Modification Form for Permit BIO-LHRI-047

Permit Holder: Andy Babwah

Approved Personnel
(Please stroke out any personnel to be removed)

Maryse Ahow

Macarena Pampillo

Cynthia Pape

Additional Personnel
(Please list additional personnel here)

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. Give the full name - do not abbreviate.
Approved Microorganisms	E. coli (DH5 alpha), E.coli (Top 10), XL1-Blue super-competent cells	
Approved Primary and Established Cells	Human (primary): placenta. Human (established): HEK 293, HTR-8/Svneo, MDA-MB-231, MDA-MB-435S, MCF-10A, PC-3, PZ-HPV-7, JEG-3, GripTite 293 MSR, ARIP, AR42J, MEF wild type, MEF, Barr 1 deficient,	LβT2 αT3
Approved Use of Human Source Material	Human chorionic gonadotropin - purified	
Approved Genetic Modifications (Plasmids/Vectors)	[plasmids]: pEGFP-C3, pECFP-C1, pEYFP-C1, pcDNA3.1/Hygro(+). SV 40 large T antigen	
Approved Use of Animals	Mus musculus	
Approved Biological Toxin(s)	Cholera toxin	

LβT2 and αT3 are GnRH neuronal cell lines. We are using These lines to study the roles of Kisrpeptin and GnRH in regulating the HPG-axis.

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE STORED, USED AND DISPOSED OF.*

As the principal investigator, I have ensured that all of the personnel named on the form have been trained. I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca.

Signature of Permit Holder: _____ Aug 22, 2011

Current Classification: 2 **Containment Level for Added Biohazards:** _____

Date of Last Biohazardous Agents Registry Form: Oct 22, 2010

Date of Last Modification (if applicable): _____

BioSafety Officer(s): _____ (Peter J. Ferguson, Ph. D.)

Chair, Biohazards Subcommittee: _____ **Date:** _____

LβT2 cells: Recently, targeted expression of the SV40 T antigen with the rat LHβ-subunit gene regulatory region was used to generate transgenic mice. An immortalized cell line (LβT2 cells) was derived from a tumor generated in a LHβ-Tag mouse, in a manner analogous to the preparation of the αT3-1 cells (188). These cells express both the α - and β-subunits of LH as well as GnRHR, estrogen receptors, and estrogen-inducible progesterone receptors. However, the FSHβ-subunit is not expressed.

0163-769X/97/$03.00/0
Endocrine Reviews

Vol. 18, No. 1
Printed in U.S.A.

Studies of Gonadotropin-Releasing Hormone (GnRH) Action Using GnRH Receptor-Expressing Pituitary Cell Lines*

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I. Introduction

THE regulation of normal mammalian sexual maturation and reproductive function requires the integration and precise orchestration of hormonal regulation at the hypothalamic, pituitary, and gonadal levels. GnRH is a decapeptide synthesized in neurosecretory cells in the preoptic area of the hypothalamus. GnRH is secreted into the hypophysial portal circulation and is transported to the anterior pituitary gland, where it binds to receptors on a specific pituitary cell type, the gonadotrope, to modulate the synthesis and secretion of the gonadotropins, LH and FSH. Gonadotropins, in turn, are secreted into the systemic circulation and act on the gonads to regulate steroidogenesis and gametogenesis. LH stimulates ovulation and corpus luteum formation in females and androgen secretion in males; FSH stimulates the growth and maturation of ovarian follicles in females and spermatogenesis in males. Gonadal steroids and peptides, in turn, are secreted into the systemic circulation and act to modulate hypothalamic and pituitary function in both positive and negative feedback loops (1, 2).

Research into the neuroendocrine control of reproductive function by GnRH has undergone an explosion in the past 25 yr, marked first by the isolation and chemical characterization of GnRH (3–5). This led to the development of both agonist and antagonist analogs, resulting in rapid advances in our basic understanding as well as clinical applications to the treatment of disorders such as prostate cancer, endome-

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*This work was supported by NIH HD-19938 (to W.W.C.), HD-19899 (MERIT; to P.M.C.), and HD-33001 (to U.B.K.), an American Society for Reproductive Medicine–Serono Research Grant (to U.B.K.), and a Medical Research Council of Canada Clinician-Scientist Award (to U.B.K.).

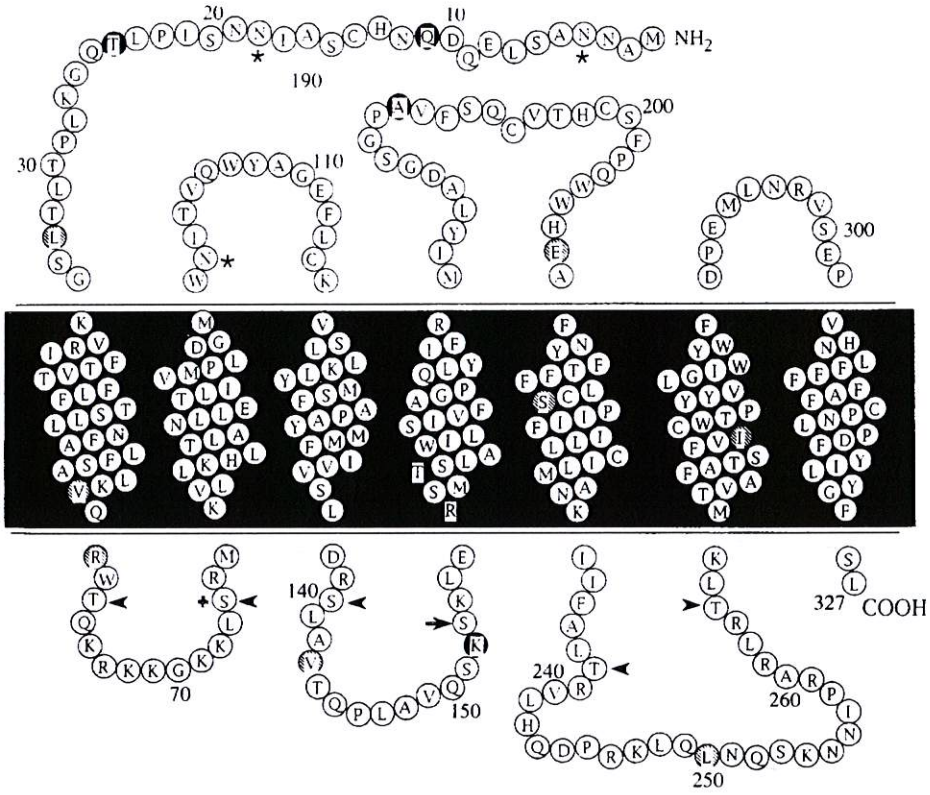

FIG. 1. Model of the rat GnRHR. Amino acid residues in *black* represent nonconserved amino acids between the rat and mouse GnRHR; *shaded* amino acid residues are nonidentical but conserved between the two species. *Asterisks* denote potential glycosylation sites. Potential phosphorylation sites are indicated for protein kinase C (*arrowheads*), casein kinase II (*arrow*), and protein kinase A (*cross*). [Reprinted with permission from U. B. Kaiser *et al*: *Biochem Biophys Res Commun* 189:1645–1652, 1992 (13) (Fig. 2A).]

triosis, precocious puberty, and infertility (6, 7). More recently, the molecular cloning of cDNAs encoding receptors for GnRH (GnRHR)[1] was achieved, first in mouse (8, 9) and subsequently in human, rat, cow, and sheep (10–17). The availability of the GnRHR cDNA has allowed studies leading to further understanding of the mechanisms of GnRH action.

Primary anterior pituitary cells are comprised of a heterogeneous population of well differentiated, secretory cell types. These include somatotropes, which secrete GH; lactotropes, which secrete PRL; corticotropes, which secrete ACTH as well as other hormones derived from the peptide precursor, POMC, including MSH, lipotropins, endorphins, and enkephalin; thyrotropes, which secrete TSH; and gonadotropes, which secrete LH and/or FSH (18, 19). Several anterior pituitary cell types produce more than one of the anterior pituitary hormones; for example, LH and FSH are often colocalized to the same cell, as are GH and PRL. More recently, there has been evidence of colocalization of GH with LH or FSH (20) .

A major hindrance to progress in our understanding of the mechanisms of neuroendocrine control of reproduction at the hypothalamo-pituitary level is the lack of an ideal cell model for these studies. Historically, such studies have been performed *in vivo* in a variety of animal models and *in vitro* in dispersed primary pituitary cell cultures. These studies are limited by the heterogeneity of anterior pituitary cell types; gonadotropes make up only 6–15% of anterior pituitary secretory cells in adult animals (21). In addition, anterior pituitary cells cannot be propagated in culture systems, thus limiting the feasibility of many studies. Recently, a number of immortalized pituitary cell lines have been used as models for studies of the mechanisms of action of GnRH and its receptor.

Several aspects of the GnRHR and its signaling properties have been reviewed previously (22–30). Past reviews have focused on the molecular mechanisms of action of GnRH and the signaling properties of the GnRHR in primary pituitary cells. In this review, we will focus on studies of GnRH action using GnRHR-expressing pituitary cell lines as model systems. The results of these studies will be compared with what

[1] Abbreviations used: GnRHR, GnRH receptor; TRHR, TRH receptor; SV40, simian virus-40; PMA, phorbol myristic acid; PKC, protein kinase C; PKA, protein kinase A; PLC, phospholipase C; IP, inositol phosphate; $[Ca^{2+}]_i$, ionized intracellular calcium concentration; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; GSE, gonadotrope-specific element; SF-1, steroidogenic factor-1; MIS, Mullerian inhibiting substance; PGBE, pituitary glycoprotein hormone basal element; GnRH-RE, GnRH-responsive element; αLUC, α-subunit gene promoter/luciferase reporter fusion gene; PRLLUC, PRL gene promoter/luciferase reporter fusion gene; LHβLUC, LHβ subunit gene promoter/luciferase reporter fusion gene; FSHβLUC, FSHβ subunit gene promoter/luciferase reporter fusion gene; CAT, chloramphenicol acetyltransferase; E_2, estradiol.

is known about GnRH signaling in primary pituitary cells. In addition, we will focus on the role of the GnRHR pathway in the regulation of gene expression.

II. GnRHR Structure Analysis

The GnRHR cDNA encodes a 327- to 328-amino acid protein with seven putative membrane-spanning domains, characteristic of the family of G protein-coupled receptors (Fig. 1) (31). Interestingly, the GnRHR lacks the typical intracellular carboxyl terminus, making it one of the smallest receptors with the seven-transmembrane segment motif. The lack of a carboxyl-terminal tail domain is a unique feature of the GnRHR among G protein-coupled receptors.

Northern blot analysis using the mouse GnRHR cDNA as a probe reveals the presence of at least two hybridizing mRNAs, approximately 4.3 kb and 2 kb in size, in the murine gonadotrope-like cell line, αT3-1 (described below) (8, 9, 32). mRNAs of similar sizes are present in other species as well. An additional mRNA approximately 5.0–5.5 kb in size is present in rat and sheep pituitaries, and a smaller 1.3-kb mRNA is also detected in sheep pituitaries (13, 16). It is not clear whether the differences between αT3-1 cells and rat and sheep pituitaries reflect species differences or differences between primary gonadotropes and an immortalized cell line. The presence of these multiple transcripts raises the possibility that alternative functional forms of the GnRHR may exist.

Cloning of the mouse and human GnRHR genes reveals the presence of two introns (Fig. 2) (33, 34). The introns in the mouse gene occur in the sequences encoding the fourth transmembrane helix and the third intracellular loop. The human gene has the same structure, with the introns interrupting the coding sequences at the same locations, although the introns appear to vary in size. Both the human and the mouse appear to have only a single GnRHR gene, as determined by Southern blot analysis. Analysis of multiple cDNA clones obtained from αT3-1 cells revealed the presence of at least four alternative transcripts, derived largely by alternate splicing (34). It is possible that these alternative transcripts account for some of the additional bands seen on Northern blot analysis. However, these alternative transcripts are less abundant than the original cDNA clone and appear to encode nonfunctional, truncated GnRHRs.

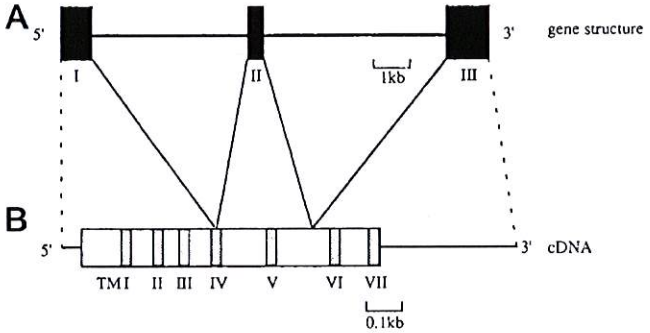

FIG. 2. Schematic representation of the human GnRHR gene. A, Exon-intron localization. The *shaded boxes* indicate exons and the *intervening lines* indicate introns. B, The structure of the human GnRHR cDNA. The *open box* indicates the protein-coding regions, and *hatched boxes* are the putative transmembrane domains. [Reprinted from *Mol Cell Endocrinol* 103:R1-R6, (Fig. 1, C and D), N. C. Fan *et al.*, "The human gonadotropin-releasing hormone (GnRH) receptor gene: cloning, genomic organization and chromosomal assignment" 1994 (33) with kind permission from Elsevier Science Ireland Ltd., Bay 15K, Shannon Industrial Estate, Co. Clare, Ireland.]

The 5′-flanking region of the mouse GnRHR gene has been cloned, and its transcriptional start sites have been defined (35). A major transcriptional start site was identified 62 nucleotides upstream of the translational start site, which does not appear to use a TATA box. Other minor transcriptional start sites were also detected; 1.2 kb of the 5′-flanking sequence fused to a luciferase reporter gene appears to be sufficient to direct high levels of expression when transiently transfected into αT3-1 cells. Some expression also occurred in the rat somatolactotropic GH_3 pituitary cell line, whereas only low levels of expression occurred in a placental cell line, JEG-3, and in a kidney fibroblast cell line, CV-1. These data suggest that this region of the GnRHR gene confers pituitary-specific, and, to a large extent, gonadotrope-specific expression. 5′-Deletion analyses indicate the presence of sequences between −500 and −400 relative to the translational start site that appear to activate GnRHR gene expression in the αT3-1 cell line (36).

The 5′-flanking region of the human GnRHR gene has also been cloned and sequenced (37). Five consensus TATA boxes were identified, distributed within a 700-nucleotide region, and multiple transcriptional start sites were detected associated with these TATA sequences. These transcriptional start sites reside further upstream than the major transcriptional start site identified in the mouse, although the mouse 5′-flanking sequence also reveals several putative TATA boxes. These findings raise the possibility of species-specific or tissue-specific transcription initiation sites. The 3′-end of the human GnRHR gene has also been sequenced, revealing five classical polyadenylation signals (37). The large 3′-untranslated sequence likely accounts for the greatest portion of the major mRNA species observed by Northern blot analysis.

III. Studies of GnRH Action in αT3-1 Cells

A. Derivation of αT3-1 cells

A fusion gene containing 1.8 kb of 5′-flanking sequences of the human glycoprotein hormone α-subunit gene linked to the protein-coding sequences of the simian virus-40 (SV-40) T antigen oncogene was used to generate transgenic mice. Mice carrying this fusion gene developed tumors of the anterior pituitary. The αT3-1 cell line was derived from a pituitary tumor in such a mouse. Cells from this tumor were dispersed and maintained in monolayer culture. Stable cultures were established, and monoclonal cell lines were derived and characterized (38). These cells have provided a continuous cell model system for the study of the GnRHR and GnRH action, as well as for cell-specific expression of the α-subunit; indeed, the availability of αT3-1 cells was critical for the molecular cloning of cDNAs encoding the GnRHR (8, 9).

B. Characterization of αT3-1 cells

αT3-1 cells express α-subunit mRNA. In addition, α-subunit protein is synthesized and secreted by these cells. The cells do not express TSHβ, GH, PRL, or POMC genes, the hormones expressed in other, nongonadotrope anterior pituitary cell types. However, neither LHβ nor FSHβ subunit mRNA, expressed in primary pituitary gonadotropes, is expressed in the αT3-1 cells. The cells respond to GnRH with an increase in α-subunit mRNA levels, whereas levels remain unchanged after exposure to TRH. The GnRH response is time- and dose-dependent and blocked by a GnRH antagonist, consistent with action through the GnRHR (38). Furthermore, GnRH binding and expression of GnRHR mRNA in αT3-1 cells have been shown (39). αT3-1 cells also bind activin A and express mRNAs for the activin receptor types I, II, and IIB, as well as for the inhibin βB-subunit (40, 41). The expression of the gonadotropin α-subunit and GnRHR in αT3-1 cells is consistent with their derivation from the gonadotrope lineage; however, they fail to express the full complement of gonadotrope-specific proteins, specifically the LHβ and FSHβ subunits. This suggests that αT3-1 cells are derived from precursor cells that were not fully differentiated into gonadotropes. This is supported by observations that α-subunit expression occurs early in ontogeny before LHβ or FSHβ (42, 43). The presence of GnRH responsiveness indicates that these cells likely arose after the expression of GnRHR; GnRH-binding sites have been reported to appear, albeit at very low levels, several days earlier in development than the β-subunits (44).

C. GnRH binding

Specific, high-affinity binding sites for GnRH have been identified in αT3-1 cell membrane preparations (39). A GnRH analog binds to these sites with a dissociation constant of 0.50 nM, similar to that measured in normal mouse (0.51 nM) and rat (0.20 nM) anterior pituitary. The total number of binding sites for GnRH is 1.6 pmol/mg protein, about 5 times higher than in normal mouse (0.33 pmol/mg) and rat (0.31 pmol/mg) anterior pituitary (Table 1) (39). However, one must take into account that αT3-1 cells represent a homogeneous cell population, in which all the cells express the GnRHR and bind the GnRH analog, whereas anterior pituitary cells are a heterogeneous cell population, in which only approximately 10% of the cells, the gonadotropes, express the GnRHR. Therefore, the estimated number of GnRH-binding sites on αT3-1 cells is approximately 50% of the number on primary gonadotropes.

TABLE 1. Comparison of the GnRH receptor on mouse, αT3-1, and rat anterior pituitary membrane homogenates

	Mouse	αT3-1	Rat
K_d (nM)	0.51 (0.22–1.2)	0.50 (0.38–0.67)	0.20 (0.14–0.30)
R^o (pmol/mg)	0.33 (0.19–0.67)	1.6 (1.3–1.9)	0.31 (0.24–0.41)

K_d, Dissociation constant of [D-Ala^6,Me-Leu^7,Pro^9-NEt]-GnRH; R^o, total number of binding sites. The 95% confidence limits are given in *parentheses*. [Adapted with permission from F. Horn *et al.*: *Mol Endocrinol* 5:347–355, 1991 (39) (Table 1) © The Endocrine Society.]

D. GnRHR regulation

1. Homologous regulation by GnRH. Homologous ligand regulation of the GnRHR has been shown to occur *in vivo* in rats (45, 46) as well as *in vitro* in cultured rat anterior pituitary cells (47). Similarly, exposure of αT3-1 cells to 10^{-10} or 10^{-8} M GnRH for 20 min has been shown to induce a 50% increase in the number of GnRHRs 24 h later, as determined by GnRH-binding studies (32). This appears to occur at a posttranscriptional level, as GnRHR mRNA levels were unchanged. Interestingly, treated αT3-1 cells with increased GnRH-binding capacity showed a corresponding increase in cellular GnRHR mRNA "activity." That is, αT3-1 RNA was injected into *Xenopus* oocytes, and the GnRH-stimulated Cl^- current was quantitated by voltage clamp recording of the response to GnRH. The evoked current, a measure of the levels of functional GnRHR translated from the injected mRNA, was almost 2-fold higher in oocytes injected with RNA from treated αT3-1 cells compared with controls. These data suggest that GnRH regulates GnRHR numbers in αT3-1 cells by altering GnRHR mRNA translational efficiency. Similarly, prolonged exposure of αT3-1 cells to continuous high concentrations of GnRH, 1 μM for 24 h, resulted in a decrease in GnRH-binding sites to 25% of control levels, no change in GnRHR mRNA levels, but a decrease in GnRH-induced currents in oocytes injected with RNA isolated from the down-regulated cells (48). The changes in GnRH binding in response to GnRH are qualitatively similar to those seen in primary pituitary cells, but this novel mechanism has not yet been shown to occur in primary gonadotropes; indeed, it has been shown that GnRH can regulate GnRHR mRNA levels in primary pituitary cells (49). Hence, it is unclear whether this mechanism of modulation of GnRHR mRNA translational efficiency is unique to αT3-1 cells or is a generalized phenomenon. Alarid and Mellon (50) also found no change in GnRHR mRNA levels in αT3-1 cells in response to continuous exposure to a GnRH agonist for 1–24 h. In contrast, Catt and co-workers (51) showed that exposure of αT3-1 cells to GnRH or a GnRH agonist resulted in a time- and dose-dependent reduction in the level of GnRHR mRNA. Nevertheless, the reductions in mRNA levels were less pronounced than the decreases in receptor number, consistent with the involvement of additional, posttranscriptional mechanisms.

2. Regulation by gonadal steroid hormones. Estradiol has been shown to reduce GnRHR number in αT3-1 cells, as determined by GnRH-binding studies, without significantly altering the dissociation constant (K_d) (52). This inhibitory effect of estradiol is dose- and time-dependent. A reduction in GnRHR number was measurable after 24 h of exposure to estradiol and was maximal after 4–5 days. The EC_{50} of the estradiol effect was approximately 10^{-11} M. In primary cultures of rat pituitary cells, estradiol can both increase (chronic exposure) and decrease (short-term exposure) GnRH binding (53, 54). In ovine pituitary cultures, estradiol increased GnRH binding by 10 h, and this increase was maintained up to 48 h (55). Thus, there appear to be some differences in the responses of αT3-1 cells and primary gonadotropes to estradiol. These discrepancies may be attributable to differences between physiological cellular responses of αT3-1 cells and primary gonadotropes; alternatively, the up-regulation of

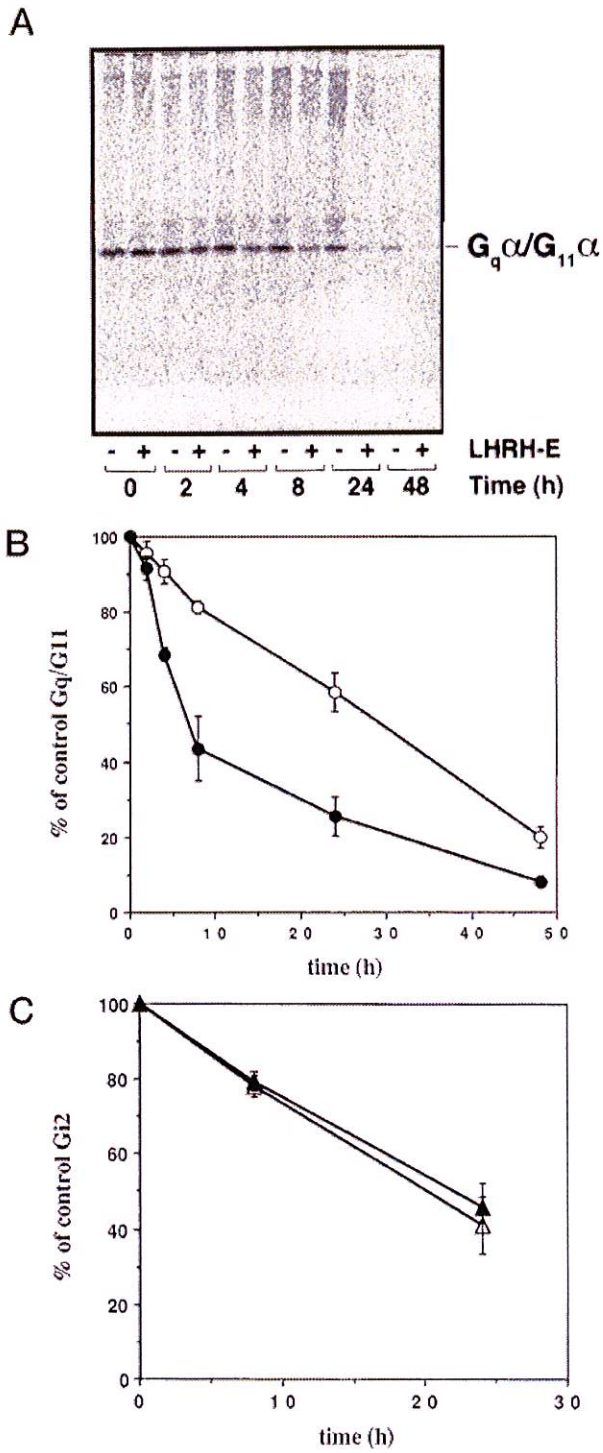

FIG. 3. The turnover of $G_q\alpha/G_{11}\alpha$ and $G_i2\alpha$ in control and LHRH-E-treated αT3-1 cells. A, Autoradiograph of a pulse-chase experiment with [^{35}S]methionine in αT3-1 cells treated or not for various times with LHRH-E. The turnover of $G_q\alpha/G_{11}\alpha$ was monitored in αT3-1 cells in the presence (+) or absence (−) of LHRH-E (1 μM) as described. Immunoprecipitates using antiserum CQ ($G_q\alpha/G_{11}\alpha$) were subjected to SDS-PAGE, and the resulting gel was exposed to a phosphor storage plate for 48 h. The indicated ^{35}S-labeled band was not present in immunoprecipitations done with preimmune serum (data not shown). B, Quantitative analysis of the effect of LHRH-E on the turnover of $G_q\alpha/G_{11}\alpha$. Data such as that presented in panel A were quantitated and are displayed as means ± SEM of four individual experiments. ○, Control; ●, LHRH-E treated. C, LHRH-E treatment does not alter the turnover of $G_i2\alpha$. Samples such as those of panel A were immunoprecipitated with the anti-$G_i2\alpha$ antiserum, SG, and exposed to a phosphor storage plate; the images were analyzed as for panel B. Data represent the means ± SEM of three experiments. △, Control; ▲, LHRH-E treated. LHRH-E, des-Gly^{10}-[D-Ala^6] LH-releasing hormone ethylamide. (The term LHRH used in this figure is synonymous with GnRH used elsewhere.) [Reprinted with permission from B. H. Shah *et al*: *Proc Natl Acad Sci USA* 92:1886–1890, 1995 (64) (Fig. 3).].

GnRHR number seen in primary cultures may occur indirectly, involving steroid hormone effects on cells other than gonadotropes.

3. Regulation by gonadal peptides. Activin A increases GnRHR mRNA levels in αT3-1 cells in a time- and dose-dependent fashion, with maximal stimulation occurring after 24–48 h of exposure (40). This stimulation of GnRHR mRNA levels by activin A occurs at the transcriptional level, as indicated by nuclear run-off and transient transfection experiments. Furthermore, pretreatment of αT3-1 cells with activin A is able to enhance GnRH-induced activation of the gonadotropin α-subunit promoter, suggesting that activin A may have a functional role in modulating the responsiveness of the gonadotrope to GnRH by increasing the expression of the GnRHR. Follistatin is able to block the effects of activin on the GnRHR gene, possibly by binding to and inactivating activin. These data are consistent with data in primary pituitary cells, demonstrating stimulation of the synthetic rate of GnRHRs by activin A (56). In contrast, recent data demonstrated that activin A blocked the stimulatory effect of GnRH on α-subunit promoter activity in αT3-1 cells; whether this was a receptor or postreceptor effect was not determined (57) .

4. Regulation by second messenger activators. In an attempt to identify possible regulators of GnRHR, αT3-1 cells were treated with the second messenger activators, phorbol myristic acid (PMA) and forskolin (50). These agents activate the signal transduction pathways of a multitude of potential effectors that might regulate GnRHR. PMA, a phorbol ester that activates protein kinase C (PKC), had no effects on GnRHR mRNA levels in αT3-1 cells. However, forskolin, which activates adenylyl cyclase, leading to increases in intracellular cAMP levels and hence activation of protein kinase A (PKA), decreased GnRHR mRNA levels by up to 6-fold. This effect was maximal after 8 h, but was transient, with GnRHR mRNA levels returning to control levels by 24 h after treatment. Correlation with GnRH binding is not yet known. Thus, in αT3-1 cells, factors that activate the PKA pathway may decrease GnRHR mRNA levels, whereas activation of the PKC pathway appears to have no effect. In contrast, activation of PKC appears to play a role in mediating up-regulation of the GnRHR by GnRH in primary rat pituitary cells (27, 58, 59).

E. Intracellular second messengers

Studies of signal transduction pathways activated by GnRH in αT3-1 cells have included studies of G protein coupling, generation of inositol phosphates, stimulation of increases in intracellular calcium concentration, activation of

PKC, generation of cAMP, and activation of mitogen-activated protein kinases. The majority of studies have observed the responses to a single pulse of GnRH or to continuous GnRH; the responses to pulsatile administration of GnRH have not yet been described.

1. G protein coupling. Activation of the GnRHR by GnRH has long been known to result in the activation of heterotrimeric GTP-binding (G) proteins. Therefore, when the GnRHR cDNA was cloned, it was no surprise to find that it encoded a protein predicted to be a member of the family of cell surface, seven-transmembrane domain, G protein-coupled receptors (31). Because GnRH actions are generally not affected by cholera or pertussis toxin, a novel G protein (G_p) was suggested to mediate receptor activation. Using an antibody to the common $G_{q\alpha}/G_{11\alpha}$ carboxy-terminal sequence, it has been shown that GnRH activation of phospholipase C (PLC) in αT3-1 cells requires GnRHR coupling to G_q, G_{11}, or both (60). Sustained exposure of αT3-1 cells to a GnRH agonist results in the specific down-regulation of cellular levels of both $G_{q\alpha}$ and $G_{11\alpha}$ (Fig. 3) (61–63). This was attributable to enhanced proteolysis of the activated G proteins; there was no change in $G_{q\alpha}$ or $G_{11\alpha}$ mRNA levels (64). Sustained activation of PKC with the phorbol ester, PMA, was unable to mimic the GnRH agonist-mediated down-regulation of $G_{q\alpha}$ and $G_{11\alpha}$, and inhibition of PKC with the selective inhibitor chelerythrine did not prevent this effect of GnRH, suggesting that the down-regulation of the G protein α-subunits is a direct result of activation of the G protein, and does not require activation of a downstream second messenger-activated protein kinase. Interestingly, the rate of decay of $G_{q\alpha}/G_{11\alpha}$ in the presence of GnRH agonist had two components: an initial rapid rate and a slower secondary phase. It is possible that the initial fast decay rate occurring upon receptor occupancy is reduced to a lower rate with desensitization of the receptor response; alternatively, the fast decay rate may be dependent on the fraction of the cellular G protein that becomes activated upon occupancy of the GnRHR, whereas the lower decay rate depends on the residual G protein pool. The down-regulation of $G_{q\alpha}$ and $G_{11\alpha}$ may, in turn, be a component of the desensitization of the cellular response to GnRH upon sustained exposure to GnRH or to an agonist.

2. Inositol phosphates (IPs). Activation of the pertussis toxin-insensitive G proteins of the G_q family results in stimulation of PLCβ activity, leading to the breakdown of phosphoinositide to inositol phosphates and diacylglycerol. Therefore, the coupling of the GnRHR to G_q and G_{11} would lead one to expect that activation of the GnRHR by GnRH or GnRH agonists would give rise to elevated intracellular concentrations of IPs. Indeed, intracellular concentrations of IPs increased within 30 sec following exposure of αT3-1 cells to a GnRH agonist and continued to accumulate, reaching a maximum after 20 min (Fig. 4) (39, 61). The IP responses were pertussis toxin-insensitive. Levels of inositol 1,4,5-trisphosphate, the immediate product of the cleavage of phosphatidylinositol 4,5-bisphosphate (the major substrate of PLCβ), were rapidly but transiently stimulated after exposure of αT3-1 cells to GnRH. Levels increased within 10 sec, reached a maximum after 30 sec, and returned to basal values after 60 sec. The accumulation of IPs in response to GnRH was inhibited by estradiol. The maximum levels of IPs attained were decreased, and estradiol caused a rightward shift in the dose-response relationship for GnRH-stimulated IP accumulation. This suggests that estradiol reduces GnRHR number and also reduces the efficiency with which the residual receptors are able to activate PLC (52). Estradiol has been shown to regulate levels of G proteins in rat pituitaries; hence, down-regulation of $G_{q\alpha}$ and $G_{11\alpha}$ levels may contribute to this effect (65).

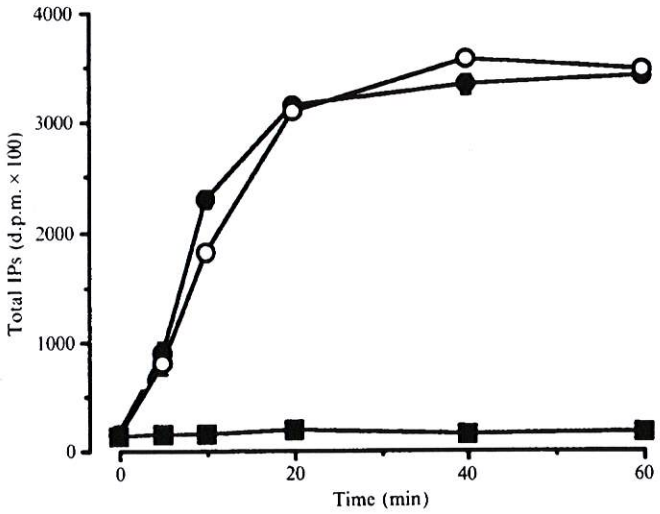

FIG. 4. Time course of total IP production in unstimulated αT3-1 cells (■) or cells stimulated with either GnRH (GnRH; 10 μmol/liter (●) or the GnRH agonist, buserelin (10 nmol/liter; (○). Monolayer cultures were incubated for the times indicated and total IPs were measured. Results are the mean ± SD of triplicate determinations in three separate experiments. [Reproduced by permission of The Journal of Endocrinology, Ltd. From L. Anderson *et al*: *J Endocrinol* 136:51–58, 1993 (61) (Fig. 1).].

3. Intracellular calcium. Intracellular calcium concentrations ($[Ca^{2+}]_i$) increase rapidly in αT3-1 cells after exposure to GnRH. $[Ca^{2+}]_i$ started to increase by 5 sec following GnRH exposure, with the majority of cells showing a maximal response within 15 sec. Thereafter, $[Ca^{2+}]_i$ decreased, although there was a prolonged secondary phase of the GnRH-induced calcium response, with levels increased up to 11 min after the addition of GnRH (Fig. 5) (61, 66). Thus, GnRH augments calcium currents in αT3-1 cells, with a functionally similar response to that reported in primary gonadotropes. Primary gonadotropes have at least two types of voltage-sensitive calcium channels, resembling T- and L-type calcium channels and giving transient and sustained currents, respectively (67). Like T-type current, the transient current in αT3-1 cells was activated by low voltage and rapidly inactivated, and, like L-type current, the sustained current was activated by high voltage and dihydropyridine-sensitive (39, 68). Precise measurements of $[Ca^{2+}]_i$ have been done in single, fura-2-loaded αT3-1 cells by dual wavelength fluorescence microscopy, as well as in cell suspension by spectrofluorometric analysis, and in single indo-1 AM-loaded cells (66, 69). These studies revealed a biphasic rise in $[Ca^{2+}]_i$

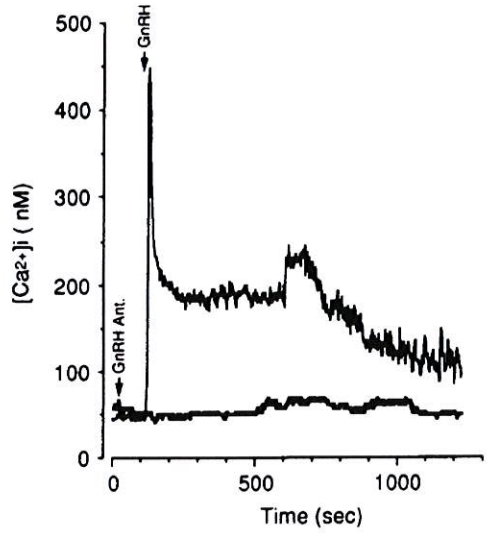

FIG. 5. The effect of GnRH (10^{-8} M, t = 118 s, n = 6, *upper trace*) alone or after pretreatment with a GnRH antagonist (10^{-6} M for 2 min, *lower trace*, n = 10) on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$, Ionized intracellular calcium concentration. [Reprinted from *Mol Cell Endocrinol* 86:167–175, Fig. 1, L. Anderson *et al.*, "Characterization of the gonadotropin-releasing hormone calcium response in single αT3-1 pituitary gonadotroph cells" 1992 (66) with kind permission from Elsevier Science Ireland Ltd., Bay 15K, Shannon Industrial Estate, Co. Clare, Ireland.].

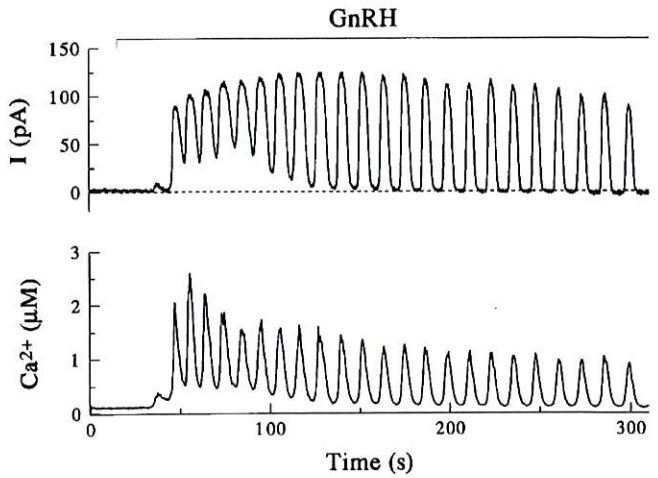

FIG. 6. GnRH-induced oscillations of outward K^+ current and $[Ca^{2+}]_i$. The K^+ current is measured under voltage clamp conditions at −50 mV, and $[Ca^{2+}]_i$ is measured simultaneously with 50 μM indo-1 in the pipette. GnRH (2 nM) is perfused in the bath during the period marked with a *bar*. The opening of K^+ channels is strictly synchronous with $[Ca^{2+}]_i$ elevations. I, Current; Ca^{2+}, ionized calcium concentration. [Reprinted with permission from B. L. Hille *et al*: *Recent Prog Horm Res* 50:75–95, 1995 (25) (Fig. 3).]

in response to 10^{-8} to 10^{-7} M GnRH. The initial calcium response was complete within seconds and involved primarily an IP_3-mediated rise in cytosolic calcium due to release from intracellular stores. Importantly, the peak elevation in $[Ca^{2+}]_i$ was around 500 nM, above the threshold for activation of exocytosis (24). The smaller secondary plateau phase lasted several minutes and primarily involved the influx of extracellular calcium through specific, dihydropyridine-sensitive, L-type, PKC-activated channels. The biphasic nature and duration of the calcium response in αT3-1 cells is similar to the response obtained in studies using enriched gonadotrope preparations. In single αT3-1 cells exposed to increasing doses of GnRH, from 10^{-10} to 10^{-6} M, amplitude-modulated calcium responses were elicited, with no indication of $[Ca^{2+}]_i$ oscillations or frequency modulation. This finding contrasts with observations in primary pituitary gonadotropes, in which GnRH induces prominent $[Ca^{2+}]_i$ oscillations and frequency-modulated calcium signaling (Fig. 6) (25, 70). An additional difference observed in the calcium response of αT3-1 cells compared with primary gonadotropes is that activation of PKC exerts only a negative feedback effect on calcium entry in αT3-1 cells, whereas in cultured primary pituitary gonadotropes, PKC activators cause transient activation of calcium entry, followed by an inactivation phase (69, 70). This effect in αT3-1 cells is similar to that observed in the rat somatolactotropic GH_3 cell line (71). Although the reasons for these differences between the immortalized cell lines and primary pituitary cells are not known, it is possible that calcium channels in αT3-1 cells and GH_3 cells are spontaneously active and undergo inactivation in a Ca^{2+}- and PKC-dependent manner.

4. Protein kinase-C (PKC). The cleavage of phosphoinositides by phospholipase C produces 1,2-diacylglycerols in addition to inositol trisphosphates. Diacylglycerols activate PKC, which results in the translocation of PKC from the cytosol to the plasma membrane. After exposure to GnRH, a portion of intracellular PKC is translocated in αT3-1 cells (39). PMA, a potent activator of PKC, caused an even more pronounced translocation of the enzyme. The effects of GnRH on PKC in αT3-1 cells is similar to that observed in primary pituitary cells *in vivo* and *in vitro* (72, 73). αT3-1 cells contain PKC α-, ε-, and ζ-isoforms, as detected by immunostaining (74). By Northern blot analysis, mRNAs for PKCα and -β, but not -γ, were detected. Exposure of αT3-1 cells to a GnRH agonist resulted in a dose-dependent increase in PKCβ, but not PKCα, mRNA levels. This response was mimicked by PMA. The calcium ionophore, ionomycin, stimulated the expression of both PKCα and PKCβ mRNA levels. Removal of intra- or extracellular calcium or inhibition of PKC abolished the effect of GnRH, indicating that GnRH-induced PKCβ gene expression is Ca^{2+}-dependent and autoregulated by PKC (75).

5. cAMP. No significant change in cAMP levels could be detected in αT3-1 cells after treatment with a GnRH agonist, even in the presence of a phosphodiesterase inhibitor to prevent the degradation of cAMP (39). This is in contrast to the rise in cAMP levels that has been observed in whole pituitaries (76). This difference may lie in the possible need for the presence of testosterone for this response; the GnRH-induced rise in cAMP levels was observed in intact male rats only (77). Others have not been able to detect significant changes in cAMP levels after GnRH treatment of primary gonadotropes (78).

6. Mitogen-activated protein kinases (MAPKs). MAPKs, also known as extracellular signal-related kinases (ERKs), are a family of serine/threonine protein kinases that are rapidly activated in response to a wide variety of stimuli (Fig. 7)

(79–83). Several members of the MAPK family have been identified, including $p42^{mapk}$ (ERK2) and $p44^{mapk}$ (ERK1). Stimuli for their activation include growth factors, many of which have receptors with intrinsic protein tyrosine kinase activity. MAPKs are involved in transmitting extracellular growth and differentiation signals into the cell nucleus, resulting in an array of transcriptional and mitogenic effects. Recent evidence indicates that some G protein-coupled receptors can activate the MAPK family of enzymes and that MAPKs may also be involved in nonproliferative signaling cascades (84–87). G protein-coupled receptors appear to activate MAPK through Ras-dependent and -independent pathways, and both Gα- and Gβγ-subunits appear to be variably involved. These findings have led several investigators to study the ability of the GnRHR to activate MAPK and the role of MAPK in mediating cellular effects of GnRH (88–93).

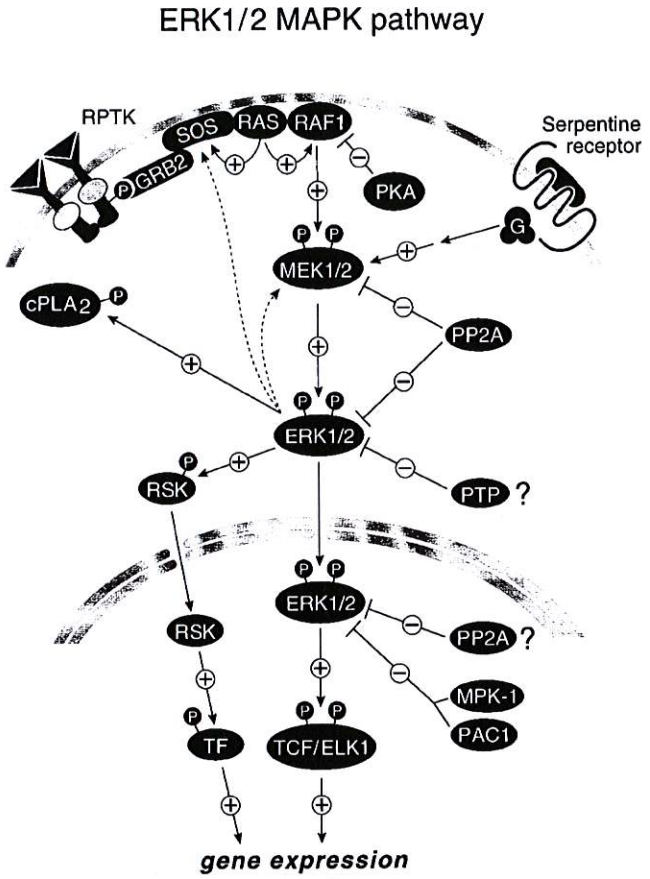

FIG. 7. ERK1/ERK2 MAPK pathway. A schematic illustration of the MAPK pathway. RPTK, receptor protein tyrosine kinase; ERK, extracellular signal-related kinase; MEK, mitogen activated protein kinase (MAPK)/ERK; PKA, protein kinase A; $cPLA_2$, cytosolic phospholipase A_2; PP2A, protein phosphatase 2A; G, G protein; PTP, protein tyrosine phosphatase; RSK, ribosomal S6 kinase; TF, TCF, ELK1, transcription factors; MKP1, PAC1, protein phosphatases. [Reprinted with permission from T. Hunter: *Cell* 80:225–236, 1995 (79) (Fig. 1). © 1995 by Cell Press].

Stimulation of αT3-1 cells with GnRH resulted in phosphorylation of both ERK1 and ERK2, and rapid and sustained activation of both, as assayed by their ability to phosphorylate myelin basic protein (91, 92, 94). Stimulation of enzyme activity was detected within 5 min after the addition of GnRH and remained elevated for 60 min. A maximal activation of 4- to 5-fold was achieved, at a GnRH concentration of 100 nM. Activation of ERK1 and ERK2 was blocked by treatment of αT3-1 cells with a GnRHR antagonist, Antide, demonstrating that activation of the MAPK signal transduction cascade by GnRH is receptor-mediated (92). Activation of MAPK by GnRH was comparable to that observed in response to PMA. Furthermore, PMA pretreatment for 24 h to deplete phorbol ester-sensitive forms of PKC blocked the activation of ERK1 by GnRH. These data suggest that the activation of MAPK by GnRH may involve activation of PKC (91). MAPK activity was also stimulated, although to a lesser extent, by GnRH in primary cultures of male rat pituitary cells. The lower level of activation probably reflects the heterogeneity of the pituitary cell population. Thus, it appears that the MAPK signal transduction pathway is activated by GnRH in both αT3-1 cells and primary pituitary gonadotropes. Interestingly, treatment of αT3-1 cells with pertussis toxin blocked GnRH-induced MAPK activation, suggesting that this signaling pathway is coupled to the pertussis toxin-sensitive G_i or G_o pathway. This provides evidence for G_i/G_o-mediated signal transduction by GnRHR in addition to G_q-mediated signal transduction (88, 90).

F. α-Subunit gene expression

1. Cell-specific expression. αT3-1 cells have proven to be a useful cell model for the isolation and characterization of transcription factors that appear to be involved in mediating gonadotrope-specific expression of the α-subunit gene (Fig. 8). Some of these factors may be involved in mediating stimulation of α-subunit gene expression by GnRH as well. However, because these factors appear to be more important for basal or tissue-specific α-subunit gene expression rather than GnRH-stimulated expression, they will be mentioned only briefly here.

The element in the α-subunit promoter that has been best

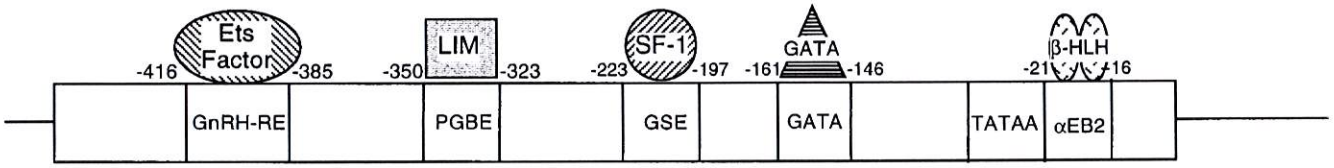

FIG. 8. *cis*-Acting elements and transcription factors important for cell-specific and regulated expression of the glycoprotein hormone α-subunit gene that have been characterized in αT3-1 cells. GnRH-RE, GnRH response element; PGBE, pituitary glycoprotein hormone basal element; GSE, gonadotrope-specific element; αEB2, E-box; LIM, LIM homeodomain protein; SF-1, steroidogenic factor 1; βHLH, basic helix-loop-helix protein.

characterized as a basal, tissue-specific enhancer is the gonadotrope-specific element (GSE). The GSE sequence, TGACCTTG, occurs upstream of the placenta-specific elements, at positions −215/−208 in the mouse α-subunit gene, and is highly conserved among mouse, human, rat, cow, and horse species (95). The GSE is bound by a 54-kDa protein, steroidogenic factor-1 (SF-1) (96). SF-1 was first identified by its ability to bind to and coordinately regulate the expression of genes encoding enzymes in the corticosteroid biosynthetic pathway (97, 98). Subsequently, it has also been shown to bind to and regulate the aromatase and Mullerian-inhibiting substance genes in gonadal tissues (99, 100). Disruption of the gene encoding SF-1 in mice precludes adrenal and gonadal development and also results in the selective loss of expression of gonadotrope-specific markers, including LHβ, FSHβ, and GnRHR mRNAs, and a reduction in α-subunit mRNA levels (101, 102). Thus, SF-1 appears to be important for function of the reproductive axis at multiple levels. Treatment of SF-1-deficient mice with exogenous GnRH stimulates expression of LHβ and FSHβ, suggesting that SF-1 is not necessary for GnRH stimulation of gonadotropin gene expression (103) .

An additional putative basal enhancer, referred to as the pituitary glycoprotein hormone basal element (PGBE), has been identified at −344/−300 of the mouse α-subunit gene (104). The PGBE is able to direct expression of the α-subunit promoter to cells of both gonadotrope and thyrotrope lineages, but not to placenta. A member of the LIM (lin-11, isl-1, mec-3)-homeodomain family of transcription factors, LH-2, binds to a 14-bp imperfect palindrome within the PGBE domain *in vitro* (105). This element and factor are discussed further below.

Other elements that have been identified to play a role in expression of the α-subunit gene in αT3-1 cells include a GATA element, bound by GATA-binding proteins (106), and two E boxes, which bind members of the family of basic-helix-loop-helix-zipper proteins (107). The optimum level of α-subunit gene expression in gonadotropes is probably determined by the combined actions of widely expressed, pituitary-restricted, and gonadotrope-specific transcriptional activators that act in combination and synergistically.

2. *GnRH-stimulated expression.* Although a number of factors that may be necessary for maintenance of basal levels of gonadotrope-specific gene expression have been identified in αT3-1 cells, the identification of mechanisms for GnRH-stimulated expression have been less forthcoming. Windle *et al.* (38) have demonstrated that αT3-1 cells respond to GnRH by elevating α-subunit gene expression. A similar increase of α-subunit mRNA levels was observed in response to PMA, and this increase was not additive with GnRH, suggesting that PKC may play a role in transducing the GnRH signal to the nucleus (39). The calcium ionophore, ionomycin, also stimulates α-subunit mRNA levels. In contrast, an inhibitor of cAMP-dependent protein kinase did not affect the ability of GnRH or PMA to stimulate expression of an α-subunit promoter/luciferase reporter gene (αLUC), indicating that cAMP-dependent protein kinase is not required for transcriptional activation by GnRH (104).

The increase in α-subunit mRNA levels in response to GnRH was maximal at 12–24 h and maintained for a further 24 h (Fig. 9) (108). The observed increase in mRNA levels appears to be mediated by both an increase in α-subunit gene transcription and mRNA stability. Nuclear run-off assays demonstrated an increase in α-subunit gene transcription of 2- to 3-fold within 1 h after exposure to GnRH but returned to baseline by 12 h. GnRH also stimulated the activity of αLUC, apparent after 1 h, maximal after 4–6 h, but back to baseline by 24 h of GnRH treatment (Fig. 9). Thus, GnRH appears to stimulate a burst of α-subunit gene transcription lasting less than 4–6 h. The persistent elevation of α-subunit mRNA levels for at least 48 h suggests that the mRNA has a long half-life and/or that GnRH stabilizes the mRNA in addition to its transcriptional effects. Indeed, pulse-chase experiments showed that the half-life of the α-subunit mRNA increased from 1.2 h in the absence of GnRH to 8 h in the presence of GnRH in αT3-1 cells. Whether this mechanism also occurs in primary gonadotropes is unclear, as the half-life of α-subunit mRNA in primary pituitary cultures is 6.5 h; however, in this case both gonadotropes and thyrotropes contribute to α-subunit mRNA levels (109). Interestingly, while the stimulatory effects of GnRH on α-subunit gene transcription and mRNA levels were evident very rapidly, within 1 h after exposure to GnRH, GnRH-induced α-subunit release was detected only after a lag of 4 h of incubation (110). Thus, there appears to be dissociation between the stimulation of gene expression and exocytosis.

Transient transfection studies in αT3-1 cells with mouse or human αLUC have been used to determine DNA sequences

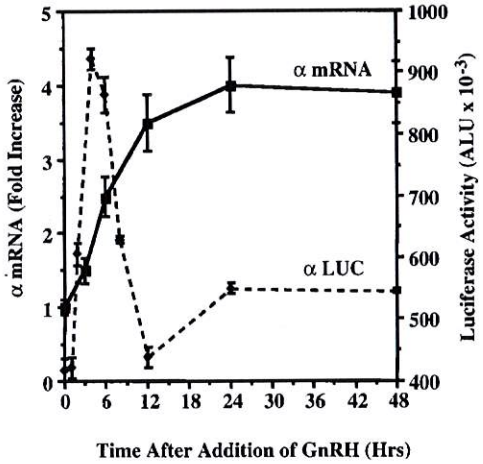

FIG. 9. Effect of GnRH on αLUC expression and α-subunit mRNA levels. αT3 cells stably transfected with αLUC were incubated in the absence or presence of GnRH (10^{-7} M) for the indicated periods of time. Cells were harvested and assayed for luciferase activity. Luciferase activity (αLUC) is expressed in arbitrary light units (ALU) and is the mean ± SEM of triplicate plates of cells. Basal expression of αLUC was 415,000 ALU. Background luciferase activity was below 120 ALU. Total RNA (5 μg) from triplicate plates of αT3 cells treated in the absence or presence of GnRH was analyzed by Northern blot for α-subunit and GAPDH mRNAs. mRNA levels were quantitated using scanning densitometry, and α-subunit mRNA levels were corrected for hybridization to GAPDH mRNA. The mean ± SEM of three separate experiments are expressed relative to the basal α-subunit mRNA level in the absence of GnRH. [Reprinted with permission from P. J. Chedrese *et al*: *Endocrinology* 134:2475–2481, 1994 (108) (Fig. 1). © The Endocrine Society.]

of the α-subunit gene that mediate transcriptional responses to GnRH. Deletion analyses indicated that deletion of sequences between −507 and −205 of the mouse α-subunit gene resulted in a decrease in responsiveness to GnRH, as well as to PMA and to cAMP (104). This region, when linked to a heterologous promoter, was capable of supporting responses to GnRH, PMA, and cAMP. Further mutational analysis revealed that mutations at positions −406/−399 and −337/−330 resulted in a decrease in the response to GnRH. Multimers of −416/−385, when linked to a minimal promoter upstream of the luciferase gene, responded to GnRH with a stimulation of luciferase activity (Fig. 10). In contrast, multimers of −344/−300 enhanced basal transcription but did not respond further to GnRH. These data suggest that GnRH responsiveness requires the cooperative interaction of two distinct sequences, an upstream GnRH-responsive element (GnRH-RE) at −416/−385, and a downstream element at −344/−300, corresponding to the location of the PGBE described above. The upstream GnRH-RE was also responsive to PMA, further supporting the role of the PKC pathway in mediating the effects of GnRH on expression of the α-subunit gene. The need for a complex response unit for the mediation of GnRH stimulation may provide a mechanism for the maintenance of appropriate, tissue-specific expression and regulation of the α-subunit gene. The involvement of a tissue-specific basal element may restrict α-subunit gene expression to the appropriate cell type, and the involvement of two elements in mediating GnRH responses may prevent the α-subunit gene from responding to activation of the PKC-signaling pathway in nongonadotrope cells and tissues.

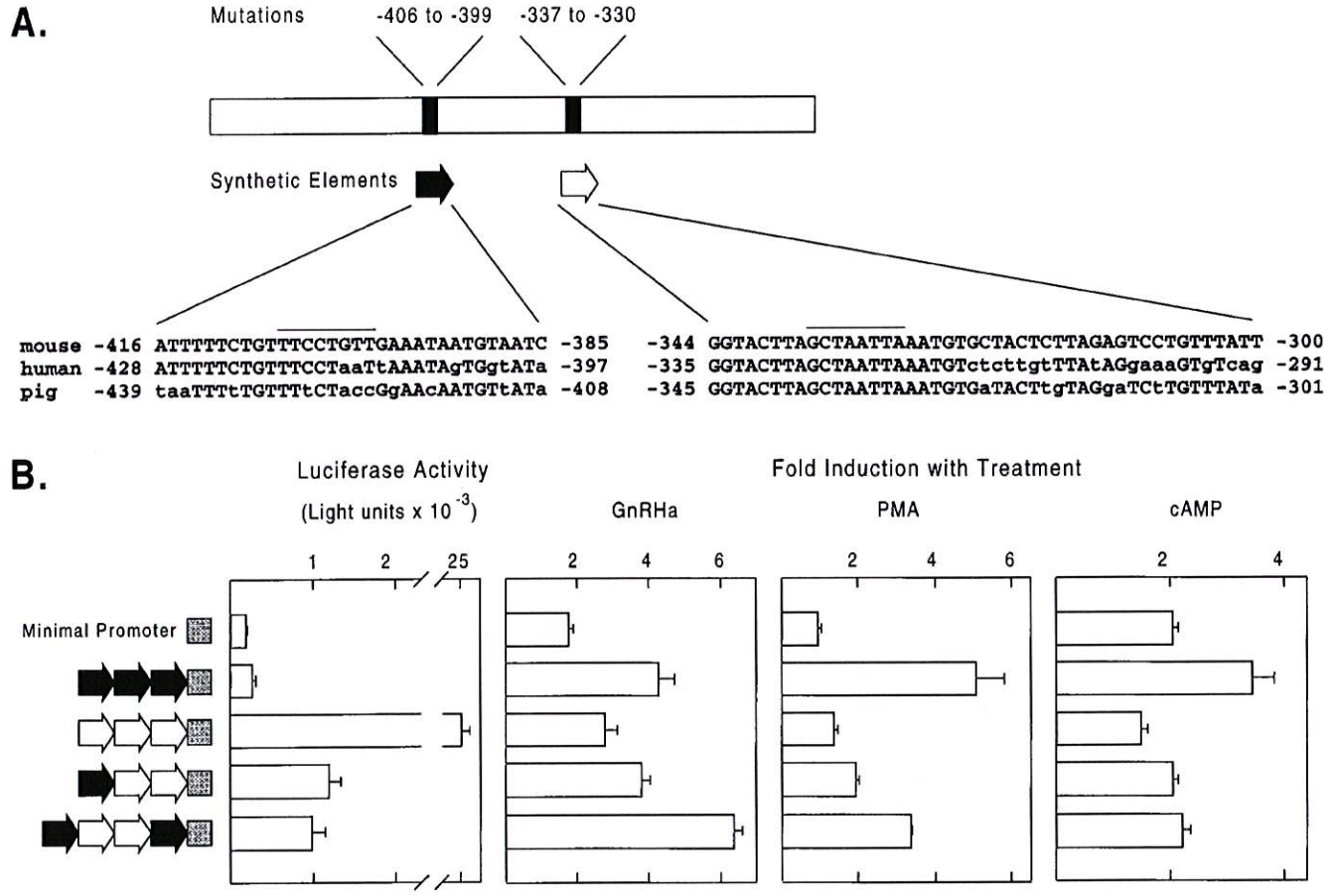

FIG. 10. Multimers of the −416 to −385 region function as a GnRH-responsive element. A, Synthetic DNA elements were prepared that included the sequences that were shown to be important by mutation analysis. The sequence of the mouse α-subunit gene, which was used as a synthetic DNA element, is aligned with the corresponding region of the human and pig α-subunit genes. Positions in which the human or pig sequence are identical to the mouse sequence are indicated by *uppercase letters*. The locations of the α-subunit sequences where mutations reduced GnRH and phorbol responses are indicated by *overbars*. B, To assess the functional properties of these elements, multimers of the synthetic DNA elements were placed upstream of a minimal promoter, which was linked to luciferase, and the reporter genes were transfected into αT3-1 cells. Cells were treated with vehicle alone, 10^{-5} M buserelin (GnRHa), 10^{-7} M phorbol myristic acid (PMA), or 0.5 mM 8-(4-chlorophenylthio)cAMP (cAMP) 18 h after transfection. Cells were collected 24 h after transfection (6 h after treatment), and luciferase activity was determined. All values are means ± SE from two to four separate experiments; each experiment included three transfections for each DNA construct. The luciferase data were normalized for transfection efficiency between experiments. Responses to different agents are indicated as the ratio of luciferase activity in the treated cells to that in vehicle-treated cells. A schematic representation of the organization of each of the constructs is shown at the *left*. The −416 to −385 element is indicated by a *black arrow*; the −344 to −300 element is indicated by a *white arrow*; the minimal promoter sequences are indicated by *gray shading*. [Reprinted with permission from W. E. Schoderbek *et al*: *J Biol Chem* 268:3903–3910, 1993 (104) (Fig. 7).].

As mentioned above, a member of the LIM-homeodomain family of transcription factors, LH-2, binds to a 14-bp imperfect palindrome within the PGBE domain *in vitro* (Fig. 8) (105). LIM-homeodomain proteins contain both a zinc finger (the LIM domain) and a homeodomain (111). The homeodomain of these factors is sufficient for specific DNA binding; the LIM domains appear not to be DNA-binding domains, but rather may function as protein-protein interaction domains to facilitate homo- or heterodimer formation. LH-2 has a restricted tissue distribution, being most abundant in αT3-1 and αTSH cells, cell lines of gonadotropic and thyrotropic origin, respectively, and in mouse brain; less abundant in whole rat pituitaries, corticotropic AtT20 cells, and somatolactotropic GH_3 cells; and undetectable in placental JEG-3 cells and in mouse liver. Cotransfection of LH-2 into COS cells showed that LH-2 is able to activate specifically the α-subunit promoter 2-fold and a 3XPGBE reporter construct 5- to 6-fold. These studies suggest that the LIM-homeodomain protein LH-2 is an activator of the glycoprotein hormone α-subunit gene in gonadotropes and thyrotropes. It is possible that another transcription factor, binding to the upstream GnRH-RE, may interact with LH-2 bound to the PGBE to mediate GnRH-induced expression of the α-subunit gene.

Another candidate factor for a role in mediating α-subunit gene expression by binding to the PGBE is mLim-3, a related member of the family of LIM-homeodomain proteins. mLim-3, also known as P-Lim or Lhx3, is a mouse gene expressed in the pituitary throughout development and in the adult, as well as transiently in the spinal cord, pons, and medulla oblongata, but with no detectable expression elsewhere. mLim-3 expression was detected in cell lines of pituitary origin, including cells representative of somatolactotropes (GH_3, GH_4C1, GC), thyrotropes (αTSH), gonadotropes (αT3), and corticotropes (AtT-20), but not in cell lines derived from peripheral, other endocrine, or neural tissues (112, 113). mLim-3 is able to bind to the PGBE sequence *in vitro* and is a strong activator of transcriptional activity of the α-subunit promoter, as well as the PRL, TSHβ, and Pit-1 promoters (112). Interestingly, it was recently reported that targeted disruption of the mLim-3 gene in mice leads to failure of growth and differentiation of the anterior and intermediate lobes of the pituitary (114). The development of all pituitary cell lineages, except the corticotropes, was affected. This suggests that mLim-3 plays an important role not only in α-subunit gene expression, but in differentiation and proliferation of nearly all the pituitary cell lineages.

Further studies of the putative GnRH-RE in the mouse α-subunit promoter have identified a core Ets factor (a family of transcription factors that have been implicated in mediating transcriptional responses to MAPK activation) binding site within the GnRH-RE, which appears to be important in mediating GnRH stimulation of α-subunit gene expression (Fig. 8) (92). Recent evidence that GnRH activates the MAPK signal transduction pathway, as discussed above, is relevant in terms of the mechanisms of transcriptional stimulation of the α-subunit gene by GnRH. Activation of the MAPK cascade by a constitutively active form of Raf kinase in αT3-1 cells leads to stimulation of the α-subunit promoter. Furthermore, inhibition of MAPK activity by kinase-defective ERK1 or ERK2, or overexpression of MAPK phosphatase 2, which dephosphorylates and inactivates MAPK, leads to the attenuation of GnRH-induced activation of the α-subunit promoter. The DNA-binding domain of Ets-2 was able to bind specifically to a site within the GnRH-RE, and a dominant negative Ets-2 expression vector reduced the ability of GnRH to stimulate expression of αLUC. These findings suggest that the Ets factor-binding site in the GnRH-RE may contribute to transcriptional stimulation of the α-subunit gene by GnRH, via activation of the MAPK pathway. In contrast, however, Sundaresan *et al.* (91) found that dominant negative mutant forms of Ras, ERK1, and ERK2 reduced basal expression of a human αLUC but had no effect on GnRH-stimulated expression. The reasons for the differences between these two studies are not clear, although Roberson *et al.* (92) used the mouse α-subunit promoter, whereas Sundaresan *et al.* used the human gene.

In addition to the studies characterizing GnRH-responsive DNA sequences in the mouse α-subunit gene using αT3-1 cells as described above, a GnRH-responsive region in the human gene was identified by transfection analyses in primary rat pituitary cell cultures (115). Deletion analyses suggested that one or more GnRH-responsive sequences reside between −346 and −244 in the human α-subunit promoter. This GnRH-responsive region does not include the GnRH-RE defined in the mouse α-subunit promoter. In contrast to the findings with the mouse α-subunit gene in αT3-1 cells, the regions of the human α-subunit gene that are important for the GnRH response appear to be distinct from those required for basal activity. Basal expression appeared to be primarily mediated through the proximal promoter and cAMP-responsive regions. These differences may reflect different mechanisms of GnRH stimulation of the human *vs.* the mouse α-subunit gene or differences in the mechanisms of regulation in αT3-1 cells *vs.* primary pituitary gonadotropes.

G. Desensitization

GnRH is secreted from the hypothalamus in a pulsatile fashion, and pulsatile GnRH stimulates LH and FSH biosynthesis and secretion (116). In contrast to the stimulatory effects of pulsatile GnRH, sustained exposure to high concentrations of GnRH reduces the response of gonadotropes to subsequent stimulation with GnRH (homologous desensitization), leading to suppression of gonadotropin secretion (117). This homologous desensitization to GnRH can occur rapidly, within the time frame of endogenous GnRH pulses (118). The mechanism of this desensitization is not known, and both receptor (119) and postreceptor (120, 121) mechanisms have been proposed. For a number of other G protein-coupled receptors, early desensitization events are thought to involve the uncoupling of the receptor from its regulatory G protein, with loss of downstream-signaling events (122). Rapid desensitization appears to involve phosphorylation by specific intracellular kinases of the third intracellular loop or the C-terminal tail (123, 124). However, the GnRHR lacks the C-terminal cytoplasmic tail as well as the third intracellular loop sequences implicated in the desensitization of other receptors (31).

αT3-1 cells have been used as a model for the study of mechanisms of desensitization to GnRH. Stimulation of αLUC activity in transfected αT3-1 cells was maximal 4–6 h after exposure to GnRH but thereafter declined, returning to levels in unstimulated control cells by 12–24 h. αLUC activity was also stimulated by a PKC activator, PMA, a calcium channel agonist, BAY K 8644, and an activator of the PKA pathway, 8-bromo-cAMP. Maximal responses to these agents also occurred after 4–6 h of exposure, although the maximal levels of activity were less than those observed in response to GnRH. A decline in αLUC activity over time with continuous exposure to these agents was particularly marked for PMA, but was also seen with BAY K 8644, whereas stimulation by 8-bromo-cAMP was maintained for at least 24 h. Pretreatment of αT3-1 cells with GnRH blocked subsequent stimulation of αLUC activity by either GnRH or PMA. In contrast, both 8-bromo-cAMP and BAY K 8644 were still able to stimulate αLUC activity after pretreatment with GnRH. These data suggest that the transcriptional stimulation of the α-subunit gene by GnRH is mediated by the PKC pathway, and that this pathway can be desensitized in αT3-1 cells by continuous exposure to GnRH. The kinetics of desensitization are difficult to infer from these studies; exposure to GnRH may incite a short burst of transcriptional activity of the α-subunit promoter, which then leads to a more gradual accumulation of the luciferase product. However, the addition of the GnRH antagonist, Antide, after treatment of the cells with GnRH resulted in a reduction of luciferase activity compared with exposure to GnRH alone, even when Antide was added up to 6 h after GnRH, indicating that some stimulation of the α-subunit promoter by GnRH was still occurring, *i.e.* the cells were not fully desensitized to GnRH. Continuous exposure of primary pituitary cells to GnRH causes rapid desensitization at the secretory level for free α-subunit as well as intact LH and FSH, evident within 15 min (125). The differences in kinetics for transcriptional and secretory desensitization may reflect different cellular mechanisms or differences between the αT3-1 cell line and primary gonadotropes.

Regulation of α-subunit gene transcription is a relatively downstream endpoint for the study of homologous GnRH desensitization. Measurements of second messengers may lead to insights into early or short-term desensitization events. GnRH treatment led to a linear increase in total IP production in αT3-1 cells over 0–15 min (126–128). Furthermore, GnRH pretreatment for 5 min did not alter subsequent stimulation of IP_3 production by GnRH 15 min later. These data indicate a lack of desensitization of the rapid GnRH-induced IP_3 response in αT3-1 cells. Pretreatment with GnRH for 1 h did reduce subsequent cellular IP accumulation in response to GnRH, but this may be attributable to a reduction in GnRHR numbers. GnRH pretreatment of αT3-1 cells for short times (5–15 min) had no effect on GnRHR number; however, treatment for 1 h with 10^{-7} M GnRH reduced GnRHR number by 48%. The affinity for GnRH was not altered. Desensitization of both the extracellular Ca^{2+}-dependent and -independent phases of the Ca^{2+} response to GnRH were observed after pretreatment with 10^{-7} M GnRH for 1 h (128). Thus, one mechanism of intermediate desensitization to GnRH may be receptor loss.

However, this does not account for rapid or early desensitization or the degree of desensitization of the Ca^{2+} response. An additional uncoupling event may occur during the pretreatment, which reduces the ability of the agonist-occupied GnRHR to elevate intracellular Ca^{2+}. Treatment of αT3-1 cells with 5-min pulses of GnRH every 15 min resulted in desensitization of the Ca^{2+} response after the first pulse in a dose-dependent manner, being evident at GnRH concentrations greater than 2×10^{-9} M (126). The mechanisms underlying this desensitization are not known but could include loss of IP_3 receptors, depletion of intracellular Ca^{2+} stores, and inactivation of Ca^{2+} channels, as has been suggested in studies of primary pituitary cells (120). The dissociation of IP production and calcium stimulation suggests that desensitization of GnRH-induced calcium mobilization is a postreceptor phenomenon occurring distal to PLC activation. The lack of the C-terminal cytoplasmic tail, implicated in the desensitization of other G protein-coupled receptors, in the GnRHR therefore appears to correlate with a lack of receptor desensitization; rather, desensitization to GnRH appears to be primarily a postreceptor phenomenon. Alternatively, αT3-1 cells may be lacking a factor(s) necessary for mediating rapid receptor desensitization in primary gonadotropes.

H. Summary of GnRH action in αT3-1 cells

The development of the αT3-1 gonadotropic cell line has enabled significant advances in our understanding of gonadotrope function and gonadotropin regulation, particularly in the areas of α-subunit gene expression and GnRHR structure and function. αT3-1 cells were critical for the initial cloning of GnRHR cDNAs as well as for elucidation of the GnRHR gene structure, confirming previous findings in primary pituitary cells which suggested that the GnRHR was a member of the G protein-coupled receptor family. The absence of a carboxy-terminal intracellular tail on the receptor was a surprising finding, which makes questions about the mechanisms of gonadotrope desensitization to GnRH all the more intriguing.

αT3-1 cells have been used to elucidate a number of components of the GnRH signal transduction pathway (Fig. 11). The GnRHR in αT3-1 cells is coupled to G proteins of the G_q/G_{11} family, leading to production of IPs and increases in intracellular calcium levels, which, in turn, leads to activation of PKC. While cAMP has, in some studies, been suggested to be activated by GnRH, and has been shown to lead to increases in expression of the α-subunit gene, there is no evidence for increases in cAMP levels in response to GnRH in αT3-1 cells. Furthermore, there is now evidence that the MAPK pathway is activated by GnRH in αT3-1 cells and may be important in the stimulation of α-subunit gene expression by GnRH.

While αT3-1 cells have proven to be invaluable for the study of GnRH action, there are some differences between αT3-1 cells and primary pituitary gonadotropes. The regulation of the GnRHR in αT3-1 cells is different from primary gonadotropes; in particular, the receptor does not appear to be markedly regulated by GnRH itself in αT3-1 cells, especially at the level of gene expression, whereas it is markedly

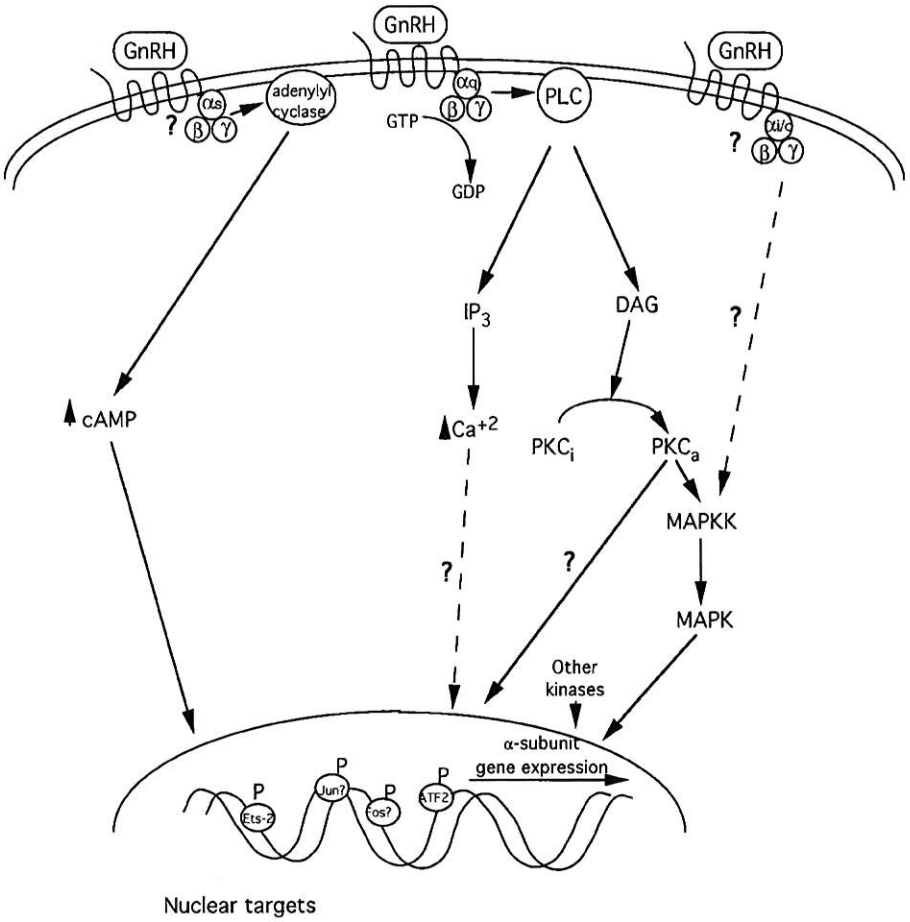

FIG. 11. Summary of known GnRH actions on α-subunit gene expression in αT3-1 cells. GnRH binds to the seven-transmembrane domain GnRHR, which is coupled to G_q/G_{11}. Activation of G_q/G_{11} activates phospholipase C, which stimulates the production of inositol triphosphate and an increase in $[Ca^{2+}]_i$, leading to activation of PKC. PKC, in turn, leads to stimulation of α-subunit gene expression, either directly, or indirectly by activating the MAPK cascade. GnRHR may also be coupled to G_s, leading to activation of adenylyl cyclase and stimulation of cAMP production, which may also influence α-subunit gene expression. Third, activation of the GnRHR may also activate the MAPK cascade via G_i.

regulated in primary pituitary cells. In addition, detailed studies of intracellular calcium profiles in response to GnRH reveal that amplitude-modulated intracellular calcium responses occur in αT3-1 cells, in contrast to primary gonadotropes, in which GnRH induces calcium oscillations and frequency-modulated calcium signaling. A major difference between αT3-1 cells and primary gonadotropes, however, is the lack of expression of the gonadotropin β-subunit genes by αT3-1 cells.

IV. Studies of GnRH Action in GH_3 Cells Transfected with the GnRH Receptor (GGH_3 Cells)

A. *Derivation of GH_3 cells*

The GH_3 cell is a well characterized pituitary cell strain established from a GH-producing rat pituitary tumor, MtT/W5, that was propagated as a transplantable rat pituitary tumor. By a method of alternate culture and animal passage, several clonal strains of epithelial cells were established (129, 130).

B. *Characterization of GH_3 cells*

These cells are somatolactotropic in origin. They secrete large amounts of GH into culture medium and stimulate body weight gain and growth after injection into normal or hypophysectomized rats (129, 130). They express PRL and GH genes and also secrete PRL and GH in a regulated fashion. GH_3 cells express TRH receptors (TRHR) and respond to TRH with an increase in PRL biosynthesis and secretion, and a reduction in GH production (131, 132). GH_3 cells do not express α-subunit, TSHβ, LHβ, FSHβ, and POMC genes, hormones expressed in other, nonsomatolactotropic anterior pituitary cell types. However, they are capable of supporting the expression of exogenous α- and TSHβ-subunit genes, introduced into the cells by transient transfection (133–138). In addition to TRHR, GH_3 cells also express somatostatin, galanin, and pituitary adenylate cyclase-activating polypeptide receptors (139–141), as well as activin types I and II receptors (142). However, GH_3 cells appear to lack functional dopamine receptors (143).

C. Derivation of GH_3 cells transfected with the GnRHR (GGH_3 cells)

GH_3 cells stably expressing the rat GnRHR (GGH_3 cells) were developed by cotransfecting GH_3 cells with a rat GnRHR expression vector (pcDNA1-GnRHR) and a pSVneo plasmid, which expresses the neomycin resistance gene, by electroporation (144). Control cells were transfected with pSVneo and pcDNA1 vector. The cells were grown in the presence of neomycin, to select for neomycin-resistant cell clones, and monoclonal cells were then expanded and tested for binding of GnRH and GnRH responsiveness. The general morphology of the stably transfected GGH_3 cell lines is indistinguishable from that of the parent GH_3 cells and of control, vector-transfected cells (145).

D. GnRH binding

Specific, high-affinity binding sites for GnRH and GnRH analogs are present on GGH_3 cells but not on the parental GH_3 cell line (127, 144, 146). Scatchard analysis of the binding of [^{125}I]Buserelin, a metabolically stable analog of GnRH, shows a K_d of $4.1 \pm 1.0 \times 10^{-8}$ M using GGH_3-1′ cells, one of the clonal strains of GH_3 cells that have been stably transfected with the rat GnRHR cDNA. Each GGH_3-1′ cell has 11,000 ± 2,800 receptors. Both GnRH agonists and antagonists are able to displace binding of [^{125}I]Buserelin to GGH_3-1′ cells (Table 2), whereas chemically unrelated peptides such as PRL, GH, and TRH do not significantly displace binding, even at high concentrations. The relative affinities of the GnRH agonists and antagonists are similar to those observed in cell cultures derived from rat pituitaries, suggesting that this receptor is similar to those expressed in primary gonadotropes, although the absolute affinities are slightly lower. Another clonal strain of stably transfected GH_3 cells, GGH_3-2 cells, had 13,000 ± 1,000 binding sites for [^{125}I]Buserelin per cell. In comparison, this cell line had 64,800 ± 3,700 specific [^{3}H]MeTRH binding sites per cell, representing binding to the TRHR, which is expressed endogenously in these cells.

The GnRHR contains Asn^{87} and Asp^{318} rather than the highly conserved Asp^{87} and Asn^{318} found in other G protein-coupled receptors. Site-directed mutagenesis was used to introduce a Asn^{318} mutation and $Asp^{87}Asn^{318}$ double mutation into the mouse GnRHR, and the mutant receptors were stably expressed in GH_3 cells. Both mutant receptors were able to bind [^{125}I]Buserelin, but IP production was attenuated (147). Furthermore, while cell surface levels of wild-type GnRHR are down-regulated upon exposure to GnRH (see below), this down-regulation was not observed for either mutant receptor. These data suggest that these mutations impair the coupling between the GnRHR and Gq, the G protein believed to be involved in IP production. Similar studies have been done using COS cells. In these studies, mutation of Asn^{87} to Asp resulted in loss of ligand binding, whereas mutation of Asp^{318} to Asn or the $Asp^{87}Asn^{318}$ double mutant were able to bind GnRH, but IP production was attenuated, similar to the results in GH_3 cells (148). However, in a similar study, also in COS cells, the $Asp^{87}Asn^{318}$ double mutant was unable to bind ligand, similar to the Asp^{87} mutant (149). The reason for the differences between these studies is not clear.

TABLE 2. Displacement of [^{125}I]buserelin binding to GGH_3 cells by GnRH analogs and other peptide and protein hormones

Agent	IC_{50} (nM) ± SEM
GnRH	18.2 ± 0.88
Lupron	0.8 ± 0.02
Lystide	0.2 ± 0.01
PRL	>1000
GH	>1000
TRH	>1000

Lupron, D-Leu^6-des-Gly^{10}-Pro^9-ethylamide-GnRH; Lystide, [Ac-D-Qal^1-D-Cpa^2-D3Pal^3-Ser^4-NicLys^6-Leu^7-ILys^8-Pro^9-D-Ala^{10}]NH_2.
[Adapted with permission from D. Kuphal *et al.*: *Endocrinology* 135: 315–320, 1994 (146) (Table 1) © The Endocrine Society.]

E. GnRHR regulation

The expression of the GnRHR in GGH_3 cells is driven by a cytomegalovirus promoter, which is not regulated by GnRH (144). The concentration of GnRHR is therefore unaffected at the transcriptional level by hormonal manipulation. This cell model may be useful, therefore, for distinguishing transcriptional from posttranscriptional regulation of cell surface GnRHR concentrations. Many G protein-coupled receptors are down-regulated by their ligands (123). Receptors are sequestered from the plasma membrane and internalized, followed by proteolytic degradation. This leads to a reduction in receptor number (homologous down-regulation) over a period of hours. In addition, the receptor may be down-regulated at the level of gene expression as well, leading to a reduction in receptor number over a period of several hours to days. Because GnRH will not affect the transcriptional rate of the GnRHR gene in GGH_3 cells, changes in cell surface GnRHR numbers would be expected to reflect translational or posttranslational regulation of the receptor by GnRH.

Indeed, the GnRHR in GGH_3 cells does undergo homologous down-regulation followed by recovery after continuous exposure to 10 nM GnRH, as determined by GnRH-binding studies (145). Down-regulation of the GnRHR was evident by 1 h of GnRH treatment, reached a nadir of 50–80% by 2–5 h, and returned to baseline levels by 7 h. This biphasic regulation of GnRHR is similar in time course and extent to that reported in primary pituitary cells (119). The ability of the receptor to be down-regulated in GGH_3 cells suggests that down-regulation does not require cell-specific components other than the receptor itself, does not involve transcriptional down-regulation (which may occur in primary gonadotropes, but over a longer time course), and does not require an intracellular C-terminal region. The mechanisms of this down-regulation of the GnRHR in GGH_3 cells remain to be elucidated. It will be interesting to determine whether the GnRHR is internalized and degraded or recycled after exposure to its ligand, as is the case for other receptors of this family, such as the TRHR (150, 151) and the β-adrenergic receptor (152).

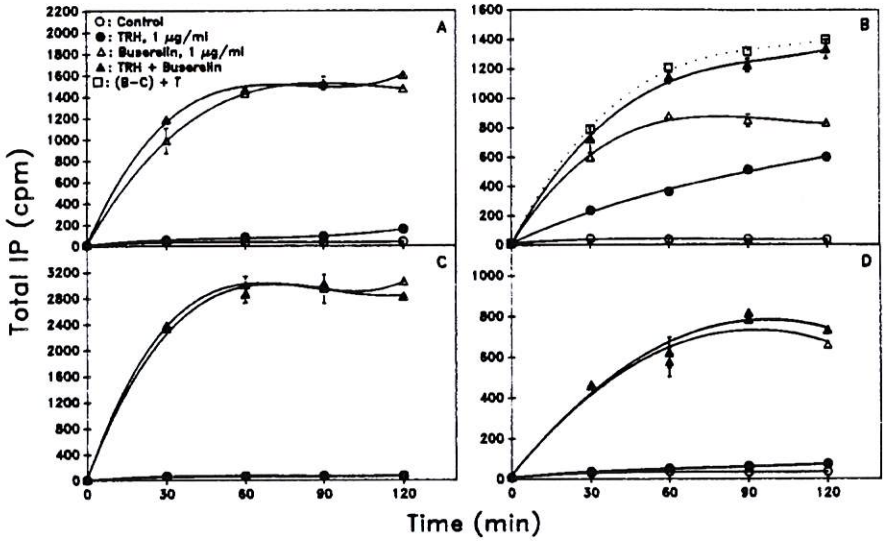

FIG. 12. Time course of IP production in untreated cells (○) or cells treated with 1 μg/ml TRH (●), 1 μg/ml of the GnRH agonist Buserelin (△), or both peptides (▲). GGH_3-1′ (A), GGH_3-2 (B), GGH_3-6 (C), or GGH_3-12 (D) cells (GGH_3 clonal cell lines) were plated and preincubated in [^{3}H]inositol. At the indicated times after the addition of peptides, total IPs were determined by ion exchange chromatography. (B − C) + T is the arithmetic result of the response measured in the presence of Buserelin (1 μg/ml) alone (B) less that in unstimulated cells (C) plus the response in the presence of TRH (1 μg/ml) alone (T). [Reprinted with permission from J. A. Janovick and P. M. Conn: *Endocrinology* 135:2214–2219, 1994 (162) (Fig. 1). © The Endocrine Society.]

F. Intracellular second messengers

1. G protein coupling. Direct studies of G protein coupling of the GnRHR in GGH_3 cells, as were done in αT3-1 cells, have not been reported. However, the TRHR, which is expressed endogenously by GH_3 cells, has been shown to be directly coupled to G proteins of the $G_{q/11}$ family (60, 153).

The βγ-subunits of a G protein can mediate signals as well as the α-subunit (154, 155). This confers to any G protein the potential for dual signaling. This has been best described for the G_i protein, *i.e.* the α-subunit inhibits adenylyl cyclase activity, whereas at higher concentrations the βγ-subunits can stimulate the activity of some adenylyl cyclase subtypes (156, 157). Similarly, G protein βγ-subunits have been shown to be capable of stimulating the β2-isoform of PLC (158). Recent data suggest that the βγ-complex of G proteins is involved in mediating GnRH effects. A 125-amino acid pleckstrin homology domain within the carboxyl terminus of β-adrenergic receptor kinase (βARK1-495/689) binds to $G_{\beta\gamma}$ (159). When transiently transfected and expressed in cell culture, this polypeptide has been shown to inhibit βγ-mediated signal transduction (160). Expression of this βARK1 fragment in GGH_3 cells inhibits basal and GnRH-stimulated IP production, cAMP release, and PRL release (161). The numbers and GnRH binding affinity of the GnRHR were unaffected by the expression of βARK1-495/689, indicating that the changes in signal transduction and PRL release are not due to a change in receptor expression or affinity. These data suggest that the βγ-complex of G proteins may play a role in mediating GnRH-stimulated signal transduction in GGH_3 cells.

2. Inositol phosphates. IP production was stimulated by a GnRH agonist (Buserelin) in GGH_3 clonal cell lines in a dose-dependent manner (145, 162). The EC_{50} was approximately 10^{-10} M GnRH. Production of IPs is an early response of GGH_3 cells to Buserelin, measurable at 15–30 min, maximal at 60 min, and maintained for at least 120 min after treatment (Fig. 12). GGH_3 cells also respond to TRH with an increase in IP production. Interestingly, the production of IPs by a GnRH agonist and by TRH were additive when maximal concentrations of both peptides were present. This suggests that GnRH and TRH may provoke IP production in GGH_3 cells by different means. This conclusion is supported by the finding that cholera toxin and pertussis toxin inhibit IP production in response to TRH, whereas they augment Buserelin-stimulated IP production. Alternatively, receptor number may be limiting the maximal IP production in response to either ligand. As is the case for αT3-1 cells, studies in GGH_3 cells have observed the responses to a single pulse of GnRH or to continuous GnRH; the responses to pulsatile administration of GnRH have not yet been described.

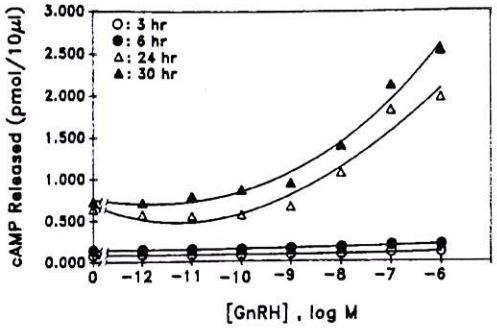

FIG. 13. cAMP production by GGH_3-1′ cells in response to GnRH. cAMP release from GGH_3-1′ cells was determined in response to GnRH for 3, 6, 24, or 30 h, as measured by RIA. [Reprinted with permission from D. Kuphal *et al*: *Endocrinology* 135:315–320, 1994 (146) (Fig. 7A). © The Endocrine Society.].

3. cAMP. Buserelin induced the release of cAMP in GGH_3 cells in a dose- and time-dependent manner (145, 146). Stimulation of cAMP production was not evident until 24 h after exposure to Buserelin (Fig. 13). The EC_{50} was approximately 10^{-8} M, about 100-fold higher than for IP generation. The stimulation of cAMP production by GnRH suggests that the GnRHR may be able to couple to G_s as well as G_q and is analogous to reports that GnRH can increase cAMP production in primary pituitary cells (76, 77).

Increased production of cAMP appears to mediate the desensitization of the PRL-secretory response to GnRH in GGH_3 cells. However, cAMP production in response to GnRH in GGH_3 cells persists even after the onset of desensitization. These data are consistent with a mechanism of desensitization that occurs distal to the production of cAMP (163).

G. Regulation of secretion

1. PRL. The TRHR and GnRHR are both members of the family of G protein-coupled receptors, and both are coupled to G proteins of the $G_{q/11}$ family (60, 153). The cellular responses to TRHR or GnRHR activation include enhanced phosphoinositide turnover, calcium mobilization, and PKC activation (24, 164, 165). Thus, the effects of TRH and GnRH appear to be mediated through the same or similar intracellular signal transduction pathways. Therefore, one might expect that GnRH would be able to stimulate PRL release in GGH_3 cells. Indeed, a GnRH agonist is able to stimulate PRL release from GGH_3 cells, whereas it does not in the parental GH_3 cells or in control cells, confirming that this response is mediated by the GnRHR, rather than by cross-activation of the TRHR by GnRH (145, 146, 162). The release of PRL in response to exposure of GGH_3 cells to a GnRH agonist was nearly linear to 96 h and was dose-dependent with an EC_{50} of approximately 10^{-8} M (Fig. 14). Cycloheximide inhibited the release of PRL in response to GnRH, indicating that this response is dependent on protein synthesis. This inhibition, and the slow time course for PRL release after GnRH stimulation, suggest that GnRH-stimulated PRL release is regulated at the level of PRL synthesis, rather than by release of stored hormone. The secretion of PRL in response to GnRH in these cells indicates that all of the components needed for coupling of the GnRHR to the activation of secretory events

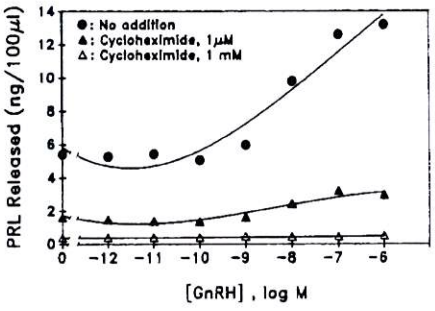

FIG. 14. Effect of the protein synthesis inhibitor cycloheximide on GnRH-stimulated PRL release from GGH_3 cells. Cells were treated with GnRH for 24 h in the presence or absence of cycloheximide. [Reprinted with permission from D. Kuphal *et al*: *Endocrinology* 135: 315–320, 1994 (146) (Fig. 8A). © The Endocrine Society.]

are present in the GGH_3 cell and suggests that no gonadotrope-specific components other than the secretory proteins themselves are necessary.

GnRH-stimulated PRL release in GGH_3 cells is potentiated by a phosphodiesterase inhibitor. This, coupled with the stimulation of cAMP production by GnRH and the stimulation of PRL release by 8-bromo-cAMP, suggests that cAMP may be a second messenger in GnRH-stimulated PRL release (146). Furthermore, GnRH-stimulated PRL release was sensitive to calcium channel inhibitors, suggesting a role for calcium as a second messenger as well (145).

The regulation of PRL by GnRH in GGH_3 cells could be interpreted to suggest that, at least in some instances, the hormonal regulation of gene expression in given pituitary cell subtypes is determined by the presence or absence of receptors for a particular hormone in that cell type, rather than or in addition to other tissue-specific intracellular factors or second messengers. A similar situation has, in fact, been observed *in vivo*. While LH and FSH are not normally regulated by TRH, in the case of gonadotrope adenomas secreting LH or FSH, their secretion is often stimulated by TRH (166, 167). Presumably, these adenomas express the TRHR, enabling a response to TRH, while normal gonadotropes do not.

2. LH and FSH (in GH_3 cells transfected with the α-, LHβ-, and FSHβ-subunit genes). GH_3 cells are able to secrete proteins through both constitutive and regulated pathways (168). To compare the mechanisms of storage and release of LH and FSH, expression vectors carrying the human LHβ or FSHβ gene in combination with the common gonadotropin α-subunit gene were transfected into GH_3 cells (169, 170). After transit and processing in the Golgi, the majority (>85%) of LH was retained intracellularly in Golgi/post-Golgi compartments, and decreased gradually with a $t_{1/2}$ of about 13 h. In contrast, FSH did not accumulate in GH_3 cells to the same extent as LH and was secreted with a $t_{1/2}$ of about 6 h. KCl or forskolin was able to stimulate LH secretion by 4- to 5-fold compared with unstimulated (basal) release, supporting the observation that LH enters a regulated secretory pathway in GH_3 cells and is stored in a secretagogue-releasable pool. FSH secretion was also stimulated by KCl or forskolin, but to a lesser extent (2.5-fold), possibly reflecting the smaller size of the stored, secretagogue-releasable pool. These studies indicate that in GH_3 cells, LH is secreted primarily through a regulated pathway, whereas the majority of FSH is released constitutively. These findings are consistent with observations in primary gonadotropes that FSH secretion is more tightly coupled to FSH biosynthesis, and that the magnitude of FSH secretion in response to secretagogues is smaller than that of LH (171–173). The effects of GnRH on LH and FSH secretion in this system, using cells in which the GnRHR is coexpressed, have not yet been studied.

3. Secretogranin-II. Secretogranin-II, an acidic glycoprotein that is a marker for the regulated pathway of secretion and a component of secretory granules, is synthesized in GGH_3 cells (174). It is released in a time- and dose-dependent manner in response to GnRH agonists, as well as to a cAMP analog. Release is inhibited by cycloheximide, an inhibitor of

protein synthesis, and by actinomycin-D, an RNA synthesis inhibitor. Interestingly, while secretogranin-II is generally thought to be a marker for the regulated pathway of secretion, it appears to be secreted in a constitutive manner in GGH_3 cells.

H. Regulation of PRL mRNA

In addition to stimulating PRL secretion, TRH also stimulates PRL gene transcription and mRNA levels, leading to an increase in PRL biosynthesis (175, 176). Analogously, PRL mRNA levels are stimulated in GGH_3 cells by GnRH (144). Treatment of GGH_3 cells with 100 nM of a GnRH agonist for 24 h resulted in the stimulation of PRL mRNA levels by 3- to 4-fold, whereas PRL mRNA levels were unchanged in response to the GnRH agonist in the parental GH_3 cells or in control cells. In the same cell line, TRH stimulated PRL mRNA levels by approximately 3-fold.

I. Regulation of expression of transiently expressed reporter genes

1. PRL vs. α-subunit gene. TRH stimulates PRL mRNA levels in GH_3 cells by increasing gene transcription, as determined by nuclear run-off assays (176). Time course experiments indicate that transcriptional activation is maximal 2 h after treatment with TRH. We therefore hypothesized that TRH and GnRH would stimulate the activity of a fusion gene comprised of the PRL promoter fused to a luciferase reporter gene (PRLLUC) in GGH_3 cells. Indeed, when PRLLUC was transiently transfected into GGH_3 cells, luciferase activity was stimulated by treatment with either TRH or a GnRH agonist in a dose- and time-dependent manner (144). In the parental GH_3 cells and in the control cell line, $pcGH_3$-1 (stably transfected with the pcDNA1 vector), luciferase activity was stimulated by TRH, but not by the GnRH agonist (Fig. 15). Time course studies using maximal doses of TRH and a GnRH agonist showed an increase in luciferase activity in response to both TRH and GnRH agonist by 2 h; maximal stimulation occurred at 4–6 h in both cases. The difference in the time courses for TRH response in nuclear run-off experiments and luciferase activities likely reflects the time needed for translation and accumulation of the luciferase enzyme in the cells after transcriptional activation. When a human αLUC was transiently transfected into GGH_3 cells, luciferase activity was also stimulated by treatment with either TRH or a GnRH agonist, whereas $pcGH_3$-1 cells and GH_3 cells responded only to TRH (144). Dose-response and time course studies for αLUC were similar to those for PRLLUC.

The stimulatory effects of TRH and the GnRH agonist, each at maximally effective doses, were additive on PRL mRNA levels and PRLLUC activity. In contrast, stimulation of αLUC by both TRH and GnRH agonist was no greater than with either one alone (144). If TRH and GnRH share the identical signal transduction pathway for the stimulation of gene expression, it would be expected that the induction of gene expression by maximally effective concentrations of one hormone would prevent any further stimulation by the other, as is the case for the α-subunit gene. Therefore, the

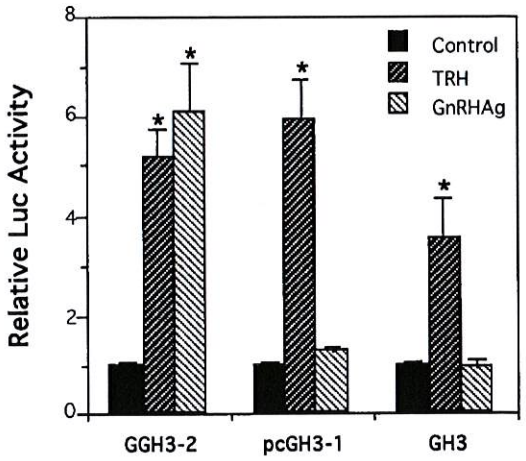

FIG. 15. Stimulation of luciferase activity by TRH and GnRHAg in GGH_3-2 cells, $pcGH_3$-1 cells, and GH_3 cells transiently transfected with PRLLuc. All cells were harvested 48 h after transfection. Cells were treated with 1 μM TRH or 100 nM GnRH agonist for the final 6 h before harvesting. Levels of luciferase activity are internally standardized according to levels of activity of RSV-β-galactosidase. Each *bar* represents the mean ± SEM for four samples. *, $P < 0.01$, significantly different from controls. [Reprinted with permission from U. B. Kaiser *et al*: *Mol Endocrinol* 8:1038–1048, 1994 (144) (Fig. 3A). © The Endocrine Society.]

signal transduction pathways used by TRH and GnRH in the regulation of PRL gene expression in GGH_3 cells may have some differences, particularly in the response-limiting step(s). These results also imply that the regulation of α-subunit and PRL gene expression by TRH and GnRH may not occur by identical pathways. The additivity of TRH and GnRH effects on PRL gene expression could also be accounted for if receptor number was the limiting factor in the pathway for either ligand. However, if this were the case, one might expect TRH and GnRH to be additive on α-subunit gene expression as well.

In time course studies, luciferase activity declined by 18–24 h of exposure to TRH or GnRH, similar to studies of αLUC in αT3-1 cells, suggesting that the signal transduction pathways became desensitized. To study this further, GGH_3 cells were pretreated with either TRH or a GnRH agonist, and then the responses of PRLLUC and αLUC to TRH and the GnRH agonist were tested. These studies show that homologous desensitization occurs, *i.e.* pretreatment with the GnRH agonist blocks subsequent stimulation of PRLLUC and αLUC activity by the agonist, and pretreatment with TRH blocks subsequent stimulation of PRLLUC and αLUC activity by TRH. In the case of the α-subunit promoter, heterologous desensitization also occurs. Stimulation of αLUC activity by TRH was blunted by pretreatment with a GnRH agonist, and conversely, there was loss of responsiveness to GnRH agonist after pretreatment with TRH. In contrast, heterologous desensitization of the PRL promoter did not occur (144). These data provide additional support that TRH and GnRH stimulate the α-subunit promoter by the same mechanism, whereas the mechanisms of transcriptional regulation of PRL by TRH and GnRH are not identical. It would be interesting to know whether similar effects would be ob-

served in αT3-1 cells transfected with the TRHR; however, these studies have not been performed.

2. α-, LHβ-, and FSHβ-subunit genes. The gonadotropin subunit genes, α, LHβ, and FSHβ, are not expressed endogenously in the somatolactotropic GH_3 cell line. As previously reported and discussed above, GH_3 cells are capable of supporting expression of transiently transfected luciferase reporter constructs driven by the rat, mouse, or human α-subunit promoter (133, 138). Furthermore, in GGH_3 cells, the α-subunit is stimulated in response to GnRH. Similarly, studies have shown that the TSHβ-subunit gene promoter, normally active in thyrotropes, can be expressed in GH_3 cells (134, 135). The rat LHβ gene promoter, fused to a chloramphenicol acetyltransferase (CAT) gene, was expressed at low levels when transiently transfected into GH_3 cells (177). Transcription was initiated correctly at the same LHβ transcriptional start site used in primary pituitary gonadotropes, as determined by RNase analysis. CAT activity was consistently induced by forskolin and by cAMP analogs, suggesting the presence of a cAMP-responsive *cis*-acting domain in the LHβ 5′-flanking region.

GH_3 cells transiently transfected with reporter genes in which regulatory regions of the human α-subunit, rat LHβ, and rat FSHβ genes are fused to the luciferase gene (αLUC, LHβLUC, and FSHβLUC, respectively) yield expression levels 250-fold higher than those of the promoterless luciferase vector for αLUC, 5-fold higher for LHβLUC, and 12-fold higher for FSHβLUC (178). Cotransfection of the GnRHR cDNA results in the additional specific stimulation of luciferase activity by a GnRH agonist, 10-fold for αLUC, 8-fold for LHβLUC, and 4-fold for FSHβLUC. TRH is also able to stimulate the expression of the gonadotropin subunit gene reporter constructs in these transfected cells, although to a lesser degree than the GnRH agonist (Fig. 16).

The magnitude of stimulation of gonadotropin subunit promoter activity in response to GnRH in these cells is influenced by the concentration of GnRHR expressed. When GH_3 cells are transiently transfected with progressively increasing amounts of GnRHR cDNA, the average number of GnRHRs expressed on the cell surface, as determined by a GnRH binding assay, also increases. When these cells are cotransfected with αLUC or LHβLUC and progressively increasing amounts of GnRHR cDNA, the degree of stimulation of luciferase activity by GnRH is increased in proportion to the numbers of GnRHR. In contrast, FSHβLUC activity is optimally stimulated when relatively low numbers of GnRHR are expressed; at higher concentrations of cell surface GnRHR, the magnitude of stimulation of FSHβLUC by GnRH is decreased (178).

Observations *in vivo* indicate that cell surface GnRHR numbers are regulated by varying GnRH pulse frequencies (47, 49, 179). The highest concentrations of cell surface GnRHR, as reflected by GnRH-binding activity, occur at a GnRH pulse frequency of every 30 min, which has also been shown to stimulate preferentially LH biosynthesis and secretion *in vivo* (180–184). Lower cell surface GnRHR numbers occur at a GnRH pulse frequency of every 2 h, which, in turn, has been shown to stimulate preferentially FSH biosynthesis and secretion. GnRHR concentration in primary pituitary cells is approximately 2- to 3-fold higher when the GnRH pulse frequency is every 30 min, compared with that at a frequency of every 2 h (179), a magnitude of change in GnRHR concentration similar to that in the GGH_3 cells which resulted in the maximal differences in LHβ and FSHβ gene expression. These observations support the hypothesis that varying GnRH pulse frequencies regulate differentially LH and FSH biosynthesis and secretion *in vivo* by regulating pituitary GnRHR numbers (Fig. 17). These data suggest that

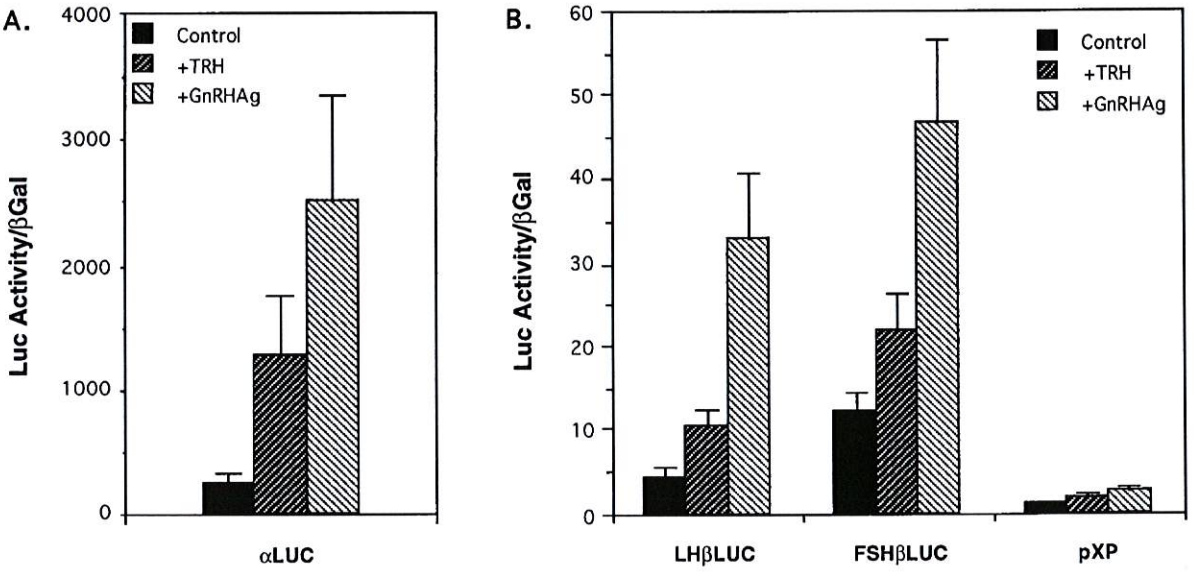

FIG. 16. Basal, TRH-, and GnRH-stimulated expression of (A) αLUC and (B) LHβLUC, FSHβLUC, and pXP2 in GH_3 cells. Cells were cotransfected with 20 μg αLUC, LHβLUC, FSHβLUC, or pXP2, 20 μg pcDNA1-GnRHR, and 9 μg RSV-βGAL. Cells were treated with 100 nM GnRHAg (GnRH agonist), 1 μM TRH, or control for 6 h before harvesting. All experiments were repeated at least three times. Each *bar* represents the mean ± SEM for nine samples, from three independent experiments. [Reprinted with permission from U. B. Kaiser *et al*: *Proc Natl Acad Sci USA* 92:12280–12284, 1995 (178) (Fig. 1).]

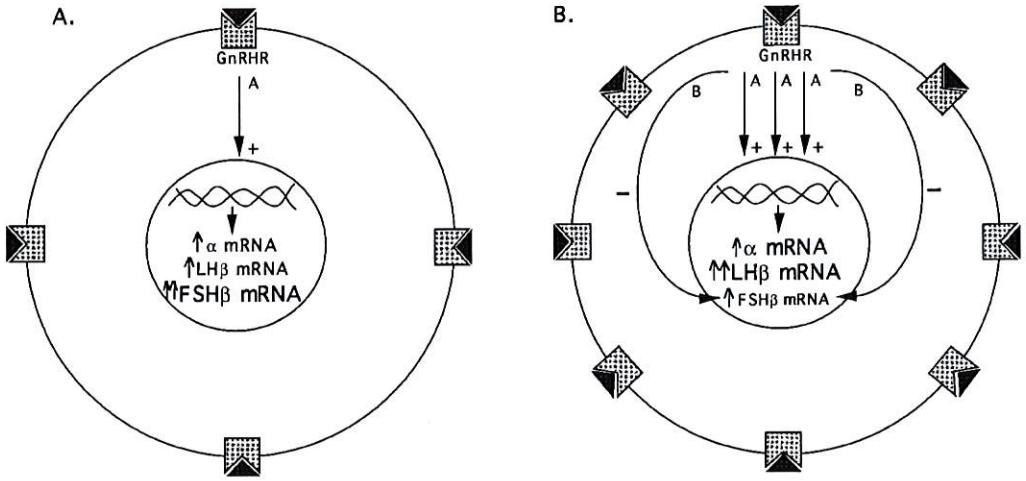

FIG. 17. Model of the mechanism of differential regulation of the gonadotropin subunit genes by GnRH at (A) low GnRH pulse frequencies, and (B) high GnRH pulse frequencies. A, At low GnRH pulse frequencies, every 2 h, GnRHR concentrations on the gonadotrope cell surfaces are relatively low. When GnRH binds to its receptors on gonadotropes, a signal transduction pathway, pathway A, is activated, resulting in the stimulation of the expression of all three of the gonadotropin subunit genes, α, LHβ, and FSHβ. B, At higher GnRH pulse frequencies, every 30 min, GnRHR concentrations on the gonadotrope cell surfaces are higher. When GnRH binds to the now greater receptor numbers on the gonadotropes, signal transduction pathway A is activated to an even greater extent, resulting in the greater stimulation of the α- and LHβ-subunit genes. In addition, a second signal transduction pathway, pathway B, is now also activated. Activation of pathway B results in the specific inhibition of the expression of the FSHβ gene, with no effects on the α and LHβ genes. The net effect is that α and LHβ gene expression is maximally stimulated at relatively high GnRH pulse frequencies, whereas FSHβ gene expression is optimally stimulated at lower GnRH pulse frequencies. [Reprinted with permission from U. B. Kaiser *et al*: *Proc Natl Acad Sci USA* 92:12280–12284, 1995 (178) (Fig. 4).]

the mechanisms by which GnRH regulates α and LHβ gene expression are distinct from those by which FSHβ gene expression is regulated. Furthermore, the signal transduction pathways activated by GnRH may be different at low *vs.* high GnRH receptor numbers. The details of the different intracellular signaling pathways activated by GnRH at low *vs.* high GnRHR numbers remain to be elucidated. It is possible that the GnRHR may couple to different G proteins at low *vs.* high cellular receptor numbers or that different signaling pathways are activated by the α- and βγ-subunits of a single G protein.

J. Summary of GnRH action in GGH_3 cells

GGH_3 cells have provided another cell model system for the study of the GnRHR and GnRH action. These cells bind GnRH and GnRH analogs specifically and with similar affinities to primary gonadotropes. Because they express both the GnRHR and the TRHR, they provide an opportunity to compare directly the signal transduction pathways and mechanisms of action of TRH and GnRH. GnRHR levels are modulated by GnRH treatment, suggesting that GnRH regulates its receptor at the posttranscriptional level. Unlike αT3-1 cells, GnRH appears to increase cAMP production as well as stimulating IP production and intracellular calcium levels in GGH_3 cells. The GnRHR may be coupled to more than one G protein in GGH_3 cells; additionally, there is evidence to suggest a role for Gβγ-subunits in mediating GnRH responses as well. GnRH is able to stimulate gene expression as well as hormone release in GGH_3 cells.

GH_3 cells have both advantages and disadvantages for the study of GnRH action. Because the parental GH_3 cell line does not express the GnRHR gene, these cells are useful for structure-function studies of the GnRHR. Wild type and mutant receptors can be transfected into GH_3 cells and their function studied without interference from endogenously expressed receptors. Such studies to date have been largely done in nonpituitary cell lines, such as COS cells (148, 149). However, unlike the GGH_3 cells, COS cells do not support gonadotropin subunit promoter activity, so that domains of the GnRHR important for mediating signals for transcriptional stimulation cannot be defined. The transfected GnRHR is driven by a heterologous viral promoter, which is not regulated by GnRH. This means that GGH_3 cells cannot be used for studies of GnRHR gene expression. However, it allows effects of GnRHR concentration on GnRH action and effects of GnRH on GnRHR desensitization to be studied independently of regulatory effects at the level of gene expression. Finally, GH_3 cells support the expression of transfected reporter genes driven by regulatory regions of the LHβ- or FSHβ-subunit genes, making them the first homogeneous, immortalized cell population available for the study of gonadotropin β-subunit gene expression and regulation by GnRH.

Nonetheless, an important caveat to studies performed in GGH_3 cells is that this is a heterologous cell expression system, and the signal transduction pathways used in these cells may differ from those used in primary gonadotropes as well as in thyrotropes. It must be remembered that GH_3 cells, while they are pituitary in origin, are derived from somatolactotropes rather than from gonadotropes, and do not express the gonadotropin subunit genes endogenously. They may, therefore, lack gonadotrope-specific factors that may be important for cell-specific and/or regulated expression of gonadotropin subunit genes. Observations made using such a cell model should be confirmed in primary pituitary cells

or *in vivo* before physiological relevance is assigned to such observations.

V. Studies of GnRH Action in Other Pituitary Cell Lines

A. RC-4B/C cells

This anterior pituitary cell line was established from a pituitary adenoma that developed spontaneously in a 3-yr-old male rat (185). These cells have the ultrastructural appearance of well differentiated anterior pituitary cells. Immunocytochemical studies showed the presence of all known anterior pituitary secretory cell types, including gonadotropes. However, the proportion of different cell types was different from that observed in primary rat pituitaries. In particular, the percentage of LHβ cells was higher than in the normal male rat pituitary, and the percentage of GH cells was lower. The percentage of FSHβ-, PRL-, ACTH-, and TSHβ-staining cells was comparable to the normal male rat pituitary. GnRH receptors were also shown to be present, with the same binding affinity for a GnRH agonist, Buserelin, as in the pituitary gland, but with 2-fold lower capacity. Interestingly, dual-staining studies revealed that many of the cells are bihormonal, producing FSHβ and PRL or LHβ and PRL (186). This cell line thus represents an additional cell model for the study of the GnRHR and GnRH action, and for gonadotrope-specific expression of the FSH and LH subunit genes. This cell model has an advantage over αT3-1 cells and GGH_3 cells in that the FSHβ- and LHβ-subunit genes are expressed endogenously. However, a disadvantage of the RC-4B/C cell line is that the cells are a heterogeneous population, so only a fraction of the cells express the gonadotropin subunit genes. Furthermore, it has not yet been shown that the gonadotropins are synthesized and secreted in a regulated manner analogous to that which occurs *in vivo*. Keri *et al.* (187) were unable to obtain activity greater than that of a promoterless reporter for a fusion gene in which the bovine LHβ promoter was fused upstream of the CAT gene. Activity remained low even after the upstream addition of a Rous sarcoma virus enhancer. Also, surprisingly, although the proportion of cells staining for LHβ and/or FSHβ are higher than in normal pituitary, the FSH and LH content of RC-4B/C cells was 70- and 800-fold lower, respectively, than that of the normal male rat pituitary gland. Thus, the validity of this cell line as a physiological model has yet to be determined.

B. LβT2 cells

Recently, targeted expression of the SV40 T antigen with the rat LHβ-subunit gene regulatory region was used to generate transgenic mice. An immortalized cell line (LβT2 cells) was derived from a tumor generated in a LHβ-Tag mouse, in a manner analogous to the preparation of the αT3-1 cells (188). These cells express both the α- and β-subunits of LH as well as GnRHR, estrogen receptors, and estrogen-inducible progesterone receptors. However, the FSHβ-subunit is not expressed. LβT2 cells probably arose later in ontogeny than αT3-1 cells and thus represent a more mature gonadotrope precursor than do the αT3-1 cells.

LβT2 cells have been used to study the effects of GnRH on intracellular Ca^{2+} concentrations. Cells stimulated with 1 μM GnRH responded with an increase in intracellular Ca levels and also had a secretory response, as measured by changes in plasma membrane capacitance (189). Furthermore, LβT2 cells responded to a 15-min pulse of 1–100 nM GnRH with a dose-dependent increase in LH secretion (188, 190). Exposure of the cells to four, 15-min, 10 nM GnRH pulses every 90 min for 4 days led to an increase in LH secretion in response to the initial GnRH pulse on each succeeding day, independent of cell number. The stimulation of LH secretion by GnRH was enhanced by steroids: the LH-secretory response to GnRH by day 4 was 4-fold in the absence of added steroids, 7-fold in the presence of 0.2 nM estrogen (E_2), 14-fold in the presence of 20 nM dexamethasone, and 15-fold in the presence of both E_2 and dexamethasone. These changes in responsiveness to GnRH appeared to be due in part to changes in GnRHR number and in part to changes in the LH synthesis/secretory pathway independent of changes in the GnRHR.

Studies of $[Ca^{2+}]_i$ and exocytosis in individual LβT2 cells have been performed using the whole-cell perforated patch clamp technique to measure plasma membrane capacitance (191). These studies show that GnRH evokes dose-dependent increases in $[Ca^{2+}]_i$ and secretion. The $[Ca^{2+}]_i$ responses to GnRH are biphasic, as in αT3-1 cells and primary gonadotropes. However, the $[Ca^{2+}]_i$ oscillations observed in primary gonadotropes at low GnRH concentrations are not observed in the LβT2 cells. The extent of the changes in $[Ca^{2+}]_i$ and exocytosis in response to GnRH were dependent on the steroid hormone background. E_2 and dexamethasone caused an increase in the peak $[Ca^{2+}]_i$ stimulated by GnRH as well as a shift toward increased sensitivity of the Ca^{2+} dependency of exocytosis. The increased $[Ca^{2+}]_i$ response may be due to an increase in GnRHR numbers (see below). The increase in GnRH-induced secretion may be due to both an increase in $[Ca^{2+}]_i$ and an increase in the sensitivity of the secretory apparatus to $[Ca^{2+}]_i$, which may, in turn, be due to effects of other second messenger pathways activated by GnRH and/or steroids.

Studies of mRNA levels indicate that LβT2 cells respond to pulsatile GnRH administration with an increase in GnRHR mRNA levels (190). However, this increase is only approximately 2-fold, compared with the much greater increase observed in primary pituitary cells (49). This difference may be due to differences in the experimental paradigm of pulsatile GnRH delivery. GnRHR mRNA levels are also increased in LβT2 cells by estradiol and dexamethasone. LHβ mRNA levels are markedly induced by pulsatile GnRH in LβT2 cells, with no effect of steroids, and α-subunit mRNA levels are unaffected by either steroids or GnRH.

The LβT2 cell line thus exhibits some characteristics consistent with those of pituitary gonadotropes, including the expression of the α, LHβ, and GnRHR genes, the biphasic stimulation of $[Ca^{2+}]_i$ by GnRH, the secretion of LH via a regulated pathway, and the regulation of LHβ and GnRHR mRNA levels in response to GnRH and steroid hormones. These cells may be useful for studying the molecular and

cellular mechanisms involved in the regulation of LH subunit gene expression and LH secretion.

VI. Future Directions

The recent availability of immortalized cell models of gonadotrope function have allowed considerable advances in our understanding of the structure and function of the GnRHR, the signal transduction pathways activated by GnRH, and molecular mechanisms of action of GnRH in terms of regulation of gene expression, hormone biosynthesis, and hormone secretion, as well as of homologous regulation of the GnRHR at both transcriptional and posttranscriptional levels. In addition, these cell models have proven useful for the study of cell-specific and regulated expression of the gonadotropin α-subunit gene in particular, and to some extent the gonadotropin β-subunit genes as well. Nonetheless, further studies to clarify the molecular mechanisms by which GnRH regulates LH and FSH subunit gene expression, hormone biosynthesis, and secretion are needed. In addition, the mechanisms of the unique responses of gonadotropes to varying GnRH pulse frequencies and amplitudes are not well understood. For these studies, we eagerly await the development and availability of a novel, immortalized gonadotrope cell line that expresses the LHβ and FSHβ-subunit genes as well as the α-subunit and the GnRHR and that has the unique responses to different modes of administration of GnRH in a manner reflecting that which occurs in primary pituitary gonadotropes.

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Approved Biohazards Subcommittee: July 9, 2010
Biosafety Website: www.uwo.ca/humanresources/biosafety/

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario (UWO) or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biological agents is described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans or involving plants, fungi, or insects that require Public Health Agency of Canada (PHAC) or Canadian Food Inspection Agency (CFIA) permits.

This form must be updated at least every 3 years or when there are changes to the biological agents being used.

Containment Levels will be established in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Public Health Agency of Canada (PHAC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Completed forms are to be returned to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190) for distribution to the Biohazards Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135 or biosafety@uwo.ca. If there are changes to the information on this form (excluding grant title and funding agencies), contact Occupational Health and Safety for a modification form. See website: www.uwo.ca/humanresources/biosafety

PRINCIPAL INVESTIGATOR	Andy Videsh Babwah
DEPARTMENT	Obstetrics & Gynaecology
ADDRESS	Victoria Research Laboratories, A4-140
PHONE NUMBER	519-685-8500 extension 55485
EMERGENCY PHONE NUMBER(S)	519-679-3135
EMAIL	ababwah@uwo.ca

Location of experimental work to be carried out: Building(s) Victoria Research Laboratories
Room(s) all 4th floor rooms A4-128 (Level 2 Tissue Culture room)

*For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to the University of Western Ontario Biosafety Officer (See Section 15.0, Approvals).

FUNDING AGENCY/AGENCIES: NSERC, ERA
GRANT TITLE(S):
Molecular and functional analysis of nuclear membrane localized GnRH-RI (NSERC Discovery)
GPR54 signaling in human placentation (ERA)

List all personnel working under Principal Investigators supervision in this location:

Name	UWO E-mail Address	Date of Biosafety Training
Maryse Ahow	mahow@uwo.ca	Jul 16 2009
Cynthia Pape	cynthia.pape@schulich.uwo.ca	Oct 11 2006
Macarena Pampillo	mpampill@uwo.ca	Sep 27 2002

Please explain the biological agents and/or biohazardous substances used and how they will be stored, used and disposed of. Projects without this description will not be reviewed.

Dr Andy Babwah's laboratory focuses on elucidating the roles of two G protein coupled receptors, GnRH-RI and GPR54, in human placentation. A wide variety of techniques are used, including biochemical techniques such as immunoprecipitation and assays to measure inositol phosphate turnover, and imaging techniques such as confocal microscopy. A number of experiments are performed using cell lines, all of which are containment level 1 or 2. The cells are handled in a biological safety cabinet and grown in a tissue culture incubator housed in a level 2 laboratory. All contaminated liquid materials are aspirated into a flask containing 10% bleach and contaminated solid plastic materials are sent to be autoclaved and incinerated. Some experiments require the overexpression of a particular protein in a mammalian cell. To that effect, bacteria cells are transformed with a DNA plasmid containing the gene of interest, then the plasmid DNA is harvested and transfected into the mammalian cells. The bacterial strains used in this lab are containment level 2 and under, and contaminated solid and liquid materials are treated as mentioned before. The overexpression of the protein of interest in bacterial or mammalian cells does not increase the containment level required for these cells. For some of the experiments performed in this laboratory, it is necessary to use cholera toxin; however, the amount handled at one time is equivalent to 20% of the LD 50 in mice, and the maximum amount stored is equivalent to 2x this value. Finally, animal experiments are performed (AUS protocol # 2008-017) according to the regulations of the regulations of the Animal Care and Use Subcommittee. The contaminated materials from these experiments are treated as mentioned before and animal remains are incinerated. All staff members of this laboratory have attended the required training courses given by OHS.

Please include a one page research summary or teaching protocol.

Early Researcher Award Program:
Title: GPR54 signaling in human placentation (ERA)

Abnormal placentation causes significant maternal and fetal morbidity and mortality. Placentation is in part regulated by a receptor called GPR54. When activated, GPR54 couples to two signaling pathways in the cell, each leading to specific cellular responses. Our studies suggest that under some disease conditions of the placenta, selective engagement of one pathway over the other can potentially reduce disease severity. Our studies are aimed at identifying cellular response that lie downstream of each pathway and to develop drugs that can selectively activate a given pathway under disease conditions. This in turn can greatly benefit maternal/fetal health-care in Ontario.

NSERC Discovery 2006-2011
Title: Molecular and Functional Analysis of Nuclear Membrane Localized GnRH-RI

The goal of my research program is to better understand G protein-coupled receptor (GPCR) signaling and the mechanisms that regulate this signaling. GPCRs are transmembrane receptors that constitute a large and diverse family of proteins whose primary function is to transduce extracellular stimuli into intracellular signals. Upon binding of their cognate ligands, which include light, odorants, neurotransmitters, hormones and chemokines, GPCRs couple to specific G α subunits (of which there are at least 18 that include Gq/11 and Gs) and activate intracellular signaling pathways. Our current understanding of GPCR signaling and the mechanisms that regulate it are perhaps best understood for the β2-adrenergic receptor and accordingly this has served as the prototypical receptor for subsequent studies. However, it is now abundantly clear that there is significant variation in GPCR signaling and the mechanisms that regulate signaling. Consequently, while the β2-adrenergic receptor has been an extremely useful model for studying GPCR biology, it is still not as representative as it was once perceived as being. Therefore, to complement the β2-adrenergic receptor as a GPCR model system, I have chosen to study the biology of the gonadotropin releasing hormone receptor (GnRH-R). This is primarily based on emerging ideas which suggest that the mammalian GnRH-RI represents one of the most evolutionary advanced members within the GPCR superfamily and its biology may be representative of numerous other GPCRs.

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? X YES O NO
(non-pathogenic and pathogenic biological agents including but not limited to bacteria and other microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)? If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? O YES X NO
If YES, please give the name of the species. _____
What is the origin of the microorganism(s)? _____
Please describe the risk (if any) of escape and how this will be mitigated:

_____
_____
_____

Please attach the CFIA permit.
Please describe any CFIA permit conditions:

_____
_____
_____

1.2 Please complete the table below:

Name of Biological agent(s)*	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
E coli (DH5 alpha)	O Yes X No	O Yes X No	O Yes X No	1	Invitrogen	X 1 O 2 O 2+ O 3
E coli (Top 10)	O Yes X No	O Yes X No	O Yes X No	1	Invitrogen	X 1 O 2 O 2+ O 3
XL1 Blue supercompetent cells	O Yes X No	O Yes X No	O Yes X No	1	Stratagene	O 1 X 2 O 2+ O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 2+ O 3

*Please attach a Material Safety Data Sheet or equivalent from the supplier.

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? X YES O NO
If no, please proceed to Section 3.0

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture:

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	X Yes O No	Human placenta	Not applicable
Rodent	O Yes X No		
Non-human primate	O Yes X No		
Other (specify)	O Yes X No		

2.3 Please indicate the type of established cells that will be grown in culture in:
2.4

Cell Type	Is this cell type used in your work?	Specific cell line(s)	Supplier / Source
Human	X Yes O No	HEK 293	ATCC (original supplier)
Human	X Yes O No	HTR-8/Svneo	Dr Lala, UWO (Anatomy and Ce Biol)
Human	X Yes O No	MDA-MB-231	ATCC (original supplier)
Human	X Yes O No	MDA-MB-435S	ATCC (original supplier)
Human	X Yes O No	MCF-10A	ATCC (original supplier)
Human	X Yes O No	PC-3	ATCC (original supplier)
Human	X Yes O No	PZ-HPV-7	ATCC (original supplier)
Human	X Yes O No	JEG-3	Dr Yang, UWO (Ob&Gyn)
Human	X Yes O No	GripTite 293 MSR	Invitrogen
Rodent	X Yes O No	ARIP	Dr Pin, UWO (Phys & Pharm)
Rodent	X Yes O No	AR42J	Dr Pin, UWO (Phys & Pharm)
Rodent	X Yes O No	MEF wild type	Dr Lefkowitz, Duke Univ, USA
Rodent	X Yes O No	MEF, Barr 1 deficient	Dr Lefkowitz, Duke Univ, USA
Rodent	X Yes O No	MEF, Barr 2 deficient	Dr Lefkowitz, Duke Univ, USA
Rodent	X Yes O No	MEF, Barr 1 and 2 deficient	Dr Lefkowitz, Duke Univ, USA
Rodent	X Yes O No	MEF wild type	Dr Offermanns, Max-Planck Institute, Germany
Rodent	X Yes O No	MEF Galpha q/Galpha11 deficient	Dr Offermanns, Max-Planck Institute, Germany
Rodent	X Yes O No	MEF Galpha 12/Galpha13 deficient	Dr Offermanns, Max-Planck Institute, Germany
Rodent	X Yes O No	GT1-7	Dr Mellon, Univ California, USA
Non-human primate	X Yes O No	COS-7	ATCC (original supplier)
Other (specif	O Yes X No		

*Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org)

2.5 For above named cell types(s) indicate PHAC or CFIA containment level required O 1 X 2 O 2+ O 3

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? X YES O NO
(human corionic gonadotropin – purified – tested for human pathogens (eg: HIV))
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	**Source/Supplier /Company Name**	**Is Human Source Material Infected With An Infectious Agent? YES/NO**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid		O Yes O Unknown		O 1 O 2 O 2+ O 3
Human Blood (fraction) or other Body Fluid		O Yes O Unknown		O 1 O 2 O 2+ O 3

Human Organs or Tissues (unpreserved)		O Yes O Unknown		O 1 O 2 O 2+ O 3
Human Organs or Tissues (preserved)		Not Applicable		Not Applicable

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? X YES O NO If no, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? X YES, complete table below O NO

Bacteria Used for Cloning *	Plasmid(s) **	Source of Plasmid	Gene Transfected	Describe the change that results from transformation or tranfection
E coli DH5alpha	pEGFP-C3	Clontech	GPR54	Cells overexpress a fusion protein (GPR54-GFP) that is a green fluorescent protein
E coli DH5alpha	pECFP-C1	Clontech	GPR54	Cells overexpress a fusion protein (GPR54-CFP) that is a cyan fluorescent protein
E coli DH5alpha	pEYFP-C1	Clontech	GPR54	Cells overexpress a fusion protein (GPR54-YFP) that is a yellow fluorescent protein
E coli DH5alpha	pcDNA3.1/Hygro (+)	Invitrogen	GPR54	Cells overexpress GPR54 and are resistant to the antibiotic hygromycin

* Please attach a Material Data Sheet or equivalent if available.
** Please attach a plasmid map.

4.3 Will genetic modification(s) involving viral vectors be made? O YES, complete table below X NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results from transduction

* Please attach a Material Safety Data Sheet or equivalent.

4.4 Will genetic sequences from the following be involved?

- HIV O YES, please specify _____ X NO
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens O YES, specify _____ X NO
- SV 40 Large T antigen X YES (COS-7 and HTR-8/Svneo cells) O NO
- E1A oncogene X YES (HEK 293 cells) O NO
- Known oncogenes O YES, please specify _____ O NO
- Other human or animal pathogen and or their toxins O YES, please specify _____ X NO

4.5 Will virus be replication defective? O YES O NO N/A

4.6 Will virus be infectious to humans or animals? O YES O NO N/A

4.7 Will this be expected to increase the containment level required? O YES O NO N/A

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials be conducted involving a biological agent? O YES X NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 6.0

5.2 If YES, please specify which biological agent will be used: _____
Please attach a full description of the biological agent.

5.2 Will the biological agent be able to replicate in the host? O YES O NO

5.3 How will the biological agent be administered? _____

5.4 Please give the Health Care Facility where the clinical trial will be conducted:_____

5.5 Has human ethics approval been obtained? O YES, number: _____ O NO O PENDING

6.0 Animal Experiments

6.1 Will live animals be used? X YES O NO If no, please proceed to section 7.0

6.2 Name of animal species to be used: Mus musculus

6.3 AUS protocol # 2008-017

6.4 Will any of the agents listed in section 4.0 be used in live animals O YES, specify: _____ X NO

6.5 Will the agent(s) be shed by the animal: O YES O NO, please justify:

_____

_____

7.0 Use of Animal species with Zoonotic Hazards

7.1 Will any animals with zoonotic hazards or their organs, tissues, lavages or other body fluids including blood be used (see list below)? O YES X No If no, please proceed to section 8.0

7.2 Please specify the animal(s) used:

- Pound source dogs O YES O NO
- Pound source cats O YES O NO
- Cattle, sheep or goats O YES, please specify species _____ O NO
- Non-human primates O YES, please specify species _____ O NO
- Wild caught animals O YES, please specify species & colony # _____ O NO
- Birds O YES, please specify species _____ O NO
- Others (wild or domestic) O YES, please specify _____ O NO

8.0 Biological Toxins

8.1 Will toxins of biological origin be used? X YES O NO If no, please proceed to Section 9.0

8.2 If YES, please name the toxin(s): Cholera toxin
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

8.3 What is the LD_{50} (specify species) of the toxin: 250 ug/kg in mice

8.4 How much of the toxin is handled at one time*? 50 ug

8.5 How much of the toxin is stored*? 0.5 mg

8.6 Will any biological toxins be used in live animals? O YES, Please provide details:_____ X NO

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

9.0 Insects

9.1 Do you use insects? O YES X NO If no, please proceed to Section 10.0

9.2 If YES, please give the name of the species. _____

9.3 What is the origin of the insect? _____

9.4 What is the life stage of the insect? _____

9.5 What is your intention? O Initiate and maintain colony, give location: _____
O "One-time" use, give location: _____

9.6 Please describe the risk (if any) of escape and how this will be mitigated:
_____
_____

9.7 Do you use insects that require a permit from the CFIA permit? O YES O NO
If YES, Please attach the CFIA permit & describe any CFIA permit conditions:
_____
_____

10.0 Plants

10.1 Do you use plants? O YES X NO If no, please proceed to Section 11.0

10.2 If YES, please give the name of the species. _____

10.3 What is the origin of the plant? _____

10.4 What is the form of the plant (seed, seedling, plant, tree...)? _____

10.5 What is your intention? O Grow and maintain a crop O "One-time" use

10.6 Do you do any modifications to the plant? O YES O NO
If yes, please describe: _____
_____

10.7 Please describe the risk (if any) of loss of the material from the lab and how this will be mitigated:
_____
_____
_____

10.8 Is the CFIA permit attached? O YES O NO
If YES, Please attach the CFIA permit & describe any CFIA permit conditions:
_____
_____

11.0 Import Requirements

11.1 Will any of the above agents be imported? O YES, please give country of origin_____ X NO
If no, please proceed to Section 12.0

11.2 Has an Import Permit been obtained from HC for human pathogens? O YES O NO

11.3 Has an import permit been obtained from CFIA for animal or plant pathogens? O YES O NO

11.4 Has the import permit been sent to OHS? O YES, please provide permit # _____ O NO

12.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biological agents in Sections 1.0 to 9.0 have been trained.

SIGNATURE _____

13.0 Containment Levels

13.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. O 1 ☒ 2 O 2+ O 3

13.2 Has the facility been certified by OHS for this level of containment?
☒ YES, permit # if on-campus BIO-LHRI-0047 Rm A4-128
O NO, please certify
O NOT REQUIRED for Level 1 containment

Level 2 certification completed by Gail Ryder on MAY 2008 (PHAC) Gail Ryder NOV 2008 (CFIA)

14.0 Procedures to be Followed

14.1 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca/

SIGNATURE _____ Date: Aug 31, 2010

14.2 Please describe additional risk reduction measures will be taken beyond containment level 1, 2, 2+ or 3 measures, that are unique to this agent.
N/A

14.3 Please outline what will be done if there is an exposure to the biological agents listed, such as a needlestick injury:

Immediately wash thoroughly with soap and water, refer to MSDS sheet, contact OH&S.

15.0 Approvals

1) UWO Biohazards Subcommittee: SIGNATURE: _____
Date: Oct 22 2010

2) Safety Officer for the University of Western Ontario
SIGNATURE: J Stanley
Date: Oct 19, 2010

3) Safety Officer for Institution where experiments will take place (if not UWO):
SIGNATURE: Gail Ryder
Date: SEPTEMBER 15, 2010

Approval Number: BIO-LHRI-0047 Expiry Date (3 years from Approval): October 21, 2013

Special Conditions of Approval:
Recommended (only):
- Store toxin in two different locations.
- Follow attached biosecurity requirements. See www.uwo.ca/humanresources/biosafety for information (also see attached) JS

Western

Biosecurity Requirements for Facilities Using Biological Agents

(1) Biological agents protected by a lock. For example, biological agents in a freezer, fridge, laboratories or other type of container must be locked after-hours/if no one present.

(2) The supervisor must ensure that each person has the qualifications and training to do the work without supervision.

(3) Visitors must be accompanied.

(4) The supervisor must keep a current inventory and a list of the location(s) where the biological agent(s) are stored and handled.

(5) Labelling to identify samples and the container in which they are stored.

(6) Notify the biosafety officer if a sample is lost, stolen, or otherwise misused.

(7) Notify Campus Community Police Services of suspicious behaviour.

There are two additional requirements for Facilities Using or Storing Biological Toxins:

(8) Do not keep on hand more than the amounts regulated by the United States Select Agents regulation: www.selectagents.gov/index.htm/

(9) For best practices, it is recommended to use or handle less than one human dose at any given time.

Subject: Re: Biological Agents Registry Form: Babwah
From: Gail Ryder <Gail.Ryder@LawsonResearch.Com>
Date: Fri, 01 Oct 2010 14:05:03 -0400
To: Jennifer Stanley <jstanle2@uwo.ca>

Hi Jennifer,

Sorry, it meant to say November 2008. It was initially certified on May 20, 2008 but then he added more pathogens so I did a reinspection on November 26, 2008. I can FAX you over the main signature sheet if you want. He is one of the labs on my list to be recertified this fall as I know his date of expiration is approaching.

Cheers,
Gail

Gail Ryder, CRSP
Research Safety Officer

Lawson Health Research Institute
South Street Hospital
375 South Street, Room A210, NR
London, Ontario, Canada N6A 4G5
Tel: (519) 685-8500 x75109
Fax: (519) 432-7367
Pager: x18059
E-mail: Gail.Ryder@LawsonResearch.com
Website: www.lawsonresearch.com

||| Jennifer Stanley <jstanle2@uwo.ca> 2010/09/30 04:25 PM >>> |||

Hi Gail

Thank you for this recent submission.

I noticed that the PHAC inspection was done in May 2008, over 2 years ago. Since PHAC inspections are normally only valid for 2 years, do you have plans to re-inspect the facility soon?

Regards
Jennifer

Western

TOXIN USE RISK ASSESSMENT

TOXIN: Cholera

PROPOSED USE (DOSE): 50 µg (use), 500 µg (storage)

LD_{50} (species): 250 µg/kg (mice)

CALCULATION:

50 ug/kg (use) X 70 kg/person = 3500 ug per person

Divide by safety factor(s) of 10 (as applicable): 350 ug per person
(one safety factor used)

COMMENTS/RECOMMENDATION:

use dose (50 µg) < lethal dose calculated (350 µg)

storage amount (500 µg) > lethal dose calculated (350 µg)

1. IDENTIFICATION OF THE SUBSTANCE/PREPARATION AND THE COMPANY/UNDERTAKING

Product code 18265017
Product name Subcloning Efficiency ™ DH5alpha™ Competent Cells

Company/Undertaking Identification

INVITROGEN CORPORATON
5791 VAN ALLEN WAY
PO BOX 6482
CARLSBAD, CA 92008
760-603-7200

INVITROGEN CORPORATION
5250 MAINWAY DRIVE
BURLINGTON, ONT
CANADA L7L 6A4
800-263-6236

GIBCO PRODUCTS
INVITROGEN CORPORATION
3175 STALEY ROAD P.O. BOX 68
GRAND ISLAND, NY 14072
716-774-6700

24 hour Emergency Response (Transport): 866-536-0631
301-431-8585
Outside of the U.S. ++1-301-431-8585

For research use only

2. COMPOSITION/INFORMATION ON INGREDIENTS

Hazardous/Non-hazardous Components
The product contains no substances which at their given concentration, are considered to be hazardous to health. We recommend handling all chemicals with caution.

3. HAZARDS IDENTIFICATION

Emergency Overview
The product contains no substances which at their given concentration, are considered to be hazardous to health

8. EXPOSURE CONTROLS / PERSONAL PROTECTION

Occupational exposure controls

Exposure limits

Engineering measures	Ensure adequate ventilation, especially in confined areas

Personal protective equipment

Respiratory Protection	In case of insufficient ventilation wear suitable respiratory equipment
Hand protection	Protective gloves
Eye protection	Safety glasses with side-shields
Skin and body protection	Lightweight protective clothing.
Hygiene measures	Handle in accordance with good industrial hygiene and safety practice
Environmental exposure controls	Prevent product from entering drains.

9. PHYSICAL AND CHEMICAL PROPERTIES

General Information

Form	Liquid

Important Health Safety and Environmental Information

Boiling point/range	**°C** No data available	**°F** No data available
Melting point/range	**°C** No data available	**°F** No data available
Flash point	**°C** No data available	**°F** No data available
Autoignition temperature	**°C** No data available	**°F** No data available
Oxidizing properties	No information available	
Water solubility	No data available	

10. STABILITY AND REACTIVITY

Stability	Stable.
Materials to avoid	No information available
Hazardous decomposition products	No information available
Polymerization	Hazardous polymerisation does not occur.

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Principle Routes of Exposure/ Potential Health effects

Eyes	No information available
Skin	No information available
Inhalation	No information available

This product has been classified according to the hazard criteria of the CPR and the MSDS contains all of the information required by the CPR

16. OTHER INFORMATION

For research use only

The above information was acquired by diligent search and/or investigation and the recommendations are based on prudent application of professional judgment. The information shall not be taken as being all inclusive and is to be used only as a guide. All materials and mixtures may present unknown hazards and should be used with caution. Since the Company cannot control the actual methods, volumes, or conditions of use, the Company shall not be held liable for any damages or losses resulting from the handling or from contact with the product as described herein. THE INFORMATION IN THIS MSDS DOES NOT CONSTITUTE A WARRANTY, EXPRESSED OR IMPLIED, INCLUDING ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR ANY PARTICULAR PURPOSE.

End of Safety Data Sheet

invitrogen

Material Safety Data Sheet

Revision Date: 18-Sep-2008

1. IDENTIFICATION OF THE SUBSTANCE/PREPARATION AND THE COMPANY/UNDERTAKING

Product code 500257
Product name TOP 10 - ONE SHOT

Company/Undertaking Identification

INVITROGEN CORPORATON
5791 VAN ALLEN WAY
PO BOX 6482
CARLSBAD, CA 92008
760-603-7200

INVITROGEN CORPORATION
5250 MAINWAY DRIVE
BURLINGTON, ONT
CANADA L7L 6A4
800-263-6236

GIBCO PRODUCTS
INVITROGEN CORPORATION
3175 STALEY ROAD P.O. BOX 68
GRAND ISLAND, NY 14072
716-774-6700

2. COMPOSITION/INFORMATION ON INGREDIENTS

Hazardous/Non-hazardous Components
The product contains no substances which at their given concentration, are considered to be hazardous to health

3. HAZARDS IDENTIFICATION

Emergency Overview
The product contains no substances which at their given concentration, are considered to be hazardous to health

Form
Suspension

Principle Routes of Exposure/ Potential Health effects

Eyes	No information available
Skin	No information available

3. HAZARDS IDENTIFICATION

Inhalation	No information available
Ingestion	No information available

Specific effects

Carcinogenic effects	No information available
Mutagenic effects	No information available
Reproductive toxicity	No information available
Sensitization	No information available

Target Organ Effects — No information available

HMIS

Health	0
Flammability	0
Reactivity	0

4. FIRST AID MEASURES

Skin contact	Wash off immediately with plenty of water
Eye contact	Rinse thoroughly with plenty of water, also under the eyelids.
Ingestion	Never give anything by mouth to an unconscious person
Inhalation	Move to fresh air
Notes to physician	Treat symptomatically.

5. FIRE-FIGHTING MEASURES

Suitable extinguishing media	Dry chemical
Special protective equipment for firefighters	Wear self-contained breathing apparatus and protective suit

6. ACCIDENTAL RELEASE MEASURES

Personal precautions	Use personal protective equipment
Methods for cleaning up	Soak up with inert absorbent material.

7. HANDLING AND STORAGE

Handling	No special handling advice required
Storage	Keep in properly labelled containers

8. EXPOSURE CONTROLS / PERSONAL PROTECTION

Occupational exposure controls

Exposure limits

Engineering measures — Ensure adequate ventilation, especially in confined areas

Personal protective equipment

Respiratory protection	In case of insufficient ventilation wear suitable respiratory equipment
Hand protection	Protective gloves
Eye protection	Safety glasses with side-shields
Skin and body protection	Lightweight protective clothing.

Hygiene measures	Handle in accordance with good industrial hygiene and safety practice
Environmental exposure controls	Prevent product from entering drains.

9. PHYSICAL AND CHEMICAL PROPERTIES

General Information

Form	Suspension

Important Health Safety and Environmental Information

Boiling point/range	°C No data available	°F No data available
Melting point/range	°C No data available	°F No data available
Flash point	°C No data available	°F No data available
Autoignition temperature	°C No data available	°F No data available
Oxidizing properties	No information available	
Water solubility	No data available	

10. STABILITY AND REACTIVITY

Stability	Stable.
Materials to avoid	No information available
Hazardous decomposition products	No information available
Polymerization	Hazardous polymerisation does not occur.

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Principle Routes of Exposure/ Potential Health effects

Eyes	No information available
Skin	No information available
Inhalation	No information available
Ingestion	No information available

Specific effects

Carcinogenic effects	No information available
Mutagenic effects	No information available
Reproductive toxicity	No information available
Sensitization	No information available

Target Organ Effects — No information available

12. ECOLOGICAL INFORMATION

Ecotoxicity effects	No information available.
Mobility	No information available.
Biodegradation	Inherently biodegradable.
Bioaccumulation	Does not bioaccumulate.

13. DISPOSAL CONSIDERATIONS

13. DISPOSAL CONSIDERATIONS

Dispose of in accordance with local regulations

14. TRANSPORT INFORMATION

IATA

Proper shipping name	Not classified as dangerous in the meaning of transport regulations
Hazard Class	No information available
Subsidiary Class	No information available
Packing group	No information available
UN-No	No information available

15. REGULATORY INFORMATION

International Inventories

U.S. Federal Regulations

SARA 313
This product is not regulated by SARA.

Clean Air Act, Section 112 Hazardous Air Pollutants (HAPs) (see 40 CFR 61)
This product does not contains HAPs.

U.S. State Regulations

California Proposition 65
This product does not contain chemicals listed under Proposition 65

WHMIS hazard class:
Non-controlled

This product has been classified according to the hazard criteria of the CPR and the MSDS contains all of the information required by the CPR

16. OTHER INFORMATION

This material is sold for research and development purposes only. It is not for any human or animal therapeutic or clinical diagnostic use. It is not intended for food, drug, household, agricultural, or cosmetic use. An individual technically qualified to handle potentially hazardous chemicals must supervise the use of this material.

The above information was acquired by diligent search and/or investigation and the recommendations are based on prudent application of professional judgment. The information shall not be taken as being all inclusive and is to be used only as a guide. All materials and mixtures may be present unknown hazards and should be used with caution. Since Invitrogen Corporation cannot control the actual methods, volumes, or conditions of use, the Company shall not be held liable for any damages or losses resulting from the handling or from contact with the product as described herein. THE INFORMATION IN THIS MSDS DOES NOT CONSTITUTE A WARRANTY, EXPRESS OR IMPLIED, INCLUDING ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR ANY PARTCULAR PURPOSE.

End of Safety Data Sheet

MATERIAL SAFETY DATA SHEET

IDENTIFICATION

Stratagene
11011 N. Torrey Pines Rd.
La Jolla, CA 92037

Date of last update: 6/07/2007
Phone #: 800-894-1304
Fax #: 858-373-5300

Part # : 1071-13
CAS # : 67-68-5

Product Name: DMSO (Comp Cells)

HAZARDOUS COMPONENTS

Chemical Name & Synonyms: Dimethyl Sulfoxide* A 10846* Deltan* Demeso* Demasorb, Demavet* Demsodrox, Dermasorb* Dimethyl Sulfoxide* Dimethyl Sulphoxide* Dimexide* Dipirartril-Tropico* DMS-70* DMS-90* DMSO* Dolicur* Domoso* Dromisol* Durasorb* Gamasol 90* Hyadur* Infiltrina* M 176* Methane, Sulfinylbis-* Methylsulfinylmethane* NSC-763* RIMSO-50* Somipront* SQ 9453* Sulfinylbis (Methane)* Syntexan* Topsym*

OSHA PEL Limits: N/A
ACGIH TLV: N/A
Other Limits Recommended: N/A

KIT	DMSO-containing component	%
200124 TKX1 Competent Cells	200124-41 TKX1 Competent Cells	<10%
200129 XL1-Red Competent Cells	200129-41 XL1-Red Competent Cells 200236-41 XL1-Blue Competent Cells	<10%
200130 XL1-Blue Subcloning-Grade Competent Cells	200130-41 XL1-Blue Subcloning Competent Cells	<10%
200131 BL21(DE3) Competent Cells	200131-41 BL-21 (DE3) Cells	<10%
200132 BL21(DE3)pLysS Competent Cells	200132-41 BL-21 (DE3) pLysS Cells	<10%
200133 BL21 Competent Cells	200133-41 BL-21 Competent Cells	<10%
200134 TKB1 Competent Cells	200134-41 TKB1 Competent Cells	<10%
200138 XL1-Blue MRF' Kan Library Pack Competent Cells	200138-41 XL1-Blue MRF' Kan Competent Cells	<10%
200150 XL2-Blue Ultracompetent Cells	200150-41 XL2-Blue Ultracompetent Cells	<10%
200151 XL2-Blue MRF'Ultracompetent Cells	200151-41 XL2-Blue MRF' Ultracompetent Cells	<10%
200152 SURE®2 Supercompetent Cells	200152-41 SURE®2 Ultracompetent Cells	<10%
200170 ABLE® Competent Cells	200171-41 ABLE® C Competent Cells 200172-41 ABLE® K Competent Cells	<10%
200171 ABLE® C Competent Cells	200171-41 ABLE® C Competent Cells	<10%
200172 ABLE® C Competent Cells	200171-41 ABLE® K Competent Cells	<10%
200180 BacterioMatch® Two-Hybrid System Reporter Strain Competent Cells	200180-41 BacterioMatch® Reporter Competent Cells	<10%
200190 BacterioMatch® II Screening Reporter Competent Cells	200190-41 BacterioMatch® II Reporter Competent Cells	<10%
200192 BacterioMatch® II Validation Reporter Competent Cells	200192-41 BacterioMatch® II Reporter Competent Cells	<10%
200229 XL1-Blue MR Competent Cells	200229-41 XL1-Blue MR Competent Cells	<10%
200230 XL1-Blue MRF' Super Competent Cells	200230-41 XL1-Blue MRF' Competent Cells	<10%
200231 SCS 1 Supercompetent Cells	200230-41 SCS1 Competent Cells	<10%
200232 AG1 Competent Cells	200232-41 AG1 Competent Cells	<10%
200233 NM522 Competent Cells	200233-41 NM522 Competent Cells	<10%
200234 JM101 Competent Cells	200234-41 JM101 Competent Cells	<10%
200235 JM109 Competent Cells	200235-41 JM109 Competent Cells	<10%
200236 XL1-Blue Supercompetent Cells	200236-41 XL1-Blue Competent Cells	<10%
200238 SURE® Competent Cells	200238-41 SURE® Competent Cells	<10%
200239 JM110 Competent Cells	200239-41 JM110 Competent Cells	<10%
200247 SCS110 Competent Cells	200247-41 SCS110 Competent Cells	<10%
200248 XL1-Blue MRF' Kan Supercompetent Cells	200248-41 XL1-Blue MRF' Kan Competent Cells	<10%

200249 XL1-Blue Competent Cells	200236-41 XL1-Blue Competent Cells	<10%
200314 XL10-Gold® Ultracompetent Cells	200315-41 XL10-Gold® Competent Cells	<10%
200315 XL10-Gold® Ultracompetent Cells	200315-41 XL10-Gold® Competent Cells	<10%
200317 XL10-Gold® KANr Ultracompetent Cells	200317-41 XL10-Gold® KANr Ultracompetent Cells	<10%
200324 96Pack® Gold Competent Cells	200324-41 96Pack® Gold Competent Cells	<10%
230130 BL21-Gold Competent Cells	230130-41 BL21-Gold Competent Cells	<10%
230132 BL21-Gold(DE3) Competent Cells	230132-41 BL21-Gold(DE3) Competent Cells	<10%
230134 BL21-Gold(DE3) PLysS competent Cells	230134-41 BL21-Gold(DE3) PLysS Competent Cells	<10%
230135 BL21-Gold(DE3) LacZ Competent Cells	230135-41 BL21-Gold(DE3) LacZ Competent Cells	<10%
230191 ArcticExpress™ Competent Cells	230191-41 ArcticExpress™ Competent Cells	<10%
230192 ArcticExpress™ (DE3) Competent Cells	230192-41 ArcticExpress™ (DE3) Competent Cells	<10%
230193 ArcticExpress™ (DE3) RIL Competent Cells	230193-41 ArcticExpress™ (DE3) RIL Competent Cells	<10%
230194 ArcticExpress™ (DE3) RP Competent Cells	230194-41 ArcticExpress™ (DE3) RP Competent Cells	<10%
230195 ArcticExpress™ RIL Competent Cells	230195-41 ArcticExpress™ RIL Competent Cells	<10%
230196 ArcticExpress™ RP Competent Cells	230196-41 ArcticExpress™ RP Competent Cells	<10%
230240 BL21-CodonPlus®-RIL Competent Cells	230240-41 BL21- CodonPlus®-RIL Competent Cells	<10%
230245 BL21- CodonPlus® (DE3)-RIL Competent Cells	230245-41 BL21- CodonPlus® (DE3) RIL Competent Cells	<10%
230246 Protein Expression Competent Cell Pack	200131-41 BL-21 (DE3) Cells 230132-41 BL21-Gold(DE3) Cells 230280-41 BL21-CodonPlus® (DE3)-RIPL Cells 230134-41 BL21-Gold(DE3) PLysS Cells	<10%
230247 Difficult Cloning Competent Cell Pack	200317-41 XL10-Gold® KANr' Cells 200152-41 Ultra Comp SURE® Cells 200172-41 ABLE® K Cells	<10%
230248 Routine Cloning Competent Cell Pack	200150-41 Ultra Comp XL2-Blue Cells 200151-41 Ultra Comp XL2-Blue MRF Cells 230325-41 SoloPack® Gold Cells	<10%
230250 BL21- CodonPlus®-RP Competent Cells	230250-41 Codon® Plus RP Competent Cells 230255-41 Codon Plus® DE3 RP Competent Cells	<10%
230255 BL21-CodonPlus® (DE3)-RP Competent Cells	230255-41 Codon Plus® DE3 RP Competent Cells	<10%
230265 BL21-CodonPlus® (DE3)-RIL-X Competent Cells	230265-41 Codon Plus® RIL (DE3) MET Cells	<10%
230275 BL21-CodonPlus® (DE3)-RP-X Competent Cells	230275-41 Codon Plus® RP (DE3) MET-Cells	<10%
230280 BL21-CodonPlus® (DE3)-RIPL Competent Cells	230280-41 BL21-CodonPlus® (DE3)-RIPL Competent Cells	<10%
230315 XL10-Gold® Ultracompetent Cells	200315-41 XL10-Gold® Competent Cells	<10%
230325 SoloPack® Gold Competent Cells	230325-41 SoloPack® Gold Cells	<10%
230350 SoloPack® Gold Competent Cells	230350-41 SoloPack® Gold SuperCompetent Cells	<10%
929236 XL1-Blue Supercompetent Cells	929236-41 XL1-Blue Competent Cells	<10%

TOXICITY DATA

Irritation Data:

SKN-RBT	10 mg/24H open Mld.	AIHAAP	23,95,62
SKN-RBT	500 mg/24H Mld.	85JCAE	-,1044,86
EYE-RBT	100 mg	TXAPA9	39,129,77
EYE-RBT	500 mg/24H Mld.	85JCAE	-,1044,86

Toxicity Data:

ORL-RAT	LD_{50}:	14,500 mg/kg	TXAPA9	15,74,69
SKN-RAT	LD_{50}:	40 gm/kg	ANYAA9	141,96,67
IPR-RAT	LD_{50}:	8200 mg/kg	FCTOD7	22,665,84
SCU-RAT	LD_{50}:	12 gm/kg	ARZNAD	14,1050,64
IVN-RAT	LD_{50}:	5360 mg/kg	TXAPA9	7,104,65
UNR-RAT	LD_{50}:	1300 mg/kg	NTIS**	AD-A159-418
SKN-MUS	LD_{50}:	50 gm/kg	ANYAA9	141,96,67
ORL-MUS	LD_{50}:	7920 mg/kg	CHTPBA	3,10,68
IPR-MUS	LD_{50}:	2500 mg/kg	RPTOAN	35,300,72
SCU-MUS	LD_{50}:	14 gm/kg	ANYAA9	141,96,67
IVN-MUS	LD_{50}:	3100 mg/kg	TXAPA9	15,74,69
ORL-DOG	LD_{50}:	>10 gm/kg	ANYAA9	141,96,67
IVN-DOG	LD_{50}:	2500 mg/kg	CNCRA6	31,7,63
ORL-CKN	LD_{50}:	12 gm/kg	JPPMAB	15,688,63
ORL-MAM	LD_{50}:	21,400 mg/kg	GISAAA	39(4),86,74
ORL-BWD	LD_{50}:	100 mg/kg	TXAPA9	21,315,72

Reviews, Standards, and Regulations:

OEL=MAK

OEL-RUSSIA: STEL 20 mg/m^3 JAN 93

OEL-SWITZERLAND: TWA 50 ppm (160 mg/m^3); SKIN JAN 93

NOHS 1974: HZD 80564; NIS 11; TNF 476; NOS 25; TNE 22461

NOES 1983: HZD 80564; NIS 29; TNF 3507; NOS 40; TNE 44947; TFE 16837

EPA GENETOX program 1988, Positive: Aspergillus-Aneuploidy; S. Cerevisiae gene conversion.

EPA GENETOX program 1988, Negative: SHE-clonal assay; Cell transform. - mouse embryo.

EPA GENETOX program 1988, Negative: Cell transform. - RLV F344 Rat embryo.

EPA GENETOX program 1988, Negative: D. melanogaster - Whole sex chrom. Loss; Host -mediated assay.

EPA GENETOX program 1988, Negative: N. crassa - aneuploidy; E. coli PolA with S9.

EPA GENETOX program 1988, Negative: Histidine reversion - Ames test; in vitro SCE-nonhuman.

EPA GENETOX program 1988, Negative: D. melanogaster sex-linked lethal.

EPA GENETOX program 1988, Inconclusive: Aspergillus - Recombination; Carcinogenicity - Mouse/Rat

EPA GENETOX program 1988, Inconclusive: D. melanogaster - reciprocal translocation.

EPA GENETOX program 1988, Inconclusive: Rodent dominant lethal; B. subtilis REC assay.

EPA GENETOX program 1988, Inconclusive: E. coli PolA without S9.

EPA TSCA Section 8(B) Chemical Inventory

EPA TSCA Section 8(D) Unpublished health/safety studies

EPA TSCA Test Submission (TSCATS) Data Base, January 1997

Target Organ Data:
Behavioral (altered sleep time)
Gastrointestinal (nausea or vomiting)
Liver (jaundice, other or unclassified)
Effects on fertility (pre-implantation mortality)
Effects on embryo or fetus (Fetotoxicity)
Specific developmental abnormalities (musculoskeletal system)

Only selected Registry of Toxic Effects of Chemical Substances (RTECS) data is presented here. See actual entry in RTECS for complete information.

RTECS #: PV6210000, Methyl Sulfoxide

Health Hazard Data

Acute Effects:
May be harmful if swallowed, inhaled or absorbed through skin.
Vapor or mist is irritating to the eyes, mucous membranes and upper respiratory tract.
Causes skin irritation.
Avoid contact with DMSO solutions containing toxic materials or materials with unknown toxicological properties. Dimethyl Sulfoxide is readily absorbed through skin and may carry such materials into the body.

Chronic Effects:
Target Organs: Eyes, Skin

To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated.

First Aid:
Eyes: In case of contact, immediately flush with copious amounts of water for at least 15 minutes.
Skin: In case of contact, immediately flush with copious amounts of water for at least 15 minutes while removing contaminated clothing and shoes.
Inhalation: Remove to fresh air. If not breathing give artificial respiration. If breathing is difficult, give oxygen.
Ingestion: Wash out mouth with water provided person is conscious.
Wash contaminated clothing before reuse.
In all cases, call a physician.

Physical Data

MF: C_2H_6OS
Boiling point.....: 189°C
Melting point.....: 18.4°C
Specific Gravity (H_2O = 1): 1.101
Vapor Density.....: 2.7
Vapor Pressure.....: 0.42 mm @ 20°C
Flashpoint.....: 185°F (85°C)
Autoignition Temperature..: 573°F (300°C)
Explosion Limits in Air:
Lower .....: 3.5%
Upper .....: 63%

Fire and Explosion Hazard Data

Extinguishing Media:

Water Spray.
Carbon Dioxide, Dry chemical powder, or appropriate Foam

Special Firefighting Procedures:

Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes.
Combustible liquid.

Unusual Fire and Explosion Hazards:

Emits toxic fumes under fire conditions.

Reactivity Data

Incompatibilities:

Acid chlorides
Phosphorus halides
Strong acids
Strong oxidizing agents
Strong reducing agents
Sensitive to moisture

Hazardous Combustion or Decomposition Products:

Toxic fumes of :

Carbon monoxide and carbon dioxide
Sulfur oxides

Stability: Stable

Hazardous polymerization: Will not occur.

Additional Information: Methyl Sulfoxide (DMSO) undergoes a violent exothermic reaction on mixing with copper wool and trichloroacetic acid. On mixing with potassium permanganate, it will flash instantaneously. It reacts violently with: acid halides, cyanuric chloride, silicon tetrachloride, phosphorus trichloride and trioxide, thionyl chloride, magnesium perchlorate, silver fluoride, methyl bromide, iodine pentafluoride, nitrogen periodate, diborane, sodium hydride, perchloric and periodic acids. When heated above its boiling point, methyl sulfoxide degrades, giving off formaldehyde, methyl mercaptan, and sulfur dioxide.

Spill or Leak Procedures

Steps to be taken if Material is Released or Spilled:

- Evacuate area.
- Wear self-contained breathing apparatus, rubber boots and heavy rubber gloves.
- Absorb on sand or vermiculite and place in closed containers until proper disposal is possible.
- Ventilate the area and wash spill site after material pickup is complete.

Waste Disposal Method:

This combustible material may be burned in a chemical incinerator equipped with an afterburner and scrubber.
Observe all federal, state and local environmental regulations.

Precautions to be Taken in Handling and Storage

Wear appropriate NIOSH/MSHA-approved respirator, chemical-resistant gloves, safety goggles and other protective clothing.
Ensure that a safety shower and eye bath are available.
Use only in a chemical fume hood.
Do not breathe vapor.
Avoid contact with eyes, skin and clothing.
Avoid prolonged or repeated exposure.
Readily absorbed through skin.
Wash thoroughly after handling.
Keep tightly closed.
Keep away from heat and open flame.
Store in a cool, dry place.

Label Precautionary Statements:

Irritant.
Irritating to eyes, respiratory system, and skin.
Combustible.
Target Organs: eyes, skin
Readily absorbed through skin.
In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
Wear suitable protective clothing.
Do not breathe vapor.

The above information is believed to be correct but does not purport to be all-inclusive and shall be used only as a guide. Stratagene shall not be held liable for any damage resulting from handling or from contact with the above product.

Cell Biology

ATCC® Number:	**CRL-1573™** Order this Item	Price:	**$256.00**
Designations:	293 [HEK-293]	Depositors:	FL Graham
Biosafety Level:	2 [CELLS CONTAIN ADENOVIRUS]	Shipped:	frozen
Medium & Serum:	See Propagation	Growth Properties:	adherent epithelial
Organism:	*Homo sapiens* (human)	Morphology:	PHOTO
Source:	**Organ:** kidney **Cell Type:** transformed with adenovirus 5 DNA		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
	Related Cell Culture Products		
Restrictions:	These cells are distributed for research purposes only. 293 cells, their products, or their derivatives may not be distributed to third parties.		
Applications:	efficacy testing [92587] transfection host(technology from amaxa Roche FuGENE® Transfection Reagents) viruscide testing [92579]		
Receptors:	vitronectin, expressed		
Tumorigenic:	Yes		
DNA Profile (STR):	Amelogenin: X CSF1PO: 11,12 D13S317: 12,14 D16S539: 9,13 D5S818: 8,9 D7S820: 11,12 THO1: 7,9.3 TPOX: 11 vWA: 16,19		
Cytogenetic Analysis:	This is a hypotriploid human cell line. The modal chromosome number was 64, occurring in 30% of cells. The rate of cells with higher ploidies was 4.2 %. The der(1)t(1;15) (q42;q13), der(19)t(3;19) (q12;q13), der(12)t(8;12) (q22;p13), and four other marker chromosomes were common to most cells. Five other markers occurred in some cells only. The marker der(1) and M8 (or Xq+) were often paired. There were four copies of N17 and N22. Noticeably in addition to three copies of X chromosomes, there were paired Xq+, and a single Xp+ in most cells.		
Age:	fetus		
	Although an earlier report suggested that the cells contained Adenovirus 5 DNA from both the right and left ends of the viral genome [RF32764], it is now clear that only left end sequences are present. [39768] The line is excellent for titrating human adenoviruses.		

Comments:

The cells express an unusual cell surface receptor for vitronectin composed of the integrin beta-1 subunit and the vitronectin receptor alpha-v subunit. [23406]
The Ad5 insert was cloned and sequenced, and it was determined that a colinear seqment from nts 1 to 4344 is integrated into chromosome 19 (19q13.2). [39768]

Propagation:

ATCC complete growth medium: The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.
Atmosphere: air, 95%; carbon dioxide (CO2), 5%
Temperature: 37.0°C
The cell line does not adhere to the substrate when left at room temperature for any length of time, therefore, live cultures may be received with the cells detached.The cells will re-attach to the flask over a period of several days in culture at 37C.

Subculturing:

Protocol:

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Preservation:

Subcultivation Ratio: A subcultivation ratio of 1:2 to 1:4 is recommended
Medium Renewal: Every 2 to 3 days
Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO
Storage temperature: liquid nitrogen vapor phase

Related Products:

Recommended medium (without the additional supplements or serum described under ATCC Medium): ATCC 30-2003
derivative: ATCC CRL-10852
derivative: ATCC CRL-12006
derivative: ATCC CRL-12007
derivative: ATCC CRL-12013
derivative: ATCC CRL-12479
derivative: ATCC CRL-2029
derivative: ATCC CRL-2368
purified DNA:ATCC CRL-1573D

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33137: Arai H, Charo IF. Differential regulation of G-protein-mediated signaling by chemokine receptors. J. Biol. Chem. 271: 21814-21819, 1996. PubMed: 8702980
33138: Huang Q, et al. Substrate recognition by tissue factor-factor VIIa. J. Biol. Chem. 271: 21752-21757, 1996. PubMed: 8702971
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92587: Standard Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal and Sporicidal Activities of

Liquid Chemical Germicides. West Conshohocken, PA:ASTM International;ASTM Standard Test Method E 2197-02.

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Cell Biology

ATCC® Number:	**HTB-26™** Order this Item	Price:	**$256.00**
Designations:	MDA-MB-231	Depositors:	R Cailleau
Biosafety Level:	1	Shipped:	frozen
Medium & Serum:	See Propagation	Growth Properties:	adherent epithelial
Organism:	*Homo sapiens* (human)	Morphology:	

Source:
Organ: mammary gland; breast
Disease: adenocarcinoma
Derived from metastatic site: pleural effusion
Cell Type: epithelial

Permits/Forms:
In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Related Cell Culture Products

Applications:
transfection host (technology from amaxa Roche FuGENE® Transfection Reagents)

Receptors:
epidermal growth factor (EGF), expressed
transforming growth factor alpha (TGF alpha), expressed

Tumorigenic:
YES

DNA Profile (STR):
Amelogenin: X
CSF1PO: 12,13
D13S317: 13
D16S539: 12
D5S818: 12
D7S820: 8,9
THO1: 7,9.3
TPOX: 8,9
vWA: 15,18

Cytogenetic Analysis:
The cell line is aneuploid female (modal number = 64, range = 52 to 68), with chromosome counts in the near-triploid range. Normal chromosomes N8 and N15 were absent. Eleven stable rearranged marker chromosomes are noted as well as unassignable chromosomes in addition to the majority of autosomes that are trisomic. Many of the marker chromosomes are identical to those shown in the karyotype reported by K.L. Satya-Prakash, et al.

Isoenzymes:
AK-1, 1
ES-D, 1
G6PD, B
GLO-I, 2
Me-2, 1-2
PGM1, 1-2
PGM3, 1

Age:	51 years adult
Gender:	female
Ethnicity:	Caucasian
Comments:	The cells express the WNT7B oncogene [PubMed: 8168088].
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Leibovitz's L-15 Medium, Catalog No. 30-2008. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Atmosphere:** air, 100% **Temperature:** 37.0°C **Protocol:**
Subculturing:	1. Remove and discard culture medium. 2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. 3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. 4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting. 5. Add appropriate aliquots of the cell suspension to new culture vessels. 6. Incubate cultures at 37°C without CO2.
Preservation:	**Subcultivation Ratio:** A subcultivation ratio of 1:2 to 1:4 is recommended **Medium Renewal:** 2 to 3 times per week **Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO **Storage temperature:** liquid nitrogen vapor phase
Related Products:	purified DNA:ATCC 45519 purified DNA:ATCC HTB-26D purified DNA:ATCC 45518 Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2008 purified RNA:ATCC HTB-26R recommended serum:ATCC 30-2020

1206: Brinkley BR, et al. Variations in cell form and cytoskeleton in human breast carcinoma cells in vitro. Cancer Res. 40: 3118-3129, 1980. PubMed: 7000337
22182: Cruciger Q, et al. Morphological, biochemical and chromosomal characterization of breast tumor lines from pleural effusions. In Vitro 12: 331, 1976.
22429: Siciliano MJ, et al. Mutually exclusive genetic signatures of human breast tumor cell lines with a common chromosomal marker. Cancer Res. 39: 919-922, 1979. PubMed: 427779
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References:

Cancer Inst. 53: 661-674, 1974. PubMed: 4412247
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23113: Huguet EL, et al. Differential expression of human Wnt genes 2, 3, 4, and 7B in human breast cell lines and normal and disease states of human breast tissue. Cancer Res. 54: 2615-2621, 1994. PubMed: 8168088
26321: Satya-Prakash KL, et al. Cytogenetic analysis on eight human breast tumor cell lines: high frequencies of 1q, 11q and HeLa-like marker chromosomes. Cancer Genet. Cytogenet. 3: 61-73, 1981. PubMed: 7272986
32272: Katayose Y, et al. Promoting apoptosis: a novel activity associated with the Cyclin-dependent kinase inhibitor p27. Cancer Res. 57: 5441-5445, 1997. PubMed: 9407946
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32489: De Vincenzo R, et al. Antiproliferative activity of colchicine analogues on MDR-positive and MDR-negative human cancer cell lines. Anticancer Drug Des. 13: 19-33, 1998. PubMed: 9474240
33021: Soker S, et al. Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-endoded domain. J. Biol. Chem. 271: 5761-5767, 1996. PubMed: 8621443

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Product Description

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Cell Biology

ATCC® Number: HTB-129™ Order this Item

Price: $264.00

Designations: MDA-MB-435S

Biosafety Level: 1

Shipped: frozen

Medium & Serum: See Propagation

Growth Properties: adherent

Organism: *Homo sapiens* (human)

Morphology: spindle shaped

Source: **Organ:** previously described as: mammary gland; breast
Disease: previously described as ductal carcinoma
Derived from metastatic site: pleural effusion

Cellular Products: tubulin; actin

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Isolation: **Isolation date:** 1976

Tumorigenic: No

DNA Profile (STR): Amelogenin: X
CSF1PO: 11
D13S317: 12
D16S539: 13
D5S818: 12
D7S820: 8,10
THO1: 6,7
TPOX: 8,11
vWA: 16,18

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Material Transfer Agreement

Technical Support

Related Cell Culture Products

Cytogenetic Analysis: modal number = 56; range = 55 to 62
The cell line is aneuploid human female (XX), with most chromosome counts in the 55 to 60 range. Normal chromosomes N6, N11, and N22 were absent, while chromosomes N7, N13, N18 and N21 were single. Most of the remainder of normal chromosomes were usually paired, but chromosome N2 was triple. Nineteen marker chromosomes were identified, with most of them formed from structural alterations of the missing copies of the normal chromosomes. Six of these markers involve regions of chromosome N7, while three are recognized as derivatives of chromosome N6. Regions of a third copy of the normal and paired chromosomes N3, N15, N17, N20 are noted in markers M1, M2, M15, and M5, respectively.

Isoenzymes: AK-1, 1
ES-D, 1
G6PD, B
GLO-I, 2
PGM1, 2
PGM3, 1

Age: 31 years adult

Gender: female

Ethnicity: Caucasian

Comments: This cell line was originally described as a spindle shaped variant of the parental MDA-MB-435 strain isolated in 1976 by R. Cailleau, et al. from the pleural effusion of a 31 year old female with metastatic, ductal adenocarcinoma of the breast. However, recent studies have generated questions about the origin of the parent cell line, MDA-MB-435, and by extension HTB-129. Gene expression analysis of the cells produced microarrays in which MDA-MB-435 clustered with cell lines of melanoma origin instead of breast [PubMed ID: 10700174, PubMed ID: 15150101, PubMed ID: 15679052]. Additional studies have since corroborated a melanocyte origin of MDA-MB-435, to which ATCChas responded by pursuing its own investigation into the identity of this cell line. The cell line to which MDA-MB-435 is reported to have been cross-contaminated with is the M14 melanoma line [PubMed ID: 12354931 and PubMed ID: 17004106].
Derivatives of HTB-129 with identities in question:
M4A4, ATCC® CRL-2914
M4A4 GFP, ATCC® CRL-2915
M4A4 LM3-2 GFP, ATCC® CRL-2916
M4A4 LM3-4 CL 16 GFP, ATCC® CRL-2917
NM2C5, ATCC® CRL-2918
NM2C5 GFP, ATCC® CRL-2919

Propagation:

ATCC complete growth medium: The base medium for this cell line is ATCC-formulated Leibovitz's L-15 Medium, Catalog No. 30-2008. To make the complete growth medium, add the following components to the base medium:

- 0.01mg/ml bovine insulin
- 0.01mg/ml glutathione
- fetal bovine serum to a final concentration of 10%

Atmosphere: air, 100%
Temperature: 37.0°C

Subculturing: **Protocol:** Remove medium, add fresh 0.25%trypsin - 0.53 mM EDTA, rinse and remove. Place flask at room temperature (or incubated at 37C) for approximately 10 minutes or until the cells detach. Add fresh medium, aspirate and dispense into new flasks.
Subcultivation Ratio: A subcultivation ratio of 1:3 to 1:6 is recommended
Medium Renewal: 2 to 3 times per week

Preservation: **Freeze medium:** Culture medium, 95%; DMSO, 5%
Storage temperature: liquid nitrogen vapor phase

Related Products: Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2008
recommended serum:ATCC 30-2020
purified DNA:ATCC HTB-129D
purified RNA:ATCC HTB-129R

References:

1206: Brinkley BR, et al. Variations in cell form and cytoskeleton in human breast carcinoma cells in vitro. Cancer Res. 40: 3118-3129, 1980. PubMed: 7000337
22429: Siciliano MJ, et al. Mutually exclusive genetic signatures of human breast tumor cell lines with a common chromosomal marker. Cancer Res. 39: 919-922, 1979. PubMed: 427779
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49803: Ross DT, et al. Systematic variation in gene expression patterns in human cancer cell lines. Nature Genetics 24: 227-235, 2000. PubMed: 10700174
89918: Ellison G, et al. Further evidence to support the melanocytic origin of MDA-MB-435. Mol. Pathol. 55: 294-299, 2002. PubMed: 12354931
90826: Sellappan s, et al. Lineage infidelity of MDA-MB-435 cells: expression of melanocyte proteins in a breast cancer cell line. Cancer Res. 64: 3479-3485, 2004. PubMed: 15150101
90828: Rae JM, et al. Common origins of MDA-MB-435 cells from various sources with those shown to have melanoma properties. Clin. Exp. Metastasis 21: 543-552, 2004. PubMed: 15679052
16173093: Rae JM, et al., MDA-MB-435 cells are derived from M14 Melanoma cells - a loss for breast cancer, but a boon for melanoma research. Breast Cancer Res. Treat. 104:13-19, 2007. PubMed: 17004106.
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Cell Biology

ATCC® Number:	**CRL-10317™** Order this Item	Price:	**$264.00**
Designations:	MCF 10A	Depositors:	Michigan Cancer Foundation
Biosafety Level:	1	Shipped:	frozen
Medium & Serum:	See Propagation	Growth Properties:	adherent
Organism:	*Homo sapiens* (human)	Morphology:	epithelial
Source:	**Organ:** mammary gland; breast **Disease:** fibrocystic disease **Cell Type:** epithelial		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

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Related Cell Culture Products

Isolation:	**Isolation date:** August 22, 1984
Applications:	transfection host (Roche FuGENE® Transfection Reagents)
Tumorigenic:	No
DNA Profile (STR):	Amelogenin: X CSF1PO: 10,12 D13S317: 8,9 D16S539: 11,12 D5S818: 10,13 D7S820: 10,11 THO1: 8,9.3 TPOX: 9,11 vWA: 15,17
Isoenzymes:	AK-1, 1 [23084] ES-D, 1 [23084] G6PD, B [23084] GLO-I, 1-2 [23084] PGM1, 1-2 [23084] PGM3, 1 [23084]
Age:	36 years
Gender:	female
Ethnicity:	Caucasian

The MCF 10A cell line is a non-tumorigenic epithelial cell line. [21968]
The line was produced by long term culture in serum free medium with low Ca++

concentration. [21968]
MCF 10A was derived from adherent cells in the population. [21968]
Cells derived from a floating population are available (see MCF 10F, ATCC CRL-10318). [21968]
The cells are positive for epithelial sialomucins, cytokeratins and milk fat globule antigen. [21968]
They exhibit three dimensional growth in collagen, and form domes in confluent cultures. [21968]

Comments:

Thus far, the cells have shown no signs of terminal differentiation or senescence. The line is responsive to insulin, glucocorticoids, cholera endotoxin, and epidermal growth factor (EGF). [21968]
By electron microscopy the cells display characteristics of luminal ductal cells but not of myoepithelial cells. [23085]
They also express breast specific antigens as detected by positive reaction with MFA-Breast and MC-5 monoclonal antibodies. [23085]
The calcium content of the medium exerts a strong effect on the morphology of the cells. [22248]

Propagation:

ATCC complete growth medium: The base medium for this cell line is MEBM, which is supplied as part of the MEGM Bullet Kit available from Clonetics Corporation, Catalog No. CC-3150. To make the complete growth medium, add the following components to the base medium: All MEGM SingleQuot additives that are supplied with the kit except the GA-1000 (BPE 13 mg/ml, 2 ml; hydrocortisone 0.5 mg/ml, 0.5 ml; hEGF 10 ug/ml, 0.5 ml; insulin 5 mg/ml, 0.5 ml); 100 ng/ml cholera toxin (sold separately).
Temperature: 37.0°C

Subculturing:

Protocol: Remove medium and rinse monolayer with PBS (ATCC Cat# 30-2200).Add 3.0 ml 0.05% trypsin, 0.53 mM EDTA and incubate at 37C for 15 minutes. To neutralize trypsin, add 3 ml solution of 0.1% soybean trypsin inhibitor.Centrifuge cell suspension at 125 xg for 5 to 10 minutes.Resuspend cell pellet in complete culture medium. Add appropriate aliquots of cell suspension to new culture vessels.
Subcultivation Ratio: A subcultivation ratio of 1:3 to 1:4 is recommended
Medium Renewal: Every 2 to 3 days

Preservation:

Freeze medium: Complete growth medium supplemented with 7.5% (v/v) DMSO
Storage temperature: liquid nitrogen vapor phase

Related Products:

derived from same individual:ATCC CRL-10318
derived from same individual:ATCC CRL-10780
derived from same individual:ATCC CRL-10781

References:

21968: Soule H, McGrath CM. Immortal human mammary epithelial cell lines. US Patent 5,026,637 dated Jun 25 1991
22025: Pauley RJ, et al. Immortal human mammary epithelial cell sublines. US Patent 5,206,165 dated Apr 27 1993
22248: Soule HD, McGrath CM. A simplified method for passage and long-term growth of human mammary epithelial cells. In Vitro Cell. Dev. Biol. 22: 6-12, 1986. PubMed: 2418007
23084: Soule HD, et al. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res. 50: 6075-6086, 1990. PubMed: 1975513

23085: Tait L, et al. Ultrastructural and immunocytochemical characterization of an immortalized human breast epithelial cell line, MCF-10. Cancer Res. 50: 6087-6094, 1990. PubMed: 1697506

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Cell Biology

ATCC® Number:	**CRL-1435™** Order this Item	Price:	**$256.00**
Designations:	PC-3	Depositors:	ME Kaighn
Biosafety Level:	1	Shipped:	frozen
Medium & Serum:	See Propagation	Growth Properties:	adherent (The cells form clusters in soft agar and can be adapted to suspension growth)
Organism:	*Homo sapiens* (human)	Morphology:	epithelial 
Source:	**Organ:** prostate **Tumor Stage:** grade IV **Disease:** adenocarcinoma **Derived from metastatic site:** bone		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

Related Cell Culture Products

Applications:	transfection host (technology from amaxa Roche FuGENE® Transfection Reagents)
Tumorigenic:	YES
Antigen Expression:	HLA A1, A9
DNA Profile (STR):	Amelogenin: X CSF1PO: 11 D13S317: 11 D16S539: 11 D5S818: 13 D7S820: 8,11 THO1: 6,7 TPOX: 8,9 vWA: 17
Cytogenetic Analysis:	The line is near-triploid with a modal number of 62 chromosomes. There are nearly 20 marker chromosomes commonly found in each cell; and normal N2, N3, N4, N5, N12, and N15 are not found. No normal Y chromosomes could be detected by Q-band analysis.
Age:	62 years adult
Gender:	male
Ethnicity:	Caucasian
Comments:	The PC-3 was initiated from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old male Caucasian. [22363] The cells exhibit low acid phosphatase and testosterone-5-alpha reductase activities.

Propagation:

ATCC complete growth medium: The base medium for this cell line is ATCC-formulated F-12K Medium, Catalog No. 30-2004. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.
Atmosphere: air, 95%; carbon dioxide (CO2), 5%
Temperature: 37.0°C
Protocol:

Subculturing:

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Preservation:

Subcultivation Ratio: A subcultivation ratio of 1:3 to 1:6 is recommended
Medium Renewal: 2 to 3 times per week
Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO
Storage temperature: liquid nitrogen vapor phase

Related Products:

Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2004
recommended serum:ATCC 30-2020

References:

22363: Kaighn ME, et al. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest. Urol. 17: 16-23, 1979. PubMed: 447482
22470: Chen TR. Chromosome identity of human prostate cancer cell lines, PC-3 and PPC-1. Cytogenet. Cell Genet. 62: 183-184, 1993. PubMed: 8428522
26302: Ohnuki Y, et al. Chromosomal analysis of human prostatic adenocarcinoma cell lines. Cancer Res. 40: 524-534, 1980. PubMed: 7471073
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32488: Geiger T, et al. Antitumor activity of a PKC-alpha antisense

oligonucleotide in combination with standard chemotherapeutic agents against various human tumors transplanted into nude mice. Anticancer Drug Des. 13: 35-45, 1998. PubMed: 9474241
32916: Su ZZ, et al. Surface-epitope masking and expression cloning identifies the human prostate carcinoma tumor antigen gene PCTA-1 a member of the galectin gene family. Proc. Natl. Acad. Sci. USA 93: 7252-7257, 1996. PubMed: 8692978

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Cell Biology

ATCC® Number:	**CRL-2221™** Order this Item	Price:	**$338.00**
Designations:	PZ-HPV-7	Depositors:	DM Peehl
Biosafety Level:	2 [CELLS CONTAIN PAPOVAVIRUS]	Shipped:	frozen
Medium & Serum:	See Propagation	Growth Properties:	adherent
Organism:	*Homo sapiens* (human)	Morphology:	epithelial

Source:
Organ: prostate
Tissue: epithelium
Cell Type: human papillomavirus 18 (HPV-18) transformed

Permits/Forms:
In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Related Cell Culture Products

Tumorigenic: N0

DNA Profile (STR):
Amelogenin: X,Y
CSF1PO: 11,12
D13S317: 12,14
D16S539: 11,12
D5S818: 9,13
D7S820: 9
THO1: 7,9
TPOX: 8,11
vWA: 17

Cytogenetic Analysis: at low passages maintained the diploid karyotype of the normal parental cells but by passage 99 the karyotype had changed to near-triploid.

Age: 70 years adult

Gender: male

Ethnicity: Caucasian

Comments:
PZ-HPV-7 was derived from epithelial cells cultured from normal tissue from the peripheral zone of the prostate.
The cells were transformed by transfection with HPV18 DNA.
Incorporation of HPV18 DNA was confirmed by polymerase chain reaction.
Specific amplification of a 160-base pair fragment of the HPV18 E6 transforming region was noted.
Immunocytochemical analysis showed expression of keratins 5 and 8 and also the early region 6 (E6) oncoprotein of HPV.
The cells are negative for prostate specific antigen (PSA).

Propagation:
ATCC complete growth medium: The base medium for this cell line is provided by Invitrogen (GIBCO) as part of a kit: Keratinocyte Serum Free Medium (K-SFM), Kit Catalog Number 17005-042. This kit is supplied with each of the two additives required to grow this cell line (bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF). To make the complete growth

	medium, you will need to add the following components to the base medium: • 0.05 mg/ml BPE - provided with the K-SFM kit • 5 ng/ml EGF - provided with the K-SFM kit. NOTE: Do not filter complete medium. **Temperature:** 37.0°C
Subculturing:	**Protocol:** Remove spent medium, add fresh 0.25% trypsin, 0.53 mM EDTA solution; let the culture set incubate at 37C for two minutes. Neutralize the trypsin with 0.1% soybean trypsin inhibitor, and gently dislodge the cells by agitating or tapping the flask. Centrifuge the cell suspension at 1000 rpm for 10 minutes, resuspend the pellet in fresh medium, aspirate and dispense into new flasks. **Subcultivation Ratio:** A subcultivation ratio of 1:3 is recommended **Medium Renewal:** Every 2 to 3 days
Preservation:	**Freeze medium:** Complete growth medium, 85%; fetal bovine serum, 10%; DMSO, 5% **Storage temperature:** liquid nitrogen vapor temperature
Related Products:	purified DNA:ATCC CRL-2221D
References:	23116: Weijerman PC, et al. Lipofection-mediated immortalization of human prostatic epithelial cells of normal and malignant origin using human papillomavirus type 18 DNA. Cancer Res. 54: 5579-5583, 1994. PubMed: 7923200

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Cell Biology

ATCC® Number:	**HTB-36™** Order this Item	Price:	**$268.00**
Designations:	JEG-3	Depositors:	G Kohler
Biosafety Level:	1	Shipped:	frozen
Medium & Serum:	See Propagation	Growth Properties:	adherent epithelial
Organism:	*Homo sapiens* (human)	Morphology:	
Source:	**Organ:** placenta **Disease:** choriocarcinoma		
Cellular Products:	human chorionic gonadotropin (hCG), human chorionic somatomammotropin (placental lactogen); progesterone		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		

Related Cell Culture Products

Applications:	transfection host (Roche FuGENE® Transfection Reagents)
Tumorigenic:	YES
DNA Profile (STR):	Amelogenin: X,Y CSF1PO: 11,12 D13S317: 9,11 D16S539: 13,14 D5S818: 10,11 D7S820: 10,12 THO1: 9,9.3 TPOX: 8 vWA: 16
Cytogenetic Analysis:	This is a hypertriploid human cell line. The modal chromosome number is 71, occurring at 34%, and polyploidy at 2.6%. The t(4;11)(p15;q13), i(13q), t (10p15q), del(18)(q21), and 6 other markers are common to most cells, and two other markers are found in some. Giant satellites are seen in one N14, and two N22. N2, N5, and N9 have 4 copies, and N7, N13, N18, N21 and X a single copy. A single Y chromosome is detected by Q-band examination.
Isoenzymes:	AK-1, 1 ES-D, 1 G6PD, B GLO-I, 1-2 PGM1, 1 PGM3, 1-2
Comments:	This is one of six clonally derived lines isolated from the Woods strain of the Erwin-Turner tumor by Kohler and associates. The cells are able to transform steroid precursors to estrone and estradiol

ATCC complete growth medium: The base medium for this cell line is ATCC-

Propagation:	formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Temperature:** 37.0°C
Subculturing:	**Subcultivation Ratio:** A subcultivation ratio of 1:4 to 1:6 is recommended **Medium Renewal:** 2 to 3 times per week Remove medium, and rinse with 0.25% trypsin, 0.03% EDTA solution. Remove the solution and add an additional 1 to 2 ml of trypsin-EDTA solution. Allow the flask to sit at room temperature (or at 37C) until the cells detach. Add fresh culture medium, aspirate and dispense into new culture flasks.
Preservation:	**Freeze medium:** Culture medium, 95%; DMSO, 5% **Storage temperature:** liquid nitrogen vapor phase
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2003 recommended serum:ATCC 30-2020
References:	22536: Fogh J, et al. Absence of HeLa cell contamination in 169 cell lines derived from human tumors. J. Natl. Cancer Inst. 58: 209-214, 1977. PubMed: 833871 22539: Fogh J, et al. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. J. Natl. Cancer Inst. 59: 221-226, 1977. PubMed: 327080 23377: . . Acta Endocrinol. Suppl. 153: 137-153, 1971. 32288: Landers JE, et al. Translational enhancement of mdm2 oncogene expression in human tumor cells containing a stabilized wild-type p53 protein. Cancer Res. 57: 3562-3568, 1997. PubMed: 9270029 32564: Roesler WJ, et al. The alpha-isoform of the CCAAT/enhancer-binding protein is required for mediating cAMP responsiveness of the phosphoenolpyruvate carboxykinase promoter in hepatoma cells. J. Biol. Chem. 271: 8068-8074, 1996. PubMed: 8626491 58051: Kohler PO, Bridson WE. Isolation of hormone-producing clonal lines of human choriocarcinoma. J. Clin. Endocrinol. 32: 683-687, 1971.

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GripTite™ 293 MSR Cell Line

SKU# R795-07

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Description:
The GripTite™ 293 MSR Cell Line is a genetically engineered Human Embryonic Kidney (HEK 293) cell line that expresses the human macrophage scavenger receptor and strongly adheres to standard tissue culture plates for dependable results. Developed from a 293-H subclone, GripTite™ 293 MSR cells show the same fast cell growth, high transfection efficiency, and high-level expression characteristics of the parental 293-H cells. Unlike most 293 cells, GripTite® 293 MSR cells dont wash away during the repeated manipulations of routine tissue culture or plate washing protocols (Figure 1). Their superior adherence enables reliable reproduction of ligand-binding, enzymatic, or immunofluorescence assay results.

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Contents and Storage:
The GripTite™ 293 MSR Cell Line is provided as two tubes of a frozen stock of 3 x 10^6 cells, each in 1 ml of 90% complete media and 10% DMSO. Store in liquid nitrogen. Liquid Geneticin® Selective Antibiotic (20 ml) is supplied as a 50 mg/ml solution. Store at -20°C or 2°C to 8°C. Versene (100 ml) contains 0.2 g/L EDTA 4Na in phosphate-buffered saline. 1X concentration is 0.2 g/L=0.53 mM. Store at 2°C to 8°C. Guaranteed stable for 6 months when properly stored.

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Cell Biology

ATCC® Number:	**CRL-1674™**	Order this Item	Price:	**$417.00**
Designations:	ARIP		Depositors:	NW Jessop, RJ Hay
Biosafety Level:	1		Shipped:	frozen
Medium & Serum:	See Propagation		Growth Properties:	adherent
Organism:	Rattus norvegicus (rat)		Morphology:	epithelial

Source:
Organ: pancreas
Strain: Wistar
Tissue: exocrine
Disease: pancreatic tumor

Cellular Products: exocrine enzymes (low levels)

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Related Cell Culture Products

Tumorigenic: No

Propagation:
ATCC complete growth medium: The base medium for this cell line is ATCC-formulated F-12K Medium, Catalog No. 30-2004. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.
Atmosphere: air, 95%; carbon dioxide (CO2), 5%
Temperature: 37.0°C
Protocol:

Subculturing:
1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:3 to 1:6 is recommended
Medium Renewal: Every 2 to 3 days

Preservation:
Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO
Storage temperature: liquid nitrogen vapor phase

Related Products:

Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2004
recommended serum:ATCC 30-2020

References:

22185: Jessop NW, Hay RJ. Characteristics of two rat pancreatic exocrine cell lines derived from transplantable tumors. In Vitro 16: 212, 1980.
22884: Cockell M, et al. Identification of a cell-specific DNA-binding activity that interacts with a transcriptional activator of genes expressed in the acinar pancreas. Mol. Cell. Biol. 9: 2464-2476, 1989. PubMed: 2788241
22978: Roux E, et al. The cell-specific transcription factor PTF1 contains two different subunits that interact with the DNA. Genes Dev. 3: 1613-1624, 1989. PubMed: 2612907
51602: Hui H, et al. Glucagon-like peptide 1 induces differentiation of islet duodenal homeobox-1-positive pancreatic ductal cells into insulin-secreting cells. Diabetes 50: 785-796, 2001. PubMed: 11289043
90276: Silver K, Yao F. ARIP cells as a model for pancreatic beta cell growth and development. Pancreas 22: 141-147, 2001. PubMed: 11249068

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Cell Biology

ATCC® Number:	**CRL-1492™** Order this Item	Price:	**$268.00**
Designations:	AR42J	Depositors:	NW Jessop
Biosafety Level:	1	Shipped:	frozen
Medium & Serum:	See Propagation	Growth Properties:	adherent epithelial
Organism:	Rattus norvegicus (rat)	Morphology:	

Source:
Organ: pancreas
Strain: Wistar
Tissue: exocrine
Disease: tumor

Cellular Products: amylase and other exocrine enzymes [22185]

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

Related Cell Culture Products

Applications: transfection host(Roche FuGENE® Transfection Reagents)

Receptors: insulin, expressed
glucocorticoid, expressed

Tumorigenic: Yes

Comments: Secretory activity is inducible by glucocorticoid stimulation, and is accompanied by extensive re-organization of the endoplasmic reticulum.

Propagation:
ATCC complete growth medium: The base medium for this cell line is ATCC-formulated F-12K Medium, Catalog No. 30-2004. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 20% .
Atmosphere: air, 95%; carbon dioxide (CO2), 5%
Temperature: 37.0°C
Growth Conditions: The cells cells grow slowly, in clusters. They tend to pile up and appear refractile.

Protocol: Monolayer never becomes confluent. Subculture when patches of cells start forming "domes".

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

Subculturing:

4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. To remove trypsin-EDTA solution, transfer cell suspension to centrifuge tube and spin at approximately 125 xg for 5 to 10 minutes.Discard supernatant and resuspend cells in fresh growth medium. Add appropriate aliquots of cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:3 to 1:4 is recommended
Medium Renewal: Every 3 to 4 days. May need to only add media initially, do not fluid change until cells attach well.

Preservation:

Freeze medium: Complete growth medium supplemented with an additional 30% (v/v) fetal bovine serum and 10% (v/v) DMSO
Storage temperature: liquid nitrogen vapor phase

Related Products:

Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2004
recommended serum:ATCC 30-2020

References:

22185: Jessop NW, Hay RJ. Characteristics of two rat pancreatic exocrine cell lines derived from transplantable tumors. In Vitro 16: 212, 1980.
22384: Longnecker DS, et al. Transplantation of azaserine-induced carcinomas of pancreas in rats. Cancer Lett. 7: 197-202, 1979. PubMed: 509403
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48308: Adell T, et al. Role of the basic helix-loop-helix transcription factor p48 in the differentiation phenotype of exocrine pancreas cancer cells. Cell Growth Differ. 11: 137-147, 2000. PubMed: 10768861
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48311: Negre F, et al. Autocrine stimulation of AR4-2J rat pancreatic tumor cell growth by glycine-extended gastrin. Int. J. Cancer 66: 653-658, 1996. PubMed: 8647628
48312: Bertrand V, et al. Inhibition of gastrin-induced proliferation of AR4-2J cells by calcium channel antagonists. Int. J. Cancer 56: 427-432, 1994. PubMed: 7508895
57433: Mashima H, et al. Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. J. Clin. Invest. 97: 1647-1654, 1996. PubMed: 8601630
57434: Palgi J, et al. Transcription factor expression and hormone production in pancreatic AR42J cells. Mol. Cell. Endocrinol. 165: 41-49, 2000. PubMed: 10940482
90275: Mashima H, et al. Formation of insulin-producing cells from pancreatic acinar AR42J cells by hepatocyte growth factor. Endocrinology 137: 3969-3976, 1993. PubMed: 8756573
90276: Silver K, Yao F. ARIP cells as a model for pancreatic beta cell growth and development. Pancreas 22: 141-147, 2001. PubMed: 11249068

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Cell Biology

ATCC® Number:	**CRL-1651™** Order this Item	Price:	**$264.00**
Designations:	COS-7	Depositors:	Y Gluzman
Biosafety Level:	2 [Cells Contain SV-40 viral DNA sequences]	Shipped:	frozen
Medium & Serum:	See Propagation	Growth Properties:	adherent fibroblast
Organism:	*Cercopithecus aethiops*	Morphology:	
Source:	**Organ:** kidney **Cell Type:** SV40 transformed		
Cellular Products:	T antigen		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
	Related Cell Culture Products		
Applications:	transfection host(technology from amaxa Roche FuGENE® Transfection Reagents)		
Virus Susceptibility:	SV40 (lytic growth); SV40 tsA209 at 40C; SV40 mutants with deletions in the early region		
Comments:	This is an African green monkey kidney fibroblast-like cell line suitable for transfection by vectors requiring expression of SV40 T antigen. This line contains T antigen, retains complete permissiveness for lytic growth of SV40, supports the replication of ts A209 virus at 40C, and supports the replication of pure populations of SV40 mutants with deletions in the early region.The line was derived from the CV-1 cell line (ATCC ® CCL-70?) by transformation with an origin defective mutant of SV40 which codes for wild type T antigen.		
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Atmosphere:** air, 95%; carbon dioxide (CO2), 5% **Temperature:** 37.0°C		
Subculturing:	**Protocol:** 1. Remove and discard culture medium. 2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. 3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach		

may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:4 to 1:8 is recommended
Medium Renewal: 2 to 3 times per week

Preservation:

Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO
Storage temperature: liquid nitrogen vapor phase

Related Products:

parental cell line:ATCC CCL-70
0.25% (w/v) Trypsin - 0.53 mM EDTA in Hank' BSS (w/o Ca++, Mg++):ATCC 30-2101
Cell culture tested DMSO:ATCC 4-X
Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2002
recommended serum:ATCC 30-2020

References:

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33137: Arai H, Charo IF. Differential regulation of G-protein-mediated signaling by chemokine receptors. J. Biol. Chem. 271: 21814-21819, 1996. PubMed: 8702980
33163: Hsieh CM, et al. APEG-1, a novel gene preferentially expressed in aortic smooth muscle cells, is down-regulated by vascular injury. J. Biol. Chem. 271: 17354-17359, 1996. PubMed: 8663449
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Frontiers in Neuroendocrinology xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Frontiers in Neuroendocrinology

journal homepage: www.elsevier.com/locate/yfrne

Review

Hypothalamic cell lines to investigate neuroendocrine control mechanisms

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ARTICLE INFO

Keywords:
Hypothalamus
Neuronal cell line
Neuroendocrinology
Gene expression
Signal transduction
Molecular techniques
Cell biology
Energy homeostasis
Reproduction
Circadian rhythms

ABSTRACT

The hypothalamus is the control center for most physiological processes; yet has been difficult to study due to the inherent heterogeneity of this brain region. For this reason, researchers have turned towards cell models. Primary hypothalamic cultures are difficult to maintain, are heterogeneous neuronal and glial cell populations and often contain a minimal number of viable peptide-secreting neurons. In contrast, immortalized, clonal cell lines represent an unlimited, homogeneous population of neurons that can be manipulated using a number of elegant molecular techniques. Cell line studies and in vivo experimentation are complementary and together provide a powerful tool to drive scientific discovery. This review focuses on three key neuroendocrine systems: energy homeostasis, reproduction, and circadian rhythms; and the use of hypothalamic cell lines to dissect the complex pathways utilized by individual neurons in these systems.

1. Introduction

The hypothalamus maintains the homeostasis of our internal environment and is the control center for all endocrine functions (Fig. 1). It is situated below the thalamus, posterior to the optic chiasm and surrounds the third ventricle; access to the ventricle allows it to integrate signals from circulating factors that cross the blood-brain barrier (BBB). A study by Faouzi et al. indicates that there is differential accessibility to circulating factors within the hypothalamus. They have shown evidence that one of the ventral regions of the hypothalamus, the arcuate nucleus, does not require peripheral factors to cross the BBB, but instead may have neuronal projections that extend outside of the BBB [53]. The regulatory actions of the hypothalamus can be divided into three categories: control of internal homeostasis, endocrine system regulation and autonomic nervous system regulation. Within these three categories there are numerous critical physiological functions regulated by the hypothalamus. Internal homeostasis covers the regulation of water balance, temperature, circadian rhythms and energy and glucose homeostasis [60]. The latter two processes, circadian rhythms and energy and glucose homeostasis, are two areas that are heavily studied, especially as the hypothalamus has recently been considered a key region in the pathogenesis of obesity and diabetes [62]. With regard to endocrine system regulation the hypothalamus, via the pituitary gland, regulates stress, growth, metabolism and reproduction [60]. The third category, autonomic nervous system regulation, includes the control of blood pressure, gut motility and respiration [60]. As the hypothalamus controls these numerous vital tasks, a perturbation of the delicate regulatory balance can lead to detrimental effects resulting in major health problems.

The hypothalamus contains multiple nuclei, which are comprised of a complex network of neurons. Within this complex array of neurons there are distinct neuronal phenotypes, each expressing a specific complement of neuropeptides, neurotransmitters and receptors [52]. An understanding of the control mechanisms of the unique hypothalamic, peptidergic neurons is critical before we can understand how the brain achieves its diverse control of basic physiological functions. The cellular mechanisms involved in this process are not clearly understood, mainly due to the complexity of the in vivo hypothalamic architecture. Numerous studies have been undertaken to map the afferent connections between distinct hypothalamic nuclei and neurons, utilizing methodology such as double- and triple-label immunocytochemistry, in situ hybridization and retrograde tracing [43,47,51,74,145]. These studies are useful to generate an emerging picture of the potential cellular communication within the hypothalamus, but are not comprehensive and do not address the molecular mechanisms involved in gene regulation and cellular signaling. In order to overcome the complexity of in vivo models, numerous labs have attempted hypothalamic cell line generation. Historically, it was difficult to establish immortalized hypothalamic cell lines, due to the lack of naturally occurring tumors and the inherent difficultly of transforming or immortalizing highly differentiated neurons

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doi:10.1016/j.yfrne.2009.03.005

Please cite this article in press as: C.M. Mayer et al., Hypothalamic cell lines to investigate neuroendocrine control mechanisms, Front. Neuroendocrinol. (2009), doi:10.1016/j.yfrne.2009.03.005

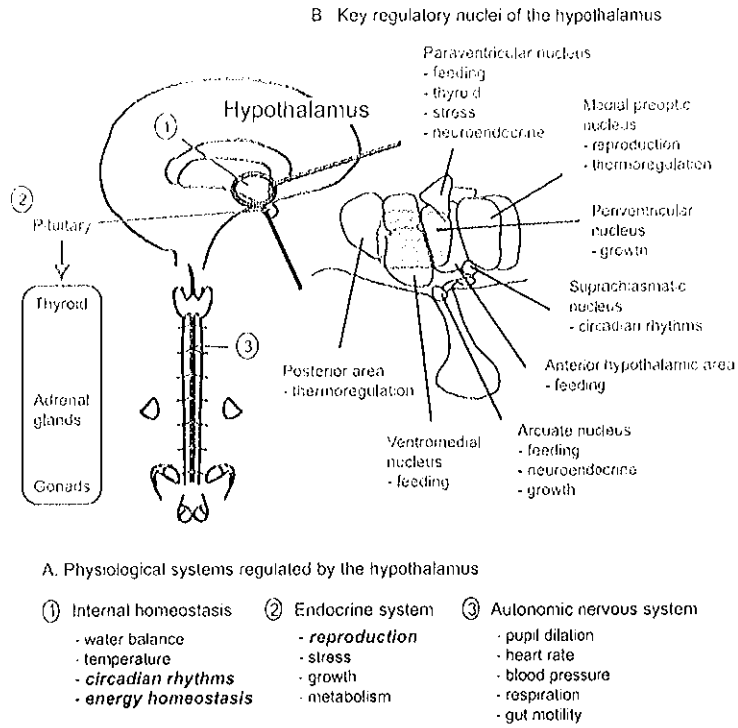

Fig. 1. Hypothalamic function. (A) The hypothalamus regulates numerous functions, all of which can be categorized under three headings: internal homeostasis, endocrine regulation and autonomic nervous system regulation. The three functions specifically discussed in this review, circadian rhythms, energy homeostasis and reproduction, are italicized. (B) Within the hypothalamus these processes are controlled by discrete nuclei, each with a different complement of phenotypically distinct neurons.

from primary culture [28]. Cell lines from the peripheral nervous system were established from neuroblastomas, such as the Neuro2A, and pheochromocytomas, such as the PC12 cell line; however, these models do not truly represent differentiated central nervous system neurons (CNS). For instance, the murine N1E 115 neuroblastoma cell line is routinely used as a CNS-derived neuronal model, although it was originally generated from a spontaneous tumor on the spinal cord [9]. Recently, cell models from the hypothalamus have been developed and have proven to be invaluable towards understanding the cellular biology of specific neuroendocrine cells. The number of cell models from the hypothalamus and from the entire brain, consist of a few isolated cell types and represent an infinitesimal percentage of the neuronal phenotypes represented within the brain. For this reason, our group and other labs have been continually working on developing new hypothalamic, neuronal cell models. In this review, we will be summarizing current hypothalamic cell lines in use and will examine how they have enriched our understanding of hypothalamic function, with a focus on three important neuroendocrine topics: energy homeostasis, reproduction and circadian rhythms.

2. Hypothalamic cell lines

Non-transformed primary hypothalamic cultures are difficult to maintain, have a short life span and represent a heterogeneous neuronal and glial cell population; often these cell populations contain a minimal number of healthy peptide-secreting neurons. On the other hand, immortalized, clonal cell lines represent an unlimited homogeneous population of specific neuronal cell types. Additionally, they offer a model with fewer uncontrolled variables than the in vivo situation and are maintained in a controlled and homogeneous condition. Classical in vivo approaches cannot firmly establish the direct action of an agent on specific hypothalamic neurons or on neuropeptide transcription, mainly because the cell receives input from other neurons. As well, little is known about the molecular mechanisms involved in intracellular signaling, promoter regulation, regulation of gene transcription, or regulation of secretion in native neurons due to the complexity and difficulty of studying molecular events in vivo. The use of cell lines provides a simpler model to begin these investigations. Since few studies have been performed in vivo, researchers are unable to state whether neuronal cell lines function identically to native neurons. For this reason, caution must be taken when extrapolating theories from the cell line to the in vivo model. As well, cell lines lack the complexity and integrated network of neuronal connections and signaling. Despite these limitations, cell line studies can be used to understand the in vivo model by having a clearer idea of further studies to pursue with, as well as confirming molecular events. From the current studies available that have looked at effects such as hormonal regulation of gene expression or receptor activation, most studies have found that the results from cell lines replicate that of in vivo studies. Cell lines also provide a good model to screen different neuronal phenotypes for the expression of specific genes or proteins; studies that are difficult in vivo due to the numerous phenotypes of neurons present in a given hypothalamic area. Because of this, limited characterization of native hypothalamic neurons has been performed, and though hypothalamic cell lines express a compilation of neuropeptides, receptors and signaling molecules, it is unconfirmed that this differs from in vivo. It was originally shown from in vivo studies that the GnRH neuron only expressed one of the estrogen receptor (ER) isoforms, ERalpha. Using the GnRH cell line, GT1-7, researchers found that both isoforms ERalpha and beta were expressed [128], after which it was confirmed that both isoforms are present in the native GnRH neuron [143]. This exemplifies how cell lines can be utilized to clarify in vivo studies and although hypothalamic cell lines express

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numerous factors not yet shown in native neurons, this may be due to a lack of in vivo studies.

For the reasons presented above, researchers have turned towards immortalized cell models for detailed molecular and mechanistic studies. In 1885 Wilhelm Roux established basic tissue culture techniques but it was not until 1940 when the first immortal cell line was developed by Earle: the L Strain cells [48,55]. Since then numerous cell lines have been developed from many different tissues; although the first attempt at immortalizing neurons was not until 1974, performed by Shaw et al. [42]. They infected primary hypothalamic cells from embryonic mice day 14 with intact simian virus 40, a DNA virus that contains the oncogene, large T antigen (SV40 T-Ag), creating an immortalized cell population labeled HT9. Unfortunately, these cells were morphologically similar to precursor cells and were not fully differentiated neurons. In 1984, Cepko et al. [27] developed retroviral shuttle vectors allowing for the introduction of DNA sequences into mammalian cells, utilizing SV40 T-Ag mediated replication. It allowed for increased efficiency of gene transfer and contained a gene conferring resistance to specific antibiotics, such as neomycin, allowing for selection of infected cells. This technology has permitted researchers to retrovirally infect primary cells with an immortalizing oncogene and selectively propagate them. This is one of the key technologies, along with the development of transgenic mice expressing oncogenes, which has allowed for the development of cell lines. In the following subsections we will discuss how these technologies were utilized to develop gonadotropin-releasing hormone (GnRH), suprachiasmatic nucleus (SCN) and other general hypothalamic cell lines.

2.1. GnRH expressing cell lines

One of the first fully differentiated hypothalamic cell lines was a GnRH expressing cell model developed by Mellon et al. in 1990 [104]. They created a transgenic mouse utilizing 5′ flanking DNA of the rat GnRH gene to target expression of SV40 T-antigen in GnRH neurons. They obtained nine transgenic mice that expressed the GnRH-SV40 T-Ag gene, none of which were fertile. Two of these mice developed anterior hypothalamic tumors. From one of the female mice, portions of the tumor were removed for cell culture, ultimately becoming the GT-1 cell line population. Following serial dilutions of the GT-1 cells, they developed three homogeneous cell populations, labeled GT1-1, GT1-3 and GT1-7. These cells expressed neuronal morphology and secreted GnRH when depolarized. These cells became one of the most highly utilized neuronal cell models for studies related to not only GnRH, but also basic neuronal function, as they represented one of the few appropriate neuronal models available.

Currently, there are now four GnRH expressing cell models: the GT1, GN, Gnv and GRT cells. The GN cells were developed utilizing a similar method as the GT1s, except they used the 5′ flanking region of the human GnRH gene [122]. Also, the T-Ag driven tumors developed before migration of the GnRH neurons from the olfactory placode to the hypothalamus was complete and thus the cells are considered to be immature GnRH neurons. One of their mice, a male mouse, contained T-Ag and GnRH expressing cells, from which two cell lines were created, the NLT and Gn11 cells. Interestingly, there were distinct phenotypic differences between the two lines, as the NLT cells expressed GnRH at levels 10-fold higher than the Gn11 cells, while the Gn11s expressed a splicing variant of the GnRH gene lacking exon 2. The last two GnRH cell lines, the Gnv and GRT cells, were developed using quite different methods.

The Gnv cells were developed by Salvi et al. [136] and were one of the first attempts to create cell lines from adult hypothalamic cultures. Isolated hypothalami from 10 to 12 weeks old rats were infected using two lentiviral vectors. The first vector expressed a tetracycline (Tet) transactivator gene driven by the GnRH promoter and the second vector expressed the v-myc oncogene and puromycin resistance gene fused with a Tet-responsive element. These vectors allowed for two things: the Tet-transactivator gene to only be expressed in GnRH neurons and the conditional activation of the v-myc oncogene with Tet treatment. They developed 12 clones of which clones 3 and 4, named Gnv-3 and Gnv-4, expressed the highest levels of GnRH and were thus selected for future studies. These cells have a phenotype of mature neurons and secrete GnRH in a pulsatile pattern.

The GRT cells, developed by Wolfe et al. [164], are the most recent hypothalamic cell lines and were also immortalized using conditional activation of an oncogene. They created transgenic mice expressing a Tet-regulated, GnRH promoter driven T-Ag hybrid gene. At 4 months of age, mice were treated with doxycycline and hypothalami were dissected for cell culture at 5 months. The cells were passaged in doxycycline containing media, which allowed for proliferation of the cells. The GRT cell line expresses and secretes GnRH, although at lower levels than the GT1-7 cells.

2.2. SCN cell lines

Motivated in a similar fashion as reproductive researchers, scientists with a circadian background desired a cell culture model of the SCN. In 1999, Earnest et al. developed the first SCN cell line [49]. They isolated SCN from embryonic rat hypothalamic, day 15 and 16, and infected them with a retrovirus expressing the adenovirus 2-adenovirus five hybrid E1A 12s sequence (12S E1A), an oncogene, and a neomycin resistance gene. The infected cells were selected using geneticin and two cell lines were subcloned: the SCN2.2 and SCN1.4. Both lines expressed neuronal markers and SCN specific peptides, such as VIP and GRP. These cell lines have become the most well studied SCN cell models.

Recently, two other SCN cell lines have been developed: the N14.5 and RS182 cells. The N14.5 cells were created using novel technology [102]. They isolated cells from the venterolateral region of the SCN, from transgenic rats expressing the temperature sensitive (ts) SV40 T-Ag. The SV40 T-Ag gene is only activated at 33 °C, thus once the primary SCN neurons were isolated and grown at 33 °C, SV40 T-Ag was activated and the cells began to proliferate. When grown at 39 °C, proliferation is arrested and the cells differentiate further. This cell line is another example of a conditionally activated SV40 T-Ag cell line. The N14.5 cell line expresses neuronal markers and the SCN marker, VIP.

The RS182 cells were also created using tsSV40 T-Ag expressing rats, except the rats were crossed with Per1 promoter driven luciferase gene expressing [85]. SCN cells were isolated from these rats at embryonic day 19 and grown at 33 °C. They established 512 cell lines of which 17 displayed stable Per1 oscillations, visualized by luciferase protein expression, indicative of intrinsic circadian gene cycling. From the 17 lines, the RS182 cells had the highest amplitude of cycling and were selected for further studies.

2.3. A Wide array of other hypothalamic neurons

The GnRH and SCN cells are only a few neuronal cell types from the hypothalamus. Thus other groups, including ours, have developed cell lines that are representative of the enormous range of cell types in the hypothalamus. Two groups have utilized retroviral gene transfer and SV40 T-Ag to immortalize rat embryonic cell lines. In 1990, Rasmussen et al. developed the RCF-8 and 12 and RCA-6 cell lines [123]. These lines are responsive to estrogen and the RCA-6 cells express NPY and IGF-1. Although these cells were developed in 1990, few studies have utilized these models and they have not been further characterized them. In 2003, Kasckow et al. [83] also retrovirally infected embryonic day 19 rat hypotha-

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lami, producing cell lines. They screened the cells and identified one line, IVB, which expressed CRH, for further studies. From their initial experiments, they have found that this cell line serves as a model for parvocellular CRH neurons.

Recognizing the need for mouse cell lines representative of other unique hypothalamic neurons, our group initially established 38 embryonic, clonal hypothalamic mouse cell lines [18]. We utilized retroviral infection of SV40 T-Ag of primary hypothalamic cell cultures from fetal mice day 15, 17 and 18 to produce a heterogeneous mix of cells, which were further subcloned into homogeneous cell populations. The cells are currently labeled as mHypoE-'clone number' to distinguish them from other newly created cell lines and for clarity, although it should be noted that in previous studies they were labeled as N-'clone number'. These cells have been well characterized and screened for over 28 neuroendocrine markers, while the number of clonal lines has now increased to over 60. The cell lines also express mature neuronal markers and exhibit neuronal morphology (Fig. 2). Each of the cell lines expresses a unique complement of receptors and neuropeptides and has a distinctive morphology. Some key neuropeptides expressed in these cells are neuropeptide Y (NPY), agouti-related peptide (AgRP), proopiomelanocortin (POMC), cocaine- and amphetamine-related transcript (CART), neurotensin (NT), melanin concentrating hormone (MCH), vasoactive intestinal peptide (VIP) and corticotopin-releasing hormone (CRH), among many others (see Table 1).

Embryonic cell lines may or may not accurately represent the adult, fully-differentiated neuron due to their unique developmental and physiological roles, and this is difficult to study without appropriate models. Therefore, our group has recently devised a novel method to immortalize neurons from the adult mouse (D.D. Belsham et al., submitted for publication). In order to retrovirally incorporate the oncogene SV40 T-Ag into the cellular genome, cells must be dividing. As such, we utilized the nerve growth factor, ciliary neurotrophic factor (CNTF), to treat primary cell culture from adult mouse hypothamali, inducing neurogenesis and cell division. Following 10 days of CNTF treatment, the cells were retrovirally infected with SV40 T-Ag and the infected cells were selected with geneticin. The cells express markers of mature neurons and exhibit neuronal morphology. We have established over 50 cell lines, labeled mHypoA-'clone number', and are currently characterizing these lines for expression of pertinent neuropeptides,

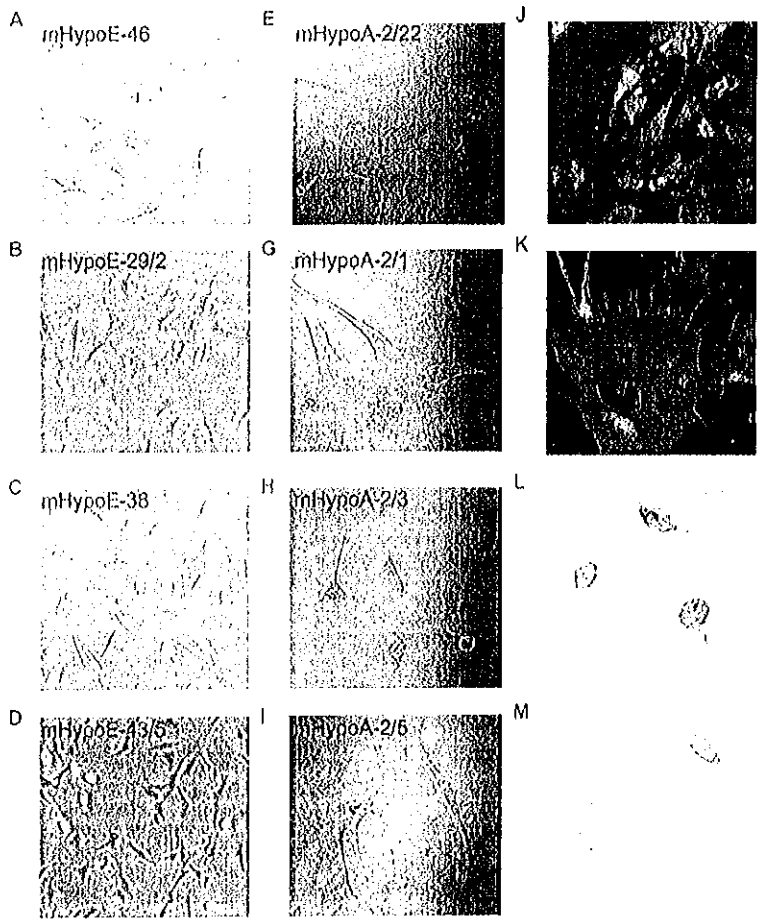

Fig. 2. Imaging of embryonic and adult hypothalamic cell lines Examples of hypothalamic cell lines, both embryonic and adult, are illustrated within this figure. (A–D) The embryonic mHypoE-46, -29/2, -38 and -43/5 were imaged using phase contrast microscopy. These four lines were used in studies described in the energy homeostasis section and the mHypoE-38 line was also used in studies described in the reproduction section. (E–I) The adult mHypoA-2/22, -2/1, -2/3 and -2/5 were imaged using confocal differential interference contrast microscopy. (J) The embryonic mHypoE-38 neurons were imaged using fluorescent confocal microscopy after immunocytochemical analysis with anti-ghrelin sera (green); nuclei were counterstained with propidium iodide (red) (originally published in [56]). (K) The adult mHypoA-2/12 neurons were imaged using fluorescent microscopy after immunocytochemical analysis with an antibody against NPY (green); nuclei were counterstained with DAPI (blue). (L and M) The mHypoE-36/1 neurons were imaged using DAB staining during immunocytochemical analysis with antibodies against neurofilament (NF) and neurotensin (NT) (originally published in [10]) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

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Table 1
Genes expressed in hypothalamic cell lines.

Gene	mHypoE-29/2	mHypoE-38	mHypoE-29/4	mHypoE-39	mHypo-36/1	mHypoE-46	GT1-7	GnV3	GnV4	SCN2.2
Agouti-related peptide	+	+	+	+	+	+	+			
Androgen receptor	–	–	–	–	–	+	+			
Arginine vasopressin		+					+			+
Arginine vasopressin receptor 1a	+	+	+	+	+	+	+			
Arginine vasopressin receptor 1b	+	+	+	+	+	+	+			
Cocaine and amphetamine related transcript	–	+	–	+	+	+	+			
Cannabinoid receptor 1			–	+	–	+	+			
Ciliary neurotrophic factor receptor	+	+	–	+	+	+	+	+	+	
Corticotropin releasing hormone receptor 1	+	–	+	+	+	–	+			
Corticotropin releasing hormone receptor 2	+	–	+	+	+	+	+			
Corticotropin releasing hormone	–	+	–	+	–	+	–			–
Dopamine transporter	–	+	–	+	+	+	+			
Dipeptidylpeptidase 4	–	–	–	–	+	+	+			
Estrogen receptor α	+	+	–	+	+	+	+	+	+	
Estrogen receptor β	+	+	+	+	+	+	+	+	+	
Galanin	–	–	–		+	+	–			
Galanin-like peptide	–	–	+	+	–	–				
Gastrin-releasing peptide	+	–	+	+	+	–	+			–
Glucokinase	+	+	+	+		+	+			
Glial fibrillary acidic protein		–	–	–	–	–	–	–	–	–
Ghrelin	+	+	+	+	+	+	+			
Growth hormone secretagogue receptor	+	+	+	+	+	+	+	+	+	
Glucagon-like peptide receptor 1	–	–		+	+	–	+			
Glucagon-like peptide receptor 2	–	–	–	–	+	–	+	–	–	
Glucagon receptor	–	–	–	–	–	+	–	–	–	
Glucocorticoid receptor	+	+	+	+	+	+	+			
Glucose transporter 1	+	+	+	+	+	+	+			
Glucose transporter 2	–	–	–	+	–	–	+			
Glucose transporter 3	+	+	+	+	+	+	+			
Glucose transporter 4	+	+	+	+	+	+	+			
Gonadotropin inhibitory hormone	+	+	+	+	+	+	+			
Insulin-like growth factor 1	+	+	+	+	+	+	+			
Insulin-like growth factor 1 receptor	+	+	+	+	+	+	+			
Insulin II	–	–	–	+	–	–				
Insulin receptor	+	+	+	+	+	+	+			
Insulin receptor substrate 2	+	+	–	+	–	+	+			
KISS-1 metastasis-suppressor	–	–	–	–	+	–	–			
KISS-1 metastasis-suppressor receptor	+	+	+	+	+	+	+			
Leptin receptor	+	+	+	+	+	+	+			
Melanocortin 4 receptor	–	–	–	+	–	+	+			
Melanin concentrating hormone	+	+	+	+	+	+	+			
Melanin concentrating hormone receptor 1	+	+	+	+		+	+			
Melatonin receptor 1							+			+
Melatonin receptor 2							+			+
Neuropeptide peptide Y	–	+	+	–	–	+	–			
Neuropeptide peptide Y receptor Y1		+	+	+	+		–			
Neuropeptide peptide Y receptor Y2		+	+	+	+		+			
Neuropeptide peptide Y receptor Y4		+	+	+	+		+			
Neuropeptide peptide Y receptor Y5		–	+	+	–		–			
Neuron specific enolase	+	+	+	+	+	+	+	+	+	+
Neurotensin	+	–	+	+	+	+	+			
Neurotensin receptor 1	–	–	–	+	+	–	–			
Orexin receptor 1	+	+	+	+	+	+	+		–	
Orexin receptor 2	+	–	+	+	+	+	+	+	+	
Oxytocin		+					+			–
Proopiomelanocortin	+	–	–	–	–		+	–	–	
Proglucagon	–	–	–	+	+	–	+	–	–	
Serotonin receptor 1b	+	+	+	+	+	+	+	–	–	
Serotonin receptor 2a	+	+	+	+	+	+	–			
Serotonin Receptor 2c	–	–	–	–	–	–	+	–	–	
Somatostatin		+	+	+	+	+				+
Splicing factor 1	+	+	+	+	+	+	+			
Suppressor of cytokine signaling 3	+	+	+	+	+	+	+			
Spermiogenesis specific transcript on the Y	+	+	+	–	–	+	–			
Signal transducer and activator of transcription 3	+	+	+	+		+	+			
Signal transducer and activator of transcription 5A	+	+	+	+		+	+			
Signal transducer and activator of transcription 5B	+	+	+	+		+	+			
Sulfonylurea receptor 1		–	–				+			
Sulfonylurea receptor 2	–	–	–	+		+	–			
Syndecan 3	+	+	+	+	+	+	+	+	+	
T-antigen	+	+	+	+	+	+	+			

(continued on next page)

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Table 1 (continued)

Gene	mHypoE-29/2	mHypoE-38	mHypoE-29/4	mHypoE-39	mHypo-36/1	mHypoE-46	GT1-7	GnV3	GnV4	SCN2.2
Urocortin	+	-	+	+	+	+	+			
Vasoactive intestinal peptide	+	+	+	+	+	+	+			+
Vasoactive intestinal peptide receptor 1	-	-		+	+	+	-			
Vasoactive intestinal peptide receptor 2	+	+	+	+	+	+	+			

'+' indicates that the gene is expressed; '-' indicates that it is not expressed; blank entry indicates that the presence/absence is unknown.

receptors, and neurotransmitter systems. These lines will be important to understand the control mechanisms utilized by mature neurons in terms of basic physiological functions and stimulus control, and can be used for direct comparisons with neurons of embryonic origin. We continue to develop new models for neuroendocrine research and our repertoire now includes comparable models from the embryonic mouse (mHypoE, previously called N-xx), male and female adult mouse (mHypoA), embryonic rat (rHypoE), and adult mouse pituitary (mPitA). Eventually, we hope to be able to provide a representative cell model for virtually all hypothalamic research involving specific neuropeptide-expressing neurons from our mixed cell populations.

3. Energy homeostasis

The hypothalamus was first considered important for the regulation of feeding after lesions in the ventromedial hypothalamus and lateral hypothalamus led to hyperphagia and obesity or aphagia and starvation, respectively [135]. Based on this work, the hypothalamus is known to be the primary center for food intake

Table 2
Key studies performed using hypothalamic cell lines.

	Study	Key findings	Cell line(s) used
Energy homeostasis	Lee et al. [90]	Glucose decreases AgRP by increasing ATP and inhibiting AMPK	GT1-7
	Cheng et al. [34]	Glucose regulates AgRP through NADH production	mHypoE-38
	Li et al. [94]	GLUT2 overexpression decreases AgRP through increased ATP and inhibition of AMPK	GT1-7
	Mayer et al.	Insulin represses NPY and AgRP through MAPK MEK/ERK pathway	mHypoE-46
	Frago et al. [58]	GHRH-6 regulates IGF-1 and NPY through an PI3K-Akt independent pathway	RCA-6
	Anderson et al. [8]	Increased intracellular calcium activates CAMKK2, activating AMPK and increasing NPY	mHypoE-38
	Fick et al. [56]	Insulin regulates ghrelin through the PI3K-Akt and MAPK MEK/Erk pathways	mHypoE-38
	Cui et al. [40]	Leptin regulates neurotensin through JAK-STAT3 pathway	mHypoE-36, -39
	Miroshamsi et al. [110]	Leptin and insulin activate K_{ATP} channels through PI3K	GT1-7
	Ning et al. [115]	Leptin inhibits PTEN, increasing PIP3 and decreasing F-actin	GT1-7, mHypoE-29/4
	Kaszubska et al. [84]	PTP1B negatively regulates leptin signaling	GT1-7
	Mayer et al.	Prolonged insulin exposure induces neuronal insulin resistance by degrading IR and IRS1, and serine phosphorylating IRS1	mHypoE-46
	Fox et al. [57]	Leptin activates PC1/3 transcription via Nhlh2 and STAT3	mHypoE-29/2
Reproduction	Roy et al. [128]	Estrogen decreases GnRH gene expression through ERα	GT1-7
	Pak et al. [118]	Estrogen provides negative feedback on GnRH promoter activity	GT1-7
	Shakil et al. [139]	Androgen decreases GnRH expression via nuclear ARs and increases GnRH secretion via membrane ARs	GT1-7
	Krsmanovic et al. [89]	Autocrine regulation of GnRH promotes switch from basal to surge -like GnRH secretion	GT1-7
	Quaynor et al. [121]	Kisspeptin increases GnRH secretion in an autocrine fashion	GT1-7
	Bowe et al. [21]	NPY stimulates GnRH secretion	GT1-7
	Roy et al. [129]	Melatonin decreases GnRH secretion and gene expression	GT1-7
	Igaz et al. [78]	Insulin increases GnRH expression	GT1-7
	Yu et al. [167]	cAMP and PKC induce GnRH secretion	GT1-7
	Titolo et al. [150]	Estrogen differentially regulates NPY depending on ratio of ERα and ERβ	mHypoE-38, -42
	Jacobi et al. [80]	Estrogen increases kisspeptin and GRP54	GT1-7
	Luque et al. [97]	NPY stimulates kisspeptin expression	mHypoE-6
	Belsham et al. [15]	NMDA and NO repress GnRH expression	GT1-7
Circadian rhythms	Balsalobre et al. [11]	Serum shock synchronizes cultured rat fibroblasts	Rat-1 fibroblasts
	Balsalobre et al. [12]	Glucocorticoids synchronize cultured rat fibroblasts	Rat-1 fibroblasts
	Balsalobre et al. [13]	cAMP, Ca^{2+}, and protein kinase C (PKC) affect rhythmicity and synchronization	Rat-1 fibroblasts
	Izumo et al. [79]	Temperature compensation and real time imaging of circadian rhythms	Rat-1 fibroblasts
	Welsh et al. [158]	Peripheral cells in culture cycle over 24 h, but lose synchronicity	Rat-1 fibroblasts
	Akashi et al. [2]	MAP kinase cascade is involved in resetting of the clock	NIH-3T3 fibroblasts
	Hirayama et al. [73]	Mutant BMAL1 protein could not restore rhythmicity when not acetylated by CLOCK	BMAL1 mutant MEFs
	Earnest et al. [49]	Establishment of adenoviral E1A immortalized SCN2.2 cell lines	SCN2.2
	Earnest et al. [50]	SCN2.2 neurons restore behavioral rhythms when transplanted into SCN-lesioned rats	SCN2.2
	Allen et al. [4]	SCN2.2 neurons synchronize rat-1 fibroblasts in culture	SCN2.2
	Allen et al. [6]	SCN and peripheral clocks respond to different entraining stimuli	SCN2.2, rat-1 fibroblasts
	Chappell et al. [33]	Altered circadian function affects GnRH secretion	GT1-7
	Roy et al. [129]	GT1-7 neurons express functional melatonin receptors and melatonin alters GnRH gene expression	GT1-7
	Gillespie et al. [65]	GT1-7 neurons express functional clock genes	GT1-7

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and body weight regulation. Within the hypothalamus there are specific areas that are involved with energy homeostasis, namely the arcuate nucleus (ARC) and the paraventricular nucleus (PVN) [82]. These regions of the hypothalamus are comprised of orexigenic and anorexigenic signals. There are numerous orexigenic and anorexigenic neuropeptides that are involved in the regulation of feeding. Some of the peptides expressed by these neuropeptidergic neurons include: NPY, AgRP, MCH, galanin, Orexin, alpha-MSH, CRH, CART, and NT [92,138]. Energy homeostasis is maintained through regulation of these neuropeptidergic neurons by peripheral signals. These signals include hormones such as leptin and insulin, and nutrients such as glucose and free fatty acids [162]; in concert they act to either repress or stimulate feeding neurons, altering secretion and gene expression. In the following subsections, we will look at how hypothalamic cell lines have been used to study the regulation of neuropeptide gene expression, peripheral hormone signaling and the regulation of intracellular signaling molecules.

3.1. Neuropeptide regulation

One of the key steps in altering feeding responses is the change in neuropeptide expression. Nutrients, like glucose, and peripheral hormones, like leptin and insulin, act in the hypothalamus to alter the expression of feeding-related neuropeptides. In vivo studies using knockout animals have explored the importance of individual neuropeptides and the role they play in feeding regulation. As well, in vivo studies have looked at the overall response of the hypothalamus to specific hormones and nutrient signals, but it is in the in vitro model where researchers have begun to define the molecular mechanisms involved in the regulation of neuropeptide gene expression.

Neuropeptide Y and agouti-related peptide are two key potent orexigenic peptides, as indicated by the adult NPY/AgRP neuron knockout mice [68,98], which exhibit extreme aphagia and eventual starvation. In vitro studies have recently been investigating the intracellular mechanisms involved in the regulation of these neuropeptides. Glucose was shown to regulate NPY and AgRP expression in vivo, although the intracellular mechanisms were not known [32,156]. Lee et al. used the GT1-7 cells to study how glucose regulates NPY/AgRP [90]. GT1-7 treatment with glucose decreased AgRP mRNA expression and increased ATP levels. In response to the rising ATP levels, they found that phospho-AMPK, an intracellular fuel sensor, decreased. An inhibitor of glucose metabolism, 2-deoxy-D-glucose (2DG), decreased ATP while increasing AgRP mRNA and phospho-AMPK. To determine if ATP was directly involved with AgRP regulation, they depleted cellular ATP using sodium azide, which affects mitochondrial oxidation. The depletion of ATP led to an increase in AgRP. Next they studied if AMPK was directly involved and found that an AMP analog, AICAR, increased phospho-AMPK, as AMPK is activated by an increase in the ratio of AMP to ATP, and in turn, AgRP expression. Lastly they used a dominant-negative AMPK construct and found that it prevented the 2-DG induced increase in AgRP. This study indicates that glucose levels affect AgRP expression by changes in ATP levels, which lead to activation of inhibition of AMPK. Cheng et al. [34] followed up on this study using the N-38 cells (now known as mHypoE-38) which endogenously express NPY and AgRP. They confirmed that glucose inhibits AgRP expression and decreases cellular ATP levels. Although they found that ATP and AMPK were not involved in the regulation of AgRP. Instead they found that a GAPDH inhibitor, iodoacetate, which decreases the levels of NADH, increased AgRP mRNA and decreased ATP. As well, inhibition of AMPK with RNA interference, induced AgRP mRNA at 10 mM glucose and AICAR inhibited AgRP. They proposed that glucose regulates AgRP through the production of NADH by GAPDH and regulation of the C-terminal binding protein. These two studies indicate that glucose may regulate AgRP dissimilarly in different neuronal populations, supporting the need for studies at the individual neuronal level. One of the key steps in glucose sensing is the uptake of glucose into cells. Li et al. used the GT1-7 cells to study the role of the glucose transporter 2 (GLUT2) in the regulation of AgRP by glucose [94]. They found that GLUT2 overexpression increased ATP levels leading to inhibition of AMPK and a decrease in AgRP mRNA expression. As well, overexpression of GLUT2 blocked the 2DG-mediated increase in AgRP mRNA levels. This study further illustrates that neuropeptides, such as AgRP, can be regulated by glucose. Taken together with the previous two studies, these studies indicate that researchers are just beginning to understand the mechanisms utilized by glucose to regulate feeding-related neurons and that neuronal cell lines provide an important model in which this may be investigated.

Along with glucose, peripheral hormones are known to regulate NPY and AgRP gene expression. One of these key hormones is insulin. In order to understand how the mechanisms through which insulin regulates NPY and AgRP, our lab has used a cell line that expresses these peptides, mHypoE-46 (C.M. Mayer and D.D. Belsham, submitted for publication). Insulin treatment decreased NPY and AgRP mRNA levels and induced phosphorylation of Akt and Erk1/2. Using inhibitor analysis we determined that the MAPK MEK/Erk pathway is involved in insulin regulation of NPY and AgRP mRNA expression. Lin et al. [95] studied the effects of another peripheral hormone enterostatin, an anorexigenic peptide found in the pancreas, gastric mucosa and specific brain regions, on AgRP expression. They found that enterostatin decreased AgRP mRNA levels in GT1-7 cells. This data was presented in conjunction with in vivo data and used to confirm the direct actions of the peptide on neurons. Ghrelin is a peripheral hormone produced by the stomach, thought to counteract the anorexigenic effects of leptin. Using a ghrelin receptor agonist and the RCA-6, NPY expressing cell line, Frago et al. [58] studied the actions of the agonist on NPY and IGF1 expression. They found that the agonist, growth hormone-releasing peptide-6 (GHRH-6) increased insulin-like growth factor 1 (IGF-1) and NPY mRNA expression and induced phosphorylation of Akt. IGF-1 treatment also increased NPY mRNA expression. Interestingly, inhibition of the PI3 K-Akt pathway using LY294002 did not affect GHRH-6 regulation of IGF-1 and NPY. This study indicates that the PI3 K-Akt pathway does not mediate the effects of GHRH-6 on IGF-1 and NPY.

Cell lines have also been used to study the effects of intracellular signaling molecules on NPY regulation. In conjunction with in vivo studies, Anderson et al. [8] used the N-38 (mHypoE-38) cell line to provide more direct evidence linking CAMKK2, a serine/threonine protein kinase, to pathways regulating NPY. Ionomycin treatment, which increases intracellular calcium, activates CAMKK2 and induces phosphorylation of AMPK in the N-38 cells. Inhibition of CAMKK2 blocked the ionomycin-induced increase in phospho-AMPK. As well, ionomycin increased NPY mRNA expression and the CAMKK2 inhibitor attenuated this increase. This study indicated that intracellular calcium increases activate CAMKK2, leading to phosphorylation of AMPK and increased NPY mRNA.

Cell lines have allowed for the analysis of the NPY, AgRP and GnRH promoters. There are excellent reviews on GnRH promoter analysis [19,66,114], and therefore this will not be further covered in this review. However, relatively little has been reported about the regulation of the NPY promoter [7,96,108,109,159], and these studies were only performed in heterologous cell models. It is known from bilateral neural transection experiments and antisense data that the NPY neurons responsible for the reproductive and orexigenic effects of NPY lie within defined regions of the hypothalamus, including the arcuate nucleus [81,133,134], which clearly are not represented by any of the tumor-derived cell lines

Please cite this article in press as: C.M. Mayer et al., Hypothalamic cell lines to investigate neuroendocrine control mechanisms, Front. Neuroendocrinol. (2009), doi:10.1016/j.yfrne.2009.03.005

previously used for NPY studies. Using the GT1-7 cell line, our lab transiently transfected NPY 5′ flanking gene luciferase reporter constructs and found a repressor region between −867 and −1078 [103]. Three protein binding regions were determined with DNase I footprint analysis and the region between −943 and −922 were further analyzed using electrophoretic mobility shift assays (EMSA). This revealed that four different transcription factor-DNA complexes formed with GT1-7 nuclear proteins and that two of these proteins were the Oct-1 and Pbx-1 transcription factors. We are now using the cell lines derived in our laboratory expressing endogenous NPY from appropriate hypothalamic nuclei to study the direct regulation of the NPY promoter by hormones and to map these effects to distinct regions of the NPY gene 5′ regulatory region. This type of analysis has already been performed on the AgRP gene in the N-38 cell line, in which a novel SNP was found allowing functional dimorphism [10]. As exemplified above, cell lines provide a useful model in which to examine 5′ regulatory regions and to identify transcription factor binding regions involved in the regulation of a gene of interest. As well, using phenotypically different cell lines one can begin to compare cell type specific transcription factors involved in differential expression of neuropeptides.

Ghrelin, a potent orexigenic hormone, is secreted by the stomach and acts in the hypothalamus to stimulate NPY/AgRP neurons. Ghrelin is also expressed in the brain, possibly having a more local action. Our lab found that one of our hypothalamic cell lines, mHypoE-38, expressed preproghrelin [56]. This cell line was used to determine if peripheral hormones, like insulin, could regulate brain ghrelin. We found that insulin decreased preproghrelin and phosphorylated Akt and Erk1/2. The MAPK-MEK/Erk1/2 pathway inhibitor, PD98059, attenuated the insulin-mediated decrease in preproghrelin, while the PI3 K inhibitor, LY294002, upregulated gene expression. This study indicated that insulin directly regulates brain ghrelin and the MAPK MEK/Erk and PI3 K-Akt pathways are involved in the regulation of preproghrelin.

Along with study of orexigenic peptides, hypothalamic cell lines have also been used to study the regulation of specific anorexigenic factors; in particular, neurotensin (NT) and insulin. Neurotensin is expressed in the CNS and digestive tract and is involved in a number of physiological processes, including feeding. Our group utilized the N-39 and N-36/1 cells (mHypoE-39 and mHypoE-36/1) to analyze the effects of leptin, insulin and alpha-melanocyte stimulating hormone (alpha-MSH) on NT gene expression [40]. Treatment of the two cell lines with leptin, insulin and alpha-MSH increased NT gene expression. Promoter analysis revealed a leptin responsive region located in the NT 5′ flanking region between −250 and −391. This region contained STAT3 responsive elements and chromatin immunoprecipitation (ChIP) showed binding of STAT3 to this region. In order to determine if STAT3 was involved in leptin-mediated NT regulation, the cells were transfected with dominant negative STAT3 constructs. The dominant negative construct attenuated leptin-induced increases in NT mRNA. This study found that leptin, insulin and alpha-MSH directly regulate NT gene expression and that leptin does so through the STAT3 transcription factor.

Insulin is mainly produced in pancreatic beta cells, although there is also some evidence for expression in the rodent brain. Two of our cell lines, mHypoE-39 and mHypoE-46, express the rodent Ins2 gene, which is highly homologous to the single human insulin gene [99]. We found that mouse and rat Ins2 5′ flanking gene reporter constructs were active in these cell lines, while the human construct was not. Treatment of the mHypoE-39 with glucose increased Ins2 mRNA levels, while exendin four (a GLP-1 R agonist) decreased Ins2 mRNA. As well, we found that exendin four increased cAMP levels indicating that the GLP-1 receptor is active in our cell lines. Through this study we show that rodent Ins2 is expressed in neuronal cells and can be potentially regulated by central or peripheral hormones.

3.2. Peripheral hormone signaling

Although determining the regulation of neuropeptides by hormones and nutrient signals is important, an understanding of the mechanisms through which these hormones and nutrients signal in neurons is critical to gaining an insight into the pathogenesis of diseases such as diabetes and obesity. For these mechanistic studies, hypothalamic cell lines will play an invaluable role. Over the last few years, researchers have begun utilizing cell lines to work out the signaling pathways employed by insulin and leptin. Leptin and insulin are known to activate the classic signaling pathways PI3 K-Akt and MAPK MEK/Erk1/2 in neurons. Mirshamsi et al. [110] further studied the linkage between the PI3 K-Akt pathway and other intracellular signaling mediators. Using the GT1-7 cell line they confirmed that insulin and leptin phosphorylate Akt, STAT3, Erk1/2 and GSK3. The PI3 K inhibitor LY294002 inhibited leptin- and insulin-mediated Akt and Erk1/2 phosphorylation. Interestingly, they found that leptin and insulin increased activation of the K_{ATP} channel, as well as increasing the phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), the downstream product of PI3 K. Next they investigated the cellular mediators involved in the activation of the K_{ATP} channel. They found that leptin induces reorganization of actin filaments, causing an increase in G-actin and a decrease in F-actin. This effect was dependent on PI3 K, as the PI3 K inhibitors, LY294002 or wortmannin, attenuated leptin-mediated actin reorganization. From this study, they proposed that leptin and insulin activate the K_{ATP} channel through PI3 K and that leptin may utilize actin reorganization to mediate this action. This study was followed up by the same lab, in which they analyzed the role of the endogenous PI3 K pathway antagonist, PTEN, in leptin and insulin signal transduction [115]. They used the GT1-7 cell line along with the leptin sensitive N-29/4 (mHypoE-29/4) cell line for these studies. They found that decreased levels of PTEN, through siRNA knock down, led to increased PIP3 levels. As well, increasing PTEN levels with protein expression constructs did not alter F-actin levels alone, but prevented a leptin-mediated decrease in F-actin. The authors hypothesized that a decrease in PTEN leads to an increase in PIP3 via PI3 K, which causes a decrease in F-actin. Leptin did not alter PI3 K activity, whereas insulin increased it and PIP3 levels, but did not affect F-actin. From this they stated that changes in F-actin were not dependent upon activation of PI3 K. This study then went on to show that leptin phosphorylates PTEN leading to its inactivation, which causes an increase in PIP3 levels and a decrease in F-actin.

Our lab explored the signaling pathways activated by leptin in a NT expressing cell line, N-39 (mHypoE-39) [41]. We found that leptin increased phospho-STAT3, -Erk1/2, -p38 and -ATF1. To determine if these pathways are utilized by leptin to affect downstream NT gene expression, the N-39 cells were treated with leptin and specific pathway inhibitors. The p38 inhibitors, SB203580, SB202190 and SB239063 all attenuated the effects of leptin on NT. Applying EMSA and ChIP we determined that leptin induces binding of ATF-1 and c-fos to the NT promoter, both downstream signaling proteins of the MAPK p38 pathway.

The negative regulator of insulin signaling, protein tyrosine phosphatase 1B (PTP1B), is implicated in the regulation of feeding. Kaszubska et al. [84] used the GT1-7 cell line to study if it affects leptin signaling in neurons. Leptin increased phospho-STAT3 and activated a STAT3 responsive luciferase reporter construct. Over-expression of PTP1B attenuated these leptin actions. Microarray analysis revealed that the over-expression of PTP1B decreased the number of genes upregulated by leptin. This study indicated that PTP1B does negatively regulate leptin signaling in neurons.

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Glucose signaling is active in hypothalamic neurons, although the cell types that are glucose-responsive are not yet fully defined. Exploiting an anorexigenic-POMC expressing cell line, N-43/5 (mHypoE-43/5), our lab determined if POMC neurons are glucose-responsive [26]. Glucose depolarized the cells and caused an increase in cellular calcium levels, indicative of functional glucose-sensing machinery. As well, glucose altered intracellular signaling proteins, noted by decreased phospho-AMPK and -ACC levels. We also analyzed if changes in glucose levels affected the response of the N-43/5 cells to leptin and insulin. In low glucose conditions, leptin and insulin decreased phospho-AMPK, an effect that was absent in high glucose. This study indicates that the POMC expressing cell line, N-43/5, is responsive to glucose and that changes in glucose levels alter the cellular response to other hormones.

Along with determining hormonal and nutrient signal transduction pathways, we found that hypothalamic cell lines could be utilized to probe the intracellular changes in pathological states. Using the mHypoE-46 cell line we studied the effects of hyperinsulinemia on neurons and analyzed the mechanisms involved in the development of cellular insulin resistance (C.M. Mayer and D.D. Belsham, submitted for publication). Prolonged exposure to insulin caused cellular insulin resistance, noted by attenuation of PI3 K-Atk pathway activation by insulin. Insulin induced phospho-Akt and -S6 K, as well as IRS1 serine phosphorylation. As well, long term treatment with insulin decreased IRS1 and insulin receptor (IR) protein levels. In order to determine the pathways involved with the decrease in insulin-mediated Akt phosphorylation, we used inhibitors for lysosomal (3-methyladenine) and proteasomal (epoximicin) degradation pathways, as well as an mTor-S6 K pathway inhibitor (rapamycin). We found that prolonged exposure to insulin decreases IR levels through a lysosomal pathway and IRS1 levels through a proteasomal degradation pathway. As well, insulin increased IRS1 serine phosphorylation through the mTor-S6 K pathway. Phosphorylation of IRS1 on serine residues is known to decrease insulin signal transduction. Interestingly, treatment with any one of the inhibitors in the presence of high insulin concentrations restored insulin signaling. This study indicates that prolonged insulin exposure causes cellular insulin resistance through lysosomal degradation of IRs, proteasomal degradation of IRS1 and serine phosphorylation of IRS1.

3.3. *Regulation of intracellular signaling molecules*

This last subsection of feeding looks at how hypothalamic cell lines have been used to study the regulation of intracellular signaling molecules. Four current studies on this topic have been reported. In the first study Fox et al. [57] used the POMC expressing N-29/2 (mHypoE-29/2) cell line to analyze the regulation of proconvertase 1/3 (PC1/3) expression. PC1/3 is required in POMC neurons in order to process the prohormone, POMC, into active peptides such as alpha-MSH and beta-endorphin. They found that leptin stimulated PC1/3 promoter transcription, using a 5′ flanking PC1/3 gene reporter construct, and required the presence of two transcription factors, Nhlh2 and STAT3. A ChIP assay revealed that Nhlh2 binds to the PC1/3 5′ flanking region. Using site directed mutagenesis, they found that STAT3 sites within the PC1/3 5′ flanking area were required for leptin activation of PC1/3 transcription. Lastly, a modified ChIP assay was used to determine that STAT3 and Nhlh2 heterodimerize and interact with the PC1/3 promoter. Thus, leptin activates PC1/3 transcription via the transcription factors Nhlh2 and STAT3.

Exploiting the N-38 (mHypoE-38) cell line in combination with a novel protein–protein interaction determining method, MAPPIT, Wauman et al. [157] analyzed the interaction between the leptin receptor (ObR) and IRS4. IRS4 is a member of the IRS family of proteins, which are required by insulin for signal transduction and may also be involved with leptin signaling, and is highly expressed in the hypothalamus. IRS4 was found to interact with the ObR in N-38 cells and phosphorylation of the ObR at tyrosine 1077 is required for this interaction. As well, the authors found that IRS4 interacted with other intracellular signaling molecules: the p85 subunit of PI3 K, phospholipase C and SOCS2, 6 and 7. This study indicates that IRS4 may serve a function in leptin signaling.

In the third study, Brown et al. [23] analyzed the effects of adipokines on SOCS3 expression. Adipokines are produced in adipose tissue, like leptin, but are also secreted in certain regions of the brain. The authors examined the central role of two adipokines, resistin (rstn) and fasting-induced adipose factor (FIAF), using a hypothalamic cell line that expresses the two factors, N-1 (mHypoE-1). They found that resistin treatment decreased both FIAF and SOCS3 mRNA levels and that over-expression of rstn in the N-1 cells had a similar effect. Conversely, decreased in FIAF levels via siRNA did not affect rstn or SOCS3 levels. This study indicates that resistin has a novel paracrine/autocrine effect upon FIAF and SOCS3 in neurons.

The last study used the GT1-7 cell model to evaluate the effect of leptin and alpha-MSH upon melanocortin four receptor (MC4R) expression [67]. The MC4R is part of the anorexigenic melanocortin pathway and is activated by alpha-MSH. Gout et al. found that leptin and alpha-MSH act directly on neurons to increase MC4R mRNA expression. This study took advantage of the in vitro model to reinforce the direct actions of leptin and alpha-MSH on MC4R expression, confirming experiments performed in in vivo studies.

The studies above exemplify how appropriate cell models can be used to dissect the intricate molecular events utilized by individual neurons to control basic aspects of physiology by sensing central and peripheral signals. The generation of the cell lines with a clonal, homogeneous population of neurons allows the use of technologies not yet possible in the whole brain. These discoveries will allow for more detailed and directed studies in the whole animal and with perseverance, a confirmation of the cellular events determined in vitro in the in vivo situation.

4. Reproduction

The reproductive system is regulated by a complex interaction of neuropeptides and peripheral hormones acting upon the hypothalamic–pituitary–gonadal (HPG) axis. Situated at the peak of the HPG axis are the gonadotropin-releasing hormone (GnRH) neurons. GnRH is secreted by these neurons and acts upon pituitary gonadotropes inducing the secretion of the gonadotropic hormones LH and FSH into portal circulation where they ultimately act on the ovaries to stimulate ovulation. Estrogen produced in the ovaries regulates reproduction by controlling GnRH synthesis and secretion by acting through both positive and negative feedback mechanisms on the HPG axis.

GnRH neurons represent a small population estimated at 400–1000 neurons, which are found scattered throughout the preoptic and anterior hypothalamus [137]. GnRH neurons are regulated through autocrine mechanisms and by many extracellular signals including neurotransmitters, steroid hormones and peptide hormones. In addition, they receive paracrine inputs from many different neuronal phenotypes including steroid-sensitive neurons such as the neuropeptide Y (NPY) and kisspeptin neurons.

GnRH controls reproduction through secretory actions on pituitary gonadotrophes. GnRH is secreted in rhythmic pulses which are required for reproductive maturation and homeostasis [59], and surges, which are responsible for inducing ovulation [93]. While the function of GnRH in reproduction is well documented, the complex regulation of GnRH expression and secretion are not fully understood, mainly due to the difficulty of studying these

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mechanisms using in vivo models. Over the past 18 years and after approximately 300 publications researchers have used the immortalized GnRH cell lines, GT1, GN11 and Gnv3 to advance our understanding of GnRH regulation to a degree that would not have been attainable solely using classical in vivo approaches. The GT1 cell model has proven to be an excellent model to study the regulation of GnRH, as GT1 neurons mimic GnRH secretion in vivo. GT1 neurons not only basally secrete GnRH in a rhythmic pulsitile manner [160], but also acutely secrete increased levels of GnRH in response to depolarization [104]. The immature GnRH cell line, GN11, was found to express [122] and modestly secrete GnRH [168]. The recently developed conditionally immortalized GnRH cell line, Gnv3, was found to secrete GnRH in a pulsitile manner and secretion is acutely increased following NMDA stimulation [136]. In the following subsections we will look at the role these cell lines have played in studying GnRH neuronal regulation.

4.1. Hormonal regulation of GnRH

Hormonal regulation of GnRH synthesis and secretion through negative and positive feedback mechanisms is crucial for the maintenance of normal reproductive function. Estrogen regulates normal tonic GnRH secretion through negative feedback mechanisms [59], while inducing the preovulatory LH surge by positive feedback mechanisms [93]. Over the past 30 years scientists have debated whether estrogen regulates GnRH by acting directly on GnRH neurons itself or indirectly through estrogen-responsive interneurons. The latter view was supported by several immunocytochemical studies that showed GnRH neurons of several species in vivo lack estrogen receptors (ERs) [70–72,91,142,148]. However, because the brain contains few GnRH neurons that are scattered throughout several nuclei, classical immunocytochemical approaches are likely not sensitive enough to detect expression of ERs. As a way of overcoming this inherent in vivo obstacle, several groups utilized the clonal GT1 neuronal cell line to investigate whether functional ERs are expressed in GnRH neurons. Indeed, several groups demonstrated that estrogen binds to receptors in GT1-1 cells [119] and detected functional ERα [25,128,140] and ERβ [128] receptors in GT1-7 cells. In light of these findings, Skynner et al. used the advanced technique of single-cell multiplex RT-PCR to demonstrate for the first time that GnRH neurons in vivo express ERβ mRNA [144]. More recently Hu et al. showed that both fetal and adult GnRH neurons in vivo express both ERα and ERβ mRNA [75].

Confirmation that ERs are expressed in GnRH neurons prompted groups to begin to investigate the direct actions of estrogen on GnRH and the mechanisms mediating these effects by using the GT1 cell lines. 17β-estradiol was shown to repress GnRH mRNA expression [21,128], an effect that was mimicked by HPTE, an ERα agonist/ERβ antagonist [128]. This effect was blocked by the non-selective ER antagonist ICI 182,780 [21,128], but not by the selective ERβ antagonist, R,R-THC [21], suggesting that ERα mediates 17β-estradiol-induced repression of GnRH mRNA expression. The phytoestrogen, coumestrol, was found to decrease GnRH mRNA expression in GT1-7 cells, an effect that is likely mediated by ERβ, as R,R-THC blocked the effect of coumestrol [21]. Investigation of ERβs role in regulating the GnRH promoter found that GT1-7 cells transfected with splice variants of ERβ increased promoter activity in a ligand-independent manner. When these transfected cells were treated 17β-estradiol the increased promoter activity was attenuated, suggesting that ERβ acts as a transcription factor for the GnRH promoter and that estrogen provides negative feedback on GnRH promoter activity [118]. 17β-estradiol was also found to rapidly inhibit cyclic adenosine monophosphate (cAMP) production and GnRH secretion, which suggested the involvement of a G_i-coupled membrane ER [112].

The effects of 17β-estradiol on the electrophysiological properties of GnRH neurons have been studied using GT1-7 cells. 17β-estradiol was found to augment Ca^{2+} activated potassium channels [116] and modulate potassium currents [54] through ERs. More specifically, the effect on Ca^{2+} activated potassium channels was mediated by ERβ, as the ERβ agonist, 2,2-bis(4-hydroxyphenyl)-propionitrile (DPN) mimicked the effect while a selective ERα agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) did not and the effect was blocked by ERβ, but not ERα, knockdown by RNA interference [116].

Aside from estrogen the effects of other steroid hormones on GnRH expression have also been investigated using cell lines. The sex steroid precursor dehydroepiandrosterone (DHEA) was also found to inhibit GnRH mRNA expression, an effect that could not be attributed to the metabolism into 17β-estradiol since the enzyme aromatase is not present in GT1 neurons [39]. When treated with the androgen 5α-dihydrotestosterone (DHT), GnRH mRNA expression was repressed in GT1-7 cells, an effect that was mediated by androgen receptors (AR) [16]. Further experiments revealed that DHT and testosterone decreases GnRH mRNA expression while the membrane-impermeable BSA-conjugated testosterone (T-3-BSA) did not, suggesting that DHT and testosterone act through nuclear ARs to regulate gene expression [139]. Conversely, GnRH secretion and intracellular calcium were rapidly increased by DHT, testosterone and T-3-BSA, suggesting that membrane androgen receptors mediate these effects. DHT also blocked the forskolin-induced increase in cAMP production via membrane androgen receptors coupled to the inhibitory G-protein, G_i [139].

4.2. Autocrine, peptide and second messenger regulation of GnRH

GnRH neurons express functional GnRH receptors [88] suggesting the possibility of autocrine regulation. GnRH was found to induce the expression of the immediate early gene, c-FOS, through a protein kinase C (PKC) mechanism in GT1-7 cells [29]. Using the GnRH agonist, buserelin, basal GnRH secretion, promoter activity, and mRNA levels were decreased in GT1-1 cells [35]. Furthermore, GnRH was found to increase mobilization of intracellular calcium and to decrease GnRH secretion frequency, but, increase pulse amplitude [88]. These findings suggest that autocrine regulation of GnRH promotes a switch from basal to surge-like release of GnRH.

Paracrine regulation of GnRH by afferent neurons including, neuropeptide Y (NPY) and kisspeptin neurons has been suggested based on neuroanatomical and pharmacological studies. Neuroanatomical studies provide evidence that NPY-producing [154] and kisspeptin-producing [37,120] neurons project to or have terminals in close proximity to the preoptic GnRH neurons, respectively. Pharmacological studies demonstrate that NPY [14,38,86,132,165] and kisspeptin [101,105] increase GnRH secretion in vivo. Although these studies suggest that NPY and kisspeptin are capable of acting directly on GnRH neurons, indirect actions through other afferent neurons cannot be ruled out. Utilizing GT1-7 neurons, Besecke et al. demonstrated that NPY increases secretion of GnRH through direct actions on GnRH neurons, likely through the Y1 NPY receptor and intracellular calcium mobilization [20]. The GT1 cell line expresses the kisspeptin receptor, G-coupled protein 54 (GPR54) [80,121]. Recently kisspeptin-10 has been shown to rapidly increase GnRH secretion [80,121] and more specifically increase both the frequency and amplitude of GnRH secretory pulses from GT1-7 neurons [121]. Furthermore, kisspeptin-10 was shown to increase GnRH mRNA expression [80].

Many other peptides have been demonstrated to regulate GnRH secretion, mRNA expression and promoter activity in GT1 neurons including melatonin, retinoic acid, insulin and prolactin. Melatonin

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was found to decrease GnRH secretion [131] and decrease GnRH mRNA expression in a 24-cyclical manner [130] and is described below in the circadian section of this review. Retinoic acid was found to increase GnRH secretion, mRNA expression and promoter activity [36]. Insulin has been shown to induce increases in GnRH mRNA expression, c-FOS mRNA expression and activate phosphoinositide 3-kinase (PI3 K) and ERK1/2 in GnV3 cells [78]. Prolactin was found to decrease GnRH secretion in GT1 cells [106].

Specific second messenger pathways have been found to be involved in the regulation of GnRH secretion and mRNA expression. Activation of calcium, cAMP [167], PKC (protein kinase C) [161,167], and PKA (protein kinase A) [24,161] pathways were found to induce GnRH secretion from GT1 neurons. Interestingly, the activation of calcium, cAMP, and PKC decreased GnRH mRNA expression [167].

The regulation of GnRH gene expression and secretion, as illustrated above, is highly complex and occurs through several avenues. Autocrine signals promote the switch from basal to surge-like secretion of GnRH inducing ovulation, while paracrine signals such as melatonin and insulin control reproductive function according to season/photoperiod and nutritional status. Reproductive function during pregnancy, postpartum, and lactation is regulated in part by prolactin effects on GnRH. Although regulation of normal reproductive function is complex, studies from clonal cell lines have begun to characterize the key signaling mechanisms.

4.3. Regulation of the GnRH afferents NPY and kisspeptin

Neuropeptides are integral to the regulation of reproduction in part by mediating indirect effects of estrogen on GnRH neurons through paracrine signaling. In vivo studies demonstrate that estrogen affects NPY expression [1,141]. However, because it was uncertain whether these effects were direct or indirect, by using immortalized neuronal cell lines developed in our laboratory we were able to investigate the direct actions of estrogen on the NPY neuron. Using N-38 (mHypoE-38) and N-42 (mHypoE-42), neuronal cells lines that express NPY, ERα, and ERβ we investigated the effects of 17β-estradiol on the regulation of ERs and NPY mRNA expression. In N-38's, 17β-estradiol regulated ERα mRNA and protein expression in a biphasic manner, beginning with an initial increase, but was later repressed by 17β-estradiol, while ERβ slowly increased expression over time (Fig. 3A) [150]. NPY mRNA expression was initially repressed and overtime was greatly increased by 17β-estradiol (Fig. 3B). These results raise the intriguing possibility that NPY mRNA expression in N-38 is dependent on the ERα/ERβ ratio and that the increase in ERβ levels is an important factor in the increase in NPY mRNA expression. Interestingly, the effects of 17β-estradiol on ERα and ERβ expression in N-42 neurons were strikingly different, as the expression of both receptors was steadily repressed. Additionally, the estrogen-mediated repression of NPY in N-38 was mapped to the 5′ regulator region. Finally, by using small-interfering RNA knockdown of each ER subtype the repression of NPY mRNA gene expression by 17β-estradiol in NPY expressing N-38 neurons was found to be mediated by both ERs, while the induction was solely through ERβ. Further investigations into the signaling mechanisms activated by 17β-estradiol in N-38, NPY neurons, demonstrated that Akt and ERK1/2 are rapidly activated by 17β-estradiol (Fig. 3C and D) and that the PI3 K and MAPK pathways are both involved in the activation of these signaling molecules. Furthermore, the selective ERα agonist, PPT, activated Akt, ERK1/2 and CREB (Fig. 3C and D). Importantly, activation of the PI3 K and MAPK pathways were found to mediate the 17β-estradiol-induced repression and induction of NPY mRNA levels (Fig. 3E) [151]. This indicates that early membrane signaling events may potentiate or amplify the long-term transcriptional response.

The differing effects observed in the above study between the mHypoE-42 and mHypoE-38 clonal cell populations can be attributed to the differing phenotypes of the two cell lines, indicating that they are different neuronal subtypes. In vivo, it is unknown how many subtypes of NPY neurons exist, nor for any other neuropeptide neuronal phenotype. Each clonal cell line developed may represent a different subtype of a specific neuropeptide and may thus allow for studies investigating how each neuronal subtype differs in both the mechanisms through which it is regulated and the mechanisms through which it can signal to and regulate other neurons. GT1-7 neurons, although classically known for their GnRH-secreting characteristic, also basally secrete kisspeptin [121]. Not only does kisspeptin increase GnRH secretion in GT1-7 cells, but, GnRH decreases kisspeptin secretion, suggesting that autocrine regulation of these systems in the GT1-7 cell involves negative feedback mechanisms [121]. The effect of 17β-estradiol on Kiss and GPR54 mRNA levels in GT1-7 cells was investigated and Jocabi et al. found that long term exposure (24 h) to 17β-estradiol increased mRNA expression levels of both genes [80]. Because NPY has been implicated as a very important metabolic regulator of reproduction Luque et al. investigated the potential role of NPY in kisspeptin regulation. Initial studies using NPY knockout mice demonstrated that in the absence of NPY, kisspeptin mRNA expression was attenuated, suggesting a regulatory role, either directly or indirectly, for NPY on kisspeptin [97]. By using the hypothalamic cell line, N-6 (mHypoE-6), the potential direct effects of NPY on kisspeptin expression were investigated. N-6 cells were confirmed to express NPY receptors, Y1-Y6, GPR54, and kisspeptin and were found to express higher levels of kisspeptin mRNA when treated with NPY [97].

The use of immortalized, clonal cell lines has been instrumental towards our current understanding of GnRH neuronal function. These studies have allowed a detailed study of the molecular events in the whole animal. An excellent example of this is the finding that the GnRH neuron was controlled by the glutamate, nitric oxide, cGMP signaling pathway in the GT1-7 neurons [15,17]. A number of years later, it was confirmed that the specific knockout of neuronal nitric oxide synthase indeed had major effects on the reproductive axis and this could be traced to a direct action at the level of GnRH synthesis [69]. Similarly, using the information gained from this important model system [15,100,146], researchers have been able to return to the animal model with renewed focus, resulting in new insights into the role of GnRH neurons in normal reproductive physiology [22,87,113,117].

5. Circadian rhythms

The rhythmic nature of our environment dictates that there will be a changing availability of resources at any given time. In this dynamic setting it is essential for organisms to maintain cellular and behavioral homeostatic efficiency. To maintain this optimal efficiency, an innate cellular rhythm generating machinery has evolved that enables organisms to adapt to these cyclic environmental changes by preparing cells for periodic stimuli during the 24-h day. The rhythm generator exists in even the simplest cyanobacteria and has become more complex as the changing needs of the organisms have evolved. As evolution progressed and multicellular eukaryotic organisms could no longer rely on direct cellular photic stimulation, organized neural and humoral machinery developed. In higher animals, such as mammals, these signals are coordinated by a core circadian oscillator or master clock. This master clock is situated within the mammalian suprachiasmatic nucleus (SCN) of the hypothalamus. Signals from the SCN lead to rhythm entrainment within other cells allowing for a healthy response to external stimuli through smooth operation of the circadian system.

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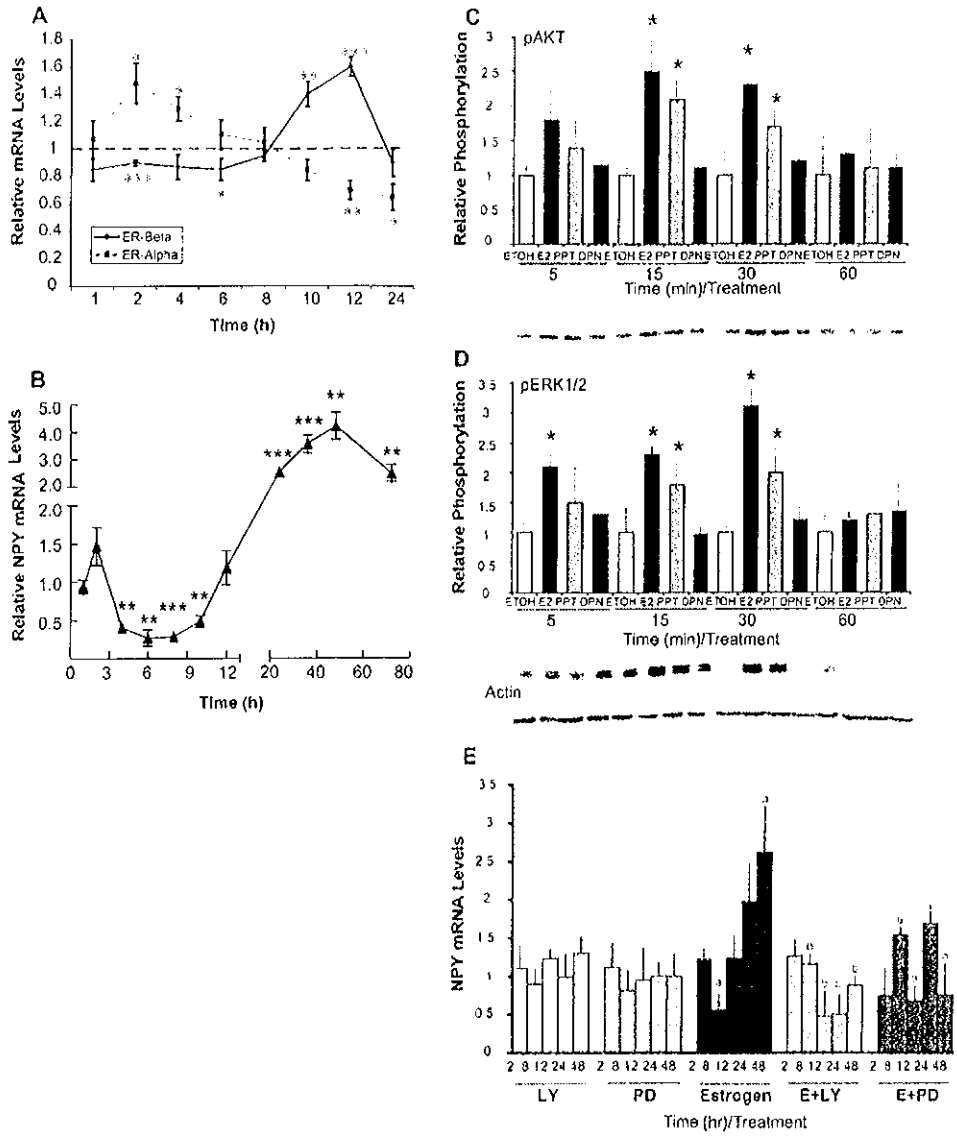

Fig. 3. Effect of 17β-estradiol on ERα, ERβ, and NPY mRNA expression in mHypoE38 (N-38) neurons and the role of the PI3K and MAPK signaling pathways. (A) ERα and ERβ mRNA expression pattern in mHypoE38 (N-38) neurons treated with 10 nM 17β-estradiol over a 24-h time course as determined by real time RT-PCR (originally published in [150]). (B) Biphasic NPY mRNA expression pattern in mHypoE38 neurons treated with 10 nM 17β-estradiol over a 72-h time course as determined by real time RT-PCR (originally published in [150]). (C) Activation of Akt kinase. (D) Activation of ERK1/2 kinase following treatment of vehicle (ETOH), 17β-estradiol (E2), the selective ERα agonist: PPT, and the selective ERβ agonist: DPN over a 60-min time course as determined by Western blot analysis (originally published in [151]). (E) Inhibition of the 17β-estradiol-induced changes in NPY mRNA expression by the selective PI3-kinase inhibitor: LY294002 (LY) and the MAP kinase inhibitor: PD98059 (PD) as determined by real time RT-PCR (originally published in [151]).

The circadian system is distinguished by positive and negative transcription/translation cycles within the cell and posttranslational signal transduction cascades (Fig. 4A). Approximately, 10% of the transcriptome is controlled by the circadian rhythm generator [107,147]. While this number does not initially seem significant, the transcripts controlled in a rhythmic fashion are the rate-limiting enzymes for virtually every process within the cell. Additionally, in an elegant study on the hepatic proteome, Reddy et al. showed that over 20% of proteins within the cell are cyclic [125]. Interestingly, nearly half of these proteins lacked an oscillating transcript suggesting that post-translational processing occurs in a circadian and rhythmic fashion [124]. That we have elucidated this much about the nature of circadian biology is due largely to studies at the cellular level.

5.1. Early cell-based circadian research

During the 1970s and through the 1980s avian pinealocytes were extracted and cultured to study the mechanics of the circadian system within vertebrates. Dissociated pinealocyte cultures demonstrated that individual cells could exhibit oscillatory properties, express photoreceptors and synthesize melatonin [44–46]. However, the pinealocyte model has yet to be efficiently transformed and so interventions at the molecular level in the pinealocyte have yet to be performed. Much of the current knowledge of the biochemical circadian system owes its ancestry to initial studies in *Neurospora crassa* and *Drosophila melanogaster*. The molecular clock was first characterized within bread mould and the fruit fly in the early 1980s. Classical and molecular genetic research

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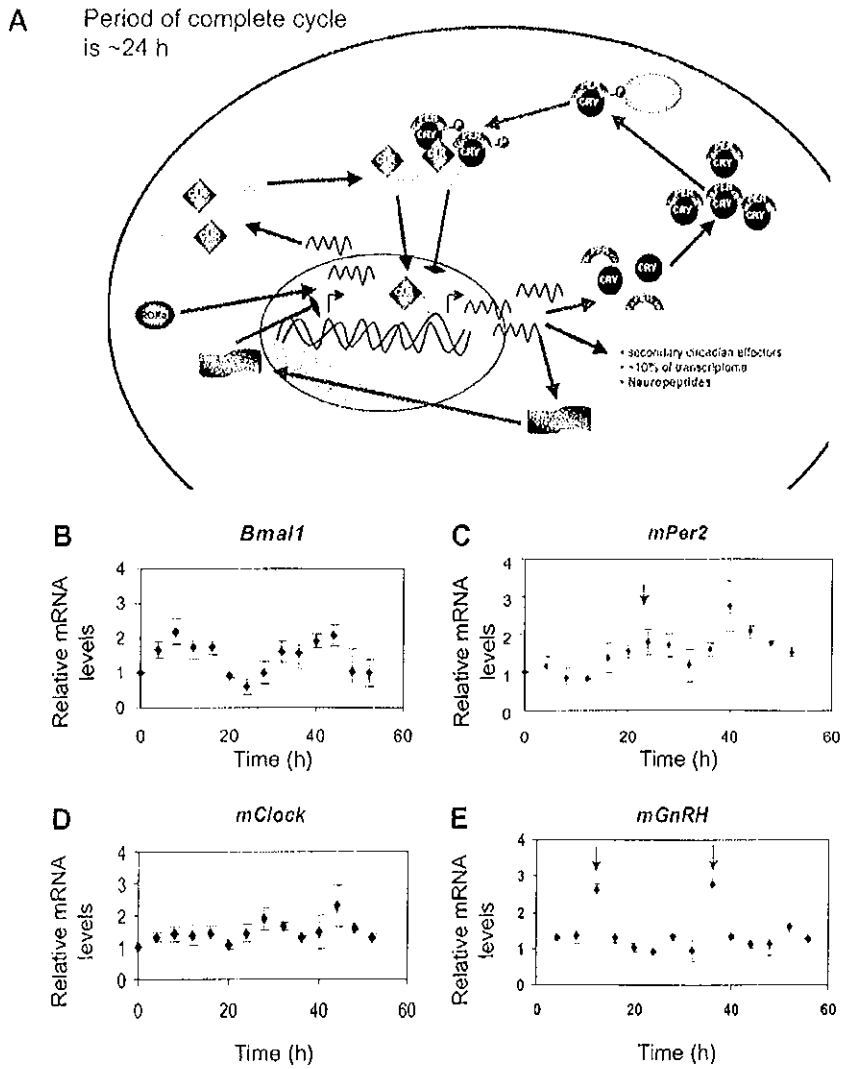

Fig. 4. Circadian regulation within hypothalamic neuronal models. (A) Schematic diagram of the basic transcriptional/translational molecular clock. Bmal1 and Clock are transcribed and translated. They heterodimerize and translocate to the nucleus where they initiate the transcription of the period (per) and cryptochrome (cry) genes, Rev-Erbα, secondary circadian effectors and approximately 10% of the transcriptome. Per and Cry proteins heterodimerize and build up in the cytoplasm where they are phosphorylated by casein kinase 1ε (CK1ε). They then inhibit the Bmal1/Clock heterodimer, thereby inhibiting their own transcription. Rev-Erbα also inhibits Bmal1 transcription. Eventually levels of Per, Cry and Rev-Erbα diminish and the cycle starts again, approximately 24 h later. (B–E) Gene expression profiles that demonstrate a circadian rhythmicity of clock gene and GnRH mRNA levels within the GT1-7 neurons (originally published in [63]).

yielded the first mammalian homolog of the fly system in 1997, the protein CLOCK. Further genomic research revealed many more mammalian homologs indicating that a mammalian model was required for continued research into mammalian circadian rhythmicity. As a result, mammalian retinal cells and SCN explants were cultured, as it was recognized that these areas exhibited innate oscillatory properties and had measurable endocrine outputs such as arginine vasopressin. But primary retinal and neuronal cultures are heterogeneous and only suitable for single interventions that often lack a genetic basis unless the primary culture came from a knock out or transgenic mouse model. With the advent of cell line development this has rapidly changed the understanding of the mammalian circadian system.

5.2. Clocks in peripheral cell lines

Initially circadian studies in cell lines were not thought to be advantageous as the circadian clock did not appear to cycle. However, in 1998 it was discovered that immortalized rat-1 fibroblasts could be synchronized with a serum bolus [11]. After synchronization, the fibroblasts demonstrate predictable patterns of clock gene transcription for up to three 22.5 h periods. From this study it was ascertained that a serum shock could mimic light-induced immediate early gene expression in a non-photoreceptive cell model. Balsalobre et al. then went on to prove that glucocorticoids could reset and synchronize rat-1 cultures, leading to the discovery that unlike central oscillators which are responsive to phase shifting at certain periods, peripheral oscillators retain the ability to be phase reset throughout different periods of the day [12]. Further, this group identified numerous signaling pathways including those for cAMP, Ca^{2+}, and protein kinase C (PKC) as being capable of eliciting changes in rat-1 fibroblast circadian gene expression and rhythmicity [13]. Two separate groups stably transfected the rat-1 fibroblast cell lines with reporter constructs for real time imaging. Through this technique it was successfully proven that the rhythms within the rat-1 lines are indeed true circadian rhythms,

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as they exhibit robust temperature compensation [79] and that individual peripheral cells in culture do cycle over 24 h, but lose their synchrony with their neighbours through lack of cell coupling [158]. This model was then expanded using the murine NIH-3T3 fibroblasts where it was discovered that the MAP kinase signal transduction pathway was also involved in the setting of the peripheral circadian clock in response to a potent PKC activator 12-O-tetradecanoylphorbol-13-acetate (TPA) administration and/or serum bolus [2]. Further work with the NIH-3T3 fibroblasts elaborated on the mechanisms of nucleocytoplasmic shuttling of BMAL1 [149], circadian temperature compensation [152], the importance of casein kinase 1 epsilon (CK1ε) to the stability of the period proteins [3] and that prostaglandin E2 could be used as a zeitgeber in cell culture studies [153]. Additional fibroblast-based studies involving the use of mouse embryonic fibroblast (MEF) cells has allowed for elucidation of alterations in the circadian system within transgenic mouse models, an innovation that removes the necessity for plasmid transfection or transduction. Notably, Hirayama et al. used MEF cells from BMAL1 knock out mice to prove that a mutant BMAL1 protein could not restore rhythmicity when it is unable to be acetylated by CLOCK [73].

Relatively little has been elucidated in the field of circadian rhythms in other tissue specific peripheral immortalized lines. In our lab, Chalmers et al. used the MOVAS-1 murine aortic smooth muscle cell line to clarify the expression of circadian rhythms in vasculature remodeling genes and the similarity in the circadian expression of these genes in vivo [31]. We also went on to characterize the expression of neuroendocrine genes in the heart and further elucidated the circadian profiles of proopiomelanocortin gene expression in the healthy and hypertrophied heart and MOVAS-1 cells [30].

Human cell lines have been used in cancer studies to elaborate on the link between circadian rhythmicity and cell cycle progression [155]. These cell lines include the HEK293 kidney cells, HeLa cervical cancer cells and the NHF-1 fibroblastic cells. Further, a group has recently established a stably transfected human retinal pigment epithelial (hTERT) cell line that expresses luciferase under the control of the BMAL1 promoter to elaborate on the nature of retinal circadian rhythms and photoresponsiveness [166].

5.3. Clocks in SCN neuronal cell lines

In addition to the important innovations accomplished through the use of peripheral cell lines, in 1999 Earnest et al. successfully established embryonic rat SCN cell lines, named the SCN 1.4 and 2.2 cells, that exhibit the functional characteristics of SCN neurons and exist in immortalized culture [49]. These cells are a heterogeneous population of cells and exhibit pacemaker potential and rhythmic expression of clock gene mRNAs, 2-deoxyglucose (2-DG) uptake and bone-derived neurotropic factor (BDNF) expression, much like the intact SCN [50]. These cells were further characterized and found to express a broad range of clock genes, and circadian regulatory pathways [76]. Additionally, the SCN2.2 cells were able to be phase shifted with a glutamatergic stimulus, similar to the SCN in vivo [77].

Of key importance, the SCN2.2 neurons were also able to restore behavioral rhythms when transplanted into arrhythmic, SCN-lesioned rats; an effect that was not reproducible with immortalized mesencephalic or fibroblastic cells [50]. This finding represents an incredibly important innovation in cell line usage. While the SCN2.2 neurons are immortalized, they have retained enough of their neuronal SCN phenotype to function appropriately in the in vivo context. This marks a fundamental keystone in the potential usage of immortalized cell lines in the treatment of in vivo disorders.

Further work with the SCN2.2 cells has highlighted valuable information about the study of circadian rhythms. The SCN2.2 cells were able to synchronize rat-1 fibroblasts in co-culture through diffusible signals [4]. While in co-culture, the SCN2.2 cells conferred rhythms within the rat-1 fibroblasts in their metabolic and clock gene expression with a 4 h delay, similar to the phase-shift delay seen between the SCN and periphery in vivo. Interestingly, only co-culture with the SCN2.2 was able to confer metabolic rhythmicity in the rat-1 fibroblasts; whereas serum shock was only able to synchronize gene expression. This effect suggests that diffusible signals from the SCN are required for metabolic cyclicity, whereas serum bolus only confers a synchronization of the molecular clock. The SCN2.2 cells were then assayed with real time analysis of a human c-fos reporter gene to catalog their responses to serum and potassium chloride (KCl). It was determined that the SCN2.2 cells exhibited a similar pattern of c-fos reactivity as the SCN in vivo [5]. Allen et al. then proceeded to assay the importance of CLOCK in the SCN2.2 and rat-1 fibroblast lines by transfecting the cell lines with anti-sense RNA for CLOCK. Subsequently, they found that disruption of CLOCK in the SCN2.2 neurons altered cyclicity of period gene expression and 2-DG uptake. They also reiterated these findings in the rat-1 cell lines, but then surprisingly found that even with antisense inhibition of CLOCK the rat-1 cell lines could be synchronized to cycle with a serum shock indicating that the SCN and periphery have different entrainable stimuli [6].

Other groups have elucidated a variety of important circadian findings that bolster the applicability of the SCN2.2 cells as a circadian model including: the circadian expression of nicotinamide adenine dinucleotides within the SCN2.2 cells [163], the functionality of melatonin receptors within the SCN2.2 cells [127], a rhythmicity of PKC with melatonin administration [126], the importance of voltage-dependent calcium channels in SCN and SCN2.2 rhythmicity [111] and a temporal desensitization of MT2 receptors with melatonin administration that allows a cyclic sensitivity of the SCN to melatonin [61]. All of these studies have helped to reiterate findings found within the explanted SCN, however, now exist in a model that has vastly greater potential for genetic and molecular intervention.

Recently, two additional SCN cell lines have been generated: the N14.5 cells, which are SV40-temperature sensitive ventrolateral SCN neurons developed for glutamatergic/photic entrainment studies [102]; and the per1-luciferase expressing RS182 cells [85]. Further circadian research with these new models is forthcoming and will likely shed further light on the field of circadian rhythms.

5.4. Circadian studies in Non-SCN neuronal cell lines

Owing to the relative shortage of neuronal cell lines for this type of work, there is a limited amount of data about the cyclicity of the circadian clock within non-SCN neuronal cell lines. Much of the existing data from non-SCN neuronal cell lines was elucidated using the immortalized GnRH-expressing GT1-7 cells. Chappell et al elucidated that GnRH secretion is altered by perturbation of the molecular clock. Over-expression of a dominant-negative Clock-Delta19 protein reduced mean pulse frequency of GnRH secretion. Further, over expression of mCry1 increased pulse amplitude of GnRH secretion, but did not affect overall frequency [33]. Research from our lab showed that the GT1-7 neurons contain functional melatonin receptors on the GT1-7 GnRH neurons and we clarified the actions of melatonin on the gene expression of GnRH over 24 h. We mapped the regions of melatonin responsiveness on the GnRH promoter and showed the first evidence of direct melatonin action on GnRH neurons [64,130]. We further elucidated the signaling cascades of melatonin within these pulsatile neuronal models. We were able to determine that melatonin signaling acts through multiple pathways including inhibition of the forskolin-

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induced increase in cAMP and activation of PKC, MAPK and the immediate early genes. We also determined that these pathways are involved in the melatonin-mediated decrease in GnRH secretion [131]. Finally, we went on to fully characterize the circadian gene expression and protein profiles within the GT1-7 neurons. The GT1-7 neurons express clock, BMAL1, timeless (tim), period1 (per1), period2 (per2), cryptochrome1 (cry1) and cryptochrome2 (cry2). Of these transcripts BMAL1, per1, per2 and GnRH mRNA expression was assayed over 54 h and the transcripts were found to cycle over 24 h (Fig. 4B–E). Accordingly, the protein levels of BMAL1 oscillated as well [63].

Further studies from our laboratory have exploited the novel generation of an array of clonal hypothalamic neuronal models [18]. We have successfully characterized the circadian profiles within multiple cell lines including the mHypoE-44, -42, -36/1, -36/2 and -39 neuronal models and have found that they represent an excellent model for neuronal circadian research outside the SCN. These cell lines express phenotypically distinct expression profiles of neuropeptides, receptors and signaling molecules. In addition to characterizing the clock gene profiles of these neurons – clock, BMAL1, timeless (tim), period1 (per1), period2 (per2), cryptochrome1 (cry1) and cryptochrome2 (cry2) – we have also generated a circadian gene expression profile for a number of important neuropeptides including the orexigenic: neuropeptide Y (NPY), preproghrelin and agouti-related peptide (AgRP); and the anorexigenic corticotropin-releasing hormone (Crh), neurotensin (NT) and neuromedin U (NMU) (L.J. Fick and D.D. Belsham, unpublished data). Interestingly, there appears to be inductive, but not cyclic, expression in NPY and NT gene expression following serum shock, but AgRP cycles over 24 h. Interestingly, both Crh and NMU exhibit ultradian (<24 h) rhythms. Preproghrelin oscillates, but without a fixed period. These are the first models to demonstrate direct rhythmicity within a single neuronal phenotype. We further elucidated the role of nutrient signals on the circadian system and discovered that palmitate, a 16-carbon saturated fatty acid, blunted clock cyclicity within the mHypoE-44 neurons and elevated orexigenic neuropeptide gene expression. Additionally, fructose administration reduced NMU gene expression, indicating a role for sugars in the abrogation of anorexigenic neuropeptide signaling. Further research into the mechanisms of these findings is currently under way. The use of cell lines will ultimately allow a characterization of the molecular mechanisms controlling circadian neuronal gene expression, whether the classic clock genes are directly involved in this process, and how these rhythmic patterns of expression can be disturbed by peripheral and central signals, perhaps leading to circadian disruption and disease.

6. Conclusion

Elucidating the functions of the hypothalamus is essential to generating an understanding of how the brain orchestrates many important processes vital to life. The hypothalamus is the seat of neuroendocrine control and as such is responsible for the regulation of feeding, reproduction and the coordination of circadian rhythmicity (Fig. 5). However, the location and heterogeneic nature of the hypothalamus precludes easy determination of its mechanisms within in vivo situation. This state of affairs has been rapidly altered with the advent of hypothalamic cell lines. Clonal and nucleus specific hypothalamic cell lines have permitted the clarification of many neuronal mechanisms that until now had been virtually impossible to determine. As discussed in this review, the knowledge gained from using cell lines representative of a single neuronal phenotype include analyses of neuropeptide gene expression, genetic intervention using RNAi and plasmid transfection, secretion, signal transduction, 5′ regulatory region control, ion channel function, peripheral hormone and factor responsiveness, and global microarray and proteomic data. While technology closes the gap between current in vivo techniques and future assays which will allow for single cellular analysis in situ, in the interim, cell lines have proven themselves invaluable as the workhorses of molecular biology within the hypothalamus and throughout the entire organism.

This review has highlighted the roles of hypothalamic cell lines in advancing the fields of feeding, reproduction and circadian rhythms (Table 2). While these three fields are the most heavily studied and have an essential impact on the health sector, further research with hypothalamic cell lines should yield novel information about these and the many other vital and ubiquitous functions of the hypothalamus. Future studies made possible by the advent of hypothalamic cell lines may include elaboration on gene expres-

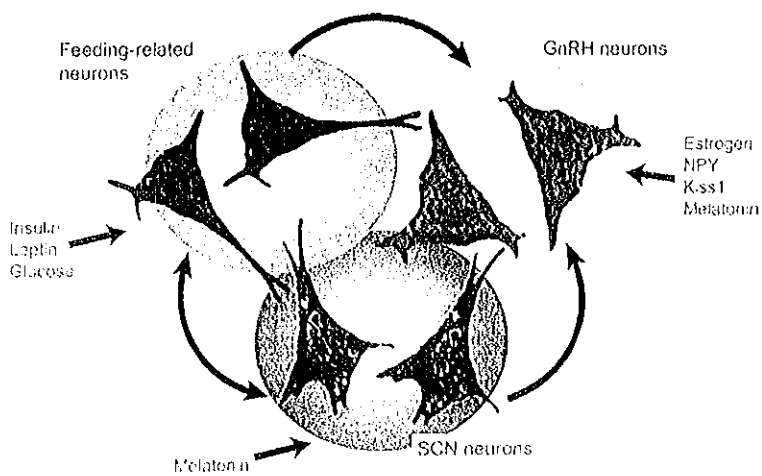

Fig. 5. Summary of hypothalamic neuronal interaction. The hypothalamus contains a complex interacting network of neurons. This network includes feeding-related, SCN and GnRH neurons. Feeding-related neurons can integrate peripheral signals, including insulin, leptin and glucose, as well as communicate to GnRH and SCN neurons. The SCN neurons receive input from melatonin and other neurons, such as the feeding-related neurons, and communicate to both the feeding-related and GnRH neurons. In contrast GnRH neurons only receive input from other hypothalamic neurons, including the feeding-related and SCN neurons. As well, they integrate signals from neuropeptides and hormones, including NPY, Estrogen, Kiss1 and melatonin. This figure indicates the integration between the neurons within these three systems and shows the relation between the hypothalamic areas regulating energy homeostasis, circadian rhythms and reproduction.

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sion, signal transduction in neurons, drug discovery, receptor cloning and characterization, ion channel function, phenotypic profiling of individual neuronal cell types, neuron–neuron interactions and communication, and numerous others. Cell line studies and in vivo research are complementary to each other; and, as demonstrated within this review, both models have been used effectively in concert to elucidate the mechanisms of our physiology. Cell line usage is an invaluable asset that can expand upon the molecular basis of knowledge gathered in the in vivo situation. Cell lines can be used for hypothesis clarification or as a preliminary tool upon which to base hypothesis. Together cell lines and in vivo research represent different, but equally important methods for achieving the same goal – knowledge of our physiology and an understanding of how we can use that information to advance health and treat disease.

Acknowledgments

We acknowledge generous funding from Canadian Institutes for Health Research, Natural Sciences and Engineering Research Council, Heart and Stroke Foundation of Ontario, Canadian Diabetes Association, Canada Foundation for Innovation, and the Canada Research Chairs Program to DDB. CMM and LJF were supported by Natural Sciences and Engineering Research Council Post-graduate Studentships, as well as a Banting and Best Diabetes Center Studentship (LJF) and an Ontario Graduate Studentship (LJF).

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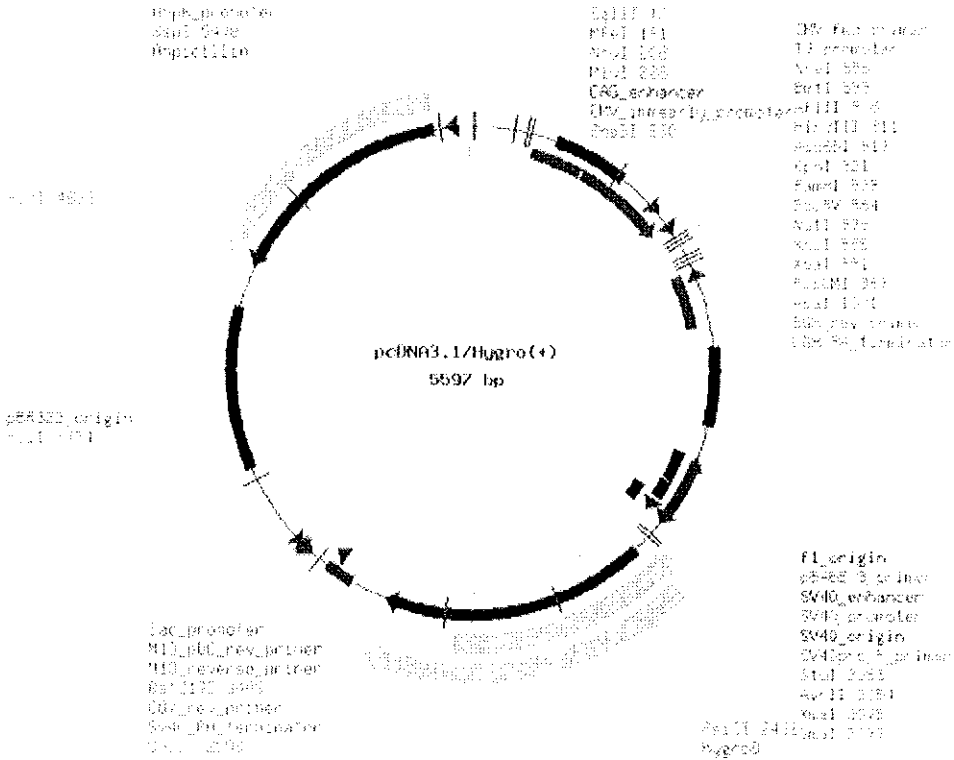
Please cite this article in press as: C.M. Mayer et al., Hypothalamic cell lines to investigate neuroendocrine control mechanisms, Front. Neuroendocrinol. (2009), doi:10.1016/j.yfrne.2009.03.005

addgene **Vector Database**

Vector Database is a list of plasmid backbones from publications and several companies, including cloning, mammalian expression, bacterial expression, and lentiviral and retroviral plasmids. The database is compiled by Addgene, and hosted on LabLife. LabLife does not sell or distribute any of the plasmids listed in this catalog.

Plasmid Name	pcDNA3.1/Hygro(+)
Alt Names	pcDNA 3.1 Hygro (+)
Source/Vendor	Invitrogen
Plasmid Type	Mammalian
Viral/Non-viral	Nonviral
Stable/Transient	Transient
Constitutive/Inducible	Constitutive
Promoter	CMV
Expression Level	High
Plasmid Size	5597
Sequencing Primer	T7 Fwd
Sequencing Primer Sequence	5'd[TAATACGACTCACTATAGGG]3'
Bacterial Resistance	Ampicillin
Mammalian Selection	Hygromycin
Notes	Differs from other pcDNA3.1 in drug resistance; +/- refers to orientation of f1 ori.
Catalog Number	V87020
Plasmid Sequence	View Sequence


pcDNA3.1/Hygro(+)
5597 bp
CAG_enhancer
f1_origin
SV40_enhancer
SV40_origin
lac_promoter
Hygro

1. IDENTIFICATION OF THE SUBSTANCE/PREPARATION AND THE COMPANY/UNDERTAKING

Product code 350675
Product name pcDNA3.1/Hygro (+)

Contact manufacturer
INVITROGEN CORPORATON
1600 FARADAY AVENUE
PO BOX 6482
CARLSBAD, CA 92008
760-603-7200

INVITROGEN CORPORATION
2270 INDUSTRIAL STREET
BURLINGTON, ONT
CANADA L7P 1A1
800-263-6236

GIBCO PRODUCTS
INVITROGEN CORPORATION
3175 STALEY ROAD P.O. BOX 68
GRAND ISLAND, NY 14072
716-774-6700

2. COMPOSITION/INFORMATION ON INGREDIENTS

Hazardous/Non-hazardous Components
The product contains no substances which at their given concentration, are considered to be hazardous to health

3. HAZARDS IDENTIFICATION

Emergency Overview
The product contains no substances which at their given concentration, are considered to be hazardous to health.

Form
Solid

Principle Routes of Exposure/
Potential Health effects

Eyes	No information available
Skin	No information available
Inhalation	No information available
Ingestion	No information available

Specific effects

Carcinogenic effects	No information available
Mutagenic effects	No information available
Reproductive toxicity	No information available

Sensitization	No information available
Target Organ Effects	No information available

4. FIRST AID MEASURES

Skin contact	Wash off immediately with plenty of water
Eye contact	Rinse thoroughly with plenty of water, also under the eyelids.
Ingestion	Never give anything by mouth to an unconscious person
Inhalation	Move to fresh air
Notes to physician	Treat symptomatically

5. FIRE-FIGHTING MEASURES

Suitable extinguishing media	Dry chemical
Special protective equipment for firefighters	Wear self-contained breathing apparatus and protective suit

6. ACCIDENTAL RELEASE MEASURES

Personal precautions	Use personal protective equipment
Methods for cleaning up	Soak up with inert absorbent material

7. HANDLING AND STORAGE

Handling	No special handling advice required
Storage	Keep in properly labelled containers

8. EXPOSURE CONTROLS / PERSONAL PROTECTION

Occupational exposure controls

Exposure limits

Engineering measures — Ensure adequate ventilation, especially in confined areas

Personal protective equipment

Respiratory protection	In case of insufficient ventilation wear suitable respiratory equipment
Hand protection	Protective gloves
Eye protection	Safety glasses with side-shields
Skin and body protection	Lightweight protective clothing
Hygiene measures	Handle in accordance with good industrial hygiene and safety practice
Environmental exposure controls	Prevent product from entering drains

9. PHYSICAL AND CHEMICAL PROPERTIES

General Information

Form — Solid

Important Health Safety and Environmental Information

Boiling point/range	°C No data available	°F No data available
Melting point/range	°C No data available	°F No data available
Flash point	°C No data available	°F No data available
Autoignition temperature	°C No data available	°F No data available
Oxidizing properties	No information available	

Water solubility	No data available

10. STABILITY AND REACTIVITY

Stability	Stable.
Materials to avoid	No information available
Hazardous decomposition products	No information available
Polymerization	Hazardous polymerisation does not occur

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Principle Routes of Exposure/ Potential Health effects

Eyes	No information available
Skin	No information available
Inhalation	No information available
Ingestion	No information available

Specific effects

Carcinogenic effects	No information available
Mutagenic effects	No information available
Reproductive toxicity	No information available
Sensitization	No information available

Target Organ Effects — No information available

12. ECOLOGICAL INFORMATION

Ecotoxicity effects	No information available.
Mobility	No information available.
Biodegradation	Inherently biodegradable.
Bioaccumulation	Does not bioaccumulate.

13. DISPOSAL CONSIDERATIONS

Dispose of in accordance with local regulations

14. TRANSPORT INFORMATION

IATA

Proper shipping name	Not classified as dangerous in the meaning of transport regulations
Hazard Class	No information available
Subsidiary Class	No information available
Packing group	No information available
UN-No	No information available

15. REGULATORY INFORMATION

International Inventories

U.S. Federal Regulations

SARA 313
Not regulated

Clean Air Act, Section 112 Hazardous Air Pollutants (HAPs) (see 40 CFR 61)
This product contains the following HAPs:

U.S. State Regulations

California Proposition 65
This product contains the following Proposition 65 chemicals:

WHMIS hazard class:
Non-controlled

This product has been classified according to the hazard criteria of the CPR and the MSDS contains all of the information required by the CPR

16. OTHER INFORMATION

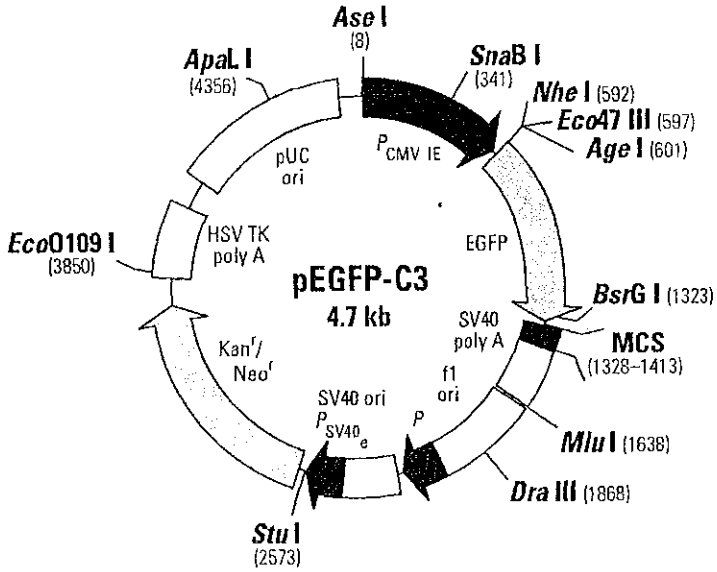
This material is sold for research and development purposes only. It is not for any human or animal therapeutic or clinical diagnostic use. It is not intended for food, drug, household, agricultural, or cosmetic use. An individual technically qualified to handle potentially hazardous chemicals must supervise the use of this material.

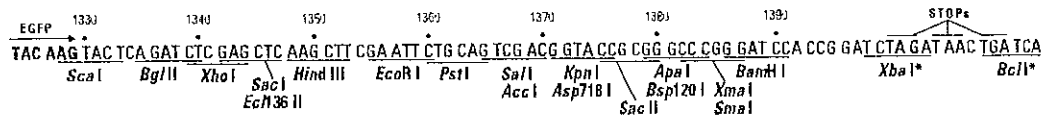
The above information was acquired by diligent search and/or investigation and the recommendations are based on prudent application of professional judgment. The information shall not be taken as being all inclusive and is to be used only as a guide. All materials and mixtures may be present unknown hazards and should be used with caution. Since Invitrogen Corporation cannot control the actual methods, volumes, or conditions of use, the Company shall not be held liable for any damages or losses resulting from the handling or from contact with the product as described herein. THE INFORMATION IN THIS MSDS DOES NOT CONSTITUTE A WARRANTY, EXPRESS OR IMPLIED, INCLUDING ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR ANY PARTCULAR PURPOSE.

End of Safety Data Sheet

pEGFP-C3 Vector Information PT3052-5

GenBank Accession #: U57607 Catalog #6082-1





Restriction Map and Multiple Cloning Site (MCS) of pEGFP-C3. All restriction sites shown are unique. The *Bcl* I site cannot be used for fusions since it contains an in-frame stop codon. The *Xba* I and *Bcl* I sites (*) are methylated in the DNA provided by BD Biosciences Clontech. If you wish to digest the vector with these enzymes, you will need to transform the vector into a *dam*⁻ host and make fresh DNA.

Description:

pEGFP-C3 encodes a red-shifted variant of wild-type GFP (1–3) which has been optimized for brighter fluorescence and higher expression in mammalian cells. (Excitation maximum = 488 nm; emission maximum = 507 nm.) pEGFP-C3 encodes the GFPmut1 variant (4) which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences (5). Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site (6) to further increase the translation efficiency in eukaryotic cells. The MCS in pEGFP-C3 is between the EGFP coding sequences and the SV40 poly A. Genes cloned into the MCS will be expressed as fusions to the C terminus of EGFP if they are in the same reading frame as EGFP and there are no intervening stop codons. SV40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3' end of the EGFP mRNA. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T-antigen. A neomycin resistance cassette (Neor), consisting of the SV40 early promoter, the neomycin/ kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex virus thymidine kinase (HSV TK) gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette expresses kanamycin resistance in *E. coli.* The pEGFP-C3 backbone also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production.

(PR29969; published 03 October 2002)

Use:
Fusions to the C terminus of EGFP retain the fluorescent properties of the native protein allowing the localization of the fusion protein *in vivo*. The target gene should be cloned into pEGFP-C3 so that it is in frame with the EGFP coding sequences, with no intervening in-frame stop codons. The recombinant EGFP vector can be transfected into mammalian cells using any standard transfection method. If required, stable transformants can be selected using G418 (7). pEGFP-C3 can also be used simply to express EGFP in a cell line of interest (e.g., as a transfection marker).

Location of Features:
- Human cytomegalovirus (CMV) immediate early promoter: 1–589
  Enhancer region: 59–465; TATA box: 554–560
  Transcription start point: 583
  C→G mutation to remove *Sac* I site: 569
- Enhanced green fluorescent protein gene
  Kozak consensus translation initiation site: 606–616
  Start codon (ATG): 613–615; Stop codon: 1408–1410
  Insertion of Val at position 2: 616–618
  GFPmut1 chromophore mutations (Phe-64 to Leu; Ser-65 to Thr): 805–810
  His-231 to Leu mutation (A→T): 1307
  Last amino acid in wild-type GFP: 1327–1329
- MCS: 1328–1413
- SV40 early mRNA polyadenylation signal
  Polyadenylation signals: 1546–1551 & 1575–1580; mRNA 3' ends: 1584 & 1596
- f1 single-strand DNA origin: 1643–2098 (Packages the noncoding strand of EGFP)
- Bacterial promoter for expression of Kan^r gene
  –35 region: 2160–2165; –10 region: 2183–2188
  Transcription start point: 2195
- SV40 origin of replication: 2439–2574
- SV40 early promoter
  Enhancer (72-bp tandem repeats): 2272–2343 & 2344–2415
  21-bp repeats: 2419–2439, 2440–2460 & 2462–2482
  Early promoter element: 2495–2501
  Major transcription start points: 2491, 2529, 2535 & 2540
- Kanamycin/neomycin resistance gene
  Neomycin phosphotransferase coding sequences:
  Start codon (ATG): 2623–2625; stop codon: 3415–3417
  G→A mutation to remove *Pst* I site: 2805
  C→A (Arg to Ser) mutation to remove *Bss*H II site: 3151
- Herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal
  Polyadenylation signals: 3653–3658 & 3666–3671
- pUC plasmid replication origin: 4002–4645

Primer Locations:
- EGFP-N Sequencing Primer (#6479-1): 679–658
- EGFP-C Sequencing Primer (#6478-1): 1266–1287

Propagation in *E. coli*:
- Suitable host strains: DH5α, HB101, and other general purpose strains. Single-stranded DNA production requires a host containing an F plasmid such as JM109 or XL1-Blue.
- Selectable marker: plasmid confers resistance to kanamycin (30 µg/ml) to *E. coli* hosts.
- *E. coli* replication origin: pUC
- Copy number: ≈500
- Plasmid incompatibility group: pMB1/ColE1

References:

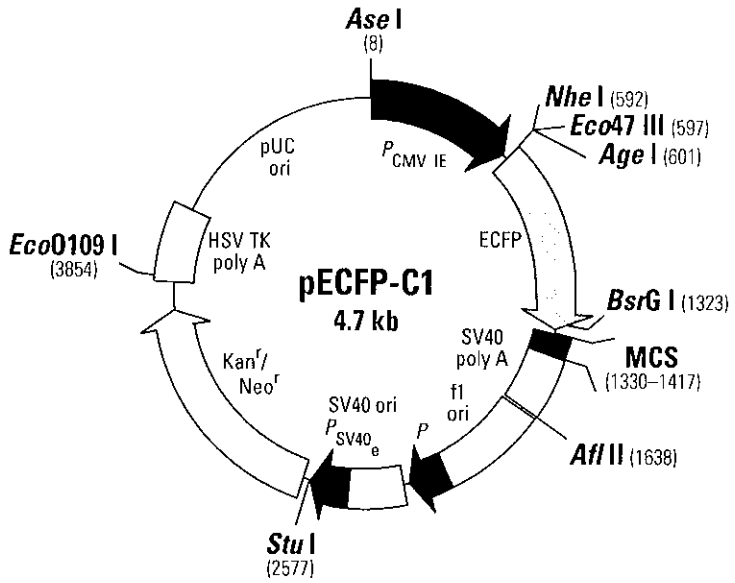
1. Prasher, D. C., *et al.* (1992) *Gene* **111**:229–233.
2. Chalfie, M., *et al.* (1994) *Science* **263**:802–805.
3. Inouye, S. & Tsuji, F. I. (1994) *FEBS Letters* **341**:277–280.
4. Cormack, B., *et al.* (1996) *Gene* **173**:33–38.
5. Haas, J., *et al.* (1996) *Curr. Biol.* **6**:315–324.
6. Kozak, M. (1987) *Nucleic Acids Res.* **15**:8125–8148.
7. Gorman, C. (1985) In *DNA Cloning: A Practical Approach, Vol. II*, Ed. Glover, D. M. (IRL Press, Oxford, UK) pp. 143–190.

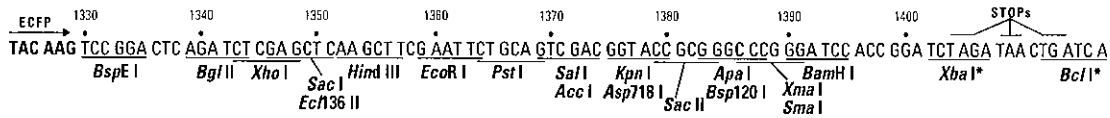
Note: The attached sequence file has been compiled from information in the sequence databases, published literature, and other sources, together with partial sequences obtained by BD Biosciences Clontech. This vector has not been completely sequenced.

Vector Information

pECFP-C1 Vector Information PT3259-5

Catalog #6076-1





Restriction map and multiple cloning site (MCS) of pECFP-C1. Unique restriction sites are in bold. The *Xba* I and *Bcl* I sites (*) are methylated in the DNA provided by BD Biosciences Clontech. If you wish to digest the vectors with these enzymes, you will need to transform the vector into a *dam*⁻ host and make fresh DNA.

Description

pECFP-C1 encodes an enhanced cyan fluorescent variant of the *Aequorea victoria* green fluorescent protein gene (GFP). The ECFP gene contains six amino acid substitutions. The Tyr-66 to Trp substitution gives ECFP fluorescence excitation (major peak at 433 nm and a minor peak at 453 nm) and emission (major peak at 475 nm and a minor peak at 501 nm) similar to other cyan emission variants (1–3). The other five substitutions (Phe-64 to Leu; Ser-65 to Thr; Asn-146 to Ile; Met-153 to Thr; and Val-163 to Ala) enhance the brightness and solubility of the protein, primarily due to improved protein-folding properties and efficiency of chromophore formation (2, 4, 5).

In addition to the chromophore mutations, ECFP contains >190 silent mutations that create an open reading frame comprised almost entirely of preferred human codons (6). Furthermore, upstream sequences flanking ECFP have been converted to a Kozak consensus translation initiation site (7). These changes increase the translational efficiency of the ECFP mRNA and consequently the expression of ECFP in mammalian and plant cells.

The MCS in pECFP-C1 is between the ECFP coding sequence and the stop codon. Genes cloned into the MCS will be expressed as fusions to the C-terminus of ECFP if they are in the same reading frame as ECFP and there are no intervening in-frame stop codons. ECFP with a C-terminal fusion moiety retains the fluorescent properties of the native protein and thus can be used to localize fusion proteins *in vivo*.

The vector contains an SV40 origin for replication and a neomycin resistance (Neor) gene for selection (using G418) in eukaryotic cells. A bacterial promoter (*P*) upstream of Neor expresses kanamycin resistance in *E. coli*. The vector backbone also provides a pUC19 origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production.

(PR29964; published 03 October 2002)

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Mountain View, CA 94043
Technical Support (US)
E-mail: tech@clontech.com
www.clontech.com

The recombinant ECFP vector can be transfected into mammalian cells using any standard transfection method. If required, stable transfectants can be selected using G418 (8). pECFP-C1 can also be used simply to express ECFP in a cell line of interest (e.g., as a transfection marker).

Location of features

- Human cytomegalovirus (CMV) immediate early promoter: 1–589
  Enhancer region: 59–465; TATA box: 554–560; transcription start point: 583
  C→G mutation to remove *Sac* I site: 569
- Enhanced cyan fluorescent protein gene
  Kozak consensus translation initiation site: 606–616
  Start codon (ATG): 613–615; stop codon: 1408–1410
  Insertion of Val at position 2: 616–618
  ECFP mutations (Phe-64 to Leu; Ser-65 to Thr; and Tyr-66 to Trp): 805–813; Asn-146 to Ile: 1051-1053; Met-153 to Thr: 1072–1074; Val-163 to Ala: 1102-1104
  His-231 to Leu mutation (A→T): 1307
  Last amino acid in ECFP coding region: 1327–1329
- MCS: 1330–1417
- SV40 early mRNA polyadenylation signal
  Polyadenylation signals: 1550–1555 & 1579–1584; mRNA 3' ends: 1588 & 1600
- f1 single-strand DNA origin: 1647–2102 (Packages the noncoding strand of ECFP.)
- Bacterial promoter for expression of Kan^r gene.
  –35 region: 2164–2169; –10 region: 2187–2192
  Transcription start point: 2199
- SV40 origin of replication: 2443–2578
- SV40 early promoter
  Enhancer (72-bp tandem repeats): 2276–2347 & 2348–2419
  21-bp repeats: 2423–2443, 2444–2464 & 2466–2486
  Early promoter element: 2499–2505
  Major transcription start points: 2495, 2533, 2539 & 2544
- Kanamycin/neomycin resistance gene
  Neomycin phosphotransferase coding sequences:
  Start codon (ATG): 2627–2629; stop codon: 3419–3421
  G→A mutation to remove *Pst* I site: 2809
  C→A (Arg to Ser) mutation to remove *Bss*H II site: 3155
- Herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal
  Polyadenylation signals: 3657–3662 & 3670–3675
- pUC plasmid replication origin: 4006–4649

Primer Locations:

- EGFP-N Sequencing Primer (#6479-1): 679–658
- EGFP-C Sequencing Primer (#6478-1): 1266–1287

Propagation in *E. coli*:

- Suitable host strains: DH5α, HB101, and other general purpose strains. Single-stranded DNA production requires a host containing an F plasmid such as JM109 or XL1-Blue.
- Selectable marker: plasmid confers resistance to kanamycin (30 µg/ml) to *E. coli* hosts.
- *E. coli* replication origin: pUC
- Copy number: ≈500
- Plasmid incompatibility group: pMB1/ColE1

References:

1. Heim, R., *et al.* (1994) *Proc. Natl. Acad. Sci. USA* **91**:12501–12504.
2. Heim, R. & Tsien, R. Y. (1996) *Curr. Biol.* **6**:178–182.
3. Miyawaki, A., *et al.* (1997) *Nature* **388**:882–887.
4. Cormack, B., *et al.* (1996) *Gene* **173**:33–38.
5. Yang, T. T., *et al.* (1996) *Nucleic Acids Res.* **24**:4592–4593.
6. Haas, J., *et al.* (1996) *Curr. Biol.* **6**:315–324.
7. Kozak, M. (1987) *Nucleic Acids Res.* **15**:8125–8148.
8. Gorman, C. (1985) In *DNA Cloning: A Practical Approach, Vol. II*, Ed. Glover, D. M. (IRL Press, Oxford, UK), pp. 143–190.

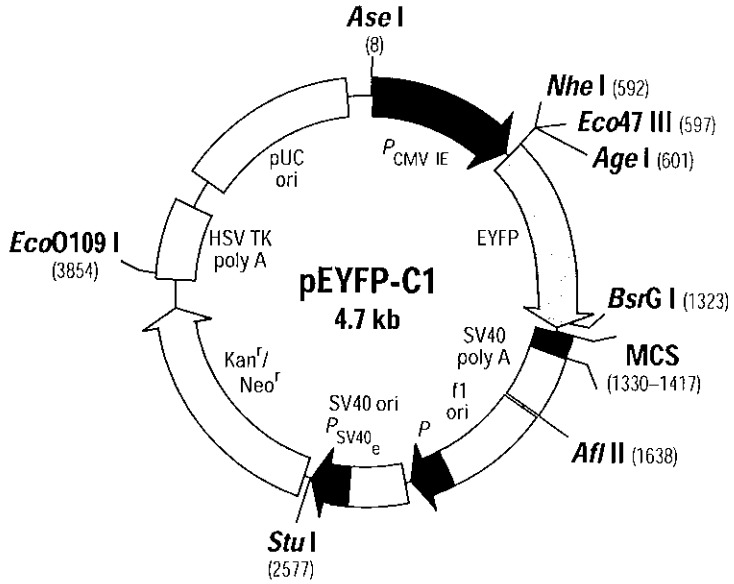
Note: The attached sequence file has been compiled from information in the sequence databases, published literature, and other sources, together with partial sequences obtained by BD Biosciences Clontech. This vector has not been completely sequenced.

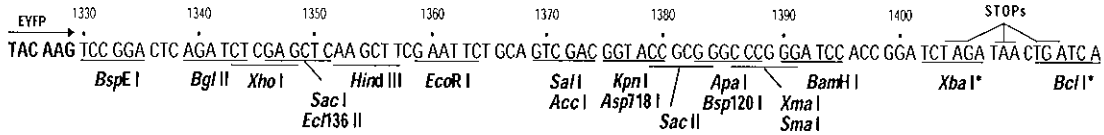
Vector Information

pEYFP-C1 Vector Information

PT3176-5

Catalog #6005-1





Restriction map and multiple cloning site (MCS) of pEYFP-C1. All restriction sites are shown are unique. The *Xba* I and *Bcl* I sites (*) are methylated in the DNA provided by BD Biosciences Clontech. If you wish to digest the vectors with these enzymes, you will need to transform the vector into a *dam*⁻ host and make fresh DNA.

Description:

pEYFP-C1 encodes an enhanced yellow-green variant of the *Aequorea victoria* green fluorescent protein (GFP). The EYFP gene contains the four amino acid substitutions previously published as GFP-10C (1): Ser-65 to Gly; Val-68 to Leu; Ser-72 to Ala; and and Thr-203 to Tyr. The fluorescence excitation maximum of EYFP is 513 nm; the emission spectrum has a peak at 527 nm (in the yellow-green region). When excited at 513-nm, the E_m of EYFP is 36,500 $cm^{-1}M^{-1}$ and the fluorescent quantum yield is 0.63 (1), resulting in a bright fluorescent signal. The fluorescence observed is roughly equivalent to that from EGFP.

A mixture of EYFP- and EGFP-expressing cells can be sorted by flow cytometry using a single excitation wavelength (i.e., 488 nm). EYFP emission is detected using a 525-nm dichroic shortpass mirror and a 530/30-nm bandpass filter; EGFP emission is detected using a 510/20-nm bandpass filter.

In addition to the chromophore mutations, EYFP contains >190 silent mutations that create an open reading frame comprised almost entirely of preferred human codons (2). Furthermore, upstream sequences flanking EYFP have been converted to a Kozak consensus translation initiation site (3). These changes increase the translational efficiency of the EYFP mRNA and consequently the expression of EYFP in mammalian and plant cells.

The MCS in pEYFP-C1 is between the EYFP coding sequence and the stop codon. Genes cloned into the MCS will be expressed as fusions to the C-terminus of EYFP if they are in the same reading frame as EYFP and there are no intervening in-frame stop codons. EYFP with a C-terminal fusion moiety retains the fluorescent properties of the native protein and thus can be used to localize fusion proteins *in vivo*.

The vector contains an SV40 origin for replication and a neomycin resistance (Neo^r) gene for selection (using G418) in eukaryotic cells. A bacterial promoter (*P*) upstream of Neo^r expresses kanamycin resistance in *E. coli*. The vector backbone also provides a pUC19 origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production. The recombinant EYFP vector can be

(PR29944; published 03 October 2002)

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transfected into mammalian cells using any standard transfection method. If required, stable transformants can be selected using G418 (4). pEYFP-C1 can also be used simply to express EYFP in a cell line of interest (e.g., as a transfection marker).

Location of features:

- Human cytomegalovirus (CMV) immediate early promoter: 1–589
  Enhancer region: 59–465; TATA box: 554–560; transcription start point: 583
  C→G mutation to remove *Sac* I site: 569
- Enhanced yellow fluorescent protein (EYFP) gene:
  Kozak consensus translation initiation site: 606–616
  Start codon (ATG): 613–615; stop codon: 1408–1410
  Insertion of Val at position 2: 616–618
  GFP-10C mutations (Ser-65 to Gly: 808–810; Val-68 to Leu: 817–819; Ser-72 to Ala: 829–831; Thr-203 to Tyr: 1222–1224)
  His-231 to Leu mutation (A→T): 1307
  Last amino acid in wild-type GFP coding region: 1327–1329
- MCS: 1330–1417
- SV40 early mRNA polyadenylation signal:
  Polyadenylation signals: 1550–1555 & 1579–1584; mRNA 3' ends: 1588 & 1600
- f1 single-strand DNA origin: 1647–2102 (Packages the noncoding strand of EYFP.)
- Bacterial promoter for expression of Kan^r gene:
  –35 region: 2164–2169; –10 region: 2187–2192
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- Selectable marker: plasmid confers resistance to kanamycin (30 µg/ml) to *E. coli* hosts.
- *E. coli* replication origin: pUC
- Copy number: ≈500
- Plasmid incompatibility group: pMB1/ColE1

References:

1. Ormö, M., *et al.* (1996) *Science* **273**:1392–1395.
2. Haas, J., *et al.* (1996) *Curr. Biol.* **6**:315–324.
3. Kozak, M. (1987) *Nucleic Acids Res.* **15**:8125–8148.
4. Gorman, C. (1985) In *DNA Cloning: A Practical Approach, Vol. II*, Ed. Glover, D. M. (IRL Press, Oxford, UK), pp. 143–190.

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