled for 1.5 hr. with ^{35}S-methionine.

Figure 7. Time course of accumulation of newly synthesized prolactin in the culture medium.

Cells were maintained in serum-free medium containing 10 nM bromoergocryptine or 40 ng/ml hydrocortisone for three days prior to labeling. Aliquots of the medium were denatured with SDS and analyzed on SDS-polyacrylamide gels.

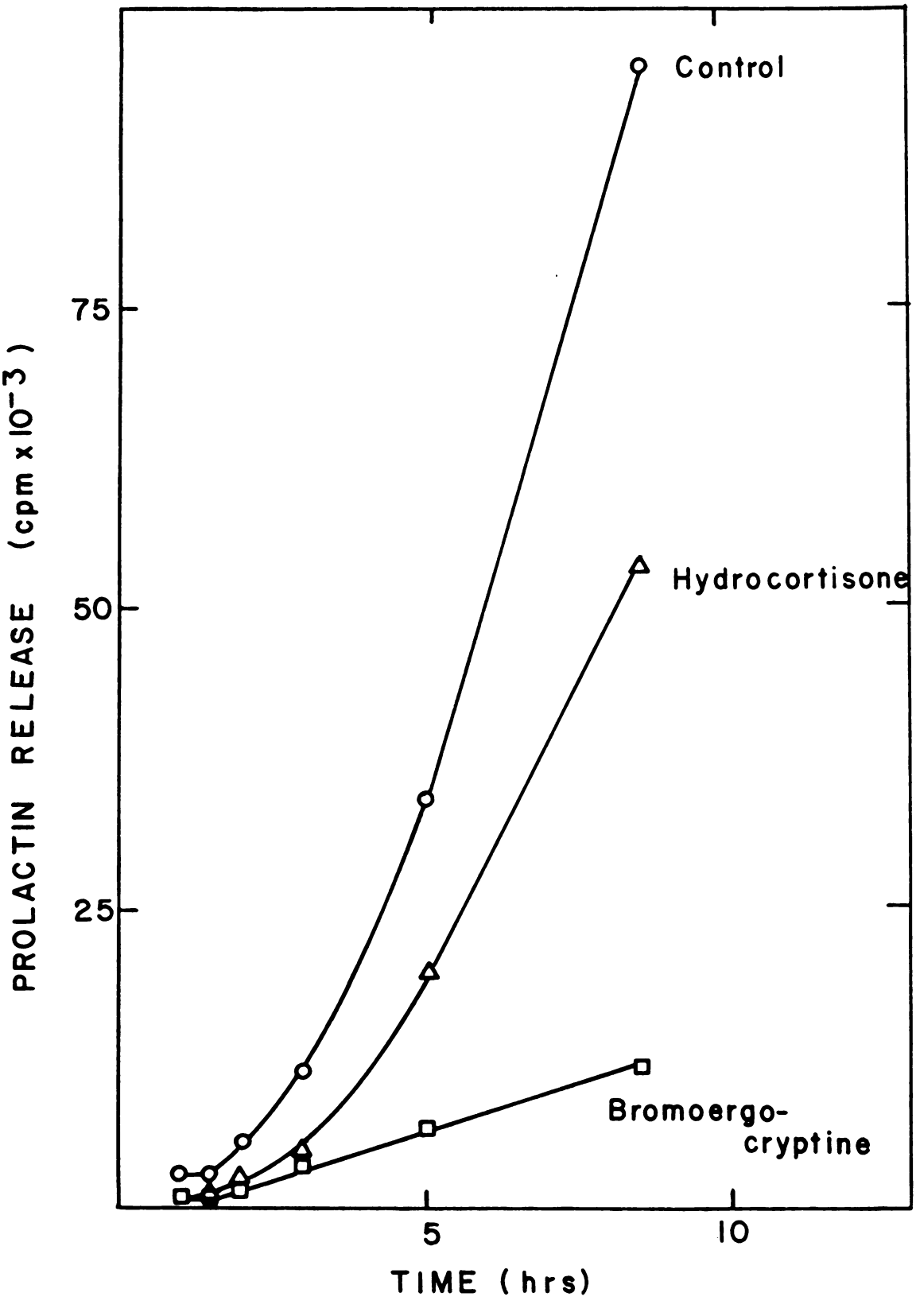

Control
Hydrocortisone
Bromoergo-
cryptine
75
50
25
PROLACTIN RELEASE (cpm x 10⁻³)
5
10
TIME (hrs)

Figure 7

Several other media were also tested. It was suggested (Mauer, personal communication) that while D-val MEM maintained cell viability and PRL and GH production, responsiveness to exogeneously added hormones was diminished. Rat primary cells respond well in serum-free Dulbecco's medium with insulin, transferrin and HEPES. The results using this medium with a variety of hormones are shown in Fig. 8. After two days of treatment there has been no effect on PRL levels and new GH was not synthesized under any condition even though GH protein was still present in these cells as shown by Coomassie blue staining of the gel. At seven days there was still no effect on PRL levels (data not shown). Other methods for preparing cells from the pituitary gland (Miller et al., 1977) were also evaluated, but had no noticible effect on hormone responsiveness.

Serum-free pituitary cell cultures maintained in D-valine MEM responded to hydrocortisone and ergocryptine. The magnitude of the responses at the level of protein synthesis were not as dramatic as the secretory response but may reflect what is happening in the intact animal. Omission of serum from the medium does eliminate concerns about introducing unknown serum hormones to the cultures.

Fetal pituitary cell cultures were also established in view of the possibility that hormone responses might be more dramatic in tissue from animals that had a shorter history of previous exposure to circulating hormones. The bovine pituitary differentiates throughout gestation and mRNA levels change throughout development (Nilson et al., 1980). Fetal cultures were established using methods similar to those used for adult tissues and their response to a variety of hormones was tested (Figure 9 and Figure 10 A-D) It was found that

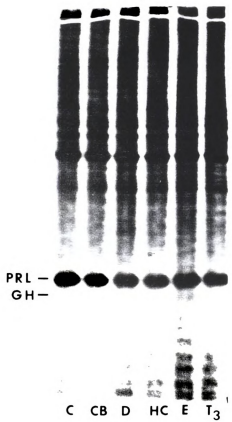


Figure 8. Protein Synthesis in Serum-Free Dulbeccos Medium.

Tissue was prepared in CMF-HBSS (Miller, et al., 1977) and grown on Poly-D-lysine coated cell culture dishes in Dulbeccos medium, with insulin and transferrin (C). Additions to the medium were; 10^{-8}M Bromoergocryptine (CB), 5 x 10^{-7} M Dexamethasone (D), 10^{-8} M Hydrocortisone (HC), 10^{-8} M Estrogen (E), or 3 x 10^{-8} M Triiodothyronine (T).

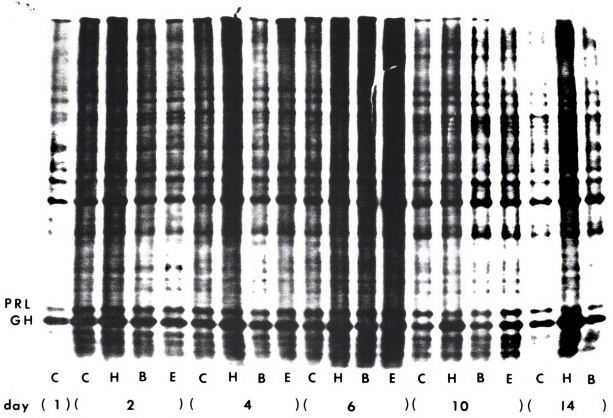


Figure 9. Protein Synthesis in Cell Cultures From Fetal Pituitaries.

Primary cell cultures were prepared from bovine fetuses of mixed gestational ages. Cells were maintained in serum-free D-valine MEM, supplemented with insulin, transferrin and HEPES. Treatment with hormones was begun one day after plating cells. HC-hydrocortisone, 40 ng/ml; CB-CB154(a dopamine agonist), 10 nM; E-estrogen, 50 ng/ml. At days indicated below the figure, cells were labeled with ^{35}S-methionine for 90 min. Total cell lysates were prepared and 12 ul lysate/lane was analyzed directly on SDS-PAGE (13% acrylamide).

Figure 10. PRL and GH synthesis in Fetal Pituitaries.

Fetal cultures maintained in serum-free Swims medium containing D-valine, 2 ug/ml insulin, 5 ug/ml transferrin, with or without hydrocortisone (HC).

(A) %PRL = cpm in PRL divided by the total cpm recovered from the gel.

(B) %GH = cpm in GH divided aby the total cpm recovered from the gel.

(C) Total cpm in PRL protein (% PRL x cpm in total protein).

(D) Total cpm in GH protein (% GH x cpm in total protein).

(E) Total ^{35}S-Methionine incorporation into cellular protein.

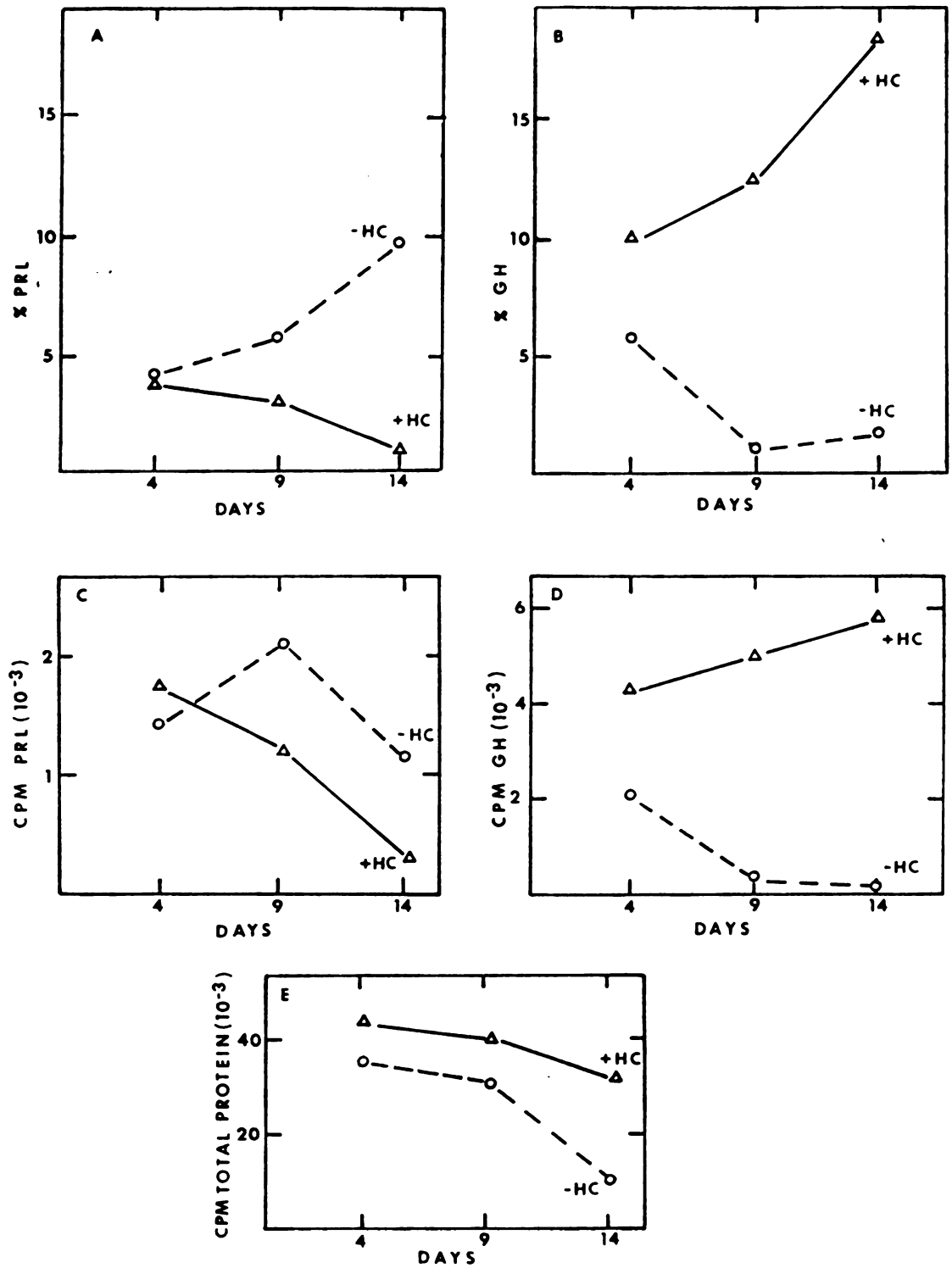

A
% PRL
15
10
5
-HC
+HC
4
9
14
DAYS
B
% GH
15
10
5
+HC
-HC
4
9
14
DAYS
C
CPM PRL (10-3)
2
1
-HC
+HC
4
9
14
DAYS
D
CPM GH (10-3)
6
4
2
+HC
-HC
4
9
14
DAYS
E
CPM TOTAL PROTEIN (10-3)
40
20
+HC
-HC
4
9
14
DAYS

Figure 10

the fetal pituitary synthesizes much more GH than PRL which is consistent with the very high level of growth during fetal development (Figure 9). In these cultures hydrocortisone had the effect of maintaining the viability of the culture [as measured by continued protein synthesis, (Figure 10E)] as well as maintaining GH synthesis. Synthesis of GH in cultures containing hydrocortisone was very high (18% of total protein at 14 days) and increased steadily throughout the culture period (Figure 10D). While the effects of hydrocortisone on PRL synthesis were dramatic (Figure 10A), the effect of hydrocortisone on PRL synthesis could not be studied in this system since the cultures did not survive well without it (Figure 10C and E). Neither estrogen nor bromoergocryptine had any effect on the synthesis of PRL or GH (Figure 9) in these cultures.

To study the stability of specific mRNAs in cultures, a method to quantitate small amounts of radiolabeled individual mRNAs using plasmid DNA bound to nitrocellulose filters was characterized. Hybridization and wash conditions were tested and adjusted using pituitary polysomal Poly A+ RNA that was labeled with ^{32}P using polynucleotide kinase (Figure 11A). The assay shows that specific binding increases linearly with increased amounts of RNA indicating that the filters are not saturated. This was an important control since prolactin and growth hormone represent a large percentage of the Poly A+ RNA in the pituitary.

To test this assay for sequences present at much lower levels a different gene product was used. It was essential to use another bovine sequence for this work since it had been determined that stringent wash conditions were necessary to produce reliable results

Figure 11. Evaluation of Hybridization Assay Using Filter-bound Plasmid.

A. Pituitary polysomal poly A+ RNA was labeled with ^{32}P using polynucleotide kinase. The labeled RNA was hybridized to filters containing plasmid DNA; pPRL(○), pGH(△) and (▲) duplicate hybridization reactions, and pBR322(□).

B. & C. Plasmid bound filter hybridization using <u>in vivo</u> ^{3}H-uridine labeled RNA. Transformed cells were labeled in culture for two hours, cytoplasmic poly A+ RNA was prepared and hybridized to filters containing α-tubulin (○), pGH or pBR322 (□).

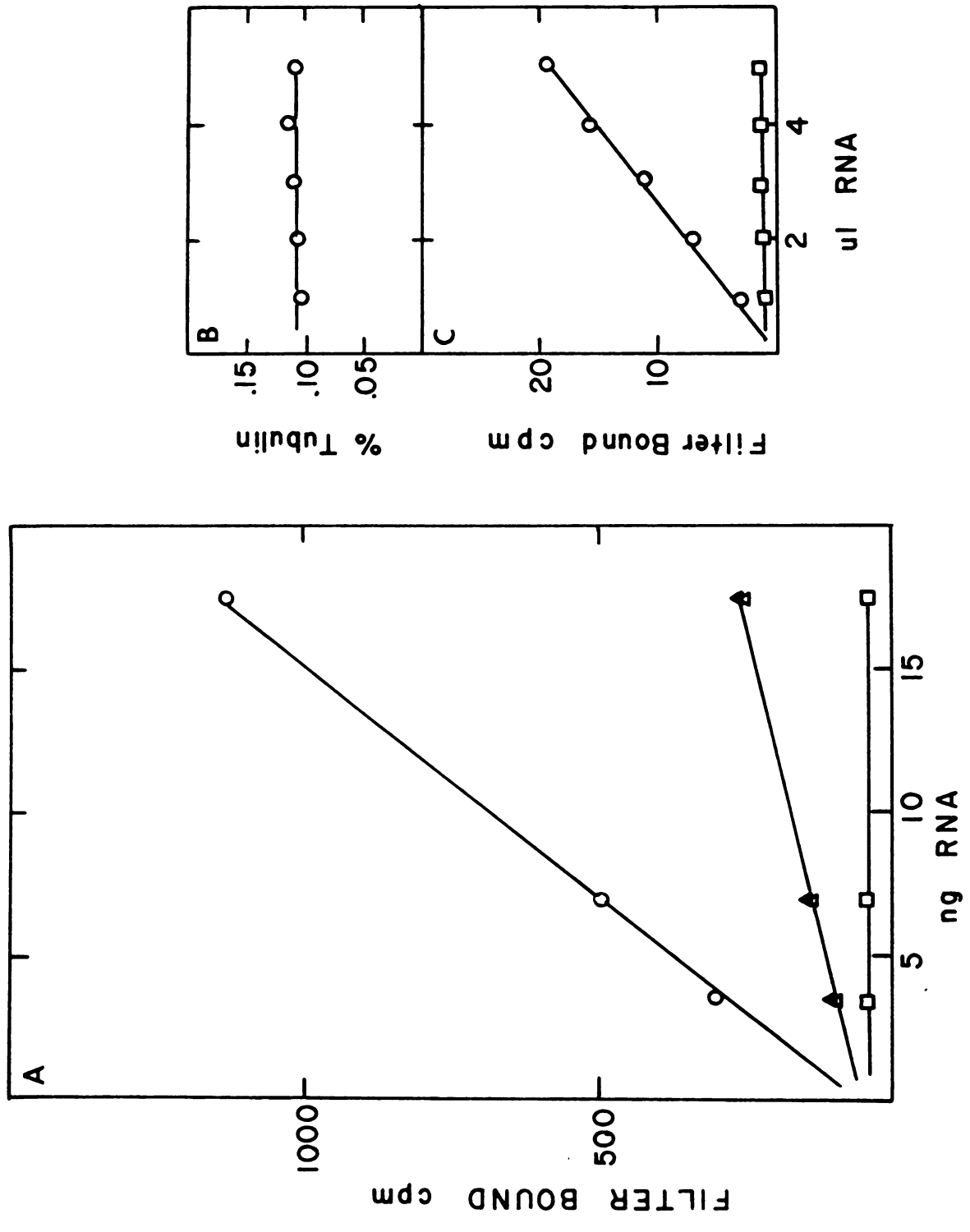

A
FILTER BOUND cpm
1000
500
5
10
15
ng RNA
B
% Tubulin
.15
.10
.05
C
Filter Bound cpm
20
10
2
4
ul RNA

Figure 11

with this assay. A plasmid containing chicken α-tubulin was used to screen a bovine cDNA library made from pituitary gland Poly A+ RNA. A bovine -tubulin clone was identified which contained an 1100 bp. insert. This clone was presumed to be authentic α-tubulin because the initial screen used washes at 0.2 X SSC at 55°C, but the insert was not sequenced. In CHO cells α-tubulin represents approximately 1% of the mRNA and has a turnover of 2-10 hr, depending on the levels of unpolymerized tubulin in the cells (Cleveland et al., 1981). The filter hybridization assay was repeated using Poly A+ and Poly A- RNA from ^{3}H-uridine labeled transformed fetal bovine cells (discussed below). Of the Poly A+ RNA, 0.1% bound (Figure 11B) while 0.01% of the Poly A- RNA bound to the α-tubulin plasmid. Filters containing GH or pBR322 plasmid showed background binding (0.0006%). This assay was also linear over several RNA concentrations (Figure 11C)

To determine the turnover of PRL, GH, and α-tubulin in primary pituitary cultures pulse-chase analysis was attempted. Cells were labeled for 3.5 hrs. with ^{3}H-uridine and a chase was performed with medium containing cold uridine and cytidine. Cytoplasmic RNA was prepared from cultures at time intervals after the start of the chase (Figure 12). Although preliminary experiments had indicated that this chase procedure was adequate, it is clear that the chase did not become effective until after 15 hr. During the time period between 15 and 27 hour incorporation into total RNA was constant whereas the amount of label in Poly A+ RNA declined by nearly 50%. In contrast, PRL and GH mRNA showed a 1.2 and 1.4 fold increase, respectively (Figure 13) during this interval. Tubulin mRNA was also stable in this experiment but did not accumulate to the level of PRL mRNA even

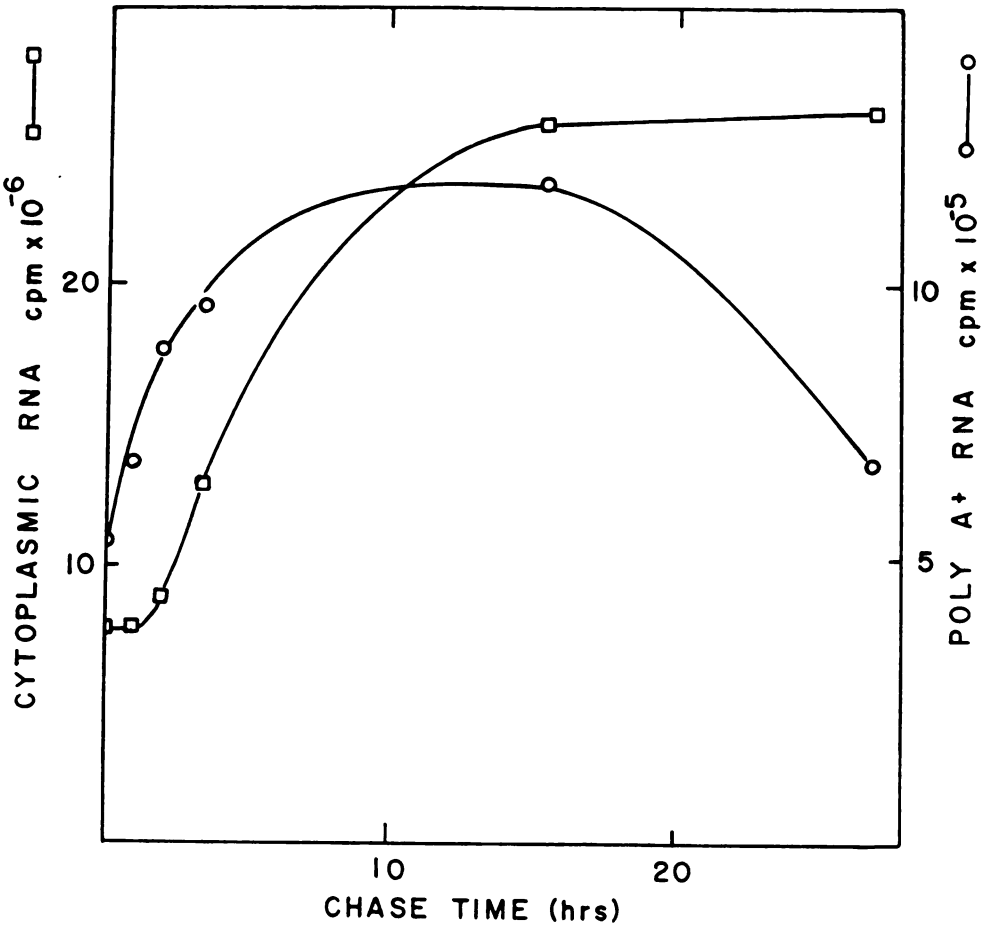


Figure 12. Pulse-Chase of Primary Pituitary Monolayer Cultures.

Cells were labeled for 3.5 hr. with 240 uCi ^{3}H-uridine/5 x $^{6}10$ cells. At the times indicated, cytoplasmic RNA was extracted from 10^{7} cells. An aliquot from each time point was hybridized to oligo dT cellulose and the amount of poly A+ RNA determined.

Figure 13. Specific mRNA Binding to Nitrocellulose Filters During Chase.

Total RNA from Figure 12 was hybridized to filters. Filters were washed and eluted as described in Materials and Methods.

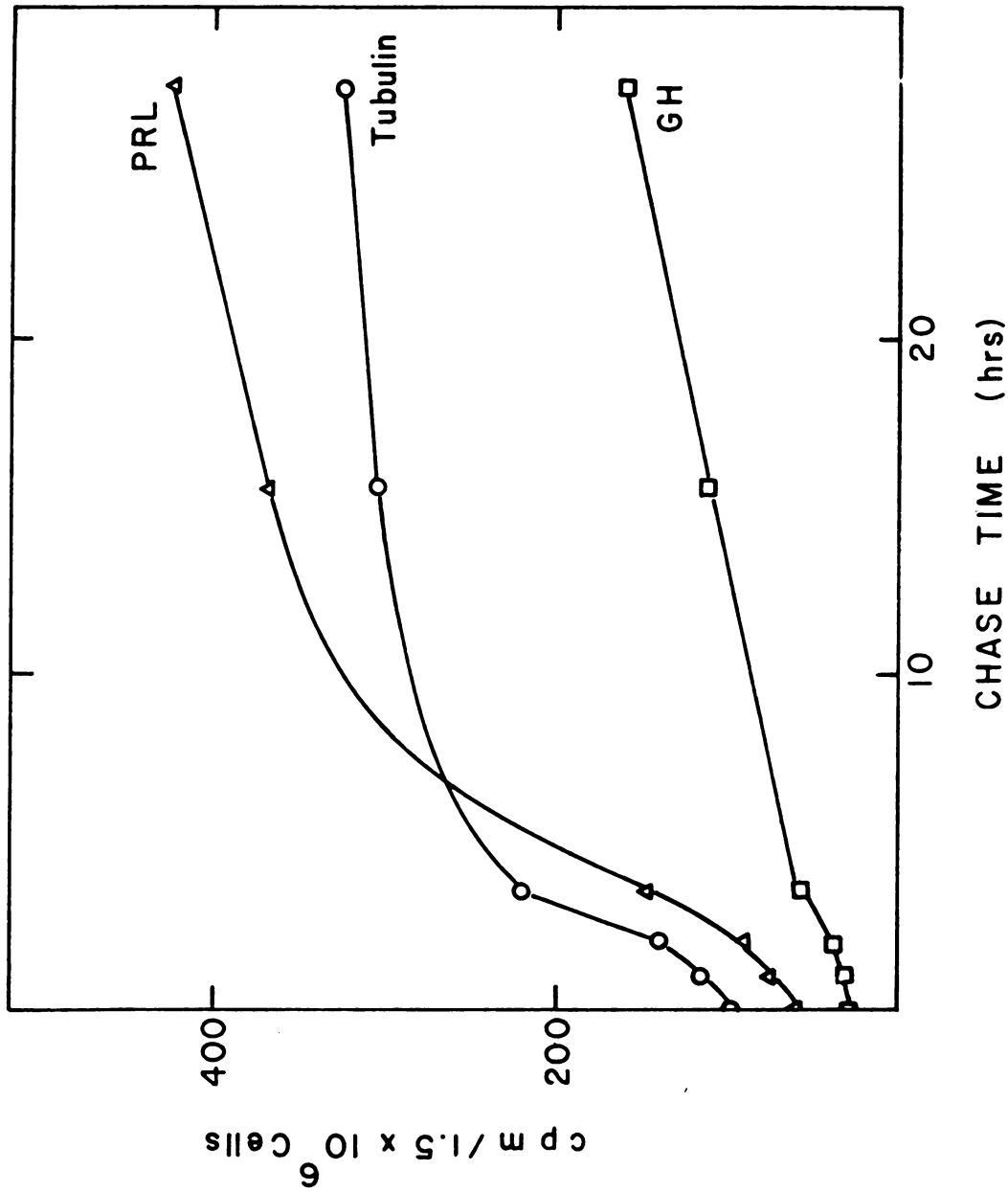


Figure 13

though tubulin attained a higher level during the 2 hour pulse. Interestingly, this preliminary evidence showed that PRL and GH had lower turnover rates than total RNA which would be primarily rRNA. These data indicated that the mRNA for PRL and GH was very stable and even under effective chase conditions would be very difficult to measure in these non-dividing primary cultures. This is in agreement with the observations that the levels of PRL and GH mRNA, as measured by protein synthesis, change very little and rather slowly in response to hormones which control their secretion. In the bovine pituitary cell any control of PRL and GH mRNA levels by alteration of mRNA stablility does not result in dramatic changes and would be very difficult to quantitate.

The unusual stability of PRL and GH mRNAs in primary cell cultures was intriguing. The extreme stability of these mRNAs could be due in part to the metabolism of the non-dividing lactotrophs and somatotrophs which synthesize and secrete these proteins. Alternatively, the stability of these mRNAs could be a result of a basic sequence difference from other mRNAs. Therefore, I decided to identify other mRNAs of varying stabilities in cells of bovine pituitary origin for comparison with PRL and GH mRNAs.

Studies on less stable mRNAs are not possible in these primary cell cultures since the cell population is so heterogeneous. If mRNAs other than PRL or GH are studied they could be produced by a subset of cells that are behaving differently than the lactotrophs and somatotrophs for which these cultures were optimized. Conditions for the maintenance of other cell types may not be optimal. Cell death in a small subset of cells may result in an alteration of the apparent

half-life of any mRNA they synthesize. To approach this problem we attempted to develop SV40 transformed bovine cells which were capable of producing PRL or GH.

We needed to use fetal cell cultures for SV40 transformation since this virus requires cell division to establish transformation. In the adult gland cell division and differentiation has ceased, but in the fetus the pituitary cells are still proliferating. Somatotrophs were kept viable and functional by including hydrocortisone in the growth medium during establishment of the transformation.

Dr. Robert Lyons established several clonal cell lines following SV40 transformation. As expected, many of these cell lines show very different phenotypes. Papova virus transformation can lead to a varity of phenotypes but since the original tissue had a variety of cell types also, it was hoped that the morphological variation might be due to differences in the parental cells. Several cell lines were chosen for further studies. One cell line had the morphology of an epithelial cell, and appeared to contain secretory granules. There was preliminary evidence for GH mRNA production in these cells. We expected to use these lines to study regulation of GH gene expression in a homogeneous cell population.

To obtain mRNAs from various stability classes I constructed a cDNA library from the cell line described above. From this library I hoped to identify a number of bovine mRNAs with varying stabilities to compare with the very stable GH mRNA. The primary objective of this approach was to obtain a number of bovine mRNA clones in each of several stability classes. The metabolism of these mRNAs could be

compared with that of the GH mRNA. Following identification of clones representing different stability classes these bovine sequences could ten be examined to determine how they differ from the PRL and GH mRNAs. The cDNA library would also be used to identify clones representing mRNAs which accumulated to different levels in two transformed cell lines which showed very different mophologies. The problem we planned to address was: if two mRNAs reach different steady-state levels in two cell types, is this accomplished primarily through transcriptional control or by stability differences.

A cDNA library was constructed using mRNA from an SV40 transformed cell line named 5A5, as described in Materials and Methods. The transformation efficiency was 9×10^6 colonies/ug insert DNA or 3×10^6 colonies/ug insert + vector DNA. Approximately 600 colonies were transferred to gridded plates and to duplicate filters. Preliminary screening was attempted using mRNA from cells labeled in culture with $^{32}PO_4$ or ^{3}H-uridine for short and long periods of time. Messenger RNA from each was prepared and used for hybridization to duplicate filters containing clones from the cDNA library. Phosphate labeling always resulted in very high backgrounds, and ^{3}H-uridine produced too little signal to be useful. I decided that I would have to assume that mRNA abundance was sometimes a reflection of RNA stability and screen the library for abundant, moderately abundant, and rare mRNAs.

To screen for sequences of varying abundance poly A+ RNA from 5A5 cells and a morphologically different cell line, 8A3, was used to make labeled cDNA using reverse transcriptase with random primers. Clones representing highly abundant mRNAs were easily identified, and

were the same in 8A3 and 5A5 mRNA. A few clones seemed to represent higher levels of mRNA in one or the other cell line. These were chosen for further study along with some which showed low levels of hybridization to the mRNA from either cell line.

Approximately 30 clones were chosen for further study. These clones had an average insert size of 350 nt with a range of 50-1300 nt. The clones with larger inserts were chosen for further analysis (Table 3).

Northern analysis was used to determine the sizes of mRNA these cDNAs represented and to give some indication of their relative abundance in a transformed cell line (8A3) and polysomal Poly A+ RNA prepared from intact pituitary glands (Figure 14). The RNA from 8A3 cells was partially degraded resulting in the ladder effect seen in these RNA samples. RNA from cell line 5A5 was also analyzed. It was more degraded than 8A3 RNA but showed a similar intensity of signal. Several of the cDNA clones represented mRNAs which were very abundant (1/112, 1/105, 3/10, 2/71) as judged by their intensity relative to α-tubulin. Some were moderately abundant (5/14, 5/38) and some were not detectable on Northern blots (1/44, 5/48, 5/90). Most were present in RNA from all three cell types. Clone 5/14 showed no hybridization to pituitary gland RNA. The sizes of the mRNAs varied from 700-2000 bp (Table 3). Clone 1/16 is an interesting exception and will be discussed in Part II. Two clones, 3/10 and 1/105, showed the same size and pattern on Northern analysis and were subsequently shown to contain the same sequence.

Southern analysis showed that most of these cDNA clones represent single copy genes (Fig. 15; Table 3). There were two

TABLE 3

Characterization of Pituitary cDNA Clones

Clone	Insert size(bp)	cDNA screen abundance	Southern copy #	Northern Analysis		Kinetic Analysis (ppm of total RNA)			
				size(bp)	abundance	primary 2 hr.	5A5 cells 1 hr.	5A5 cells 2 hr.	$t_{\frac{1}{2}}$
p1/16	1250	low	highly repet	5000	moderate	375	33	13	45 min.
p1/44	360	low	mod repet	N.D.	low	0	2		
p1/105	240	high	single	1900	high				
p1/112	280	high	single	680	high	72	26	66	4.5 hr.
p2/71	540	moderate	single	1000	high-mod	59	24	25	8 hr.
p3/10	450	high	single	1900	high	216	90	228	6.5 hr.
p3/67	180	low-mod		N.D.	low				
p5/14	300	moderate		1150	moderate	86	27	54	8.5 hr.
p5/39	300	moderate	single	1650	moderate	9	4		
p5/48	850	low	single	N.D.	low	14	4		
p5/90	400	low		N.D.	low	29	2		
α-tub.	1600			2000	mod-high	26	12	26	8 hr.

N.D. Not detectable

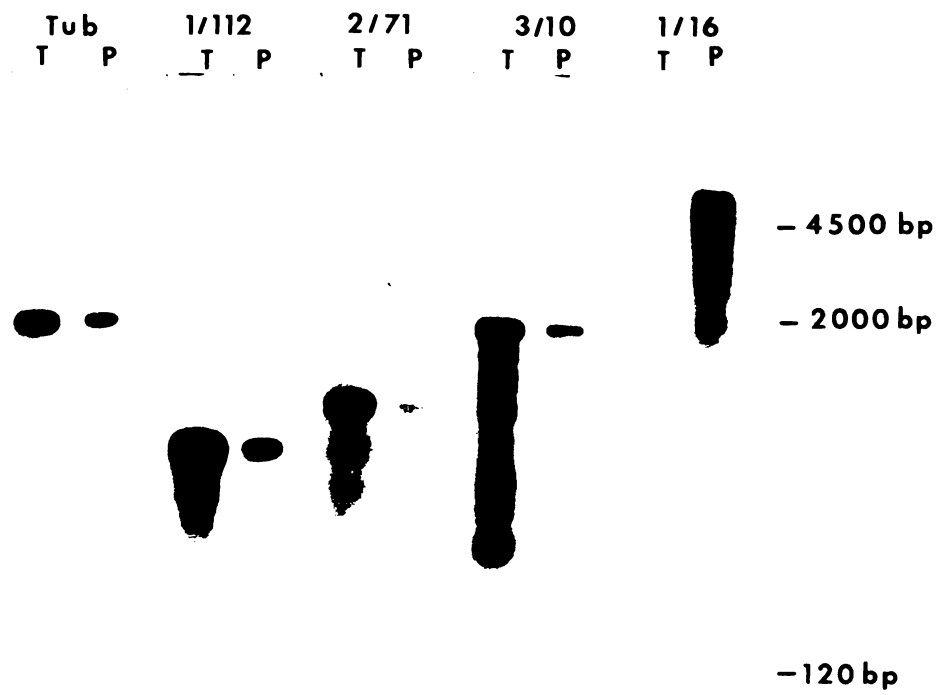


Figure 14. Northern Analysis of RNA Corresponding to cDNA Clones.

Plasmid DNA from the indicated clones was nick-translated to a specific activity of 1-2 x 10^8 cpm/ug DNA and used to probe northern blots of Poly A+ RNA (1 ug/lane) from; T, Cytoplasmic Poly A+ RNA from SV40 transformed 8A3 cells or P, Pituitary polysomal poly A+ RNA.

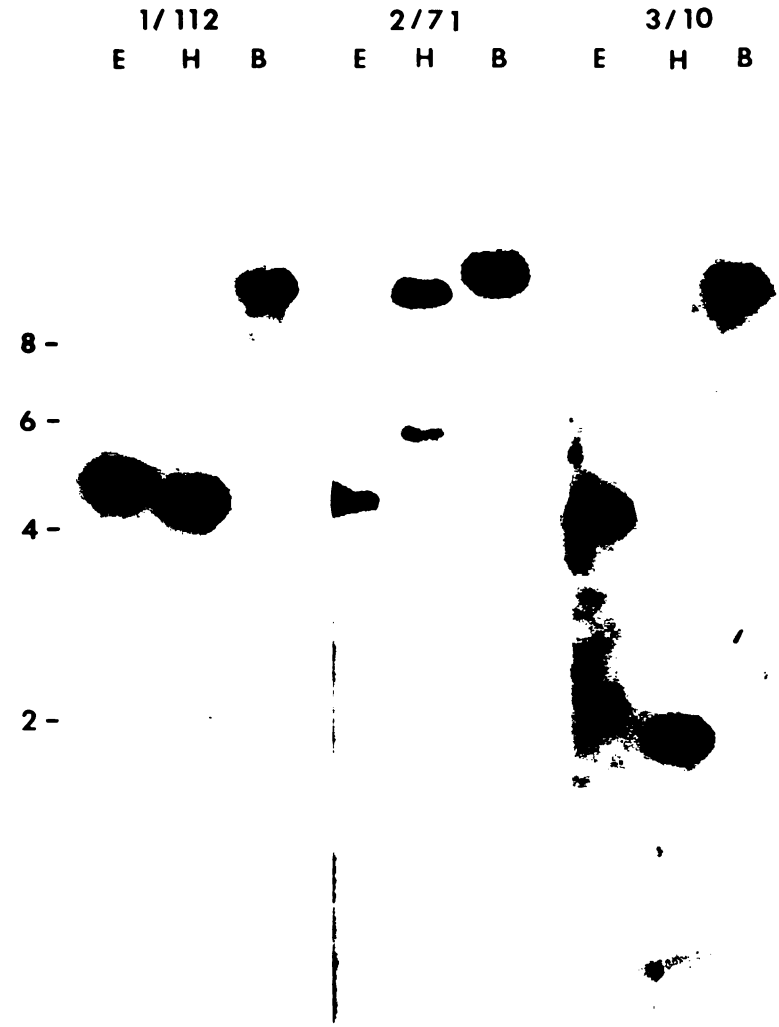


Figure 15. Southern Blot of 5A5 DNA Probed with cDNA Clones.

Ten micrograms of high molecular weight DNA from 5A5 cells was digested with EcoRI (E), HindIII (H), or Bam HI (B) and resolved on 0.7% agarose in TAE, blotted, and hybridized three days at 65°C in 6X SSC/2X Denhardts with 2 x 10^6 cpm/ml nick-translated cloned DNA.

exceptions; clone 1/44 probably contains a sequence which is moderately repetitive and clone 1/16 contains a sequence which is highly repetitive (data not shown).

I attempted to study the turnover of these mRNAs in primary cell cultures and in transformed cells. In one experiment with primary pituitary cell cultures, analysis of the RNA by filter hybridization showed they did not synthesize detectable levels of PRL and GH (data not shown). A two hour pulse revealed that these cells did show appreciable synthesis of some of mRNA corresponding to the other cDNA clones tested (Table 3).

5A5 cells were used to study the metabolism of mRNA corresponding to a number of the cDNA clones. To investigate mRNA turnover in these cultures pulse-chase analysis was performed. At two hours of pulse the various mRNAs displayed a wide range of accumulations (Table 3). A preliminary comparison of RNA from cultures labeled for short time periods shows a very similar pattern of abundance in the primary cell culture and transformed cells.

The half-life of the clones which showed the highest levels of label was determined (Fig 16, Table 3). All clones except p1/16 showed moderately long half-lives of 4-8 hrs. Clone p3/10 which was very abundant according to the initial library screen, has the highest transcription rate of the clones studied and a relatively rapid turnover (6.5 hr).

Clone p1/16 contains sequences which hybridize to a single copy, nonrepetitive gene. But it also contains a repetitive element which is frequently found in introns in hnRNA and to a lesser extent in the 3′ untranslated regions of mRNA. RNA hybridizing to this

Figure 16. Decay Kinetics of cDNA Clones.

Cell cultures were pulsed for 2 hrs with ^{3}H-uridine and chased in medium containing unlabeled uridine and cytidine. Each time point was analyzed, in triplicate, for binding to each cloned cDNA. Filter-bound pBR322 DNA was used a background and was subtracted prior to determination of parts per million (ppm) bound.

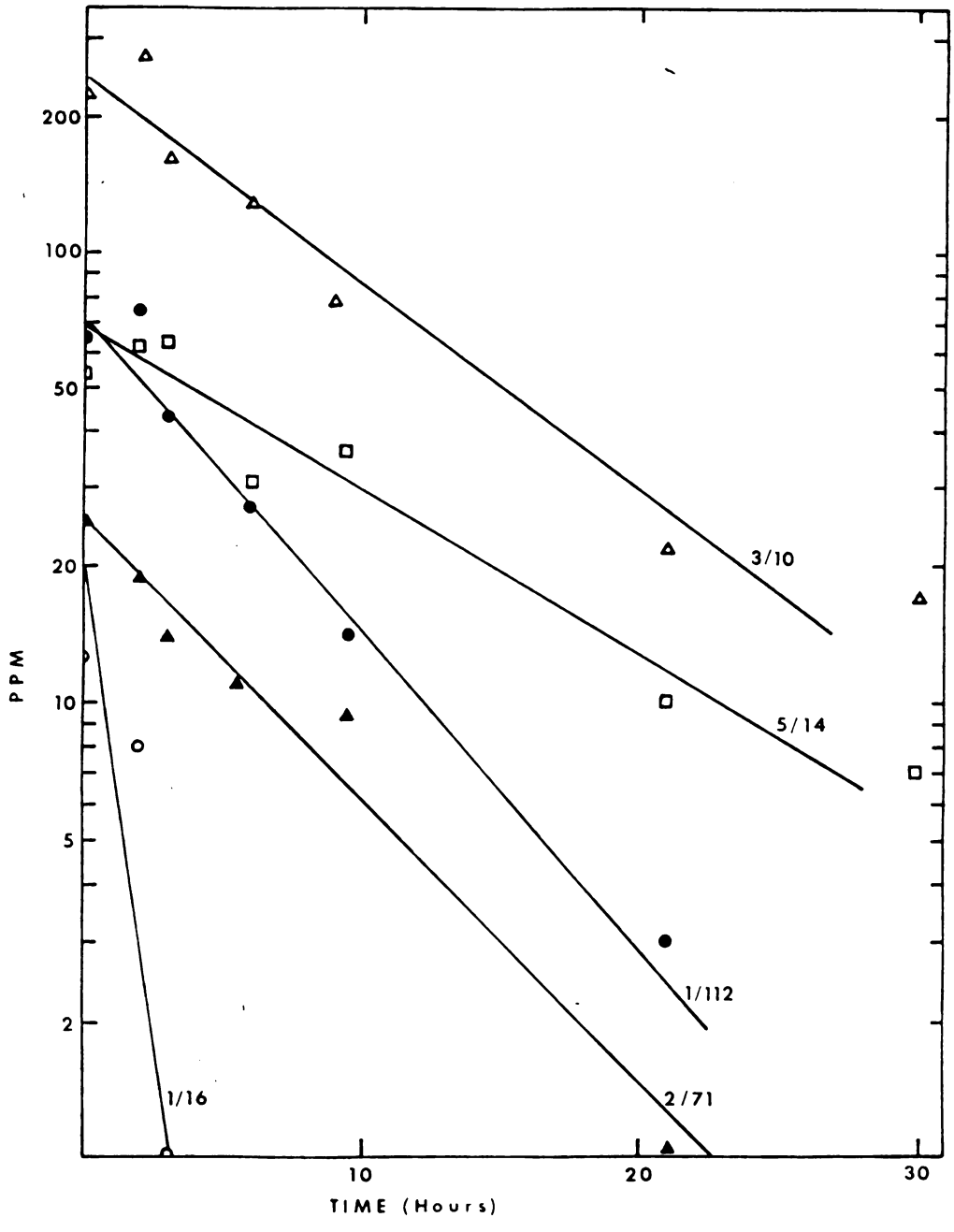


Figure 16

sequence turns over very rapidly (45 min.).

DISCUSSION

A bovine primary cell culture system for the study of PRL and GH synthesis was established and optimized. To maintain long-term PRL and GH synthesis, D-valine had to be substituted for L-valine in all the growth media tested. This replacement had the effect of suppressing cell division in these cultures. When growth was allowed to procede, the cultures were dominated by a single cell type within 5-7 days of initial plating. PRL and GH production declined as soon as confluence was reached. Although fibroblast overgrowth is frequently a problem in primary cultures, the cells which proliferated in these cultures were probably not fibroblasts. Serum-free medium will not support fibroblast growth without a number of additional factors, including fibroblast growth factor. In our serum-free cultures with L-valine, cell division occurred readily with the concomitant loss of PRL and GH production seen in serum containing medium with L-valine. The cells which overgrew these primary cultures grew in a 'cobblestone' pattern characteristic of epithelial cells. Lactotroph and somatotrophs probably do not divide in primary cell cultures from adult tissue but do remain viable and responsive for at least 30 days.

Our primary cell culture system is consistently sensitive to hydrocortisone or dexamethasone when serum is pretreated with charcoal or when serum-free medium is used. In serum-free medium a more complete shut-off of growth hormone production was observed in the

absence of hydrocortisone. Charcoal treatment may not remove all the glucocorticoids and does not remove T_3. Addition of hydrocortisone to these cells resulted in a 3-5 fold increase in GH synthesis in adult and fetal cultures. There are conflicting reports concerning the effects of hydrocortisone on GH production. In some systems hydrocortisone by itself produces little or variable response, while in others it has been shown to induce 2-5 fold increases in GH production (reviewed by Bancroft et al., 1985). In rat cells GH production responds to T_3 stimulation but these bovine pituitary cells never exhibited this response.

PRL synthesis in this primary culture system was reliably inhibited by hydrocortisone. This is in agreement with results using transformed rat GH cell lines (Evans et al., 1982). In previous experiments with primary cell cultures from either rats (Lieberman et al., 1978) or sheep (Vician et al., 1979) no hydrocortisone repression of PRL synthesis was detected. Shull and Gorski (1986) have postulated that only transformed cells display an inhibition of PRL synthesis in the presence of hydrocortisone. We now describe a primary cell culture system in which PRL synthesis is inhibited by corticosteroids. It is possible that differences in culture conditions and media may account for the varying observations. Alternatively, bovine cells may respond to hormonal stimulation differently than rat or ovine cells.

Using ovine primary cultures Vician et al. (1979) found that addition of estrogen doubled PRL synthesis. We were not able to demonstrate an estrogen stimulation of PRL synthesis in our bovine primary cultures. These cells did, however, respond to estrogen with

a 70% increase in secretion of PRL (Padmanabhan et al., 1982). Similar results were obtained using bromoergocryptine, a dopamine agonist. This drug very effectively blocked secretion of PRL from primary pituitary cells. Using similar conditions, decreases in PRL synthesis were not always observed.

Bovine primary pituitary cells respond predictably to hormonal stimuli at the level of secretion. Changes in protein synthesis were observed primarily in response to hydrocortisone but not estrogen or bromoergocryptine. It is possible that dissociation of the pituitary gland could lead to changes in differentiated function of these cells and that they respond slightly differently when in association with other cells (Clayton, et al., 1985). It is also possible that bovine cells respond differently to hormones than do the rat cells on which most of the mRNA synthesis studies have been performed.

PRL and GH mRNA synthesis and turnover were studied in these cultures using pulse-chase analysis. In these primary cells the chase did not become effective until 15 hrs after its initiation. It is not unusual for incorporation into cytoplasmic RNA to increase after initiation of chase due to continued transport of nuclear sequences to the cytoplasm. When this occurs, however, it is usually on the order of only 1-3 hr. It is possible that cells which are not dividing do not respond rapidly to typical chase conditions. Between 15 and 27 hours of chase, label in poly A+ RNA decreased nearly 50% while ^{3}H-uridine in total RNA remained constant. During this time when poly A+ RNA as a whole decayed 50%, PRL and GH mRNA did not decay but instead increased slightly. The mRNAs for these proteins showed kinetics very similar to total or rRNA in these primary cultures indicating that

they are very stable messenger RNAs. This correlates well with evidence from changes in levels of synthesis of these proteins. Under conditions of stimulation or inhibition, new steady-state protein levels are not acheived for several days. This would be expected of mRNAs whose half-lives are very long.

Since the half-lives of PRL and GH were unusually extended in these cells it was of interest to determine what factors contributed to this extreme stability. One approach to this question was to compare GH and PRL mRNA to other bovine mRNAs of various stability classes. To begin this study, a cDNA library was prepared from a transformed bovine pituitary cell line which initially appeared to produce GH.

Results from cDNA clones of bovine mRNAs are basically descriptive. The majority (75%) of the clones examined by Southern analysis represented unique genes (Table 3). One had a highly repetitive short interspersed sequence and another contained a moderately repetitive sequence.

Northern analysis detected only abundant or moderately abundant sequences. The mRNA represented by these clones varied in size from less than 700 bp to 2000 bp (Table 3). The insert sizes (Table 3) in these plasmids are approximately 1/4 to 1/2 the full length of the mRNA they represent. Most clones probably contain the 3'end of the messenger RNA since the average size of the first strand produced by reverse transcriptase in the cDNA cloning was 500-600 bp. Second strand synthesis went to completion with an average mass of 1200 to 1400 bp (data not shown). Therefore, the reverse transcriptase copied part of the mRNA starting at the poly A tail, and

then the Klenow polymerase made a complete copy of that fragment.

Preliminary kinetic analysis of some of these clones was performed in primary pituitary cell cultures and in transformed bovine pituitary cells (Table 3). Clones which were determined to be of low abundance on either Northern analysis, or in the initial library screen using ^{32}P-cDNA copied from transformed cell mRNA, were labeled to very low levels in the 1 hr or 2 hr pulse. Interestingly, the levels of labeling attained in the primary pituitary cells at two hr. were very similar to those found in the transformed cells at two hr.

In the transformed cells, the most abundant mRNAs incorporated enough label during the pulse to allow determination of their turnover rates. The half-lives of these mRNAs varied from 4.5 to 8.5 hr.

One clone, p3/10, was very abundant. It hybridized very strongly on the initial cDNA library screens. Of the approximately 20 clones analyzed in more detail two were found to contain homologous sequences (p3/10 and p1/105). This mRNA probably has a very high transcription rate since it contained 2-3 X more ^{3}H-uridine than any of the other mRNAs at 2 hours in both primary and transformed cells. On the other hand, it has an intermediate turnover rate of 6.5 hr (Table 3). This mRNA could be interesting to compare to PRL and GH since it accumulates to high levels in these cells but seems to do so by a very different mode than do GH and PRL mRNA.

RNA which hybridized to clone p1/16 had a short half-life of 45 min. This clone contained a short interspersed repetitive element (characterized in Part II) These repetitive elements are frequently found as part of Pol II transcripts, usually in intron sequences and occasionally in the 3′ untranslated end of mature mRNAs (Jelinek and

Schmid, 1982). The labeled RNA sequences hybridizing to clone p1/16 could represent hnRNA which may have contaminated the cytoplasmic RNA preparation. This would be consistent with the larger size of the RNA sequences hybridizing to this clone in a Northern analysis. Alternatively, mature mRNA containing a repetitive element in an untranslated region may be turned over very rapidly in these cells.

REFERENCES

Ambesi-Impiombato, C., Parks, A.B. and Coon, H. 1981. Proc. Natl. Acad. Sci. USA 77:3455-3459.

Bancroft, F.C., 1981. In "Functionally Differentiated Cell Lines" (Sato, G. ed.) 47-60. Alan R. Liss. Inc., New York.

Bancroft, C., Gick, G.G., Johnson H.E. and White B.A. 1985. Biochemical Action of Hormones 12:173-213.

Barinaga, M., Yamamoto, G. Revier, C. Vale, W., Evans R., and Rosenfeld M.F. 1983. Nature 306:84-85.

Barnes, D. and Sato, G. 1980. Anal. Biochem. 102:255-270.

Berger, S.L., and Cooper, H.L. 1975. Proc. Nat. Acad. Sci. USA. 72:3873-3877.

Brock, M.L., and Shapiro, D.J. 1983. Cell 34:207-214.

Carneiro, M., and Schibler, U. 1984. J. Mol. Biol. 178:869-880.

Caron, M.G., Beaulieu, M., Raymond, V., Gagne, B., Prouin, J., Lefkowitz, R.J. and Labrie F. 1978. J. Biol. Chem. 253:2244-2253.

Carrillo, A.J., Pool, T.B., and Sharp, Z.D. 1985. Endocrinology 116:202-206.

Clayton, D.F., Harrelson, A.L., and Darnell, J.E. 1985. Mol. Cell. Biol. 5:2623-2632.

Cleveland, D.W., Lopata, M.A., Sherline, P., and Kirschener, M.W. 1981. Cell 25:537-546.

Cleveland, D.W. and Havercroft, J.C. 1983. J. Cell Biol. 91:919-924.

Cleveland, D.W. 1987. J. Cell Biol. 104:381-383.

Cronin, M.J., Roberts, J.M., and Weiner, R.I. 1978. Endocrinology 103:302-309.

Dannies, P.S., and Tam, S.W. 1982. In "Cellular Regulation of Secretion and Release" (Conn, P.M. ed.) 529-568 Academic Press,

New York.

Dannies, P.S. and Tashjian 1973. J. Biol. Chem. 248:6174-6179.

Dannies, P.S. 1985. Biochemical Action of Hormones 12:289-310

Darnell, J.E. 1982. Nature 297:365-371

Derman, E., Krauter, K., Walling, L., Weinberger, C., Ray, M., and Darnell, J.E. 1981. Cell 23:731-739.

DiPasolo, T., Carmichael, R., Labrie, F., and Raynaud, J.P. 1979. Mol. Cell. Endocrinol 16:99-112.

Dobner, P.R., Kawasaki, E.S., Yu, L.-Y., and Bancroft, F.C. 1981. Proc. Natl. Acad. Sci. USA 78:2230-2234.

Diamond, D.J. and Goodman, H.M. 1985. J. Mol. Biol. 181:41-62.

Evans, R.M., Birnberg, N.C., and Rosenfeld, M.G. 1982. Proc. Natl. Acad. Sci. USA 79:7659-7663.

Farquhar, M.G., 1977. In "Comparative Endocrinology of Prolactin" (H.D. Dellman, J.A. Johnson, and D.M. Klachko, eds.) 37-94 Plenum, New York.

Friderici, K., Oh, S-Y., Ellis, R., Guacci, V., and Fluck, M.M.. 1984. Virology 137:67-78.

Gay, D.A., Yen, T.J., Lau, J.T.Y., and Cleveland, D.W. 1987. Cell 50:671-679.

Gick, G.G. and Bancroft, C. 1985. J. Biol. Chem. 160:7614-7618.

Gersten, B.E. and Baker, B.L. 1970. Am. J. Anat. 128:1-20.

Gilbert, S.F. and Migeon, B.R. 1975. Cell 5:11-17.

Graves, R.A., Pandey, N.B., Chadchoy, N., and Marzluff, W.F. 1987. Cell 48:615-626.

Graves, R.A. and Marzluff, W.F. 1984. Mol. Cell. Biol. 4:351-354.

Greenberg, J.R. 1972. Nature 240:102-104.

Greenberg, M.E., Hermanowaki, A.L., and Ziff, E.B. 1986. Mol. Cell. Biol. 6:1050-1057.

Guyette, W.A., Matuski, R.J., Rosen, J.M. 1979. Cell 17:1013-1023.

Harpold, M.M., Evans, R.M., Salditt-Georgieff, M., and Darnell, J.E. 1979. Cell 17:1025-1035.

Heintz, N., Sive, H.L., and Roeder, R.G. 1983. Mol. Cell. Biol. 3:539-550.

Jelinck, W.R. and Schmid, C.W. 1982. Ann. Rev. Biochem. 51:813-844.

Jones, T.R., and Cole, M.D. 1987. Mol. Cell. Biol. 7:4513-4521.

Laemmli, U. 1970. Nature 227:680-686.

Lau, L.F. and Nathans, D. 1987. Proc. Natl. Acad. Sci. USA 84:1182-1186.

Laverriere, J.N., Morin, A., Tixier-Vidal, A., Truong, A.T., Gourdji, D., and Martial, J.A. 1983. EMBO J. 2:1493-1499.

Lieberman, M.E., Maurer, R.A., and Gorski, J. 1978. Proc. Natl. Acad. Sci. USA 75:5946-5949.

Lieberman, M.E., Maurer, R.A., Claude, P., and Gorski, J. 1982. Mol. Cell. Endocrinol. 25:277-294.

MacLeod, R.M., Fontham, E.H., and Lehmeyer, J.E. 1970. Neuroendocrinology 6:283-294.

Maniatis, T., Fritsch, E.F., and Sambrook, J. 1982. "Molecular Cloning -- A Laboratory Manual". Cold Spring Harbor Laboratory N.Y.

Martial, J.A., Baxter, J.D., Goodman, H.M., and Seeburg, P.H. 1977. Proc. Natl. Acad. Sci. USA 74:1816-1820.

Maurer, R.A., 1980. J. Biol. Chem. 255:8092-8097.

Maurer, R.A., 1981. Nature 294:94-97.

Maurer, R.A., 1982a. Endocrinology 110:1507-1514.

Maurer, R.A., 1982b. Endocrinology 110:1515-1520.

Maurer, R.A., 1982c. Endocrinology 110:1957-1963.

Maurer, R.A., 1982d. in "Cellular Regulation of Secretion and Release" (Conn, P.M. ed.) 267-292 Academic Press, New York.

McKnight, G.S. and Palmiter, R.D. 1979. J. Biol. Chem. 254:9050-9058.

Meites, J. and Clemens, J.A., 1972. Recent Prog. Horm. Res. 28:165-221.

Miller, W.L., Knight, M.M., Grinek, H.J., and Gorski, J. 1977. Endocrinology 100:1306-1316.

Murdoch, G.H., Potter, E., Necolaisen, A.K., Evans, R.M., and Rosenfeld, M.G. 1982. Nature 300:192-194.

Murdoch, G.H., Rosenfeld, M.G., and Evans, R.M. 1982b. Science 218:1315-1317.

Murdoch, G.H., Evans, R.M., and Rosenfeld, M.G., 1985. Biochemical Action of Hormones 12:35-68.

Nilson, J.H., Barringer, K.J., Convey, E.M., Friderici, K.A., and Rottman, F.M. 1980, J. Biol. Chem. 255:5871-5885.

Nyborg, J.K., Nguyen, A.P., and Spindler, S.R., 1984. J. Biol. Chem.

Padmanabhan, V., Friderici, K.H., Convey, E.M., and Rottman, F.M. 1982. Mol. Cell. Endocr. 28:613-626.

Pandey, N.B. and Marzluff, W.F. 1987. Mol. Cell. Biol. 7:4557-4559.

Paek, I. and Axel, R. 1987. Mol. Cell. Biol. 7:1496-1507.

Perrone, M.H., Greer, T.L., and Hinkle, P.M. 1980. Endocrinology 106:600-605.

Piechaczyk, M., Yang, J.Q., Yang, Blanchard, J.M., Jeanteur, P., and Marcu, K.B. 1985. Cell 42:589-597.

Pittenger, M.R. and Cleveland, D.W. 1985. J. Cell Biol. 101:1941-1952.

Potter, E., Nicolaisen, A.K., Ong, E.S., Evans, R.M., and Rosenfeld, M.G. 1981. Proc. Natl. Acad. Sci. USA 78:6662-6666.

Puckett, L., Chambers, S., and Darnell, J.E. 1975. Proc. Natl. Acad. Sci. USA 72:389-393.

Raymond, V., Beulieu, M., Labrie, F., and Boissur, J. 1978. Science 200:1173-1175.

Rivier, J., Speiss, J., Thorner, M., and Vale, W. 1982. Nature 300:276-278.

Roberts, B.E. and Patterson, B.M. 1973. Proc. Nat. Acad. Sci. USA 70:2330-2334.

Ross, J., and Kobs, G. 1986. J. Mol. Biol. 188:579-593.

Ross, J., and Pizarro, A. 1983. J. Mol. Biol. 167:607-617.

Samuels, H.H., Stanley, F., and Shapiro, L.E. 1979. Biochemistry 18:715-721.

Schumperli, D. 1986. Cell 45:471-472.

Shapiro, D.J., and Brock, M.L. 1985. "Biochemical Actions of Hormones" Vol XII. Academic Press, Inc.

Shaw, G., and Kamen, R. 1986. Cell 46:659-677.

Shull, J.D., and Gorski, J. 1986. Vitamins and Hormones 43, 197-249.

Singer, R.H., and Penman, S. 1973. J. Mol. Biol 78:321-334.

Spradling, A.C., Digan, M.E., Mahowold, A.P., Scot, M., and Craig, E.A. 1980. Cell 19:905-915.

Stanley, F., and Samuels, H.H. 1984. J. Biol. Chem. 259:9768-9775.

Steck, P.A., Voss, P.G., and Wang, J.L., 1979. J. Cell. Biol. 83:562-575.

Vale, W., Rivier, C., and Brown, M.A. 1977. Ann. Rev. Physiol 39:473-525.

Vician, L., Shupnik, M.A., and Gorski, J. 1979. Endocrinology 104:736-743.

Vollach, V., and Housman, D. 1981. Cell 23:509-514.

White, B.A., and Bancroft, F.C. 1983. J. Biol. Chem. 258:4618-4622.

Yaffe, B.M., and Samuels, H.H. 1984. J. Biol. Chem. 259:6284-6291.

PART II

CHARACTERIZATION OF A BOVINE REPETITIVE ELEMENT

LITERATURE REVIEW

The eukaryotic genome is a mixture of single copy sequences interspersed with sequences that are repeated many times. In mammals these repetitive sequences may account for 10-30% of the genomic DNA (Laird, 1971). Repetitive sequences can be broadly classified as highly repetitive ($>10^6$ copies per genome) or moderately repetitive (10^3 - 10^5 copies per genome) (Britten and Kohn, 1968).

Highly repetitive DNA consists of tandemly repeated short sequences. These segments frequently have buoyant densities in cesium chloride gradients that differ from the bulk of an organism's DNA so that they form satellite bands and are present mainly at centromeric and telemeric positions on the chromosome (Jelinek and Schmid, 1982). No transcriptional activity has been described from this type of repetitive DNA.

Moderately repetitive DNA is widely distributed throughout the genome, and is interspersed in unique sequences. These repetitive sequences are divided into two classes according to their length; long (6-7 kb) and short (<500 kb). Mammalian genomes contain a long interspersed element known as the LINE (L1) family (Singer, 1982), whose best studied members are the Kpn family in primates and BamHI (MIF1) family in the mouse. Only a single LINE family has been found in each mammalian species studied (Weiner et al., 1986). The LINE families of different species are very homologous over a portion of

their length, somewhat homologous in other portions and show species specific divergence over the rest. Family members are heterogeneous in size, frequently showing a variable truncation of what has been designated the "5'" end. There can also be internal rearrangements, including deletions and inversions. Several human and mouse segments contain open reading frames over 600 bp long near the 3' end, in the region of mouse and human homology (Singer and Skowronski, 1985). The extreme 3' end of the elements in the L1 family is variable in length and rich in A-residues. These elements are apparently mobile in the genome and make target site duplications at the site of insertion.

Little is known about the function(s) of long interspersed repeats. Some may contain functional genes capable of producing proteins. They could also influence the expression of neighboring genes either by enhancing (Leuders et al., 1984) or silencing (Laimins et al., 1986) their transcription. It has also been suggested that L1 units could foster rearrangements both within L1 elements and within adjacent genomic regions (Singer and Skowronski, 1985).

Short dispersed repeats (SINES) consist of families of sequences approximately 70-300 bp that are present at over 100,000 copies per genome. Each family is a set of elements that are closely similar in sequence and length but are not identical. The first sequence of this type was described in the human genome and named the "alu family" (Houck et al., 1979). This is the major family of repetitive elements in humans and typifies the short interspersed repetitive elements found in many species.

In general the alu sequences are 300 bp in length and consist of an imperfect dimer of two directly repeated, approximately 130 bp

units with a 31 bp insertion in the second monomer. The nucleotide sequences of different members of this family tend to differ from a consensus sequence by about 14% (Schmid and Shen, 1986). The alu sequence is highly defined at the 5′ end and contains a variable length A-rich sequence interrupted by other nucleotides at the 3′ end. In the genome this whole structure is flanked by direct repeats of 8 to 20 base pairs that are unique for each alu insert.

Alu family members are fairly evenly dispersed throughout the human genome, and occur on the average every 5000-8000 bp (Jelinek and Schmid, 1982). They have been found both within RNA polymerase II transcription units and between transcription units (Fritsch et al., 1980). Alu sequences are heavily represented in hnRNA molecules and comprise as much as 18-25% of the mass of HeLa hnRNA (Federoff et al., 1977, Jelinek et al., 1978). Poly A+, polyribosome-associated cytoplasmic HeLa cell RNA also contains these sequences, but at a lower frequency per molecule than hnRNA (Jelinek et al., 1978). Apparently alu units can occur both within introns and in the 5′ or 3′ noncoding regions of messenger RNAs.

Alu family sequences also contain the A and B block promoter elements necessary for RNA polymerase III transcription (Duncan et al., 1981, Fowlkes and Shenk, 1980). One or more A blocks lie close to the 5′ end of the element (positions 5 and 31) and a B block is found between position 70 and 100. Alu sequences can be transcribed in vitro or in vivo by RNA polymerase III to yield low molecular weight RNAs (Duncan et al., 1979, Allan and Paul, 1984).

A summary of the salient features of the alu family are diagrammed below.

All mammalian species contain short interspersed repetitive elements which show varying homologies or structural similarities to the human alu family. In mice there are two major SINE families designated B1 and B2 which occur at about 10^5 copies per genome (Kramerov et al., 1977, 1979). The 130 bp B1 repeat is homologous to the first momomer of human Alu, and shares homology with the polyoma viral replication origin (Karayev et al., 1982). B2 resembles B1 in its general structure and organization but has a different concensus sequence. Both have a split Pol III promoter, 3' poly A sequence and are flanked by direct repeats (Krayev et al., 1982). B1 and B2 are found in both hnRNA and mRNA as part of RNA Pol II transcription units (Kramerov et al., 1982, Ryskov et al., 1983).

Another repetitive sequence found in rodents is the rat ID sequence (Sutcliffe et al., 1982). This is an abundant (10^5 copies/genome) SINE in rats, while the homologous sequence in mice is present at 10^4 copies/genome (Sapienza and StJacques, 1986). ID sequences are 82 bp long, have a split Pol III promoter and a 3' poly A tail of variable length.

ID sequences were originally thought to be part of pol II transcription units that were only expressed in brain nuclei

(Sutcliffe et al., 1984b, Milner et al., 1984), and it was speculated that they could possibly function as some sort of tissue specific control signal for neural specific genes. Subsequent studies have shown, however, that ID sequences are present in the hnRNA synthesized in other tissues at similar levels to that found in brain hnRNA (Owens et al., 1985, Sapienza and St-Jacques, 1986).

In addition to being expressed as part of larger Pol II transcription units, short interspersed repetitive elements from humans (Duncan et al., 1979), rodents (Haynes and Jelinek, 1981), cows (Furth and Su, 1986) and lower vertebrates (Matsumoto et al., 1983, Endoh and Okada, 1986) can all function as templates for RNA polymerase III in in vitro transcription systems. In these systems the RNA products are usually longer than the repetitive sequence being examined. One end of the transcript corresponds exactly to the 5' end of the repetitive unit but the 3' end generally terminates outside the element yielding an RNA transcript longer than the actual repetitive unit.

To date, Pol III trancription from repetitive elements in vivo has been demonstrated primarily in rodent cells (Haynes and Jelinek, 1981, Scott et al., 1983, Singh et al., 1985, Sutcliff et al., 1984a), where expression of these elements shows an intriguing tissue specific expression. BC1 and BC2 are the Pol III transcription products of rat ID sequences (Sutcliffe,1984a, Owens et al., 1985). These small cytoplasmic RNAs are found only in brain and neural tissue and in certain established cell lines (McKinnon et al.,1987). Their expression begins in rat brain immediately after birth and is found in large amounts 5-10 days postnatally.

Pol III transcription of the mouse repetitive B1 element has not been shown *in vivo* but the B2 element is transcribed under certain circumstances. B2 transcription in mouse cells is induced by transformation with SV40 (Scott et al., 1983, Singh et al., 1985), other transforming viruses, and carcinogens (Scott et al., 1983). It is elevated in early embryos and undifferentiated embryonal carcinoma (EC) cells and decreases as development proceeds or upon *in vitro* differentiation of EC cells (Murphy et al., 1983,). These B2 sequences are found to be induced by serum in secondary cultures of mouse embryo fibroblasts (Edwards et al., 1985). It appears, therefore that the mouse B2 pol III product is induced in response to growth stimulation of cells.

Short interspersed repetitive elements in the ruminant genome share some but not all of the structural features seen in other species studied to date. In goats and cattle there are about 10^5 copies/genome of a 120 bp repetitive unit (named A or BCS) which shows limited homology to the replication origin of papova viruses. These elements are flanked by direct repeats and have a sharply defined 5′ end but do not have an A-rich 3′ tail, possessing instead short tandem repeats of variable length in the 3′ region. There has been no evidence of a Pol III split promoter in this A sequence (Watanabe et al., 1982, Rogers, 1985). The ruminant genome also contains an additional repeat of 73 bp called the C repeat (Schon et al.,1981). This unit has the typical Pol III promoter but does not have a poly A tail. To date the C element has only been found paired with A elements.

How alu type sequences may have dispersed throughout the

eukaryotic genome is suggested by the flanking direct repeats at their borders and by their general structure, including the pol III promoter and Poly A sequences. These features are typical of retroposons (Rogers 1985, Weiner et al., 1986) where RNA sequences transcribed from short repeated sequences are reverse transcribed and reinserted into the genome at another site.

The alu/B1 family is thought to derive from 7SL RNA by one or more internal deletions of the 7SL RNA sequence followed in primates by dimerization (Weiner et al., 1986). The 7SL RNA itself is highly conserved throughout evolution and is a component of the RNP required for cotranslational secretion of membrane and secretory proteins. In rodents there is another RNA (4.5S) which is also homologous to a portion of the B1 repetitive sequence. The function of this RNA is not known but it is found hydrogen bonded to mRNA and hnRNA in mouse cells (Schoeniger and Jelinek, 1986).

Although the Alu/B1 family arose from 7SL RNA, many other families of SINEs are apparently derived from tRNAs. There is, of course, homology between the internal Pol III promoters of SINEs and tRNA. For some families of repetitive elements homology to tRNA extends beyond the promoters and into the adjoining region. Such homology was first noted between the goat C family and a cysteine tRNA (Rogers, 1985). The rat ID, mouse B2, rabbit C and bovine 73 bp repeats were also found to display homologies to tRNA, although sequence divergence within the repetitive elements has led to disagreement on which is the exact parental tRNA species (Daniels and Deninger 1985, Lawrence et al., 1985, Sakamoto and Okada 1985). In all cases the tRNA homology lies at the 5′ end of the element as

expected for an internal Pol III promoter. In the case of elements which are longer than a typical tRNA sequence the 3' portion may have originated as a read-thru transcript from the tRNA gene.

There are also interesting composites of repetitive elements. The prosimian, Galagos, has a repetitive family called monomer which is 68% homologous to met tRNA and is frequently found in combination with the right monomer of the alu family. The three repetitive families in Galagos; alu, alu/monomer, and monomer are all present in this prosimian genome in approximately equal portions (Daniels and Deninger 1985). Goats and cattle have a similar situation in which the C repeat is found linked to the 5' end of the A sequence (Schon et al.,1981, Watanabe et al., 1982). Alternatively, A sequences are linked to each other other with the sequence $(CACTTT)_3CATGCATT$ repeated between them (Watanabe et al, 1982, Skowronski et al., 1984).

Before discussing the possible functions of SINE sequences it is instructive to consider how they may have evolved. SINE sequences are relatively homologous within each species but are often characteristically different between species. To account for this phenomenon it has been suggested that the expansion of these repetitive sequences within the genome began quite recently in evolutionary time and may be continuing today (Weiner et al., 1986). Mutations occurring when there were a few copies in the genome (founder sequences) would alter the consensus sequence somewhat between two species. Once the copy number increased it would be difficult for subsequent mutations to alter the consensus. It is not known whether the expansion is exponential (each new element can retro-transpose as easily as its parent) or linear (a smaller number

of elements functioning as active retroposons).

The possible functions of these small but numerous inhabitants of the vertebrate genome have been the focus of a great deal of speculation. Early suggestions involved control of gene expression or origins of DNA replication (Britten and Davidson, 1969). It is difficult to reconcile an overriding function such as DNA replication, where the cellular machinery is well conserved, with sequences which are so heterogeneous between species. On the other hand having dispersed Pol III promoters in the genome could conceivably serve some generalized function (Daniels and Deninger, 1985).

The possibility that repetitive sequences function in the tissue or differentiation stage specific control of gene expression has been eagerly pursued. Particularily tantalizing is the fact that SINEs are found as part of Pol II transcription products, both as hnRNA and mRNA. The rat brain ID sequence was proposed as an example of tissue specific expression of a repetitive element (Sutcliffe et al, 1984b), but it now appears that the specificity lies not in the expression of Pol II products (Owens et al., 1985) but of Pol III products to yield low molecular weight RNAs found only in brain and certain cell lines (McKinnon et al., 1987, Owens et al., 1985). Initial observations in mouse systems gave indications that B2 sequences might be associated with genes expressed only in transformed cells (Scott, et al. 1983, Murphy, et al. 1983) Again most of the increase in cellular levels of these sequences was due to an increase in Pol III transcription of the B2 repetitive element (Singh et al. 1985). The significance of this Pol III stimulation in rodent cells is not known. Recently Clemens (1987) observed that a small region of

the B2 consensus sequence is complementary to the AU rich sequence at the 3' end of certain inducible mRNAs. This AU sequence has been shown to be responsible for the short half-life of some transiently expressed mRNAs in human and animal cells (Shaw and Kamen, 1986). Clemens (1987) proposes that changes in the composition of the B2 RNA population might differentially regulate the susceptibility of individual mRNAs to nucleolytic degradation in the cytoplasm.

The main reason for doubting a functional role for alu-like sequences is their extreme lability in evolution. Related species can have divergent consensus sequences and different mammalian orders have entirely different consensuses (Rogers, 1985). Apparently SINEs evolved after many of the basic cellular control systems were determined. The individual locations of repetitive elements in expressed mRNAs are also not conserved between species, i.e. rat growth hormone has a SINE in an intron, human does not (Malissen et al., 1982). The mouse MAK gene has been found to have a B1 element in its 3' untranslated region in some but not all strains of mice. The repetitive unit does not appear to alter the expression of this gene in any way (King et al., 1986).

It is possible that repetitive sequences have no physiological function but act as selfish DNA (Orgel and Crick, 1980, Doolittle and Sapeinza, 1980). In this model, sequences which have Pol III promoters are able to retrotranspose and have been selected only for their efficiency to do so. It is possible that repetitive elements are found more frequently in genes that are expressed in an undifferentiated cell since these may be the ones more likely to be expressed in the germ line, and active chromatin in the germline may

be more accessible to retroposition (Weiner el al., 1986).

Somewhere between these extremes is a suggestion by Hess et al. (1983) that one consequence of insertion of a repetitive element may be blockage of gene conversion in stretches of homologous DNA. So far, careful examination of the globin family in a number of species has supported this proposal (Schimenti and Duncan, 1984, Spence et al., 1985, Brunner et al., 1986). Insertion of a repetitive element between duplicated genes might disrupt the gene correction processes and allow genes to drift independently, thus permitting modified or divergent function in what was originally one gene.

Retroposition may help maintain the complexity and fluidity of eukaryotic genomes (Weiner et al., 1986). The abundance of transposable elements suggests that the excess DNA is not subject to strong negative selection. The wealth of genetic variation may serve as raw material for positive selection, as well as for negative selection and neutral drift (Rogers, 1985).

INTRODUCTION

A cDNA library was constructed from an SV40-transformed bovine pituitary cell line. From this library a clone (pB1/6) was isolated which contained a repetitive sequence. Since ruminant repetitive elements which have been described show some unusual structural features this clone was characterized to determine the nature of its repetitive sequence. Transcription of repetitive elements from a number of species has been demonstrated under certain conditions of cell growth or differentiation. The expression of this bovine repetitive element was examined in both normal and transformed cells.

MATERIALS AND METHODS

Construction of cDNA library and DNA analysis.

Construction of the cDNA library, preparation of genomic cellular DNA and Southern blot analysis were described in part one of this thesis.

Dot blot analysis of species homology.

DNA from various vertebrates was prepared by phenol/chloroform extraction and ethanol precipitation. Concentrations of DNA solutions were determined by absorbance at 260 nm. Hamster and human DNA was a gift from Dr. Paul Bates. Serial dilutions of DNA ranging from 2 ug to 0.06 ug were prepared in 100 ul final volume. To each sample 100 ul of NaI solution (5 g/2 ml hot water) was added, the samples were incubated at 95^{o}C for 20 min., and applied directly to nitrocellulose paper which had been soaked in 6X SSC using a dot blot apparatus. The filter was washed three times for 5 min in water, 3 X 5 min in 70% Ethanol and 10 min in acetic anhydride solution (0.25 ml/100 ml 0.1 M triethanolamine). The filter was prehybridized for 1 hr in 6X SSC/5X Denhardts and hybridized 14 hr in 6X SSC/5X Denhardts containing 2x 10^{6} cpm/ml of nick translated pB1/16 plasmid. A low stringency wash (1X SSC/0.1% SDS at 65^{o}C for 1 hr.) was performed first and the filter was exposed to film for 2 hr and 11 hr. Then a high stringency wash (0.4X SSC/0.1% SDS at 65^{o}C for 1 hr) was performed and the filter was reexposed to film for 2 hr.

Sequencing.

pB1/16 plasmid DNA (10 ug) was digested for 3 hrs. using 20 units Msp I. The resulting fragments were end labeled by adding 40 uCi dCT^{32}P (Amersham 3000 Ci/mMole) and 2 units Klenow polymerase to the restriction reaction and allowing the reaction to proceed 15 min at room temperature. 2 nmoles of each dNTP was added and the reaction was continued for 5 min. The labeled fragments were ethanol precipitated, digested for 3 hrs with 20 units of PstI and resolved on a 1.2% agarose gel in TAE buffer. An 840 bp fragment corresponding to the large PstI to MspI portion of the insert was eluted according to the method of Girvitz (Maniatis et al., 1982).

Chemical sequencing was performed according to Maxam and Gilbert (1980).

Northern analysis.

RNA was prepared from the 5A5 cell line (described in part I) and MDBK cells (Madin-Darby bovine kidney). Total Poly A+ RNA was prepared by lysing newly confluent 100 mm dishes in 2 ml binding buffer (0.5M NaCl, 10 mM Tris pH 7.5, 1 mm EDTA, 0.2% SDS) plus 200 ug/ml Proteinase K. Viscosity was reduced by aspirating the lysate several times thru a syringe fitted with an 18 gauge needle and the samples were digested at 37^{o}C for 45 min. Oligo dT cellulose (BRL) was added at a final concentration of 5 mg/ml and the suspension was agitated at room temperature for 1 hr. The oligo dT cellulose was pelleted by centrifugation, resuspended in binding buffer, transferred to a quick sep column (Isolabs) and washed 1 time with binding buffer, followed by one wash with binding buffer containing 0.1 M NaCl. Poly A+ RNA was

eluted with two washes of 10 mM Tris pH 7.6/1 mM EDTA. RNA was precipitated with ethanol in the presence of carrier tRNA and resuspended in 50 ul northern sample buffer per 100 mm dish of cells.

Total RNA was prepared from these cell lines by lysis with Quanidinium isothiocyanate and pelleting in CsCl (Maniatis et al., 1982, Chirgwin et al., 1979,).

Gel analysis was performed on a 1.1% agarose gel as described in part 1 of this thesis. RNA was transferred to nitrocellulose, the filter was baked, prehybridized in 2.5X Denhardts, 3X SSC, 50 mM $NaPO_4$, 50 % formamide, 50 ug/ml salmon sperm DNA at 42°C and hybridized for 20 hr in the same buffer containing 5% dextran sulfate, 0.1% SDS and 10^6 cpm/ml nick translated pB1/16. The filter was washed at 65°C with 2X SSC/0.5% SDS.

Cell Cultures.

Cell line 5A5 was derived by SV40 transformation of bovine primary pituitary cells and was the gift of Dr. Robert Lyons. MDBK cells are nontransformed cells derived from bovine kidney.

RESULTS

Southern blot analysis and mapping of pB1/16

Genomic DNA prepared from cell line 5A5 was digested with several restriction enzymes and analyzed by Southern blot analysis using nick translated plasmid pB1/16 as a probe (Figure 1A). The results indicated that pB1/16 contained a repetitive element which was highly interspersed in the bovine genome.

Plasmid pB1/16 was mapped using a variety of restriction enzymes to determine the size and location of the repetitive sequence. Total plasmid DNA or the purified PstI insert fragment was digested, resolved on agarose gel and blotted. The blot was then probed with nick-translated 5A5 genomic bovine DNA to determine which fragments contained sequences which were highly represented in the bovine DNA (Figure 2A, B). The resulting map (Figure 2C) revealed that there was one small (<200 bp) repetitive sequence and that it was contained entirely within the pB1/16 insert.

To determine the nature of the rest of pB1/16 the 700 bp HinfI fragment which did not contain the highly repetitive sequence was used to probe a Southern blot of 5A5 DNA (Figure 1B). The HinfI fragment apparently represents a single or low copy sequence in the bovine genome.

Homology of the repetitive sequence to sequences in other species was examined. DNA from a variety of organisms ranging from fish

Figure 1. Southern Blot analysis.

Ten micrograms of DNA prepared from 5A5 cells was digested with EcoRI (lane R), BamHI (lane B) or HindIII (lane H), resolved on a 0.7% agarose gel and blotted. Panel A was probed with nick-translated pB1/16 plasmid DNA (10^8cpm/ug, 10^7 cpm in 5 ml 2X SSC/1X Denhardts). Panel B was probed with nick-translated purified PstI-HinfI fragment from pB1/16 insert DNA (see Figure 2).

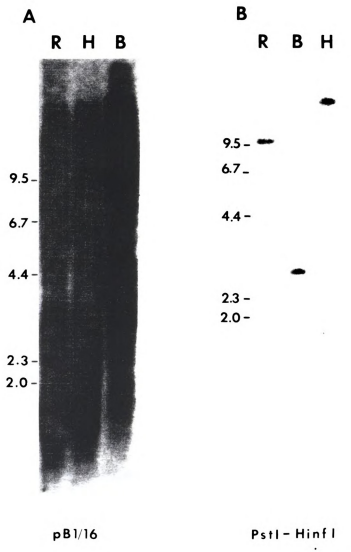


Figure 1

Figure 2. Map of pB1/16.

Plasmid DNA (0.5 ug/lane) was digested with a variety of restriction enzymes, resolved on a 1.5% agarose gel in TAE buffer, stained with ethidium bromide (Panel A) and transferred to nitrocellulose. (Panel B) The blot was probed with nick-translated 5A5 DNA (1.5 x 10^8 cpm/ug, 10^6 cpm/ml, 2X SSC/1X Denhardts, filters were not blocked with carrier DNA). Enzymes used in this example were 1; BglI, 2; PvuII, 3; PstI, 4: PstI + PvuII, 5; BglI + PvuII, 6; BglI + PstI, 7; BglI + PstI + PvuII, 8; DdeI, 9; DdeI + PvuII, 10; DdeI + BglI.

Bottom panel is restriction map of pB1/16 insert and location and size of the repetitive element (hatched region). Enzymes which do not cut within the pB1/16 insert are; AvaI, BamHI, ClaI, HaeIII, HindIII, RsaI, Sau3A, StuI, TaqI, XbaI, XhoI.

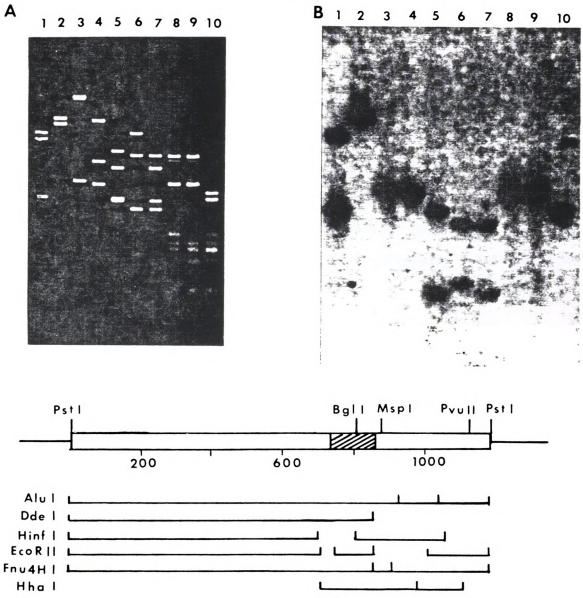

A
1 2 3 4 5 6 7 8 9 10
B
1 2 3 4 5 6 7 8 9 10
Pst I
Bgl I
Msp I
Pvu II
Pst I
200
600
1000
Alu I
Dde I
Hinf I
EcoR II
Fnu4H I
Hha I

Figure 2

to human was applied to nitrocellulose in a dot blot apparatus and probed with nick-translated pB1/16 plasmid DNA (Figure 3). The filter was washed first at low stringency (Figure 3A,B), exposed to film, and rewashed at higher stringency (Figure 3C). Of the species examined, all showed some homology at low stringency after a long exposure (Figure 3A). After short exposure times chicken was undetectable, but salmon and rat showed some homology. A small amount of hybridization to rodent DNA remained after the high stringency wash, while no hybridization to fish or human DNA was detected. Hybridization to bovine cell line DNA's was unchanged between high and low stringency washes. Similar results were obtained using Eco RI digested DNA analysed by Southern blot, where hybridization was performed in high and low stringency conditions (data not shown).

Sequence of the repetitive element in pB1/16

The repetitive region in pB1/16 was sequenced from the MspI site at the right side of the element using end filling by Klenow polymerase and Maxam-Gilbert sequencing. A sequencing gel is shown in Figure 4 and the sequence is shown in Figure 5. The repetitive region of pB1/16 is compared to the bovine repetitive A element consensus sequence determined by Watanabe et al. (1982) and Skowronski et al. (1984). The repetitive element in pB1/16 shows 4 changes for this consensus sequence (97% homology) and ends with an AGC repeat which has been shown to occur on the 5' side of certain bovine and goat repetitive sequences (Watanabe et al. 1982, Skowronski et al. 1984).

The homology with the A element ends at position 95 in Figure 5 and no homology to the 73 bp C element described by Schon et al. is

Figure 3. Homology of bovine repetitive element with DNA from other species.

DNA from the species shown in the figure was found to nitrocellulose and hybridized as described in Materials and Methods. DNA levels were Lane 1; 2 ug, lane 2; 0.35 ug, lane 3; 0.06 ug. The blot was washed first with 1x SSC/0.1% SDS for 1 hr at 65°C. Panel A shows 11 hr. exposure to film, Panel B; 2hr. The blot was rewashed in 0.4X SSC/0.1% SDS for 1 hr. at 65°C and exposed to film for 2 hr; Panel C.

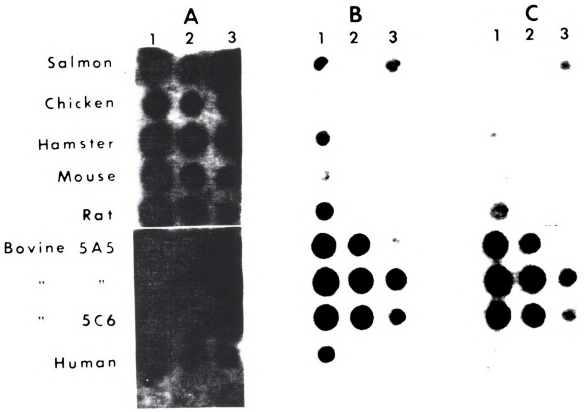

A
1 2 3
B
1 2 3
C
1 2 3
Salmon
Chicken
Hamster
Mouse
Rat
Bovine 5A5
" "
" 5C6
Human

Figure 3

Figure 4. Sequencing Gel of Bovine repetitive element.

pB1/16 plasmid was labeled with ^{32}P-dCTP and prepared as described in Materials and Methods. The chemical sequencing reactions were resolved on a 32 x 37 cm 6% acrylamide-urea gel at 50 W. The gel was dried and exposed to film for 15 hr. with an intensifing screen.

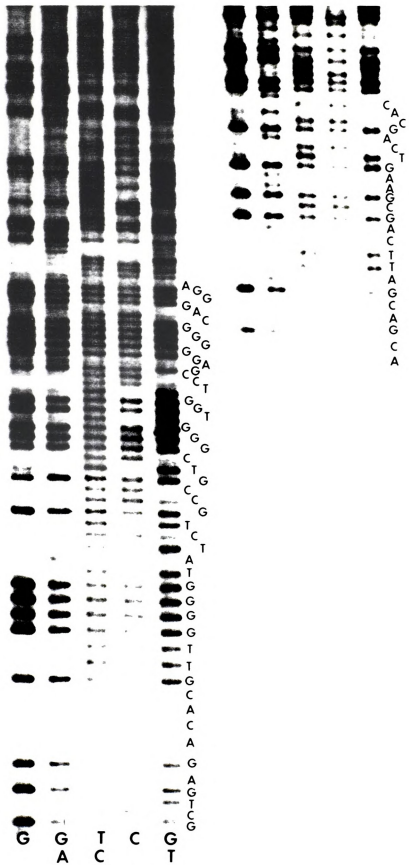


Figure 4

Figure 5. Sequence of pB1/16 in region of repetitive element.

Consensus sequence of bovine element A_R as compiled by Skowronski et al. is shown above pB1/16 sequence. Bases which differ in the A_L consensus sequence are shown above the A_R sequence. Restriction sites are indicated beneath the pB1/16 sequence. Homology to other species has been ascribed to the region between 128 and 171 in this map (Skowronski et al., 1984).


A A C A T C T G T T C T G G N N G T G C G T G T T G C G C A T G T G G T
50
G T T T G T G A C A T G C G T G T C T G C G C T T A C A G G C T C T T T
HhaI
100
C
G G A G A A G G A A A T G G
G G C A C C A T T T C C A G T G A G T C C T G G A G A A G G A A A T G G
HinfI EcoRII
C
A
C A A C C C A C T C C A G T A T T C T T G C C T G G A G A A T C C C A G
C A A - C C A C T C C A G T G T T C T T G C C T G G A G A A T C C C A G
EcoRII HinfI EcoRII
150
A
G G A C G G G G G G A G C C T G G T G G G C T G C C G T C T A T G G G G T
G G A C G G G G G G A G C C T G G T G G G C T G C C G T C T A T G G G G T
EcoRII BglI Fnu4H
200
T
C G C A C A G A G T C G G A C A C G A C T G A A G C G A C T T
T G C A C A G A G T C G G A C A C G A C T G A A G C G A C T T A G C A G
HinfI DdeI Fnu4H
C A G C A G C A G C A G C G G G
Fnu4H Fnu4H Fnu4H Fnu4H

Figure 5

found. There is no evidence of a total or partial repeat of the A element upstream of the 3′ end and there are no CACTT sequences which have been shown to link some bovine and caprine repetitive sequences (Watanabe et al., 1982, Skowronski et al., 1984,).

Transcription of bovine repetitive sequences

Transcription of repetitive elements in other species has been demonstrated under certain conditions of cell growth, differentiation, or transformation. The cell line 5A5, which was derived by SV40 transformation of bovine pituitary cells, was used to study transcription of the repetitive element found in pB1/16. A nontransformed cell line derived from bovine kidney (MBDK) was also examined.

Total RNA was prepared from cell lines 5A5 and MDBK by the method of Chirgwin et al. (1979). Total Poly A+ RNA was also prepared as described in materials and methods. Purified RNA was resolved according to size by denaturing formaldehyde gel electrophoresis, blotted and probed with nick-translated pB1/16 DNA (Figure 6). No discrete species of RNA was found which contained homology to the pB1/16 repetitive element, but a smear of high molecular weight RNA was evident in all the RNA samples. This probably represents RNA still carrying introns with repetitive sequences or mRNAs with repetitive elements in the 3′ untranslated region. Similar analysis with cytoplasmic Poly A+ RNA also showed a smear of RNA but the size range was slightly smaller (data not shown). The RNA from transformed cell line 5A5 and the nontransformed MDBK cells had similar levels of repetitive sequences.

Figure 6. Transcription of Bovine repetitive sequences.

Northern blot analysis of RNA from cell lines 5A5 and MDBK. Lanes labeled A+; Poly A+ RNA prepared as described in material and methods. Each lane contains half the RNA from a confluent 100 mm dish of cells. Lanes labeled Total; 10 ug/lane of total RNA from 5A5 and MDBK cells. Lane labeled Pit. polys. A+; Polysomal Poly A+ RNA from pituitary glands (1 ug).

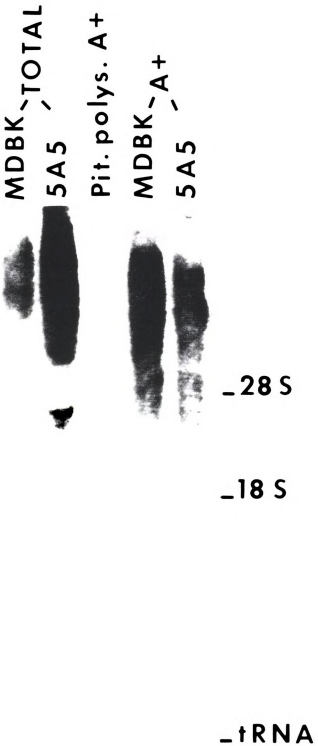


Figure 6

Repetitive sequences in some other species have been shown to be homologous to RNA polymerase III transcripts, producing RNA species in the 100 to 600 bp range. There is no evidence for small transcripts in the northern blot analysis presented here.

DISCUSSION

Previous analyses of the predominant moderately repetitive DNA sequences in ruminants have led to a number of generalizations regarding their nature. The repetitive elements in genomic DNA have been found in two types of arrangements. Dimeric sequences composed of two related 117 bp A monomers have been observed in five cases in cows and two in goats (Figure 7). In all instances the right and left monomer differed reproducibly from each other at certain positions (Skowronski et al., 1984) (Figure 5). In addition the left and right halves of the dimer are separated by a short repeating unit consisting of the sequence $(CACTTT)_3CATGCATT$. A second type of element is the A unit preceded by the 73 bp C sequence (Figure 7). To date, only one example of an isolated A unit has been described (Watanabe et al., 1982) and it has been suggested that this sequence arose by recombination between right and left monomers in a dimer unit (Skowronski et al., 1984). This conclusion was based on the observation that a portion of the sequence fit the consensus for a left monomer while the right portion fits the consensus for the right monomer.

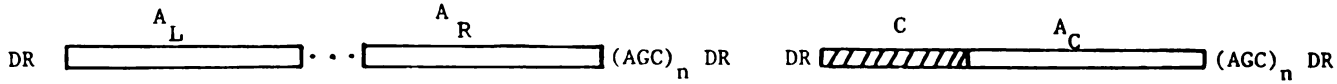
The repetitive element described in this paper is 97.4% homologous with the right monomer and contains only right monomer bases at the sites where systematic differences have been reported (Figure 5). The repetitive element in pB1/16 is terminated at the 3'

Figure 7. Organization of Bovine and Caprine repetitive elements.

Prototype elements are given in the top line. A_R; A element, right half; sequence same as that shown in Figure 5. A_L; A element, left half, differences from A_R are shown in Figure 5. A_R and A_L are linked by the sequence $(CACTTT)_3CATGCATT$ in all cases except the goat B_F which is $(CACTTT)_2CATGCATT$. C; 73 bp C element; concensus sequence is : $TGGCTCAG^{AC}TCTG^{C}CT^{G}CA^{G}TGCGGGAGACCTGGGTTCNATCCCTGGGTTGGGAAGATCCCCT$.

Data were obtained from the following sources. Goat globin; Spence et al., 1985, Schon et al., 1981, Shapiro et al., 1983. Bovine globin; Schimenti and Duncan, 1984. Bovine satellite; Skowronski et al., 1984. Bovine repetitive; Richardson et al., 1986. Bovine corticotropine-b-lipotropin precursor introns; Wantanabe et al., 1982.

PROTOTYPE



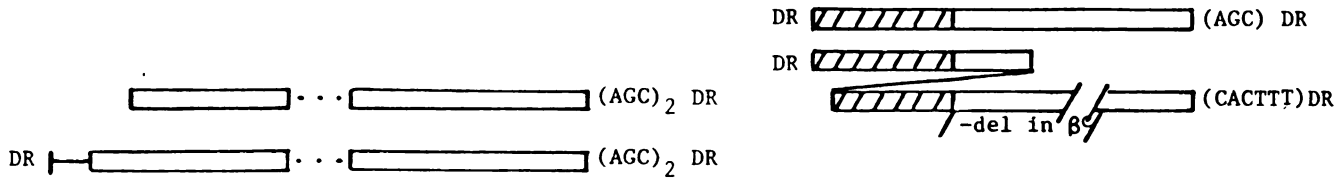
GOAT GLOBINS

ε

β^a/β^c

β^f

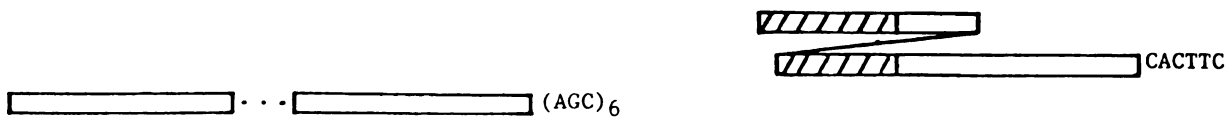
γ



BOVINE GLOBIN

β

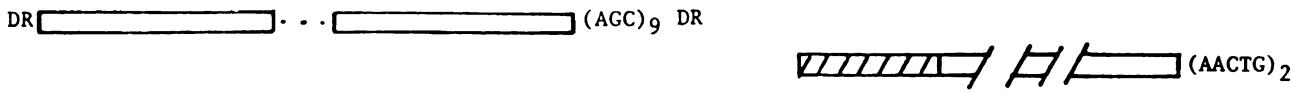
γ



BOVINE SATELLITE

S_1/S_2

S_3



BOVINE REPETITIVE

pPHB9

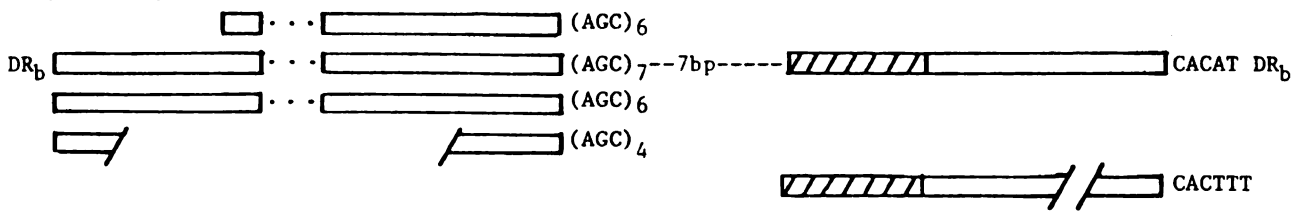
BOVINE Corticotropin-β-Lipotropin

a

b

c

d



end with a repeating $(AGC)_6$ associated with right monomers. There is no evidence of another repetitive sequence to the left of the element or of the typical CACTTT repeat found in the spacer region of the dimer. The region to the left of the repetitive element also shows no homology to the 73bp tRNA-like repeat. The repetitive element in pB1/16 is apparently an example of a third possible arrangement for repetitive elements in the bovine genome. Namely, an A unit unaccompanied by any other repetitive unit.

Repetitive elements can be transcribed by either of two RNA polymerases. Transcription by RNA Pol II can occur when the element is contained within the Pol II transcription unit. Alternatively repetitive elements can be transcribed from internal promoters by RNA Pol III. In vitro transcription of repetitive elements by Pol III has been demonstrated for a wide variety of species using either cloned DNA segments or total DNA (Matsumoto et al., 1986, Furth and Su, 1986, Kramerov et al., 1982, Duncan et al., 1979). In vivo Pol III transcription has been most clearly demonstrated in rodent cells. An RNA corresponding to the B1 sequence is transcribed when mouse cells are transformed by SV40 (Murphy et al., 1983, Singh et al., 1985). A species of cytoplasmic RNA (BC) homologous to the rat ID repetitive element is transcribed specifically in rat brain and pituitary cells (McKinnon et al., 1987). An alu repetitive sequence near the e-globin gene has been shown to produce Pol III transcripts only in the nuclei of a cell line which expressed e-globin (Allan and Paul, 1984).

Clearly, repetitive elements can be transcribed and their transcription is regulated in some way by the cell so that the RNA is expressed only under certain conditions. Bovine repetitive sequences

can be transcribed by Pol III *in vitro* (Furth and Su, 1986) to yield two species of RNA, a 120 bp sequence and a heterologous length length fraction 180-650 bp. Both showed homology to the A repetitive unit. *In vivo* transcription of these sequences was not tested by Furth and Su.

In the present study *in vivo* transcription of bovine repetitive elements was examined. Both a normal (MDBK) and transformed cell line were tested. RNA sequences of high molecular weight were found to contain repetitive elements (Figure 6) presumably as part of Pol II transcription products. No difference in the level of expression of HMW RNA species harboring repetitive sequences was observed between normal kidney and transformed pituitary cells. I found no evidence for a unique Pol III transcription product in bovine pituitary tissue, transformed pituitary cells or normal cells. Expression of the A sequence in cattle shows no analogy to expression of ID or B2 repetitive elements in rodents.

REFERENCES

Ackerman, E.J. 1983. EMBO 2:1417-1422.

Allan, M., and Paul, J. 1984. Nucl. Acids. Res. 12:1193-1200.

Britten, R.J., and Davidson, E.M. 1969. Science 165:349-358.

Britten, R.J., and Kohn, D.E. 1968. Science 161:529-540.

Brunner, A.M., Schimenti, J.C., and Duncan, C.H. 1986. Biochem. 25:5028-5036.

Bennett, 1984.

Daniels, G.R., and Deininger, P.L. 1985. Nature 317:819-822.

Clemens, M.J. 1987. Cell 49:157-158.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.F.J., and Rutter, W.J. 1979. Biochemistry 18:5294-5299.

Doolittle, W., and Sapienza, C. 1980. Nature 284:601-603.

Duncan, C., Biro, P.A., Choudary, P.V., Elder, J.T., Wang, R.R.C., Forget, B.G., DeRiel, J.K., Weissman, S.M. 1979. Proc. Natl. Acad. Sci. USA 76:5095-5099.

Duncan, C.H., Jagadeeswaran, P., Wang, R.R.C., and Weissmann, S.M. 1981. Gene 13:185-196.

Edwards, D.R., Parfett, C.L.J., and Denhardt, D.T. 1985. Mol. Cell. Biol. 5:3280-3288.

Endoh, H., and Okada, N. 1986. Proc. Natl. Acad. Sci. USA 83:251-255.

Federoff, N.B., Wellauer, P.K., and Wall, R. 1977. Cell 10:597-610.

Fowlkes, D.M., and Shenk, T. 1980. Cell 22:405-413.

Fritsch, E.F., Lawn, R.M., and Maniatis, T. 1980. Cell 19:959-972.

Furth, J.J. and Su C-Y. 1986. Biochem. J. 237:827-835.

Haynes, S.R., and Jelinek, W.R. 1981. Proc. Natl. Acad. Sci. USA 78:6130-6134.

Hess, J., Fox, M., Schmid, C., and Shen, C-K.J. 1983. Proc. Natl. Acad. Sci. USA 80:5970-5974.

Houck, C.M., Rinehart, F.P., and Schmid, C.W. 1979. J. Mol. Biol. 132:289-306.

Jelinek, W.R., Evans, R., Wilson, M., Salditt-Georgieff, M., Darnell, J.E. 1978. Biochemistry 17:2776-2783.

Jelinek, W.R., and Schmid, C.W. 1982. Ann. Rev. Biochem. 51:813-844.

King, D., Snider, L.D., and Lingrel, J.B. 1986. Mol. Cell. Biol. 6:209-217.

Kramerov, D.A., Ryskov, A.P., and Georgiev, G.P. 1977. Biochem. Biophys. Acta 475:461-475.

Kramerov, D.A., Grigoryan, A.A., Ryskov, A.P., and Georgiev, G.P. 1979. Nucl. Acids Res. 6:697-713.

Kramerov, D.A., Lekakh, I.V., Samarina, O.P. and Ryskov, A.P. 1982. Nucl. Acids Res. 10:7477-7491.

Krayev, A.S., Markusheva, T.V., Kramerov, D.A., Ryskov, A.P., Skryabin, K.G., Bayev, A.A., and Georgiev, G.P. 1982. Nucl. Acids Res. 10:7461-7474.

Laimins, L., Holmgren-Konig, M., and Khoury,G. 1986. Proc. Natl. Acad. Sci. 83:3151-3155.

Laird, C.D. 1971. Chromosoma 32:378-406.

Lawrence, C.B., McDonnell, D.P., and Ramsey, W.J. 1985. Nucl. Acids Res. 13:4239-4251.

Lueders, K.K., Fewell, J.W., Kuff, E.L., and Koch, T. 1984. Mol. Cell. Biol. 4:2128-2135.

McKinnon, R.D., Danielson, P., Brow, M.A.D., Bloom, F.E., and Sutcliffe, J.G. 1987. Mol. Cell. Biol. 7:2148-2154.

Malissen, B., Malissen, B., and Jordan, B.R. 1982. Proc. Natl. Acad. Sci. USA 79:893-897.

Maniatis, T., Fritsch, E.F., and Sambrook, J. 1982. "Molecular Cloning-A Laboratory Manual". Cold Spring Harbor Laboratory. New York.

Matsumoto, K-I., Murakami, K., and Okada, N. 1986. Proc. Natl. Acad. Sci. USA 83:3156-3160.

Matsumoto, K-I., Murakami, I., and Okada, N. 1984. Biochem. Biophys. Res. Comm. 124:514-522.

Maxam, A.M. and Gilbert, W. 1980. Methods Enzymol. 65:499.

Milner, R.J., Bloom, F.E., Lai, C., Lerner, R.A., and Sutcliffe, J.G. 1984. Proc. Natl. Acad. Sci. USA 81:713-717.

Murphey, D., Brickell, P.M., Latchman, D.S., Willison, K., and Rigby, P.W.J. 1983. Cell 35:865-871.

Owens, G.P., Chaudhari, N., and Hahn, W.E. 1985. Science 229:1263-1265.

Orgel, L., and Crick, F.H.C. 1980. Nature, 284:604-607.

Richardson, K.K., Crosby, R.M., Good, P.J., Rosen, N.L., and Mayfield, J.E. 1986. Eur. J. Biochem. 154:349-354.

Rogers, J. 1985. Int. Rev. Ctyol. 93:187-279.

Ryskov, A.P., Ivanov, P.L., Kramerov, D.A., and Georgiev, G.P. 1983. Nucl. Acids Res. 11:6541-6558.

Sakamoto, K., and Okada, N. 1985. J. Mol. Evol. 22:134-150.

Sapienza, C., and St-Jacques, B. 1986. Nature 319:418-420.

Schimenti, J., and Duncan, C. 1984. Nucl. Acids Res. 12:1641-1655.

Schon, E.A., Cleary, M.L., Haynes, J.R., and Lingrel, J.B. 1981. Cell 27:359-369.

Schmid, C.W., and Shen, C-K.J. 1986. In "Molecular Evolutionary Genetics" (MacIntyre, R.J., ed.) p323-354. Plenum. New York.

Schoeniger, L.O., and Jelinek, W.R. 1986. Mol. Cell. Biol. 6:1508-1519.

Scott, M.R.D., Westphal, K-H., and Rigby, P.W.J. 1983. Cell 34:557-567.

Shapiro, S.G., Schon, E.A., Townes, T.M., and Lingrel, J.B. 1983. J. Mol. Biol. 169:31-52.

Shaw, G., and Kamen, R. 1986. Cell 46:659-667.

Singer, M.F. 1982. Cell 28:433-434.

Singer, M.G. and Skowronski, J1 1985. Trends Biochem. Sci. 10:119-122.

Singh, K., Carey, M., Saragosti, S., and Botchan, M. 1985. Nature 314:553-556.

Skowronski, J., Plucienniczak, A., Bednarek, A., and Jaworski, J. 1984. J. Mol. Biol. 177:399-416.

Spence, S.E., Young, R.M., Garner, K.J. and Lingrel, J.B. 1985. Nucl. Acids Res. 13:2171-2186.

Sutcliffe, J.G., Milner, R.J., Bloom, F.E., and Lerner, R.A. 1982. Proc. Natl. Acad. Sci. USA 79:4942-4946.

Sutcliffe, J.G., Milner, R.J., Gattesfeld, J.M., and Lerner, R.A. 1984a. Nature 308:237-241.

Sutcliffe, J.G., Milner, R.J., Gottesfeld, J.M., and Reynold, W. 1984b. Science 225:1308-1315.

Watanabe, Y., Tsukada, T., Matake, M., Nakanishi, S., and Numa, S. 1982. Nucl. Acids Res. 10:1459-1469.

Weiner, A.M., Deininger, P.L., and Efstratiadis, A. 1986. Ann. Rev. Biochem. 55:631-661.