Use of the Regulated Secretory Pathway

to Ease Protein Product Recovery in Animal Cell Culture

by

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ABSTRACT

An experimental study was performed to determine methods to improve the cloning efficiency of the βTC3 cell line prior to obtaining clonal cell lines expressing recombinant protein. Polylysine pretreatment of the substrate was found to increase colony formation along with the use of conditioned media. Using the acquired knowledge, clonal lines were obtained from the parental (nonclonal) line, as well as from mixtures of cells expressing recombinant prolactin.

Secretion experiments were carried out on the clonal lines to determine whether the recombinant prolactin could be used in a controlled secretion production scheme. Results showed the recombinant prolactin to be partially sorted to the regulated secretory pathway, however the native insulin appeared to be preferentially sorted by the cells.

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CHAPTER 1 INTRODUCTION

1.1 Protein Production

1.1.1 Animal Cells vs. Bacteria

Because bacteria grow quickly (doubling time in minutes) in simple media formulations, are easy to transform and clone, and produce generous amounts of a desired protein, they are the first choice for the production of most proteins. However, animal cells possess the irreplaceable ability for post-translational processing. That is, many proteins require accurate disulfide bond formation, specific proteolytic cleavage of precursor proteins, and other modifications such as glycosylation (addition of sugar residues) and phosphorylation to function properly. As bacteria lack the ability to carry out these modifications, eukaryotic systems are called to service for production of many therapeutic or diagnostic proteins that require posttranslational processing for biological activity.

Unfortunately, eukaryotic cells grow slowly (doubling time in hours) while requiring complex medium supplements such as growth factors and

animal sera. The lack of a cell wall results in sensitivity to shear stress and many mammalian cell lines require a surface substrate for anchoring (anchorage dependence), complicating scale-up. And finally, eukaryotic systems often prove to be difficult to transfect and clone.

A consequence of the growth hormone requirements of eukaryotic cells is that large amounts of serum proteins are added to medium and subsequently interfere with downstream protein purification steps. Although progress is being made in the development of defined serum-free media to alleviate this problem, research in our lab has focused on an alternative solution that has the potential to directly yield not only a high purity but also a high titer product.

The Controlled Secretion Process (CSP) utilizes highly specialized cells derived from exocrine or endocrine glands, capable of regulating the secretion of certain synthesized proteins, to uncouple cell growth and protein synthesis from protein harvesting. Before outlining CSP in detail, a brief review of these specialized cells and their secretory characteristics will be presented.

1.1.2 Regulated vs. Constitutive Secretion

Endocrine and exocrine glands are both factory and warehouse from which hormones vital to the proper functioning of an organism are produced and sequestered until needed. **Endocrine** cells deliver hormones via the bloodstream, while **exocrine** cells supply target organs indirectly (i.e. via a duct). These cells have the rare ability to sequester mature processed protein internally until stimulated by external secretion inducing agents, referred to as secretagogues or secretion agonists.

As previously mentioned, this **regulated secretion** is potentially valuable for industrial production schemes, where cells could be grown in conventional serum-containing medium, while storing newly synthesized protein for subsequent secretion into a second, protein-free "harvest" medium. With this production scheme, collected product does not have to be purified from the serum proteins present in the growth medium. Also, by reducing the volume of the "harvest" medium, the product could be obtained at higher concentrations than would be possible if the product was secreted into growth medium, where a larger volume is required (to supply adequate amounts of nutrients, absorb waste products, etc.).

When posttranslational processing is involved, which is likely for candidates of animal cell production, a further advantage may be gained by collecting protein secreted via the regulated secretory pathway. That is, secretory granules undergo a maturation process, during which covalent modifications occur (i.e. proteolysis, in the case of insulin). During stimulated secretion, mature granules are secreted and predominantly fully processed protein is recovered. For example, while proinsulin is prematurely secreted via basal unstimulated secretion, insulin is the predominant secreted form during stimulated secretion of pancreatic *beta* cells (see Section 1.4.3). In summary, an increase in purity, titer, and product quality relative to conventional methods of protein production can potentially be realized with a controlled secretion process.

To fully exploit the unique secretory properties of these cells, knowledge of their *in vivo* role, as well as protein transport, processing and mechanisms of secretion is crucial. Because the model line under consideration for use in protein production is of pancreatic origin, the pancreas will be examined in some detail.

The pancreas exhibits both endocrine and exocrine secretion while performing its assigned role. Attached to the outer surface of the stomach, the pancreas manufactures digestive (exocrine) enzymes for the gut, as well as endocrine hormones, such as insulin. Insulin is synthesized by clusters of cells (Islets of Langerhans), located throughout the pancreas, and functions to lower blood sugar levels. Although islet cells make up only one percent by weight of the pancreas, they are responsible for the release of at least three other major hormones. Glucagon counteracts insulin (raises blood sugar levels); pancreatic polypeptide regulates pancreatic digestive enzyme release; somatostatin inhibits release of all islet hormones. Interestingly, studies with fluorescent labeled antibodies specific to the various islet hormones have shown that each is produced by a separate islet cell population. The insulin producing subpopulation, called *beta* cells, comprise about seventy percent of the islet cell population, and are the origin of the murine insulinoma βTC3 line, currently under study in our lab for use with the Controlled Secretion Process (Efrat *et al.*, 1988).

Insulin is not continuously secreted into the blood stream by *beta* cells. Instead, insulin is released only when triggered by appropriate signals (e.g.

high glucose blood levels). Rather than wait until periods of high demand before initiating insulin synthesis, *beta* cells stock insulin in electron dense secretory granules. This **regulated secretion** is potentially valuable for industrial production schemes, where cells could be grown in serum-containing medium and store synthesized protein intracellularly for subsequent secretion in a separate, highly defined (protein-free) "harvest" medium. The premise of this project is to exploit the secretion phenomenon exhibited by endocrine or exocrine cells (i.e. pancreatic *beta* cells), by artificially controlling protein secretion in a manner that facilitates downstream purification steps.

The Controlled Secretion Process utilizes endocrine or exocrine cells to uncouple cell growth and protein synthesis from protein harvesting. Potential industrial cell lines are **transformed** cell lines of exocrine or endocrine origin; that is, they have been immortalized for continuous passage.

Obtaining a detailed understanding of the secretory pathway by which proteins are processed is critical to the development and success of a controlled secretion process. Before describing the proposed controlled

secretion process, a review of the cellular pathway of secreted proteins will be presented.

1.2 The Secretory Pathway

1.2.1 Signal Sequence for RER Translocation

The processing pathway for secreted (and membrane) proteins begins on the cytoplasmic side of the Rough Endoplasmic Reticulum (RER), where peptide chains are translated from mRNA templates and concurrently (or co-translationally) translocated (transferred across) through the RER membrane to the lumen where processing begins. Translocation to the RER occurs only for those proteins containing an N-terminal "signal sequence" consisting of about 15 to 29 amino acids (Blobel *et al.*, 1980) and has been shown to be mediated by a signal recognition particle (SRP) and docking protein (DP) (Meyer *et al.*, 1982; Walter and Blobel, 1981). Upon translocation, the signal sequence is promptly cleaved by a signal peptidase (Blobel and Dobberstein, 1975; Jackson and Blobel, 1977) and core glycosylation of glycoproteins initiated (Katz *et al.*, 1977; Lingappa *et al.*, 1978; Rothman and Lodish, 1977). The signal sequences for many secretory proteins have been identified,

including rat preproinsulin and preprolactin (McKean and Maurer, 1978; Ullrich *et al.*, 1977; Villa-Komaroff *et al.*, 1978); the "pre" prefix refers to the protein prior to cleavage of the signal sequence.

1.2.2 Transport to the Golgi

From the RER, secretory proteins are transported in vesicles to the Golgi Complex (Kaiser and Schekman, 1991; Lodish *et al.*, 1983; Nakano and Muramatsu, 1989; Rexach and Schekman, 1991; Ruohola *et al.*, 1988), which consists of a *cis, medial,* and *trans* compartment, in order of proximity to the RER and sequence of processing. Vesicular traffic transports proteins between the Golgi compartments (Orci *et al.*, 1986a; Rothman *et al.*, 1984; Rothman and Orci, 1990), where further modifications (e.g. glycosylation) may occur.

1.2.3 Glycosylation

Glycosylation is a common modification of extracellular protein of eukaryotes and involves the covalent linkage of carbohydrate chains to the peptide. Significantly, many products of modern biotechnology and the pharmaceutical industry are glycosylated (Berman and Lasky, 1985). The carbohydrate composition may reach as high as 60% (Sharon, 1975), and the

structure may be linear or branched. The linkage between oligosaccharide and protein can be either N-glycosidic (carbohydrate attached to an asparagine residue), or O-glycosidic (carbohydrate linked to the hydroxyl of serine, threonine, or occasionally 4-hydroxy-proline).

The biological role of carbohydrates is thought to include protection against proteolytic degradation (Schauer, 1985), formation or maintenance of protein conformation, control of clearance rate from the plasma, involvement in the immune response (Tsai *et al.*, 1977), and secretion or mobilization of certain proteins to the cell surface (Powell *et al.*, 1987). Because of the importance of these carbohydrate moieties to the proper functioning or bioactivity of the glycosylated protein (or glycoprotein), the extent and pattern of glycosylation of a potential glycoprotein must be examined for each potential host cell line. As many glycoproteins are naturally produced as a mixture of glycoforms (the same peptide backbone with different attached oligosaccharide groups), obtaining a recombinant product with a desired glycosylation profile may prove to be extremely difficult. Also, environmental factors (i.e. pH, glucose concentration, etc.) that vary during a production process may adversely affect the glycosylation pattern. Ideally, the

production process would be designed so that conditions are optimal for producing a product with the desired glycosylation and bioactivity.

1.2.3 Exocytosis: Constitutive vs. Regulated Secretion

From the *trans* Golgi, constitutive secretory vesicles emerge and continuously fuse with the plasma membrane, releasing their contents in a process known as exocytosis (Orci *et al.*, 1986b; Rothman and others, 1984; Rothman and Orci, 1990). Endocrine and exocrine cells possess an additional class of post-Golgi vesicles that do not fuse with the plasma membrane shortly after budding, but rather remain in the cell until an external signal (specific to that cell type) is present. In *beta* cells, elevated glucose levels in the bloodstream is one of a variety of chemical signals that triggers exocytosis of insulin-containing secretory granules

The means by which *beta* cells are able to secrete some proteins continuously, while segregating others (i.e. proinsulin) in regulated secretory granules for future secretion is not well understood. Many animal cells are known to carry distinct classes of vesicles, which differ in their pH, proteolytic activity, and site of fusion within the cell, as well as their reliance on external signals for release (Burgess and Kelly, 1984; Kelly, 1985;

Tartakoff and Vassalli, 1978). For example, some epithelial cells possess vesicles that allow for protein transport selectively to either the apical or basolateral surface (Rodriguez-Boulan *et al.*, 1985).

Endocrine and exocrine cells have been shown to contain two different classes of vesicles that originate in the Golgi. By electron microscopic antibody studies, it was shown that proteins destined for regulated secretion (referred to as **regulated secretory** proteins) are found in rough (protein-coated) electron dense post-Golgi secretory vesicles; these vesicles undergo a maturation process that entails acidification, proteolytic processing (e.g. proinsulin to insulin), and shedding of the protein coat. The coats of the dense secretory granules, were found to consist of the protein, clathrin, which has also been identified on the rims of Golgi cisternae in several types of secretory cells (Aggeler *et al.*, 1983; Louvard *et al.*, 1983). Regulated secretory proteins were not found in a second class of smooth translucent vesicles, which were found to carry proteins that the cell continuously secretes and in fact are seen by electron microscopy to fuse with the plasma membrane in the absence of external stimulus.

The AtT-20 cell line has been often studied as a model system of regulated secretion. This mouse pituitary cell line stores adrenocorticotropic hormone (ACTH) in granules and relinquishes the protein only under appropriate stimulus (Gumbiner and Kelly, 1981; Mains and Eipper, 1981). At the same time, constitutively secreted proteins such as laminin, a basement membrane component, are found to reside exclusively in vesicles that fuse continuously with the plasma membrane (Gumbiner and Kelly, 1982; Moore *et al.*, 1983). Similar experiments with pituitary GH3 cells (Green and Shields, 1984), PC-12 cells (Schweitzer and Kelly, 1985), and pancreatic *beta* cells (Orci *et al.*, 1987), have confirmed the presence of two distinct intracellular pathways for secretory proteins–the **constitutive or nonregulated** route for continuously secreted protein, and the **regulated** pathway for proteins whose secretion is dependent on the presence of external signals.

Antibody experiments by Orci et al (1988), reveal that the **constitutive** secretory protein, hemagglutin, and **regulated** secretory protein, proinsulin, coexist in the majority of Golgi cisternae; however, hemagglutin is not found in the dilated region of the *trans* Golgi where

proinsulin is concentrated (Orci *et al.*, 1987; Orci *et al.*, 1988). Thus, the site of divergence of the two pathways appears to be the *trans* Golgi. The fact that both constitutive and regulated secretory proteins enter the ER by a common mechanism (via a signal peptide), are processed in the same compartments in the Golgi, yet emerge from the trans-Golgi in separate vesicles suggests the existence of a discriminatory mechanism by which the two classes of proteins are physically sorted.

1.2.4 Existence of a Sorting Protein

Some researchers speculate that a sorting protein, or "sortase", is involved in the discrimination between the two classes of secretory proteins, in which case, a logical conclusion would be that a recognition site exists on the sortase with affinity for one class of secretory protein. Constitutive secretion is thought to occur by a bulk flow mechanism; that is, a protein is secreted constitutively by default, unless "recognized" by the putative "sortase" to belong to the class of regulated secretory proteins.

This line of reasoning is supported by work of Burgess and Kelly (1984), who demonstrated that in cells blocked from synthesizing proteoglycans, the resulting (protein-free) glycosoaminoglycan (GAG) chains

are nevertheless secreted in constitutive vesicles (Burgess and Kelly, 1984). Additional evidence is obtained from gene fusion studies in which a constitutive protein fused to a regulated secretory protein, is diverted to the regulated pathway (Moore and Kelly, 1986). Thus, recognition of **regulated** secretory protein appears to be the dominant mechanism for determining protein targeting.

Morphological identification of regulated proinsulin shows it to be associated with the Golgi membrane (Orci *et al.*, 1984), perhaps bound by a sortase. In fact, in the recent literature, a protein known to be secreted via a **regulated** secretory pathway was employed in the isolation of a sortase candidate. Chung et al (1989), used regulated peptide hormones as affinity ligands to purify a set of 25-kilodalton proteins from canine pancreatic tissue (Chung *et al.*, 1989). The Golgi membranes were first isolated by a differential centrifugation process; Following membrane solubilization and centrifugation, the golgi lysate was passed over a column containing a sepharose 4B resin coupled to (regulated secretory) sheep prolactin. Significantly, the purified hormone binding proteins (HBK25's) were found to have affinity for other regulated secretory hormones, while demonstrating no

binding capacity for nonregulated proteins, suggesting that HBK25's indeed recognize only the regulated secretory class of protein.

Significantly, DNA transfection experiments have demonstrated an apparent conserved sorting machinery between endocrine and exocrine cells of diverse tissue and species origin. Specifically, AtT-20 cells were found to accurately sort recombinant human growth hormone (Moore and Kelly, 1985), human and rat proinsulin (Moore *et al.*, 1983; Orci and others, 1987), and rat trypsinogen (Burgess *et al.*, 1985). Hence, regulated secretory proteins appear to contain a common domain, recognized by the sorting machinery that diverts them from the constitutive to the regulated secretion pathway. Once identified, this domain might be used in gene fusion studies to convert an otherwise constitutively secreted protein to the regulated secretory pathway. This would potentially enable the controlled secretion process to be applicable to the production of many industrially important proteins.

Effective use of the regulated secretory pathway for protein harvesting will depend on the ability to efficiently control exocytosis of regulated secretory protein. To intelligently manipulate the secretion process, a

thorough understanding of the molecular mechanisms by which cells are induced to secrete is essential.

1.2.5 Secretion Stimulus via Signal Transduction

The means by which a cell is "stimulated" by external signaling agents to secrete sequestered protein is a complicated, poorly understood process and yet, should be efficiently manipulated in the application of CSP. Secretion of protein product must occur only when desired, and then rapidly, to complete exhaustion of internal stores.

Extracellular signaling agents rely on the presence of intracellular "second messengers" (i.e. cAMP, cGMP, Ca^{2+}, IP_3, diacylglycerol (DAG) etc.) to relay the signal into the cell cytoplasm. Activated protein kinases catalyze the transfer of phosphate from ATP to serine, threonine, or tyrosine residues in a host of other proteins (Greengard, 1978), usually increasing their biological activity (Hanks *et al.*, 1988). Phosphorylation by protein kinases is, in turn, counteracted by the action of phosphatases, which function to return a cell from the activated to the resting state (Cohen, 1988).

A general pattern has emerged in a wide variety of "excitable" cell types, following the binding of extracellular signal (ligand) to cell receptor, involving protein kinase activation, increased phosphoinositide turnover, membrane depolarization, elevated cytosolic calcium and exocytosis (Berridge and Irvine, 1984; Dave *et al.*, 1987; Go *et al.*, 1987; Hoenig *et al.*, 1989; Malaisse *et al.*, 1981).

In the case of pancreatic *beta* cells, it is unclear how glucose initiates the signal that leads to release of insulin. Glucose manifests its presence by increasing the ATP/ADP ratio, thereby decreasing activity of ATP-sensitive potassium channels that maintain the membrane potential. The resulting membrane depolarization opens voltage-dependent calcium channels (Ashcroft *et al.*, 1987; Henquin and Meissner, 1981), which leads to an increase in cytosolic calcium, a prerequisite for secretory activity (Rasmussen and Barrett, 1984). The precise role of calcium in secretory granule release still awaits elucidation, but has been postulated to involve a contractile event, or granule mobility due to changes in cytosol viscosity (Trifaro, 1978; Trifaro *et al.*, 1985). The calcium binding protein calmodulin has also been suggested as a possible mediator of secretion stimulation (Trifaro and Fournier, 1987).

Numerous studies have examined the role of calcium channels in insulin release (Al-Mahmood *et al.*, 1986; Findlay and Dunne, 1985; Janis and Scriabine, 1983; Mailaisse-Lagae *et al.*, 1984). For example, various calcium channel blockers, such as verapamil (Devis *et al.*, 1975) and nitrendipene (Hoenig and others, 1989) have been examined for their effect on insulin secretion and found to be secretion antagonists. In contrast, agents that block the action of phosphodiesterase or increase the activity of adenylate cyclase (i.e. increase the intracellular cAMP concentration), potentiate or amplify glucose induced secretion.

Understanding of the above signal transduction mechanisms by which secretion is mediated can be applied to the selection of agents added to media to exert either a secretion stimulating or antagonistic effect, depending on the stage in the controlled secretion process. That is secretion antagonists could be supplied in growth media to prevent premature secretion of newly synthesized, stored product, while agonists in secretion media stimulate exocytosis (see below).

1.3 Controlled Secretion Process

As mentioned above, the premise behind the Controlled Secretion Process is to capitalize on the secretion phenomenon of endocrine or exocrine cells (i.e. pancreatic *beta* cells), by artificially controlling protein secretion to facilitate downstream purification steps. To accomplish this, a two phase cycle is implemented: a recharging (synthesis) phase and a discharging (secretion) phase. In optimizing this process, separate growth and secretion media must be designed with emphasis on preventing premature secretion during the growth phase, and maximizing secretion during the harvest phase.

1.3.1 Recharging phase

Specifically, the cells are grown and maintained in a conventional serum-containing **recharging medium**, with complex growth factors and nutrients as in traditional cell culture, with the addition of agents designed to inhibit secretion. During this growth or recharging phase the cells synthesize secretory proteins which, following processing through the ER and Golgi, are sorted to clathrin coated secretory granules. Further processing, such as proteolytic cleavage may occur but, in the absence of secretion agonists and in the presence of secretion antagonists included in the recharging medium,

there is minimal release to the cell exterior. After a period of approximately twenty hours, the cells reach their storage capacity and are ready for "harvesting". At this point, a fifteen minute rinsing phase is initiated in which the growth medium is removed along with cell waste products and metabolites.

1.3.2 Discharging Phase

The Discharging or Secretion Phase is implemented by contacting the cells with a highly defined **secretion medium**, containing only secretion agonists and osmotic balancing salts. A period of intense secretory activity follows, as stored protein is released from secretion granules into the medium. By supplying secretion agonists in a defined solution, free of serum and constitutively secreted proteins and other complex medium supplements, the protein product is obtained in relatively pure form, as opposed to current practice in industry where desired products must be purified from growth media. A prepurified and preconcentrated product solution reduces the number of downstream unit operations, resulting in a lower cost of production.

After the two hour secretion period, cellular stores are exhausted and following a quick rinse to remove residual secretagogues, the recharging phase is reentered. Ideally, many cycles of production and harvesting can be completed before cell productivity and secretion efficiency decline. One of the obstacles encountered is an apparent desensitization of the cells to secretion agonists with each cycle. Research in our lab has focused on the development of secretion and recharging media formulations for use with the murine pancreatic βTC3 cell line.

1.4 Previous Work on the Controlled Secretion Process

1.4.1 First Attempt

Sambanis first investigated the idea of protein production via regulated secretion using the mouse pituitary AtT-20 cell line (Sambanis *et al.*, 1990). This line synthesizes endogenous proopiomelanocortin (POMC) which is processed through the regulated secretory pathway and cleaved to lower molecular weight peptides, including Adrenocorticotropic Hormone (ACTH). In addition to the original (parental) line, AtT-20 lines that constitutively express recombinant human growth hormone (hGH), or insulin

have been created. Both proteins are secreted by the regulated secretory pathway in their native cell lines, and were shown to be sorted to the regulated pathway in the host AtT-20 line as well. Significantly, the results of these transfection studies suggests that regulated secretory proteins are recognized by a universal mechanism.

Sambanis found that both the hGH and insulin lines secreted their respective product protein at rates above basal, under stimulus by the secretion agonist 8-bromo-cyclic AMP; However, the cells prematurely secreted a large fraction of newly synthesized protein during periods of recharging (i.e. following a round of induced secretion); thus, the time needed to replenish the cells was much longer than would be predicted based on cellular rates of protein synthesis. Also, the growth of undifferentiated foci (and poor attachment of the insulin producing cells), limited the number of possible secretion and recharging cycles.

Although the AtT-20 line has been well characterized as an experimental system for studying the regulated secretory pathway, a line with more promising secretory features was adopted for further study. The murine pancreatic insulinoma βTC3 line was chosen due to its increased

storage capacity and rate of protein synthesis. This line was cultured from tumors that heritably developed from pancreatic *beta* cells in transgenic mice. These cells were reported to synthesize insulin at much higher rates than other transformed insulinoma lines and were found to secrete at high rates in response to glucose.

1.4.2 Advances in Secretion, Recharging Media

Gustavo Grampp, a doctoral student, developed recharging media (VLC-DMEM) for use with βTC3 cells that minimized premature secretion of newly synthesized insulin while replenishing depleted stores (i.e. following a cycle of induced secretion) (Grampp, 1992). This was accomplished by repressing intracellular calcium concentration, while maintaining high glucose-induced insulin synthesis rates. That is, because elevated cytosolic calcium is a prerequisite to secretion, media were prepared with low calcium content. In addition, Verapamil, a calcium channel blocker, was employed to further limit ability of the cell to import extracellular calcium. Therefore, high glucose concentrations could be used (inducing high insulin synthesis rates), without triggering secretion. In fact, while βTC3 cells secreted as much as 50% of newly synthesized insulin in unmodified growth medium,

only 10% was secreted with VLC-DMEM. Thus, a recharging media was devised that successfully prevented unwanted secretion and thereby shortened the time needed to refill the cells following an episode of induced secretion (about 20 hours).

In addition, Grampp designed secretion media (CI-DMEM), to artificially induce cellular exocytosis of stored insulin in βTC3 cells. Of course, high glucose concentrations were used as the main secretion agonist. As opposed to recharging media, the calcium concentration was elevated, while Verapamil was excluded to allow the calcium channels to function. Carbachol, a muscarinic agonist, which acts to raise cytoplasmic calcium concentration by indirectly triggering release of calcium stored in the endoplasmic reticulum was also employed. Elevated intracellular cAMP levels are known to amplify glucose induced secretion; Therefore, IBMX, which inhibits degradation of cAMP was included in the secretion medium (CI-DMEM). With the above agents working in concert, βTC3 cells could be stimulated to secrete at an initial rate of more than 800 μU/h per 10^5 cells and release 80% of the (1200 μU/10^5 cells) intracellular insulin in just two hours.

1.4.3 Improved Protein Quality via Regulated Secretion

As mentioned previously, an advantage of using a controlled secretion process is that in cases where covalent modifications of a secretory peptide are necessary for biological activity, the processed form can be selectively recovered via stimulated release of mature secretory granules. Using HPLC, Grampp demonstrated 90% proteolytic conversion of long-term stored insulin. Following a two hour discharge in CI-DMEM, proinsulin accounted for only 14% (molar basis) of the total secreted insulin related peptides. In contrast, during experiments in unmodified growth medium, as much as 50% of the secreted insulin related peptides consisted of immature proinsulin. Hence, a marked improvement in quality of harvested protein was observed with controlled secretion.

1.4.4 Large Scale Production Potential

To investigate the potential for large scale production, Grampp collaborated with Applegate, who developed single-pass and recycle ceramic monolith reactors for high-density culture of anchorage dependent lines. βTC3 cells were shown to grow to high densities in both reactor types (Grampp, 1992). In addition, insulin concentration and purity were improved

by a factor of 10 and 100 respectively, during controlled secretion. However, the specific productivity was estimated to be only 10% of that observed in small-scale T-flask experiments. Since cell density was inferred from lactic acid production rates, which may differ from reactor to T-flask (as oxygenation efficiency varies), the actual productivity may be much higher.

1.5 Expression of Recombinant Protein

Previous work (see above) indicated that the βTC3 line had potential for use in controlled secretion production schemes. However, the βTC3 line could not be considered a viable host cell line, unless foreign proteins, in addition to endogenous insulin, can be stably expressed. An additional objective was to demonstrate that βTC3 cells can process and secrete in a regulated manner, (regulated secretory) proteins in addition to (endogenous) insulin. While the synthesis and secretory features of native insulin are favorable, it was not known whether other regulated secretory proteins would be handled as competently by βTC3 cells. Significantly, DNA transfection experiments have demonstrated an apparent conserved sorting machinery between endocrine and exocrine cells of diverse tissue and species origin (see

section 1.2.3). Therefore, we hypothesized that regulated secretory proteins from a given endocrine or exocrine cell line would be similarly secreted in βTC3 cells. Of course, the ultimate accomplishment would be regulated secretion of proteins that are secreted constitutively in their native cell line. This may soon become possible once the putative sorting "signal" possessed by regulated secretory proteins is discovered and could be used to divert constitutively secreted proteins to the regulated pathway (i.e. in a fusion protein).

Obtaining a cell line expressing recombinant protein requires transfection with foreign DNA encoding the desired protein. The expression vector components must be carefully chosen to insure recognition by the *beta* cell transcription apparatus. Of special importance is the promoter/enhancer placed in the 5' flanking region of the encoded protein. Before further discussion on the expression of non-native protein in βTC3 cells, a brief review of the origin of the cell line and regulation of gene expression will be given. The βTC (*beta* tumor cell) lines were derived from insulinoma tumors heritably developed in mice expressing the transgene, SV40 large T antigen (Efrat and others, 1988). Because the transgene was placed under control of

the enhancer and promoter region of the rat insulin II gene, the tumors develop in a tissue-specific manner--that is, in *beta* cells where the insulin promoter and enhancer regions are actively transcribed (Hanahan, 1985). As stated earlier, *beta* (β) cells are located in clusters of cells in the endocrine portion of the pancreas, termed the Islets of Langerhans, along with α,δ, and PP cell types producing glucagon, somatostatin, and pancreatic polypeptide respectively.

1.5.1 Selection of Model Recombinant Protein

Because the primary goal is expression of a foreign gene in the βTC3 line, the actual protein chosen is not of great significance. However, to demonstrate regulated secretion of a foreign protein, a protein whose secretion is regulated in its native cell type must be chosen. We hypothesize that this protein will also be secreted via the regulated secretory pathway in βTC3 cells, based on the conserved nature of the sorting machinery between different endocrine cell types (see Section 1.2.4).

For ease of analysis, an economical, commercial assay should be available for the transfected protein. Human growth hormone, prolactin, and thyroid stimulating hormone (TSH) are all secreted by the regulated pathway

and can be assayed using commercially available kits. In addition, the gene must be available for use in an expression vector. Human growth hormone is a well studied regulated secretory protein and would therefore be suitable for expression in the βTC3 line; however, because it is not glycosylated, carbohydrate processing in βTC3 cells could not be evaluated. Thus, a glycosylated protein, such as prolactin, would be a more interesting protein to study. Fortunately, the gene for baboon prolactin was kindly provided by Genzyme.

1.5.2 Expression vector

Efficient expression of the baboon prolactin gene in βTC3 cells depends on the selection of appropriate eukaryotic promoter/enhancer elements, as well as mRNA splice/donor sites, and polyadenylation sequences. Almost universally, the SV40 donor/acceptor splice and polyadenylation signal sequences are used with eukaryotic systems.

The obvious promoter/enhancer to drive transcription of the desired gene is the rat insulin I or II promoter, which is known to be transcriptionally active in the *beta* cell. From the many transient expression studies conducted

with primary islets, HIT, and βTC3 cells, much is known regarding the transcription efficiencies in *beta* cells of various eukaryotic promoter and enhancer combinations, as well as the role of discrete *cis*-acting elements within the rat insulin I and II promoter/enhancer region.

1.5.3 Promoters, Enhancers Functional in *beta* Cells

Most of the transcription studies conducted with pancreatic *beta* cells have been concerned with identifying critical domains of the rat insulin I and II enhancer and promoter region (5' flanking DNA), responsible for *beta* cell specific expression as well as inducible transcription in the presence of glucose and other energy sources. Some of the studies investigated the effect of various agents (such as glucose) and conditions on insulin production rates and mRNA levels. More recently, experiments have been designed to identify specific sequences in the control region (5' flanking DNA) of the insulin gene responsible for the cell specific and glucose induced transcription.

Because investigators required only transient expression of transfected genes to compare different constructs, stable, long-term expression had not yet been demonstrated. These past experiments do however indicate

combinations of enhancer-promoter elements that can be used to achieve high levels of transcription in βTC3 cells.

Although the majority of the research has been done with the HIT (SV40 T antigen transformed Syrian Hamster insulinoma) line, a few researchers also conducted parallel experiments with βTC3 cells with identical results; consequently, it will be assumed that qualitative results obtained with HIT cells apply equally to the βTC3 line.

Also, promoters driving expression of control proteins were usually cotransfected (along with the mutant hybrid insulin promoter/enhancer CAT reporter construct) in the insulin transcription studies to normalize for transfection efficiency variation and could be utilized to drive *beta* cell transcription. For example, the Simian Virus 40 (SV40) promoter was used to promote transcription of β-Galactosidase for normalization of CAT activities in one case. Consequently, no difficulties were anticipated in obtaining transcription of foreign genes in βTC3 cells, using any of the above promoter/enhancer combinations.

Since βTC3 cells were derived from mice, studies performed with rats and mice will be most useful in determining the most appropriate DNA

control elements, as well as which transfection techniques that have been successfully used. Unlike other mammals, rats and mice carry two non-allelic copies of the insulin gene, denoted rat insulin I and II (Lomedico *et al.*, 1979). The ancestral rat insulin II gene contains two introns and is similar in structure to other mammalian insulin genes. The rat insulin I copy, which contains only one intron, is thought to be the result of integration of a partially processed transcript of the ancestral (rat insulin II) gene.

The rat insulin genes, which differ by two amino acids in the B chain, as well as two amino acids in the C peptide, are expressed and secreted in equal amounts even at different glucose-induced cellular states (Giddings and Carnaghi, 1988). The B chain of the rat insulin I replaces a serine and methionine in the ancestral rat insulin II B chain with a proline and lysine, respectively. The promoter/enhancer regions in the 5' flanking regions of both genes have been the subject of recent studies and will be examined separately.

By understanding the mechanisms by which transcription of the insulin gene is controlled, it may be possible to selectively repress endogenous βTC3 insulin expression, while maintaining synthesis of recombinant protein

under control of some but not all of the promoter/enhancer elements of the insulin gene. It should also be remembered that the cells were transformed by SV40 large T antigen, under control of the rat insulin II enhancer/promoter region, which must also be considered, when targeting the insulin genes for repression.

1.5.4 The Rat Insulin I Enhancer/Promoter

In 1985, (Edlund *et al.*, 1985) used HIT, BHK cells in deletion experiments on the insulin enhancer region linked to the TK promoter and Chloramphenicol Acetyltransferase (CAT) reporter gene. Two regions important for CAT activity were identified; if either of the octamer elements between -112 and -104 and -233 and -241 (numbered relative to the insulin transcription start site) were absent, CAT expression was only 22-38% relative to intact rat insulin I enhancer expression; if both these Insulin Control Elements (ICE's) were deleted, CAT activity was reduced to only 3 to 4%.

Another study utilized synthetic oligonucleotide block replacement mutants of the insulin I enhancer region linked to CAT and confirmed that

these two octamer regions were essential for transcriptional activity (Karlsson *et al.*, 1987). The nucleotide sequence 5'-GCCATCTG'-3' was the same for both octamers and contained the consensus CANNTG sequence for binding of the Basic Helix-Loop-Helix class of transcriptional factors (N denotes an arbitrary nucleotide). Substitution of the GCCAT region with GCCAAT, the consensus sequence bound by nuclear factor I, reduced enhancer (CAT) activity by 12%. Subsequent DNAse protection experiments identified five protected regions, (E1-E5), of which E4 and E5, labeled the NIR and FAR box, were the previously described Insulin Control Elements (ICE's) GCCATCTG. Furthermore, gel mobility-shift analysis revealed a *beta* cell-specific Insulin Enhancer-Binding Factor (IEF1). As expected, mutations in the ICE that eliminated binding of IEF1 also failed to exhibit enhanced transcription of CAT reporter gene.

In 1990 two different labs reported the isolation from *beta* cell cDNA libraries of a protein that binds to the ICE. The Insulin Enhancer Binding Protein (IEBP1) gene was cloned and sequenced (Shibasaki *et al.*, 1990), and found to have homology with the DNA binding Basic Helix-Loop-Helix transcription factors, such as the Immunoglobin (Ig) enhancer binding

proteins E12 and E47. In fact, of the 59 amino acids (238 to 296) that compose the Helix-Loop-Helix domain, 58 amino acids were identical to those of the human E47. Repetition of leucine at every seventh position from amino acid 89 to 117 resembles the "leucine zipper" present in these regulatory proteins. It would seem that the *beta* cell-specific ICE binding factor had been isolated; however, (using the IEBP1 cDNA as a probe) IEBP1 mRNA was found in the rat insulinoma (RINr) but also in the rat hepatoma H35 line, indicating that IEBP1 is not uniquely present in *beta* cells.

At the same time, a βTC1 library was found to contain the gene encoding an ICE binding factor with 80% homology with the E12/E47 protein and 98% homology with the helix-loop-helix domain of E47 (Walker *et al.*, 1990). Using the cloned cDNA insert as a probe, a 3.5 kb mRNA was detected in a range of pancreatic (βTC1, HIT) and nonpancreatic (Ltk- fibroblast, mouse spleen) cell lines. This protein enhanced transcription of the rat insulin I enhancer driven CAT reporter gene in HIT cells, but does not appear to be the *beta* cell-specific (ICE binding) factor previously observed in gel mobility-shift analysis.

Philippe et al identified an octamer region between -186 and -172 (5'-TGTT GTCC-3') very similar to a cAMP Responsive Element (CRE) 5'-TGAC GTCC-3' in the enhancer region of the somatostatin gene (Philippe and Missotten, 1990). A 43 kDa CRE binding factor was subsequently isolated and found to be similar to the CREB factor that binds the somatostatin CRE. A separate region between -196 and -247 imparts glucose responsiveness to a hybrid rat insulin I enhancer/TK promoted CAT expression vector (German *et al.*, 1990). Glucose (16 mM) induces expression 10-fold compared to a 2 mM glucose concentration. Interestingly, this effect is reduced by two-thirds by the calcium channel blocker Verapamil.

1.5.5 The Rat Insulin II Enhancer/Promoter

The rat insulin II enhancer/promoter region has also been examined and found to share some elements with the rat insulin I, such as the presence of a singular insulin control element (ICE). Deletion, linker-scanning mutations with HIT cells confirm the enhancer properties of the ICE element between -100 and -91, which presumably binds to the same Basic Loop-Helix-Loop proteins that bind to the rat insulin I enhancer (Crowe and Tsai, 1989). Proteins present in HIT were found to bind to sequences from -87 to -76 and

-54 to -45. The -54 to -45 binding factor, which was also present in HeLa extracts, was subsequently discovered to be the previously characterized Chicken Ovalbumin Upstream Promoter (COUP) factor (Hwung *et al.*, 1988). Linker-scanning deletion of this region decreased reporter CAT activity to 15%. The COUP factor is not needed for expression with the rat insulin I enhancer.

Another lab demonstrated that a multimer DNA construct containing three copies of the ICE (-106 to -91) was sufficient to confer *beta* cell-specific expression with βTC1 cells. An Insulin Activating Factor (IAF) unique to *beta* cells, was isolated and found to bind the ICE at high salt (200 mM KCl) concentrations (Whelan *et al.*, 1990). As expected, ICE mutations bound poorly by IAF resulted in low CAT (reporter protein) expression.

Linker-scanning mutagenesis of the enhancer region linked with the chicken ovalbumin promoter/CAT gene confirmed the importance of the ICE, and defined an additional region between -124 and -111 whose absence resulted in 4% CAT activity in HIT M2.2.2 cells (Hwung, 1990). Deletion mutations revealed that negative regulation may be involved in *beta* cell-specific expression. Deletion of the -217 to -196 region relieved negative

regulation in HeLa cells, although deletion of the -238 to -110 region reduced CAT activity ten-fold (Cordell *et al.*, 1991). Interestingly, a ubiquitous factor was found to bind the -109 to -106 region; Deletion of this element eliminated CAT activity (obtained by removing the -217 to -196 portion) in HeLa cells.

In summary, both the rat insulin I and II enhancer/promoter are active in *beta* cells, and certain *cis*-acting elements within this 5' flanking DNA can partially, or completely confer *beta* cell-specific expression.

1.6.6 Repression of Insulin gene

In contrast to the positive acting transcription factors, several researchers report proteins that repress rat insulin II enhancer driven expression. When the XGPT gene was placed under control of the -333 to -59 region of the rat insulin II enhancer, and cotransfected in HIT cells along with the adenovirus E1A gene, XGPT activity was reduced (Stein and Ziff, 1987). This protein is known to stimulate transcription of specific cellular genes (i.e. heat shock protein, *beta*-tubulin), but more importantly, to suppress SV40, and polyoma enhancers, the Ig heavy-chain enhancer, as well

as the two rat insulin enhancers. Of these genes whose expression is repressed by E1A, all contain the consensus GTGGTTT or GTGGAAA.

Similarly, the Insulin Activating Factor previously described (binds to the ICE of the rat insulin II enhancer) is bound to and repressed by Id. Id is a protein known to bind and repress basic HLH proteins, such as E12 (Cordle *et al.*, 1991). IAF is also bound by the antibody to E12, which further supports the idea that IAF is the same basic Helix Loop Helix protein cloned and sequenced in rat insulin I enhancer studies. Id was shown to reduce 3-fold the CAT activity driven by the rat insulin II enhancer and 5.88-fold transcription of the *beta*-globin gene under control of an ICE multimer in HIT and βTC1 cells.

Ultimately, the production of native insulin in the βTC3 line will be highly undesirable, for at least two reasons. Most obvious is the fact that synthesis of insulin consumes energy and amino acids that could be used in the production of recombinant protein. Also, a limited volume is available both in the processing organelles in the secretory pathway (ER, Golgi, etc.) and the secretory granules where the protein is stored; hence, the more insulin that is synthesized, processed, and stored by the cells, the lower the

capacity of the cell becomes for production and storage of the recombinant protein.

A mechanism for repressing native insulin gene expression in βTC3 cells to facilitate foreign gene expression appears to be available, either by repression of the ICE by the protein Id, or the GTGGTTT or GTGGAAA sequence by the adenovirus E1A factor. As previously mentioned, these proteins have been shown to specifically block transcription of genes under control of the rat insulin I and II promoter/enhancer region. Because these proteins are believed to act on specific DNA sequences (i.e Id blocks activity of the ICE), the promoter/enhancer chosen for expression of the foreign protein must not contain the same targeted elements of the insulin enhancer. Of course, a vector designed for expression in *beta* cells in conjunction with either of the above insulin transcriptional repressors must lack the insulin promoter/enhancer sequences targeted for transcriptional repression. For example, the multimer mutant enhancer containing three copies of the ICE could not be employed for transcription of the recombinant gene, in conjunction with the protein Id for repression of native insulin. However,

these issues are not of concern at the present, where the goal is to establish expression of foreign proteins and not to repress native insulin synthesis.

1.5.7 Inducibility of Insulin Promoter/Enhancer

Similar to the rat insulin I gene, Glucose (16.7 mM) was found to increase (rat insulin II enhancer promoted) T antigen mRNA levels (in βTC3) cells by a factor of 2.85 relative to a Krebs Ringer Buffer control (Efrat *et al.*, 1991). As in the study of the rat insulin I gene, a channel blocking agent, D600 in this instance, eliminated the glucose induced effect. Neither cAMP increasing agent, forskolin (50 μM) or IBMX (0.5 mM), had a significant effect. In HIT cells however, preproinsulin mRNA levels increased by a factor of four between 2 and 20 mM glucose and doubled further in the presence of the adenyl cyclase activator forskolin (Hammonds *et al.*, 1987).

In the human insulin gene four regions are DNAse protected by the cAMP Responsive Element Binding Protein (CREBP1) (Inagaki *et al.*, 1992). The somatostatin CRE binding protein (CREB), which binds to the CRE consensus (TGACGTCA) sequence, is phosphorylated in response to elevated cAMP levels and activates transcription. The c-jun protein which forms a heterodimer with CREBP1 and binds the CRE with high affinity, represses

cAMP induction; this inhibitory effect was found to be relieved by mutated CREs. the human insulin CRE2 (TGACGACC) is very similar to the TGACGTCC between -177 to -184 in the rat insulin enhancer, which binds to a nuclear factor in *beta* cells.

CHAPTER 2 THESIS OBJECTIVES

The design of this thesis is to apply the controlled secretion process to the production of recombinant proteins in the βTC3 line.

2.1 Stably Expressing Clonal Line

The first objective was to obtain a clonal cell line, stably transfected with the gene encoding a regulated secretory protein. Accomplishment would be evidenced by synthesis of the protein following cloning, as detected by assay (as well as resistance to the selection marker).

2.2 Verification of Regulated Secretory Properties

Once a stably transfected line exists, secretion experiments can be conducted to determine if the recombinant protein is processed through the secretory pathway or merely constitutively secreted. If sorting to the regulated pathway occurs, as expected, parameters relating to the efficiency of controlled secretion (i.e. rate of stimulated vs. unstimulated secretion, maximum stored protein, etc.) can be calculated and compared with those from the base case with native insulin.

CHAPTER 3 CLONING EXPERIMENTS

The isolation of a successfully transfected clonal βTC3 cell line expressing recombinant protein depends on the ability to culture βTC3 cells at very low densities. As previously mentioned, the parental βTC3 line is nonclonal (cultured directly from tumors), and it was therefore not known if the specific productivity of the line could be improved by isolating an insulin producing clone. That is, because the βTC3 line was cultured directly from tumors, other cell types (i.e. fibroblasts) are likely to exist intermingled with the *beta* cell population. It is known that the proportion of βTC3 cells producing insulin decreases with increasing passage number. This phenomenon could be due to overgrowth of fibroblasts and would be highly undesirable in long term cycled controlled secretion schemes. Fibroblast cells do not contribute to insulin synthesis, but nevertheless consume nutrients and oxygen and consequently, lower the specific productivity. Contamination by fibroblasts has been found to be reduced in cultures grown at high density and high serum media. In addition, some researchers report that by selectively removing the more tightly attached (fibroblast) cells, the *beta* cell population can be enriched. However, isolating an insulin producing clone

would be the only sure method of eliminating the fibroblast subpopulation. Hence, experiments were conducted to develop protocols for improving cloning efficiencies. The specific insulin storage capacity and secretion efficiencies of this line was determined and compared with those obtained with parental βTC3 cells (see Chapter 5).

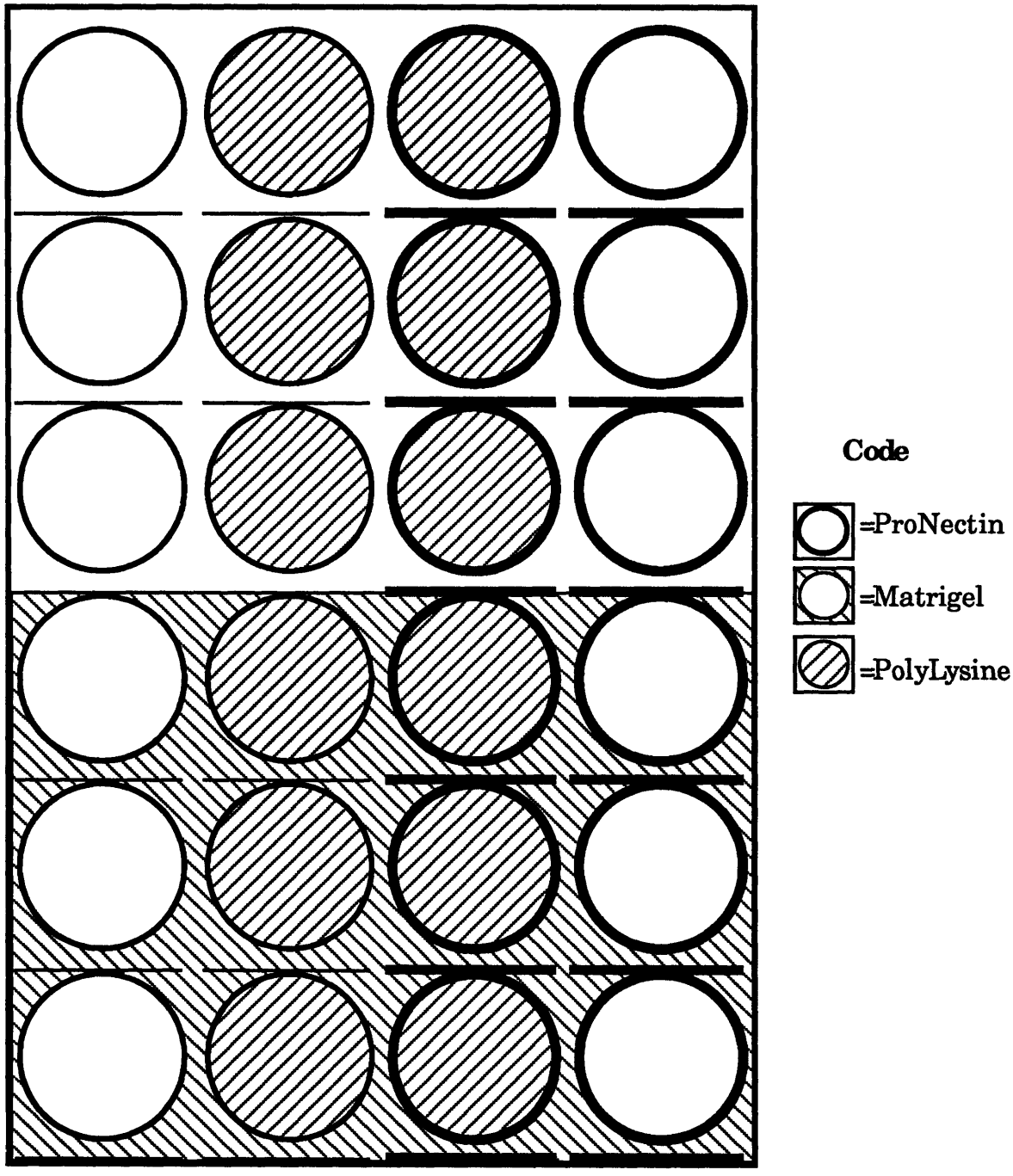
3.1 Treatment of Substrate

Possible treatments to enhance cell growth at low densities include the coating of the substrate with attachment factors, or other components that simulate the basement membrane upon which polarized cells proliferate. For example, polylysine (5 mg/25 cm^2) is reported to increase plating efficiencies of human fibroblasts (McKeehan and Ham, 1976). Polylysine, being positively charged, is believed to assist attachment and spreading of the (negatively) charged cells to the substrate. Fibronectin coating of substrate has been reported to increase plating efficiencies in a variety of cell types (Barnes and Sato, 1980).

Three different substrate treatments were tested alone and in combination, and found to facilitate cloning–Polylysine, Pronectin (a

synthetic peptide), and Matrigel, a basement membrane simulating formulation (see Figure 3.1). Cells were plated at low densities and incubated for two weeks. While the control wells (no substrate treatment) did not support cell growth and formation of colonies, all remaining wells supported the growth of at least one colony. Polylysine was selected for future substrate treatment experiments, since it is inexpensive and easy to apply. The optimal amount of polylysine per substrate area was not clear due to conflicting recommendations in the literature. Clearly, a lower limit exists where not enough polylysine is present to improve cell attachment and growth; likewise, an upper limit may also exist, where excess polylysine may redissolve in growth media and exert a toxic effect.

Figure 3.1 Combinatorial Pretreatment of 24 well plate



24 well plates were pretreated with various combinations of Pronectin (10 μg/well), Matrigel (10.1 μg/well), and polylysine (10 μg/well), with the exception of the three upper leftmost control wells.

3.2 Use of Conditioned Medium

In addition to substrate treatment, the use of conditioned media (supernatant removed from high density cultures) has been shown to increase cell survival rates. The use of one part conditioned medium to two parts cloning medium is recommended for low density cultures, especially for cells that exhibit autocrine secretion of growth factors (Freshney, 1987). Conditioned Medium was obtained by contacting 50% confluent cultures with fresh medium and collecting media after 48 hours. Centrifugation at 10,000 g for 20 minutes, followed by filtration of the supernatant through a 0.2 micron filter yields a sterile solution; cloning medium was obtained by mixing one part filtered supernatant to two parts normal DMEM. Also, media was supplemented with 10% (v/v) fetal bovine serum, which is generally considered better than calf or horse serum for clonal cell densities.

Cells were cultured in 96-well plates at an inoculum of 10 cells per well and fed with conditioned media (see Table 3.1); about 33% of the wells contained at least one colony at five weeks. This represented a 100% increase from the control case of only 17% for wells supplied with regular growth

medium. A similar increase in cloning efficiency resulted from pretreatment of the wells with polylysine (2 $\mu g/cm^2$) prior to inoculation. When polylysine pretreatment and conditioned medium were used in conjunction, 50% of the wells grew colonies—three times as many wells as the control. The results showed that the effect of using both conditioned medium in conjunction with polylysine substrate treatment are additive and together increase the plating efficiency by 200%.

Table 3.1 Effect of Polylysine and/or Conditioned Medium

Med. Type	Pretreatment	# of Colony-containing Wells	Percent of Total Wells
Regular Medium	+ Polylysine	11 of 60	17%
Regular Medium	- Polylysine	20 of 60	33%
Conditioned Med	+ Polylysine	20 of 60	33%
Conditioned Med	- Polylysine	30 of 60	50%

3.3 Cloning Efficiency Improvement by Polylysine

Preliminary experiments with 24 well plates revealed the effects of various substrate treatments (polylysine, matrigel, pronectin) on cell/colony growth and morphology; however, no quantitative results were obtained. Because preliminary results indicated a beneficial effect with polylysine substrate treatment, an experiment was designed to determine which concentration of polylysine is optimal for stimulating cell attachment and colony growth.

A stock solution of 0.2 mg/ml polylysine was prepared and diluted to appropriate concentrations so that 16.7 μl solution per well (on a 96-well plate) resulted in polylysine densities of 0.2, 1, and 10 $\mu g/cm^2$. Three 96-well plates were prepared containing symmetrical quadrants pretreated at three polylysine densities, as well as a control quadrant with no pretreatment. The symmetrical treatment was required to eliminate edge effects. That is, past experiments revealed that colonies do not form as readily in the peripheral wells (i.e. due to evaporation of water from outer wells).

Cells were trypsinized, counted and diluted and the three plates inoculated with 1, 5 and 50 cells per well, respectively. The wells were fed with conditioned medium containing one part supernatant from dense βTC3 cultures to two parts fresh DMEM. Medium was changed weekly and after two weeks colonies could be seen under a microscope. The plate inoculated with 50 cells per well was inspected for the presence of colonies and the results recorded. In addition, some wells were sacrificed at six and seven weeks for cell counts. Colonies were suspended in 20 or 30 μl of trypsin and pipetted vigorously to disperse the cells. Following the addition of an equal amount of trypan blue solution, the cells were vortexed and counted on a

hemacytometer slide. About 10 to 12 µl was required to fill each side of the hemacytometer, so replicate counts could be done with about 20 to 25 µl.

Table 3.2 lists the results at six and seven weeks as well as a combination of both (including a combined estimate of the number of cells the wells sacrificed at seven weeks would have contained at six weeks, based on calculated growth rates). Because twice as many wells were sacrificed at six weeks, the averages were weighted accordingly. The average number of colonies formed per well was calculated for the plate inoculated with 50 cells per well. Interestingly, all three polylysine plating treatments resulted in about 9 colonies per well (an estimated cloning efficiency of 20%). By comparison, the control wells contained only 5.7 colonies per well (12.6% cloning efficiency). Thus, treatment with polylysine (.2 to 10 $\mu g/cm^2$) almost doubled the survival rate of cells at an inoculum of 50 cells per well (about 150 $cells/cm^2$). The plates with 5 and 1 cells per well contained very few colonies, so a statistical analysis was not possible.

Table 3.2: Effect of Polylysine on Colony Growth

(6 Weeks)				
Polylysine: ($\mu g/cm^2$)	0.0	0.2	1.0	10.0
# Cells/Well:	39698	41215	41395	29735
# Cols./Well:	4.7	8.7	8.7	8.2
Cloning Efficiency:	10.4 %	19.2 %	19.3 %	18.2 %
% Viable	58.0 %	52.6 %	37.2 %	43.5 %
#Cells/Colony:	7866	4475	4743	3359
Doubling Time (h)	79	84	84	87
(7 Weeks)				
# Cells/Well:	89720	116373	98053	129893
# Cols./Well:	7.6	11.2	9.8	10.4
Cloning Efficiency:	16.9 %	24.9 %	21.8 %	23.1 %
% Viable	16.1 %	18.7 %	13.9 %	15.6 %
#Cells/Colony:	11988	11020	8492	8316
Doubling Time (h)	85	86	87	85
(6 Weeks-Est.)				
# Cells/Well:	37727	42279	40118	36032
# Cols./Well:	5.7	9.5	9.1	8.9
Cloning Efficiency:	12.6 %	21.1 %	20.1 %	19.9 %
#Cells/Colony:	6726	4305	4425	3798

Three 96-well plates were pretreated with three polylysine densities (0.2, 1, and 10 $\mu g/cm^2$) as well as a control quadrant with no pretreatment and inoculated with 50 cells/well . The wells were fed weekly with conditioned medium and wells sacrificed for cell counts at six and seven weeks.

Although the control wells averaged fewer colonies, the number of total cells per well was almost identical to the 0.2 and 1 $\mu g/cm^2$ wells (about 40,000 cells). Demonstrating a possible toxicity at higher polylysine concentrations, the 10 $\mu g/cm^2$ wells supported the growth of only about 30,000 cells per well. Also, the data revealed a decrease in viability with increasing polylysine concentration. After seven weeks of growth, the growth rate did not appear to have leveled off although the viability drop would indicate otherwise.

Assuming each colony had formed from a single cell, doubling times were calculated to be about 84 hours (or about 3.5 days) which corresponds to a specific growth rate of .0088 per h. This agrees well with the growth rate in T-flasks where cells are split one to four following about a week of growth and is slightly higher than that reported by Grampp (.007 per h) (Grampp, 1992).

The fact that the same number of cells were supported by different numbers of colonies could be due to a limiting nutrient effect when too many colonies are present in the well. Alternatively, (positively) charged polylysine could increase the cloning efficiency by aiding cell attachment of the negatively charged cells, but lower the growth rate due to a slight toxicity effect.

3.4 Effect of Laminin on Cloning Efficiency

Similar experiments were performed using Laminin as a plating factor and the results shown in Table 3.3.

Table 3.3: Effect of Laminin on Colonies per Well

Laminin 96 well plate

	H	G	F	E	D	C	B	A
1	0	0	0	1.5	1.5	0.5	0.5	0
2	0	2	5	4	8	4	4	0.5
3	0	1	4	3	5	5	5	0.5
4	2	2	6	13	6	7	8	0
5	1	3	6	5	5	2	3	0
6	0	2	2	4	2	2	0	0
7	1	2	3	3	1	4	0	0
8	0	1	5	3	1	2	0	0
9	2	1	6	5	2	4	0	0
10	4	2	4	2	4	2	0	0
11	2	3	4	2	1	0	0	0
12	0	0	0	0	0	0	0	0

Average # Colonies per Well

5 CELLS/WELL

CONTROL	10 µg/cm2 POLYLYS
1.0	1.7
1 µg/cm2 LAMININ	10 µg/cm2 LAMININ
0.7	0.0

50 CELLS/WELL

CONTROL	10 µg/cm2 POLYLYS
5.2	4.4
1 µg/cm2 LAMININ	10 µg/cm2 LAMININ
2.5	2.1

The column number and row letter give the coordinate of the well; the number in each well represents the number of colonies present upon inspection at 5 weeks. Note: boldface wells were inoculated with 50 cells/well, instead of 5 cells/well to determine effect of inoculum on cloning efficiency.

The control wells inoculated with 50 cells/well averaged about 5 colonies per well (10% cloning efficiency) which is similar to results obtained for the control wells in the polylysine experiment (see section 3.1). Surprisingly, laminin exhibited a negative effect on the cloning efficiency, such that no colonies grew at 10 $\mu g/cm^2$ laminin (5 cells per well inoculum).

3.5 Parental βTC3 Clonal Line

Because some wells in the laminin experiment (see previous section) contained only one colony, it was possible to isolate clonal lines of βTC3 cells. In particular six different wells contained colonies large enough to propagate. At six weeks, the cells were replated into a well of the same size that had been pretreated with polylysine. The cells were fed with conditioned medium and after two weeks, the surviving lines (three) replated in 24 well plate wells. After an additional week the cells were split one to two (from one well to two). Finally, ten weeks from inoculation, the fastest growing line was split to a T25 and large quantities of cells cultured and frozen for stock.

By examining the specific insulin productivity of this parental clone, it should be possible to draw conclusions about the presence of contaminating

fibroblasts in the nonclonal parental line. The elimination of cells not producing insulin should theoretically raise the specific insulin storage capacity of the population. Grampp reported a maximum capacity of between 1300 and 1500 μU Insulin related peptides per 10^5 cells (Grampp, 1992). On the other hand, if the cells gradually lose their differentiated capability to synthesize and store insulin with increasing passage number, then the clonal line, having been cultured for at least twenty-five doubling periods during the cloning process, would surely produce less insulin than the parental line.

While the clonal βTC3 line was at the 24-well plate stage, βTC3 (nonclonal) populations stably expressing the gene for baboon prolactin were obtained by Keqin Chen (see section 4.1). With the cloning of the parental line already accomplished, there was no reason to believe a clonal line could not be isolated from these prolactin producing mixtures.

3.6 Prolactin βTC3 Clonal Line

3.6.1 Transfection by K. Chen

Although transient expression of foreign DNA had been achieved for this cell line (see section 1.5.3), stable integration of a foreign gene had not

yet been demonstrated. Keqin Chen, a postdoc, constructed expression vectors containing the baboon prolactin gene and transfected the cells. Chen placed the gene for baboon prolactin under control of the murine insulin promoter II, (pINS II), known to be functional in the pancreatic *beta* cell (see section 1.5.4). In addition, Chen prepared an expression vector with the constitutive cytomegalovirus promoter (pCMV). Resistance to the protein synthesis inhibitor G418 (or Geneticin) was encoded by the selection marker gene.

Using intermittent selection for resistance to G418 and regrowth of surviving cells, Chen was able to obtain cell populations stably expressing prolactin, using either promoter (see above). Experiments indicated that the level of expression of the pINS II promoter was about 10 times that obtained with the pCMV promoter. For this reason, cell populations expressing prolactin under the pINS II promoter were chosen for cloning.

3.6.2 Glycosylation of Prolactin in βTC3 cells

Because prolactin is modified by glycosylation (addition of sugar residues), Chen investigated the forms of prolactin secreted under basal and stimulated secretion. He discovered that the nonglycosylated form was

preferentially secreted during episodes of regulated secretion, while the glycosylated prolactin remained inside the cell. At first glance, this result seems contrary to the case of native insulin where the mature (proteolytically processed) form is the predominant form secreted during stimulation. One explanation might be that the glycosylated form is not recognized by the sorting machinery and is therefore constitutively secreted.

3.6.3 Cloning of Prolactin Clones

Until clonal prolactin producing cell lines were obtained from the genetically variable mixture of cells, specific productivities of the subpopulations could not be ascertained. Based on previous experience, 0.2 $\mu g/cm^2$ polylysine was chosen as a substrate pretreatment to augment growth at low densities.

3.6.4 Screening for Prolactin Producing Colonies

Four 96-well plates were inoculated with cells synthesizing prolactin under control of the insulin promoter (about 10 cells per well) to yield an estimated one colony per well (based on a 10% cloning efficiency). After 6 weeks, supernatants from colony-containing wells were tested for the presence of prolactin (see Figure 3.2). Of the 17 wells testing positive for

prolactin, only a fraction (five) contained only one colony and were therefore suitable for propagating a clonal line. Of the five potential clones, one survived (Clone 1G9) to yield sufficient amounts to freeze for secretion experiments.

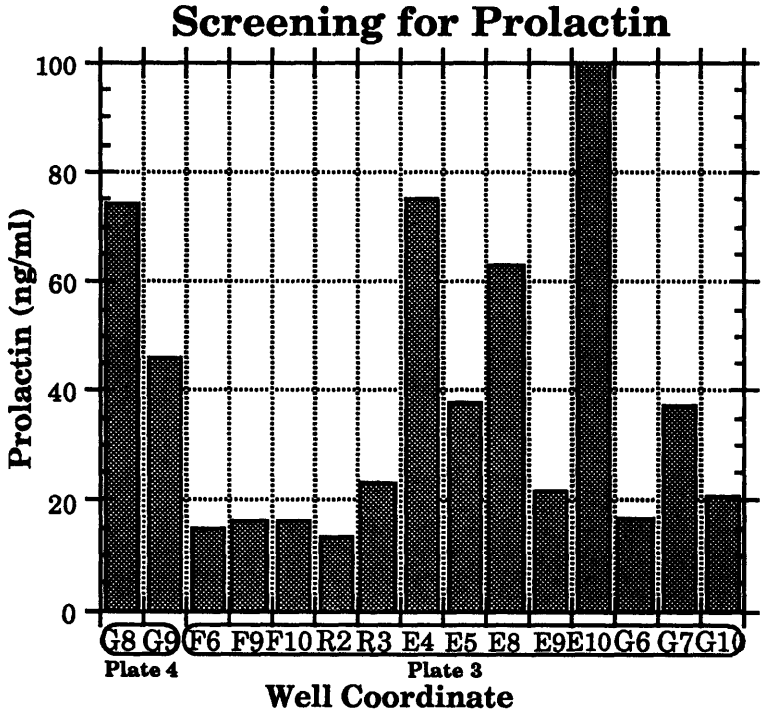


Figure 3.2 Prolactin Levels of Colony Containing Wells

CHAPTER 4 SECRETION EXPERIMENTS

To measure the potential of the prolactin producing clone for use in controlled secretion production schemes, the efficiency of sorting into the secretory pathway and cellular storage capacity was determined. Secretion experiments were carried out in an identical manner to those done by Grampp in characterizing the secretory dynamics of native insulin (Grampp, 1992), to allow comparison between the parental cell line and the prolactin producing clonal line. Hence secretion (CI-DMEM) and recharging (VLC-DMEM) media for all experiments were prepared as specified by Grampp, unless otherwise noted (Grampp, 1992).

4.1 Comparison of Insulin Secretion of βTC3 cell lines

At this stage, five different βTC3 lines/mixtures existed:

1. The original, parental βTC3 nonclonal line.
2. A clone of the parental line.
3. The prolactin producing nonclonal mixture obtained from Keqin Chen under control of the pIns II promoter.
4. The clonal line (1G9) producing prolactin under control of the pIns II promoter.
5. The prolactin producing nonclonal mixture obtained from Keqin Chen under control of the pCMV promoter.

A secretion experiment was designed to measure the insulin (and prolactin where appropriate) specific productivities and controlled secretion efficiencies of the five lines. Approximately $2x10^6$ cells of each line or mixture were plated into a T25 flask and cultured in regular DMEM for several days before the start of the experiment. Following two 3 ml rinses with DMEM base, 4 ml Secretion media (CI-DMEM, see Grampp thesis) were added to each flask and supernatants collected periodically. Interestingly, both clonal lines outperformed their nonclonal counterparts (see Figure 4.1). The nonclonal prolactin secreting mixtures appear to have a population of non-insulin-producing cells that is removed by cloning. Unfortunately, prolactin could not be quantified due to the low expression levels and relatively low cell density (about $2x10^6$ cells per T25). For this reason, cells were grown to higher densities in subsequent experiments to increase the prolactin levels assayed for.

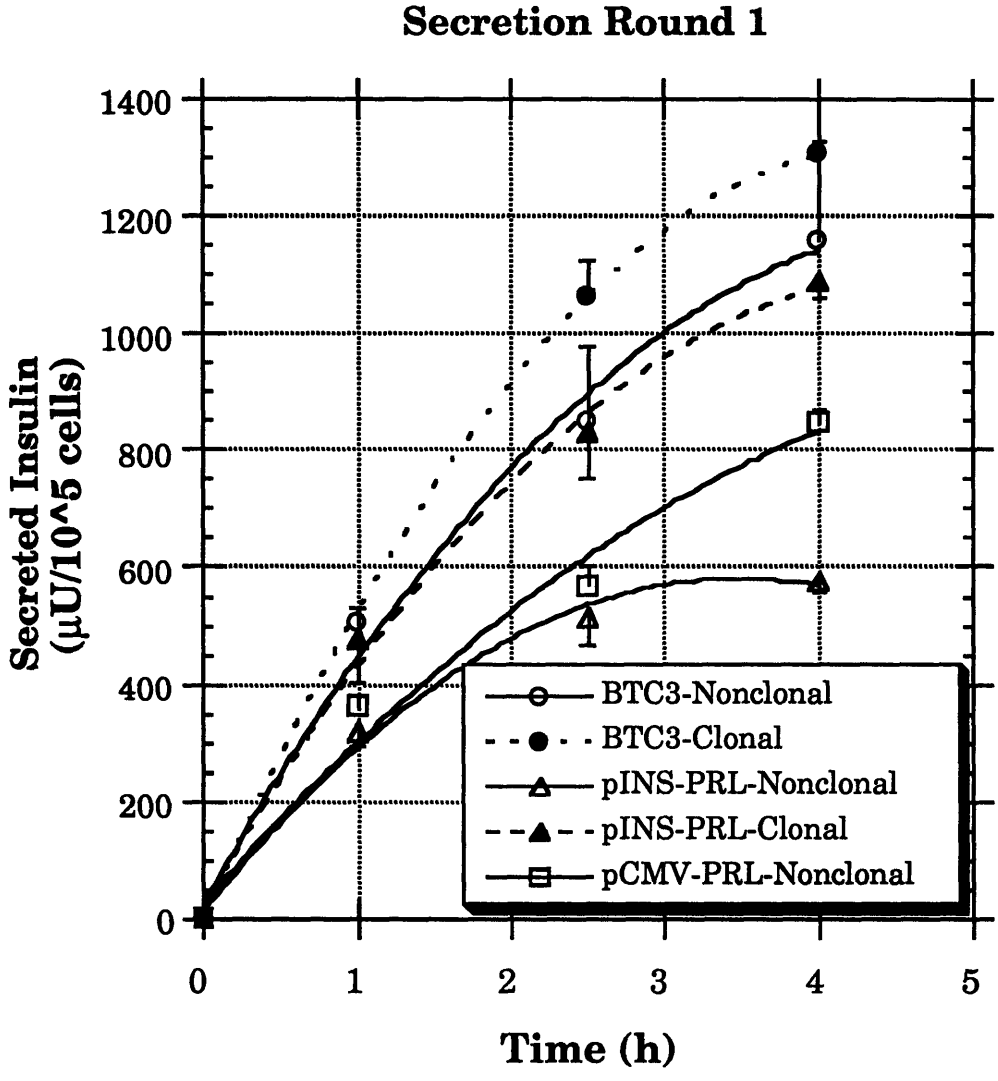


Figure 4.1 Comparison of Induced Secretion of the Five βTC3 line

4.2 Regulated Secretion of Prolactin

An experiment was designed to measure the induced and uninduced secretion rates of a prolactin producing clone. In this case, a clonal line obtained by Dr. Chen was chosen to facilitate comparison of data.

Verapamil in VLC-DMEM recharging media results in a marginal (10%) increase in recharging efficiency and appears to have lasting effects on the calcium channel which lower induced secretion rates following multiple secretion cycles (Grampp, 1992). Therefore, to reexamine the necessity of including verapamil in recharging media, standard VLC-DMEM (containing verapamil and low calcium) was used with half of the flasks and low calcium LC-DMEM (identical to VLC-DMEM, but without verapamil) with the remaining during the recharging phase.

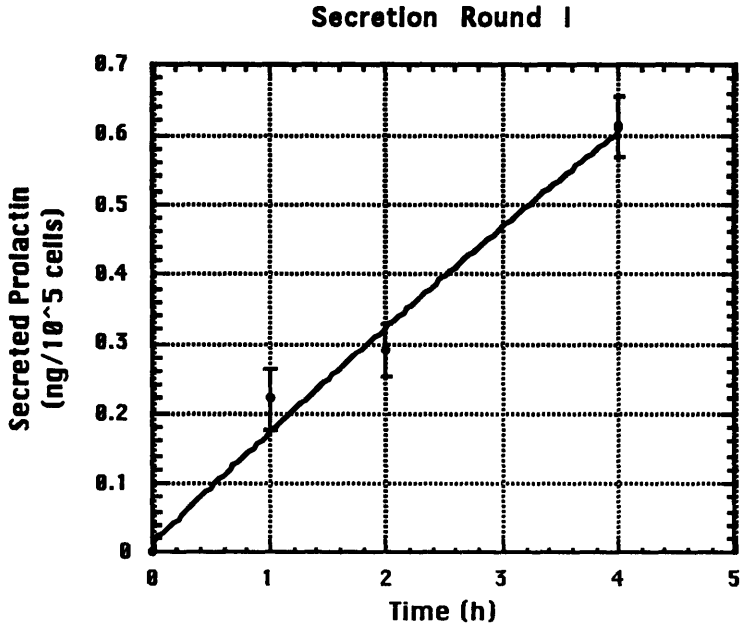
Cells were grown to a density of $1x10^7$ cells per T25 in normal DMEM, rinsed with DMEM base and stimulated for 4 hours in 4 ml CI-DMEM (Round I). The cells were then rinsed and recharged for 20 hours in one of the two recharging mediums prior to the second round of induced secretion (Round II). Periodically, supernatants were collected and flasks sacrificed for

intracellular assay (see Figures 4.2 and 4.3). The insulin profile (data not shown) resembled those obtained in previous βTC3 experiments.

The cells secreted about 0.6 ng prolactin per 10^5 cells during the first secretion round and actually secreted more during the second round (about 0.9 ng prolactin per 10^5 cells). This is not surprising given the intracellular prolactin profile during the experiment. That is, the cells contained only about 0.35 ng prolactin per 10^5 cells at the beginning of round I, which was much lower than the approximately 0.7 prior to round II. Consequently, most (75%) of the prolactin secreted in round I (0.60 ng per 10^5 cells) must be accounted for by fresh synthesis (0.116 ng/h per 10^5 cells) and only 25% (0.15 ng per 10^5 cells) from depletion of intracellular stores. The above estimated synthesis rate agrees well with the steady state secretion data–11.25 ng per 10^5 cells was secreted during the 96 hours prior to the start of the experiment, yielding a synthesis rate of .117 ng/h per 10^5 cells (neglecting cellular degradation).

The presence of verapamil caused a 14% decrease in basal secretion during recharging (3.64 vs. 3.27 ng per 10^5 cells), which corresponds with

results obtained by Grampp for insulin, although the difference in secreted prolactin was not reflected in cellular content (Grampp, 1992).



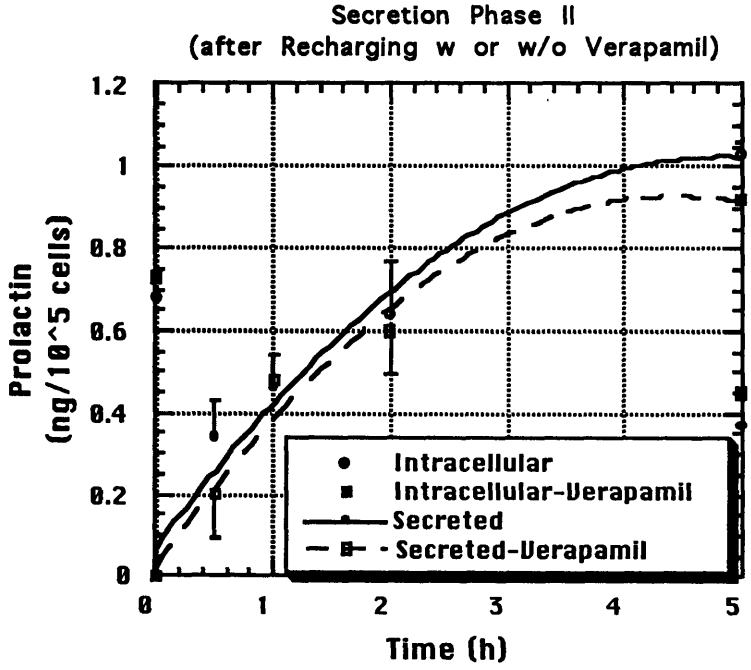
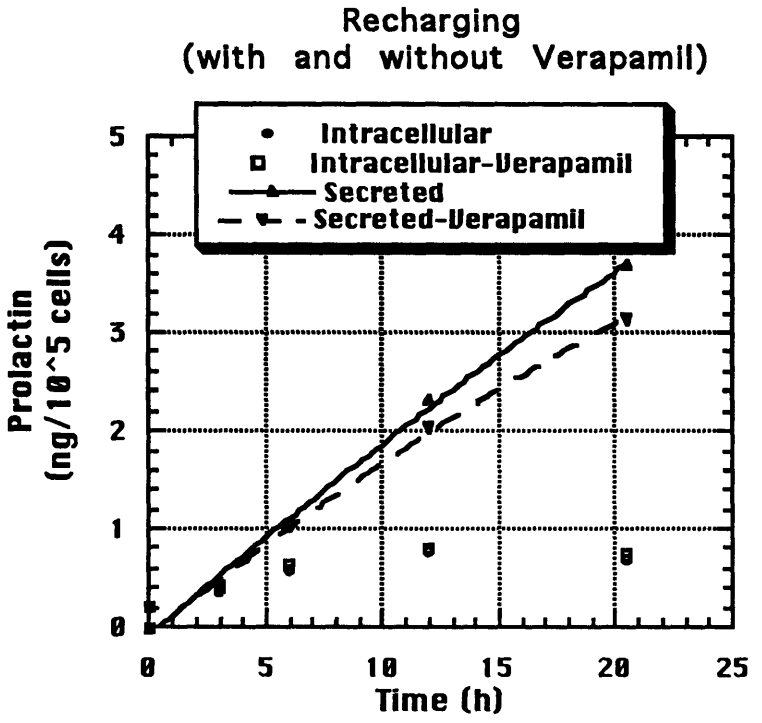


Figure 4.2 Induced Secretion of Prolactin: A). Round I & B). II



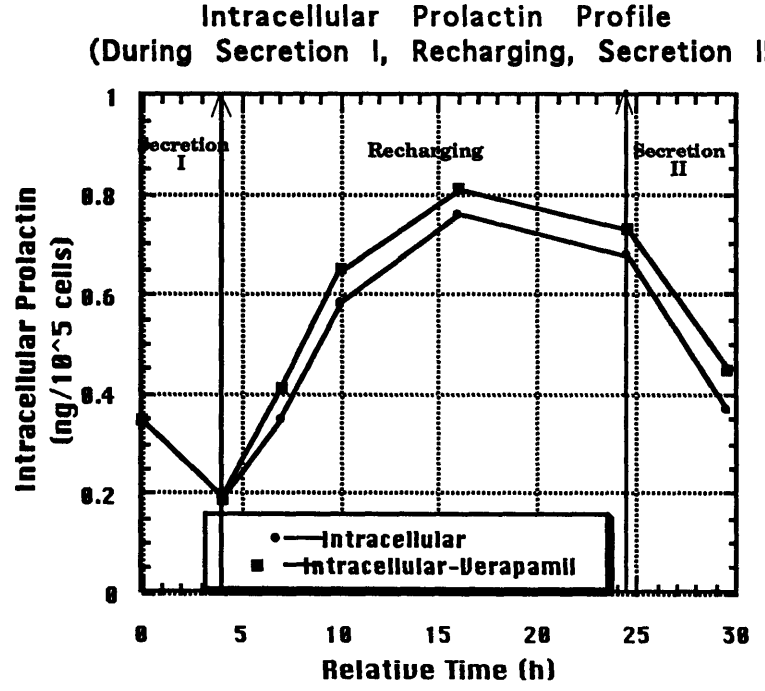


Figure 4.3 A). Recharging & B) Complete Profile for Prolactin

On the one hand, a fraction of newly synthesized prolactin appears to be sorted to the regulated pathway, since the intracellular content increases during recharging. However, prolactin was secreted at an induced rate of only 67% over basal. This is not simply due to inadequate depletion of stored prolactin, but ineffective storage of newly synthesized prolactin as well. One hypothesis is that insulin is sorted preferentially over prolactin in βTC3 cells.

CHAPTER 5 CONCLUSION

The purification and analysis of secretory granules could provide clues to the intracellular storage locus of intracellular prolactin. The presence of prolactin in secretory granules would represent positive confirmation of sorting to the regulated secretory pathway in βTC3 cells. As protocols have been developed for purification of secretory granules and are published in the literature, this type of analysis should be easily done. Alternatively, electron microscopy could be used in conjunction with gold-labeled antibodies (of both prolactin and insulin) to confirm their joint presence in secretory granules, but this would be costly and require more time and expertise.

In addition, experiments in which insulin synthesis is repressed may provide some insight into the effect of insulin on the sorting efficiency of prolactin in these cells. As described earlier, protein factors have been identified that repress the insulin promoter and could therefore be used to study this aspect (see Section 1.6.6). Of course, the cell line expressing prolactin under control of the pCMV promoter (not the insulin promoter) should be used in these studies to avoid inhibiting expression of prolactin.

The study of regulated secretion for protein production provides an interesting alternative to conventional technologies. The above results demonstrate that the βTC3 line can be used to produce foreign regulated secretory proteins. If the level of expression of foreign genes could be increased (with concurrent decrease in native insulin production), the βTC3 line might be considered a serious candidate for production of proteins via controlled secretion production schemes.

The versatility of the controlled secretion process may by limited only by the imagination and genetic engineering expertise of researchers in constructing novel genes and vector systems. For example, proteins normally not processed by the secretory pathway might be linked (in the form of a fusion protein) to a secretory protein and in this way be produced via a controlled secretion process. Furthermore, proteolytically processesing in the secretory granules might be employed to deliver the functional protein, by inserting dibasic residues between the two fused proteins. Similarly, multi-repeating genes encoding neuropeptides might be processed through the secretory pathway and undergo proteolysis to functional peptides in secretion granules. Still more issues than those mentioned will need to be resolved

before such optimistic forecasting may be made. Thus, it may be many years before a final verdict is reached on the subject of the Controlled Secretion Process.

CHAPTER 6 BIBLIOGRAPHY

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