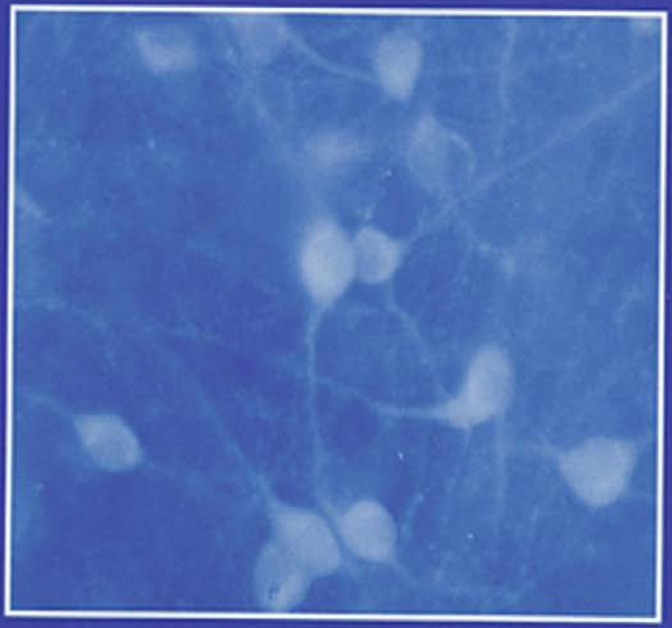
INTERNATIONAL REVIEW OF CYTOLOGY

A SURVEY OF CELL BIOLOGY

Edited by
Kwang W. Jeon



Volume 213

ACADEMIC PRESS

International Review of Cytology
A Survey of Cell Biology

VOLUME 213

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VOLUME 213

ACADEMIC PRESS
An Elsevier Science Imprint
San Diego San Francisco New York Boston London Sydney Tokyo

Front cover photograph: Immunofluorescence micrograph of MCH neurons. (For more details, see color insert figure 6.6)

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Academic Press
An Elsevier Science Imprint.
525 B Street, Suite 1900, San Diego, California 92101-4495, USA
http://www.academicpress.com

Academic Press
32 Jamestown Road, London NW1 7BY, UK
http://www.academicpress.com

International Standard Book Number: 0-12-364617-0

PRINTED IN THE UNITED STATES OF AMERICA
02 03 04 05 06 07 EB 9 8 7 6 5 4 3 2 1

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GABA and GABA Receptors in the Central Nervous System and Other Organs

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γ-Aminobutyrate (GABA) is a major inhibitory neurotransmitter in the adult mammalian brain. GABA is also considered to be a multifunctional molecule that has different situational functions in the central nervous system, the peripheral nervous system, and in some nonneuronal tissues. GABA is synthesized primarily from glutamate by glutamate decarboxylase (GAD), but alternative pathways may be important under certain situations. Two types of GAD appear to have significant physiological roles. GABA functions appear to be triggered by binding of GABA to its ionotropic receptors, $GABA_A$ and $GABA_C$, which are ligand-gated chloride channels, and its metabotropic receptor, $GABA_B$. The physiological, pharmacological, and molecular characteristics of $GABA_A$ receptors are well documented, and diversity in the pharmacologic properties of the receptor subtypes is important clinically. In addition to its role in neural development, GABA appears to be involved in a wide variety of physiological functions in tissues and organs outside the brain.

KEY WORDS: GABA, GAD, $GABA_A$ receptor, $GABA_B$ receptor, Metabolism, Polyamine, Development.

I. Introduction

Approximately 30 years have passed since γ-aminobutyrate (GABA), which is a well-known amino acid present in bacteria and plants, was first recognized as an inhibitory neurotransmitter in the mammalian brain. To date, dozens of molecules, including monoamine, acetylcholine, amino acids, and peptides, have

been classified as neurotransmitters. In the 1970s, neurotransmission was thought to be controlled by two major systems: the monoaminergic system and the cholinergic system. In addition to these systems, amino acids such as GABA and glycine have also received attention. It was in the latter half of the 1970s that many neuropeptides were recognized to be involved in neurotransmission, and much attention was given to these neuropeptides. During that period, interest in amino acids diminished. GABA has recently regained attention because of the dramatic progress in brain research that has revealed the diversity of GABA receptors and their physiological functions. The functions of GABA receptors in the endocrine system (Racagni and Donoso, 1986), various central nervous system (CNS) diseases (Tanaka and Bowery, 1996), and peripheral tissues and organs are being studied and should clarify the physiological functions of GABA. The importance of GABA research is reflected in the number of scientific publications related to this molecule. A MEDLINE search of papers with the key word GABA yielded approximately 1000 references per year from 1980 to 1985, 400 references per year from 1986 to 1990, and more than 1600 references per year from 1991 to 1999. During 2000, approximately 150 references related to GABA were added per month. It has been hypothesized that GABA is a multifunctional molecule that has different functions in the nervous system that are situation dependent. GABA influences neural migration (Behar *et al.,* 1994, 1996), acts as a neurotrophic factor (Barbin *et al.,* 1993; Obata, 1997), and facilitates neurite extension (Behar *et al.,* 1998).

In this review, we first discuss how GABA is synthesized in the brain, with emphasis on the metabolic interrelation of GABAergic and glutamatergic neurons and glial cells, on the rate-limiting enzyme glutamate decarboxylase (GAD), and on polyamines in alternative GABA synthesis pathways. This is followed by a description of $GABA_A$ receptors and a brief description of $GABA_B$ receptors. Finally, we discuss the possible roles of the GABA system in brain development and peripheral tissues. This review will not address the pharmacological aspects of the GABA system because this has been covered elsewhere (Macdonald and Olsen, 1994; Sieghart, 1995; Kerr and Ong, 1995; Mehta and Ticku, 1999). We have also omitted discussion of the spinal cord for the same reason (Malcangio and Bowery, 1996).

II. Metabolic Pathways of GABA

A. Discovery of GABA and Its Precursor in the Brain of Vertebrates

GABA was first identified in mammalian brain by Awapara *et al.* (1950) and by Roberts and Frankel (1950). In their reports, Awapara *et al.* suggested that a novel enzyme was responsible for catalyzing the conversion of glutamate to GABA. When brain homogenates of rat and rabbit were incubated with glutamate, the

decrease in glutamate levels paralleled the increase in GABA levels. Roberts and Frankel and Udenfriend (1950) then demonstrated that glutamate is a precursor of GABA. In 1951, very high levels of GAD activity were observed in the mouse brain (Roberts and Frankel, 1951).

B. Biosynthesis of Glutamate

Biosynthesis of glutamate in the brain is tied closely to glucose metabolism. The brain is separated from the vascular system by the blood–brain barrier (BBB), and although glucose and essential amino acids can cross the BBB via specific transporters, the nonessential amino acid glutamate and its precursor glutamine cannot. Therefore, in the brain, glutamate is synthesized from glucose. It has been shown that the labeled carbon atoms in ^{14}C-glucose are rapidly incorporated into glutamic acid and GABA (Shimada *et al.,* 1973). The route for this conversion is cytoplasmic glycolysis followed by the tricarboxylic acid (TCA) cycle in the mitochondria. In this pathway, α-ketoglutarate, which is an intermediate of the TCA cycle and an important precursor of glutamate, is generated (Fig. 1). It is interesting that glutamate, an important excitatory neurotransmitter, is a precursor of GABA, which is an inhibitory neurotransmitter.

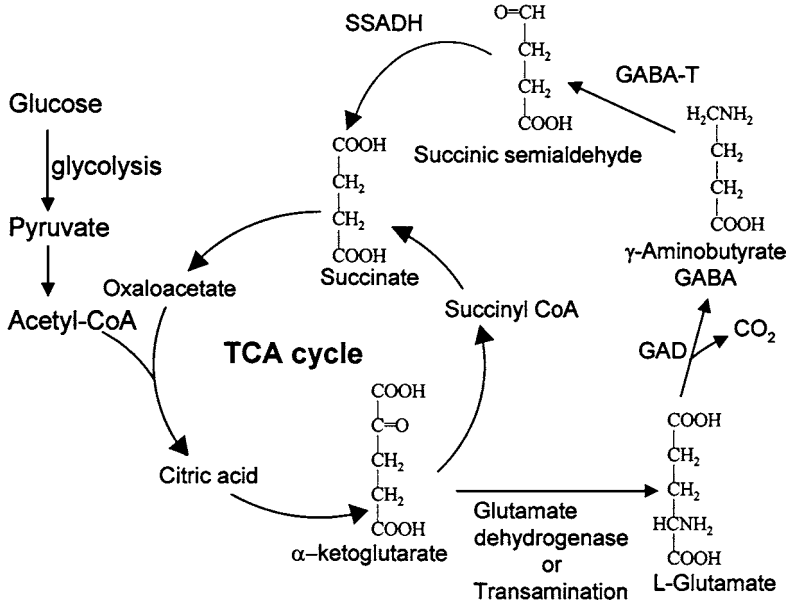

FIG. 1 Major metabolic pathway of GABA. GAD, glutamate decarboxylase; GABA-T, γ-aminobutyrate transaminase; SSADH, succinic semialdehyde dehydrogenase.

C. Major Pathway of GABA Metabolism: GABA Shunt

As shown in Fig. 1, the major pathway of GABA metabolism involves a route from α-ketoglutarate generated by the TCA cycle to succinate via glutamate, GABA, and succinic semialdehyde. This pathway bypasses the TCA cycle and is called the GABA shunt. The first step of this pathway is production of glutamate from α-ketoglutarate. Glutamate is converted to GABA by a decarboxylation reaction catalyzed by GAD. GAD is the rate-limiting enzyme in GABA synthesis, and it requires pyridoxal phosphate (PLP) as its cofactor (Roberts and Kuriyama, 1968). The reaction from glutamate to GABA is essentially irreversible. GABA catabolism is catalyzed by GABA transaminase (GABA-T), which produces succinic semialdehyde (SSA) from GABA with stoichiometric conversion of α-ketoglutarate to glutamate. The recycling of this glutamate for GABA synthesis will be described later in relation to metabolic compartment theory. In the second step of GABA catabolism, SSA is rapidly oxidized by succinic semialdehyde dehydrogenase (SSADH), and it enters the TCA cycle as succinate. GABA-T and SSADH are localized in the mitochondria. GABA-T catalyzes a reversible reaction, but SSADH activity is typically higher than that of GABA-T. The K_m of SSADH for SSA is very low (Pitts, 1965; Baxter, 1970). Therefore, GABA-T and SSADH catalyze GABA catabolism (Roberts and Hammerschlag, 1972; Tunnicliff, 1986).

The GABA shunt may function to allow GABA utilization as an energy source (Baxter, 1970). However, in terms of energy the GABA shunt pathway is not equivalent to conversion of α-ketoglutarate to succinate in the TCA cycle. During metabolic conversion of α-ketoglutarate to succinate by the TCA cycle, one ATP and one NADH are generated, whereas the GABA shunt generates only one NADH. Because only 8–10% of the flow through the TCA cycle is directed through the shunt (Patel *et al.,* 1970), the main function of the GABA shunt is thought to be synthesis of GABA. This is supported by the fact that the shunt is not distributed uniformly throughout the brain; the rate-limiting enzyme in GABA synthesis, GAD, is found almost exclusively in the inhibitory neurons that release GABA as a neurotransmitter (Kugler, 1993).

As described previously, GABA is synthesized only by GABAergic neurons in the brain. In contrast, the GABA catabolic enzyme GABA-T is found primarily in glial and endothelial cells (Fonnum, 1985). Because immunohistochemical studies have shown that inhibition of GABA-T leads to GABA accumulation in astrocytes (Bull and Blomqvist, 1991), it is thought that GABA synthesis and degradation occur in neurons and glial cells, respectively (Palmi *et al.,* 1991). Another series of experiments yielded similar results. In the brain, the TCA cycle has both anabolic and catabolic functions—synthesis of amino acids including glutamate and production of energy by metabolism of acetyl-CoA, respectively. To sustain these functions, TCA cycle intermediates must be transferred from outside. One mechanism for this transfer concerns pyruvate carboxylase activity, an enzyme that

catalyzes formation of oxaloacetate from pyruvate by CO_2 fixation (Patel, 1974). The enzyme is found in astrocytes but not in neurons (Cheng *et al.,* 1978; Yu *et al.,* 1983; Shank *et al.,* 1985). Thus, it is reasonable to hypothesize that products released by the TCA cycle in astrocytes can be used as substrates in the synthesis of new neurotransmitters in neurons. The α-ketoglutarate $\rightarrow$ glutamate $\rightarrow$ glutamine route in astrocytes and glutamine $\rightarrow$ glutamate $\rightarrow$ GABA route in GABAergic neurons have been proposed as new GABA synthesis routes by Peng *et al.* (1993). Astrocytes contain glutamine synthetase that catalyzes the reaction from glutamate to glutamine; however, neurons do not produce this enzyme (Norenberg and Martinez-Hernandez, 1979). Peng *et al.* (1993) and Waagepetersen *et al.* (2000) described the relations of astrocytes and GABAergic neurons to excitatory glutamatergic neurons. Glutamate released by glutamatergic neurons is metabolized into glutamine in astrocytes, and this glutamine is then transported to GABAergic neurons in which it is used in GABA synthesis. These findings suggest that activation of excitatory neurons is followed by activation of inhibitory neurons. It is worth noting that the metabolic compartmentation theory predicts one-way movement of GABA from glial cells to neurons. To equalize carbon skeleton and nitrogen between cells, both must be returned to neurons from glial cells. Glutamine is a carrier of nitrogen atoms. However, because glutamine contains two nitrogen atoms, a surplus of one nitrogen atom for each glutamine returned to the neuron can be assigned. A novel route has been described in glutamatergic neurons, which receive glutamine (Waagepetersen *et al.,* 2000). In addition to glutamine, lactate is taken up by glutamatergic neurons and is oxidized to form pyruvate. Glutamine in glutamatergic neurons forms ammonia, which is used in the subsequent production of glutamate. The pyruvate and glutamate thus produced form alanine, which is then taken up by glial cells. The same process may apply to GABAergic neurons, but this has not been established. However, it is reasonable to think that glial cells supply certain carbon skeletons when neurons do not receive excessive amounts of nitrogen from glial cells. A summary of the GABA metabolic compartment among neurons and glial cells is shown in Fig. 2. It should be emphasized that glial cells supply both carbon skeletons and nitrogen to neurons for GABA synthesis.

D. GAD Isoforms

In the GABA shunt, GABA is synthesized from glutamate by GAD. Mammalian species express two isoforms of GAD, GAD65 and GAD67, where the numbers refer to the molecular mass in kDa. The two GAD isoforms are products of two distinct genes. The GAD65 gene is located on chromosome 10, and the GAD67 gene is located on chromosome 2 in human (Brilliant *et al.,* 1990; Bu *et al.,* 1992). In the brain, GAD67 is responsible for synthesis of >90% of GABA, whereas the major GAD isoform in pancreatic B cells is GAD65 (Christgau *et al.,* 1992). Although the two GAD isoforms are produced by many of the same GABAergic

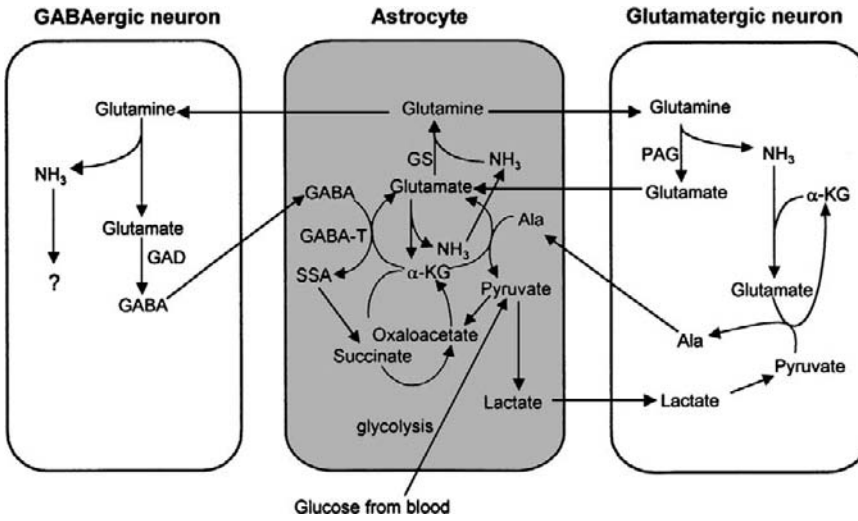

FIG. 2 Simplified schematic drawing of possible metabolic interactions among GABAergic neuron, astrocyte, and glutamatergic neuron. GS, glutamine synthetase; GABA-T, GABA transaminase; PAG, phosphate-activated glutaminase; GAD, glutamic acid decarboxylase; SSA, succinic semialdehyde; GDH, glutamic acid dehydroxynase.

neurons (Esclapez *et al.,* 1994; Dirkx *et al.,* 1995), there are differences in subcellular distribution. Immunohistochemical and subcellular fractionation analyses have demonstrated that GAD65 is preferentially localized near neuronal synaptic vesicles, whereas GAD67 is a soluble cytosolic protein (Soghomonian and Martin, 1998). This suggests that GAD65 contributes to GABA release from axon terminals (Martin and Rimvall, 1993; Asada *et al.,* 1996). GAD65 and GAD67 mRNAs show significant differences in expression levels in different brain regions. GAD65 mRNA is more abundant in structures of the visual system, including the lateral geniculate nuclei, superior colliculi, and olivary pretectal nuclei. In contrast, GAD67 mRNA is more abundant in neocortex, the granular layers of the olfactory bulbs, lateral and medial septa, globus pallidus, inferior colliculi, and cerebellar cortex (Feldblum *et al.,* 1993). Additionally, it was found in rat that hippocampal area CA1 and dentate gyrus contain GAD65 almost exclusively (Sloviter *et al.,* 1996). Moreover, GAD65 and GAD67 are expressed outside the brain. For example, GAD67 is expressed in nonneural cells in rat testis, and GAD65 is produced in the epithelium of oviduct (Tillakaratne *et al.,* 1992, 1995). Both GAD65 and GAD67 mRNAs are expressed in pancreatic B cells, but only GAD67 mRNA is expressed in spleen (Faulkner-Jones *et al.,* 1993). Phylogenetically, the GAD65 and GAD67 genes are derived from a common ancestral gene that existed between 400 and 560 million years ago (Bosma *et al.,* 1999). Because this is the only known case of two genes encoding an enzyme needed for the synthesis of a transmitter

substance, it is important to clarify the role of GADs to understand the physiologic functions of GABA.

Studies of GAD65 knockout mice showed that lack of GAD65 does not change brain levels of GABA, GAD67 activity, or animal behavior, with the exception of a slight increase in susceptibility to seizures induced by picrotoxin and pentylenetetrazol, which are $GABA_A$ receptor antagonists (Asada *et al.,* 1996). In contrast, complete loss of GAD67 mRNA and protein causes a dramatic decrease in GAD activity and GABA content in the cerebral cortex to 20% and 7%, respectively (Asada *et al.,* 1997). However, no discernible histological defects are observed in the cerebral cortex. These mice died due to complications from severe cleft palates soon after birth. These data indicate that GAD65 cannot compensate for GAD67 and that GAD67 may be involved in cell development outside the brain. Pharmacologic and genetic studies have suggested the involvement of GABA in palate development. Administration of the GABA potentiator diazepam to pregnant mice causes cleft palate in their offspring (Miller and Becker, 1975). Application of GABA to 14.5-day-old mouse embryos in short-term culture also inhibits palate development, whereas picrotoxin, a GABA antagonist, stimulates palate development and reverses the effect of GABA (Wee and Zimmerman, 1983). Moreover, cleft palate is observed in pink-eyed cleft palate mutant mice, which are thought to be defective in the β_3 subunit of $GABA_A$ receptors (Culiat *et al.,* 1995). Similar to GAD67 knockout mice, the brains of pink-eyed cleft palate mice appeared histologically normal despite very low levels of GABA (Ji *et al.,* 1999).

The two isoforms of GAD differ in their interactions with the cofactor, PLP. The enzymatic activity of GAD65 is more responsive to PLP than is that of GAD67 (Erlander *et al.,* 1991). GAD65 is present in an apoenzyme form in the brain, whereas GAD67 is an active PLP bound holoenzyme (Erlander *et al.,* 1991; Kaufman *et al.,* 1991). The mechanism controlling GABA synthesis may involve regulation of GAD activity via interaction with PLP (Martin and Rimvall, 1993).

GAD is composed of two domains—a N-terminal domain that contains the information required for targeting of the protein to intracellular membrane compartments (Christgau *et al.,* 1992; Solimena *et al.,* 1993; Dirkx *et al.,* 1995) and a C-terminal domain that contains the catalytic center (Qu *et al.,* 1998). The first 100 N-terminal amino acids differ substantially between the two GAD isoforms. GAD65 and GAD67 associate with membranes, but their means of association appear to differ. Association of GAD65 is thought to be mediated by the palmitoylated N-terminal domain, although palmitoylation does not appear to be essential for membrane association (Christgau *et al.,* 1992). Membrane association of GAD67 appears to involve formation of heterodimers between the N-terminal domains of the two isoforms (Dirkx *et al.,* 1995), although only a fraction of membrane-bound GAD67 is part of heterodimer with GAD65 (Kanaani *et al.,* 1999). It was recently shown that GAD65 is not required for membrane anchoring of GAD67 and that GAD67 homodimer can interact with membrane in an unknown manner (Kanaani *et al.,* 1999).

GAD protein levels can be regulated transcriptionally and translationally, but the mechanisms differ for GAD65 and GAD67 (Szabó *et al.,* 1996; Pinal *et al.,* 1997; Yanagawa *et al.,* 1997). GABA might regulate GAD67 protein expression (Rimvall *et al.,* 1993; Rimvall and Martin, 1994). Furthermore, it is unclear whether GADs in peripheral tissues are identical to those in the brain. GADs in the oviduct and testis may be identical to those in the brain (Tillakaratne *et al.,* 1992), whereas those in the kidney and liver may be different (Denner *et al.,* 1987; Huang *et al.,* 1990). This may be due to developmental differences because oviduct and testis are derived from the neural crest, whereas kidney and liver are mesenchymal in origin.

E. Alternative Pathways of GABA Synthesis

1. Polyamine Route

The metabolic pathway for polyamines is shown in Fig. 3. The name "polyamine" indicates that a molecule contains numerous amines (-NH_2) or imines (-NH-). Polyamines include putrescine, which contains two amines, spermidine, which contains three amines/imines, and spermine, which contains four amines/imines. In mammals, the precursors of polyamines are ornithine and *S*-adenosylmethionine (SAM), which is produced from methionine. Decarboxylation of ornithine is catalyzed by ornithine decarboxylase (ODC), a PLP-dependent enzyme (Seiler, 1987), and leads to formation of putrescine. SAM is converted to decarboxylated *S*-adenosylmethionine (dcSAM) by SAM decarboxylase (SAMDC). The aminopropyl residues of dcSAM are then transferred to putrescine or spermidine to form spermidine and spermine, respectively. Two aminopropyltransferases, spermidine synthase and spermine synthase, catalyze these reactions, respectively.

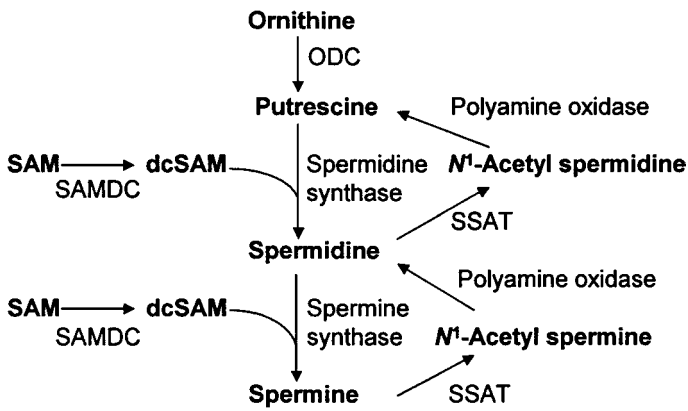

FIG. 3 Interconversion of polyamines in mammals. ODC, ornithine decarboxylase; SAM, *S*-adenosylmethionine; SAMDC, SAM decarboxylase; dcSAM, decarboxylated SAM; SSAT, spermidine/spermine N^1-acetyltransferase.

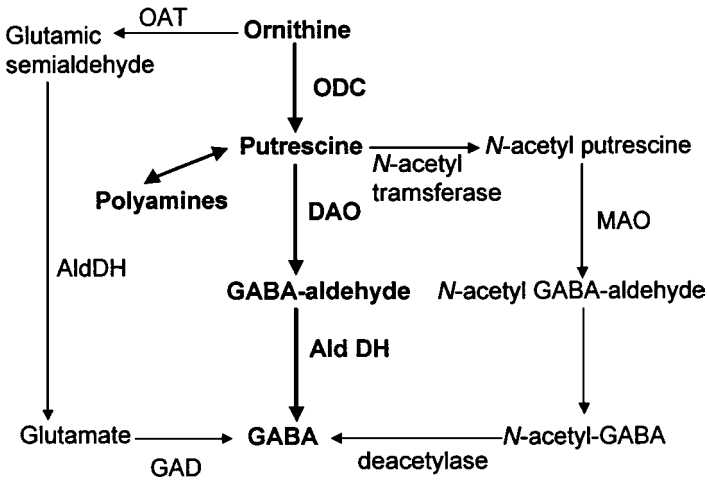

FIG. 4 Biosynthesis of GABA from ornithine and putrescine. AldDH, aldehyde dehydrogenase; DAO, diamine oxidase; MAO, monoamine oxidase; ODC, ornithine decarboxylase; OAT, ornithine aminotransferase.

In mammals, ODC and SAMDC are the rate-limiting enzymes in polyamine synthesis.

Catabolism of polyamine results in the conversion of spermine into spermidine and spermidine into putrescine. At each step, aminopropyl residues are removed by polyamine oxidase from spermine and spermidine, both of which are acetylated by spermidine/spermine N^1-acetyltransferase (Seiler, 1987).

The pathway of GABA synthesis from putrescine is shown in Fig. 4. Oxidative deamination of putrescine is catalyzed by diamine oxidase (DAO) and leads to formation of GABA aldehyde, which is oxidized to GABA by aldehyde dehydrogenase. This pathway of GABA formation appears to occur exclusively to peripheral tissues because DAO activity has not been detected in the brain. Konishi *et al.* (1977) reported that in comparison with total GABA levels in the adult rat brain, levels of putrescine-derived GABA were negligible, and Seiler (1980) calculated that only 2 or 3% of GABA may be derived from putrescine in adult mouse brain under physiological conditions. However, Seiler reported that in the mouse brain, which has very low DAO activity, putrescine is acetylated and then monoacetylputrescine can be converted to GABA by monoamine oxidase. In epileptic DBA/2J mouse brains, GABA turnover is accelerated by increased GABA-T activity, but GAD activity and GABA levels are normal (Ciesielski *et al.,* 1985). These results may be attributed to accelerated formation of GABA from putrescine, as has been shown with cultured astrocytes obtained from epileptic mouse brain (Laschet *et al.,* 1992).

Outside the brain, DAO is present in all mammalian peripheral tissues studied except lung of rat and rabbit (Rao *et al.,* 1986), and intestine has the highest levels

(Tsuji and Nakajima, 1978; D'Agostino *et al.,* 1984). In the gastrointestinal tract, DAO is distributed unevenly. Little or no activity is detected in the stomach, and the activity gradually increases through the duodenum and jejunum. It then decreases to a low level in the colon (Kim *et al.,* 1969; Kusche *et al.,* 1978). Intestinal DAO is associated almost exclusively with the epithelial mucosa (Bieganski *et al.,* 1983). The intestinal epithelium is a dynamic, actively proliferating tissue with a high rate of cell renewal. Mucosal DAO activity is associated with well-differentiated, mature cells at the tip and middle of the intestinal villus. Intestinal epithelium is also characterized by high ODC activity, a level much higher than that of other tissues. The distribution of ODC within the intestinal tract is identical to that of DAO, with minimal activity in the stomach, maximal activity in the small intestine, and low activity in the colon (Ball and Balis, 1976). As with DAO, ODC activity is much higher in maturing cells of the villus tip than in proliferating cells of the crypt areas (Baylin *et al.,* 1978). Such localization of ODC in mature nondividing cells enables one to speculate that the synthesis of putrescine is linked to the production of GABA rather than to its conversion to polyamines.

Recent immunohistochemical study demonstrated that antropyloric gastrin cells in which GAD is undetectable produce GABA via DAO-catalyzed oxidation of putrescine (Hardt *et al.,* 2000).

2. Ornithine Route

GABA is formed from ornithine via two pathways. Ornithine is converted to putrescine by ODC, and the putrescine is then metabolized to GABA by way of the polyamine pathway. The other route via glutamate (Murrin, 1980; Yoneda *et al.,* 1982; Shimosato *et al.,* 1995) is shown in Fig. 4. Because ornithine aminotransferase and GAD are found in liver and kidney (Kalita *et al.,* 1976), it is possible that this pathway is active in peripheral tissues.

3. Arginine Route

Arginine oxidase catalyzes the conversion of arginine to 4-guanidobutyramide, which is then converted to α-guanidobutyrate in a reaction catalyzed by amidinohydrolase. Amidinohydrolase also produces GABA and urea. The reaction catalyzed by amidinohydrolase is the same as that by arginase, which catalyzes conversion of arginine to ornithine. The route from GABA to succinate is a major pathway for arginine degradation in organisms that lack arginase (Bender, 1985).

4. Homocarnosine Route

Homocarnosine (γ-aminobutyryl-L-histidine) is a dipeptide composed of histidine and GABA. Three other histidyl dipeptides are found in vertebrate tissues—carnosine (β-alanyl-L-histidine), anserine (β-alanyl-L-π-methylhistidine), and ophidine or balanine (β-alanyl-L-τ-methylhistidine). Homocarnosine is found at high concentrations in human cerebrospinal fluid (CSF) and brain in comparison to

concentrations in other mammal (Kish *et al.,* 1979; Jackson *et al.,* 1994), whereas other histidyl peptides are present at high concentrations in mammalian skeletal muscle and are widely distributed in other tissues in smaller amounts (Crush, 1970). Immunohistochemical analyses have shown that homocarnosine is localized in GABAergic neurons in human brain (Jackson *et al.,* 1994). The physiologic function of homocarnosine is unclear, but histidyl dipeptides, including homocarnosine, may serve as endogenous antioxidants in skeletal muscle and brain (Kohen *et al.,* 1988). In contrast, it is also possible that these dipeptides act as prooxidants that enhance oxidation of free 2′-deoxyguanosine by H_2O_2 in the presence of Ni(II) (Aruoma *et al.,* 1989; Datta *et al.,* 1993). Because vigabatrin, a selective GABA-T inhibitor and antiepileptic, elevates GABA and homocarnosine levels in the brain and CSF (Riekkinen *et al.,* 1989; Ben-Menachem *et al.,* 1991; Petroff *et al.,* 1999), homocarnosine appears to function as an inhibitory neuromodulator (Datta *et al.,* 1993). It has also been suggested that histidyl dipeptides modulate activation of G proteins by G protein-coupled receptors (Roberts and Wendel, 1996).

Homocarnosine is synthesized from GABA and histidine by homocarnosine–carnosine synthetase (Skaper *et al.,* 1973). In adult brain, glutamate and putrescine are the sources of GABA for homocarnosine synthesis, although putrescine is the preferential substrate for GABA formation in this pathway (Konishi *et al.,* 1977; Makletsova *et al.,* 1992). The rate of conversion of putrescine into homocarnosine is seven times greater than that from glutamate in adult rat brain. Moreover, in early postnatal developing rat brain (1, 7, and 21 days), putrescine is the only significant source of GABA for homocarnosine synthesis (Makletsova *et al.,* 1992).

In human serum, carnosinase, which hydrolyzes carnosine and anserine, is present (Perry *et al.,* 1968). Serum carnosinase, which is distinct from tissue carnosinase, is found in human CSF and is the only enzyme that hydrolyzes homocarnosine in humans (Lenney *et al.,* 1983). Serum carnosinase is likely synthesized in the brain, secreted into the CSF, and then carried into the blood stream (Jackson *et al.,* 1991). Localization of serum carnosinase in the human retina, choroid plexus, and many brain regions has been demonstrated by immunohistochemistry (Datta *et al.,* 1993), and it has been hypothesized that homocarnosine is the source for GABA production in particular neurons in the human brain. However, it must be pointed out that the human brain presents a striking contrast to the rodent brain. Histidyl dipeptides are present in glial cells rather than neurons in the rat and mouse (Biffo *et al.,* 1990). Furthermore, homocarnosine is not hydrolyzed in rat brain (Ziesler *et al.,* 1984; Lenney, 1990), and the rat and mouse do not have serum carnosinase (Jackson *et al.,* 1991).

III. GABA Receptors

GABA can interact with three types of receptors: $GABA_A$, $GABA_B$, and $GABA_C$. The $GABA_A$ and $GABA_C$ receptors are members of the ligand-gated Cl^- channel

superfamily and mediate the fast inhibitory activity of GABA (Macdonald and Olsen, 1994; Rabow *et al.,* 1995; Sieghart, 1995). $GABA_B$ receptors belong to the large family of GTP-binding protein-coupled receptors and regulate the K^+ and Ca^{2+} channels that mediate the long-term inhibitory actions of GABA (Kerr and Ong, 1995).

A. $GABA_A$ Receptors

1. GABA as an Inhibitory Neurotransmitter

In general, GABA is considered an inhibitory neurotransmitter in the vertebrate brain. This is based on the observation that in most cases the electrochemical potential of Cl^- in neurons are low compared to that of the extracellular environment. When GABA binds to the $GABA_A$ receptor, it opens the associated Cl^- channel and allows flow of Cl^- into the neuron. Therefore, GABA hyperpolarizes the neuronal membrane and makes the cell less reactive to excitatory neurotransmitters. However, if the electrochemical potential of Cl^- within the neuron is higher than that of the extracellular environment, the electrochemical gradient pushes Cl^- ions out of the cell, which results in depolarization of the membrane potential (Sieghart, 1995). In this case, GABA may act as an excitatory neurotransmitter. In neonatal neurons the Cl^- gradient is opposite that in mature neurons; therefore, $GABA_A$ receptor activation leads to membrane depolarization (Bureau *et al.,* 1999).

2. Structure of $GABA_A$ Receptors

A schematic of the $GABA_A$ receptor–Cl^- ion channel complex and its subunits is shown in Fig. 5. The $GABA_A$ receptor is a pentamer that is a combination of various subunits (Macdonald and Olsen, 1994; Nayeem *et al.,* 1994; Sieghart, 1995; Barnard *et al.,* 1998; Mehta and Ticku, 1999). Each subunit is composed of a large extracellular N-terminal domain and four hydrophobic membrane-spanning domains (M1–M4) followed by the extracellular C-terminal domain. This structure resembles those of nicotinic acetylcholine receptors, glycine receptors, and 5-hydroxytryptamine receptors (Karlin and Akabas, 1995). The five $GABA_A$ receptor subunits form a quasisymmetric structure around the ion channel, and the M2 region is thought to line the wall of the channel pore (Olsen and Tobin, 1990; Chebib and Johnston, 2000). Each $GABA_A$ receptor subunit also has a large intracellular loop that may be a target for protein kinases and may be required for subcellular targeting and membrane clustering of the receptors, perhaps by anchoring the receptor to the cytoskeleton (Betz, 1990; Olsen and Tobin, 1990; Macdonald and Olsen, 1994).

3. $GABA_A$ Receptor Subunits

$GABA_A$ receptor subunits have been divided into six classes—α, β, γ, δ, ε, and π—according to their amino acid sequences. In addition, several isoforms of the

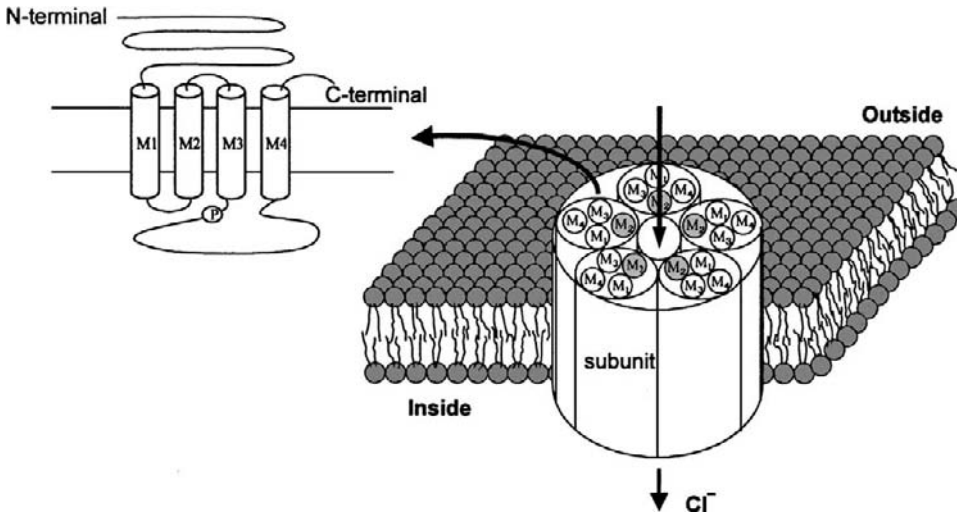

FIG. 5 Schematic representation of $GABA_A$ receptor and its subunit.

α, β, and γ subunits have been cloned. The amino acid homology within each subunit class is approximately 60–80%, but that between the different classes is approximately 30% (Olsen and Tobin, 1990; Chebib and Johnston, 2000). To date, the 15 $GABA_A$ receptor subunits identified are α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, and π. Recently, a new subunit subclass, θ, was reported (Bonnert *et al.,* 1999). These subunits are encoded by unique genes, although some genes are located on the same chromosome. In humans, the δ subunit is located on chromosome 1 (Sommer *et al.,* 1990); the α_2, α_4, β_1, and γ_1 subunits on chromosome 4; the α_1, α_6, β_2, and γ_2 subunits on chromosome 5 (Schantz-Wilcox *et al.,* 1992); the α_5, β_3, and γ_3 subunits on chromosome 15 (Knoll *et al.,* 1993; Greger *et al.,* 1995); and the α_3, β_4, and ε subunits on the X chromosome (Rogner *et al.,* 1994; Levin *et al.,* 1996; Wilke *et al.,* 1997). In mouse, the α_5, β_3, and γ_3 subunits are located on chromosome 7 (Wagstaff *et al.,* 1991, Culiat *et al.,* 1993; Nakatsu *et al.,* 1993), and the α_2, β_1, and γ_1 subunits on chromosome 11 (Buckwalter *et al.,* 1992). The γ_4 subunit has been found in chick brain (Harvey *et al.,* 1993), but the corresponding subunit has never been found in mammals. Alternative splicing variants of the γ_2 subunit (γ_{2L} and γ_{2S}) have been identified in bovine, human, and rat brains (Whiting *et al.,* 1990). These variants differ in the presence or absence of eight amino acids in the large intracellular loop between M3 and M4 membrane spanning domains that include a protein kinase C phosphorylation site. Similar variants have been found in chicken β_2 (β_{2L} and β_{2S}) and β_4 (β_{4L} and β_{4S}) (Bateson *et al.,* 1991; Harvey *et al.,* 1994). Two splice variants of the N-terminal region of the α_6 subunit (α_{6L} and α_{6S}) have also been reported (Korpi *et al.,* 1994).

In addition to the subunits described previously, three ρ subunits (ρ_{1-3}) have been reported. It is not clear whether ρ subunits can assemble with other $GABA_A$

subunits into functional $GABA_A$ receptors. Receptor assemblies derived from these ρ subunits are classified as $GABA_C$ (Barnard *et al.,* 1998; Johnston, 1994). The genes that encode the ρ_1 and ρ_2 subunits are located on human chromosome 6 and mouse chromosome 4 (Cutting *et al.,* 1992), and the ρ_3 subunit is located on human chromosome 3 (Bailey *et al.,* 1999).

Each $GABA_A$ receptor subunit has a unique pattern of expression in the mammalian brain (Laurie *et al.,* 1992a; Fritschy and Mohler, 1995; Rabow *et al.,* 1995). With respect to π subunit, the mRNA has been detected in several human and rat tissues and is particularly abundant in the uterus. The π subunit can assemble with α, β, and/or γ subunits, and the presence of the π subunit confers unique interactions with the endogenous steroid, pregnanolone (Hedblom and Kirkness, 1997). ρ subunits are expressed predominantly in human retina (Enz *et al.,* 1995, 1996; Ogurusu and Shingai, 1996) and the human brain (Enz and Cutting, 1999).

4. $GABA_A$ Receptor Diversity

Because $GABA_A$ receptors are pentameric assemblies derived from a combination of various subunits, a very large number of receptor subtypes can be formed. Receptor diversity, however, is significantly restricted, although the mechanisms governing the differential assembly and subcellular targeting of receptors subtypes are unknown. Studies of heterologous expression systems have shown that functional $GABA_A$ receptors contain at least one α, one β, and one γ subunit isoform (Levitan *et al.,* 1988; Malherbe *et al.,* 1990a; Sigel *et al.,* 1990; Verdoorn *et al.,* 1990) and that most of the pentameric combinations are $2\alpha2\beta1\gamma$, $2\alpha1\beta2\gamma$ or $1\alpha2\beta2\gamma$ (Sieghart, 1995; Barnard *et al.,* 1998; Costa, 1998; Mehta and Ticku, 1999). The δ or ε subunits are thought to be assembled into $GABA_A$ receptors in place of γ subunits. The π subunit is found outside the CNS in organs such as uterus, prostate gland, thymus, and lung (Hedblom and Kirkness, 1997).

5. GABA Binding Site

GABA binding to $GABA_A$ receptors opens a Cl^- channel. Both high- and low-affinity GABA binding sites have been found in the $GABA_A$ receptor, and the K_d values are 10 and 240 n*M*, respectively (Van Ness *et al.,* 1982). However, because micromolar concentrations of GABA may be necessary for physiological effects due to binding to $GABA_A$ receptors (Cash and Subbarao 1987a,b; Kardos and Cash, 1990), the existence of additional very low-affinity GABA binding sites is assumed (Sieghart, 1995). One $GABA_A$ receptor can possess as many as five GABA binding sites. These binding sites show high affinities for GABA in the unoccupied state, but with increasing occupation of these sites with GABA the affinities of the remaining unoccupied sites may be reduced because of allosteric restrictions. Under physiological conditions, high- and low-affinity GABA binding sites in the synaptic cleft may be occupied by GABA without opening the channel.

In fact, under physiological conditions, only two or three GABA binding sites with affinities of approximately 100 μM are unoccupied per $GABA_A$ receptor (Cash and Subbarao 1987a,b; Kardos and Cash, 1990). The concentration of GABA in the synaptic cleft may reach 500 μM (Maconochie *et al.,* 1994). The GABA binding sites of the $GABA_A$ receptor are assumed to be contained in the N-terminal extracellular domains of both α and β subunits (Amin and Weiss, 1993; Smith and Olsen, 1995).

6. Functional Significance of $GABA_A$ Receptor Diversity

Why do so many $GABA_A$ receptor subtypes exist? $GABA_A$ receptors are of great importance because they play a pivotal role in the regulation of brain excitability, and the heterogeneity is thought to provide the bases for flexibility in signal transduction and drug-induced allosteric modulation. The $GABA_A$ receptor contains binding sites for several clinically important drugs, including benzodiazepines, barbiturates, and neurosteroids. These binding sites may be formed by certain combinations of subunits.

a. Benzodiazepine Binding Site Benzodiazepines (BZs) are a class of drugs that share a common chemical structure and have antianxiety, antiepileptic, muscle relaxant, and hypnotic effects. More than 2000 BZ compounds have been synthesized. The observation that BZs enhance the inhibitory action of GABA (Study and Barker, 1981) led researchers to determine that the target was the GABA receptor (Tallman *et al.,* 1978). Thus, the $GABA_A$ receptor has also been called the $GABA_A$/BZ receptor. In the absence of GABA, however, BZs cannot open Cl^- channels (Polc, 1988). Two types of BZ receptors have been described: the central type and the peripheral type. Central-type BZ receptors associate with certain $GABA_A$ receptor subtypes, whereas peripheral-type BZ binding sites are located on the outer mitochondrial membrane and have no relation to $GABA_A$ receptors (Verma and Snyder, 1989). Most BZs bind to central-type BZ receptors with similar affinities, but BZ receptors show different affinities for different BZs. Therefore, the central-type BZ receptors have been further classified into type I and type II receptors. Type I receptors are more sensitive to CL218872 and 1,4-benzodiazepine 2-oxo-quazepam than are type II receptors, and they are the predominant $GABA_A$/BZ receptors in the brain (Pritchett *et al.,* 1989; Lüddens *et al.,* 1995). Type II receptors are enriched only in the hippocampus, striatum, and spinal cord (Olsen and Tobin, 1990). The α and γ subunits of $GABA_A$ receptors are responsible for BZ binding (Sigel and Buhr, 1997); the α subunit in type I receptor is α_1, and those in the type II receptor are α_2, α_3, or α_5 (Pritchett *et al.,* 1989; Pritchett and Seeburg, 1990). Following targeted disruption of the γ_2 subunit gene, 94% of BZ binding sites are absent in neonatal mouse brains, whereas the number of GABA sites is only slightly reduced (Günther *et al.,* 1995). However, the actual endogenous ligand for BZ receptors has not been found.

b. Other Binding Sites Pentobarbital affects $GABA_A$ receptor-mediated responses in several ways. At low micromolar concentrations, it enhances GABA-evoked responses; at high micromolar concentrations, it opens Cl^- channels directly, even in the absence of GABA. At millimolar concentrations, it reduces GABA-evoked responses (Akaike *et al.,* 1987). Pentobarbital binding sites in $GABA_A$ receptors are located near the middle of the M2 region of the β_3 subunit (Serafini *et al.,* 2000).

Picrotoxin is a convulsant and noncompetitive GABA antagonist. Electrophysiological studies of recombinant $GABA_A$ receptors have revealed that picrotoxin interacts with both resting and GABA-bound receptors, although the affinity for the latter is 10-fold greater than that for the former. Picrotoxin appears to behave as an allosteric modulator rather than as an open channel blocker (Dillon *et al.,* 1995). Because point mutations in the M2 region of the α and β subunits of the $GABA_A$ receptor abolish the block of GABA-evoked chloride currents by picrotoxin, the binding site may be within the channel pore (Gurley *et al.,* 1995).

Neurosteroids are synthesized in the central and peripheral nervous systems from cholesterol or steroidal precursors imported from peripheral sources (Baulieu, 1998). These precursors include dehydroepiandrosterone and pregnenolone and their sulfate esters and progesterone. Steroids do not exhibit absolute $GABA_A$ receptor subunit specificity (Puia *et al.,* 1990), but $GABA_A$ receptors with the α_6 subunit exhibit a reduced steroid effect (Puia *et al.,* 1993). The α_6 subunit is confined to granule cells of the cerebellum, an observation that may be important when considering the behavioral actions of steroids. Furthermore, γ_1 subunit coexpressed with $\alpha_1\beta_1$ subunits showed greater enhancement of GABA-evoked currents than coexpression with either γ_2 or γ_3 (Puia *et al.,* 1993). It is interesting that the γ_1 subunit is expressed at high levels in glial cells, which are a major site of neurosteroid synthesis in the brain. $GABA_A$ receptors on such cells are known to be steroid sensitive (Chvátal and Kettenmann, 1991), and the intriguing possibility exists that such locally produced steroids act as endogenous modulators of $GABA_A$ receptors on glial cells (Lambert *et al.,* 1995).

The direct inhibitory action of Zn^{2+} on GABA-activated currents is well documented (Draguhn *et al.,* 1990; Kilic *et al.,* 1993; White and Gurley, 1995). Apart from these substrates, various drugs interact with $GABA_A$ receptors and modulate receptor actions (Sieghart, 1995), including ethanol (Harris *et al.,* 1995; Macdonald, 1995).

7. Modulation of $GABA_A$ Receptors by Phosphorylation

Alternative mechanisms can create diversity in the $GABA_A$ receptor. Phosphorylation is a common mechanism for the regulation of receptor function and may play an important role in synaptic plasticity (Swope *et al.,* 1992). All $GABA_A$ receptor subunit subtypes contain consensus substrate sequences for some kinases, including

cAMP-dependent protein kinase A (PKA), calcium-activated, phospholipid-dependent protein kinase C (PKC), protein tyrosine kinase, and Ca^{2+}/calmodulin-dependent kinase type II (CAM kinase II) (Bureau *et al.,* 1999). These sites are located in the large cytoplasmic loop between the third and fourth transmembrane domains (Macdonald, 1995; Bureau *et al.,* 1999), although the phosphorylation consensus sequences are dependent on subunit subtype and species (Rabow *et al.,* 1995). $GABA_A$ receptor functions can be modulated by phosphorylation (Browning *et al.,* 1990; Kirkness *et al.,* 1989), but this appears to either enhance or reduce GABA-activated currents (Moss *et al.,* 1992, 1995; McDonald *et al.,* 1998). These differences in functional effects may reflect underlying heterogeneity in $GABA_A$ receptor structures. A critical role for β subunits in mediating positive or negative regulation of the $GABA_A$ receptor following PKA-induced phosphorylation has been demonstrated (Moss and Smart, 1996; Smart, 1997; McDonald *et al.,* 1998; Nusser *et al.,* 1999). Consequently, the $GABA_A$ receptor may be regulated by intracellular cAMP levels. PKC is widely distributed in various tissues, and the enzyme is most concentrated in the CNS (Minakuchi *et al.,* 1981).

It has been shown that endogenous phosphorylation is required to prevent decreases in GABA-mediated Cl^- currents (Stelzer *et al.,* 1988; Gyenes *et al.,* 1994). The types of phosphatases and kinases involved in these mechanisms are not clear, but endogenous kinase activities appear to be associated with α and γ_2 subunits (Bureau *et al.,* 1999). Recent data have suggested that a membrane-associated phosphatase other than calcineurin counteracts endogenous phosphorylation of $GABA_A$ receptors (Minier *et al.,* 2000). The activity of the endogenous kinase depends strictly on the presence of divalent cations: Mg^{2+}, Mn^{2+}, and Ca^{2+} (Bureau and Laschet, 1995; Minier *et al.,* 2000). Furthermore, endogenous phosphorylation of the $GABA_A$ receptor is modulated by polyamines. Spermine and, to a lesser extent, spermidine and putrescine modulate endogenous phosphorylation of purified $GABA_A$ receptors (Bureau *et al.,* 1999).

8. $GABA_A$ Receptor Clustering in Postsynaptic Membrane

Synaptic function depends not only on controlled release of neurotransmitter but also on regulation of the numbers and locations of receptors in the postsynaptic membrane. These receptors are deeply embedded in the membrane bilayers. To restrict the free movement of receptors and to concentrate them at the postsynaptic membrane, association of receptors with the cytoskeleton by peripheral membrane proteins called anchor or linker proteins is necessary. At the neuromuscular junction, a 43-kDa peripheral membrane protein called rapsin (receptor-associated protein at synapse) is essential for nicotinic acetylcholine receptor clustering and normal neuromuscular junction development (Sanes and Lichtman, 1999). Moreover, glycine receptors in the vertebrate CNS are linked to a specific intracellular protein called gephyrin (Triller *et al.,* 1985), which is a 93-kDa protein originally

identified by copurification with strychnine-sensitive glycine receptors (Schmitt *et al.,* 1987). Glycine receptors and $GABA_A$ receptors belong to the same protein superfamily (Betz, 1990), although these receptors differ from each other pharmacologically (Young and Snyder, 1973, 1974). In the vertebrate CNS, glycine and GABA have primarily inhibitory actions, but GABAergic transmission is used throughout the CNS, and glycine-mediated inhibition has a more limited distribution that predominates in the brain stem and spinal cord (Vannier and Triller, 1997).

There is growing evidence that gephyrin also plays a role in clustering $GABA_A$ receptors at synaptic sites (Cabot *et al.,* 1995; Sassoè-Pognetto *et al.,* 1995; Craig *et al.,* 1996; Todd *et al.,* 1996). Analyses of mice with a targeted disruption of the γ_2 subunit gene revealed that this subunit is essential for synaptic localization and clustering of major γ_2 subunit-containing $GABA_A$ receptor subtypes (Essrich *et al.,* 1998). Another report of γ_3 transgenic mice showed that the γ_3 subunit is functionally equivalent to the γ_2 subunit in its contribution to the postsynaptic localization and function of γ_3 subunit-containing $GABA_A$ receptors (Baer *et al.,* 1999). Furthermore, brain sections and neurons from gephyrin knockout mice show total loss of postsynaptic clustering of $GABA_A$ receptor α_2 and γ_2 subunits (Kneussel *et al.,* 1999a). These data suggest that gephyrin interacts directly with $GABA_A$ receptor subunits; however, gephyrin does not copurify with $GABA_A$ receptors (Meyer *et al.,* 1995), which contrasts with its strong interaction with glycine receptors (Pfeiffer *et al.,* 1982; Graham *et al.,* 1985). Additionally, attempts to define a direct interaction between $GABA_A$ receptors and gephyrin have not been successful (Meyer *et al.,* 1995), and cotransfection studies have shown only weak colocalization of β_3, but not other $GABA_A$ receptor subunits, with gephyrin (Kirsch *et al.,* 1995). Recently, two novel gephyrin binding proteins have been identified, but their roles in receptor anchoring are unknown (Kneussel and Betz, 2000). Kneussel *et al.* (1999a) suggested that gephyrin is not required for plasma membrane insertion of $GABA_A$ receptors because the GABA-induced current was only marginally reduced in cultured hippocampal neurons isolated from gephyrin-deficient mice. They also observed an accumulation of intracellular $GABA_A$ receptor microclusters in the absence of gephyrin, suggesting enhanced receptor internalization. These observations suggest that gephyrin may be necessary to stabilize inhibitory postsynaptic membrane specializations in addition to its role in inhibitory receptor clustering (Kneussel and Betz, 2000).

Recently, a new protein, GABARAP ($GABA_A$-receptor-associated protein), which attaches stably to subsynaptic microtubules (Wang and Olsen, 2000), was found to interact with the γ_2 subunit of $GABA_A$ receptors (Wang *et al.,* 1999). GABARAP mRNA and protein were detected in peripheral tissues, such as heart, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Wang *et al.,* 1999). GABARAP is thought to bind to the large intracellular loops of the $GABA_A$ receptor subunits, which have phosphorylation sites for both protein kinase C and tyrosine kinases (Kirsch *et al.,* 1996). Therefore, phosphorylation may regulate the interactions of $GABA_A$ receptor subunits with GABARAP. Indeed, the cell

surface localization of $GABA_A$ receptors may be modulated by a protein kinase C-dependent mechanism (Smart, 1997; Connolly *et al.,* 1999). Analysis of the linker protein–receptor complex could improve our understanding of the mechanisms of the transport, localization, internalization, and degradation of $GABA_A$ receptors. A recent review addressed these topics (Barnes, 2000).

9. Distribution of $GABA_A$ Receptor Subunits in the Brain

Each $GABA_A$ subunit has a unique distribution pattern in the brain. To date, immunohistochemical studies of α_1 (Fritschy *et al.,* 1992, 1994; Gao *et al.,* 1993, 1995; Fritschy and Mohler, 1995), α_2 (Marksitzer *et al.,* 1993; Benke *et al.,* 1994; Fritschy and Mohler, 1995), α_3 (Gao *et al.,* 1993, 1995; Fritschy and Mohler, 1995), α_5 (Thompson *et al.,* 1992; Fritschy and Mohler, 1995), α_6 (Thompson *et al.,* 1992), β_2 (Benke *et al.,* 1994; Fritschy and Mohler, 1995; Gao *et al.,* 1995), β_3 (Fritschy and Mohler, 1995), γ_2 (Benke *et al.,* 1991; Fritschy and Mohler, 1995; Gao *et al.,* 1995), and δ (Fritschy and Mohler, 1995) and *in situ* hybridization studies of α_1 (Malherbe *et al.,* 1990b; MacLennan *et al.,* 1991; Tietz *et al.,* 1993), α_2 (MacLennan *et al.,* 1991), α_3 (Malherbe *et al.,* 1990b), α_4 (MacLennan *et al.,* 1991; Wisden *et al.,* 1991), α_5 (Malherbe *et al.,* 1990b; Tietz *et al.,* 1993), α_6 (Kato, 1990; Laurie *et al.,* 1992a; Varecka *et al.,* 1994), γ_1 (Herb *et al.,* 1992), γ_2 (Shivers *et al.,* 1989; Herb *et al.,* 1992; Tietz *et al.,* 1993), γ_3 (Herb *et al.,* 1992), δ (Shivers *et al.,* 1989), and ε (Whiting *et al.,* 1997) have been completed. Northern analyses of α_2 (Khrestchatisky *et al.,* 1991), α_4 (Ymer *et al.,* 1989), and α_6 (Kato, 1990) have also been done. Laurie *et al.* (1992a,b) and Wisden *et al.* (1992) reported systematic *in situ* hybridization studies of 13 $GABA_A$ receptor subunit mRNAs ($\alpha_{1–6}$, $\beta_{1–3}$, $\gamma_{1–3}$, and δ) in embryonic, neonatal, and adult rat brains.

The main characteristics of distribution of $GABA_A$ receptor subunits in adult mammalian brains are as follows: (i) The α_1, β_2, β_3, and γ_2 subunits are widely distributed and show the same distribution pattern (Fritschy and Mohler, 1995); (ii) the α_2, α_3, α_5, and δ subunits are limited to certain brain regions (Fritschy and Mohler, 1995); (iii) the α_1 subunit is expressed in regions where the α_2 subunit is absent or expressed at low levels (Benke *et al.,* 1994; Fritschy and Mohler, 1995); (iv) the α_3 subunit is expressed in regions where the α_1 subunit is expressed at low levels (Benke *et al.,* 1994; Gao *et al.,* 1993; Fritschy and Mohler, 1995); (v) the α_4 subunit is abundant in hippocampus (MacLennan *et al.,* 1991; Fritschy and Mohler, 1995); (vi) the α_5 subunit is expressed in certain regions linked to memory function (Fritschy and Mohler, 1995); (vii) the α_6 subunit is expressed almost exclusively in cerebellar granule cells (Laurie *et al.,* 1992a; Thompson *et al.,* 1992; Varecka *et al.,* 1994); and (viii) the ε subunit is expressed only in parts of the hypothalamus and hippocampus (Whiting *et al.,* 1997).

These studies indicated that each $GABA_A$ subunit has a unique distribution pattern in the brain. However, a paucity of information exists regarding the cellular distribution of $GABA_A$ receptor subunit proteins *in situ*. To understand the

functional and pharmacological significance of $GABA_A$ receptor heterogeneity, determination of the receptor subtypes present on GABA-receptive neurons *in situ* is essential. Electron microscopy studies, which are very labor-intensive, are needed for this. Moreover, immunohistochemical labeling of $GABA_A$ receptor is sensitive to fixation for both light and electron microscopic procedures (Sassoè-Pognetto *et al.,* 2000). Laser scanning microscopy, which has high resolving power, may be necessary to gain information at the cellular level (Gao and Fritschy, 1994; Gao *et al.,* 1995; Mohler *et al.,* 1995; Nusser *et al.,* 1996; Todd *et al.,* 1996; Waldvogel *et al.,* 1999; Fujiyama *et al.,* 2000).

B. $GABA_B$ Receptors

The inhibitory effects of GABA have long been thought to be mediated exclusively by $GABA_A$ receptors sensitive to bicucullin. In the early 1980s, the term $GABA_B$ was used to describe a bicucullin-insensitive and baclofen-sensitive GABA receptor (Bowery *et al.,* 1980; Hill and Bowery, 1981). $GABA_B$ receptors are metabotropic receptors and, thus, are pharmacologically as well as electrophysiologically distinct from the $GABA_A$ receptor (Kerr and Ong, 1995).

1. $GABA_B$ Receptor Subunits

To date, molecular cloning has identified two main $GABA_B$ receptor subunits termed $GABA_B$ R1 and $GABA_B$ R2, which are encoded by distinct genes (Isomoto *et al.,* 1998; Kaupmann *et al.,* 1998b). Purification of the putative $GABA_B$ receptor protein was first reported by Nakayasu *et al.* (1993). Kaupmann *et al.* (1997) succeeded in isolating the cDNAs encoding two rat $GABA_B$ receptor proteins, $GABA_B$ R1a and $GABA_B$ R1b. Rat $GABA_B$ R1a and $GABA_B$ R1b comprise 960 and 844 amino acids, respectively, and these two isoforms are N-terminal splice variants. Amino acid sequence analysis indicated that $GABA_B$ R1a and $GABA_B$ R1b are members of the G protein-coupled receptor family. Moreover, similarities to metabotropic glutamate receptors (Kaupmann *et al.,* 1997), a Ca^{2+}-sensing receptor (Brown *et al.,* 1993), and vomeronasal receptor (putative pheromone and taste receptors) (Herrada and Dulac, 1997; Matsunami and Buck, 1997) were also noted. Subsequently, two additional splice variants ($GABA_B$ R1c and $GABA_B$ R1d) were identified in the rodent (Isomoto *et al.,* 1998; Pfaff *et al.,* 1999). The human homologs of $GABA_B$ R1a and $GABA_B$ R1b were also identified (Peters *et al.,* 1998; White *et al.,* 1998). A novel $GABA_B$ receptor subunit, $GABA_B$ R2, was identified simultaneously by three groups (Kaupmann *et al.,* 1998a; Jones *et al.,* 1998; White *et al.,* 1998). Hydrophobicity analysis of the rat $GABA_B$ R2 protein, which consists of 941 amino acids, suggests that it has a topological organization typical of a G protein-coupled receptor. The amino acid sequence is 35% identical to that of $GABA_B$ R1b. The native $GABA_B$ receptor may be a heterodimer of $GABA_B$ R1 and $GABA_B$ R2 through an interaction between their intracellular

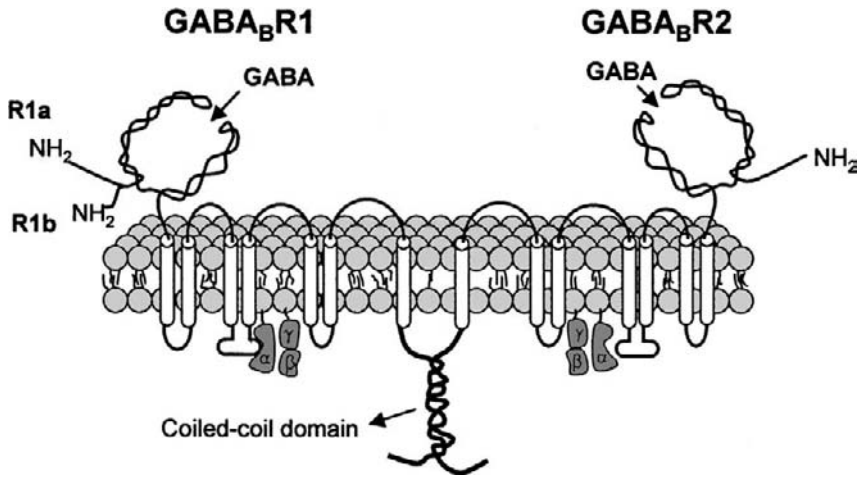

FIG. 6 Schematic representation of $GABA_B$ receptors. The native $GABA_B$ receptors may represent a heterodimer of $GABA_B$ R1 and $GABA_B$ R2.

C-terminal tails, possibly through coiled coil domains as shown in Fig. 6 (Jones *et al.,* 1998; Kuner *et al.,* 1999; Marshall *et al.,* 1999; Ng *et al.,* 1999). In contrast, the limited distribution of $GABA_B$ R2 receptor mRNA in comparison to that of $GABA_B$ R1 mRNA in rat brain suggests that there are regions where $GABA_B$ receptors are composed of $GABA_B$ R1 and possibly other unidentified family members (Clark *et al.,* 2000). The significance of heterodimers with respect to receptor activation and signaling is not yet fully understood.

2. Functions of $GABA_B$ Receptors

$GABA_B$ receptors play a critical role in long-term inhibition of synaptic transmission (Bowery, 1993). In the brain, $GABA_B$ receptors are located both pre- and postsynaptically. Activation of presynaptic $GABA_B$ receptors inhibits neurotransmitter release from presynaptic terminals (Bowery *et al.,* 1980; Bowery, 1993). Presynaptically, $GABA_B$ autoreceptors have been reported to control the release of GABA, whereas $GABA_B$ heteroreceptors regulate the release of glutamate, noradrenalin, dopamine, 5-hydroxytryptamine, substance P, cholecystokinin, and somatostain (Kaupmann *et al.,* 1997). This inhibition is mediated by a decrease in presynaptic calcium influx due to inhibition of presynaptic voltage-gated Ca^{2+} channels (Mott and Lewis, 1994; Wu and Saggau, 1997; Kaupmann *et al.,* 1998a; Takahashi *et al.,* 1998). Activation of postsynaptic $GABA_B$ receptors activates K^+ channels and causes hyperpolarization of the postsynaptic membrane (Kerr and Ong, 1995). $GABA_B$ receptors also inhibit the activity of adenylate cyclase (Kerr and Ong, 1995). A review of the pharmacological characteristics of $GABA_B$ receptors was published by Bowery and Enna (2000).

3. Distribution of $GABA_B$ Receptors in the Brain

In the brain, the pattern of $GABA_B$ receptor distribution is distinct from that of $GABA_A$ receptors, although there are many regions where both receptors are present in comparable amounts. Distribution of $GABA_B$ R1 receptor mRNA in the adult rat brain has been analyzed by *in situ* hybridization (Lu *et al.,* 1999). A high level of mRNA is observed in the medial habenula; the septohippocampal, periventricular, suprachiasmatic, and supraoptic nuclei; Purkinje cells in the cerebellum; and pyramidal and granule cells of the hippocampus and dentate gyrus. There is little or no $GABA_B$ R1 mRNA in the stratum radiatum and stratum orience of the hippocampus, the molecular and granular layers of the cerebellum, and the molecular layer of the cortex. Distribution of $GABA_B$ R1a and R1b in the rat brain was recently studied immunocytochemically (Poorkhalkali *et al.,* 2000). In the cerebellum, $GABA_B$ R1a is preferentially localized in the granule cell layer, whereas R1b is mostly found in Purkinje cells and the molecular layer. Distribution of $GABA_B$ R2 receptor mRNA in the rat brain has also been analyzed by *in situ* hybridization (Durkin *et al.,* 1999; Clark *et al.,* 2000). High levels of $GABA_B$ R2 mRNA are present in the cerebral cortex, thalamus, and cerebellum; low levels occur in the hypothalamus, and $GABA_B$ R2 mRNA is absent in the caudate/putamen.

C. $GABA_C$ Receptors

Like $GABA_A$ receptors, $GABA_C$ receptors are pentameric Cl^- channels composed of ρ subunits (ρ_{1-3}) (Barnard *et al.,* 1998; Ogurusu and Shingai, 1996); however, their kinetic and pharmacologic characteristics differ dramatically. In comparison to $GABA_A$ receptors, $GABA_C$ receptors have greater sensitivity to GABA, smaller currents, and do not desensitize. $GABA_C$ receptors are insensitive to the $GABA_A$ receptor antagonist bicucullin and the $GABA_B$ receptor agonist baclofen. However, it has been proposed that the ρ subunit-containing receptors should be classified as a specialized set of $GABA_A$ receptors because ρ subunits are structurally part of the family of $GABA_A$ receptor subunits (Barnard *et al.,* 1998). $GABA_C$ receptors are expressed predominantly in the vertebrate retina. Excellent reviews of the $GABA_C$ receptors are available (Johnston, 1994; Bormann and Feigenspan, 1995; Enz and Cutting, 1998; Feigenspan and Bormann, 1998).

IV. GABA System and Development of Brain

GABA is one of the first neurotransmitters detected during development of the CNS and is thought to play a role in neural development.

A. Appearance of GABA, GAD, and GABA Receptors during Development of the CNS

1. GABA

Immunocytochemical studies have clearly demonstrated that GABAergic cells appear early in embryonic development. The first GABA-immunoreactive neurons are observed on Embryonic Day 13 (E13) or E14 in rat cerebral neocortex, and by E15 the number of GABA-immunoreactive neurons has increased significantly (Van Eden *et al.,* 1989; Fiszman *et al.,* 1993). On Day 16, GABAergic neurons are present in all zones of the cortex, although there are regional differences in concentrations (Van Eden *et al.,* 1989). The number of GABAergic cells in the cortical plate increases after E19, whereas the numbers in the intermediate and subventricular zones decrease. In human cerebral cortex, the first GABA-immunoreactive cells are observed at 6.5 gestational weeks, before the appearance of the cortical plate (Zecevic and Milosevic, 1997). The early distribution and later laminar locations of GABAergic neurons and fibers correspond well to the distributions described in rodents, suggesting that the early appearance of GABAergic neurons in the neocortex is a common feature across species (Zecevic and Milosevic, 1997). GABA-positive neurons in all zones of the developing neocortex appear to be transient cell populations, especially in the early phases of cortical development (Van Eden *et al.,* 1989; Zecevic and Milosevic, 1997).

2. GAD

In contrast to the relatively high levels of GABA, GAD and GABA-T activities throughout the brain are quite low at birth (Coyle and Enna, 1976). In addition, GABA is found in embryonic brain before significant GAD activity is detectable (Van den Berg *et al.,* 1965; Roberts and Kuriyama, 1968; Seiler *et al.,* 1980; Lauder *et al.,* 1986). These findings suggest that GABA may be synthesized by an alternative pathway in the embryo. Significant conversion of glutamate into GABA is observed only after Day 8 of chick development, whereas the maximal rate of transformation of putrescine into GABA occurs between Days 6 and 8; rates are slow after Day 12 (Sobue and Nakajima, 1978). Because the timing of maximal putrescine degradation corresponds to that of early differentiation of neuroblasts into neuronal cells, production of GABA may be essential for cellular differentiation (Seiler, 1981).

GAD activity is low during the early stages of brain development and increases in parallel with synaptogenesis (Van den Berg *et al.,* 1965). A study of the postnatal expression of the two GAD isoforms in the rat cerebellum showed that GAD65 mRNA is expressed later than GAD67 mRNA (Greif *et al.,* 1991). Most development of the cerebellar cortex occurs after birth in rodents and continues for at least 1 month. GAD65 mRNA is expressed in Purkinje cells on Postnatal Day 7 (P7),

a time when the first synapses between climbing fibers and Purkinje cells are being formed. The expression of GAD67 mRNA in the cerebellar cortex parallels the pattern of histogenesis; GAD67 mRNA is first expressed in a subset of Purkinje cells at P0, and by P7 it is present in most Purkinje cells. The similar developmental expression patterns of GAD67 and GAD65 mRNA in other cerebellar cells suggest that GAD67 may have trophic and metabolic functions (Greif *et al.,* 1991).

3. GABA Receptors

The first studies of the ontogenesis of GABA receptors in the rat brain were done by Coyle and Enna in 1976. They reported that GABA receptors are present in the embryo at E15, although the levels remain low after birth (25% of adult values) until P8, when GABA receptor levels increase dramatically. The time courses for GAD and GABA receptor expression are similar. This contrasts with GABA, which is synthesized earlier in development than both GABA receptors and GAD.

B. GABA as a Neurotropic Factor

The development of the nervous system is a complicated process of migration of nerve cells, growth of neurites (axons and dendrites), and establishment of synaptic connections. Factors controlling these processes are critical for the establishment of appropriate contacts between the various parts of the nervous system. One hypothesis that addresses the specificity of axonal growth involves contact guidance (Rakic, 1988). According to this hypothesis, a neuron migrates along the fibers of radial glial cells that extend from the mitotic zone to the pial surface and act as scaffolds. However, many neurons are known to migrate without the guidance of glial fibers (Walsh and Cepko, 1992; O'Rourke *et al.,* 1995). The migration of these neurons may be mediated by neurotropic factors, which are chemicals that directly influence the direction of neuronal growth. Soluble molecules released from the target of the neuronal growth, known as chemotaxins or chemoattractants, are one such factor type. Axonal growth cones are expected to migrate according to the concentration gradients of such molecules. The axonal growth cone becomes the presynaptic terminal, and the dendritic growth cones become the postsynaptic elements (Vaughn *et al.,* 1974; Rees, 1978). The best characterized chemotaxin is nerve growth factor (NGF), which is needed for the survival of sympathetic and sensory neurons during development. Exogenously supplied NGF can influence the direction of nerve growth (Levi-Montalcini, 1976, 1987). Therefore, NGF may act as both a neurotropic and a neurotrophic factor. Neurotrophic factors are chemicals that are important for the continued survival, growth, and differentiation of neurons. GABA is possibly a chemotaxin (Behar *et al.,* 1994, 1996). GABA is expressed in the marginal zone and subcortical plate of the embryonic neocortex (Van Eden, 1989; Meinecke and Rakic, 1992; Ma and Barker, 1995) during the

time when neural migration occurs. Release of GABA from growth cones isolated from rat embryonic cortical tissue has been described (Gordon-Weeks *et al.,* 1984; Taylor and Gordon-Weeks, 1989; Taylor *et al.,* 1990), and growth cones isolated from neonatal rat forebrain are capable of taking up and releasing GABA before they contact their target (Gordon-Weeks *et al.,* 1984; Taylor and Gordon-Weeks, 1989). GABA induces migration of rat embryonic spinal neurons *in vitro* (Behar *et al.,* 1994). *In vitro* studies conducted by Behar *et al.* (1996) showed that cells derived from E14 cortices of rat do not migrate in response to GABA, but GABA stimulates migration of neurons from E15 and E16 onward. Two concentrations of GABA stimulate motility. Femtomolar concentrations of GABA stimulate neuronal migration along a concentration gradient (Chemotaxis), whereas micromolar concentrations initiate chemokinesis (increased random movement). Chemotactic responses to femtomolar GABA appear to involve all three classes of GABA receptors because the response is partially inhibited by antagonists for each receptor. However, the chemokinetic responses to micromolar amounts of GABA involve only the $GABA_B$ and $GABA_C$ receptors because the response is not blocked by the $GABA_A$ receptor antagonist bicucullin. Behar *et al.* (1996) showed that the majority of cells with chemokinetic responses to micromolar levels of GABA express GAD67, whereas cells exhibiting chemotaxis to femtomolar GABA do not express GAD (Behar *et al.,* 1998). It was also suggested that GABA-induced cell movement is mediated by intracellular Ca^{2+} because the movement can be blocked by a Ca^{2+}-chelating agent (Behar *et al.,* 1996). Thus, GABA and muscimol, a $GABA_A$ receptor agonist, elevate intracellular calcium levels in embryonic and newborn mammals (Yuste and Katz, 1991; Lin *et al.,* 1994).

Although GABA-accumulating glial cells and glial precursor cells are present in early postnatal and prenatal rat occipital neocortex (Chronwall and Wolff, 1980), little information is available about the physiological role of GABA in glial cell growth.

C. GABA Inhibits DNA Synthesis

LoTurco *et al.* (1995) showed that GABA causes a significant decrease in the incorporation of [^{3}H]thymidine into E16 and E19 rat cortical explants but not E14 explants. This difference may be due to the absence of functional GABA receptors at E14 because application of GABA does not induce depolarization in ventricular zone cells at E14, and the GABA-induced decrease in DNA synthesis is blocked by the GABA antagonist bicucullin. In adult cortical neurons, activation of $GABA_A$ receptor increases the membrane conductance for Cl^-, causing membrane hyperpolarization and reduced neuronal excitability. However, application of GABA to ventricular zone cells typically causes membrane depolarization (LoTurco *et al.,* 1995). This may be explained by the high intracellular Cl^- concentration in ventricular zone cells. LoTurco *et al.* also showed that GABA-induced inhibition in

DNA synthesis is depolarization dependent, although the mechanism by which GABA-induced depolarization inhibits DNA synthesis is unknown. An increase in intracellular Ca^{2+} concentrations has been suggested to play a role in this pathway. GABA decreases Ca^{2+} levels in mature hypothalamic neurons *in vitro* and depresses cellular activity through the opening of Cl^- channels, whereas GABA applied to embryonic hypothalamic neurons causes a dramatic and rapid increase in intracellular Ca^{2+} (Obrietan and van den Pol, 1995) via voltage-sensitive Ca^{2+} channels (Yuste and Katz, 1991). The majority (75%) of developing neurons in each brain region show an increase their Ca^{2+} response to GABA. In contrast, GABA elicits no Ca^{2+} increase in older neurons (>18 days *in vitro*) (Obrietan and van den Pol, 1995). Ca^{2+} has a wide variety of effects in developing brain, in which it modulates the rate and direction of neuritic growth (Mattson and Kater, 1987) and influences gene expression (Vaccarino *et al.,* 1992; Bading *et al.,* 1993). Together, these findings suggest that $GABA_A$ receptor-mediated signal transduction modulates neuronal development.

D. GABA as a Neurotrophic Factor

In vitro studies have indicated that GABA has a trophic effect on certain neural cells. Spoerri (1988) has shown that GABA facilitates proliferation and differentiation of cultured embryonic chick cortical and retinal neurons. GABA and muscimol have no significant effect on Purkinje cells in culture, but bicucullin reduces the number of cells due to degeneration (Obata, 1997). In the presence of bicucullin, cultured embryonic hippocampal neurons display a remarkable reduction in the number of primary neurites and branching points (Barbin *et al.,* 1993). Wolff *et al.* (1978) demonstrated that GABA facilitates synaptogenesis of the principal neurons of the superior cervical ganglion of adult rat.

V. GABA Systems outside the CNS

A. Tissue Distribution of GABA and GAD

GABA, GAD, and GABA-T have been found in a variety of tissues outside the brain and spinal cord. Quantitative and semiquantitative data for GABA, GAD, and GABA-T levels in peripheral tissues in mammals have been summarized by Tanaka (1985) and Erdö and Kiss (1986). The tissues include blood, blood vessels, spleen, heart, skeletal muscle, gastrointestinal tract, liver, pancreas, kidney, urinary bladder, male and female reproductive organs, lung, pituitary, thyroid, adrenal gland, thymus, salivary gland, skin, and sympathetic ganglia. In addition to these quantitative data, the distributions of GABA and GAD have been investigated with

TABLE I

Putative Functions of GABA in Tissues outside the CNS

Tissue	Function	Reference
Superior cervical ganglion	Synaptogenesis	Wolff *et al.* (1979), Dames *et al.* (1985), Joó *et al.* (1987)
Optic nerve	Modulate axonal excitability	Sakatani *et al.* (1992)
Enteric nervous system	Control of gastrointestinal motility	Tsai *et al.* (1993), Krantis *et al.* (1998), Zhou and Galligan (2000)
Nodose ganglion	Control of swallowing	Broussard and Altschulter (2000)
Trigeminal ganglion	Modulate sensory information	Stoyanova *et al.* (1998)
Pituitary	Control hormone secretion	Racagni and Donoso (1986)
	Regulate gene expression of proopiomelanocortin	Loeffler *et al.* (1986)
Pineal gland	Modulate melatonin synthesis	Rosenstein *et al.* (1990), Wakabayashi *et al.* (1991)
	Serotonin release	Chuluyan *et al.* (1992)
Carotid body	Chemoreceptive	Oomori *et al.* (1994)
Heart	Modulator in sinus node	Matsuyama *et al.* (1991)
Palate	Regulate palate reorientation	Wee and Zimmerman (1983)
Mandible	Craniofacial development	Ben-Shachar *et al.* (1988)
Lung	Modulate airway tonus and secretion	Shirakawa *et al.* (1987), Tohda *et al.* (1998)
Kidney	Modulate calcium and/or potassium transport	Erdö (1990)
	Induce vasoconstriction at the afferent arteriole	Monasterolo *et al.* (1996), Fujimura *et al.* (1999)
Urinary bladder	Modulate detrusor muscle	Shirakawa *et al.* (1988), Chen *et al.* (1994)
Adrenal	Catecholamine release	Amenta *et al.* (1988c), Kataoka *et al.* (1988), Busik *et al.* (1996), Kitayama *et al.* (1990)
Stomach	Mucoprotective effect	Erdö *et al.* (1989)
	Stimulate gastrin and/or somatostatin release	Harty and Franklin (1986), Koop and Arnold (1986)
	Induce acid secretion	Tsai *et al.* (1987)
Intestine	Stimulate electrolyte transport	MacNaughton *et al.* (1996)
Pancreas	Regulate insulin, somatostatin, and glucagon secretion	Sorenson *et al.* (1991)
Liver	Modify hepatobiliary growth and development	Minuk (1990)
Spleen	Affect T cell function	Bergeret *et al.* (1998), Tian *et al.* (1999)
Salivary gland	Regulate salivary secretion	Shida *et al.* (1995)
Uterus	Modulate of uterine contractility	Riesz and Erdö (1985)
Ovary	Regulate blood flow and hormone secretion	Erdö *et al.* (1985)
Testis	Regulate androgen production	Ritta and Calandra (1986)

immunohistochemistry (Kenny and Ariano, 1986; Erdö *et al.,* 1986; Oomori *et al.,* 1993; Pokorski and Ohtani, 1999; Szabat *et al.,* 1992; Vincent *et al.,* 1983; Wolff *et al.,* 1986; Dobó *et al.,* 1992), and expression of GAD mRNA has been studied with *in situ* hybridization histochemistry (Cram *et al.,* 1995; Katarova *et al.,* 2000; Kosaka *et al.,* 1994; Petersen *et al.,* 1993; Tillakaratne *et al.,* 1992).

B. Distributions of GABA Receptors outside the Brain

Pharmacological, biochemical, and autoradiographic lines of evidence indicate that specific receptors for GABA are not restricted to the CNS. Tissues and cells that express $GABA_A$ receptors include stomach G cells (von Blankenfeld *et al.,* 1995), dorsal root ganglion (Persohn *et al.,* 1991; Ma *et al.,* 1993), optic nerve (Sakatani *et al.,* 1992), nodose ganglion of the vagus nerve (Ashworth-Preece *et al.,* 1997), trigeminal ganglion (Kondo *et al.,* 1994), enteric nervous system (Krantis *et al.,* 1995; Poulter *et al.,* 1999), adrenal gland (Parramon *et al.,* 1994; Akinci and Schofield, 1999), salivary gland (Shida *et al.,* 1995), small intestine (Napoleone *et al.,* 1991; Akinci and Schofield, 1999), liver (Minuk *et al.,* 1987), kidney (Amenta *et al.,* 1988b), male and female reproductive tissues (Amenta *et al.,* 1988a; Erdö and Maksay, 1988; Akinci and Schofield, 1999), pancreas (von Blankenfeld *et al.,* 1995), pituitary (Boué-Grabot *et al.,* 1995), lung (Shirakawa *et al.,* 1987), and heart sinus node (Matsuyama *et al.,* 1993). $GABA_B$ receptors are also found in many peripheral tissues, including male and female reproductive tissues (Erdö *et al.,* 1984; Ritta *et al.,* 1991), nodose ganglion of the vagus nerve (Sherff and Mulloney, 1996), stomach, adrenal gland, pituitary, spleen, kidney (Belley *et al.,* 1999; Castelli *et al.,* 1999), and small intestine (Nakajima *et al.,* 1996).

C. Putative Functions of GABA in Peripheral Tissues

Recently, a great deal of attention has been given to the function of GABA outside the CNS. Although the GABA system is present in most peripheral tissues that have been studied, the precise functional roles of peripheral GABA are less clear. The putative functions of GABA in peripheral tissues are summarized in Table I.

VI. Concluding Remarks

It is thought that GABA exerts its inhibitory effect via acting $GABA_A$ receptors in adult mammalian brain. However, the effect is not uniform, and it is complicated by heterogeneity of the subunit composition of receptors, by modulation of various intrinsic and extrinsic chemical substances, and by subunit phosphorylation.

Moreover, the diversity of neurons must be considered. Pharmacological research on $GABA_A$ receptor heterogeneity, which was not addressed in this review, has made it possible to produce many CNS therapeutic drugs that have few side effects.

The interaction between the $GABA_A$ receptor and the cytoskeleton appears to have important implications in the formation of neural networks. To date, GABA system research has concentrated on physical aspects, whereas future research will require greater focus on function. Data and techniques from fields other than molecular biology will have to be introduced. Another important direction of GABA research will be to examine the evolutionary relationships of molecules in this system. Selection of specific neuronal networks during the process of evolution may be correlated with brain reward systems that involve neuropeptides, ethanol, and the endogenous ligands that correspond to benzodiazepines. Ontogenetically, disturbances of the GABA system may be the underlying causes of several psychiatric disorders.

Another important aspect is the possible participation of GABA in cell proliferation, differentiation, and growth. These are roles that are independent of the neurotransmitter function. Most of the so-called classical neurotransmitters, including GABA, are also present in organisms that do not possess nervous systems. It is well-known that a large number of nonneuronal cells also contain GABA. In this review, we emphasized the metabolic correlation between GABA and polyamines that may play significant roles in cell differentiation and development.

Because many nonneuronal cells, including immature neurons, produce $GABA_B$ receptors in addition to $GABA_A$ receptors, research on $GABA_B$ receptors may increase our understanding of GABA-related signal transduction. It is also likely that studies of GABA system knockout mice will contribute to our knowledge of this system. These lines of study will pave the way to better understanding GABA functions because we now know that GABA is not a classical neurotransmitter and its actions are not limited to nervous function.

Acknowledgments

The invaluable contributions of Drs. H. Hayashi (Biochemistry Department), R. Yoshida (2nd Physiology Department), and Fangyu Wang (Anatomy Department) of Osaka Medical College and Carlos E. de Carvalho (Department of Science for Laboratory Animal Experimentation, Research Institute for Microbial Diseases, Osaka University) to this review are gratefully acknowledged. In addition, we thank T. Kanayama and T. Miyahara for their secretarial help.

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Neuroendocrine Control of Pheromone Biosynthesis in Moths

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Prevalent among the Lepidoptera, as in many other insect orders, species-specific pheromones are synchronously produced and released for mate finding. Pheromone biosynthesis activating neuropeptide (PBAN) is a neuropeptide widespread throughout the class Insecta. Although its role in the several different orders of insects has not been fully elucidated, its regulatory role in Lepidopteran pheromone biosynthesis has been strongly implicated. The biosynthesis, gene expression, distribution, and release of PBAN have been studied in several moth species. This review discusses PBAN's mode of action as a pheromonotropic neurohormone at the organism, tissue, and cellular levels. The discussion includes an overview on PBAN structure–activity relationships, its target tissue identification, its putative receptor proteins, and the second messengers involved in signal transduction and the key regulatory enzymes in the pheromone biosynthetic pathway that may be influenced by PBAN. Finally, the review includes a discussion of various mediators and inhibitors of the pheromonotropic action due to PBAN.

KEY WORDS: PBAN/pyrokinin/myotropins, Immunocytochemistry, ELISA, Radioimmunoassay, Pheromone gland, Receptor proteins, Photoaffinity labeling, Cyclic AMP, Juvenile hormone, Octopamine, Sex peptide.

I. Introduction

The process of reproduction represents the defining feature of all life-forms. Thus, the events concerned with reproduction may be considered as the ultimate objective of all other life processes. Species diversity, relying on the exchange of genetic

information, therefore depends on the successful meeting between two individuals of the same species. Many insect species use species-specific sex pheromones for mate finding. Among the moth species, generally nocturnal and active during the night (scotophase), sex pheromones are prevalent. These are volatile chemical substances, synthesized and emitted by one partner (usually the female) and perceived by the opposite sex. Emission of pheromone by female moths occurs during calling behavior in which the female moth exposes its pheromone gland by extruding its ovipositor tip. Perception of these chemical substances triggers stereotypic orientation responses in the members of the opposite sex. The evolutionary and ecological success of the insect therefore depends on its ability to initiate and terminate pheromone biosynthesis, and the successful outcome of these behavioral events depends on their synchronization. In many moth species this synchronization is achieved by neuroendocrine mechanisms that, in turn, are influenced by various environmental and physiological events (temperature, photoperiod, host plants, mating(s), hormones, neurohormones, and neuromodulators).

This review considers the available evidence concerning the regulation of sex pheromone production at the level of the organism, tissue, and cell and attempts at providing a unifying hypothesis for the apparent variations among different moth species.

II. Reproductive Behavior in Moths

Females of many moth species are attractive to males during specific periods of the photoperiod. In these moths, therefore, pheromone production and emission are controlled by an endogenous circadian rhythm that is entrained by photoperiodic cues. Additionally, some moths (e.g., *Helicoverpa zea*) have been shown to delay production of pheromone until a suitable host plant is found for egg laying (Raina, 1988). These facts suggest that a regulatory mechanism must play an important role in the synchronization of mating behavior.

Raina and Klun (1984) were the first to discover that sex pheromone production is regulated by a neurohormone in *H. zea*. The neurohormonal activity was found in homogenates of brain complexes [consisting of brain–subesophageal ganglia–corpora cardiaca–corpora allata (Br-SOG-CC-CA) complexes] during both the photophase and the scotophase. This activity was detected in the hemolymph only during the scotophase, thereby indicating a hormonal function. The neurohormone was termed pheromone biosynthesis activating neuropeptide (PBAN). The activity was present in brain complexes of both male and female *H. zea* as well as in females of other moth species (*Ostrinia nubilalis* and *Lymantria dispar*) and in the cockroach (*Blatella germanica*). Many workers subsequently detected PBAN-like activity in neural tissues of several other moth species as well as in other insect orders (Table I).

TABLE I
The Presence of PBAN-like Activity and/or Immunoreactivity in Insect Species

Lepidopteran family	Species	Reference
Noctuidae	*Helicoverpa zea*	Raina and Klun (1984)
	Heliothis phloxiphaga	Raina and Klun (1984)
	Helicoverpa armigera	Rafaeli and Soroker (1989a)
	Helicoverpa assulta	Choi *et al.* (1998a,b)
	Heliothis virescens	Raina *et al.* (1987)
	Heliothis peltigera	Altstein *et al.* (1990)
	Chrysodeixis chalcites	Altstein *et al.* (1990)
	Agrotis segatum	Zhu *et al.* (1995)
	Agrotis ipsilon	Duportes *et al.* (1998)
	Spodoptera frugiperda	Raina *et al.* (1987)
	Spodoptera littoralis	Rafaeli and Soroker (1989a)
	Spodoptera latifascia	Jacquin-Joly and Descoins (1996)
	Spodoptera descoinsi	Jacquin-Joly and Descoins (1996)
	Mamestra brassicae	Bestmann *et al.* (1989)
	Pseudaletia separata	Cusson and McNeil (1989)
	Trichoplusia ni	Zhao and Haynes (1997)
Pyralidae	*Diaphania nitidalis*	Raina *et al.* (1987)
	Ostrinia nubilalis	Raina *et al.* (1987)
	Eldana saccharina	Jacquin-Joly and Descoins (1996)
	Plodia interpunctella	Rafaeli and Gileadi (1995a)
Lymantriidae	*Lymantria dispar*	Raina *et al.* (1987)
Sphingidae	*Manduca sexta*	Raina *et al.* (1987)
Gelechiidae	*Pectinophora gossypiella*	Rafaeli and Klein (1994)
Tortricidae	*Choristonura fumiferana*	Delisle *et al.* (1999)
	Choristonura rosaceana	Delisle *et al.* (1999)
Pieridae	*Pieris brassicae*	Jacquin-Joly and Descoins (1996)
Insect order		
Orthoptera	*Blatella germanica*	Raina *et al.* (1987)
	Locusta migratoria	Sreng *et al.* (1990)
Diptera	*Mayetiola destructor*	Foster *et al.* (1991)
	Sarchophaga bullata (*larvae*)	Zdarek *et al.* (1997)
	Anastrepha suspensa	Teal (1998)

The regulatory mechanisms of pheromone production (physiological response) do not depend on calling behavior (behavioral response), although both responses may be synchronized and entrained to the photoperiod. In some species (*Sesamia nonagrioides, Plodia interpunctella, Ephestia cautella,* and *Helicoverpa* spp.), pheromone production and calling behavior are synchronous and peak pheromone production occurs during peak periods of calling behavior (Bablis and Mazomenos 1992a; Coffelt *et al.,* 1978; Raina, 1988). However, in other moth species pheromone titers either remain high throughout the 24 h (*Trichoplusia ni* and *Pectinophora gossypiella*) (Shorey and Gaston, 1965; Rafaeli and Klein, 1994) or are high before calling behavior (*Platynota stultana*) with rates of production remaining low during calling (Sower *et al.,* 1972; Bjostad *et al.,* 1980; Webster and Carde, 1984). An early study conducted on *Antheraea polyphemus* (Sasaki *et al.,* 1983) demonstrated that females with a blood-tight ligature between the third and fourth abdominal segments exhibited normal calling behavior while maintaining an intact ventral nerve cord (VNC). The results indicated that a blood-borne factor was not involved in calling behavior. Injections of Br-SOG extracts or synthetic PBAN during the photophase can initiate pheromone biosynthesis but do not initiate calling behavior (Raina, 1993). Moreover, studies conducted in our laboratory on *H. armigera* showed that treatment with biogenic amines (octopamine and analogs) will inhibit pheromone production during the scotophase (normal peak hours of pheromone production and calling behavior) but will not inhibit calling behavior (Rafaeli and Gileadi, 1995b; Rafaeli *et al.,* 1997; see Section IV.C.1). However, the close correlation between neurosecretion and calling behavior, elegantly shown in a recent study on *Bombyx mori* (Ichikawa, 1998), suggests that the efferent neural mechanism associated with the movements of calling behavior may be closely coupled with the neural mechanisms which control neurosecretion.

Mating influences both calling behavior and pheromone production. Moths exhibit different mating strategies: Some mate only once, whereas others mate more than once. Female moths that mate only once in their lifetime generally exhibit cessation of pheromone production and are not receptive to males, exhibiting a switch to oviposition behavior after mating (Raina *et al.,* 1986b). On the other hand, species that mate more than once exhibit a temporary cessation of calling behavior and pheromone production (Shorey and Gaston, 1965; Raina *et al.,* 1986b; see Section IV.C.2). Thus, from initial observations it may seem that moth species have evolved different mechanisms for synchronizing mating behavior.

III. Biochemistry of Pheromone Biosynthesis Activating Neuropeptides

A. Isolation and Identification of PBANs

Progress in the area of hormonal control of pheromone production has been made to a large extent due to the development of a simple but very sensitive bioassay

that involved ligation of females between head and thorax followed by injection of the test material into the abdomen. The pheromone gland was then excised and extracted for pheromone quantification (Raina and Klun, 1984). PBAN was thus first purified from Br-SOG-CC-CA complexes of *H. zea* (Raina *et al.,* 1987). Inactivation was demonstrated by digestion with trypsin or carboxypeptidase Y but not with aminopeptidase M, thus indicating the proteinaceous nature of the neurohormone and that it may have a blocked NH_2 terminus. Subsequent identification and sequence determination revealed a peptide consisting of 33 amino acid residues and that had a molecular weight of 3.9 kDa (Raina *et al.,* 1989b). Synthetic Hez-PBAN (*H. zea;* nomenclature according to Raina and Gade, 1988) was shown to activate pheromone production in many lepidopteran species and endogenous pheromonotropic activity was found in many moths and other insect species (Table I). Pheromonotropic neuropeptides were isolated and sequenced from two other moth species: *B. mori* (Bom-PBAN-I) (Kitamura *et al.,* 1989) and *L. dispar* (Lym-PBAN) (Masler *et al.,* 1994). A second PBAN was reported in *B. mori* (Bom-PBAN II) with an identical sequence except for an extra arginine residue at the amino terminus (Table II) (Kitamura *et al.,* 1990). The sequences of PBAN in *Helicoverpa assulta, Agrotis ipsilon,* and *Mamestra brassicae* were deduced from the cDNA (Table II). The pheromonotropic peptides are representatives of a PBAN/pyrokinin/myotropin family of peptides that bear the FXPRL-NH_2 motif (Table III). These have been shown to have pheromonotropic activity in lepidopteran species (Fonagy *et al.,* 1992b), whereas they have been found to be responsible for a variety of physiological and behavioral functions in other insects, including contraction of the locust oviduct (Schoofs *et al.,* 1991), myotropic activity of Orthopteran hindgut (Schoofs *et al.,* 1991; Nachman and Holman, 1991), egg diapause in the silkworm (Imai *et al.,* 1991), acceleration of pupariation in the fleshfly larvae (Nachman *et al.,* 1997), and melanization and reddish coloration in moth larvae (Matsumoto *et al.,* 1990, 1992b; Altstein *et al.,* 1996).

B. PBAN Gene Expression

The PBAN gene was characterized from *H. zea* and *B. mori*. Hez-PBAN genomic DNA contains a 70-amino acid precursor with the PBAN domain (Davis *et al.,* 1992). The full-length cDNA has a 766-bp sequence encoding 194 amino acids, which includes four peptide domains in addition to Hez-PBAN (Ma, 1994; Ma *et al.,* 1996) (Table IV). In *B. mori,* the polyprotein precursor from the cDNA encodes 192 amino acids and possesses not only the PBAN sequence but also four additional peptides with a common FXPRL (or K) L sequence in their C termini (Imai *et al.,* 1991; Kawano *et al.,* 1992; Sato *et al.,* 1993).

Three additional peptides with the FXPRL (or K) L sequence at the C termini deduced from cDNA (Has-PBAN, Agi-PBAN, and Mab-PBAN) also have sequence homology to those of *H. zea* and *B. mori* (Sato *et al.,* 1993; Ma, 1994; Ma *et al.,* 1996) (Table IV). The amino acid sequence of Has-SGNP I (subesophageal

TABLE II

Alignment of Isolated and Deduced Sequences of PBANs

Species	Family	Endogenous PBAN	PBAN sequence
Helicoverpa zea	Noctuidae	Hez-PBAN (Raina and Klun, 1984)	L S D D M P A T P A D Q E M Y R Q D P E Q I D S R T K Y F S P R L NH_2
Helicoverpa assulta	Noctuidae	Has-PBAN (Choi *et al.,* 1998b)	L S D D M P A T P A D Q E M Y R Q D P E Q I D S R T K Y F S P R L NH_2[a]
Bombyx mori	Bombycidae	Bom-PBAN I (Kitamura *et al.,* 1989)	L S E D M P A T P A D Q E M Y Q P D P E E M E S R T R Y F S P R L NH_2
Bombyx mori	Bombycidae	Bom-PBAN II (Kitamura *et al.,* 1990)	R L S E D M P A T P A D Q E M Y Q P D P E E M E S R T R Y F S P R L NH_2
Lymantria dispar	Lymantridae	Lyd-PBAN (Masler *et al.,* 1994)	L A D D M P A T M A D Q E V Y R P E P E Q I D S R N K Y F S P R L NH_2
Agrotis ipsilon	Noctuidae	Agi-PBAN (Duportes *et al.,* 1999)	L A D D T P A T P A D Q E M Y R P E P E Q I D S R T K Y F S P R L NH_2[a]
Mamestra brassicae	Noctuidae	Mab-PBAN (Jacquin-Joly *et al.,* 1998)	L A D D M P A T P A D Q E M Y R P D P E Q I D S R T K Y F S P R L NH_2[a]

[a](Sequence deduced from cDNA).

TABLE III
The PBAN/Pyrokinin Peptide Family

Peptide	Residue position
Hez-PBAN[1]	L S D D M P A T P A D Q E M Y R Q D P E Q I D S R T K Y F S P R L NH_2
Has-PBAN[2]	L S D D M P A T P A D Q E M Y R Q D P E Q I D S R T K Y F S P R L NH_2
Bom-PBAN-I[3]	L S E D M P A T P A D Q E M Y Q P D P E E M E S R T R Y F S P R L NH_2
Bom-PBAN-II[4]	R L S E D M P A T P A D Q E M Y Q P D P E E M E S R T R Y F S P R L NH_2
Lyd-PBAN[5]	L A D D M P A T M A D Q E V Y R P E P E Q I D S R N K Y F S P R L NH_2
Agi-PBAN[6]	L A D D T P A T P A D Q E M Y R P E P E Q I D S R T K Y F S P R L NH_2
Mab-PBAN[7]	L A D D M P A T P A D Q E M Y R P D P E Q I D S R T K Y F S P R L NH_2
Pss-PT[8]	K L S Y D D K V F E N V E F T P R L NH_2
Lem-PK[9]	pE T S F T P R L NH_2
Lom-PK[9]	pE D S G D G W Q Q P F V P R L NH_2
Lom-MT-I[9]	G A V P A A Q F S P R L NH_2
Lom-MT-II[9]	E G D F T P R L NH_2
Lom-MT-III[10]	R Q Q P F V P R L NH_2
Lom-MT-IV[104]	R L H E N G M P F S P R L NH_2

[1]Raina *et al.* (1989b), [2]Choi *et al.* (1998b), [3]Kitamura *et al.* (1989), [4]Kitamura *et al.* (1990), [5]Masler *et al.* (1994), [6]Duportes *et al.* (1999), [7]Jacquin-Joly *et al.* (1998), [8]Matsumoto *et al.* (1992a), [9]Nachman and Holman (1991), [10]Schoofs *et al.* (1991).

neuropeptide I), with 24 amino acids, corresponds to that of the diapause hormone in *B. mori* (Imai *et al.,* 1991; Sato *et al.,* 1993). This peptide has been observed in the SOG and CC with similar localization of PBAN-related peptides in *H. zea* (termed PGN-23; Ma *et al.,* 1996). The functional significance of this peptide, PBAN-encoding gene neuropeptide (PGN-23), in *H. zea* and *H. assulta* (Has-SGNP I) is unclear because these species do not exhibit egg diapause; facultative diapause occurs in the pupal stage. Another presumptive peptide, deduced from the cDNA, has 18 amino acids (termed β-SGNP, SGNP III, or PGN-18) and has a similar amino acid sequence as that of Pss pheromonotropin, isolated from *Pseudaletia separata* larvae (Matsumoto *et al.,* 1992a; Table III), but is less similar to the homologous 17-amino acid β-SGNP of *B. mori* (Sato *et al.,* 1993). The two other peptides encoded in the cDNA—SGNP II or PGN-7, with 7 amino acids, and SGNP IV or PGN-8, with 8 amino acids—have a high homology to α- and γ-SGNP of *B. mori* (Table IV).

Northern hybridization was performed to determine the site of PBAN mRNA. Expression of PBAN mRNA has been investigated in the central nervous system in *B. mori* (Sato *et al.,* 1993, 1994), *H. zea* (Ma, 1994), and *H. assulta* (Choi *et al.,* 1998b). In *B. mori, in situ* hybridization revealed a total of 12 positive cells

TABLE IV

Alignment of Sequences of Four Additional FXPRL Presumed Peptides Encoded by the PBAN Genomic cDNA

Species	Diapause hormone (SGNP I; PGN-23)	α-SGNP (SGNP II; PGN-7)	β-SGNP (SGNP III; PGN-18)	γ-SGNP (SGNP IV; PGN-8)	Reference
Helicoverpa zea	NDVKDGAA–SGAHSDRLGLWFGPRL	VIFTPKL	SLAYDDKSFENVEFTPRL	TMNFSPRL	Davis *et al.* (1992), Ma (1994), Ma *et al.* (1996)
Helicoverpa assulta	NDVKDGAA–SGAHSDRLGLWFGPRL	VIFTPKL	SLAYDDKSFENVEFTPRL	TMNFSPRL	Choi *et al.* (1998b)
Bombyx mori	TDMKD–ESDRGAHSERGALWFGPRL	IIFTPKL	SVA–KPQTHES LEFIPRL	TMNFSPRL	Imai *et al.* (1991), Sato *et al.* (1993).
Agrotis ipsilon	NDVKDGGADR GAHSDRGGMWFGPRL	VIFTPKL	SLSYEDKMFDNVEFTPRL	TMNFSPRL	Duportes *et al.* (1999)
Mamestra brassicae	---------- GLWFGPRL	VIFTPKL	SLAYDDKVFENVEFTPRL	TMNFSPRL	Jacquin-Joly *et al.* (1998)

which were localized as a cluster at three distinct regions along the ventral middle line of the SOG (Sato *et al.,* 1994). Similar clusters of neurosecretory cells were localized in *H. zea* by Northern hybridization experiments and the presence of a 0.8-kb Hez-PBAN transcript in the SOG of adults, pupae, and Days 0 and 1 final-instar larvae was demonstrated (Ma *et al.,* 1998). Low levels of Hez-PBAN mRNA were revealed in other parts of the central nervous system, including the brain, thoracic ganglia, and abdominal ganglia, by reverse transcriptase-polymerase chain reaction (Ma *et al.,* 1998). This study thus showed that the Hez-PBAN gene was expressed predominantly in the SOG and at relatively low levels in other parts of the central nervous system. Similarly, in *H. assulta* a single mRNA species of about 0.75 kb was detected from poly(A^+) RNA isolated from Br-SOG of 1- to 3-day-old female or male adults (Choi *et al.,* 1998b). The single hybridization signal showed a similar level of expression in both sexes.

C. Biosynthesis, Distribution, Release, and Metabolism of PBAN

In earlier work on *H. zea* and *H. armigera,* most of the PBAN-like biological activity was found to be associated with the Br-SOG (Raina *et al.,* 1987; Rafaeli *et al.,* 1991, 1993). The spatial and temporal distributions of PBAN were studied using a radioimmunoassay (RIA) and correlated to bioactivity in *H. armigera* (Rafaeli *et al.,* 1991, 1993). In these studies, we demonstrated immunoreactivity and bioactivity in extracts of Br-SOG, CC, thoracic ganglia, abdominal ganglia, and terminal abdominal ganglia (TAG) from various ages (0- to 4-day-old females) and photoperiods. The distribution and quantity of PBAN immunoreactivity in Br-SOG of adult *H. zea* moths (Kingan *et al.,* 1992; Ma *et al.,* 1996) as well as the immature stages (Blackburn *et al.,* 1992; Ma *et al.,* 1996) were also examined using enzyme-linked immunosorbent assay (ELISA) and immunocytochemical methods. Immunocytochemical studies revealed immunoreactive cells in the SOG of *H. zea* along the ventral midline: one cluster in mandibular (4 cells), one in maxillary (12–14 cells), and one in labial (4 cells) neuromeres (Kingan *et al.,* 1992). These results support the hypothesis that PBAN is mainly synthesized in the SOG.

Subsequent to the previously mentioned studies, similar ELISA as well as immunoblotting analyses of PBAN immunoreactivity were performed on other moth species: *Heliothis peltigera, Chrysodeixis chalcities, Spodoptera littoralis, S. latifascia, S. descoinsi, Eldana saccharina, Pieris brassicae, H. assulta,* and *Agrotis ipsilon* (Gazit *et al.,* 1992; Jacquin-Joly and Descoins, 1996; Marco *et al.,* 1996; Choi *et al.,* 1998a; Duportes *et al.,* 1998). In many of these studies, PBAN production in the Br-SOG was shown to be independent of age, present at similar levels, and produced both in females and in males from different photoperiods. Apparently, homologous cell groups containing PBAN-like immunoreactivity were also found in *Manduca sexta* (Homberg *et al.,* 1991; Davis *et al.,* 1993, 1996);

Mamestra brassicae, Locusta migratoria (Orthoptera), *Periplaneta americana* (Orthoptera), *Leucophaea madera* (Orthoptera), and *Neobellieria* (*Sarcophaga*) *bullata* (Dipteran) (Tips *et al.,* 1993); *B. mori* (Sato *et al.,* 1993, 1998; Ichikawa *et al.,* 1995); and *L. dispar* (Golubeva *et al.,* 1997). A similar pattern of localization was also reported for locustamyotropin-like immunoreactivity (belonging to the same peptide family; Table III) in the central nervous system (CNS) of *L. migratoria* (Schoofs *et al.,* 1991). The presence of PBAN-like immunoreactivity in the immature stages as well as adult moths (both males and females) and nonmoth insect species suggests that the principal structure of the neurosecretory system in the SOG is conserved but may serve more than one function.

The previous discussion clearly defines the distribution pattern of PBAN in the moths. However, the releasing site(s) of PBAN remains to be defined and various modes of transport have been suggested (Raina, 1996). One hypothesis suggests that PBAN may be released into the hemolymph from the CC, thereby acting as a neurohormone. Another hypothesis suggests that PBAN travels via the VNC to the target tissue (pheromone gland) in the ovipositor tip. Anatomical evidence exists for both the release through neurohemal structures (such as the CC) and axonal transport via the VNC to release sites presumably in the region of the pheromone gland (Kingan *et al.,* 1992; Davis *et al.,* 1996; Golubeva *et al.,* 1997). Immunoreactive cells of the mandibular and maxillary cluster were shown to project into the maxillary nerve and two additional pairs of axons from the maxillary cluster projected to the VNC. Members of the labial cluster projected to the retrocerebral complex (CC and cephalic aorta) via the nervus corpus cardiaci III (NCC III) (Kingan *et al.,* 1992). Those axonal innervations leading to the CC and those leading to the VNC may be synchronized to the external stimuli (photoperiod and mating), thereby ensuring that pheromone biosynthesis and release (manifested through calling behavior) occur at the required time as suggested by Ichikawa (1998).

The former hypothesis, suggesting that PBAN may be released into the hemolymph, is based on results that demonstrated biological and/or immunological activity in hemolymph samples in *H. zea* (Raina and Klun, 1984; Ramaswamy *et al.,* 1995), *M. brassicae* (Jacquin *et al.,* 1994), and *Spodoptera littoralis* (Fabrias *et al.,* 1994; Marco *et al.,* 1996). The fact that PBAN is effective when injected into the hemocoel (Raina and Klun, 1984; Raina *et al.,* 1989a; Nagasawa *et al.,* 1994) or when fed to female moths (Raina *et al.,* 1994b) also provides circumstantial evidence for its transport in the hemolymph. High-performance liquid chromatography (HPLC) analysis in *H. zea,* however, suggested that the PBAN-like activity in the hemolymph might differ in molecular weight from that of Hez-PBAN (Ramaswamy *et al.,* 1995), but this was not the case in *M. brassicae* (Iglesias *et al.,* 1998). On the other hand, a difference in HPLC profile may indicate that PBAN is degraded in the hemolymph. A study utilizing a tritium-labeled bis-norleucine analog of Hez-PBAN showed that hemolymph of *M. sexta* and *H. zea* contain peptidases capable of inactivating circulating PBAN at a rate of 2–10 fmol/min/μ1 hemolymph (Weirich *et al.,* 1995).

Based on ELISA determinations the presence of 5–10 fmol/female of PBAN-like activity in the hemolymph of scotophase *S. littoralis* females was suggested (Fabrias *et al.,* 1994; Iglesias *et al.,* 1998). A similar level of PBAN-like activity was found in hemolymph collected from adult female *M. brassicae* (Jacquin *et al.,* 1994; Iglesias *et al.,* 1998, 1999). Although these levels were insufficient at eliciting a stimulatory response, Fabrias *et al.* (1994) showed that gland cultures *in vitro* are stimulated to produce pheromone after repeated transfer to new drops of hemolymph. This may support the hypothesis that a continuous release of PBAN at low levels is necessary for pheromone biosynthesis and may explain previous unsuccessful attempts by other workers to show pheromonotropic activity in the hemolymph. In a recent study on *B. mori* (Ichikawa, 1998), short- and long-term firing patterns of neurosecretory cells, presumed to release pheromonotropic neuropeptides, were examined. Evidence showed a clear correlation of pheromone titers in virgin and mated females to calling behavior, the circulation of hemolymph, and diel periodicity. This evidence supports the hypothesis that pheromonotropic neuropeptides function as circulating neurohormones.

In favor of the latter hypothesis, pheromone production in some species (*L. dispar, S. littoralis,* and *M. brassicae*) is terminated on severing the VNC (Thyagaraja and Raina, 1994; Iglesias *et al.,* 1998) as well as on decapitation. In contrast to other moths, in *L. dispar* PBAN injection to decapitated females did not successfully recover pheromone production. However, if the VNC was severed (posterior to the thoracic ganglia) PBAN injection directly into the VNC caused a stimulation of pheromone production. Therefore, when PBAN was delivered directly to the posterior cut end of the VNC or injected into the TAG, or when the TAG was stimulated electrically, a significant stimulation of pheromone production occurred. These results suggest that in this species PBAN travels down the VNC. A similar effect of VNC transection was observed in *Pectinophora gossypiella* (Rafaeli and Klein, 1994). However, it should be emphasized that severing the VNC must occur posterior to the thoracic ganglia for stimulation by PBAN. This point may be indicative of a descending inhibitory signal. It may well be that VNC transection eliminates a decending inhibitory signal, which negates the stimulatory action of PBAN, and on severing the VNC this signal is prevented from reaching its target tissue. Indeed, pheromonostatic effects occur during the photophase and after mating and may travel along this route (see Section IV.C).

Teal *et al.* (1989) showed that pheromone production in *H. zea* depends on the presence of an intact VNC; however, contradicting results were observed in the same species, in which VNC transection did not inhibit pheromone production in virgin females (Kingan *et al.,* 1995). A report with data on *O. furnacalis* indicated that the TAG was not involved in PBAN activation (Qui and Cao, 1989). VNC transection in *A. ipsilon* (Zhu *et al.,* 1995) and in *O. nubilalis* (Ma and Roelofs, 1995a) likewise did not influence pheromone production. In fact, the latter study showed that removal of the entire abdominal VNC did not affect the response of the operated females to exogenous PBAN (Ma and Roelofs, 1995a). Indeed, preparations

of ovipositor tips *in vitro* (shown first in *H. armigera;* Soroker and Rafaeli, 1989) were responsive to brain extracts and Hez-PBAN (see Section IV.A.2). Moreover, the removal of the CC-CA (presumptive neurohemal source of PBAN) inhibited pheromone production by *O. nubilalis* females (Ma and Roelofs, 1995a).

It can be argued that removal of the VNC or isolation of the pheromone gland *in vitro* do not remove nerve endings closely associated with the target tissue, which may arborize and terminate in neurohemal structures. The demonstration that the TAG directly innervates the pheromone gland in *H. zea* and *Heliothis virescens* (Christensen *et al.,* 1991), the presence of PBAN immunoreactivity in the TAG of *H. armigera* (Rafaeli *et al.,* 1991, 1993) and *S. littoralis* (Marco *et al.,* 1996), as well as the demonstration of terminal arborizations of PBAN immunoreactivity in the TAG in *H. zea* (Kingan *et al.,* 1992) all may suggest that PBAN is released from the TAG locally at the proximity of the target tissue. Studies were conducted on *H. armigera* pheromone gland cultures *in vitro* that were subjected to high K^+ depolarization so as to induce release of local neurosecretory material at nerve endings. This treatment did not induce the stimulation of pheromone production nor did it prevent the normal pheromonotropic response to exogenously applied Hez-PBAN (Rafaeli *et al.,* 1996). Moreover, cobalt anterograde/retrograde filling studies conducted on *O. nubilalis* females did not show direct neural connections between the TAG and the pheromone gland (Ma and Roelofs, 1995a). Estimated values of PBAN immunoreactivity in the TAG of *H. armigera* showed only detectable levels during the photophase (5–20 fmol/tissue by RIA) (Rafaeli *et al.,* 1991, 1993) and low levels in both photophase and scotophase in *S. littoralis* (4–7 fmol/tissue by ELISA) (Marco *et al.,* 1996). The presence of immunoreactivity only during the photophase may be interpreted as an indication of release from the TAG during the scotophase and an accumulation during the nonactive periods of the photoperiod; however, this was not observed in all species. On the other hand, since much higher levels of PBAN immunoreactivity were observed in the CC—70 fmol/gland (RIA; Rafaeli *et al.,* 1991, 1993), 64–67 fmol/gland (ELISA; Marco *et al.,* 1996), and 240 fmol/gland (ELISA; Kingan *et al.,* 1992)—the CC seems to be a more likely candidate for a neurohemal organ. Axons originating from PBAN-producing SOG cells travel the entire length of the VNC and contain PBAN-like activity. It should be noted that the low levels of PBAN immunoreactivity found along the VNC and associated ganglia may also be a result of weak cross-reactivity with similar peptides from the PBAN/pyrokinin/myotropin family and may not be a true reflection of actual PBAN levels. Indeed, colocalization of the myotropins was demonstrated in the cells and axons in the CNS of some species (Tips *et al.,* 1993). In addition, through HPLC fractionation of *H. zea* neural tissue followed by ELISA detection of PBAN antigen, it was demonstrated that the thoracic ganglia contained pheromonotropic PBAN-like activity that was different from that of Hez-PBAN by HPLC analysis (Ma *et al.,* 1996). Nevertheless, *in situ* hybridization histochemistry revealed a pair of ventral midline neurons in each thoracic ganglion and some in abdominal ganglia including the TAG of adults

(Ma *et al.,* 1998), indicating the expression (if at a low level) of the PBAN gene in these parts of the nervous system.

IV. Biological Activities of PBAN

A. Pheromonotropic Mode of Action of PBAN

1. Structure–Activity Relationships

Structure–function studies using synthetic PBANs and partial sequence peptides showed that the C-terminal pentapeptide sequence (FXPRL amide, Xaa=Ser, or Thr) is the minimum sequence essential for stimulation of pheromone production in the pheromone gland of female *H. zea* and *B. mori* (Raina and Kempe, 1990, 1992; Kuniyoshi *et al.,* 1991). The concentrations of these minimum sequences required for stimulation of pheromone biosynthesis were 100- to 1000-fold higher than that of the full PBAN sequence which was active at 1–10 pmol/insect (Kitamura *et al.,* 1989; Raina and Kempe, 1990). This suggests that other parts of the PBAN sequence may be involved in the interaction with the receptor. PBAN may contain two or three Met residues (depending on the species) and it was shown in *H. zea* and *B. mori* that oxidized PBAN was more potent than unoxidized PBAN (Raina *et al.,* 1991; Kuniyoshi *et al.,* 1992). These structure–activity studies were performed using *in vivo* bioassays involving injection into the hemocoel of ligated female moths. The increased activity of oxidized PBAN may thus be due to an increased stability against proteolytic deactivation. Analogs of the C-terminal sequence containing a blocked N terminus showed enhanced activity, perhaps as a result of stability to proteolytic enzymatic activity when injected into the hemocoel (Kuniyoshi *et al.,* 1992).

Some of the insect neuropeptides belonging to the pyrokinin/PBAN family (Table IV) have been demonstrated to have pheromonotropic activities in moths (Sreng *et al.,* 1990; Fonagy *et al.,* 1992b). In fact, the myotropins Lem-PK, and Lom-PK, and Lom-MT I were shown to be 10-fold more potent than Bom-PBAN I in *B. mori* pheromone production (Kuniyoshi *et al.,* 1992). Comparable structure–activity studies were performed on hindgut/oviduct myotropic activity (Nachman *et al.,* 1986), cuticular melanization (Matsumoto *et al.,* 1992a,b; Altstein *et al.,* 1996), diapause (Matsumoto *et al.,* 1992b), and pupariation (Zdarek *et al.,* 1998). The C-terminal pentapeptide minimum sequence was both essential and sufficient in eliciting activity in all these studies except for pupariation. In the latter study, the minimum sequence for a 10% response was identified as the C-terminal tripeptide PRL amide, but for maximum response the tetrapeptide TPRL amide was identified as the active core sequence (Zdarek *et al.,* 1998).

Nuclear magnetic resonance studies were performed on the hexapeptide analog of the C-terminal sequence of PBAN, [D-Phe^{29}]PBAN 28-33 (Wang *et al.,* 1994),

and the full PBAN sequence (Clark and Prestwich, 1996). Wang *et al.* (1994) reported that the C-terminal hexapeptide fragment of PBAN, in a solution of DMSO and water, adopts a type II β-turn structure that may result from the conformational averaging over a type I β-turn and an extended conformation of the peptide. In the less polar environment, which the peptide could experience in receptor surroundings, a type I β-turn conformation encompassed the C-terminal region (Clark and Prestwich, 1996). In addition, no interaction between the C-terminal turn and the rest of the peptide molecule occurred, indicating that the C-terminal turn indeed represented the important conformation recognized by the PBAN receptor. Using a combination of spectroscopic and molecular dynamics techniques, Nachman and coworkers (1991) found that a rigid, cyclic pyrokinin/PBAN analog cyclo-[NTSFTPRL] has a type I β-turn in the active core region of the molecule. Using this conformationally constrained octapeptide analog of PBAN, a significant retention of pheromonotropic and myotropic bioactivity was obtained (Nachman *et al.,* 1993). This suggests that its active core conformation resembles that adopted by the linear peptide at the receptor site. Thus, the receptors responsible for these different physiological responses share certain binding requirements as identified by this rigid, well-defined backbone structure. Nevertheless, comparative activity studies of the different physiological responses suggest that selective binding capabilities occur (Nachman *et al.,* 1993). Cyclic backbone analogs, based on [Arg^{27}-D-Phe^{30}] PBAN 27–33 NH_2, were also developed and various PBAN agonists as well as antagonists to injected PBAN were found to be active at high concentrations (1 nmol/insect) (Altstein *et al.,* 1999a).

2. Delineation of the Target Tissue

In all the studies mentioned so far, be they structure–activity relationship studies or studies involving identification, purification, and sequencing of PBAN neuropeptides, biological activity was detected using moths in an *in vivo* bioassay in which peptides were injected into the hemocoel of insects not producing pheromone (decapitated or intact females during the photophase). This bioassay has proved to be simple and relatively efficient for isolation and identification of pheromonotropic peptides. However, using this bioassay does not identify the target tissue and the mode of action at the receptor site.

Early structural evidence implicated the glandular epithelium between the eighth and ninth abdominal segments of the ovipositor tips as the possible site of pheromone production in females of several species of moths (Percy and Weatherston, 1974; Teal *et al.,* 1983; Aubrey *et al.,* 1983). Through the development of a sensitive *in vitro* bioassay, we were able to provide evidence regarding the direct action of PBAN in the stimulation of pheromone biosynthesis. Using preparations of ovipositor tips *in vitro,* our studies pioneered the demonstrations that brain extracts and synthetic Hez-PBAN, administered in the incubation medium, could stimulate pheromone production in the moths *H. armigera* and *H. zea* (Soroker and

Rafaeli, 1989; Rafaeli *et al.,* 1990, 1993). Pheromone gland activation involved the production of the main pheromone component (Z-11 hexadecenal), which was analyzed using gas chromatography (GC), radio GC, radio HPLC, and radio thin-layer chromatography (Rafaeli *et al.,* 1990, 1991, 1993; Rafaeli, 1994). The obtained response was specific to the pheromone gland and independent of other abdominal tissues (Rafaeli, 1994; Rafaeli *et al.,* 1996). Pheromone glands were responsive irrespective of the physiological or chronological age and the photoperiod of the donor adult females (Rafaeli *et al.,* 1993). Even mated females, having ceased to produce pheromone *in vivo* (see Section IV.C.2), were responsive to stimulation *in vitro* (Rafaeli *et al.,* 1993). On the basis of these studies, we hypothesized that this tissue denotes the main target tissue for the pheromonotropic action of PBAN.

Evidence has since accumulated regarding several other species of moths—*B. mori, H. zea, S. litura, O. nubilalis, Plodia interpunctella, Thaumetopoea pityocampa*—thereby confirming our hypothesis (Arima *et al.,* 1991; Jurenka, 1996; Fonagy *et al.,* 1992a; Ma and Roelofs, 1995a,b; Rafaeli and Gileadi, 1995a; Fabrias *et al.,* 1995). In contrast, in some moths (*T. ni, Argyrotaenia velutinana,* and *Agrotis segatum*) pheromone gland cultures *in vitro* were reported not to respond to brain extracts (Tang *et al.,* 1989; Zhu *et al.,* 1995). These workers failed to test synthetic PBAN. In crude brain homogenates, there could be interfering pheromonostatic compounds.We have observed inhibitory influences on pheromone biosynthesis using crude methanol extracts of Br-SOG complexes that contained high protein levels. After dilution of these extracts a stimulatory effect could be obtained (unpublished observations). When homogenates are injected into the whole body, dilution and enzymatic activity in the hemolymph enable the insect to overcome any inhibitory influences. Therefore, conclusions that pheromone glands do not respond to PBAN directly may be imprecise and should be reexamined using synthetic neuropeptides on these species. In fact, later studies conducted on *A. velutinana,* while showing the requirement of a bursal factor for a pheromonotropic response, reported a smaller but significant increase in pheromone production upon incubation in the presence of synthetic PBAN alone and in the presence of other peptides of the PBAN/pyrokinin family (Jurenka *et al.,* 1991, 1994; Fabrias *et al.,* 1992).

Some moths (*T. ni, P. gossypiella,* and *A. velutinana*) differ from other moth species studied in that pheromone biosynthesis is continuous and the sex pheromone components become only partially depleted during scotophase as females call (Hunt and Haynes, 1990; Rafaeli and Klein, 1994; Jurenka, 1996). During the photophase the quantity of stored pheromone in *T. ni* increases as biosynthesis continues without emission. Decapitation for 24 h (Tang *et al.,* 1989) or forcible eversion (Zhao and Haynes, 1997) of the pheromone glands fail to deplete the gland of stored pheromone, and therefore injection of PBAN does not stimulate pheromone production (Tang *et al.,* 1989). The rate of pheromone volatilization from forcibly extruded glands, however, was shown to be lower than the rate during scotophase. It is thus not a simple correlate to the gland's content. Zhao and Haynes

(1997) demonstrated that *T. ni* head extracts as well as Hez-PBAN, when injected during the photophase, stimulate the rate of pheromone emission. These results may indicate that PBAN is also involved in the control of emission, including the transport of pheromone reserves to the surface for the pheromone gland.

In the pink bollworm, *P. gossypiella,* although PBAN or a PBAN-like factor is involved in the control of pheromone production, its exact target tissue is unclear. As is the case in *T. ni,* decapitation for 24 h or injections of PBAN during the midphotophase do not affect pheromone titers in *P. gossypiella*. However, unlike in *T. ni,* decapitation for longer periods (72 h) successfully depleted pheromone titers and pheromone production could be restored (although not to the full extent) after injections of Hez-PBAN or head extracts. *In vitro* incubations of pheromone glands in this species were not responsive to either Hez-PBAN or head extracts, although PBAN did induce the stimulation of *de novo* fatty acid synthesis (Rafaeli and Klein, 1994).

Delineation of the PBAN response to the intersegmental tissues situated between the eighth and ninth abdominal segments of the ovipositor tips was demonstrated in *H. armigera, H. virescens,* and *H. zea* females (Rafaeli and Gileadi, 1995, 1996; Jurenka, 1996). A recent study of the structural organization of the sex pheromone gland in *H. zea* demonstrated that pheromone was produced by an almost complete ring of columnar cells situated between the eighth and ninth abdominal segments. This study revealed distinct ultrastructural features that correlated with periods of pheromone production (Raina *et al.,* 2000). In *B. mori,* isolation of functional and viable pheromone gland cell clusters from the intersegmental membrane using papain enzymatic digestion to remove the cuticular layer was recently reported (Fonagy *et al.,* 2000). These cell clusters produced pheromone (bombykol) in response to applied pheromonotropic peptide (TKYFSPRL amide) (Fonagy *et al.,* 2000).

From these *in vitro* studies the delineation of pheromonotropic activity to the intersegmental membranes is clear. We can conclude, therefore, that the intersegmental membranes, which are situated between the eighth and ninth abdominal segments of the moth, represent a target tissue for PBAN in the majority of insect species studied to date and presumably are the tissues that contain putative receptors for PBAN. It should be noted, however, that the functional diversity of this peptide family suggests that there may be several sites of action (Nassel, 1993).

3. Identification of Receptor Proteins

Conclusive proof of the direct action of PBAN on the intersegmental membrane as the tissue responsible for pheromone biosynthesis awaits demonstration of specific binding of PBAN to specific receptor proteins present in these intersegmental membrane tissues.

To study the mode of action of PBAN at the receptor level and for subsequent receptor purification, we synthesized a biologically active photoaffinity-biotinylated

PBAN analog (*N*-[*N*-azido-tetrafluorobenzoyl] biocytinyloxy1-succinimide-PBAN) (Atf-Bct-NHS-PBAN) (Rafaeli and Gileadi, 1997, 1999a). The biological activity of purified Atf-Bct-NHS-PBAN was confirmed using both *in vivo* and *in vitro* pheromonotropic bioassays. In addition, Atf-Bct-NHS-PBAN stimulated adenylate cyclase activity and elevated cellular cAMP levels in a manner similar to those in response to Hez-PBAN (see Section IV.A.4). These observations indicate that Atf-Bct-NHS-PBAN is a full agonist of PBAN action in pheromone glands and may be used to study PBAN receptors by employing avidin coupled to various reporter groups. Using crude membrane preparations of the pheromone glands we demonstrated binding of this biotinyl photoaffinity PBAN ligand to a protein in the 50-kDa range. This protein was successfully displaced in the presence of saturating levels (1000-fold) of synthetic Hez-PBAN (Rafaeli and Gileadi, 1997).

A similar strategy was employed for the identification of possible PBAN receptors in *H. zea* (Elliott *et al.,* 1997) in which a photoaffinity label with a tritium tag for visualizing PBAN-binding proteins and/or receptors was utilized. The photoaffinity label consisted of synthetic Hez-PBAN derivatized on lysine^{27} with benzoyldihydrocinnamic acid using *N*-hydroxysuccinimide ester (BzDC-NHS) as a cross-linker. In addition to pheromone glands, various tissues were prepared from *H. zea,* including Br-SOG, VNC, and thoracic muscles. Both the supernatant and pellet fractions were analyzed for competitive photoaffinity labeling. Two proteins with molecular weights of approximately 100 and 115 kDa were labeled by [^{3}H]BzDC-PBAN and displaced by excess unlabeled PBAN, indicating competitive binding. Labeling of these two proteins was decreased by 80% in the presence of 1000-fold excess unlabeled PBAN. These proteins were labeled in the supernatant of Br-SOG, VNC, and thoracic muscles (thoracic muscles had the highest abundance) and were found to a lesser extent in the 100,000*g* pellet fractions. The signal in pheromone glands prepared from *H. zea,* however, was very weak, which indicates that these proteins may be present in pheromone glands but at a much lower concentration (R. Jurenka, personal communication). However, neither protein was found in gut or ovarian tissue. The presence of these proteins in the cytoplasm of neural and muscular tissue may indicate that the 100- and 115-kDa proteins are indeed PBAN receptors that have been internalized by receptor-mediated endocytosis following binding to PBAN. Alternatively, they may be binding proteins that are involved in transport of peptides within cells.

A binding assay was developed in *H. peltigera* but only low-affinity binding was observed in the micromolar range (5×10^{-6} *M*) (Altstein *et al.,* 1999b). The binding characteristics of a hormone should parallel its biological activity such that the concentration of hormone required for half-maximal binding is within the normal physiological range of the concentration of the circulating hormone (Goldsworthy *et al.,* 1981). PBAN levels in the hemolymph have been reported to be at the lower concentration of 5–10 fmol/female (Fabrias *et al.,* 1994; Jacquin *et al.,* 1994; see Section III.C). If we assume a hemolymph volume of 50 μl (estimation from volume determinations in *H. zea;* Raina *et al.,* 1994b), this concentration is equivalent

to 0.2×10^{-9} *M* (n*M* range). In addition, maximal responses in an *in vitro* preparation are obtained using 5×10^{-9} *M* Hez-PBAN (0.05 pmol/10 μl/intersegment; A. Rafaeli, personal observations). In general, binding sites vary in their affinity for a hormone, and often two populations of such sites exist—a small number of high-affinity sites and a larger number with low-affinity for the hormone (Goldworthy *et al.,* 1981). We and others have hypothesized that binding sites for Hez-PBAN may also fall into these categories (Raina and Kempe, 1992; Rafaeli, 1994; Rafaeli and Gileadi, 1996). The physiological significance of such an arrangement is not completely understood. Perhaps the low-affinity binding sites represent nonspecific binding and are not true receptors; their function may be to maintain a local high concentration of hormone similar to that of the "true" receptors. It is therefore important that we discriminate between likely physiological effects and those which are pharmacological (require hormone titers beyond the physiological range). Notwithstanding, it does mean that high-affinity receptors are yet to be characterized for PBAN.

4. Second Messenger Mediation

A specific receptor (i) receives a signal (of both neural and hormonal origin) on an extracellular domain, (ii) transfers and amplifies the message across the cell membrane by way of second messengers, and (iii) affects a biochemical pathway through the action of enzymes and their products. This signaling process occurs in most cell types, and each cell can have its own unique combination of these signaling mechanisms.

Research concerning the cellular events which occur in the pheromone glands as a result of PBAN stimulation have revealed the involvement of calcium and 3′,5′ cyclic-AMP (cAMP) as second messengers (Rafaeli and Soroker, 1989b; Jurenka *et al.,* 1991, 1994; Fonagy *et al.,* 1992c, 1999; Rafaeli, 1994; Matsumoto *et al.,* 1995; Ma and Roelofs, 1995b) (Table V).

Evidence for the involvement of cAMP in pheromonotropic activity of *H. armigera, H. zea,* and *H. virescens* derived from several sources. Pharmacological compounds, such as cAMP analogs, forskolin (an adenylate cyclase activator), and isobutyl methyl xanthine (IBMX; a phosphodiesterase inhibitor), all stimulated *in vitro* pheromone biosynthesis (Rafaeli and Soroker, 1989b; Rafaeli *et al.,* 1990; Jurenka *et al.,* 1991; Jurenka, 1996). In addition, intracellular cAMP levels were elevated as a result of PBAN stimulation (Rafaeli and Soroker, 1989b; Rafaeli *et al.,* 1990). On the other hand, various cyclic nucleotide analogs and IBMX failed to mimic the *in vitro* pheromonotropic action of PBAN in isolated pheromone glands of *B. mori* (Fonagy *et al.,* 1992c) and *O. nubilalis* (Ma and Roelofs, 1995b). However, in *B. mori* these cAMP analogs did induce pheromone production in decapitated females. Additionally, IBMX potentiated the pheromonotropic action of Bom-PBAN or calcium ionophore on isolated pheromone glands (Fonagy *et al.,* 1992c), but this was not apparent in isolated pheromone glands of *O. nubilalis*

(Ma and Roelofs, 1995b). The reason for the failure for cAMP analogs to mimic pheromone production *in vitro* in the previously mentioned systems is unclear. It may be due to species variation, but it may also be due to different incubation conditions for the pheromone gland. Future determinations of intracellular cAMP levels as a result of PBAN stimulation in these insects may clarify this anomaly.

Extracellular calcium is essential for pheromonotropic activity (Jurenka *et al.,* 1991, 1994; Fonagy *et al.,* 1992c, 1999; Rafaeli, 1994; Ma and Roelofs, 1995b; Matsumoto *et al.,* 1995). Moreover, omission of calcium from the incubation medium completely abolished the PBAN-induced increase in intracellular cAMP levels (Soroker and Rafaeli, 1995). The inorganic calcium channel blockers lanthanum, cobalt, nickel, and, to a lesser extent, manganese were shown to inhibit the production of pheromone in response to PBAN in the presence of extracellular calcium (Jurenka *et al.,* 1991, 1994). The voltage-gated calcium channel blockers verapamil and nifedipine did not inhibit PBAN stimulation, but the organic calcium channel blocker SKF-96365 (a voltage- and receptor-activated calcium channel blocker) was shown to inhibit pheromonotropic stimulation in *H. virescens* (Jurenka, 1996). These results indicate that a receptor-activated calcium channel is involved in the pheromonotropic response. Additionally, calcium release as a result of ionophores (A23187, ionomycin, and thapsigargin) duplicated the pheromonotropic effect of PBAN on pheromone production (Jurenka *et al.,* 1991, 1994; Fonagy *et al.,* 1992c; Soroker and Rafaeli, 1995; Rafaeli and Gileadi, 1996) and intracellular cAMP synthesis (Soroker and Rafaeli, 1995; Rafaeli and Gileadi, 1996). This confirmed that calcium is involved in pheromone biosynthesis and that there is a cross talk between the two messengers since the increase in calcium, as a result of the ionophores, induced the increase in cAMP. Stimulation of pheromone biosynthesis by cAMP analogs was shown to be independent of calcium (Rafaeli and Soroker, 1995). This reinforces the hypothesis that cAMP stimulation occurs downstream of the calcium influx (Jurenka *et al.,* 1991; Rafaeli and Soroker, 1995) (Fig. 1; see color insert).

It is not clear whether the calcium channels activated by PBAN open as a result of the action of inositol 1,4,5-triphosphate (IP_3), as is the case in many cell types (Berridge, 1984, 1993). IP_3 and diacylglycerol (DAG) are generated through the action of phospholipase C (PLC) on the lipid precursor phosphatidylinositol 4,5-bisphosphate (Berridge, 1984, 1993). DAG, when stimulated by PLC, stimulates protein kinase C (PKC). PLC can be stimulated directly by agonist-bound receptors or through a G protein (Berridge, 1993). The role of IP_3 and DAG in the pheromone gland is unclear. Lithium chloride, which inhibits the recycling of inositol phosphate, caused a reduction in the pheromonotropic action of PBAN in *H. armigera* (Rafaeli, 1994). In addition, PKC may play a role in the signal transduction pathway in *H. armigera* since phorbol-12-myristate 13-acetate, a PKC activator, also stimulated pheromone biosynthesis in *H. armigera* (Soroker and Rafaeli, 1995). However, this could not be reproduced in *S. litura* (Matsumoto *et al.,* 1995) and has yet to be demonstrated in other insect species. The specific

TABLE V

Effect of Pharmacological Agents Affecting Signal Transduction on the Pheromonotropic Action of PBAN[a]

Compound	Pharmacological action	Effect on pheromone production *in vitro*[b]						
		H. armigera[1]	*H. zea*[2]	*H. virescens*[7]	*A. velutinana*[4]	*B. mori*[3]	*S. litura*[5]	*O. nubilalis*[6]
Forskolin	Adenylate cyclase activator	⇑	?	?	?	—	—	?
Forskolin + ionophore	Enhancement of ligand	?	?	?	?	⇑⇑	?	?
IBMX	Phosphodiesterase inhibitor	⇑	?	?	?	—	—	?
IBMX + PBAN IBMX + ionophore	Enhancement of ligand	?	?	?	?	⇑⇑	?	?
8-Br-cAMP dbcAMP SpcAMP	cAMP analogs	⇑	⇑	⇑	⇑	—	—	—
8-Br-cGMP	cGMP analog	—	?	?	?	—	?	?
RpcAMP	cAMP antagonist	—	?	?	?	?	?	?
H8, H89	PKA inhibitors	—	?	?	?	—	?	?
Staurosporine	PKC inhibitor	—	?	?	?	—	?	?
PMA	PKC activator	⇑	?	?	?	—	—	?
OAG	PKC activator	⇑	?	?	?	?	?	?
A23187 Ionomycin thapsigargin	Calcium ionophores	⇑	⇑	⇑	⇑	⇑	⇑	⇑
Verapamil nifedipine	Voltage-gated channel blockers	?	—	—	?	?	?	?
Lanthanium, cobalt, nickel	Calcium channel blockers	?	⇓	⇓	⇓	⇓	⇓	⇓
SKF-96365	Voltage & Receptor channel blocker	?	⇓	⇓	?	?	?	?

Lithium chloride	1,3,4-IP_3 (pathway) inhibitor	⇓	?	?	?	?	?	?
Compound 48/80	PLC inhibitor	?	?	?	?	—	?	?
W12, W7	CaM blockers	⇓	?	?	?	⇓	?	?
Calmidazolium	CaM blocker	—	?	?	?	?	?	?
Trifluoperazine	CaM blocker	?	?	?	?	⇓	⇓	?
KN-62	CaM-dependent protein kinase II	?	?	?	?	—	?	?
pNPP	General phosphatase inhibitor	?	⇓	⇓	?	⇓	⇓	?
NaF $\geq$ 10 m*M*	General phosphatase inhibitor	⇓	?	?	?	⇓	⇓	?
NaF 1–2 m*M*	G-protein activator	⇑	?	?	?	?	?	?
Okadaic acid Calyculin	Specific phosphatase 2 inhibitors	?	?	?	?	—	?	?
Cyclosporin A FK 506	Specific phosphatase 2B inhibitors	?	?	?	?	⇓	?	?

[a]Abbreviations used: ⇑, stimulation; ⇓, inhibition; ?, not tested; ⇑⇑, enhancement;—, no effect; A23187, calcimycin; 8-Br-cAMP, 8 bromo adenosine-3′,5′-cyclic monophosphate; dbcAMP, dibutyryl adenosine-3′,5′-cyclic monophosphate; SpcAMP, Sp isomer adenosine-3′,5′-cyclic monophosphothionate; 8-Br-cGMP, 8 bromo adenosine-3′,5′-cyclic monophosphate; RpcAMP, Rp isomer adenosine-3′,5′-cyclic monophosphothionate; CaM, calmodulin; Compound 48/80, poly-*p*-methoxyphenethylmethlamine; FK506, tacrolimus; H8, *N*-[2-methylamino)ethyl]-5-isoquinoline sulfonamide; H89, *N*-[2-((*p*-bromocinnamyl) amino)ethyl]-5-isoquinoline sulfonamide; IBMX, isobutylmethyl xanthine; IP_3, inositol 1,4,5-triphosphate; KN-62, (*S*)-5-isoquinoline sulfonic acid; NaF, sodium fluoride; pNPP, *p*-nitrophenyl phosphate; OAG, 1,2,-dioleolyl *sn* glycerol; PMA, phorbol-12-myristate 13-acetate; SKF-96365, 1-[β-[3-(4-methoxy-phenyl) propoxy]-4-methoxyphenethyl]-1*H*-imidazole; W12, *N*-(4-aminobutyl)-2-naphthalene sulfonamide; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide.

[b]References: [1]Rafaeli and Soroker (1989, 1993), Rafaeli (1994), Soroker and Rafaeli (1995), Rafaeli and Gileadi (1996); [2]Jurenka *et al.* (1991), Jurenka (1996); [3]Fonagy *et al.* (1992c); [4]Jurenka *et al.* (1994); [5]Matsumoto *et al.* (1995); Fonagy *et al.* (1999); [6]Ma and Roelofs (1995b); [7]Jurenka (1996).

inhibitor of PLC, compound 48/80, also failed to inhibit the pheromonotropic effect in *B. mori* (Matsumoto *et al.,* 1995).

In *H. armigera,* cAMP plays an important role in pheromonotropic action only when the tissues are challenged with low (0.1–0.5 pmol/10 μl/pheromone gland), physiological levels of PBAN. This was concluded from the fact that inhibition of the cAMP pathway using aminergic compounds was ineffective at higher levels of PBAN (10 pmol/10 μl/pheromone gland (see Section IV.C.1) Rafaeli and Gileadi, 1996). We therefore hypothesize that the PBAN receptor has two affinity sites responding to high and low levels of PBAN. Both high and low levels initiate the intracellular influx of calcium through the opening of the calcium channel, which in turn activates adenylate cyclase initiating a cAMP cascade. Aminergic inhibition of the cAMP pathway can be overcome by using the calcium pathway alone at higher PBAN concentrations. A similar phenomenon was observed using ionophore-induced pheromonotropic activity such that although adenylate cyclase activity was inhibited, the pheromone stimulatory action was unaffected (Rafaeli and Gileadi, 1996). Demonstration that cAMP is involved in the signal transduction of pheromone biosynthesis indicates that a cAMP-dependent protein kinase is involved that will phosphorylate key enzymes in the biosynthetic pathway. The use of pharmacological inhibitors of cAMP-dependent protein kinases, however, failed to inhibit pheromonotropic activity of PBAN in both *H. armigera* and *B. mori* (Soroker and Rafaeli, 1995; Matsumoto *et al.,* 1995).

It is suggested that free calcium, entering the cell, binds to calmodulin to form a complex, thereby activating adenylate cyclase and/or phosphoprotein phosphatases. Calmodulin (CaM) is a multifunctional calcium-binding protein that mediates the effects of calcium on numerous enzymes and intracellular processes. It is neither tissue specific nor very species specific and its structural and functional properties have been highly conserved (Klee *et al.,* 1980). CaM was recently characterized from pheromone glands of *B. mori* (Iwanaga *et al.,* 1998), and it was revealed that CaM has an identical amino acid sequence to that of *Drosophila* CaM (Smith *et al.,* 1987). CaM inhibitors *N*-(4-aminobutyl)-2-naphtalene sulfonamide (W-12), *N*-(6-aminohexyl)-5-chloro-1-naphtalene sulfonamide (W-7), and trifluoperazine inhibited the expected increases observed with PBAN in both *H. armigera* and *B. mori* (Soroker and Rafaeli, 1995; Matsumoto *et al.,* 1995).

The phosphoprotein phosphatase inhibitors sodium fluoride (NaF) and *p*-nitrophenyl phosphate (pNPP) also inhibited *in vitro* pheromone biosynthesis in *B. mori* (Matsumoto *et al.,* 1995). pNPP also inhibited pheromone production *in vitro* in *H. zea* and *H. virescens* (Jurenka, 1996). However, both pNPP and NaF are general phosphatase inhibitors. In fact, pNPP is a general substrate for phosphatases, whereas NaF is a general inhibitor when administered at high concentrations ($\geq$10 m*M*). On the other hand, NaF at lower concentrations (1–2 m*M*) activates adenylate cyclase by stimulating the enzyme through a G protein (Howlett *et al.,* 1979) by its action on the G_α subunit (Northup *et al.,* 1983). In *H. armigera,* NaF, when tested at 10 m*M,* also inhibited pheromone production *in vitro,* but at lower concentrations of 1 or 2 m*M* it stimulated both pheromone production

and intracellular cAMP levels, thereby implicating the involvement of G proteins in pheromonotropic activity (Rafaeli and Gileadi, 1996). The G protein involved in pheromonotropic activity is not cholera toxin sensitive (G_s) (as identified in vertebrate systems) since cholera toxin did not affect pheromonotropic activity (Rafaeli and Gileadi, 1996). The more specific inhibitors of phosphoprotein phosphatase I and 2A (okadaic acid and calyculin, respectively) failed to suppress pheromone production in *B. mori* (Matsumoto *et al.,* 1995); however, the specific inhibitors of phosphoprotein phosphatase 2B (cyclosporin A and FK 506) exerted an inhibitory influence on pheromonotropic activity (Fonagy *et al.,* 1999), thus implicating phosphatase 2B in the regulation of pheromonotropic activity. In *H. zea,* pNPP also inhibited pheromone production in response to the calcium ionophore A23187 and the cAMP analog 8-Br-cAMP, indicating that the phosphatases occur downstream in the transduction pathway from the actions of calcium and cAMP (Jurenka, 1996) (Fig. 1).

5. Influence on the Pheromone Biosynthetic Pathway

The sex pheromones used by nocturnal Lepidoptera are composed of blends of aliphatic molecules that vary in chain length, geometry, degree and position of double bonds, and functional groups. The pheromone blends comprise structurally related compounds formed by various actions of specific Δ-desaturases and coupled to chain shortening or elongation, reduction, acetylation, and oxidation of a common fatty acyl precursor. The biosynthetic pathway has been determined in many moth species (Bjostad and Roelofs, 1981, 1983; Morse and Meighen, 1987; Soroker and Rafaeli, 1989; Rafaeli *et al.,* 1990, 1993; Jurenka *et al.,* 1991; Jurenka, 1996; Tillman *et al.,* 1999) utilizing labeled precursor fatty acids in combination with the identification of possible fatty acid intermediates. These studies have demonstrated the *de novo* synthesis of the sex pheromones of moths from acetate both *in vivo* and *in vitro*. Generally, biosynthesis commences with the production of palmitic (16:acid) or stearic (18:acid) acids from acetyl-CoA involving acetyl-CoA carboxylase and fatty acid synthetase (Fig. 2). The production of fatty acid analogs is followed by double-bond positioning as a result of the action of unique desaturases to make mono- and diunsaturated fatty acids (Δ-5; Foster and Roelofs, 1996; Δ-9, Löfstedt and Bengtsson, 1998; Δ-10; Foster and Roelofs, 1988; Δ-11, Roelofs and Bjostad, 1984; Δ-12, Jurenka, 1997; Δ-13, Arsequell *et al.,* 1990; and Δ-14, Zhao *et al.,* 1990). Subsequent chain shortening through specific chain-shortening enzymes results in the correct chain length fatty acid, and depending on the functional group of the pheromone, a reductase, an acetyltransferase, or an oxidase will produce the final pheromone blend. In certain moths pheromone gland desaturases give rise to (E) fatty acids for the production of insect-specific pheromone blends (Bjostad *et al.,* 1980; Zhao *et al.,* 1990; Navarro *et al.,* 1997; Jurenka, 1997). Recently, the isolation of a cDNA encoding a pheromone gland-specific Δ-11 desaturase was reported in the cabbage looper moth (*T. ni*) with a predicted molecular mass of 40,240 Da (Knipple *et al.,* 1998).

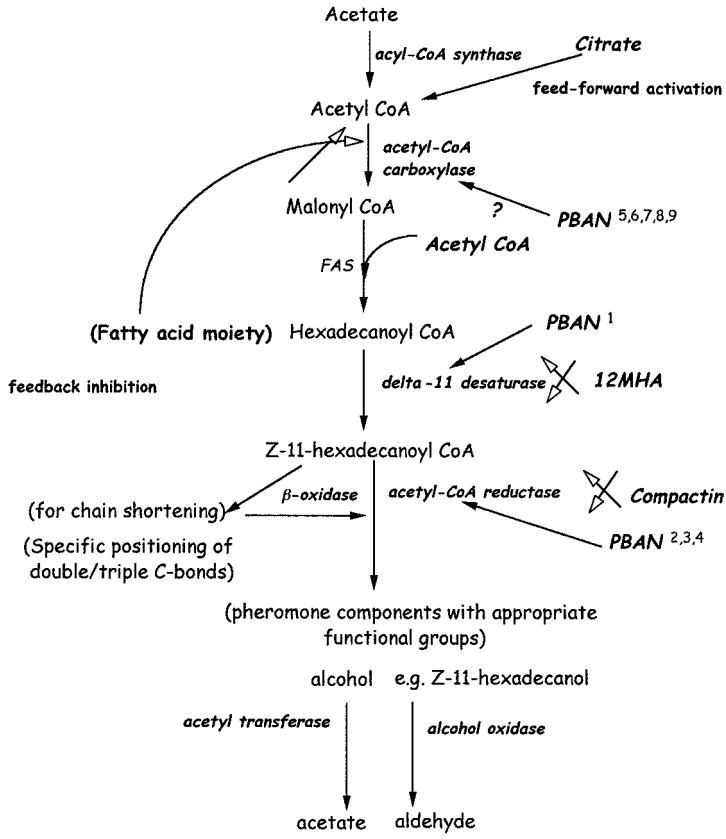

FIG. 2 Generalized scheme of the biosynthetic pathway involved in moth pheromone production. Crossed arrows indicate inhibitory influence. [1]Bestmann *et al.* (1989), [2]Gosalbo *et al.* (1992), [3]Fabrias *et al.* (1994), [4]Fonagy *et al.* (1999), [5]Tang *et al.* (1989), [6]Jurenka *et al.* (1991), [7]Jurenka (1997), [8]Jacquin *et al.* (1994), [9]Zhu *et al.* (1995).

Two acyl-CoA desaturases clones were also isolated from pheromone gland cDNA of *B. mori* showing 61% identity to *T. ni* Δ-11 desaturase (Yoshiga *et al.*, 2000).

The effect of PBAN on the different steps in the biosynthetic pathway has been investigated in several lepidopteran species but conclusive evidence of a unifying mode of action for PBAN has not been established. It was reported that Δ-11 desaturase was under the control of PBAN in *M. brassica* (Bestmann *et al.*, 1989), but this was refuted by Jacquin *et al.* (1994), who showed that PBAN had no effect on Δ-11 desaturation in this moth. In the processionary moth, *Thaumetopoea pityocampa,* it was also shown that PBAN had no effect on Δ-11 desaturase since treatment with the Δ-11 desaturase inhibitor 12-MHA (12,13-methylenehexadec-12-enoic acid) did not affect pheromonotropic activity (Arsequell *et al.*, 1989;

Gosalbo *et al.,* 1992). In this study, using a labeled enzyme intermediate precursor of the pheromone of *T. pityocampa* in combination with 12-MHA treatment, it was shown that PBAN affects the reduction step to the alcohol precursor. It was also shown that acetylation of the alcohol precursor was unaffected by PBAN. Two other studies using *S. littoralis* (Martinez *et al.,* 1990; Fabrias *et al.,* 1994) and *B. mori* (Arima *et al.,* 1991; Osawa *et al.,* 1995; Fonagy *et al.,* 1999) also suggested that reduction of the fatty acyl moiety to alcohol is controlled by PBAN. In *A. velutinana* (Tang *et al.,* 1989), *H. zea* (Jurenka *et al.,* 1991), *Cadra cautella, S. exigua* (Jurenka, 1997), and *M. brassicae* (Jacquin *et al.,* 1994), it was demonstrated that PBAN controls pheromone biosynthesis by regulating a step in or a step prior to fatty acid biosynthesis. Additionally, in *A. segatum* (Zhu *et al.,* 1995) studies suggested that PBAN has no effect on the final biosynthetic steps, including reduction and acetylation. On the other hand, PBAN did have a positive effect on the production of pheromone components from exogenously applied fatty acids in this species. This implies that PBAN affects pheromone production at the beginning of the biosynthetic pathway from precursors in the form of fatty acids or the respective Z-11–16 moieties as suggested by Jurenka *et al.* (1991).

Once the intermediates are formed, they continue their course in the biosynthetic pathway to produce the end products. That is, once PBAN has activated a rate-limiting enzyme in the pathway, other enzymatic reactions continue to the formation of the pheromone component(s). From the available data, it appears that the rate-limiting step for pheromonotropic peptides in moths may be either the biosynthesis of fatty acids or the reduction of fatty acids, or it may have a dual effect on different enzymes.

B. Mediators in Pheromonotropic Activity

The response of a target tissue to a given concentration of a hormone may be subject to considerable control by other factors, such as the stage of somatic and/or sexual development and differentiation, the time of day (or month or year), and previous endocrine experience. There are many mechanisms by which target responses could be modulated. The rate of excretion or inactivation of the hormones, the presence or absence of receptors for particular hormones, changes in the number of receptors, interaction with other hormones, neurohormones, or neurotransmitters, or the mechanisms of second messenger action can all play a role in modulation and fine-tuning of the tissue's responses.

1. Nervous System

The role of the nervous system in pheromone biosynthesis in moths is not clearly understood. The widespread distribution of pheromonotropic peptides, as discussed previously, suggests that they may be mobilized from both central and

peripheral release sites. In several moths, including *L. dispar* (Tang *et al.,* 1987; Thyagaraja and Raina, 1994), *H. virescens* (Christensen *et al.,* 1991), *S. littoralis* (Marco *et al.,* 1996), and *M. brassicae* (Iglesias *et al.,* 1998), an intact VNC was reported to be necessary for pheromone biosynthesis in intact moths. On the other hand, as discussed in Section IV.A.2, in many species, even some of those mentioned previously, isolated pheromone glands responded to locally applied synthetic PBAN in an *in vitro* culture.

Christensen and coworkers (1991, 1992, 1994; Christensen and Hildebrand, 1995) proposed that the biogenic monoamine, octopamine, acting as neurotransmitter may be involved as an intermediate messenger during the stimulation of sex pheromone production in *H. virescens*. Electrical stimulation of the connectives anterior to the TAG or nerves posterior to the TAG resulted in the stimulation of pheromone biosynthesis (Christensen *et al.,* 1991). Moreover, using a male electroantennogram bioassay to monitor the pheromone released into the air, it was demonstrated that electrical stimulation of the VNC leads to release of pheromone (Christensen *et al.,* 1994). This response was eliminated if the terminal nerves were severed, indicating that the neural connections between the TAG and pheromone gland are essential for pheromone release. Octopamine stimulated pheromone production when injected into intact females during midphotophase. In addition, only at the onset of scotophase did both octopamine and PBAN stimulate pheromone biosynthesis in the absence of the VNC (Christensen *et al.,* 1991). The effects of octopamine were only evident at a critical period that coincided with peak levels of this amine in the tissue (Christensen *et al.,* 1992). These workers suggested that octopamine is involved in the regulation of pheromone production and that PBAN's role lies in the stimulation of octopamine release at nerve endings. They showed that some neurons in the TAG are depolarized and activated by bath application of PBAN (Christensen and Hildebrand, 1995) but did not demonstrate a direct effect on octopamine levels or release. However, contradicting results concerning VNC transection and octopamine-stimulated pheromone production were obtained for the same species as well as for other moth species (Ramaswamy *et al.,* 1995; Rafaeli *et al.,* 1996; Park and Ramaswamy, 1998; Deslisle *et al.,* 1999). Ramaswamy and coworkers (1995) showed that PBAN did not significantly affect octopamine levels in either the TAG or the pheromone glands. A modulatory role for octopamine is nonetheless suggested by our research on *H. armigera,* although our investigations determined that octopamine exerts inhibitory effects (pheromonostatic) on the pheromonotropic action of PBAN (see Section IV.C.1).

2. Bursal Factors

The mediation by a bursal factor was hypothesized in the redbanded leafroller moth, *A. velutinana*. In intact moths the involvement of PBAN was established; however, isolated pheromone glands responded to a lesser degree to exogenously applied PBAN than did abdomen cultures (Jurenka *et al.,* 1991). Subsequent examinations

revealed that the bursa copulatrix was essential for a full stimulatory response to PBAN and an aqueous extract of bursa copulatrix recovered full pheromonotropic activity *in vitro*. The involvement of a bursal factor in pheromonotropic activity has been reported only in *A. velutinana* and the related tortricids *Choristoneura fumiferana* and *C. rosaceana* (Delisle *et al.,* 1999). On the other hand, in the related tortricid moths *Epiphyas postvittana* and *Planotortrix octo* the bursa copulatrix does not appear to contain a factor that stimulates pheromone production or to enhance the PBAN-stimulated response (Foster and Roelofs, 1994). In addition, in the noctuid moth *H. armigera,* removal of the bursa from abdomen cultures did not affect the pheromonotropic activity of PBAN (Rafaeli, 1994). Future identification of the bursal factor will contribute greatly to the complete understanding of its role as mediator in pheromone biosynthesis control.

3. Juvenile Hormone

Juvenile hormones (JHs), produced in the corpora allata, are important regulators of insect development and diapause. In adults they play a key role in the control of reproductive endocrinology (Ramaswamy *et al.,* 1997). Studies involving the migratory moths *P. unipuncta* and *A. ipsilon* have shown that JH is also involved in the regulation of female sex pheromone production and its release (Cusson and McNeil, 1989; Gadenne, 1993; Picimbon *et al.,* 1995). Since migration is often associated with adult diapause an adaptive strategy would be to arrest not only reproduction but also reproductive behavior. In *P. unipuncta* it was shown that a low JH production level, which occurs early in the adult life (pre-reproductive period), coincides with the absence of both calling behavior and pheromone production. The subsequent increase in the rates of JH biosynthesis correlates with the onset of pheromone production and release (Cusson *et al.,* 1994). In both insects, studies revealed that JH was essential for pheromone biosynthesis and calling behavior since allatectomized females failed to produce pheromone or call (Cusson *et al.,* 1994; Gadenne, 1993; Picimbon *et al.,* 1995). Treatment with fenoxycarb, a JH analog, induced earlier calling, mating, and oviposition by *A. ipsilon* females and caused recovery of ovarian maturation, calling, and mating in allatectomized females (Gadenne, 1993). These results show a direct intervention of JH in the initiation of calling and mating behaviors. These workers suggested that JH induced these behavioral changes by the induction of PBAN release from the CNS and not by controlling PBAN production or priming of PBAN target organs. However, no studies were performed on the competence of isolated pheromone glands to PBAN during the migratory and nonmigratory periods in these migratory species. Conversely, reproductive behavior was apparently unaffected by the removal of CA in other (but nonmigrant) species of female moths (Sasaki *et al.,* 1983; Hollander and Yin, 1985; Tang *et al.,* 1987; Park and Ramaswamy, 1998). On the other hand, in studies conducted on *P. stultana* (Webster and Carde, 1984) application of JH generated an inhibitory effect. Studies conducted in our laboratory on pharate adults

of *H. armigera,* however, showed a clear correlation between CA maturation and the competence of pheromone glands to the stimulatory action of PBAN (Fan *et al.,* 1999a). Moreover, the presence of JH II *in vitro* primed pheromone glands of pharate adults to respond to PBAN and induced earlier pheromone production by intact newly emerged females (Fan *et al.,* 1999b). These results indicate that JH is required for the primary induction of gland competence to PBAN stimulation, perhaps by upregulating gene expression of proteins required for pheromone production and/or PBAN receptor proteins. PBAN, on the other hand, functions as the circadian messenger. Research currently being conducted in our laboratory aims to answer some of these questions. It is thus obvious from the evidence that has accumulated to date that the JH effect can only be observed during a pre-reproductive period, be it during the pupal stage or during the reproductive diapause of migratory moths. Most of the research on pheromonotropic activity has been conducted on short-lived moths with short pre-reproductive periods. If the JH effect is only evident during a defined short window period, this may explain the lack of evidence in the other moths investigated.

C. Pheromonostatic Effects on PBAN Action

1. Photophase-Induced Inhibition of Pheromone Biosynthesis

Injections of PBAN to decapitated female moths causes a linear increase in the production of pheromone with time. On the other hand, when these injections are administered to intact females during the photophase hours, there is a linear increase in pheromone production for the first two or three hours followed by a significant decrease (Raina *et al.,* 1987; Matsumoto *et al.,* 1990; Teal *et al.,* 1993; Rafaeli *et al.,* 1997). This phenomenon led to our speculation that an endogenous inhibitory feedback for pheromone biosynthesis may be responsible for the modulation of the responsiveness of pheromone glands to PBAN during the photophase.

Subsequent studies on two moth species (*H. armigera* and *P. interpunctella*) suggested that octopamine plays an important role as a neuromodulator of the pheromonotropic action, causing significant inhibition of the response to PBAN (Rafaeli and Gileadi, 1995b; Rafaeli *et al.,* 1997, 1999; Rafaeli, 1995). Inhibition of pheromone biosynthesis was observed in intact moths during the photophase in the presence of synthetic PBAN. Inhibition was also evident during the scotophase on the endogenous pheromonotropic activity whereby treatment with octopamine prevented females from producing normal pheromone titers (Rafaeli *et al.,* 1997). *In vitro* studies showed that this inhibitory influence was limited to the intersegmental membrane. The inhibitory action of octopamine on the pheromone glands was evident on the production of the main pheromone component as well as the fatty acid fraction. This inhibition was due to a reduction in biosynthesis and not to an effect on release or degradation of the pheromone component (Rafaeli

and Gileadi, 1995), and it was reversible in the presence of aminergic antagonists, such as phentolamine, yohimbine, and chlorpromazine (Rafaeli and Gileadi, 1995b, 1996; Rafaeli *et al.,* 1997). The pheromonostatic action of octopamine was shown to be a result of its inhibitory effect on the second messenger transduction mechanism of the cell due to an interaction with a separate receptor. Since pertussis toxin removed the inhibitory influence of octopamine, this receptor may be a G-inhibitory-linked receptor (Rafaeli and Gileadi, 1996) (Fig. 1).

2. Mating-Induced Inhibition of Pheromone Biosynthesis

Males of several species of insects transfer substances to females during copulation that induce oviposition and inhibit receptivity to additional matings. Such substances have been reported in Diptera, Orthoptera, Lepidoptera, and Coleoptera (Gillot, 1988; Raina *et al.,* 1994a). Mating-induced termination of sex pheromone production has been studied in many moth species: *H. zea* (Raina, 1989; Teal *et al.,* 1989), *H. virescens* (Ramaswamy and Cohen, 1992), *L. dispar* (Giebultowiz *et al.,* 1991), *S. nonagriodes* (Bablis and Mazomenos, 1992b), *A. velutinana* (Jurenka *et al.,* 1993), *E. postvittana* (Foster, 1993), *B. mori* (Ando *et al.,* 1996), and *P. interpuctella* (Rafaeli and Gileadi, 1999b). In these studies, a permanent or transient decline of sex pheromone production after mating was observed and dependent on whether the species exhibited single or multiple matings (Shorey *et al.,* 1968; Webster and Carde, 1984; Raina *et al.,* 1986b, 1994a; Coffelt and Vick, 1987; Giebultowicz *et al.,* 1991; Foster, 1993; Rafaeli and Gileadi, 1995a, 1999b; Ando *et al.,* 1996). In addition to the termination of sex pheromone production, calling behavior ceases and oviposition behavior is initiated. The effect of mating on pheromone production may involve different mechanisms: mechanosensory events that occur during or after copulation, the presence of viable sperm in the spermathecae, or the transfer of pheromonostatic factors.

In *L. dispar* an initial transient suppression was found to be a result of the mechanical pressure during copulation, and permanent suppression resulted from an adequate supply of sperm in the spermatheca of the female (Giebultowicz *et al.,* 1991; Raina *et al.,* 1994a). Thus, it appears that in this species signals evoked by physical stimulation and transfer of sperm cause the termination of pheromone production through neural pathways. Similar observations were made from a study on *B. mori* in which the presence of fertile eupyrene spermatozoa was shown to be essential for accelerated oviposition (Karube and Kobayashi, 1999a,b). These studies did not include injections of male accessory gland extracts, so the inhibitory influences of humoral components cannot be ruled out.

In *H. zea* a pheromonostatic peptide (PSP) causing depletion of sex pheromone was found in extracts of male accessory glands (Raina and Stadelbacher, 1990). PSP was isolated and characterized from the accessory gland and duplex of male *H. zea* (Kingan *et al.,* 1995). The purified pheromonostatic peptide, when injected into virgin females, evoked the depletion of sex pheromone within 2 h. It was

characterized as a 57-amino acid chain containing a single disulfide bridge, blocked at the N terminus with pyroglutamate and amidated at the C terminus.

Early studies on the transfer of male-derived factors during copulation were performed on the fly, *Drosophila melanogaster* (Chen, 1984, 1991). In *D. melanogaster* postmating responses (suppression of female receptivity and increased oviposition/ovulation) in the female were shown to be induced by several components derived from the male reproductive system. These included a 36-amino acid peptide, sex peptide (SP), derived from the male accessory gland (Chen *et al.,* 1988; Kubli, 1996). A second 31-amino acid peptide, Dup99B, with a high degree of sequence homology to SP (Ottiger *et al.,* 2000), derived from the male ejaculatory duct. SP and Dup99B both induce female nonreceptivity and ovulation. In addition, SP was shown to activate adult *D. melanogaster* CA *in vitro* to produce JH (Moshitzky *et al.,* 1996) as well as uptake of vitellogenin by mature oocytes (Soller *et al.,* 1999). The third peptide, Acp28Aa (Herndon and Wolfner, 1995), stimulates oviposition during the first day of oviposition, and a fourth large glycoprotein, Acp36De, is hypothesized to participate in sperm storage (Wolfner, 1997). No sequence homology occurs between PSP (moth derived) and the peptides derived from male *D. melanogaster*. However, the synthesis of PSP has not been successfully accomplished. Recently, we studied the effect of synthetic *D. melanogaster* SP and Dup99B on both the activity of CA and pheromone biosynthesis in the moth *H. armigera* (Fan *et al.,* 1999a, 2000). *Drosophila melanogaster* SP was found to stimulate JH biosynthesis by *H. armigera* CA *in vitro* in a similar fashion to its action on *D. melanogaster* CA. In addition, SP inhibited pheromonotropic activity induced by PBAN in isolated pheromone glands and decapitated females as well as endogenous pheromonotropic activity in females during the scotophase. Structure–activity studies determined that the N terminus of SP is responsible for CA activation and that the C terminus is responsible for inhibition of pheromonotropic activity (pheromonostasis) (Fan *et al.,* 2000).

The relationship between activation of JH production and pheromonostasis is unclear. JH titers in *H. virescens* were shown to be stimulated during mating, resulting in an increase in egg production (Shu *et al.,* 1998). A similar increase in JH postmating was observed in the coding moth *Cydia pomonella* (Webb *et al.,* 1999) and in the torticid moths *C. fumiferana* and *C. rosaceana* (Cusson *et al.,* 1999). Application with JH or analogs did not inhibit pheromone production in the moth (Y. Fan, S. Applebaum and A. Rafaeli, unpublished observations) and did not mimic postmating events (Webb *et al.,* 1999). In contrast, in *P. stultana* (Webster and Carde, 1984), a JH analog inhibited pheromone production and was implicated in this postmating decline. Shirk *et al.* (1980) found that JH is stored in the accessory gland of adult male *H. cercopia* and is transferred to females during copulation. Similar observations showing high JH levels in the accessory glands of male *H. zea* were reported (Bhaskaran *et al.,* 1988). Park and coworkers (1998) speculated that JH stored in the accessory glands might be transferred by males to enhance fecundity and/or inhibit sexual receptivity in mated females. However, in the tortricid moths it was shown that the males displayed a very limited

ability to convert JH acid to JH and failed to produce JH *in vitro* or *in vivo* (Cusson *et al.,* 1999). Topical applications of JH analog to virgin females were demonstrated to induce early oviposition similar to that observed in mated females [*Ephestia cautella* (Shaaya *et al.,* 1991) *H. virescens* (Ramaswamy *et al.,* 1997), *T. ni* (Campero and Haynes, 1990), and *P. stultana* (Webster and Carde, 1984)] but had the opposite effect in the moth *C. pomonella* (Webb *et al.,* 1999). Mating is inhibited in *P. interpunctella* reared on a JH-supplemented diet as a result of a reduction in calling behavior (Oberlander *et al.,* 1975). However, SP injections in *H. armigera* virgin females during the scotophase, although inhibiting pheromone titers, did not inhibit calling behavior. Ecdysones were also implicated in the inhibition of sexual receptivity. Ramaswamy and Cohen (1992) reported a significant decrease in the pheromone titer of virgin *H. virescens* females treated with 1 μg 20-hydroxyecdysone. However, these observations could not be confirmed in *H. virescens* and *H. zea* (Teal *et al.,* 1990; Raina *et al.,* 1994a). The significance of ecdysones in suppression of receptivity is therefore unclear.

In *H. zea,* pheromonostasis after mating was also found to be dependent on an intact VNC. Females with a transected VNC before mating produced levels of pheromone similar to those of virgins (Kingan *et al.,* 1995), thus indicating that a neural signal is also important. It may well be that pheromonostasis occurs at two levels: humoral inactivation at the level of the target tissue and neural inactivation by inhibiting release of PBAN. Neural inactivation of pheromone production in mated females was confirmed in *A. velutinana* (Jurenka *et al.,* 1993), *E. postvittana* (Foster, 1993), *H. virescens* (Ramaswamy *et al.,* 1996), and *B. mori* (Ando *et al.,* 1996; Ichikawa *et al.,* 1996), in which a VNC-transected female still produced pheromone after mating. It is therefore hypothesized that the inactivation of pheromone biosynthesis is also induced by a neural signal, which travels up through the VNC from the abdomen to the head, thereby preventing release of PBAN (Kingan *et al.,* 1995; Ando *et al.,* 1996).

Studies on the effect of age on pheromone production indicated that older virgin females produce less pheromone than younger females (Raina *et al.,* 1986b). These older females also exhibit increased oviposition behavior (Teal *et al.,* 1990; Giebultowicz *et al.,* 1990), much like that observed after mating. This transience to a senescence type of behavior has been attributed to a humoral factor present in the bursa copulatrix, ovaries, and hemolymph. The nature of this factor has not been elucidated, but a similar factor was also detected in young mated females (Teal *et al.,* 1990) and is not a JH- or ecdysone-like factor. It is hypothesized that factors from both the males and the females may act in concert to inhibit pheromone production after mating, with the male factor causing a transient inhibition and the female factor maintaining low pheromone titers on subsequent nights (Teal *et al.,* 1990). The decline of pheromone production was attributed to a decline in fatty acid reductase activity as evidenced by a build up of precursor as the female ages (Foster and Greenwood, 1997).

Termination of sexual receptivity in moths can be regarded as a process consisting of several facets, including the local inhibition of pheromone biosynthesis,

breakdown of pheromone components, the inhibition of neuropeptide (PBAN) release and/or the tropic factor(s) controlling its release, inhibition of the regulatory process (neural) of calling behavior, accelerated production of eggs, accelerated ovulation of mature eggs, increased muscular movements of the genital tract for increased egg deposition (oviposition), and sperm storage control. It therefore seems feasible that suppression of mating behavior may be controlled by a combination of several regulators. Indeed, in *D. melanogaster* recent reports indicate that a variety of mating factors are involved in reproductive physiology and behavior (Wolfner, 1997).

V. Concluding Remarks

The regulatory processes involved in pheromone production have been studied in several moth species. The neuropeptide PBAN appears to be involved in the stimulation of pheromone production in the majority of moths that have been studied. It is a member of the pyrokinin/PBAN family of neuropeptides, widespread in several insect orders. Its members serve several diverse functions. Comparision of chemical structures of the pyrokinin/PBAN neuropeptide family indicates some common features and it is therefore not surprising that cross-reactivity has been revealed. This fact may also contribute to the confusion regarding the site of PBAN action, although much evidence has accumulated indicating its direct role on the pheromone-producing cells associated with the ovipositor tip of female moths. Normal activation of pheromone production may involve several interdependent mechanisms, including both neural and humoral modulators. These modulators may be differentially activated but closely coupled depending on physiological and environmental factors.

Future studies that address the molecular mechanisms of hormonal control will undoubtedly reveal receptor proteins for PBAN and the regulatory mechanisms of the genes that encode them. Elucidation of rate-limiting enzymes in the biosynthetic pathway and a deeper understanding of the second messengers involved will provide basic models to address the function and targeting of peptide hormones.

Future understanding of the modulatory mechanisms for hormonal control, their interactions, and their gene regulation will be important in understanding insect reproductory behavior and speciation and ultimately in designing ways to control the fertility of unwanted insect pests.

Acknowledgments

Work from the author's laboratory described in this review was funded by Binational Agricultural Research and Development Grants US-0784-84, IS-1645-89C, and IS-2978-98R; Israel National Academy of Science and Humanities Grants 281/88, 221/95-3, and 357/98-2; and DFG Tri-lateral

grant (Deutsche Forschungsgemeinschaft, Germany). I thank Carina Gileadi for being a devoted and dedicated research assistant, Mina Litvin for her great care of our insect culture, and all present and past graduate students, postdoctoral fellows, and collaborators. I extend my gratitude to Dr. Ashok Raina (Formosan Termite Research Unit, USDA, ARS, New Orleans, LA) for helpful comments on an earlier version of the manuscript and for allowing me to include some of his latest relevant findings.

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Gene Transfer to Salivary Glands

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This article provides a review of the application of gene transfer technology to studies of salivary glands. Salivary glands provide an uncommon target site for gene transfer but offer many experimental situations likely of interest to the cell biologist. The reader is provided with a concise overview of salivary biology, along with a general discussion of the strategies available for gene transfer to any tissue. In particular, adenoviral vectors have been useful for proof of concept studies with salivary glands. Several examples are given, using adenoviral-mediated gene transfer, for addressing both biological and clinical questions. Additionally, benefits and shortcomings affecting the utility of this technology are discussed.

KEY WORDS: Salivary glands, Gene transfer, Gene therapy, Gene therapeutics, Adenovirus, Biotechnology, Exocrine glands.

I. Introduction

Transferring genes to cells is not a new concept (Wolff and Lederberg, 1994a). Conceptually, the potential clinical and investigational applications of transferring genes have been recognized and discussed for at least 40 years. However, the rise of recombinant DNA technology has made this concept a fairly common reality. Certainly, the applications of gene transfer technology to address biological problems have changed the practice of everyday laboratory science. Research laboratories in virtually every biomedical science discipline use such common tools as gene cloning, the polymerase chain reaction, cell transfections and selections, etc. to address significant, specifically related, categorical questions.

Similarly, in the past decade the use of gene transfer for clinical purposes has markedly increased. Many bench laboratory studies have steadily progressed through the preclinical, animal model phase to reach the clinic. The number of

approved clinical protocols has grown considerably (Anonymous, 1999). Clinical gene transfer was initially considered for relatively few major organ target tissues and envisioned primarily as a tool to address severe, life-threatening disorders, particularly cancers that were refractory to all other treatments, or inherited monogenic disorders. However, recently, it has become clear that clinical applications of gene transfer extend well beyond these important initial concerns with severe mortal risk. Now, it is not at all unusual to consider using gene transfer for morbid conditions affecting quality of life issues and for acquired disorders. The only requirement common to all potential clinical applications of gene transfer is the lack of any effective conventional therapy.

Many tissues not considered as being appropriate targets a decade ago are now foci of active research with gene transfer tools. Salivary glands are one such tissue. Although salivary glands are subjected to significant pathology (e.g., irradiation damage during treatment for head and neck cancer and Sjogren's syndrome, an autoimmune exocrinopathy), most of the sequelae from these conditions are morbidities that are inconvenient, unpleasant, and uncomfortable but not fatal. Additionally, the transfer of somatic genes to salivary glands can also be used as a tool to better understand the mechanisms by which these glands form and secrete saliva. In this article, we provide a review of the reported applications of gene transfer to salivary glands, including strategies employed, examples of biological and clinical questions addressed, as well as important recognized problems limiting full application. Although there are now several laboratories employing gene transfer technology with salivary glands (Barka and Van der Noen, 1996; Baum and O'Connell, 1999; Goldfine *et al.,* 1997; Wang *et al.,* 1997), we mostly rely on studies conducted in our laboratory for the examples shown (Table I).

TABLE I
Types of Genes Transferred to Mammalian Salivary Glands *in Vivo*

Gene	Function	Reference
β-Galactosidase	Bacterial reporter gene	Mastrangeli *et al.* (1994)
Luciferase	Insect reporter gene	Zheng *et al.* (2000a)
Human α_1-antitrypsin	Protease inhibitor	Kagami *et al.* (1996)
Human kallikrein	Protease	Baum *et al.* (1999)
Human growth hormone	Endocrine hormone	X. He *et al.* (1998)
Human interleukin-10	Cytokine	Wang *et al.* (2000)
Human histatin 3	Anticandidal protein	O'Connell *et al.* (1996)
Human aquaporin 1	Membrane water channel	Delporte *et al.* (1997a)
Human E2F-1	Transcription factor	Lillibridge and O'Connell (1997)

II. Biology of the Salivary Glands

A. General Introduction

1. Saliva

The primary role of salivary glands is to secrete saliva. Saliva is essential for the maintenance of oral health (Dowd, 1999; Mandel, 1987, 1989). Although a lack of saliva is not life threatening, patients suffering from xerostomia (dry mouth) due to salivary hypofunction experience a serious decline in their quality of life due to several factors (see Section VI.A). Saliva is a complex mixture of proteins, electrolytes, and water. Saliva provides the principal protective fluid for the exposed tissues of the mouth, pharynx, and esophagus. Saliva has important roles in mucosal lubrication, tissue repair including enamel remineralization, gustation, facilitating speech, control of the oral microbial population (bacterial, viral, and fungal), and food bolus formation and translocation.

2. Secretion of Saliva

Individual major and minor salivary glands secrete saliva into the oral cavity, collectively forming whole or mixed saliva. In humans, ~0.75–1.5 liters of whole saliva is formed daily. Each of the major salivary glands (submandibular, parotid, and sublingual glands) exists in pairs, and together during stimulation the major gland secretions account for ~95% of the volume of whole saliva (Hand, 1980). Typically, this stimulation occurs during food consumption to facilitate deglutition. The remaining stimulated saliva is supplied by the many minor salivary glands that are found throughout the oral cavity. At other times, salivary secretion is considered to be unstimulated or resting. This resting saliva provides much of the protective functions noted previously. Minor salivary glands contribute up to 30% of the unstimulated saliva.

There is considerable structural diversity among the different major and minor salivary glands within a species and among homologous glands of different species (Pinkstaff, 1979; Tandler, 1978; Young and van Lennep, 1978). Anatomically, however, salivary glands can be divided into two regions—acinar (secretory endpiece) and ductal. Within each region different epithelial cell types can be found: Serous, mucous, and seromucous cells are located in the acinar region, and intercalated, striated, and excretory cells are located in the ductal region. Granular epithelial cells rich in growth factors are also found in the ducts of rat and mouse submandibular glands. Although the distribution of cell types among glands exhibits species variation, in most mammals the parotid gland is considered to be predominantly of the serous type (serous acinar cells), whereas the submandibular and sublingual glands are mixed (serous, seromucous, and mucous acinar cells). Minor glands are primarily mucous. Serous secretions are watery, whereas mucous

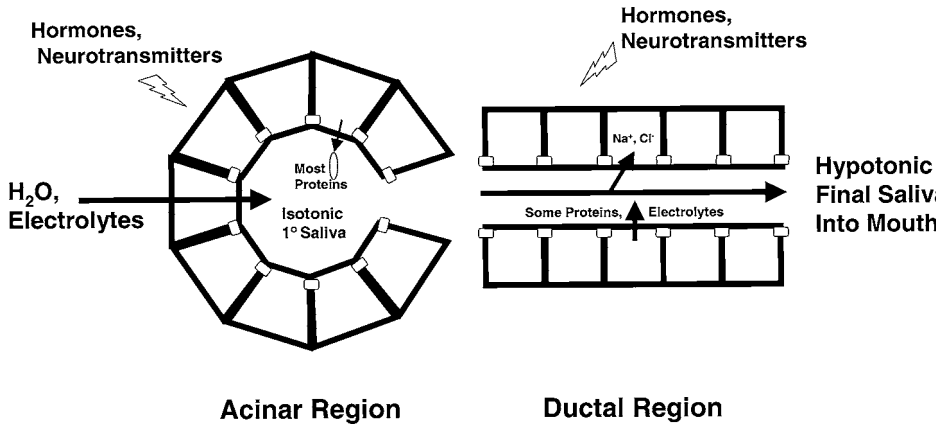

FIG. 1 Schematic depiction of the anatomical division of salivary glands into acinar and ductal regions (modified from Baum, 1993).

and seromucous secretions are more viscous due to their mucin content. In humans, minor salivary glands provide ~70% of the secreted mucous (Cook *et al.,* 1994).

The anatomical division of salivary glands into acinar and ductal regions reflects the well-established two-stage mechanism of saliva formation (Baum, 1993; Cook *et al.,* 1994; Nauntofte, 1992; Turner *et al.,* 1993; Turner, 1993a,b) (Fig. 1). During the first stage a primary saliva is formed by a neurotransmitter-stimulated secretion of electrolytes, water, and proteins into the lumen of salivary acini. Virtually all of the water and most of the exocrine proteins secreted into the oral cavity enter the gland at this stage. The electrolyte composition resembles plasma, and it is isotonic. During the second stage, the primary saliva is modified as it transits the ducts to the oral cavity. Ductal modifications include the considerable reabsorption of NaCl and a modest secretion of K^+, HCO_3^-, and some proteins into saliva. Little or no change in saliva volume occurs during ductal transit because the ducts are relatively impermeable to water. Thus, the final saliva is hypotonic with respect to plasma. The tonicity of the final saliva depends on the rate of saliva flow through the ducts, with faster flow rates leading to reduced NaCl reabsorption and thus increasing tonicity.

B. Ion Transport by Salivary Glands

Water flow into the lumena of salivary glands is driven by neurotransmitter-evoked osmotic gradients across acinar epithelia. The electrolyte composition of saliva is then modified by the ducts, primarily as a reabsorption of NaCl. Both processes are discussed later.

C. Generation of Osmotic Gradients across Acinar Cells

The mechanisms by which osmotic gradients are generated across acinar epithelial cells have been described previously (Cook *et al.,* 1994; Melvin, 1999; Nauntofte, 1992; Turner *et al.,* 1993; Turner, 1993a,b). These mechanisms employ ion transporters localized to either basolateral or apical membranes of acinar cells (Fig. 2). Three mechanisms are shown in Fig. 2 (Turner *et al.,* 1993; Turner, 1993a,b). All of the mechanisms can operate concurrently in the same gland and perhaps in the same cell. Mechanism 1 is more efficient than mechanisms 2 and 3. It is not clear why mechanisms with different efficiencies operate concurrently in the same gland. The first mechanism is discussed in detail. The other two mechanisms are discussed as variants of the first mechanism.

1. Mechanism 1

To generate an osmotic gradient capable of driving water transport, acinar epithelial cells bring about an elevation of NaCl in the lumen of salivary acini. The transport of NaCl into the acinar lumen is triggered by a neurotransmitter-evoked, Ca^{2+}-stimulated opening of basolateral K^+ channels and apical Cl^- channels in acinar cells. Ca^{2+} stimulation causes K^+ and Cl^- to flow down their electrochemical gradients and out of the cell to the interstitium and lumen, respectively. As a result, Cl^- accumulates in the acinar lumen. To preserve electrical neutrality, Na^+ moves to the lumen, presumably via a paracellular pathway from the interstitium via the tight junctions. Water then flows into the lumen in response to the accumulation of NaCl, resulting in primary (isotonic) saliva formation.

To provide for the Ca^{2+}-stimulated flow of K^+ and Cl^- out of the acinar cell, intracellular K^+ and Cl^- are accumulated above electrochemical equilibrium while the acinar cell is in a resting state (i.e., little or no neurotransmitter-evoked Ca^{2+} signal). This accumulation is a result of the actions of two basolateral ion transporters, the Na^+/K^+-ATPase and the Na^+–K^+–2 Cl^- cotransporter. The energy for this accumulation is provided by the hydrolysis of ATP. Thus, a basolateral Na^+/K^+-ATPase causes an accumulation of intracellular K^+ by coupling the extrusion of Na^+ with the uptake of K^+ via ATP hydrolysis. The stoichiometry is such that three Na^+ are extruded from the cell and two K^+ enter the cell for each molecule of ATP that is hydrolyzed. The Na^+–K^+–2 Cl^-cotransporter responds to the ATP-dependent loss of intracellular Na^+ by directing an uptake of three Na^+ for each ATP hydrolyzed. Because the stoichiometry of the cotransporter is 1 Na^+:1 K^+: 2 Cl^-, Six Cl^-, and three more K^+ also enter the cell for each molecule of ATP hydrolyzed.

The overall stoichiometry of the gradient across the acinar cell is such that hydrolysis of one ATP molecule provides for the secretion of six Cl^- into the acinar lumen. The Ca^{2+}-stimulated secretion of ions continues as long as the

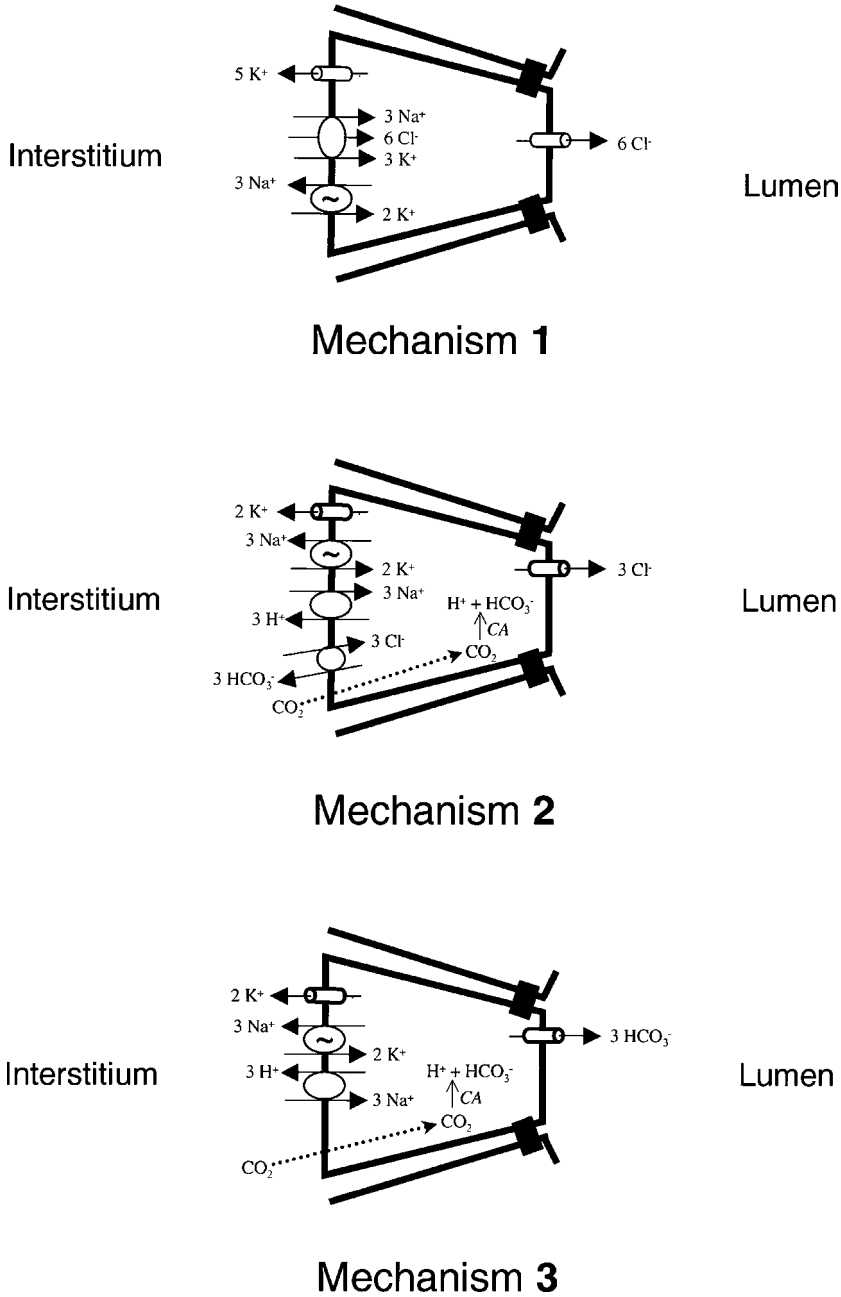

FIG. 2 Schematic depiction of the mechanisms by which osmotic gradients are generated across salivary acinar cells (modified from Turner, 1993b).

neurotransmitter stimulus is applied. When the Ca^{2+} stimulus ceases, however, the Cl^- and K^+ channels close, and the cell returns to the resting state. Of course, water flow follows ion secretion, and it also ceases when the channels close.

2. Mechanism 2

This mechanism of saliva formation is similar to mechanism 1. Thus, a neurotransmitter-evoked Ca^{2+} signal opens apical Cl^- channels and basolateral K^+ channels, Cl^- accumulates in the acinar lumen, Na^+ enters the lumen in response to the luminal Cl^-, and water enters the lumen in response to NaCl accumulation. ATP hydrolysis by a basolateral Na^+/K^+-ATPase again provides the energy for the accumulation of intracellular Cl^- and K^+ above electrochemical equilibrium. However, only three Cl^- are secreted into the acinar lumen per molecule of ATP hydrolyzed because the Na^+–K^+–2 Cl^- cotransporter does not participate in the intracellular accumulation of Cl^- and K^+. This secretory system is thus less efficient than that described in mechanism 1.

Accumulation of intracellular K^+ and Cl^- involves the participation of a basolateral Na^+/K^+-ATPase, basolateral Na^+/H^+ and Cl^-/HCO_3^- exchangers, and intracellular carbonic anhydrase (CA). In this mechanism, Ca^{2+}-stimulated opening of Cl^- channels and subsequent Cl^- release leads to an increased Cl^- uptake by the Cl^-/HCO_3^- exchanger. The accompanying cytosolic acidification due to HCO_3^- loss is buffered by the Na^+/H^+ exchanger, which extrudes H^+ using the Na^+ gradient generated by the Na^+/K^+-ATPase. CA replenishes the carbonic acid secreted in the process.

3. Mechanism 3

This mechanism differs from mechanism 1 because HCO_3^-, rather than Cl^-, is secreted into the acinar lumen. Thus, a neurotransmitter-evoked Ca^{2+} signal stimulates the opening of a basolateral K^+ channel and a HCO_3^- permeable apical channel (possibly the same channel as used by Cl^-). K^+ and HCO_3^- flow down their electrochemical gradients, emptying into the interstitial space and acinar lumen, respectively. The HCO_3^- is supplied by the CA conversion of CO_2 and water to carbonic acid. The H^+ thus generated is extruded by a basolateral Na^+/H^+ exchanger, again using the Na^+ gradient generated by the basolateral Na^+/K^+-ATPase.

D. Ductal Reabsorbtion of NaCl

During saliva formation, the ducts reabsorb NaCl by transporting the salt from the ductal lumen to the interstitium. Ion transport mechanisms for NaCl reabsorption are less well understood than those involved in the formation of osmotic gradients

by acinar cells, and they are likely to vary according to gland type, cell type, and species. Models of ductal electrolyte transport accounting for known aspects of NaCl reabsorption have been presented, however, (Cook *et al.,* 1994; Melvin, 1999). According to these models, Na^+ and Cl^- entry pathways on the luminal surface involve Na^+ and Cl^- channels as well as paired Na^+/H^+ exchange and Cl^-/HCO_3^- exchange activities. The exit of Na^+ to the interstitium occurs via a basolateral Na^+/K^+-ATPase, whereas Cl^- exits via a basolateral Cl^- channel. Other apical and basolateral transporters are also involved. Ion flux across the tight junctions is considered unlikely (Cook *et al.,* 1994).

E. Signaling

The secretion of proteins, electrolytes, and water by salivary glands is under the control of the autonomic nervous system (Baum, 1987, 1993; Baum and Wellner, 1999; Cook *et al.,* 1994). In general, a purely parasympathetic stimulation produces a high flow rate and saliva that is watery. In contrast, a slow flow rate is usually elicited by sympathetic stimulation, and the saliva is much more viscous due to a substantial secretion of mucins and other proteins (Schneyer *et al.,* 1972). Neurotransmitter receptor populations vary among glands within a species and between homologous glands from different species. Receptors can be found in both the acinar and the ductal regions of salivary glands (Cook *et al.,* 1994); however, most studies have been concerned with neurotransmitter signaling in acinar cells.

Activation of muscarinic–cholinergic and α- and β-adrenergic receptors provides the major mechanism by which saliva formation is stimulated (Baum, 1987, 1993; Baum and Wellner, 1999; Cook *et al.,* 1994; Quissell *et al.,* 1992). However, activation of cognate receptors by nonadrenergic, noncholingeric agonists (e.g., vasoactive intestinal polypeptide, substance P, and ATP) can also elicit secretion (Baum and Wellner, 1999). In addition, functional salivary gland receptors for serotonin and γ-aminobutyric acid have been reported (Baum and Wellner, 1999).

1. Parasympathetic Stimulation

Parasympathetic stimulation is the major physiological mechanism responsible for high rates of fluid secretion. Muscarinic receptors on the basolateral membranes of salivary acinar and ductal epithelial cells are activated by acetylcholine released from parasympathetic nerve fibers. Muscarinic receptors are G protein-coupled receptors containing seven plasma membrane spanning domains. The amino and carboxyl termini are located on the extra- and intracellular sides of the membrane, respectively. To date, five muscarinic receptor subtypes (m_1–m_5) have been cloned (Eglen *et al.,* 1999). A sixth subtype might also exist (Eglen *et al.,* 1999). In

rodent salivary glands, muscarinic-stimulated fluid secretion is mediated by the m_3 subtype receptor (Culp *et al.,* 1996; Dai *et al.,* 1991; Watson *et al.,* 1996), although the m_1 subtype also appears to play a role in mouse sublingual gland (Culp *et al.,* 1996).

To stimulate fluid secretion, acetylcholine binds to the m_3 subtype muscarinic receptors in acinar cells, activating phopholipase C via the $G_{q/11}$ family of G proteins (Sawaki *et al.,* 1993). Phospholipase C cleaves membrane-bound phosphatidylinositol 1,4-bisphosphate, generating diacylglycerol (DAG) and 1,4,5-inositol trisphosphate (IP_3). DAG is a known activator of protein kinase C; however, its role in the muscarinic stimulation of salivary fluid secretion is uncertain (Ambudkar, 2000). IP_3 is the primary secondary messenger for muscarinic stimulation. Thus, IP_3 binds to the IP_3 receptor in the endoplasmic reticulum, and Ca^{2+} is released from the endoplasmic reticulum into the cytosol via the IP_3 receptor, which also serves as a Ca^{2+} channel. The increase in the cytosolic concentration of Ca^{2+} stimulates the opening of apical Cl^- channels and basolateral K^+ channels, leading to the formation of an osmotic gradient across acinar epithelia and, ultimately, fluid secretion (discussed previously). Although release of intracellular Ca^{2+} into the cytosol initiates fluid secretion, sustained fluid secretion requires the entry of extracellular Ca^{2+} into the cytosol, possibly involving the transient receptor potential protein 1 (Trp1) (Liu *et al.,* 2000; see Section V).

2. Sympathetic Stimulation

Norepinephrine released from sympathetic nerves activates α- and β-adrenergic receptors. This signal is primarily responsible for the regulated exocytosis of secretory proteins from acinar cells (e.g., amylase from parotid glands and mucin from submandibular glands) (Quissell *et al.,* 1992; Quissell, 1993). Similar to the muscarinic receptors discussed previously, adrenergic receptors are coupled to large G proteins, and thus they share a common overall structure—that is, seven membrane spanning domains connected by six extramembranous loops, with the amino and carboxyl termini residing on the extra- and intracellular sides of the membrane, respectively. In rodent salivary glands, α_1- and β_1-subtype receptors are the major mediators of the sympathetic norepinephrine signal (Au *et al.,* 1977; Ito *et al.,* 1982; Pointon and Banerjee, 1979).

Binding of norepinephrine to the β_1-subtype adrenergic receptor in the basolateral membrane of the acinar cell activates adenylate cyclase via a coupled G_s protein. Adenylate cyclase utilizes ATP as a substrate and generates cAMP and inorganic pyrophosphate. Overwhelming evidence indicates that cAMP is the primary cytosolic messenger mediating β-adrenergic-stimulated protein secretion (Baum and Wellner, 1999). Several lines of evidence also indicate that cAMP-stimulated protein secretion occurs via activation of protein kinase A (Baum and Wellner, 1999).

The final steps in cAMP-stimulated protein secretion involve the release of the proteins from secretory granules to the extracellular milieu. This process possibly involves a fusion of the granule membrane with the apical plasma membrane, preceded by plasma membrane docking and activation steps. Such a series of steps occurs, for example, during the stimulated release of neurotransmitters from neurons and during the stimulated release of norepinephrine from chromaffin cells (Chen and Scheller, 2001; Morgan and Burgoyne, 1997). In this regard, VAMP-2, a participant in the docking/fusion of vesicles/granules with plasma membranes, has been shown to be a participant in cAMP-stimulated protein secretion in rat parotid glands (Fujita-Yoshigaki *et al.,* 1996, 1998; Takuma *et al.,* 1997, 2000).

In addition to increasing the concentration of cAMP in acinar cells, norepinephrine stimulation increases $[Ca^{2+}]_i$ via activation of the α_1-adrenergic receptor. The mechanism by which norepinephrine increases $[Ca^{2+}]_i$ is essentially the same as that described earlier for $[Ca^{2+}]_i$ increases evoked by muscarinic stimulation. Thus, binding of norepinephrine to the α_1-adrenergic receptor stimulates a $G_{q/11}$ protein-coupled activation of phospholipase C, generating IP_3. Binding of IP_3 to the IP_3 receptor in the endoplasmic reticulum releases intracellular Ca^{2+} from the endoplasmic reticulum to the cytosol, followed by the entry of extracellular Ca^{2+}. The norepinephrine-evoked increase in acinar cell $[Ca^{2+}]_i$ causes stimulation of salivary water flow due to a Ca^{2+}-triggered opening of apical Cl^- channels and basolateral K^+ channels in acinar cells. The water flow stimulated by norepinephrine is usually considerably less than that caused by muscarinic stimulation.

F. Protein Secretion Stimulated by cAMP + Ca^{2+}

Increases in $[Ca^{2+}]_i$ and cytosolic cAMP content can independently stimulate amylase release from parotid acinar cells, although the rate of Ca^{2+}-stimulated release is significantly less. Costimulation can result in amylase secretion that is greater than the sum of the secretions obtained from independent stimulations (Baldys-Waligorska *et al.,* 1987; Yoshimura and Nezu, 1992). It has been suggested that cAMP and Ca^{2+} promote protein secretion via a common pathway (Quissell, 1993). A model for salivary protein secretion has been proposed (Fujita-Yoshigaki, 1998) based on docking/priming/fusion models for the stimulated secretion of other moieties, such as neurotransmitters from nerve cells and norepinephrine from chromaffin cells (Chen and Scheller, 2001; Morgan and Burgoyne, 1997). Thus, during the salivary secretory process, cAMP has been proposed to promote the docking of granules containing secretory proteins with the plasma membrane. After a priming step, Ca^{2+} has been proposed to promote the fusion of the granules with the plasma membrane, releasing the secretory proteins into the extracellular milieu.

G. Water Permeability

During primary saliva formation there is a transepithelial movement of water across acinar cells into the lumina of salivary glands. This pathway of water flow accounts for virtually all the salivary water because the ducts are comparatively water impermeant. The transit of water across acinar epithelia can potentially occur via two distinct pathways: (i) a paracellullar pathway in which water flows between adjacent acinar cells, crossing tight junctions, and entering the acinar lumen, and (ii) a transcellular pathway in which water first enters acinar cells by crossing the basolateral membranes, and then it exits the cells into the lumina of the glands by crossing the apical membranes.

In an effort to more precisely understand the pathway(s) water follows during primary saliva formation, investigators have studied acinar cell membrane water permeabilities. In one study, an estimate of the water permeability of the acinar cell membranes in intact rabbit submandibular glands was made using a proton nuclear magnetic resonance method (Steward *et al.,* 1990). The results suggested that the rate of water flux across the membranes is insufficient to fully account for the known production rate of the primary salivary fluid. Thus, it was suggested that a substantial fraction of water flow into this gland occurred via a paracellular route.

Results of two other studies suggested that water flow across apical acinar membranes of mouse salivary glands is essential for normal saliva production. The apical membranes of the salivary acinar cells contain aquaporin-5 (AQP5), a water channel protein that facilitates the flow of water across membranes in response to an osmotic gradient (He *et al.,* 1997; Nielsen *et al.,* 1997). Interestingly, in one study it was found that transgenic mice deficient in AQP5 exhibited markedly defective saliva secretion (Ma *et al.,* 1999). The deficiency appeared to be due to decreased water flow and not to an impairment of ion transport. In another study it was found that AQP5 was missorted to basal membranes in minor salivary gland acinar cells from patients suffering from Sjogrens syndrome, a disease characterized by a deficiency in saliva formation (Steinfeld *et al.,* 2001). The missorting of AQP5 was suggested to contribute to defective saliva formation.

AQP5-mediated water flow across apical acinar cell membranes might provide for water flow into salivary glands via either paracellular or transcellular water transit pathways. Aquaporins AQP2, AQP3, and AQP5 have been suggested to provide for transcellular water flow in other systems (King *et al.,* 2000). In these cases, different AQPs are located on apical and basolateral membranes of the same epithelial cell, and water is presumed to flow through the cell via these AQPs in response to an osmotic gradient. In salivary acinar cells, a definitive role for AQPs located in basolateral membranes has not been established.

It is possible that water flow along the paracellular pathway requires the shrinkage of acinar cells, which is know to occur during primary saliva formation

(Nauntofte, 1992). Thus, AQP5 might promote paracellular water flow by providing for water flux across the acinar cell apical membranes during cell shrinkage (Nakahari *et al.,* 1998; Nauntofte, 1992). Of course, it is possible that during saliva formation, AQP5-mediated water flow across apical membranes promotes both transcellular and paracellular water flow into the gland. Currently, we do not know the exact contributions of each pathway to saliva formation.

H. Target Cell Kinetics

The expression of transgene products by salivary cells depends on several factors, including whether the transgene is stably integrated into the host genome. In cells in which a transgene is not stably integrated, loss of transgene expression (i.e., transient gene expression) will occur as a result of cell division. The kinetics of salivary epithelial cells have been investigated, although not definitively. Currently, the consensus is that mature salivary acinar and ductal cell populations are not considered to belong to the static or expanding class of cell populations but rather to the "slow renewing" class (Denny and Denny, 1993). Thus, for example, in parotid glands of male adult rats the acinar and striated duct cells have estimated average life spans of 200 days. Acinar and granular duct cells of adult mouse submandibular glands also have estimated life spans of 200 days. In young female rat submandibular glands, the life spans of the acinar and granular duct cells are estimated to be 125 and 62 days, respectively. Clearly, additional investigations of this important aspect of salivary gland biology are needed in order to understand the implications for gene transfer.

III. Strategies for Gene Delivery

A. General Approach

Deoxyribonucleic acid (DNA) was recognized as the genetic material in cells in the early 1950s. Since then, many biological scientists have considered a novel type of medical therapy using DNA. In the mid-1960s, the first speculations about the possible treatment of genetic disorders by introducing functional genes via viral-mediated gene transfer had already arisen (Wolff and Lederberg, 1994b). This scientific dream became reality in 1990 with the first phase I gene therapy clinical trial for the treatment of adenosine deaminase (ADA) deficiency (Blaese *et al.,* 1995). This was a major milestone for medicine, and many scientists were stimulated by this clinical study.

Gene transfer is basically a technique that delivers a piece of DNA into the target cells or tissues. Gene transfer can involve the delivery of intact functional genes or

short chains of nucleic acids (i.e., oligonucleotides). Gene transfer can be used to study fundamental biological functions for a specific gene *in vitro* and *in vivo.* It can also be used for therapeutic purposes; in this circumstance, it is also called gene therapy. Gene therapy can involve the introduction of a gene into cells to restore normal function. Alternatively, the target cells could be normal but are used to produce a functional protein to correct malfunctions in other cells and tissues.

Successful gene therapy does not simply deliver a gene into a nucleus. It involves understanding a disease from general clinical characteristics to molecular and cellular pathobiology, isolating and cloning of the therapeutic gene, selecting the target cell, selecting the method of delivery (*ex vivo* or *in vivo*), selecting the delivery vector (viral or nonviral), as well as addressing cytotoxicity, immune response, etc. At present, gene delivery vectors are arguably the key concern for all gene transfer applications. This section addresses the current strategies for gene delivery.

The simplest way to deliver a gene into the cells is to use naked DNA. Naked DNA, in the form of a plasmid containing the gene of interest, can be taken up and expressed by living mammalian cells. This method of gene transfer, however, is generally very inefficient, especially *in vivo.* A primary objective of current gene transfer research is the development of suitable delivery vectors for efficient, cell type-specific uptake of the therapeutic gene. An effective gene delivery vector requires several components. First, the vector must appropriately package the gene providing protection from the extracellular environment. Second, the vector must allow binding and uptake by the target cells. Third, the vector must allow the gene to be transported to the nucleus. Ideally, the vector carrying the gene of interest should integrate into the host genome and provide stable expression. The vector should also provide transcriptional control, with a promoter driving appropriate expression of the gene product and with cell type-specific regulatory elements for control. For persistence of gene expression, the vector and gene product should not be immunogenic in order to avoid immune recognition and destruction of the successfully transduced cell.

Vectors are classified as either viral or nonviral. Viruses have evolved very efficient mechanisms to introduce their DNA into recipient cells. Therefore, viral vectors have been widely used to transfer genes into target cells. Typically, essential viral genes are deleted to avoid viral replication. The gene of interest and associated regulatory elements are used to replace the deleted viral genes. The production of viral vectors must occur in a cell line engineered to express the missing viral genes. A major challenge for the use of viral vectors is to solve the problem of the immunological rejection of transduced host cells.

Nonviral vectors are based on cellular uptake of complexes between DNA and (bio)organic molecules. Cationic formulations involving lipids and polymers that enhance plasmid transport across the plasma membrane are reasonably effective *in vitro.* However, their poor efficiency *in vivo* is a limitation of nonviral vectors. In general, nonviral vectors are significantly less immunogenic than viral vectors.

Gene can be transferred into the cells *in vitro, in vivo,* and *ex vivo. In vitro* gene transfer is common as a model to investigate fundamental biological functions of genes. Both *in vivo* and *ex vivo* gene transfer can be used to study fundamental biological functions of genes and vector biology as well as applied for therapeutic purposes. *Ex vivo* gene transfer involves the removal of target cell types, followed by *in vitro* cell transduction and amplification, with subsequent reimplantation into the host. *In vivo* direct gene transfer is most useful for the treatment of disease, but it involves additional risk compared to *ex vivo* procedures.

B. Viral Vectors

Viral vectors are important tools for gene transfer. Naturally, viruses have efficient ways to get into the host cells and replicate. It is this natural relationship which allows viruses to be useful as gene transfer vectors. Currently, there is no other gene transfer method which provides comparably efficient gene transfer, especially *in vivo.* However, no single virus is suitable for all gene transfer applications. A viral vector is selected for gene transfer depending on the intended purpose (e.g., long-term or short-term gene expression), the target cell or tissue, and the method of delivery. There are two general classifications of viral vectors, DNA and RNA. DNA vectors may be classified as single-stranded or double-stranded. For example, adeno-associated viral (AAV) vectors are single-stranded DNA, whereas adenovirus is double-stranded. Moloney murine leukemia virus (MoMLV) and lentivirus are RNA vectors. Ideally, all recombinant viral vectors should be constructed as noncompetent for replication and deleted of viral structural genes, which may be cytotoxic or immunogenic. These vectors should also retain full infectious ability and allow the required level of transgene expression in the target cells.

Many viral vectors possess broad tissue tropism (e.g., adenovirus). For most gene transfer goals, however, the gene of interest should be delivered into a very specific target cell. If other nontargeted cells are also infected, collateral damage could result. Therefore, development of specific cell-targeted viral vectors is important to minimize side effects.

A targeted viral vector can be constructed by engineering the viral DNA to encode a protein that can limit entry of the vector to certain cell types. For example, fiber or penton proteins of adenovirus have been modified to redirect the vector to specific cell surface receptors (Krasnykh *et al.,* 2000). Immunologic retargeting is also possible. Cell- or tissue-specific promoters can also be used to restrict foreign gene expression to specific target cells.

Additionally, regulatory elements can be added to a viral vector when the insert capacity is available. Genes from higher eukaryotes contain introns which are removed during RNA processing. Genomic constructs have been shown to be expressed more efficiently in transgenic animals than identical constructs lacking

introns (Palmiter *et al.,* 1991; Choi *et al.,* 1991). In most situations, cDNA is directly cloned into a viral vector, but this is not a natural condition. 5′ and 3′ untranslated regions (UTRs) can also influence gene expression in viral vectors. For example, specific sequences in the 3′ UTR are involved in mRNA destabilization (Gueydan *et al.,* 1999).

Many viral vectors can be used for gene transfer. Each viral vector, however, has limitations. Recent novel developments in viral vectors focus on finding ways to avoid the disadvantages. An increasingly common practice is to combine positive features from more than one virus in a hybrid vector. Such vectors potentially offer many benefits.

1. Adenovirus

Adenoviruses (Ads) are nonenveloped DNA viruses that have been commonly used for gene transfer to salivary glands. Ads have an icosahedral structure with 20 triangular surfaces, 12 vertices, and a diameter of ∼70 nm. There are more than 50 human serotypes that are classified into six subgroups (groups A–F). The Ad capsid consists of three major proteins—hexon, penton, and fiber. The Ad genome is a linear, double-stranded DNA (∼36 kb) with a terminal protein (IP) attached covalently to the 5′ termini (Rekosh *et al.,* 1977), which have inverted terminal repeats (ITRs). The 36-kb genome contains early and late-expressed genes, which encode more than 50 proteins. There are six distinct early regions: E1a, E1b, E2a, E2b, E3, and E4. The E1 region is immediately transcribed after infection. Two predominant regulatory proteins are encoded by genes from this region. They transactivate the transcription of the other early genes and play a key role in the productive infection. E1a and E1b are necessary and sufficient for cellular transformation. The late gene products include the principal capid proteins, which are transcribed from a single promoter and are involved in virus attachment to surface cell receptors and to facilitate entry of the virus (Haddada *et al.,* 1995).

Ads bind to cells via fiber protein to a 46-kDa glycoprotein receptor that is widely expressed on different tissue types. This protein is a member of the immunoglobulin superfamily and is referred to as CAR (coxsackie adenovirus receptor) since it also mediates attachment of coxsackie B viruses (Nemerow, 2000). Recent studies have suggested that heparin sulfate proteoglycans (Dechecchi *et al.,* 2000) or sialic acid (Arnberg *et al.,* 2000) may also facilitate Ad attachment. Although fiber binding to CAR mediates virus attachment to cells, it is not sufficient to allow rapid virus uptake. For this, cells must express integrin subunits of the $\alpha_v\beta_{3/5}$ subclass which interact with the penton base to promote virus internalization (Chiu *et al.,* 1999). Following CAR binding, Ad is rapidly internalized into clathrin-coated vesicles (Nemerow, 2000). A unique feature of Ad particles is their ability to disrupt the membranes of early endosomes, thus enabling partially uncoated virus particles to enter the cytoplasm (Greber *et al.,* 1993; Dales and Chardonnet, 1973; Nakano and Greber, 2000; Suomalainen *et al.,* 1999). Ads subsequently enter

the nucleus, presumably through nuclear pores. Ad preterminal proteins possess a nuclear localization signal and are responsible for the nuclear entry (Shenk, 1996). Immediately thereafter, viral early genes (E1–E4) are transcribed. These early proteins facilitate Ad DNA replication and late transcription (Shenk, 1996). Once enough viral particles have been accumulated, one E3 protein, called death protein, causes cell lysis. Eventually, the virion particles are released from the cell and begin a new cycle of viral replication in the neighboring cells (Shenk, 1996).

Ads have a natural tropism for epithelial cells of upper and lower respiratory tracts, the gastrointestinal tract, and ocular tissue. They can efficiently infect dividing and nondividing cells including highly differentiated tissues such as salivary glands. The most commonly used Ads for constructing recombinant vectors are Ad2 and Ad5. Both are from the serotype C group, to which most adults have been exposed. Both Ad2 and Ad5 viruses are well characterized. However, their genomes rarely integrate into the host genome. Thus, these vectors are most suitable for applications requiring transient gene expression (Danthinne and Imperiale, 2000; Harui *et al.,* 1999). Neither vector has been shown to induce tumor formation in animal models (Seth, 2000).

First-generation Ad vectors lack all, or most, of the early genes E1a, E1b, and E3. Instead, a transgene expression cassette is placed in these regions. The 293 cell line, which is commonly used for Ad vector propagation, complements the E1 region defect in these vectors by supplying the E1a and E1b products *in trans.* The E3 region encodes products that counteract host defense mechanisms; these products are not essential for viral replication *in vitro,* and therefore no complementing cell line is necessary (Wold *et al.,* 1995). E1-deleted Ad vectors can accept insertions of up to 5.1 kb, whereas E1/E3-deleted viruses allow the cloning of about 8.2 kb (Danthinne and Imperiale, 2000). E1- or E1/E3-deleted vectors can be propagated and amplified to titers of $>10^{11}$ plaque-forming units (pfus)/ml in these cells.

Generation of a E1-deleted Ad vector requires performing a two-plasmid cotransfection. One plasmid is a shuttle vector, such as pACCMVpLpA (Becker *et al.,* 1994), which contains a region of the Ad genome between map units 1.3 and 9.1, along with the CMV promoter. The second plasmid provides most of the Ad genome, albeit with E1 or E1/E3 deletions (e.g., pJM17 or pBHG10, respectively). The shuttle vector and Ad vector are cotransfected into 293 cells. Homologous recombination between the two plasmids generates a recombinant genome in which the E1 region is replaced by the gene of interest. The recombinant Ad virus thus generated is replication deficient but can be propagated in the 293 cells. Recombinant Ads can be purified in a $CsCl_2$ gradient and stored frozen for an indefinite period of time.

First-generation Ad vectors can be used to transduce a variety of dividing and nondividing cells. However, as noted earlier, these vectors cause a strong immune response *in vivo* and they can be directly cytotoxic to target cells due to E4 gene products. E4 is a complex regulatory unit that encodes seven different polypeptides. For example, the E4 ORF6/7 polypeptide competes with RB proteins for

binding to the E2F transcription factor. To improve Ad vector performance, several groups have developed vectors deleted in E4, along with E1/E3. Not surprisingly, an Ad vector in which all Ad genes have been removed has also been developed (Kochanek, 1999). Such vectors are termed high-capacity, gutted, or helper-dependent. It is impossible to create a packaging cell line for such vectors. Instead, a helper-dependent vector system has been developed in which one virus (the helper) contains all the viral genes required for replication but has a conditional defect in the packaging domain making it less likely to be packaged into a virion. The second vector contains only the viral inverted terminal repeats and the normal packaging recognition signal. This allows ~30 kb of foreign DNA to be packaged.

2. Adeno-Associated Virus

Adeno-associated virus belongs to the family of the parvoviridae, which are among the smallest and structurally simplest DNA viruses. AAV also appears to be useful for gene transfer to salivary glands (Braddon *et al.,* 1998). AAV is a nonenveloped virus of 20- to 25-nm diameter and contains a linear, single-stranded DNA genome of 4680 bp with two ITRs of 145 bp each (Srivastava *et al.,* 1983). The ITR sequence also contains elements that function as a transcriptional promoter (Flotte *et al.,* 1993). Although there are canonical TATA boxes, the terminal repeats contain several Sp1 sites and strong homology with the consensus Inr site found in many genes (Flotte *et al.,* 1993).

AAV was originally isolated from stocks of adenovirus as a contaminant (Atchison *et al.,* 1965; Hoggan *et al.,* 1966). It does not productively infect cells in culture unless there is a coinfection by an unrelated helper virus such as adenovirus or herpesvirus (Buller *et al.,* 1981; Hoggan *et al.,* 1966). AAV is widespread in the human population (more than 80% of whom are seropositive), but there is no evidence that AAV is an etiological agent for human disease (Berns and Giraud, 1996). Six serotypes have been cloned. AAV-1 and AAV-4 are originally from nonhuman primates, and AAV-2, AAV-3, AAV-5, and AAV-6 are human in origin (Chiorini *et al.,* 1999a; Blacklow *et al.,* 1968; Dreizin *et al.,* 1981). Each serotype apparently has a distinct mechanism of cellular uptake (Chiorini *et al.,* 1997, 1999b). Of the six serotypes identified, AAV-2 has been most extensively studied; thus, in this section, only serotype 2 will be addressed.

In the single-stranded DNA genome there are two major open reading frames (ORFs). The *cap* ORF encodes the viral capsid proteins VP-1–VP-3 transcribed from the P_{40} promoter that assemble into particles with icosahedral symmetry. The *rep* ORF encodes the four nonstructural Rep proteins from two promoters (P_5 and P_{19}). A spliced and an unspliced mRNA species from both promoters are translated to produce the Rep proteins Rep78, Rep68, Rep52, and Rep40. The P_5 Rep proteins (Rep78 and Rep68) bind to a specific sequence (5′-GCTCGCTCGCTC-3′) found in the ITR (McCarty *et al.,* 1994; Chiorini *et al.,* 1994) and function as a site-specific

nickase, which cleaves after nt 124 (Snyder *et al.,* 1993). Both proteins have been shown to possess functions required to replicate the genome (Owens *et al.,* 1993; Smith *et al.,* 1997), modulate transcription from AAV and heterologous promoters (Horer *et al.,* 1995), and mediate site-specific integration into the human genome (Weitzman *et al.,* 1994). The P_{19} Rep proteins (Rep52 and Rep40) are required for the production of single-stranded genomes and the modulation of AAV promoters (Smith and Kotin, 1998; Pereira *et al.,* 1997).

The ITRs form T-shaped palindromic structures. The first 125 bp constitute an overall palindrome interrupted by two smaller palindromes (nts 42–62 and 64–84), one on either side of the overall axis of symmetry. The ITRs constitute an important *cis*-acting signal. They function as an enhancer, are critical for regulation of DNA, serve as the origin of DNA replication, and are required for the site-specific integration of AAV. Therefore, the ITRs encode sequences required for packaging, integration, and rescue and serve as the origins of DNA replication (McLaughlin *et al.,* 1988; Snyder, 1999).

There are two distinct intracellular phases to the AAV life cycle (Cheung *et al.,* 1980). In one, the wild-type AAV virion enters the cell in the absence of a helper virus such as Ad. Only limited viral gene expression occurs and the viral DNA is integrated into the genome of the host cell to establish a latent infection (Cheung *et al.,* 1980; Berns and Giraud, 1996). AAV has been demonstrated to preferentially integrated at a specific site in the human genome on the q arm of chromosome 19 (Kotin *et al.,* 1992; Samulski, 1993). AAV's site specificity is dependent on Rep gene products since vectors without this gene lose specificity (Duan *et al.,* 1998; Miao *et al.,* 1998). The second phase of the AAV life cycle is productive infection. This occurs in the presence of a helper virus, such as Ad or herpesvirus (Buller *et al.,* 1981; Hoggan *et al.,* 1966). In the case of Ad, infection can precede that of AAV, occur at the same time, or involve superinfection of a cell latently infected by AAV (Berns and Giraud, 1996). All early genes from Ad provide the helper functions and are involved in regulation of gene expression (Samulski and Shenk, 1988; Chang *et al.,* 1989).

Heparan sulfate proteoglycan is the primary AAV-2 receptor, mediating both attachment to and infection of target cells (Summerford and Samulski, 1998). Heparan sulfate proteoglycan is found in many cells, leading to a wide AAV target range. In a productive infection, the viral DNA is immediately uncoated and transported to the cell nucleus. The hydroxyl moiety of the 3′ terminal hairpin serves as a primer for host DNA polymerases that extend it to form duplex, dimer, and higher order concatameric replicative forms. AAV DNA is rescued from the vector backbone and replicated, and progeny virions are produced (Berns and Giraud, 1996). This rescue requires the inverted terminal repeat as well as Rep gene expression. The current model for AAV replication invokes a rolling hairpin mechanism that is initiated by the pleiotropic replicator protein Rep. Single-stranded progeny DNA is derived from the duplex concatenated replicative form (Snyder *et al.,* 1990; Muzyczka, 1992; Tal, 2000). DNA molecules of "plus" and "minus" orientations

are generated and encapsidated into separate virions in equal proportions. Plus and minus virions are equally infectious (Tal, 2000).

In the absence of Ad, all Rep proteins repress P_5 and P_{19} transcription (Kyostio *et al.,* 1994; Bueler, 1999), and host factor YY1 binds to a sequence in the AAV P_5 promoter and inhibits rep gene transcription (Lewis *et al.,* 1995; Bueler, 1999). In the presence of Ad, transcriptional repression is relieved. This requires the Ad E1a protein (Laughlin *et al.,* 1982; Bueler, 1999). Ad E1b (Samulski and Shenk, 1988), E2a (Janik *et al.,* 1989), E4, and VA RNA are required for a productive AAV infection. The E1b and E4 ORF6 proteins interact to facilitate transport of viral transcripts to the cytoplasm and are necessary for efficient accumulation of viral mRNAs (Samulski and Shenk, 1988). The E2a DNA-binding protein and the VA RNAs are mainly required for efficient translation of the AAV capsid mRNAs and to increase the steady-state level of AAV transcripts (Janik *et al.,* 1989).

AAV has become an attractive candidate for gene therapy. As noted earlier, no diseases are associated with AAV infection (Carter and Samulski, 2000). AAV particles are very stable and are resistant to many physical and chemical factors (Chiorini *et al.,* 1995). AAV vectors are able to transduce both dividing and nondividing cells. AAV vectors theoretically also have the potential for site-specific integration on human chromosome 19 (with Rep proteins present) or for random chromosomal integration (Duan *et al.,* 1998; Miao *et al.,* 1998) with Rep proteins absent. AAV vectors can also persist in cells for long periods of time in an episomal form. Finally, AAV vectors induce a modest immune response (Carter and Flotte, 1996; Jooss *et al.,* 1998; Chirmule *et al.,* 2000).

AAV viral vectors have some limitations for gene transfer applications. AAV vectors have a limited capacity of insertion (<5 kb). This can be somewhat circumvented, however, by dividing the transgene cassette into two parts and inserting these into two different AAV vectors. The partial mRNAs newly transcribed can be spliced into an intact mRNA and translated into a functional protein (Yan *et al.,* 2000; Nakai *et al.,* 2000). Another problem is that the production and packaging of recombinant AAV vectors is labor-intensive and expensive. Also, because AAV is a single-stranded DNA virus, it is necessary to synthesize a second strand to express the gene of interest. Finally, it is still not known whether rAAV integration occurs *in vivo.*

Until recently, the standard propagation system for rAAV involved a two-plasmid transient cotransfection system (Patijn and Kay, 1999). The first plasmid, a helper plasmid, provides the AAV genes (*rep* and *cap*) necessary for rAAV replication and packaging, except the ITRs. The second plasmid, the rAAV vector, contains the transgene flanked by the ITRs (Flotte *et al.,* 1996). Because the helper plasmid does not contain the AAV ITRs, that DNA cannot be packaged, thus reducing the probability of homologous recombination and therefore the generation of wild-type AAV (Samulski *et al.,* 1987). The rAAV plasmid and the AAV helper plasmid are cotransfected into a cell line (e.g., 293, Hela, or COS cells) and subsequently infected with helper adenovirus. The virions are liberated from the cells and viral

particles are purified by $CsCl_2$ density centrifugation. rAAV are dialyzed and heated (at 56°C) to inactivate any residual adenovirus. This method usually yields between 100 and 1000 rAAV particles per cell (Patijn and Kay, 1999). With this method, however, it is impossible to remove all Ad proteins, which can elicit an immune response (Yang *et al.,* 1995a). To circumvent this problem, an Ad miniplasmid was constructed containing all the necessary helper genes for efficient AAV production. This miniplasmid is then cotransfected into any of the previously mentioned cell lines, along with the rAAV plasmid and the AAV helper plasmid.

3. Retroviral Vectors

In 2000, a report was published describing two children affected by severe combined immunodeficiency (SCID)-X1 who were successfully treated by gene therapy. They received bone marrow stem cells that were transduced by the cDNA of the common cytokine receptor γ chain (Cavazzana-Calvo *et al.,* 2000). After infusion of the transduced cells, the affected children, who previously required protective isolation, had reconstituted immune function and thrived in a normal environment. The vector used in this trial was a retroviral vector, the same type as that used in the first human gene therapy described previously (Blaese *et al.,* 1995). These were retroviral vectors based on the MoMLV, and they are the most widely used vectors for gene therapy applications.

Retroviruses are lipid-enveloped particles composed of a homodimer of linear, positive-sense, single-stranded RNA genomes of 7–11 kb (Kay *et al.,* 2001). Each retrovirus contains two single-stranded RNAs. Retroviruses contain three major viral genes. *gag* encodes the major capsid protein. *pol* encodes the enzymes reverse transcriptase and integrase, which are involved in early events in viral replication, as well as a protease that is used in the processing of viral proteins. *env* encodes the envelope glycoprotein that is located on the surface of the viron and derternines the host range of the virus through a specific interaction with receptors on target cells (Hunter, 1997; Andreadis *et al.,* 1999). In addition, the retroviral genome contains *cis*-acting sequences, two long-terminal repeats, containing elements required to drive gene expression, reverse transcription, and integration into the host genome (Palu *et al.,* 2000).

Recombinant retroviruses are produced using a recombinant retroviral vector and a specially designed packaging cell line. To create the vector, the viral structural genes are deleted and replaced with the transgene(s) of interest. The vector retains the viral regulatory elements necessary for transmission and expression of the transgene(s) as well as a specifial sequence, the Ψ sequence, that mediates packaging of the retroviral vector RNA into the viron (Andreadis *et al.,* 1999). The packaging cells have been genetically engineered to supply the *env, pol,* and *gag* sequences. The recombinant vector is transfected into the packaging cells, where it is transcribed, and the resulting recombinant RNA is recognized by the structural proteins and packaged into retroviral particles that are shed from the

plasma membrane. The virus-containing medium is harvested to make stocks of recombinant retroviruses that are able to mediate gene transfer but are unable to replicate and act as an infectious unit (Andreadis *et al.,* 1999). To date, retroviral vectors have delivered genes into many cells *in vitro, ex vivo,* and *in vivo.*

Retroviral vectors are able to transduce a variety of cell types and to integrate efficiently into the genomic DNA of the recipient cells, resulting in long-term gene expression. Retroviral vectors also elicit a lower immune response compared to Ad vectors and still express the transgene at useful levels. However, retroviral vectors have distinct disadvantages that limit their applications. For example, during production there is a tendency to generate replication competent retrovirus. Second, reverse transcriptase has a limited fidelity. Third, there is a limitation for transgene size (8–10 kb). Fourth, retroviruses can only infect dividing cells. Additionally, retroviral vectors cannot be produced at high titer. The latter two disadvantages essentially restrict retroviral vectors to *ex vivo* use.

Lentiviruses, which include human immunodeficiency virus (HIV), are a type of retrovirus being developed for gene transfer applications (Vigna and Naldini, 2000). Compared to MoMLV, lentiviruses rely on active transport of the preintegration complex through the nuclear pore by the nuclear import machinery of the target cells (Bukrinsky and Haffar, 1999). This characteristic allows lentiviral vectors to infect both dividing cells and nondividing cells. A serious concern with these vectors is biosafety because the vector is derived from a virus causing AIDS. However, recently modified vectors with more deletions from the HIV-1 genome, the use of heterologous envelopes, and the expression of *gag, pol,* and *rev* proteins by heterologous promoters together reduce the risk of generating harmful recombinant viruses (Takeuchi and Pizzato, 2000).

The original reports showed that the lentiviral vectors exhibit efficient *in vivo* delivery and long-term gene expression in the central nervous system (Naldini *et al.,* 1996; Blomer *et al.,* 1997). However, direct transduction *in vivo* appears to be more sensitive to certain tissue barriers limiting vector access. Thus, lentiviral vectors in their current form seem comparatively poor at delivering genes *in vivo* into liver and muscle (Kafri *et al.,* 1997). A beneficial characteristic of lentiviral vectors is that they do not induce any significant inflammatory or vector-specific immune response (Naldini *et al.,* 1996; Blomer *et al.,* 1997). Overall, lentiviral vectors are considered to have promise for clinical applications but must be further developed.

4. Hybrid Vectors

Each of the previously discussed distinct viruses has disadvantages or limitations for gene transfer applications. To date, no single natural viral vector possesses all of the ideal requirements for gene transfer *in vivo.* Accordingly, alternative efforts have begun to create a hybrid vector that combines the positive attributes of more than one virus.

Although many different types of hybrid vector have been constructed, only a few are mentioned here. None are ideal in their present form and must be further developed. For example, several groups have generated a hybrid vector blending Ad and AAV (Fisher *et al.,* 1996; Sandalon *et al.,* 2000; Lieber *et al.,* 1999; Liu *et al.,* 1999; Recchia *et al.,* 1999; Gao *et al.,* 1998). One such vector is a recombinant Ad that contains a complete rAAV vector genome in the E1 region. This hybrid is used to deliver the rAAV genome to a packaging cell line and is adaptable to large-scale manufacturing processes. A second example developed an Ad able to integrate into the genome (Fisher *et al.,* 1996; Lieber *et al.,* 1999; Recchia *et al.,* 1999) by having AAV ITRs flanking a reporter gene cassette inserted into the E1 region of first-generation Ad vector. This hybrid vector was easily produced at high titers and was able to direct transgene integration.

Recently, several groups constructed Ad/MoMLV hybrid vectors for either of two goals (Zheng *et al.,* 2000a; Ramsey *et al.,* 1998; Feng *et al.,* 1997; Lin, 1998). One goal was to create a hybrid vector to simplify MoMLV production *in vitro* and to allow *in vivo* use. For, this, two or three recombinant Ads that contained all MoMLV components were used. These provided all the products required to generate the vector. The second goal was to use a Ad/MoMLV hybrid vector to test a hypothesis that the MoMLV 5′ and 3′ LTRs could facilitate integration of the vector into host genomic DNA and thus facilitate long-term transgene expression (Zheng *et al.,* 2000a). This design used the MoMLV integration property and Ad's ability to be easily propagated, with high titer and high infection efficiencies. The construct, AdLTR-luc, carried a 5′ MoMLV sequence [including part of env (1.5 kb), the 5′ LTR (0.57 kb), and the packaging sequence (0.63 kb)] and 3′ MoMLV sequence [containing a small part (∼0.5 kb) of env and an intact 3′ LTR flanking a luciferase reporter gene]. In this vector, there are no *gag* and *pol* sequences; therefore, no retrovirus can be generated.

5. Other Viral Vectors

In addition to Ad, AAV, and retrovirus, other viruses have been used as gene transfer vectors, including herpes simplex virus (HSV), Simian virus 40 (SV40), and Epstein–Barr virus (EBV). There are no published reports of these vectors transferring genes to salivary glands.

HSV is an enveloped double-stranded DNA virus that is naturally neurotropic and shows a preference for peripheral neurons (Fink *et al.,* 2000). The HSV capsid consists of seven structural proteins and contains the viral genome in association with core proteins. The capsid is surrounded by an amorphous matrix of proteins called the tegument (Roizman and Furlong, 1974), consisting of approximately 12 viral proteins (Roizman and Sears, 1993). The viral envelope (200 nm in diameter) is the outer shell of the virus particle and possesses at least 10 unique virus-encoded glycoproteins, many of which are targets of host immune responses (Simmons *et al.,* 1992; Wu and Morahan, 1992). HSV has a 152-kb linear viral

genome encoding 84 viral gene products. The HSV genome also contains several nonencoding, *cis*-acting sequences, including origins of replication (*ori*) and sequences in the terminal repeats essential for packaging of the HSV genome in virions.

Currently, HSV-based vectors consist of two types. One deletes IE genes to make a recombinant HSV vector, whereas the other is a HSV amplicon. HSV vectors are noncytotoxic (Samaniego *et al.,* 1998) and capable of persisting in a state similar to latency in neurons and other cell types within nonneuronal tissue (Kay *et al.,* 2001). The HSV amplicon system relies on introducing the gene of interest into a plasmid that contains a HSV origin of replication and packaging signal. This construct is then introduced into cells by transfection, concomitant with infection by a helper HSV virus, resulting in the amplicon becoming packaged into viral particles (Latchman, 1999). HSV has a broad host range, including dividing and nondividing cells. The major disadvantages of HSV include transient expression (days to weeks) and viral genes that induce cytopathic effects and immue responses (Wu and Ataai, 2000).

SV40 is a nonenveloped papova virus. It has a 5.2-kb double-stranded DNA genome. The DNA genome is circular without a terminal repeat region that characterizes the linear genomes of other vectors. The SV40 early promoter drives expression of large (Tag) and small (tag) T antigens. The late promoter is on the opposite strand and controls expression of the virus's structural proteins, VP1–VP3. Regulatory sequences, origin of replication, and packaging signals are all located within approximately 0.5 kb (Strayer and Zern, 1999).

Wild-type SV40 may replicate in human cells (Strayer and Zern, 1999; Bennett *et al.,* 1989). SV40 enters cells by pinocytosis. The pinocytic vesicle then travels directly to the nucleus, penetrating the nuclear membrane. Because of this unusual manner of cell entry, phagolysosomes are not formed and virus capsid antigens are not expected to be processed during early infection. Therefore, SV40 capsid proteins are protected from substantial immune recognition until their production in preparation for virus assembly late in infection. SV40 can integrate at random sites in the cellular genome (Strayer and Zern, 1999).

SV40 vectors are constructed by creating space in the genome to insert the gene of interest, rendering the virus replication defective and using a packaging cell line which supplies Tag *in trans* (Myers and Tjian, 1980; Tornow *et al.,* 1985; Strayer and Zern, 1999). Recombinant SV40 vectors are easily manipulated and produced at very high titer, and they are capable of providing sustained high levels of transgene expression in both resting and dividing cells. The major limitation of SV40 vectors is the size of insert able to be packaged ($\leq$5 kb) (Strayer, 1999).

EBV is a herpesvirus with a large ($\sim$172-kb) double-stranded DNA genome (Mecsas and Sugden, 1987). It is estimated that about 90% of the world's adult population is infected with EBV. The virus infects human B lymphocytes and some epithelial cells and establishes itself in the nucleus in a latent state as a circular, multicopy, extrachromosomal replicating plasmid. EBV may directly cause

B cell lymphoproliferative disease in immune deficient humans and is an etiologic agent in Burkitt's lymphoma, Hodgkin's disease, unusual T cell lymphomas, and nasopharyngeal carcinoma (Klein, 1994; Karimi and Crawford, 1995). Vectors derived from EBV can replicate extrachromosomally in cells over a long period. The viral elements required for episomal replication and nuclear retention are oriP (*cis*-acting replication origin) and EBNA-1 (Epstein–Barr nuclear antigen).

C. Nonviral Vectors

An ideal way to transfer genes would be to directly deliver the gene of interest into the nucleus of specific target cells without viral vectors. Normally, nonviral gene transfer systems are considered to be relatively safe, easy to use, and able to be produced on a large scale, with little immunogenicity and no restriction of DNA size. However, nonviral gene transfer systems have been considerably less efficient than those of viral gene transfer vectors in delivering DNA into cells. In recent years, nonviral gene transfer systems have been markedly improved and shown to be effective for transferring genes to salivary glands.

Nonviral gene transfer systems can be classified into two general types, physical and chemical. Physical methods used to deliver plasmid DNA include microinjection, particle bombardment (a "gene gun"), and electroporation. Chemical methods used to deliver plasmid DNA include cationic lipids, diethyl aminoethyl (DEAE) dextran, calcium phosphate, and controlled-release polymers. Effective nonviral gene therapy must be stable and provide good cellular access, appropriate intracellular trafficking, and nuclear retention of plasmid. The key limitations include inefficient endosomal release, instability against cytosolic nucleases, and limited nuclear entry of the plasmids (Mahato, 1999).

Direct gene transfer with naked plasmid DNA is the simplest nonviral delivery system (Wolff *et al.,* 1990). Initial reports showed that genes could be readily expressed following direct injection into skeletal muscle. Plasmid delivery directly to muscle is reasonably long-lasting and can result in therapeutic levels of transgene product. However, delivery to salivary glands yields quite transient expression (1 or 2 days) that is minimal at best (O'Connell *et al.,* 1995). The gene gun (Yang *et al.,* 1990) and *in vivo* electroporation (Rols *et al.,* 1998; Muramatsu *et al.,* 2001) have been employed to enhance the efficiency of naked DNA gene transfer. Both approaches allow DNA to directly penetrate cell membranes and bypass endosomes and lysosomes, thus avoiding enzymatic degradation. A gene gun is most suitable for relatively thin and easily accessible tissues such as skin because DNA-coated microparticles can effectively penetrate up to 2 or 3 mm from the surface of the tissue (Williams *et al.,* 1991).

The use of high-frequency, low-voltage electric pulses opens small "pores" in the cell membranes. This can increase the production and secretion of a recombinant protein from mouse skeletal muscle more than 100-fold (Rizzuto *et al.,* 1999).

In vivo electroporation has a variety of advantages. For example, many cell types can become targets, handling is easy and quickly done within a matter of seconds, repeated administration of DNA is possible, little immunogenicity is expected, and there is no constraint on the amount of DNA used. Gene transfer efficiency with *in vivo* electroporation is equivalent, or even superior, to that of *in vivo* lipofection, gene gun, and direct DNA injection methods (Muramatsu *et al.,* 2001).

Currently, the major chemical gene transfer approaches *in vivo* are lipid based or polymer based. Liposomes are microscopic vesicles composed of uni- or multilamellar lipid bilayers surrounding an aqueous compartment. Plasmids may be incorporated into anionic or neutral liposomes to ensure protection against degradation by nucleases in biological fluids and to enhance intracellular delivery (Ellens *et al.,* 1984; Mahato *et al.,* 1999). The encapsulation efficiency of plasmids, however, is very low. Liposomes that are pH sensitive, such as dioleoylphosphatidylethanolamine (DOPE), are fusogenic at acidic pH and can be used to facilitate the endosomal disruption and subsequent release of plasmids in the cytoplasm.

Since the introduction of the transfection reagent Lipofection, a cationic liposome composed of a 1:1 mixture of the cationic lipid *N*[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride) (DOTMA) and the colipid DOPE, cationic lipid-based gene transfer systems have been extensively employed and novel cationic lipid molecules are now synthesized routinely (Mahato *et al.,* 1999; Clark *et al.,* 2000; Bragonzi *et al.,* 2000). The general structure of a cationic lipid includes three parts: a hydrophobic lipid anchor group, which helps in forming liposomes (or micellar structures) and interacts with cell membranes; a linker group; and a positively charged headgroup, which interacts electrostatically with the negatively charge phosphate backbone of DNA. This headgroup promotes the condensation of plasmid DNA into a more compact structure (Mahato *et al.,* 1999). Cationic lipids may accumulate following administration, thus potentially resulting in undesirable side effects (Mahato *et al.,* 1999). However, there are only mild changes in clinical chemistries, hematology, and tissue histopathology. At high doses, cationic liposomes can lead to acute inflammation (San *et al.,* 1993; Mahato *et al.,* 1999).

Positively charged macromolecules, such as poly(L-lysine), histones, protamine, or poly(L-ornithine), can be linked to a cell-specific ligand and then bound to plasmids through electrostatic interaction. The resulting complexes retain their ability to interact specifically with target cell receptors, leading to receptor-mediated internalization of the complex into the cells (Mahato *et al.,* 1999). Such polycation-based vectors can provide a broad range of options for guiding the delivery of macromolecules into cells. Synthetic peptide-based gene transfer systems have the potential to take advantage of specific sequences able to circumvent barriers to gene transfer (Mahato *et al.,* 1999). Cationic polymers, such as polybrene and DEAE dextran, can condense large genes into smaller structures and mask the negative charges of plasmid DNA (Han *et al.,* 2000). The cationic polymer spontaneously

forms complexes with DNA because of electrostatic interactions between positively charged amine groups of the polycations and negatively charged phosphate groups of the DNA. The interaction between cationic polymer/DNA complexes and negatively charged cell membranes can enhance DNA uptake by the cells and thus increases the transfection efficiency (Han *et al.,* 2000). Polyethyleneimine (PEI) is a branched cationic polymer and has a high charge density. PEI can condense plasmids into colloidal particles that effectively transfect genes into a variety of cells *in vitro* (Boussif *et al.,* 1995). PEI may enhance intracellular trafficking by buffering the endosomal compartments, thus protecting the DNA from lysosomal degradation by endosomal DNA release through lysosomal disruption (Han *et al.,* 2000).

IV. Results in Salivary Glands

A. General Characteristics

Mammalian major salivary glands (parotid and submandibular) have several features that render them excellent targets for *in vivo* gene transfer. First, there is direct access to these glands through the excretory duct orifices (Stensen's and Wharton's) that open directly into the mouth. Second, almost all the epithelial cells in these glands are arranged as a monolayer along the ductal lumen, i.e., for all practical purposes the apical membranes of these cells are in direct contact with the mouth. This latter circumstance means essentially that each of these cells is a potential target for gene transfer when a vector is introduced to the gland through a cannulated duct. Third, the cannulation of these glands through the ductal orifices is a routine clinical procedure used for contrast radiography (sialography) and can be accomplished without anesthesia in humans. The cannulation of salivary glands in small laboratory animals (mice and rats) is more challenging than the cannulation of human glands or glands in large animals such as primates, but it is certainly quite manageable (Fig. 3, see color insert) (Mastrangeli *et al.,* 1994; Baccaglini *et al.,* 2001). Fourth, human major salivary glands are well encapsulated, a feature likely to limit undesirable dissemination of gene transfer vectors beyond the glands.

We and others (Barka and Van der Noen, 1996; Goldfine *et al.,* 1997) have used intraductal cannulation techniques to deliver many different types of transgenes to salivary glands (Table I). In rat glands, this delivery approach can be extremely efficient, with successful cannulation and gene transfer occurring about 90% of the time when optimal conditions are employed (Baccaglini *et al.,* 2001). Among the key variables necessary to consider in order to achieve this high success rate for small animal experiments are the size (length and internal diameter) of the cannula, the distance to which the cannula penetrates the duct, and the volume of buffer

in which the vector is delivered. An extremely broad array of genes have been used for salivary gland gene transfer (e.g., various reporter genes, normal serum proteins, membrane proteins, cytokines, and nuclear proteins). The vectors used have been equally broad and include adenoviral, retroviral, and adeno-associated viral vectors and a hybrid adeno-retroviral vector; cationic liposomes and direct "naked" plasmid delivery have also been used (Baccaglini *et al.,* 2001; Barka and Van der Noen, 1996; Braddon *et al.,* 1998; Mastrangeli *et al.,* 1994; O'Connell *et al.,* 1995; Zheng *et al.,* 2000a). Levels of expression vary depending on the vector used, with greatest expression, at least over the short term, obtained from adenoviral vectors.

B. Cellular Target Sites

As noted previously, almost all epithelial cells in the salivary glands are potential targets of gene transfer vectors. In practice, with mice and rats, depending on the vector type utilized, there are significant differences in the cell population that expresses the transgene, i.e., the target cells transduced or transfected. For example, adenoviral vectors can transduce almost all types of glandular parenchymal cells with the exception of the cells in the main excretory duct (Mastrangeli *et al.,* 1994; Delporte *et al.,* 1997a,b). Such adenoviral vectors (based on a replication deficient type 5 serotype) are also the most efficient vectors available for transferring genes to salivary cells, with up to 35% of glandular epithelial cells able to be transduced in rat submandibular glands (Delporte *et al.,* 1997a). Retroviral vectors (based on MoMLV) are able to infect only dividing cells, either acinar or ductal. However, since adult salivary epithelial cells divide very infrequently (Redman, 1995), only an extremely small population of cells are transduced when retroviral vectors are used to infect rat submandibular glands (estimated to be $<0.1\%$ based on the results of Barka and Van der Noen, 1996, 1997). AAV vectors (based on the type 2 serotype) are able to transduce only intralobular ductal cells when administered to murine submandibular glands (Braddon *et al.,* 1998). Finally, gene delivery using a cationic liposome preparation, GAP-DLRIE/DOPE (Baccaglini *et al.,* 2001), after intraductal cannulation leads to transfection of a small percentage of cells, almost entirely acinar cells (about 95% of transfected cells), in rat submandibular glands.

C. Transgene Expression

The extent and duration of transgene expression in transduced salivary cells depend on many factors, including the type of gene transfer vector used, the promoter employed, the nature of the transgene, the number of cells transduced or transfected, and the host response to the vector used. For example, as noted earlier,

adenoviral vectors generally transduce the greatest percentage of target cells in salivary glands, enhancing the potential for higher transgene expression compared with any other vector type utilized. Unfortunately, for these vectors host response is a major issue (discussed in detail later). Adenoviral vectors elicit a potent immune response and, consequently, unstable transgene expression in salivary glands (peak at 1–3 days, and background levels by 7–10 days; Delporte *et al.,* 1997b; Kagami *et al.,* 1998). Conversely, AAV vectors transduce far fewer cells than adenoviral vectors and generally elicit a much lower host response, but peak transgene expression can be observed for up to 2 months (the longest time point studied in salivary glands; Braddon *et al.,* 1998; Yamano *et al.,* submitted for publication).

In most of our studies, we employed the cytomegalovirus promoter/enhancer element (Pcmv) to drive transgene expression. This promiscuous viral promoter is very powerful and, in the short term, can lead to substantial levels of transgene expression. However, the Pcmv is particularly susceptible to silencing (promoter shutdown) as a strong foreign regulatory element in eukaryotic cells. Thus, the Pcmv is not ideal when long-term, stable gene transfer is desired. We compared the expression of the insect reporter gene luciferase in rat submandibular glands to comparable serotype 5 recombinant adenoviral vectors driven by Pcmv, the human kallikrein promoter (Pkall), which is relatively specific for salivary and other water transporting epithelia, the human amylase promoter (Pamy), which is relatively specific for salivary acinar cells, and the 5′ LTR from MoMLV (Pltr). The level of luciferase activity measured ~24 h post-vector delivery was greatest with the vector containing Pcmv: Pcmv > Pkall ≫ Pltr > Pamy (C. Zheng *et al.,* unpublished results).

Some transgenes lead to higher rates of expression compared to other transgenes. For example, we infected rat submandibular glands with two serotype 5, $E1^-$, replication-deficient recombinant adenoviruses encoding two different human secretory proteins. One vector encoded human tissue kallikrein (hK1), a serine protease normally produced by salivary epithelial cells (Baum *et al.,* 1999; Garrett *et al.,* 1995), whereas the other encoded human growth hormone (hGH), an endocrine hormone normally produced by the anterior pituitary gland. In both vectors the transgenes used were driven by the Pcmv. Thus, the vectors were essentially identical, except for the transgene encoded. Interestingly, rat submandibular glands on average made about 260 ng of total hK1 product but about 59 μg total hGH product (i.e., a >200-fold difference), a quite unexpected result. In the same study (Baum *et al.,* 1999), we also infected rat submandibular glands with an adenoviral vector encoding human α_1 antitrypsin (hα-1AT), a secretory protein normally made in the liver. This vector used the adenovirus serotype 2 major late promoter (Pmlp), which is not as powerful as Pcmv. Nonetheless, the expression of hα-1AT driven by Pmlp in infected submandibular glands was about 7-fold that of hK1 driven by Pcmv. Clearly, there are many considerations affecting transgene expression that we do not yet understand.

D. Problems

The problems associated with transferring genes to salivary glands can be categorized into two types; those general to the state of gene transfer technology and those relatively specific to salivary applications. The key general problems will be discussed here, whereas other problems that are more parochial to gene transfer applications to salivary glands will be discussed later. Arguably, the most critical general problem facing the gene transfer community is the lack of high-quality vectors. All the vectors currently being employed with salivary glands (and in general use) have significant shortcomings. No available vector is perfect for any current usage. Most of the reported experience with salivary gland gene transfer has come from studies using adenoviral vectors. However, adenoviral vectors are well recognized as eliciting potent host responses involving an innate response (i.e., neutrophils and macrophages) as well as both cellular and humoral immune responses (Harvey *et al.,* 2001; Kagami *et al.,* 1998; Otake *et al.,* 1998; Yang *et al.,* 1995b, 1996).

Following delivery of an adenoviral bolus to salivary glands and other target tissues, most vector DNA is lost within the first 24–48 h due to innate responses (Wolff *et al.,* 1997; Adesanya *et al.,* 1996; Wang *et al.,* 1999). This immediate response leads to profound vector dose-dependent inflammatory changes in the glands, which can initially result in a marked (∼75%) reduction in salivary fluid production (Adesanya *et al.,* 1996). Apparently, there is a threshold level for this response because a smaller viral dose (10^6 pfu) yields no overt changes in gland gross or histological appearance, whereas animals infected with $>10^8$ pfu show substantial inflammatory changes (Adesanya *et al.,* 1996). Several days after this initial, innate response, cytotoxic lymphocytes begin to target transgene-expressing cells and mediate much of their destruction. As noted earlier, gene expression from a typical first-generation recombinant adenoviral vector, such as that which we have used, peaks at 1–3 days postinfection and is at near background levels by Days 7–10 (Adesanya *et al.,* 1996; Kagami *et al.,* 1998). Subsequent attempts to redeliver vector to the glands are prevented by the presence of neutralizing antibodies, most notably IgA and IgM for salivary glands. When Kagami *et al.* (1998) infected rat parotid glands at 7-day intervals with a recombinant adenovirus encoding chloramphenical acetyltransferase, naive animals gave maximal transgene expression on Day 3 (100%). On Day 10 (3 days after the second vector dose), expression was at about 20% maximum, whereas on Day 17 (3 days after the third dose), no transgene expression was found in the glands.

Because the host immune response to adenoviral vectors is well recognized and universally experienced, there have been many attempts to blunt this response and, consequently, enhance and prolong transgene expression. For example, many studies have employed different types of immunosuppressive drugs (Bouvet *et al.,* 1998; Gilgenkrantz *et al.,* 1995; Wilson and Kay, 1995). In our studies we have

routinely used dexamethasone administered at high dose (∼1 mg/animal) intramuscularly (Adesanya *et al.,* 1996). This maneuver significantly enhances transgene expression in infected salivary glands (Adesanya *et al.,* 1996; Delporte *et al.,* 1997b). We have also successfully induced oral tolerance to adenoviral vectors in rats via multiple peroral feedings (gavage) of UV-inactivated virus (Kagami *et al.,* 1998). Tolerized animals showed prolonged and enhanced transgene expression, compared to nontolerized controls, and displayed substantial gene expression even after three intraductal administrations of vector. In this same study, mononuclear cells from the spleens of tolerized animals showed reduced proliferation in response to an adenoviral stimulus, and the population of $CD8^+$ T cells was relatively depleted. Thus, the oral tolerization of rats to adenoviral vectors can result in downregulation of Th1 responses and can considerably improve gene transfer to salivary glands.

An additional safety concern with adenoviral vector-mediated salivary gland gene transfer is that these vectors are directly cytotoxic at modest doses (*in vitro* at >50–100 infectious units/cell). Two recent studies examined this in detail *in vitro* (Wersto *et al.,* 1998; Brand *et al.,* 1999) and showed that these vectors result in a cell cycle arrest at the G_2/M phase. This is associated with the inappropriate expression of cyclins A, B1, and D and the cyclin-dependent kinase $p34^{cdc2}$, and it appears to be mediated through p53 (Wersto *et al.,* 1998; Brand *et al.,* 1999). Apparently, the adenoviral E4 gene region is responsible for this cytotoxic response (Wersto *et al.,* 1998). Thus, investigators wishing to study cellular growth associated properties must be cautious in using adenoviral vectors as experimental tools. We also studied adenoviral vector-associated cytotoxicity in salivary cells *in vitro* (Zheng *et al.,* 2000b) and showed that the toxicity is in part related to the type of transgene expressed. For example, we observed that adenoviral vectors with transgenes encoding secretory proteins yielded significantly less G_2/M arrest and reductions in cell number than comparable vectors encoding cytoplasmic reporter proteins (Zheng *et al.,* 2000b).

A further important general problem with virtually all the transgene constructs currently employed for animal and clinical gene transfer is that expression is unregulated and continuous. This problem is widely recognized and the subject of intensive research (Clackson, 2000). Most efforts have explored the use of small molecules (typically existing pharmaceuticals or their derivatives) for regulating transgene expression through a transactivator protein that binds the molecule. Sufficient progress has been made to allow widespread applications with cell culture systems for some approaches (e.g., the increasing use of tetracycline response elements in gene regulation, Freundlieb *et al.,* 1999). A different approach, dimerizer technology using a small molecule pharmaceutical to facilitate assembly of a transactivator protein, has shown promise in mimicking some physiological regulatory processes (Rivera *et al.,* 2000). These regulatory tools are not ready for clinical applications, though there is reason to be optimistic for the future (Clackson, 2000).

V. Use for Biological Questions

A. Rationale for Use of Gene Transfer by Adenoviral Vectors

1. *In Vitro*

Gene transfer in the laboratory setting, particularly the use of plasmid transfections (e.g., calcium phosphate precipitation and liposome-mediated electroporation) *in vitro* with cultured cells, is commonly used today to address a wide variety of important biological questions. The value in using viral vectors, especially adenoviral vectors, for gene transfer *in vitro* is that these vectors are highly efficient in transferring genes. Thus, by using a relatively modest dose of an adenoviral vector an investigator can routinely and reproducibly transfer the gene of interest to 100% of the target cells. Alternatively, gene transfer to a lesser fraction of cells can be reproducibly attained if desired. This approach has been helpful for our studies examining the relationship between the expression of aquaporin-1, a water channel protein, and transepithelial fluid movement (Delporte *et al.,* 1998; Hoque *et al.,* 2000).

In our experience, use of adenoviral vectors for gene transfer can offer a significant practical advantage to the investigator. Plasmid transfection techniques are inefficient and many experiments will ultimately require a subsequent selection step (e.g., antibiotic selections) in order to enrich (or make "uniform") the cell population employed to test the biological question of interest. Cell selection methods are not necessary for obtaining uniform transgene expression if adenoviral vectors are used for gene transfer. Although it is true that to construct a first-generation adenoviral vector takes time, the methods for doing so have greatly improved (T. C. He *et al.,* 1998). With the gene of interest in hand, it is possible to generate a new adenoviral vector in quantities suitable for cell culture use within 7–10 days' time.

2. *In Vivo*

A second important consideration for using adenoviral vectors in understanding biology is that experiments involving gene transfer can routinely be performed *in vivo* as well as *in vitro.* Without question, the use of cultured cell models has enormously helped the rate and extent of progress in understanding countless features of eukaryotic cell biology and pathology. However, it can be credibly argued that most cells (certainly all cell lines currently employed) grown in culture for experimental use are not entirely normal, nor are the circumstances of their "lives" normal. Hence, our ultimate understanding of true physiology requires deciphering or confirming biological mechanisms in an intact organism. Transfection techniques (e.g., use of cationic liposomes or a gene gun) can be used *in vivo,* but they are inefficient at present. Conversely, an adenoviral vector can be used *in vivo*

and lead to a large percentage of the target cells expressing the transgene. For example, we were able to transfer aquaporin-1 into about 30% of rat submandibular gland epithelial cells *in vivo* using a first-generation adenoviral vector (Delporte *et al.,* 1997a).

Every experimental technique has benefits and drawbacks. The use of adenoviral (or other viral) vectors for transferring genes to selected target tissues *in vivo* is certainly not without some shortcomings. However, as is evidenced by the examples provided next, using an adenoviral vector experimentally, along with appropriate controls, can facilitate answering some important biological questions.

B. Examples of Applying Adenoviral Vectors to Address Biological Questions

1. Endocrine Secretion

Salivary glands are considered to be classic exocrine glands. However, for much of the past century, there has been a steady literature hinting that salivary glands could also secrete in an endocrine fashion (Isenman *et al.,* 1999; Lawrence *et al.,* 1977; Leonora *et al.,* 1987). Such a role has never been proven unequivocally, and endocrine secretion is not routinely considered to be part of the physiological function of these glands. Interestingly, it is well recognized that salivary epithelial cells, like other epithelial cells, sit on a basement membrane, presumably resulting in large part from its own biosynthetic activity. Thus, to us it seemed likely that salivary cells contain an endogenous pathway that leads to secretion from the basolateral pole of these cells, in addition to the well-studied exocrine pathway across the apical membranes. Using gene transfer techniques *in vivo,* we demonstrated clearly that endocrine secretion from salivary glands occurs. In our original study (Kagami *et al.,* 1996) we employed a first-generation adenoviral vector encoding hα1AT. hα1AT is not normally produced by salivary glands. Rather, it is a product of the liver, secreted into the bloodstream. Fortunately, we were able to readily distinguish hα1AT from the homologous rat protein without any immunological cross reactivity using a very sensitive enzyme-linting immunosorbent assay.

After infection of rat submandibular glands *in vivo* with this vector, we measured the levels of the transgene product, hα1AT, in saliva, serum, and aqueous gland extracts during a week-long period. The highest concentrations of secreted hα1AT were found in saliva (average of about 100 ng/ml during the first 4 days). Serum hα1AT levels were considerably lower (average of about 10 ng/ml). When considering these data, it is important to recognize that the volume of each biological fluid is markedly different. In our work we assume that the average total volume of saliva that can be collected from an adult rat submandibular gland is 150 μl

during a 30-min period following stimulation with the parasympathomimetic drug pilocarpine. Conversely, we consider the total blood (serum) volume in adult rats to be about 8 ml. These assumptions may not be accurate for any given individual animal. However, they are a constant and the relationship between amounts of hα1AT in saliva and serum would thus be consistent. Based on such assumptions, our calculations for the actual total amount of hα1AT secreted from glands into saliva at peak values was 0.15 ml $\times$ 100 ng/ml or 15 ng. For serum, the comparable calculation was 8 ml $\times$ 10 ng/ml or 80 ng hα1AT secreted into the bloodstream, about fivefold that in saliva.

These results, along with appropriate controls, strongly suggested that rat salivary glands could secrete a significant amount of hα1AT in an endocrine manner. However, to test this notion rigorously, we performed a classical experiment in endocrine physiology. We cannulated the carotid artery (afferent blood supply) and submandibular vein (venous effluent) and measured hα1AT levels in the serum from each site. We hypothesized that if hα1AT was truly being secreted from glandular cells in an endocrine manner, then hα1AT levels in the venous effluent would always be greater than those in the arterial blood. This was directly tested following gene transfer to rat submandibular glands *in vivo.* In all seven animals studied, the venous effluent had higher concentrations of hα1AT than did the arterial blood. On average, serum from venous blood contained about 3.5-fold more hα1AT than did the arterial samples. Similar experiments were performed with rat parotid glands (three animals) and showed comparable results (Kagami *et al.,* 1996).

2. Sorting Pathways

We also asked an important, related biological question, and have begun to address it using similar adenoviral-mediated gene transfer methods. We asked if salivary glands *in vivo* were able to sort secretory proteins into polarized pathways, i.e., recognize presumptive sorting signals encoded within the protein and direct the protein out of the cell. Classically, protein secretion is considered to occur via one of two pathways based on the work of Kelly and colleagues in the 1980s (Kelly, 1985; Burgess and Kelly, 1987). Secretion is considered constitutive if a protein is secreted by a cell at a constant rate, similar to its rate of synthesis. Conversely, secretion is considered regulated if secretory proteins are stored and concentrated into vesicles or granules prior to being released in response to an appropriate extracellular signal. In salivary glands the regulated pathway leads to protein secretion into saliva, i.e., zymogen granules containing exocrine proteins fuse with the apical plasma membrane and release their contents into the forming saliva (Castle and Castle, 1998).

Virtually all previous studies that defined constitutive and regulated secretion mechanisms were conducted with *in vitro* cell line models in culture. To test if these

mechanisms were operative *in vivo,* we compared the distribution (saliva:serum) of secretory proteins encoded by transgenes delivered to rat submandibular glands via first-generation recombinant adenoviral vectors (Baum *et al.,* 1999). Most important, two distinctly different proteins, in terms of their secretory behavior, were tested: hGH and hα1AT. hGH is secreted physiologically from the anterior pituitary via a regulated pathway, whereas hα1AT is secreted primarily from the liver via a constitutive pathway. Thus, we predicted that hGH would be secreted primarily via the regulated pathway (saliva:serum distribution ratio >1), whereas hα1AT was predicted to show a distribution ratio <1. As a control protein, to determine if the presence of either protein in serum or saliva was due to tissue damage (i.e., leakage), we used an adenoviral vector encoding firefly luciferase, a sensitive reporter gene that should remain in the cytoplasm.

We found that under the conditions used for our experimental measurements (4 days postvector delivery to glands), very little luciferase was detected in these two extracellular fluids (Baum *et al.,* 1999). Importantly, we found that both of these often studied secretory proteins behaved as predicted. hGH was primarily secreted into saliva (distribution ratio $\sim$9:1), whereas hα1AT was primarily secreted into the serum (distribution ratio $\sim$1:5). Thus, salivary glands *in vivo* can distinguish between these two transgene-encoded proteins and sort them into two distinct polarized secretory pathways.

3. Aquaporin Function

For several years we have studied the possible role that the aquaporin water channel family proteins play in the mechanism(s) by which salivary fluid is secreted (Li *et al.,* 1994; Delporte *et al.,* 1996; Wellner *et al.,* 2000). Aquaporins are homotetrameric plasma membrane proteins that provide a facilitated pathway for water movement (see Section II; Agre *et al.,* 1998). At least nine mammalian homologs have been found, including two in salivary glands [aquaporin-5 by Raina *et al.* (1995) and aquaporin-8 by Koyama *et al.* (1997)]. In several of our studies, we utilized recombinant adenoviral vectors encoding aquaporins to ask biologically relevant questions.

For multiple reasons, we have had a long-standing interest in the relationship between the expression of the water channel protein, aquaporin-1, and transepithelial fluid movement (Delporte *et al.,* 1998). Specifically, we were interested in determining the number of cells in an epithelial monolayer with relatively low water permeability that were necessary to express aquaporin-1 in order to attain high levels of transepithelial fluid movement. Using an adenoviral vector encoding aquaporin-1 (AdCMVAQP1) we were able to examine this question with a model polarized salivary epithelia *in vitro* (Table II). With this vector we could easily and reproducibly transduce different numbers of cells with the aquaporin-1 cDNA. We used AdCMVAQP1 across a wide dosage range (from 0.01 to 10 infectious units per cell). We found that both aquaporin-1 expression and the number of cells

TABLE II

Effects of Infectious Dose of AdCMVAQP1 on Fluid Movement across a SMIE Cell Monolayer[a]

Vector (MOI)	0.1	0.5	1.0	5.0
	9	22	35	49

[a] Data are modified from average results presented in Delporte *et al.* (1998) and represent fluid movement across a polarized monolayer of SMIE (rat submandibular immortalized epithelial) cells. Fluid movement is expressed as μl/cm^2. MOI is the multiplicity of infection of AdCMVAQP1 used.

transduced were generally proportional to viral dose. Transepithelial fluid movement also increased proportionally. This increase in fluid movement occurred in a biphasic manner, with approximately 70% of the maximal effect (first phase) achieved at a relatively low viral dose (0.5 infectious units/cell). Under these conditions few cells in the monolayer were transduced. The implication of the results is that substantial fluid movement may only require the expression of a functional water channel protein by a minority of the cells present.

Another example of our use of recombinant adenoviral vectors to study aquaporins derives from a recent study by Wellner *et al.* (2000). This study focused on the cellular localization of aquaporin-8. The cloning of aquaporin-8 from liver was reported by Koyama *et al.* in 1997. In addition to liver, several tissues were shown to express this aquaporin isoform by RNA protection assays combined with *in situ* hybridization. However, no antibody-based protein localization was reported. We generated polyclonal antibodies to different peptide regions of aquaporin-8 and examined native salivary gland tissue. We obtained different results with an antibody directed against a C-terminal peptide (plasma membrane) than those obtained with an antibody directed at an N-terminal peptide (a nonspecific nuclear pattern).

There are no available cell lines reported to express aquaporin-8; hence, it was impossible for us to characterize our antibodies with a model system more simple than a complex native tissue. To help us, we constructed a recombinant adenovirus encoding aquaporin-8 (AdCMVAQP8). Thereafter, we used AdCMVAQP8 to infect a commonly employed epithelial cell line (293 cells) and direct the expression of aquaporin-8. In all our studies with both the C-terminal-directed and N-terminal-directed antibodies, we found that immunostaining was localized to the plasma membranes in a virtually identical pattern (Fig. 4). Similarly, use of the two antibodies to probe Western blots of electrophoresed membrane proteins from the transduced cells yielded comparable results. Thus, we concluded that both antibodies could recognize aquaporin-8 under native and denatured conditions. The implication for our studies with rat salivary glands was that the N-terminal epitope was not available to its cognate antibody (i.e., it was removed or rendered cryptic by posttranslational processing) (Wellner *et al.,* 2000).

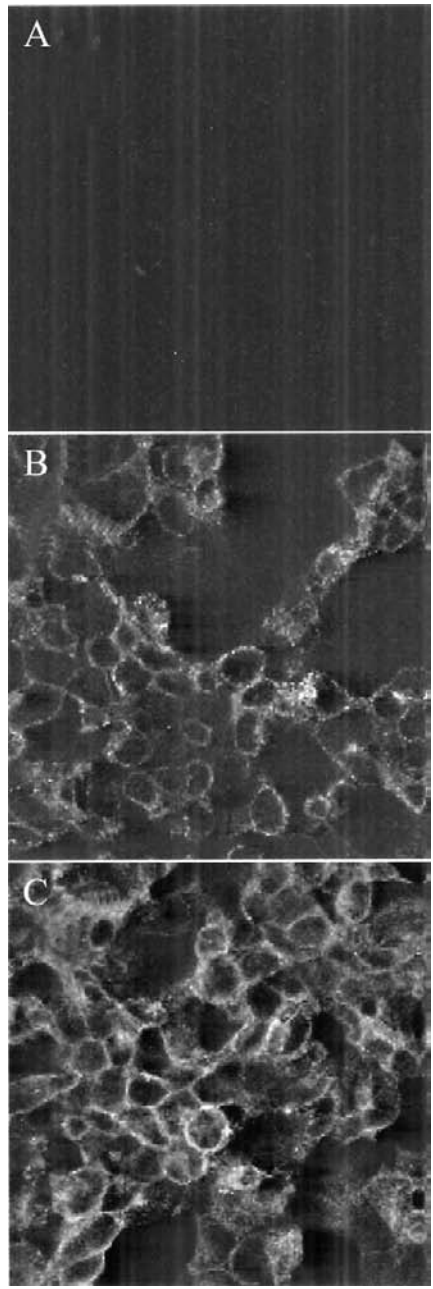

FIG. 4 Immunolocalization of rat aquaporin-8 (rAQP8) in 293 cells infected with AdCMVAQP8. One-micrometer optical sections are shown. (A) Background seen without primary antibody. (B) Cells immunostained with antibody to the C terminal of rAQP8. (C) Cells immunostained with antibody to the N terminal of rAQP8. Methods used to obtain confocal images are reported for similar experiments described in Wellner *et al.* (2000).

4. Store-Operated Calcium Entry

A recent report describes a quite different biological question addressed by employing adenoviral vectors. Singh *et al.* (2001) wished to study the role of a putative store-operated Ca^{2+} entry (SOCE) pathway in salivary gland fluid secretion. Ca^{2+} plays an essential role in intracellular signaling (Ambudkar, 2000). In normal, unstimulated circumstances levels of cytosolic-free Ca^{2+} ($[Ca^{2+}]_i$) are quite low ($\sim 10^{-7}\,M$). Following an appropriate physiological signal (e.g., for salivary glands, stimulation of muscarinic–cholinergic receptors), $[Ca^{2+}]_i$ is increased $\sim$10-fold within seconds (see Section II). Although some of this Ca^{2+} comes from intracellular stores, for sustained fluid secretion from these glands most free Ca^{2+} enters from the extracellular milieu. In nonexcitable cells the pathway by which Ca^{2+} enters is not understood (Ambudkar, 2000).

Recently, it has been suggested that homologs of the *Drosophila* Trp gene may function as a SOCE pathway. Singh *et al.* (2001) constructed a recombinant adenovirus encoding the human Trp1 (hTRP1) gene that was epitope tagged with a hemagglutinin (HA) peptide. This vector, AdCMV–hTrp1, directed the expression of HA-tagged hTrp1 in human salivary cells *in vitro,* which was readily distinguished from endogenous hTrp1. *In vitro* this viral-directed hTrp1 functioned as a SOCE pathway.

Singh *et al.* (2001) then used AdCMV–hTrp1 to infect rat submandibular glands *in vivo,* in which it directed substantial hTrp1 expression, and its appropriate localization to the basolateral membrane. Most important, the infected rat submandibular glands exhibited a fivefold increase in salivary fluid secretion after parasympathomimmetic stimulation compared to salivary secretion observed in rat glands infected with a control adenoviral vector. Also, acinar cells isolated from AdCMV–hTrp1-infected glands and stimulated *in vitro* displayed marked increases in SOCE. Together these data show that hTrp1 can function to enhance Ca^{2+} entry to salivary cells and directly demonstrate for the first time *in vivo* that SOCE has an important role in agonist-stimulated salivary fluid secretion.

VI. Use for Clinical Questions

A. Rationale for Using Gene Transfer

The only rationale for using gene transfer for clinical purposes is that there is currently no suitable conventional therapy. The particular impetus for our studies is the lack of treatment options available for patients who have lost acinar cells in their salivary glands. The two primary causes of this situation in the United States are an autoimmune disease (Sjogren's syndrome; $\sim$1 million persons, 9:1 female:male) and therapeutic radiation used in the management of a head and neck cancer ($\sim$40,000 new patients per year, predominantly males $>$50 years old).

TABLE III
Problems Associated with a Lack of Saliva Production

Dysphagia
Tooth destruction (increased dental caries)
Dysgusia
Dysarthria
Mucosal alterations (ulcerations and infections)
Pain and discomfort

As noted in Section II, acinar cells are the sole site of fluid movement in salivary glands, and they produce the vast majority (~85%) of salivary proteins. Patients with only ductal cells cannot secrete salivary fluid. Although the absence of saliva per se is not fatal, it does cause considerable morbidity and may pose some mortal risk because of the consequent dysphagia (Table III).

B. Examples of Applying Adenoviral Vectors to Address Clinical Questions

1. Radiation Damage

Our initial studies focused on the tissue damage resulting from radiation therapy because we reasoned that this would provide fewer complications than autoimmune pathology. We used irradiated rats as our experimental model (Delporte *et al.,* 1997a), although rats are known to be relatively radiation resistant compared to humans. We hypothesized that it was possible to convert the radiation surving ductal cells, which are relatively water impermeable, to a more water-permeable phenotype by the transfer of the aquaporin-1 cDNA using AdCMVAQP1. Furthermore, we suggested that these ductal cells, in the relative absence of any primary salivary fluid due to acinar cells loss, would be capable of generating a potassium bicarbonate osmotic gradient (lumen > interstitum) that could provide the driving force for transepithelial fluid movement.

In a critical series of experiments, rats were exposed to 21 Gy irradiation and followed for 4 months. After 4 months control animals, exposed only to anesthesia and a sham irradiation, displayed salivary flow rates of ~37 μ1/100 g body weight/15 min (Table IV). Irradiated animals experienced a considerable reduction in salivary flow to ~13 μl/100 g body weight/15 min. A third group of animals were treated identically to those in the sham-irradiated group; however, a control recombinant adenovirus without a transgene was administered to their submandibular glands. These animals had salivary flow rates statistically indistinguishable from those of the sham-irradiated group. Finally, a fourth group

TABLE IV
Effects of AdCMVAQP1 on Saliva Secretion from Irradiated Rat Submandibular Glands[a]

Treatment	Control virus	AdCMVAQP1
Sham	37	28
21 Gy	13	31

[a] Data are summarized from average results presented in Delporte *et al.* (1997a) and represent pilocarpine-stimulated submandibular salivary flow rates (μl/100 g body wt/15 min). Animals were either irradiated (21 Gy) or not (sham) 4 months prior to transfer of the human aquaporin-1 cDNA via AdCMVAQP1 (5×10^9 plaque-forming units/gland). The salivary flow rate of irradiated rats treated with the control virus is significantly different from all other values, which are not different from each other.

of rats were irradiated with 21 Gy, but after 4 months AdCMVAQP1 was administered to their submandibular glands. These animals also had salivary flow rates essentially identical to those of sham-irradiated animals receiving the control adenovirus (i.e., statistically indistinguishable from animals not subjected to irradiation). Thus, at least in rats, it is possible to provide a type of correction for the salivary hypofunction that results from irradiation by transferring the aquporin-1 cDNA into surviving epithelial cells.

2. Exocrine Gene Therapeutics

An obvious application for gene therapy to salivary glands is the augmentation of saliva with a transgene-encoded protein for treating a disorder of the upper gastrointestinal tract. We originally chose to address a serious complication found in immunosuppressed patients—mucosal candidiasis caused by *Candida* species resistant to the commonly used azole-type antifungal drugs (Como and Dismukes, 1994). This condition results in considerable morbidity and mortality, and there are still no suitable alternative medications available to treat this condition.

Lal *et al.* (1992) had shown that levels of the naturally occurring salivary anticandidal peptides, the histatins, were reduced in patients with AIDS and that this reduction correlated with increased oral candidiasis. Accordingly, we hypothesized that using gene transfer to overexpress histatin peptides in the saliva of AIDS patients might be a novel and effective approach to kill azole-resistant *Candida* and manage the resulting mucosal candidiasis.

We constructed a recombinant adenoviral vector encoding histatin 3 (O'Connell *et al.,* 1996), termed AdCMVH3, and infected rat salivary glands with the vector. Importantly, rodent salivary glands do not normally produce histatins, so we had no

problems with background levels of the peptide. After infection with AdCMVH3, rats began to produce copious amounts of histatin 3, with many animals secreting the peptide at levels well in excess of those found in the saliva of healthy humans (i.e., $\gg$50–100 μg/ml). Importantly, the adenoviral vector-directed histatin 3 was able to prevent germ tube formation and kill blastospores of azole-resistant *Candida* species (O'Connell *et al.,* 1996).

3. Endocrine Gene Therapeutics

The third clinical application of gene transfer to salivary glands that we pursued was systemic gene therapeutics based on our demonstration of the endocrine secretion of transgene products from the glands. To test this possibility, we chose to utilize the adenoviral vector we constructed which encodes hGH (AdCMVhGH; X. He *et al.,* 1998). It is well recognized that the current approach of treating patients with a growth hormone deficiency by the repetitive injection of recombinant hGH is inconvenient and biologically undesirable. Furthermore, the choice of hGH was useful experimentally because (i) there are excellent assays readily available to measure hGH and to distinguish it from the rat homolog and (ii) rodent GH receptors can bind to, and be activated by, hGH.

We administered AdCMVhGH to rat submandibular glands and measured serum levels of hGH as well as other serum factors whose levels could provide evidence of the systemic biological activity of the transgenic hGH [e.g., insulin-like growth factor-1 (IGF-1)]. In these experiments, we achieved supraphysiological levels of hGH in rat sera, ($\sim$15–20 ng/ml; Table V). We also detected a corresponding increase in rat IGF-1 levels in serum, directly demonstrating that an endocrine hormone secreted from salivary glands as a transgene product can have systemic biological activity. Furthermore, we also observed elevations in serum triglycerides and the blood urea nitrogen/creatinine ratio in serum, both general indicators of anabolic activity. In aggregate, our results are consistent with the notion that salivary glands may provide an unusual, and useful, target site for some applications of gene therapeutics.

TABLE V
Serological Effects of AdCMVhGH Administration to Rat Salivary Glands[a]

Treatment	hGH	IGF-1	Triglycerides	BUN/creatinine
Control	1.2	642	101	22
Vector	16.4	803	198	29

[a]Data are summarized from average results presented in X. He *et al.* (1998). The serum values for human growth hormone (hGH) and rat insulin-like growth factor-1 (IGF-1) are in ng/ml, and values for triglycerides are in mg/dl. The ratio of blood urea nitrogen (BUN) to creatinine in rat serum is also shown.

C. Status and Problems

We have demonstrated proof of concept for three potential clinical applications of salivary gland gene transfer. However, in addition to the more general problems associated with gene transfer described earlier, there are other, more tissue-specific impediments. For example, with one exception all published studies of *in vivo* salivary gland gene transfer have utilized either rats or mice. The results of murine studies indicated that inflammatory responses to adenoviral vectors described previously were greater than those observed in rats (Wang *et al.,* 1999). Unfortunately, compared with several other epithelial tissues there are relatively few studies of gene transfer to salivary glands. It is not clear whether predictions of human immune responses based on any rodent studies will be useful. Indeed, studies of gene transfer to lung tissue have suggested that immunopathology observed in rodents is not necessarily predictive of results in humans (Harvey *et al.,* 2001).

Another concern limiting applications of salivary gland gene transfer is that we still do not know the extent to which transgene expression is able to persist in transduced cells, even if an "ideal viral vector" were available. A major reason for this is that we do not accurately know the life span of salivary cells. Furthermore, we do not know whether those cells that are transduced by viral vectors are capable of dividing or are merely shed off into the duct lumen. The latter circumstance would restrict the impact of any gene transfer event in this tissue, regardless of the vector employed. There are long-standing controversies in the salivary biology literature regarding which cells in adult tissues are capable of division. One argument suggests that most cell types (acinar and ductal) are capable of division, whereas another argues that cell division is restricted to stem cells within the gland (Zajicek *et al.,* 1985; Ballagh *et al.,* 1994; Redman, 1995). These fundamental properties of salivary epithelial cells must be clarified in order to make rational biological and clinical use of gene transfer.

Similarly, another problem that is hindering salivary gland gene transfer is that we have only limited knowledge of how gene expression is regulated in salivary cells (Baum and O'Connell, 1999). There is a substantial amount of information on only one salivary promoter (human amylase; Ting *et al.,* 1992). For example, we have some knowledge of the promoters controlling the expression of the rat proline-rich protein (Lin *et al.,* 1996), mouse parotid secretory protein (Laursen and Hjorth, 1997), and human kallikrein (Chao and Chao, 1996) genes, but much more is needed. Additionally, relatively little is understood about other genetic elements involved in regulating salivary proteins (e.g., enhancers, silencers, and preferential salivary transactivating factors). Such information is critical to developing gene transfer constructs displaying safe, efficient, and specific expression in salivary target cells.

Salivary glands are capable of producing substantial amounts of protein for export. However, the mass of an individual salivary gland is much less than that of other gene transfer target tissues (e.g., liver and lung). Thus, a transduced salivary

gland is unlikely to be a satisfactory choice for the gene therapeutic target site when high levels of protein product are required. For example, in rats with modest doses of recombinant adenoviral vectors the levels of transgenic hGH expression achieved are in a range suitable for clinical applications. Conversely, similar doses of vector-encoding hα1AT produced serum levels inadequate for therapeutic purposes; at least 10-fold these levels would likely be needed to achieve any therapeutic impact. As is likely the case with all potential gene transfer target sites, for some applications salivary glands will be useful; for others, they will not.

However, there is an additional problem in using salivary glands as a target replacement tissue for hGH. This problem was uncovered through our experiments examining the ability of salivary glands to sort secretory proteins into polarized pathways. As noted previously, hGH is secreted preferentially into saliva $\sim$9:1 via the endogenous regulated pathway. This means that most of the transduced hGH produced in salivary glands is not available for physiological use. Rather, most hGH is secreted into the mouth and gastrointestinal tract, where it is not useful therapeutically. If it were possible to efficiently direct hGH into the bloodstream instead of saliva, it would theoretically be possible to decrease the virus dose administered by a log step and likely markedly reduce the untoward effects of the higher virus dose.

Finally, a major problem specific to the use of aquaporin-1 to correct irradiation-induced gland damage is that we do not understand the mechanism by which the effect is achieved in rat glands. Our original findings were consistent with our hypothesis that ductal cell fluid secretion could occur when aquaporin-1 expression was present because the cells were capable of generating a potassium bicarbonate gradient (Delporte *et al.,* 1997a). However, we have not proven this hypothesized mechanism. A particular difficulty in addressing this problem is that we need to identify, and study physiologically, living transduced cells expressing aquaporin-1. To accomplish this, we have recently succeeded in constructing a recombinant adenovirus encoding an aquaporin-1/green fluorescence protein fusion product (Hoque *et al.,* 2000). This fusion product is able to function as a water channel, albeit at $\sim$50% of the efficacy of native aquaporin-1. Nonetheless, we are now poised to examine ion transport and other functions in individual, living irradiated acinar and ductal cells transduced *in vivo.*

VII. Concluding Remarks

In this article, we reviewed the application of gene transfer technologies to salivary glands for addressing both biological and clinical questions of significance. We began by reviewing the biology of salivary glands. Although this might seem to be expected, it reflects our strong belief that the utility of this or any other technology critically depends on having a sound understanding of the biology of the tissue to

which the technique is to be applied. Thus, all our studies reflect our efforts in the context of a strong biological grounding.

We next provided the reader with a spectrum of potentially useful strategies for gene transfer, both viral and nonviral. The discussion of vector options presented here is not intended to be comprehensive but rather an overview. The reader interested in applying any particular vector type should read more specific and detailed articles on the subject. Clearly, adenoviral vectors have had the greatest impact on salivary gland studies, as they have for many other tissues. Adenoviral vectors are very useful for slowly growing or nondividing, well-differentiated cells and for establishing proof of concept. However, as we noted, adenoviral vectors have significant limitations for both biological and clinical studies. In the long run, other vector systems may prove more valuable. Additionally, because of the potential impact of gene therapy clinically, we expect that new and better vectors will be developed in the future.

We also provided the reader with a detailed description of studies conducted in salivary glands. This included a variety of general findings as well as some in response to asking specific questions, both biological and clinical. The result is an overview that we believe is representative of applications to many tissues not subjected to common study by investigators in this emerging field. Readers may or may not be interested in conducting salivary gland studies; however, we hope that this article is useful to all as an example.

Finally, we discussed problems in transferring genes to salivary glands, of which there are many. However, we view these problems as a normal part of the scientific endeavor (i.e., they are meant to be solved for progress to occur). It is clear to us that viral-mediated gene transfer offers a powerful tool for the cell biologist, especially for extending studies from cell culture models to *in vivo* studies. We hope the readers will agree and find this article helpful.

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Cell Type Specific Expression of Secretory TFF Peptides: Colocalization with Mucins and Synthesis in the Brain

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The "TFF domain" is an ancient cysteine-rich shuffled module forming the basic unit for the family of secretory TFF peptides (formerly P-domain peptides and trefoil factors). It is also an integral component of mosaic proteins associated with mucous surfaces. Three mammalian TFF peptides are known (i.e., TFF1–TFF3); however, in *Xenopus laevis* the pattern is more complex (xP1, xP4.1, xP4.2, and xP2). TFF peptides are typical secretory products of a variety of mucin-producing epithelial cells (e.g., the conjunctiva, the salivary glands, the gastrointestinal tract, the respiratory tract, and the uterus). Each TFF peptide shows an unique expression pattern and different mucin-producing cells are characterized by their specific TFF peptide/secretory mucin combinations. TFF peptides have a pivotal role in maintaining the surface integrity of mucous epithelia *in vivo*. They are typical constituents of mucus gels, they modulate rapid mucosal repair ("restitution") by their motogenic and their cell scattering activity, they have antiapoptotic effects, and they probably modulate inflammatory processes. Pathological expression of TFF peptides occurs as a result of chronic inflammatory diseases or certain tumors. TFF peptides are also found in the central nervous system, at least in mammals. In particular, TFF3 is synthesized from oxytocinergic neurons of the hypothalamus and is released from the posterior pituitary into the bloodstream.

KEY WORDS: TFF domain, Mucins, Cell migration, Apoptosis, Ulcer, Inflammation, Goblet cell, Pituitary.

I. Introduction

The "TFF domain" (trefoil factor family domain; formerly P-domain or trefoil domain; Hoffmann and Hauser, 1993a) represents an ancient shuffled module containing six conserved cysteine residues. The present nomenclature is based on an agreement at a Conference Philippe Laudat (Wright *et al.,* 1997); the single term "trefoil" is avoided because of its manyfold meanings (for example, see Ponting and Russell, 2000).

TFF modules arose well before amphibian evolution: Eggshell proteins were found in teleost fish, an epidermis-specific protein in a tunicate, and an open reading frame in a nematode (Sommer *et al.,* 1999). A linear arrangement of up to four TFF domains is found in three mammalian TFF peptides (i.e., TFF1–TFF3) and in a complex family of *Xenopus laevis* TFF peptides (known members so far are xP1, xP4.1, xP4.2, and xP2). Furthermore, TFF domains are integral constituents of a variety of mosaic proteins. All these proteins are closely associated with mucous surfaces. Examples are human zona pellucida proteins ZP1 and ZPB (Bork, 1993; Harris *et al.,* 1994; Hughes and Barratt, 1999) and the sugar-degrading enzymes sucrase–isomaltase (Tomasetto *et al.,* 1990), α-glucosidase (Hoefsloot *et al.,* 1988), and maltase–glucoamylase (Nichols *et al.,* 1998). Multiple TFF domains are found in frog integumentary mucins FIM-A.1 (formerly spasmolysin; Hoffmann, 1988; Hauser *et al.,* 1990) and FIM-C.1 (Hauser and Hoffmann, 1992) as well as in the APEG protein from *X. laevis* skin, which is a splice variant of xP2 (Hauser *et al.,* 1992). Thus, the TFF domain is an unique shuffled module always encoded by a single exon (Botzler *et al.,* 1999) belonging to the class 1-1 (Patthy *et al.,* 1994).

All TFF peptides are synthesized via precursors containing a cleavable N-terminal signal sequence typical of secretory proteins. Historically, human TFF1 (formerly pS2), containing only a single TFF domain, was the first member of the mammalian TFF peptide family discovered via cDNA cloning of an estrogen responsive gene (Masiakowski *et al.,* 1982; Jakowlew *et al.,* 1984). TFF2 (formerly spasmolytic polypeptide/SP), containing two TFF domains, was detected in porcine pancreas independently at about the same time (Jørgensen *et al.,* 1982; Thim *et al.,* 1985; Rose *et al.,* 1989). Human TFF2 was characterized later (Tomasetto *et al.,* 1990; Gött *et al.,* 1996). The high similarity of TFF1 and TFF2 was first recognized in the course of analyzing TFF domains from the frog integumentary mucin FIM-A.1 (Hoffmann, 1988). TFF3 (previously called intestinal trefoil factor/ITF or hP1.B), containing a single TFF domain, was the third member of this family recognized originally in rat intestine (Suemori *et al.,* 1991), and the human sequence was reported in 1993 (Hauser *et al.,* 1993; Podolsky *et al.,* 1993).

Figure 1 represents a compilation of human TFF domains found in TFF1-3 as well as various mosaic proteins. A minimum consensus sequence for TFF domains is outlined based on invariant amino acid residues in all three human TFF peptides. The TFF-like sequences from zona pellucida proteins and sugar-degrading enzymes each form separate subfamilies. Major hallmarks of the primary structure

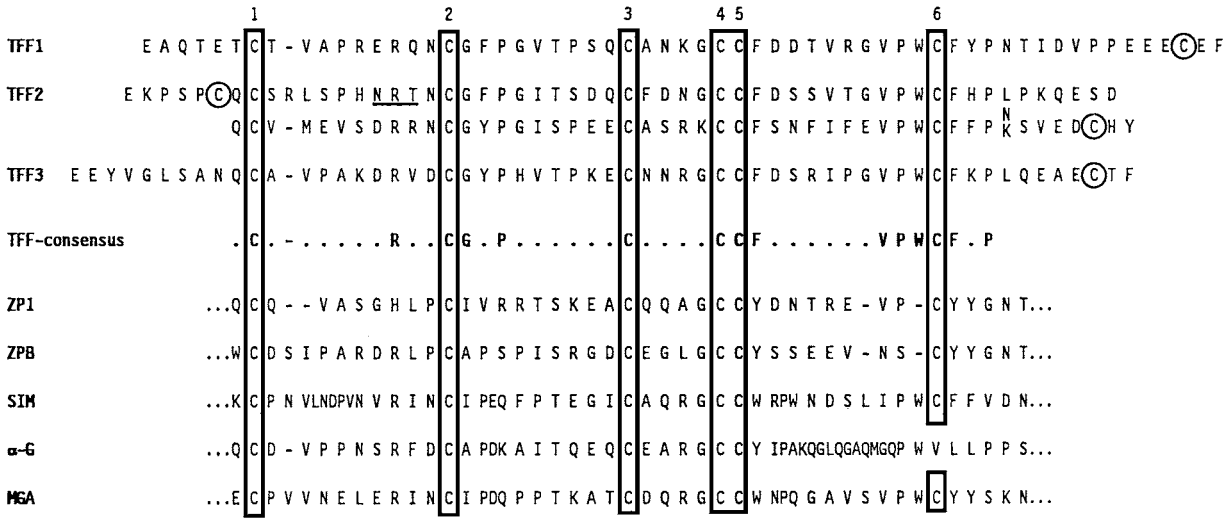

FIG. 1 Comparison of TFF domains found in human peptides and mosaic proteins. Each position given in the TFF consensus sequences is conserved in TFF1 (Jakowlew *et al.,* 1984), TFF2 (Tomasetto *et al.,* 1990; Gött *et al.,* 1996), and TFF3 (Hauser *et al.,* 1993). All further cysteine residues that do not appear in the consensus sequence are encircled. The N-glycosylation site in TFF2 is underlined. Human mosaic proteins containing a TFF-like domain are zona pellucida proteins 1 and B (ZP1 and ZPB; Harris *et al.,* 1994; Hughes and Barratt, 1999), sucrase–isomaltase (SIM; Green *et al.,* 1987), α-glucosidase (α-G; Hoefsloot *et al.,* 1988), and maltase–glucoamylase (MGA; Nichols *et al.,* 1998).

are six conserved cysteine residues, various hydrophobic amino acid residues, and an arginine residue. The conserved cysteine residues form intramolecular disulfide bridges in the order C^1–C^5, C^2–C^4, C^3–C^6 (Thim, 1989), creating three characteristic disulfide loops (L1–L3); a planar representation of this structure resembles a trefoil. The unique pattern of disulfide bridges is responsible for the remarkable resistance of TFF peptides against proteolytic degradation (Jørgensen *et al.,* 1982; Kinoshita *et al.,* 2000).

The three-dimensional structures of porcine TFF2 (Carr, 1992; Carr *et al.,* 1994; Gorman *et al.,* 1992; De *et al.,* 1994; Gajhede *et al.,* 1992, 1993; Petersen *et al.,* 1996), dimeric human TFF1 (Williams *et al.,* 2001), the monomeric C58S TFF1 mutant (Polshakov *et al.,* 1995, 1997), and human TFF3 (Lemercinier *et al.,* 2001) were determined using nuclear magnetic resonance and X-ray crystallography. A characteristic structural feature of all TFF domains is a hydrophobic cleft between loops L2 and L3. In particular, all conserved amino acid residues are either part of this pocket or are placed around it. One of the most important residues in this respect is the tryptophan residue just before C^6.

The single-copy TFF peptides TFF1 and TFF3 form disulfide-linked homo- and heterodimers via an additional cysteine residue at their C-terminal regions (Chinery *et al.,* 1995; Thim *et al.,* 1995; Chadwick *et al.,* 1997; Newton *et al.,* 2000; Moro *et al.,* 2001; Williams *et al.,* 2001). Thus, a pair of TFF domains seems to represent the functional unit for all TFF peptides. However, the spatial arrangement of TFF domains in TFF1 and TFF3 (symmetrical tail-to-tail linkage) is clearly expected to be different from that of TFF2, in which the two TFF domains are linked asymmetrically head to tail. Furthermore, the structure of TFF2 is stabilized by an additional intramolecular disulfide bridge linking the N- and C-terminal regions of the molecule.

II. Biosynthesis and Localization of TFF Peptides

The expression of TFF genes is precisely regulated in different mucous epithelia and in the brain. Consequently, the localization patterns are characteristic for each TFF peptide. The synthesis in mucous epithelia always occurs in combination with mucins. To date, the cellular localization of TFF peptides has been investigated in mammals and *X. laevis* only. Table I gives an overview of the predominant localization of TFF peptides in mucous epithelia. Only minor amounts are detectable in the brain.

A. Physiological Expression in Mammals Including Human

1. TFF1

The stomach is the major source of TFF1. This was shown for human (Rio *et al.,* 1988), rat (Itoh *et al.,* 1996), and mouse (Lefebvre *et al.,* 1993). TFF1 is present

TABLE I
TFF Peptides Detected in Mucous Epithelia of Mammals and *X. laevis*

Name	Origin	Number of TFF domains	Major localization in human and *X. laevis* epithelia
TFF1	Mammals	1	Stomach, conjunctiva
TFF2	Mammals	2	Stomach, Brunner's glands
TFF3	Mammals	1	Intestine, salivary glands, respiratory tract, conjunctiva, endocervix
xP1	*X. laevis*	1	Stomach/fundus
xP2	*X. laevis*	2	Skin
xP4.1	*X. laevis*	4	Stomach
xP4.2	*X. laevis*	4	Esophagus, stomach/fundus

in human gastric juice at varying concentrations between 30 and 100 ng/ml (Rio *et al.,* 1988). Gastric TFF1 is localized mainly within faveolae cells, i.e., the surface epithelial cells and the pits (Rio *et al.,* 1988; Piggott *et al.,* 1991; Hanby *et al.,* 1993b; Newton *et al.,* 2000), where it is expressed (Tomasetto *et al.,* 1990; Hanby *et al.,* 1993b; Lefebvre *et al.,* 1993). Human gastric faveolae are also major sites for the synthesis of the secretory mucin MUC5AC (Audie *et al.,* 1993; Ho *et al.,* 1995), indicating a colocalization of TFF1 and MUC5AC in these cells. In addition, minor amounts of TFF1 are detectable in human mucous neck cells (MNCs) (Piggott *et al.,* 1991; Hanby *et al.,* 1999).

There are conflicting reports concerning the biosynthesis of TFF1 in human Brunner's glands (Wright *et al.,* 1990b; Rio *et al.,* 1991). TFF1 is probably synthesized by the surface and upper duct cells but not in the acinar portion (Hanby *et al.,* 1993a; Khulusi *et al.,* 1995). In the mouse, TFF1 is only transiently expressed in the developing Brunner's glands but not in adult animals (Lefebvre *et al.,* 1993; Otto and Patel, 1999; Terada *et al.,* 2001).

Another physiologically important source of TFF1 is the human conjunctiva, where goblet cells synthesize this peptide (Langer *et al.,* 1999) together with the mucin MUC5AC (Gipson and Inatomi, 1997). TFF1 is a constituent of the complex eye mucus and a minor constitutent of the tear fluid.

Remarkable species differences were observed in the uterus. Little TFF1 expression was reported for the mouse (Lefebvre *et al.,* 1993), whereas the normal human uterus does not express TFF1. Only occasionally were trace amounts of TFF1 transcripts detectable in the human endocervix, and they were never detectable in the endometrium (Wiede *et al.,* 2001).

Furthermore, low-level expression of TFF1 is detectable in human sublingual and submandibular glands (Rio *et al.,* 1988; Jagla *et al.,* 1999a; Devine *et al.,* 2000), human respiratory tract (Wiede *et al.,* 1999; dos Santos Silva *et al.,* 2000), human nonlactating breasts (Piggott *et al.,* 1991; Poulsom *et al.,* 1997), and mouse

spleen, heart, and muscle (Hirota *et al.,* 1994b). In addition, variable expression was observed in the human gallbladder (Williams and Wright, 1997). The concentration of TFF1 in human serum was reported to be approximately 150 pg/ml (Higashiyama *et al.,* 1996) and in the human urine approximately 14 ng/mg creatinine (Miyashita *et al.,* 1994b).

TFF1 is also widely distributed throughout the adult rat brain, with pronounced expression in the hippocampus, frontal cortex, and the cerebellum (Hirota *et al.,* 1995). Remarkably, astrocytes, but not neurons, have been reported to synthesize TFF1 in the mouse and rat (Hirota *et al.,* 1994a,b, 1995).

2. TFF2

The stomach generally represents a major source for TFF2 in most species, e.g., human (Tomasetto *et al.,* 1990), rat (Jeffrey *et al.,* 1994), and mouse (Tomasetto *et al.,* 1990). However, porcine stomach surprisingly contains only little TFF2 (Thim *et al.,* 1982). Human TFF2 is N-glycosylated, in contrast to porcine, mouse, or rat TFF2 (May *et al.,* 2000). Gastric TFF2 is predominantly released from MNCs and cardiac and antral gland cells as shown for humans, pigs, rats, and mice (Rasmussen *et al.,* 1992; Hanby *et al.,* 1993b; Jeffrey *et al.,* 1994; Konturek *et al.,* 1997). In humans, these cells synthesize MUC6 as their major secretory mucin (Ho *et al.,* 1995; Bartman *et al.,* 1998), indicating a colocalization of TFF2 and MUC6. Furthermore, a gradient of TFF2 expression was detected in human, mouse, and rat, in which TFF2 was shown to be most abundant in the antrum (Hanby *et al.,* 1993b; Jeffrey *et al.,* 1994; Taupin *et al.,* 1995; Otto and Patel, 1999). Only weak TFF2 mRNA expression was observed at the human gastric surface epithelium, and no TFF2 peptide expression was observed (Hanby *et al.,* 1993b). Thus, TFF1 and TFF2 show complementary cellular localization patterns within the stomach. There are conflicting reports concerning the agreement of the localization of the TFF2 mRNA as determined by *in situ* hybridization with the results from immunohistochemistry (Jeffrey *et al.,* 1994; Hanby *et al.,* 1993b; Otto and Patel, 1999). TFF2 is present in the human and murine gastric juice at concentrations between 1 and 20 μg/ml with dramatic diurnal variations (May *et al.,* 2000; Tomasetto *et al.,* 2000a; Semple *et al.,* 2001).

TFF2 is also expressed in the human Brunner's glands duct epithelium (Wright *et al.,* 1990b) and in Brunner's gland acini (Hanby *et al.,* 1993a; Khulusi *et al.,* 1995; Longman *et al.,* 2000), which are known for their MUC6 synthesis (Ho *et al.,* 1995). TFF2 expression has also been detected in Brunner's glands of the pig (Rasmussen *et al.,* 1992), rat (Jeffrey *et al.,* 1994), and mouse (Lefebvre *et al.,* 1993; Terada *et al.,* 2001). Additional TFF2 immunoreactivity has been reported in columnar cells in the lower parts of the crypts from the porcine jejunum and ileum but not in goblet cells (Rasmussen *et al.,* 1992).

Considerable species differences are observed concerning panreatic TFF2 synthesis. For example, the pancreas is the major source for porcine TFF2 (Thim *et al.,*

1982), in which it is found in all acinar cells (Rasmussen *et al.,* 1992). The pancreas head contains considerably more TFF2 than the pancreas tail (Thim *et al.,* 1982). Relatively high TFF2 expression also occurs in the pancreas of mouse (Lefebvre *et al.,* 1996) and rat (Jeffrey *et al.,* 1994). In contrast, TFF2 expression in human pancreas is scarce (Welter *et al.,* 1992; Ebert *et al.,* 1999; Ohshio *et al.,* 2000).

TFF2 expression is also reported to occur in mucous acini of the rat sublingual gland (Jeffrey *et al.,* 1994). However, TFF2 transcripts are barely detectable in human sublingual glands (Jagla *et al.,* 1999a) and protein analysis failed to detect TFF2 in porcine salivary glands (Rasmussen *et al.,* 1992).

Additional sites of low-level TFF2 expression are the human gallbladder (Williams and Wright, 1997; Longman *et al.,* 2000) and the rat spleen and thymus (Cook *et al.,* 1999). A relatively high TFF2 concentration was detected in porcine plasma (Thim *et al.,* 1982).

3. TFF3

This peptide has a completely different localization pattern compared to those of TFF1 and TFF2. It is the predominant TFF peptide in most mucous epithelia, which are generally devoid of TFF1 and TFF2. In addition, TFF3 is scarce in the human (Hauser *et al.,* 1993; May and Westley, 1997a) and mouse stomach (Mashimo *et al.,* 1995; Tomita *et al.,* 1995). Only a restricted population of cells of the rat forestomach strongly express TFF3 (Chinery *et al.,* 1992).

A major source for TFF3 are goblet cells of the small and large intestine. This was shown for humans (Hauser *et al.,* 1993; Podolsky *et al.,* 1993), rats (Suemori *et al.,* 1991; Chinery *et al.,* 1992; Taupin *et al.,* 1995; Moro *et al.,* 2001), mice (Tomita *et al.,* 1995; Mashimo *et al.,* 1996; Terada *et al.,* 2001), and pigs (Fig. 2, see color insert; Jagla *et al.,* 1999b). Human intestinal goblet cells secrete the mucin MUC2 and also smaller amounts of MUC3 (Audie *et al.,* 1993; Chang *et al.,* 1994). Surprisingly, expression of MUC2 and TFF3 genes is not coordinately regulated along the longitudinal axis of the rat intestine (Matsuoka *et al.,* 1999). Maximal TFF3 expression was observed in the distal portions of the ileum and the colon, whereas MUC2 expression was highest in the proximal colon (Matsuoka *et al.,* 1999). This is consistent with the observation that TFF3 expression in the mouse does not simply correlate with the number of goblet cells (Tomita *et al.,* 1995). Secretion of TFF3 is evoked by certain neurotransmitters and inflammatory mediators (Moro *et al.,* 2001). Furthermore, the level of TFF3 transcripts did not parallel TFF3 immunoreactivity, which was highest in the rat duodenum and ileum and much lower in the colon (Taupin *et al.,* 1995). Duodenal TFF3 expression is restricted to goblet cells and was not detectable in human and rat Brunner's glands (Chinery *et al.,* 1992; Longman *et al.,* 2000).

TFF3 is also synthesized from certain human salivary glands and represents a constituent of the saliva. Relatively large amounts of TFF3 are stored only within serous cells of submandibular glands (Jagla *et al.,* 1999a). These cells synthesize

MUC7 as their predominant mucin (Nielsen *et al.,* 1996, 1997). In contrast to the immunohistochemical data, TFF3 mRNA has been reported to occur in mucous but not in serous acini of submandibular glands (Devine *et al.,* 2000). However, the highest levels of TFF3 transcripts were detectable in sublingual glands, which store only minor amounts of TFF3 (Jagla *et al.,* 1999a).

TFF3 immunoreactivity has also been reported in the rat esophagus (Taupin *et al.,* 1995). However, TFF3 has not been detected in the normal human esophagus (Labouvie *et al.,* 1999).

The human respiratory tract represents another physiologically important site of TFF3 synthesis (Wiede *et al.,* 1999; dos Santos Silva *et al.,* 2000). Secretion occurs predominantly by mucous cells of the submucosal glands (together with the mucin MUC5B) but also to a limited extent by goblet cells secreting MUC5AC and MUC5B (Hovenberg *et al.,* 1996; Wickström *et al.,* 1998; Wiede *et al.,* 1999). In contrast, goblet cells of the sheep respiratory tract contain large amounts of TFF3 (Fig. 3, see color insert). TFF3 immunoreactivity has also been reported for the rat lung (Taupin *et al.,* 1995).

TFF3 is expressed in the human uterus (Hauser *et al.,* 1993), in which it is a major secretory product of the endocervix but not the endometrium (Wiede *et al.,* 2001). The surface epithelium and gland-like structures release TFF3 together with the mucin MUC5B (Wickström *et al.,* 1998) as a constituent of cervical mucus of pre- and postmenopausal women. It is not known if TFF3 synthesis is changed during the ovulatory cycle (Wiede *et al.,* 2001).

Probably a most physiologically relevant site of TFF3 synthesis are the goblet cells of the conjunctiva. This has been shown for humans (Langer *et al.,* 1999) and pigs (Fig. 4, see color insert; Jagla *et al.,* 1999b). Together with TFF1 and MUC5AC, it contributes to the delicate eye's mucus layer and it is a minor constituent of the tear fluid.

Abundant TFF3 expression has also been reported to occur in the rat kidney (Suemori *et al.,* 1991), in which TFF3 immunoreactivity is also found (Taupin *et al.,* 1995). Variable expression was reported for the human gallbladder (Williams and Wright, 1997) and low-level expression was detected in the rat spleen and thymus (Cook *et al.,* 1999).

TFF3 is also a typical neuropeptide synthesized in the hypothalamus of at least the rat and human (Probst *et al.,* 1995, 1996). Oxytocinergic neurons in the human supraoptic and paraventricular nuclei were shown to be the sole site of synthesis. The distinct population of vasopressinergic neurons is completely devoid of TFF3 (Jagla *et al.,* 2000). Figure 5 (see color insert) illustrates the colocalization of TFF3 and oxytocin in a single neuron of the human paraventricular nucleus. These neurons project to the posterior pituitary, in which relatively large amounts of TFF3 are stored awaiting its release into the bloodstream (Jagla *et al.,* 2000). Storage of TFF3 and oxytocin occurs mainly in the same secretory vesicles (Schwarz *et al.,* 2001). Figure 6 (see color insert) shows the accumulation of TFF3 in the porcine posterior pituitary mainly around blood vessels. Remarkably, the molecular weight

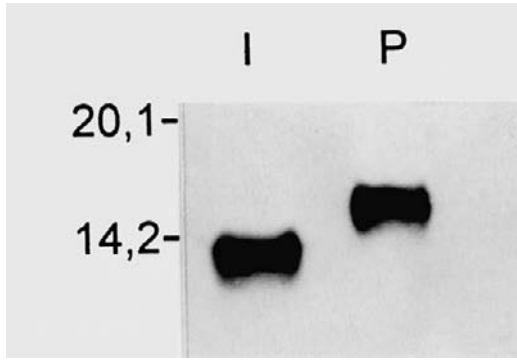

FIG. 7 Detection of TFF3 in extracts of porcine duodenum (I) or the neural lobe of the pituitary (P) after SDS–polyacrylamide gel electrophoresis (15%) and subsequent Western blot analysis using affinity-purified rabbit anti-hTFF3-2 antiserum (Wiede *et al.,* 1999) and the ECL analysis system (Amersham Pharmacia Biotech). The molecular size standard is shown on the left.

of TFF3 from the posterior pituitary as estimated from gel electrophoresis appears somewhat larger than that of corresponding material from the intestine, probably due to an unknown posttranslational modification (Fig. 7). TFF3 synthesis was also claimed to occur in tanycytes lining the third ventricle (Griepentrog *et al.,* 1999). This might explain the presence of TFF3 in human postmortem cerebrospinal fluid obtained from the third ventricle (Jagla *et al.,* 2000).

4. Coordinated Localization with Different Mucins

Taken together, a variety of mucin-secreting cells represent the predominant sites for synthesis of the three mammalian TFF peptides in the body. Each of these specialized cells secretes its specific cocktail of TFF peptides and secretory mucins, probably reflecting the quite different physiological functions of these highly specialized mucosae (Table II).

B. Developmental Expression in Mammals

TFF peptides appear generally very early during embryonic development (e.g., well before differentiation of the gastrointestinal epithelium). Thus, TFF peptides may be involved in differentiation and maturation of the gastrointestinal mucosa. This hypothesis is supported by TFF1$^{-/-}$ and TFF3$^{-/-}$ mice partly lacking a functional mucus layer (Lefebvre *et al.,* 1996; Mashimo *et al.,* 1996).

A comprehensive expression study exists for the mouse gastrointestinal tract (Otto and Patel, 1999). TFF1 expression was detected first at Embryonic Day 12 (E12) in the stomach, then it spread to the small intestine (E15) and caecum (E16). From E19, TFF1 expression remained gastric only. A similar pattern was obtained

TABLE II
Predominant TFF Peptide/Secretory Mucin Combinations Observed in Various Human Mucin-Producing Cells

Cell	TFF peptide/mucin
Gastric surface cell	TFF1/MUC5AC
Brunner's glands	TFF2/MUC6
Gastric mucous neck cell	TFF2/MUC6
Gastric antral glands	TFF2/MUC6
Intestinal goblet cell	TFF3/MUC2
Conjunctival goblet cell	TFF1 + TFF3/MUC5AC
Respiratory goblet cell	TFF3/MUC5AC + MUC5B
Respiratory submucosal glands	TFF3/MUC5B
Endocervical epithelial cell	TFF3/MUC5B
Submandibular gland: serous cell	TFF3/MUC7

for TFF2 starting at E11 from the stomach, spreading to the duodenum (E16), extending to the small intestine and caecum (E17), and becoming restricted to the stomach in the adult. TFF3 expression also started in the stomach (E11), was upregulated in the small and large intestine (E15) and the caecum (E16), and was downregulated in the stomach and Brunner's glands after E16. However, a previous expression study reported a different timescale, and there is a discrepancy concerning the disappearance of TFF2 in the adult Brunner's glands (Lefebvre *et al.,* 1993). Both studies never detected TFF1 and TFF2 expression in the developing large intestine. Surprisingly, an immunohistochemical study failed to detect TFF3 in the intestine of newborn or embryonic mice; TFF3 staining was observed only starting with P3 (Mashimo *et al.,* 1995).

In the rat, the first expression of TFF genes was reported in the gut at E15 (TFF3) and E16 (TFF1 and TFF2), respectively. The corresponding peptides were detected first at E17, i.e., before differentiation of the epithelium (Familiari *et al.,* 1998). A significant increase in TFF3 in the small intestine was observed starting with birth (E22), whereas TFF2 reached maximal concentration in the stomach after birth. Similar results were obtained from Lin *et al.* (1999), who detected TFF3 mRNA in the rat intestine at E17 and the peptide at E20, with a further increase during the weaning period. Jeffrey *et al.* (1994) detected strong TFF2 expression at E20 in the rat antrum but not in the corpus, duodenum, or pancreas.

A developmentally regulated expression was also reported for TFF1 in the rat hippocampus, where the highest expression is observed in the adult brain. Hippocampal TFF1 mRNA gradually decreased mainly in the first 3 weeks after birth. This led to the hypothesis that TFF1 may play an important role during brain development (Hirota *et al.,* 1995).

C. Physiological Expression in *X. laevis*

A complex pattern of TFF peptides was detected in amphibia that is quite different when compared with that of mammals (Table I). In *X. laevis*, the homolog of mammalian TFF1 is xP1 (Hauser and Hoffmann, 1991; Hoffmann and Hauser, 1993a) and the functional equivalent of mammalian TFF2 seems to be xP4.1 consisting of four TFF domains arranged in tandem (Hauser and Hoffmann, 1991; Jagla *et al.,* 1998). The homolog of mammalian TFF3 is not known in *X. laevis.* Furthermore, the TFF peptides xP4.2 (Botzler *et al.,* 1999) and xP2 (Hauser *et al.,* 1992) were discovered in *X. laevis,* but functional mammalian homologs are not known.

1. xP1

This peptide is expressed from gastric surface mucous cells (SMCs; Hauser and Hoffmann, 1991) in which immunohistochemical localization is also observed (Jagla *et al.,* 1998). Figure 8 (see color insert) clearly illustrates immunohistochemical localization in SMCs but not in MNCs. There is a decreasing protein gradient from the fundus to the antrum (Fig. 9; Jagla *et al.,* 1998).

Based on molecular structure and cellular localization, xP1 has to be considered to represent the *X. laevis* homolog of human TFF1.

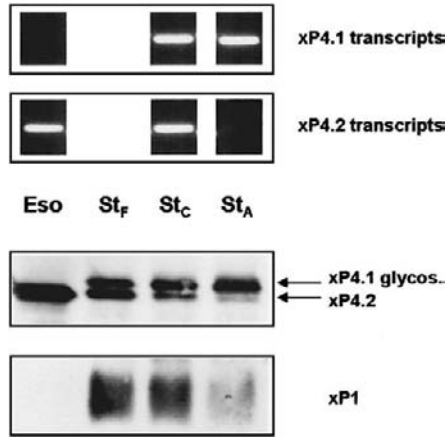

FIG. 9 Biosynthesis of xP1, xP4.1, and xP4.2 along the alimentary tract of *X. laevis*. Shown are results obtained from the esophagus (Eso), the stomach fundus (St_F), corpus (St_C), and antrum (St_A). RT-PCR analyses specifically monitor xP4.1 or xP4.2 transcripts, whereas Western blot analysis detects the xP1 and xP4 peptides, respectively [adapted from Jagla *et al.* (1998) with permission of Springer-Verlag].

2. xP4.1 and xP4.2

These two peptides share 89% similarity and are encoded by two different genes probably originating from a duplication of the *X. laevis* genome about 30 million years ago (Hauser and Hoffmann, 1991; Botzler *et al.,* 1999). One major difference is the N-glycosylation site in xP4.1, which is mutated in xP4.2. Furthermore, xP4.1 and xP4.2 show clearly different expression patterns along the alimentary tract of *X. laevis* (Jagla *et al.,* 1998; Botzler *et al.,* 1999). xP4.1 is found only in the stomach in its glyscosylated form, whereas xP4.2 is also a major secretory product of the esophagus. Gastric localization of xP4.2 shows a decreasing gradient from the fundus to the antrum (Fig. 9). xP4.2 is secreted by a specific subpopulation of esophageal goblet cells (Fig. 10, see color insert) that are characterized by their specific binding of the lectin GSA-II (Jagla *et al.,* 1998). xP4.1 and xP4.2 are mainly synthesized by MNCs and weakly by SMCs (Fig. 11, see color insert). Interestingly, expression of xP4.1 was detected only in patches of SMCs (Hauser and Hoffmann, 1991).

Furthermore, little xP4.1 is expressed in the proximal part of the intestine (Botzler *et al.,* 1999) including the duodenum. However, typical Brunner's glands are missing in *X. laevis* and xP4.1 is expected to be a secretory product of intestinal goblet cells.

Based on its strikingly similar distribution, xP4.1 might be considered to be the functional *X. laevis* homolog of TFF2; it is also N-glycosylated as human TFF2. On a structural basis, xP4.1 is unique and represents a duplicated version of TFF2. A functional human homolog of xP4.2 probably does not exist because of the anatomical differences between amphibian and mammalian esophagus, the latter being devoid of TFF2 (Hanby *et al.,* 1994).

3. xP2

X. laevis skin is the typical site of synthesis for this peptide. Here, it is probably also present in longer splice variants containing repetitive alanine- and proline-rich sequences (Hauser *et al.,* 1992). Granular glands and the epidermis show positive immunoreactivity for xP2. xP2 cannot be considered to be the functional amphibian homolog of mammalian TFF2, despite the fact that it also consists of two TFF domains.

D. Pathological Expression

1. Inflammation, Ulceration, and Wounding Response

TFF peptides are aberrantly expressed during a wide range of chronic inflammatory diseases, e.g., during ileal Crohn's disease, ulcerative colitis and duodenitis, cholecystitis (Rio *et al.,* 1991), inflammatory bowel disease (Wright *et al.,* 1993),

gastric ulcerations (Hauser *et al.,* 1993), and pancreatitis (Wright *et al.,* 1990b; Ebert *et al.,* 1999). An increased level of TFF1 expression was reported particularly during the healing of gastric ulcers (Saitoh *et al.,* 2000). Expression of TFF peptides is also a typical response after gastric mucosal damage in animal models of experimental ulceration (Alison *et al.,* 1995; Konturek *et al.,* 1997, 1998; Taupin *et al.,* 2001), in which an ordered sequence of gene expression including epidermal growth factor receptor (EGFR), c-met, EGF, transforming growth factor alpha (TGF-α), and hepatocyte growth factor was observed (Wong *et al.,* 2000). Furthermore, incisional wounding of fetal gastrointestinal tissue triggered a rapid upregulation of TFF gene expression (Otto and Patel, 1999).

During various chronic inflammatory conditions, an unique glandular structure known as the ulcer-associated cell lineage (UACL; Wright *et al.,* 1990a) is a prominent site of synthesis for all three TFF peptides (Wright *et al.,* 1990b; Hauser *et al.,* 1993). Originally described as "pyloric" or "pseudo-pyloric" metaplasia, the UACL has morphological similarities to both Brunner's glands and pyloric glands (Ahnen *et al.,* 1994; Wright, 1998). It develops from stem cells most commonly in the small intestine, particularly in Crohn's disease and duodenal ulcerations. It has also been detected in the colon, in ileal pouch mucosa of patients with pouchitis, in pancreatic ducts during chronic pancreatitis, in gallbladder during chronic cholecystitis, in the fallopian tube during chronic salpingitis, and in inflammatory nasal polypi (Wright, 1998; Pera *et al.,* 2001). The UACL is thought to represent a natural repair kit which is activated after mucosal damage. TFF genes are differentially expressed within the UACL [i.e., TFF1 (together with MUC5AC) solely in the distal ductular and surface elements, TFF2 (together with MUC6 and EGF) in the acinar and proximal ductular structures, and TFF3 (together with MUC5B, TGF-α, and lysozyme) heterogeneously throughout the gland (Wright, 1998; Longman *et al.,* 2000)].

2. Metaplasias and Tumors

Pathological expression of TFF peptides is observed in certain premalignant conditions, particularly in various types of metaplasia (Hanby *et al.,* 1993a; Khulusi *et al.,* 1995; Shaoul *et al.,* 2000) including Barrett's metaplasia (Hanby *et al.,* 1994; Labouvie *et al.,* 1999) and metaplastic polyps (Hanby *et al.,* 1993c; Hauser *et al.,* 1993; Nogueira *et al.,* 1999).

Furthermore, many epithelial and certain neuroendocrine tumors synthesize TFF peptides (Williams and Wright, 1997; May and Westley, 1997b; Ribieras *et al.,* 1998; Hoffmann *et al.,* 2001), e.g., tumors of the breast, esophagus, stomach, biliary tract, pancreas, intestine, lung, ovary, uterus, skin, and thyroid. A general prognostic value of ectopic TFF peptide expression in different tumors is still under debate. For example, TFF1 is significantly associated with prostate cancer but is absent in benign prostate hyperplasia (Colombel *et al.,* 1999). Furthermore, expression of TFF1 in a subclass of breast cancers is correlated with favorable

tumor characteristics and response to endocrine therapy (Rio and Chambon, 1990; Balleine and Clark, 1999), whereas TFF3 is significantly upregulated in certain colorectal carcinomas with a mucinous phenotype known to bear an especially poor prognosis (Taupin *et al.,* 1996).

Recently, somatic mutations and loss of heterozygosity (LOH) were also detected in the TFF1 gene in gastric tumors (Park *et al.,* 2000a). Furthermore, a recent, extensive LOH study of gastric cancers revealed a peak of LOH frequency at chromosome 21q22.3, where the TFF genes are clustered (Park *et al.,* 2000b).

E. Regulation of Gene Expression

All three human TFF genes are clustered on chromosome 21q22.3 in a head-to-tail arrangement within 50 kb in the order cen–TFF3–TFF2–TFF1–tel (Tomasetto *et al.,* 1992; Chinery *et al.,* 1996b; Gött *et al.,* 1996; Seib *et al.,* 1997; Hattori *et al.,* 2000). Transcription of all three genes is directed toward the centromere (Gött *et al.,* 1996). A syntenic 40 kb region was characterized for the mouse on chromosome 17q (Chinery *et al.,* 1996a; Burmeister and Meyer, 1997; Ribieras *et al.,* 2001), and the bovine syntenic group U10 encodes at least the TFF2 homolog (Threadgill and Womack, 1991). Organ-specific expression of TFF-genes is dependent on promoter methylation (Ribieras *et al.,* 2001). There are numerous studies structurally and functionally characterizing the promoter regions of the various mammalian and *X. laevis* TFF genes.

The expression of the clustered TTF genes is coordinatedly regulated particularly in the murine stomach. For example, 70% of transgenic TFF1$^{-/-}$ mice failed to express gastric TFF2 (but had normal pancreatic TFF synthesis; Lefebvre *et al.,* 1996) and transgenic TFF3$^{-/-}$ mice showed reduced expression of gastric TFF1 and TFF2 (Taupin *et al.,* 1999). Coordinated expression of human TFF genes was also suggested because sequences were detected common to 5′-flanking regions of all three TFF genes (Gött *et al.,* 1996; Beck *et al.,* 1998), i.e., motif I (at about −460) and motif IV (close to the TATA box). Motif I shows enhancing effects and might bind the transcription factor oct-1 (Beck *et al.,* 1998), whereas motif IV shows striking similarity to the binding site for hepatocyte nuclear factor 3/forkhead and may explain the acute phase response of TFF genes (Beck *et al.,* 1999).

Furthermore, all three human TFF genes are regulated by osmotic stress and ethanol (Lüdeking *et al.,* 1998). Expression of TFF genes in epithelial cells is dependent of a mucinsecreting phenotype which was shown for HT29 cells during various stages of differentiation (Gouyer *et al.,* 2001).

1. TFF1

Originally, this gene was identified because its expression was induced by estrogen in the human breast carcinoma cell line MCF-7 (Masiakowski *et al.,* 1982). The human TFF1 gene is about 4.5 kb long and consists of three exons (Jeltsch *et al.,*

1987). The proximal 5′-flanking region contains typical regulatory elements (e.g., a TATA box, a CAAT element, and a GC-rich motif). Further upstream, a functional AP-1-like site (between −518 and −512; Gillesby *et al.,* 1997), an estradiol responsive element (ERE; between −405 and −393) (Berry *et al.,* 1989), and a complex enhancer region responsive to EGF, the tumor promoter TPA and the protooncoproteins c-Ha-ras and c-jun were detected (between −428 and −332; Nunez *et al.,* 1989). The ERE and the TATA box are each located at the edge of a nucleosome (Sewack and Hansen, 1997). Furthermore, motif II (adjacent to the ERE and considerably matching the binding site of heat shock factor HSH-2) and motif III (close to the TATA box matching a GATA consensus sequence) were discovered due to their similiarity to sequences in the 5′-flanking region of the TFF2 gene (Gött *et al.,* 1996; Beck *et al.,* 1998). Additional regulation by several growth factors, such as insulin, insulin-like growth factor I (IGF-I), and basic fibroblast growth factor (bFGF), was reported (Cavailles *et al.,* 1989). Regulation by bFGF was proposed to occur at the posttranscriptional level, probably due to stabilization of the TFF1 mRNA (Miyashita *et al.,* 1994a). Furthermore, a repression of TFF1 expression was observed by retinoic acid (Hirota *et al.,* 1992).

Regulation of TFF1 expression by estrogens requires the presence of estrogen receptors. This explains why expression of TFF1 is restricted to a subclass of estrogen receptor-rich human breast tumors (Rio and Chambon, 1990). For example, in the hormone-dependent MCF-7 cell line, induction of TFF1 transcription is a very early event that is apparent within 15 min after addition of estradiol (Brown *et al.,* 1984). TFF1 expression is also regulated by altered estrogens and antiestrogens (Weaver *et al.,* 1988; Pilat *et al.,* 1993). However, regulation is not solely controlled by the amount of estrogen receptor. There are also complex synergistic effects with TPA and EGF (Beck *et al.,* 1997), and TPA was suggested to induce translation of the TFF1 mRNA but not its transcription (Kida *et al.,* 1993). Furthermore, hormone-dependent and -independent breast cancer cell lines differ in their DNAse I hypersensitive sites in the TFF1 gene that are induced by estrogens and/or IGF-I (Giamarchi *et al.,* 1999).

The methylation status of the TFF1 promoter region changes in different tumors and clearly affects TFF1 expression. For example, the region around the ERE is hypomethylated in estrogen receptor-rich breast tumors causing ectopic TFF1 expression (Martin *et al.,* 1995, 1997). Surprisingly, the ERE is not only recognized by the estrogen receptor but also from a methylation-sensitive DNA-binding protein (Martin *et al.,* 1998). In contrast, hypermethylation of CpG sites within the TFF1 promoter region was observed at an early stage of stomach carcinogenesis resulting in reduced TFF1 expression (Fujimoto *et al.,* 2000).

In estrogen receptor-rich human breast tumors the ERE is clearly the predominant element directly controlling TFF1 transcription (Rio *et al.,* 1987). On the other hand, TFF1 expression in the stomach is probably independent of estradiol and EGF (Campbell-Thompson, 1997; Ribieras *et al.,* 1998). Regulatory sequences responsible for stomach-specific expression of TFF1 are probably motif II and motif III (Beck *et al.,* 1998). The latter was identified as representing a GATA-6

binding sequence that activates gastric expression probably together with additional GATA sites at −174, −124, and −110 (Al-azzeh *et al.,* 2000). HNF-3 specifically activates TFF1 via motif IV, which is probably of importance during inflammatory conditions and neoplasia (Beck *et al.,* 1999).

Surprisingly, the mouse TFF1 gene is lacking a functional ERE (Terada *et al.,* 2001). This is in line with previous observations concerning the expression of TFF1 in the mouse mammary gland (Lefebvre *et al.,* 1993). TFF1 expression was also reported to be induced by extracts from cabbage (Ju *et al.,* 2000) and by ginseng root extract used in alternative medicine (Duda *et al.,* 1996).

2. TFF2

The human TFF2 gene consists of four exons. Each of the two different TFF domains is encoded by a single exon (Gött *et al.,* 1996). An unique tandemly repeated 25-bp sequence is located within intron 2, which shows diallelic polymorphism (Kayademir *et al.,* 1998). A TATA box and potential binding sites for transcription factors Myc, PEA3, and Ets-like factor are present in the proximal 5′-flanking region (Gött *et al.,* 1996). When compared with the TFF1 gene, there is no ERE but motifs II and III (a GATA binding site) are present. These motifs (Beck *et al.,* 1998), together with two particularly active GATA-6 binding sequences at −400 and −322, confer the stomach-specific expression pattern of TFF2 (Al-azzeh *et al.,* 2000). Furthermore, an aspirin responding element was mapped between −546 and −758 (Azarschab *et al.,* 2001).

Surprisingly, gastric TFF2, but not TFF1, expression was shown to be increased by estradiol in the rat (Campbell-Thompson, 1997). This might be mediated by an AP-1 site found in all TFF genes from human and *X. laevis* (Lamph, 1991; Botzler *et al.,* 1999).

3. TFF3

The human TFF3 gene spans about 3.2 kb and consists of three exons, with exon 2 encoding the TFF domain (Seib *et al.,* 1995). No canonical TATA box exists and there are potential binding sites for several transcription factors in the 5′-flanking region (e.g., SP1, AP-1, CF1 and HRE) (Seib *et al.,* 1995; Furuta *et al.,* 2001). When compared with the "gastric" genes TFF1 and TFF2, motif II and motif III are missing in the TFF3 gene (Beck *et al.,* 1998) and GATA-6 did not activate TFF3 (Al-azzeh *et al.,* 2000). The human 5′-flanking region of the human TFF3 gene does not show striking similarity to the rat TFF3 gene except for a region between −682 and −623 (Seib *et al.,* 1995).

The rat TFF3 gene spans about 4.8 kb. Surprisingly, none of the known regulatory elements demonstrated to play a role in intestine-specific expression could be identified in the sequenced 1.7 kb of the 5′-flanking region (Sands *et al.,* 1995). However, 153 base pairs of the 5′-flanking region were sufficient to direct

expression of a reporter gene in the goblet cell-like LS174T colon cancer cell line (Sands *et al.,* 1995). A 9-base pair element [goblet cell response element (GCRE)] between −154 and −118 was shown to represent the *cis*-acting element binding a nuclear transcription factor directing goblet cell-specific expression (Ogata *et al.,* 1998).

The TFF3 gene was also characterized in the mouse (Mashimo *et al.,* 1995; Chinery *et al.,* 1996a). A positive regulatory element (−181 to −170) and a negative regulatory element (−208 to −200) were characterized in addition to the GCRE. These elements were also well conserved in the human and rat TFF3 gene promoters (Itoh *et al.,* 1999). Furthermore, a goblet cell-specific silencer inhibitor (GCSI) was detected between −2216 and −2204 which contributes to a goblet cell-specific transcription of the TFF3 gene (Itoh *et al.,* 1999; Iwakiri and Podolsky, 2001). However, this might not be too surprising because of the complex expression pattern of TFF3, including goblet cells, glandular cells, and neuronal cells.

TFF3 expression in colon cancer cell lines is stimulated by neuropeptides and acetylcholine (Ogata and Podolsky, 1997) as well as by hypoxia (Furuta *et al.,* 2001); whereas short-chain fatty acids inhibit TFF3 expression (Tran *et al.,* 1998).

4. xP4.1

The *X. laevis* xP4.1 gene consists of six exons spanning about 7 kb. Each TFF domain is encoded by a separate exon (Botzler *et al.,* 1999). Its 5′-flanking region contains a typical TATA box at −31 and a consensus sequence for binding HNF-3 at −48. The latter is typically found in relative proximity to the TATA box in all mammalian TFF genes analyzed thus far (motif IV; Beck *et al.,* 1998). Most important are multiple potential GATA binding sites (one was previously termed motif III), which are thought to confer to the stomach-specific expression pattern. At position −201 there is a potential AP-1 site, which is also typically present in all other TFF genes but at variable positions. Taken together, these results are in line with the assumption that xP4.1 is the *X. laevis* homolog of mammalian TFF2 despite its different molecular structure. In the future, it would be quite interesting to analyze the promoter region of the very similar xP4.2 gene, which shows a very different expression pattern.

III. Multiple Biological Functions of TFF Peptides

As major secretory products of a variety of mucous epithelia, TFF peptides contribute in many ways to their surface integrity. This function is particularly important during inflammatory conditions and after wounding. Furthermore, TFF peptides are released from the brain in minor amounts.

A. Protection of Mucous Epithelia *in Vivo*

Numerous studies clearly document protective and healing effects of all three TFF peptides after damage mainly of the gastrointestinal tract (Hoffmann *et al.,* 2001). Here, TFF peptides were applied orally or subcutaneously after chemical damage (e.g., with indomethacin, ethanol, aspirin, or acetic acid) or restraint. A compilation of these reports is given in Table III.

Generally, systemic subcutanous application of TFF peptides to the basolateral side of the mucosa seems to be more effective than oral application. Interestingly, dimeric TFF1 was significantly more active than monomeric TFF1 (Marchbank *et al.,* 1998). There was also a synergistic effect, with EGF observed (Chinery and Playford, 1995).

Several studies with transgenic animals have been published concerning the roles of TFF peptides in protection of mucous epithelia. For example, transgenic mice overexpressing TFF1 or TFF3 in the jejunum are more resistant to indomethacin damage than are control animals (Playford *et al.,* 1996b; Marchbank *et al.,* 2001).

TABLE III
In Vivo Studies in Rats or Different Transgenic (tg) Mice on Protective or Healing Effects of TFF Peptides[a]

TFFs	Animal	Application	Damage	Reference
TFF1	tg mouse	Jejunum/tg	Indo	Playford *et al.* (1996b)
TFF1	Rat	sc	Indo	Marchbank *et al.* (1998)
TFF2	Rat	Oral, sc	Indo + restr	Playford *et al.* (1995)
TFF2	Rat	Oral	Indo, EtOH	Babyatsky *et al.* (1996)
TFF2	Rat	sc	Restr	Konturek *et al.* (1997)
TFF2	Rat	sc	Indo	McKenzie *et al.* (1997)
TFF2	Rat	Oral	Asp	Cook *et al.* (1998)
TFF2	Rat	Intrarectal	DNBS	Tran *et al.* (1999)
TFF2	Rat	iv, gastric	EtOH	McKenzie *et al.* (2000)
pTFF2	Rat	sc	Indo	McKenzie *et al.* (1997)
pTFF2	Rat	Oral, sc	Indo, merc	Poulsen *et al.* (1999)
pTFF2	Rat	iv, gastric	EtOH	McKenzie *et al.* (2000)
rTFF3	Rat	sc	Indo + restr	Chinery and Playford (1995)
rTFF3	Rat	Oral	Indo	Babyatsky *et al.* (1996)
rTFF3	tg mouse	Rectal	AcOH	Mashimo *et al.* (1996)
rTFF3	tg mouse	Jejunum/tg	Indo	Marchbank *et al.* (2001)

[a] After Damage with Indomethacin (indo), Aspirin (asp), ethanol (EtOH), dinitrobenzene sulfonic acid (DNBS), mercaptamine (merc), acetic acid (AcOH) or restraint (restr). iv, intravenous; sc, subcutaneous.

In contrast, transgenic TFF1$^{-/-}$ mice failed to develop a functional gastric mucosa and developed obligatory antropyloric adenomas, 30% of which progressed to carcinomas (Lefebvre *et al.,* 1996). Transgenic TFF3$^{-/-}$ mice were significantly more susceptible to colonic injury by oral administration of 2.5% dextran sodium sulfate when compared with wild-type mice (Mashimo *et al.,* 1996).

B. Interaction with Mucins

TFF peptides are cosecreted with mucins (Table II) and are major constituents of mucous gels (for review, see Hoffmann *et al.,* 2001). Consequently, it was postulated that TFF peptides interact with mucins as "link peptides" influencing the rheological properties of these viscous biopolymers (Hauser *et al.,* 1993; Hoffmann and Hauser, 1993a). This implies a noncovalent interaction of mucins and TFF peptides that is particularly in line with the entangled network model of mucus (Verdugo, 1990). Interaction is possible either with the cysteine-rich domains of mucins (protein–protein interaction) or with their carbohydrate moiety (lectin-like interaction). The latter has been favored based on the three-dimensional structure of porcine TFF2 (Gajhede *et al.,* 1993).

The hypothesis of a TFF peptide–mucin interaction is supported by the observations that TFF2 and TFF3 protect monolayers of T84 cells in cooperation with mucins (Kindon *et al.,* 1995) and that TFF2 decreases proton permeation through gastric mucus (Tanaka *et al.,* 1997). TFF peptides were also reported to change the rheological properties of mucous gels (Dignass *et al.,* 1994). The proposed direct interaction of TFF domains and mucins is particularly important to understand the molecular architecture of frog skin mucus probably lacking a TFF peptide but consisting of the TFF domain containing mucins FIM-A.1 and FIM-C.1 as well as the von Willebrand Factor (vWF)-type mucin FIM-B.1 (Hoffmann, 1988; Hauser *et al.,* 1990; Hauser and Hoffmann, 1992; Hoffmann and Hauser, 1993b; Hoffmann and Joba, 1995; Joba and Hoffmann, 1997).

In the meantime, the TFF1 dimer was shown to be strongly associated with gastric mucins even after caesium chloride density gradient centrifugation in the presence of 6M guanidine hydrochloride (Newton *et al.,* 2000). Furthermore, a direct interaction of TFF1 and cysteine-rich domains at the C termini of mouse Muc2 and mouse Muc5AC has been reported by means of the susceptible yeast two-hybrid system (Tomasetto *et al.,* 2000b). Probably both the B-like and the C domains homologous with vWF are involved in the direct interaction with TFF1. Based on these results, a direct interaction of TFF peptides with MUC5B but not with MUC6 is within the limits of expectation (Hoffmann *et al.,* 2001). Furthermore, a direct interaction of mucins from HT29–MTX cells and secretory peptides with a molecular weight similar to that of TFF peptides was observed (Gouyer *et al.,* 2001). Thus, conclusive studies can be expected in the future that will clearly verify or nullify one of the two hypotheses on the nature of direct TFF peptide–mucin interactions.

C. Motogenic Activity and Scattering of Cells *in Vitro*

Migration of epithelial cells is particularly observed in the gastric and respiratory mucosae after superficial injury. This rapid repair by cell migration is called "restitution" (Silen and Ito, 1985) and starts within minutes after damage and well before extensive inflammatory processes and proliferation occur (Lacy, 1988; Erjefält *et al.,* 1995). A classical *in vitro* model mimicking restitution is the cultured rat intestinal epithelial IEC-6 cells after *in vitro* wounding (McCormack *et al.,* 1992).

All TFF peptides were shown to enhance cell migration *in vitro* (motogenic effect). To date, this has been demonstrated for various gastric, intestinal, bronchial or corneal cells (for review, see Hoffmann *et al.,* 2001). An updated compilation of these data is provided in Table IV. Note that only dimeric TFF1 was active, and not monomeric TFF1 (Marchbank *et al.,* 1998), and the motogenic effect of TFF2 and TFF3 was synergistically enhanced by EGF (Chinery and Playford, 1995; Oertel *et al.,* 2001). However, monomeric TFF3 still has motogenic activity (Boxberger *et al.,* 1998; Kinoshita *et al.,* 2000; Oertel *et al.,* 2001).

TABLE IV
In Vitro Studies Demonstrating Motogenic Effects for TFF Peptides

TFFs	Cells	Reference
TFF1	HT29	Marchbank *et al.* (1998)
TFF2	IEC-6	Dignass *et al.* (1994)
TFF2	HT29	Playford *et al.* (1995)
TFF2	LIM1215	Wilson and Gibson (1997)
TFF2	Monocytes	Cook *et al.* (1999)
TFF2	HT29	Efstathiou *et al.* (1999)
TFF2	Primary oxyntic cultures	Kato *et al.* (1999)
TFF2	Primary corneal epithelial cells	Göke *et al.* (2001)
TFF2	BEAS-2B, primary bronchial epithelial cells	Oertel *et al.* (2001)
TFF3	IEC-6	Dignass *et al.* (1994)
TFF3	Monocytes	Cook *et al.* (1999)
TFF3	IEC-6	Kinoshita *et al.* (2000)
TFF3	Primary corneal epithelial cells	Göke *et al.* (2001)
TFF3	BEAS-2B	Oertel *et al.* (2001)
rTFF3	IEC-6	Dignass *et al.* (1994)
rTFF3	HT29	Chinery and Playford (1995)
rTFF3	LIM1215	Wilson and Gibson (1997)
rTFF3	Primary oxyntic cultures	Kato *et al.* (1999)
rTFF3	BEAS-2B	Oertel *et al.* (2001)

The motogenic effect of TFF peptides is probably triggered by activation of the Ras/MEK pathway stimulating phosphorylation of the MAP kinases ERK1/2 (Kinoshita *et al.,* 2000). This implies the presence of specific TFF receptors. Activation of ERK1/2 is known to be essential for migration of various cell lines, leading to phosphorylation of myosin light-chain kinase (Klemke *et al.,* 1997).

The regulation of cell motility by TFF peptides implies that TFF peptides have a major influence on restructuring of the cytoskeleton. This is in agreement with observations that TFF peptides reduce cell–cell and cell–matrix interactions and enhance scattering of cells. For example, TFF1 enhances dispersed growth in a three-dimensional (3D) collagen gel and reduces extracellular matrix degradation (Williams *et al.,* 1996). Furthermore, TFF2 promotes branching morphology of MCF-7 cells in collagen lattices (Lalani *et al.,* 1999) and TFF3-transfected cells form smaller and more dispersed colonies in a 3D collagen gel (Uchino *et al.,* 2000). TFF3 also leads to decreased cell substratum and cell–cell adhesion accompanied by decreased expression of the E-cadherin–catenin complexes (Efstathiou *et al.,* 1998). Interestingly, mutated E-cadherin described in many tumors is associated with a loss of responsiveness to the migratory effects of TFF2 (Efstathiou *et al.,* 1999). Recently, scattering and cellular invasion of src- and RhoA-transformed epithelial cells were observed after treatment with all three TFF peptides. This effect is dependent on several signaling pathways, including phosphatidylinositol-3-kinase, phospholipase C, protein kinase C, and the rapamycin target TOR (Emami *et al.,* 2001) including involvement of cyclooxygenase- and thromboxane A2 receptor-dependent signaling pathways (Rodrigues *et al.,* 2001).

Taken together, the motogenic activity of TFF peptides as well as their scattering effects synergistically support the beneficial effect of mucosal restitution. Unfortunately, the detrimental effect of metastasis is also probably promoted by TFF peptides. The latter would explain the results of certain clinical studies investigating TFF peptide-secreting tumors.

D. Antiapoptotic Effects

Inhibition of apoptosis was reported for TFF2 (Lalani *et al.,* 1999) and TFF3 (Chen *et al.,* 2000; Taupin *et al.,* 2000). This antiapototic effect required intact TFF3 dimer and phosphorylation of the EGFR (Kinoshita *et al.,* 2000). Another study observed activation of the transcription factor NF-κB triggered by TFF3 (Chen *et al.,* 2000). These results obtained *in vitro* are consistent with the *in vivo* observation that transgenic TFF3$^{-/-}$ mice have increased intestinal apoptosis (Taupin *et al.,* 2000).

In contrast to the observed clear antiapoptotic effects of TFF2 and TFF3, there is a single report on an increase in the number of apoptotic nuclei after treatment of HT29 cells with TFF3 (Efstathiou *et al.,* 1998).

E. Modulation of Inflammatory Processes

TFF peptides are pathologically expressed during various chronic inflammatory conditions. Their potential to trigger activation of NF-κB (Chen *et al.,* 2000) makes them very interesting candidates to regulate proinflammatory cytokines (e.g., interleukin-6 and interleukin-8). Furthermore, TFF3 was reported to activate cyclooxygenase-2 (COX-2) in IEC-18 cells (Tan *et al.,* 2000). COX-2 is known to play a critical role in inflammation. TFF3 also induced NO production and synthesis of the inducible nitric oxide synthase (iNOS) in IEC-18 cells (Tan *et al.,* 1999). iNOS is involved in the inflammatory response of the intestine and is regulated via NF-κB (Qu *et al.,* 2001). TFF2 and TFF3 are expressed in rat lymphoid tissues and are upregulated during experimental inflammation with bacterial wall lipopolysaccharide (LPS), indicating again that TFF peptides may be involved in the modulation of the inflammatory response (Cook *et al.,* 1999).

F. Function as Neuropeptides

TFF3 is a typical neuropeptide of oxytocinergic but not vasopressinergic neurons of the supraoptic and paraventricular nuclei (Jagla *et al.,* 2000; Schwarz *et al.,* 2001). From here, it is expected to be released into the bloodstream. The peripheral targets of this endocrine-secreted TFF3 are not known. However, TFF peptides are about two orders of magnitude more effective in preventing gastric damage *in vivo* when given systematically than when applied luminally (Playford *et al.,* 1995; Babyatsky *et al.,* 1996). This suggests that TFF receptors such as the EFGR (Playford *et al.,* 1996a) are localized to the basolateral membrane of mucous epithelia. Indeed, Poulsen *et al.* (1998) showed in the rat that TFF2 is bound to various mucus-producing cells after intravenous administration. Systemic neurohypophyseal release of TFF3 into the bloodstream could consequently represent an important physiological route for a receptor-mediated function of this peptide.

Furthermore, synthetic TFF3/monomer showed fear-modulating activities when injected into the basolateral nucleus of the rat amygdala (Schwarzberg *et al.,* 1999). This is a clear indication for behavioral effects of TFF peptides. From the highly regulated expression of TFF1 in the brain, a role for neural development may also be expected (Hirota *et al.,* 1995). In addition, synthesis of TFF1 in astrocytes may be an indication for a role in neuroinflammation.

IV. Concluding Remarks

A clearer picture of the multiple physiological functions of TFF peptides only emerged within the past few years. This picture turned out to be surprisingly complex and is probably far from being complete. However, there is much

evidence for a major role of TFF peptides in the maintenance of the surface integrity of mucous epithelia. Here, a structural role for the mucus due to interaction with mucins is evident along with various intracellular functions probably triggered via specific receptors. The receptor-mediated intracellular processes include cell migration, cell scattering, apoptosis, and inflammatory processes. Furthermore, minute amounts of TFF peptides are present in the brain as secretory products of neuronal and glial cells, particularly astrocytes. Here, they might play a role as neurotransmitters/neuromodulators and neurohormones reaching peripheral targets.

There are preliminary insights into the complex pattern of signaling cascades triggered by TFF peptides. However, clear molecular cloning data unambigously describing TFF receptors are still missing (for review, see Hoffmann *et al.,* 2001). The first report on TFF2 receptors in the rat small intestine was published in 1986 (Frandsen *et al.,* 1986). Recently, a β subunit of the fibronectin receptor and a transmembrane protein with similarity to CRP-ductin/muclin/Ebnerin were characterized due to their binding capacity of porcine TFF2 (Thim and Mortz, 2000). Thus, the characterization of putative receptors triggering the complex signaling cascades observed is clearly a major challenge for the near future. Only with this understanding can we fully discern the molecular function of this important peptide family and define its specific target cells and multiple roles in the health and disease of the body.

Acknowledgments

The authors thank M. Hinz for invaluable technical assistance, Dr. R. Zawada for his comments on the manuscript, C. Finger for excellent secretarial assistance, T. Weber for computerized processing of a figure, and Prof. K. Dietzmann, Prof. N. Parvizi (Neustadt-Mariensee), and Dr. T. Welte for support of the mapping studies illustrated in this article. Work in the authors' laboratory was supported by the Land Sachsen-Anhalt (1918A/0025H, 1918A/2587B, and 2349A/0085H to W.H.), the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (BMBF, Neuroverbund 01 ZZ 9510/Project B8 to W.H.), and the "Fonds der Chemischen Industrie" (0163615 and 0500058 to W.H.).

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Molecular Patterning along the Sea Urchin Animal–Vegetal Axis

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The molecular regulatory mechanisms underlying primary axis formation during sea urchin development have recently been identified. Two opposing maternally inherited systems, one animalizing and one vegetalizing, set up the animal–vegetal (A–V) axis. The vegetal system relies in part on the Wnt–β-catenin–Tcf/Lef signaling pathway and the animal system is based on a cohort of animalizing transcription factors that includes members of the Ets and Sox classes. The two systems autonomously define three zones of cell-type specification along the A–V axis. The vegetalmost zone gives rise to the skeletogenic mesenchyme lineage; the animalmost zone gives rise to ectoderm; and the zone in which the two systems overlap generates endoderm, secondary mesenchyme, and ectoderm. Patterning along the A–V also depends on cellular interactions involving Wnt, Notch, and BMP signaling. We discuss how these systems impact the formation of the second axis, the oral–aboral axis; how they connect to later developmental events; and how they lead to cell-type-specific gene expression via *cis*-regulatory networks associated with transcriptional control regions. We also discuss how these systems may confer on the embryo its spectacular regulatory capacity to replace missing parts.

KEY WORDS: Transcription factors, Inductive interactions, Cell fate specification, Embryonic regulation, Maternal determinants, Pattern formation, Gene regulation, β-catenin, Wnt, Notch, BMP.

I. Introduction

A century ago, Boveri (1901a,b) recognized and began experimental investigations on the animal–vegetal (A–V) polarity within the sea urchin egg. Like eggs

of many animals, sea urchin eggs have a firmly established, maternally imposed A–V polarity. Axes of polarity provide a three-dimensional coordinate system that patterns the embryo. There is much variation in the timing of establishment of different axes within species and between species and also in the mechanisms that underlie polarization (Goldstein and Freeman, 1997). In sea urchins, the ectoderm forms from the animal part of the egg (the animal pole is defined as the point of polar body extrusion), whereas the mesoderm and endoderm are derived from the vegetal part (the vegetal pole is defined as the pole opposite the animal pole). An oral–aboral (O–Ab) axis orthogonal to the A–V axis is specified in the early embryo, but it is not fixed until gastrulation (Cameron *et al.,* 1990; Hardin *et al.,* 1992; Davidson *et al.,* 1998). Many experiments indicate that patterning along the A–V axis depends on localized maternal determinants and cellular interactions, whereas differentiation of oral and aboral ectoderm requires interactions with vegetal cells. Although several speculative models have been advanced, the molecular mechanisms involved in axial patterning of the echinoid embryo have remained obscure until recently. New methods for assessing and modulating gene expression, combined with experimental manipulations of embryos, have provided much exciting new information. Here, we summarize the experimental results that provide our current understanding of the mechanisms of axial polarization and pattern formation in the sea urchin embryo, with an emphasis on important recent advances (Angerer and Angerer, 1999, 2000; Ettensohn and Sweet, 2000).

We review evidence that indicates that the establishment of the A–V axis involves cell autonomous activation of maternally specified vegetalizing and animalizing transcriptional regulatory factors. During early cleavages, maternally encoded animalizing factors, including orthologs of mammalian SoxB1, SoxB2, and Ets4, become localized exclusively to the nuclei of cells in the animal zone of the embryo destined to become ectodermal cell types. During the same time period, β-catenin becomes localized in nuclei of the cells close to the vegetal pole, cells whose descendents will produce skeletogenic and coelomic mesoderm. These animalizing and vegetalizing transcription systems overlap in nuclei of cells having an intermediate position along the A–V axis that are destined to become mesoderm, endoderm, and ectoderm. In turn, the maternally encoded transcription factors regulate the zygotic transcription of genes having spatially restricted domains of expression along the A–V axis. Some of the responding genes encode transcription factors, including those of the Krox–Krüpple class, that are involved in refining the pattern. Other responding genes encode proteins involved in intercellular signaling that have important roles in patterning the embryo. These include a Wnt8 ortholog that appears to reinforce the role of β-catenin in vegetal patterning, a BMP2/4 extracellular signal that promotes ectoderm differentiation, and a ligand for the Notch receptor that is produced by the vegetalmost blastomeres and is involved in specification of secondary mesenchyme cells, distinguishing them from endoderm.

In the intact sea urchin embryo, there is a strong correspondence between cell lineage and cell fate. Expression of "marker" genes in clones of cells sharing

fates is generally activated early in development, implying that specification of fate has occurred. We use specification as defined by Logan and McClay (1999) to indicate processes that result in distinctive cellular identities that predict their fates. A stricter definition of specification requires that clonal expression of marker genes be maintained when the specified embryo fragments are cultured in isolation (Davidson, 1989). This has sometimes been confirmed for fragments of sea urchin embryos, but regulation (in this context, the expression of genes normally restricted to other lineages) is often observed as well. Most urchin embryonic cells remain uncommitted to a fate until late in development, as assayed by cell transplantation experiments. That is, the developmental potentials of most cells remain broader than their fates in the intact embryo until late stages. Micromeres are the prominent exception to this generalization.

The cellular interactions involved in specification of cell fates in the sea urchin can be either evocative or repressive. Genes whose expression in embryos is associated with cellular specification are often temporally activated by general activating transcription factors. Negative spatial regulatory transcription factors then restrict expression to appropriate embryonic territories. The late commitment to a fate by most cells and the robust and counterbalancing regulatory networks involved in patterning the sea urchin embryo account for its remarkable capacity to regulate in response to experimental perturbations that disrupt normal cellular interactions.

II. Historical Perspective

A. Evidence for a Maternal A–V Axis of Polarity

The eggs of most sea urchin species have no obvious morphological polarity, but in some populations of *Paracentrotus lividus* a pattern of pigment granules predicts the A–V polarity (Boveri, 1901b). During meiotic maturation, which occurs long before the egg is shed, the polar bodies form at the animal pole (as in many other organisms), but they usually leave the surface of the egg before it is shed (Schroeder, 1980a). In addition, a hole on the egg jelly layer, the "jelly canal," is at the animal pole and can be detected by suspending eggs in India ink (Schroeder, 1980a,b); however, it is not a convenient marker of the animal pole for many experimental manipulations. The origin of the jelly canal is unknown, but its presence in oocytes prior to meiotic maturation suggests that it may be related to an axis-generating event in oogenesis (Schroeder, 1980a,b). The maternal pronucleus been reported to be in the animal half of the egg of *P. lividus* (Di Carlo *et al.,* 1994). If true for other species, this may allow development of a vital stain to detect A–V polarity, facilitating experimentation. In the regular sea urchins that produce feeding pluteus larvae commonly investigated by developmental biologists, the first two equal cleavages are along the A–V axis, whereas the third is equatorial, resulting in eight equal and usually indistinguishable blastomeres. At the next cleavage, the

four vegetal blastomeres cleave unequally and obliquely to produce a quartet of blastomeres at the vegetal pole—the micromeres; their sisters are the much larger macromeres. The animal blastomeres cleave equally and meridionally, forming a ring of eight mesomeres that constitute the animal half. Thus, as shown in Fig. 1A (see color insert), the fourth cleavage provides the first obvious manifestation of the A–V polarity of the embryo.

Lineage tracing experiments indicate that cells along the A–V axis have mostly predictable fates caused by a nearly invariant pattern of cleavage that results in a consistent distribution of polarized determinants and interacting cells (Hörstadius, 1939, 1973; Cameron *et al.,* 1987; Cameron and Davidson, 1991). The cell lineage of the pluteus larva of *Strongylocentrotus purpuratus* is almost completely defined and nearly invariant (Cameron *et al.,* 1987). When ready to feed, the larva has approximately 1800 cells of approximately 15 different types, most distinguishable by their expression of specialized genes. Fate maps for early embryos and the resulting pluteus larva are shown in Fig. 1. The vegetalmost micromeres differentiate into skeletogenic and coelomic mesoderm, whereas the macromeres differentiate into endodermal cells of the gut and various derivatives of the secondary mesenchyme as well as a variable number of ectodermal cells. Cells of the animal half form ectodermal epithelia distinguished by expression of oral or aboral ectoderm marker genes. The stomodeum (mouth) forms on the oral side where the invaginating archenteron fuses with the overlying ectoderm, whereas the ciliary band of neurogenic cells forms at the interface of oral and aboral ectoderm. A pair of coelomic pouches form at the tip of the archenteron as gastrulation nears completion. After feeding begins there is extensive growth and morphogenesis of the larva. Ultimately, an adult rudiment is formed on the left side of the pluteus that is released during metamorphosis.

Upon the fifth cleavage, mesomeres cleave equally and latitudinally to form two animal tiers (an1 and an2), whereas the macromeres divide equally and meridionally. The micromeres divide unequally and obliquely to form the skeletogenic large micromeres; the small micromeres cleave very slowly in embryos and are fated to form part of the coelomic pouch of the larva (Pehrson and Cohen, 1986). Upon the sixth cleavage, the animal blastomeres divide equally and meridionally, the daughters of the macromeres divide equally and latitudinally, and the large micromeres divide equally. As shown in Fig. 1B, the resulting 60-cell embryo thus consists of five tiers along the A–V axis: an1, an2, veg1, veg2, and (large and small) micromeres. These tiers of cells have more or less distinctive fates in the undisturbed embryo, as summarized in Fig. 1C. The veg2 tier gives rise to the vegetal plate, consisting of a central ring of secondary mesenchyme cells that lie at the tip of the invaginating archenteron and that are surrounded by endoderm cells that form the fore- and midguts. Early lineage studies suggested that the border between the endoderm and ectoderm corresponds to the border of the veg1 and veg2 tiers. However, more refined investigations indicate that variable numbers of veg1 cells become endoderm (of the hindgut) while others become anal ectoderm, depending

on conditional specification mechanisms discussed in Section IV.C (Ruffins and Ettensohn, 1996; Logan and McClay, 1997; Ransick and Davidson, 1998). The boundary between oral and aboral ectoderm, where the ciliary band forms, is still not determined by late blastula stage, and cells from lineages that contribute to both aboral and oral ectoderm are included in the ciliary band (Cameron *et al.,* 1993). Thus, the fate map for the 60-cell embryo is necessarily ambiguous for the boundaries of ectoderm–endoderm and oral–aboral ectoderm whose positions depend on later cellular interactions. By the 60-cell stage, founder cells for each of five polyclonal territories have formed: large and small micromeres, mesendoderm of the vegetal plate, and oral and aboral ectoderm (Davidson, 1989; Davidson *et al.,* 1998). The territories consist of contiguous cells that often express a common set of marker genes, although the lineages of cells of a territory may subsequently follow different pathways of differentiation.

Fragments derived from early embryos bisected along the equator develop very differently in isolation (Boveri, 1901b; Hörstadius, 1939). The isolated animal half arrests as an epithelial ball, the dauerblastula, that expresses some genes characteristic of specified ectoderm but usually does not form structures or express marker genes characteristic of endoderm or mesoderm. The animal half also fails to complete morphogenesis of structures normally formed by ectoderm cells of animal halves, such as the stomodeum and the ciliary band. In contrast, isolated vegetal halves often develop into a normal pluteus larva having differentiated derivatives of all three primary germ layers. Similar results are obtained when unfertilized eggs are surgically bisected along the equator prior to fertilization, indicating that the A–V axis is of maternal origin (Hörstadius, 1939, 1973; Maruyama *et al.,* 1985). These results indicate that the animal half lacks essential maternal determinants present in the vegetal half and that interactions with vegetal cells are required for the specification of the full range of fates of ectodermal cells derived from the animal half. Fragments of eggs bisected along the A–V axis, and blastomeres separated after the first or second cleavages along the A–V axis, develop into pluteus larvae, adjusting for their reduced size (Driesch, 1892). These observations indicate that maternal determinants are distributed around the A–V axis in a radially symmetric pattern, and that the oral–aboral poles have not been fixed during early cleavage.

B. Cellular Interactions and the Regulative Capacity of the Embryo

Tiers of blastomeres separated along the A–V axis at the 60-cell stage all show restricted potential according to their position along the axis when cultured in isolation but often regulate to form cell types and structures beyond their fates in the intact embryo (Hörstadius, 1973). Indicative of the regulative capacity of the embryo, none of the five tiers is required for development of an essentially normal

pluteus larva, although some extirpates may lack the full complement of secondary mesenchyme derivatives. The large micromeres in isolation give rise only to skeletogenic mesoderm, although their full differentiation requires the addition of serum to seawater, suggesting the need for permissive cellular interactions in the intact embryo. When the large micromeres are transplanted to ectopic sites or recombined with other fragments of embryos, they form only skeletogenic mesenchyme and can thus be considered to be determined. The fates of all other blastomeres (except possibly the enigmatic small micromeres) remain plastic until the late blastula or gastrula stages and can be altered by recombination with other embryo fragments. Many spectacular recombination experiments were performed and summarized by Hörstadius (1939, 1973). In most instances, they have been repeated or extended using modern methods to track cells and molecular markers of specification or differentiation; these experiments have been summarized elsewhere (Wilt, 1987; Livingston and Wilt, 1990a,b; Khaner and Wilt, 1991; Brandhorst and Klein, 1992; Davidson *et al.,* 1998). Particularly informative was Hörstadius' observation that an essentially normal pluteus can be generated from the combination of the an1 or an2 tiers with micromeres, indicating that the intervening three-fourths of the embryo is not required for patterning of the embryo. Hörstadius found that the an1 tier required more micromeres than the an2 tier to promote development of a pluteus. He presented these observations as evidence for Runnström's (1929) model whereby the A–V pattern is the result of opposing, intersecting concentration gradients of extracellular animalizing and vegetalizing influences that diffuse from the two poles. According to this model, the ratio of the two substances establishes positional information along the A–V axis that specifies cell fate.

A recent model for blastomere specification along the A–V axis was based on a cascade of short-range inductive interactions during early cleavage stages initiated by the micromeres and passing successively to more animal tiers (Wilt, 1987; Davidson, 1989; Davidson *et al.,* 1998). When micromeres are transplanted from the vegetal pole of the 16-cell embryo to ectopic sites, including the animal pole, they induce the formation of a vegetal plate (mesendoderm), sometimes producing a fully formed secondary gut and associated assemblages of skeletogenic and secondary mesenchyme (Horstadius, 1973; Ransick and Davidson, 1993). Removal of micromeres delays gastrulation, although embryos eventually regulate to form nearly normal plutei lacking some secondary mesenchyme derivatives (Ransick and Davidson, 1995; Sweet *et al.,*1999). These experiments indicate an important role for the vegetal blastomeres, particularly the micromeres, in patterning the embryo. This role will be discussed in more detail later. Although neither the opposing morphogen gradient model nor the inductive cascade model can fully explain all the experimental observations, aspects of both contribute to the emerging view of A–V patterning (Angerer and Angerer, 1999, 2000).

Cells of different territories of the early blastula begin to express marker genes that are dependent on autonomous specification or conditional specification via the cellular interactions that occur during cleavage stages. A useful test of the autonomy of specification, as well as the timing of cellular interactions required for

specification, is the expression of markers of specification in embryos dissociated into single cells at various stages. Such experiments suggest that many specification events are autonomous or depend only on very early cellular interactions (Hurley *et al.,* 1989; Stephens *et al.,* 1989; Chen and Wessel, 1996; Godin *et al.,* 1997). However, these types of investigations rarely establish whether expression of the marker gene is restricted to the appropriate cells or even the appropriate fraction of cells of the embryo. This is a significant caveat to the interpretation of autonomous specification because of the importance of suppressive interactions and negative gene regulatory events that will be discussed later. Cells expressing markers of specification other than the micromeres remain uncommitted and can often be respecified in response to experimental perturbations until late in embryonic development. For instance, the derivatives of the large micromeres ingress as primary mesenchyme cells that are skeletogenic. However, if primary mesenchyme cells are removed from embryos, some secondary mesenchyme cells convert their fate to form skeletogenic mesenchyme (Ettensohn and McClay, 1988; Ettensohn, 1992). If the archenteron is removed from a gastrulating embryo, the remaining endoderm cells are respecified to replace the missing parts of the gut (McClay and Logan, 1996). These spectacular regulative properties indicate the importance of ongoing cellular interactions and indicate that early specification events involving the differential expression of specialized genes can occur long before commitment. We next consider the mechanisms underlying these early specification events in more detail. Many of the experiments discussed in this article depend on the use of new experimental techniques to perturb gene function. These are introduced and discussed throughout the text.

This review concerns embryos of regular urchins that have radial cleavage and undergo indirect development: A swimming pluteus larva is formed that feeds, grows, forms an adult rudiment, and undergoes metamorphosis to form a juvenile. Several species are commonly used for research and their basic development is similar. However, surprising differences are sometimes observed in the response of echinoid embryos of different species to the same experimental manipulation. When these experiments have only been performed on a single species, we often identify that species; however, even when the results reported apply to multiple species, they may not apply to all.

III. Maternally Encoded Transcriptional Regulators Acting along the A–V Axis

A. Summary of the Molecular Events Regulating A–V Axis Patterning

Considerable progress has recently been made in understanding the molecular events that lead to the formation of cell types along the A–V axis. It is now

clear that cell-autonomous, maternally derived mechanisms initiate a process that gives rise to three gene-regulatory zones. These zones are defined in the early embryo by distinct sets of vegetalizing and animalizing transcription factors and their region of overlap (Angerer and Angerer, 2000). The zones are refined as development proceeds to produce the five embryonic territories of the 60-cell stage embryo and, ultimately, approximately 15 individual cell types of the pluteus larva.

Although not yet characterized at the molecular level, a cell-autonomous mechanism inherited by the egg cytoplasm leads to a wave of nuclear entry of β-catenin beginning at the vegetalmost pole of the embryo. Opposed to the β-catenin vegetal signaling system is a set of animalizing transcription factors, which include SpEts4, SpSoxB1, and SpSoxB2, whose expression is excluded from the vegetal pole of the embryo by maternally inherited mechanisms. The vegetal signaling system defines the micromere zone, which gives rise to skeletogenic (primary) mesenchyme and part of the larval coelom. The animalizing transcription factors define the mesomere zone, which leads to primitive ectoderm. Primitive ectoderm requires signaling from vegetal cells to fully differentiate into definitive oral and aboral ectoderm. The region in which vegetal and animal systems overlap defines the macromere zone, which will produce the mesendoderm (i.e., secondary mesenchyme cells and gut) and some ectoderm.

B. Vegetalization via the β-Catenin Signaling Pathway

1. Nuclear β-Catenin Specifies Mesendoderm

During the 16- to 120-cell stage, the sea urchin embryo displays a striking graded pattern of nuclear β-catenin along its A–V axis (Logan *et al.,* 1999). The vegetal pole micromeres and their immediate descendants have the highest levels, the overlying vegetal blastomeres have intermediate levels, and the animal blastomeres have little or no nuclear β-catenin. From what is known about the functions of β-catenin in other organisms (Willert and Nusse, 1998; Fig. 1D), the spatial distribution observed in sea urchins implies a role for β-catenin in early cell-fate decisions along the A–V axis. β-Catenin functions as a transcriptional activator in combination with members of the Tcf/Lef family of HMG domain proteins by interacting with Tcf/Lef at Tcf/Lef–DNA elements within the transcriptional control regions of numerous genes. It seemed likely that β-catenin would function in sea urchin embryos in an analogous fashion by interacting with Tcf/Lef to establish cell fates. The nuclear localization of β-catenin in vegetal blastomeres suggested that it had a direct role in the establishment of mesendoderm. This hypothesis was confirmed in three different laboratories by perturbing nuclear β-catenin levels and observing dose-dependent changes in mesodermal and endodermal cell-type specification (Emily-Fenouil *et al.,* 1998; Wikramanayake *et al.,* 1998; Logan *et al.,* 1999).

Expression of a hyperstable form of β-catenin by RNA injection into sea urchin eggs causes massive vegetalization, with severe exogastrulation and overproduction of secondary mesenchyme cells such as pigment and muscle. These results are virtually identical to those seen when embryos are treated with lithium ion, an agent known to inhibit GSK-3β, an intracellular serine–threonine kinase that phosphorylates β-catenin and promotes its decay. In striking contrast, depleting nuclear β-catenin pools in embryos by overexpression of truncated C-cadherin results in severely animalized embryos with no detectable vegetally derived cell types. Complementary results to those with β-catenin are obtained with overexpressed or dominant-negative forms of sea urchin GSK-3β or Tcf/Lef. An *S. purpuratus* Tcf/Lef ortholog is expressed maximally when β-catenin becomes localized to vegetal nuclei, consistent with its acting in combination with β-catenin to specify vegetal cell fates (Huang *et al.,* 2000; Vonica *et al.,* 2000).

Perhaps even more dramatic effects of perturbing β-catenin are obtained with naive animal halves. For example, when the hyperstable β-catenin form is injected into eggs and animal halves are isolated and cultured, they produce a well-defined archenteron and secondary mesenchyme cell derivatives, whereas control animal halves expressing an inactive form of β-catenin produce classical dauerblastulae (Wikramanayake *et al.,* 1998). These experiments mimic one that was first performed several decades ago, in which lithium treatment of animal halves led to the production of an archenteron (von Ubisch, 1929). Lithium ions are a strong vegetalizing agent and inhibit the activity of GSK-3β, thereby circumventing the Wnt signaling pathway and resulting in stabilization and nuclear localization of β-catenin (Klein and Melton, 1996). The perturbation experiments with β-catenin, GSK-3β, and Tcf/Lef provide compelling evidence for the vegetal nuclear β-catenin signaling system as necessary and sufficient for establishing mesendoderm fates. However, several features of β-catenin's action merit further discussion.

2. Cell Autonomous Nuclear Entry of β-Catenin

When sea urchin blastomeres are dissociated and cultured as single cells, β-catenin enters the nuclei of cells on schedule and in the same proportion of cells as that seen in intact embryos (Logan *et al.,* 1999). These experiments show that the accumulation of β-catenin in nuclei of vegetal cells is regulated cell autonomously. Furthermore, because the cultures of dissociated cells are highly dilute and transmission of extracellular signals is unlikely, the cell autonomous nuclear entry of β-catenin obviates the requirement for an upstream Wnt ligand as an initial component of the β-catenin specification process (Fig. 1D). As discussed later, a sea urchin Wnt8 ortholog has been characterized and appears to act downstream rather than upstream of the initial β-catenin signaling event, functioning to reinforce and maintain nuclear β-catenin in vegetal cells. The generation of nuclear β-catenin involved in the specification of the dorsal–ventral axis of *Xenopus* may also occur in the absence of an upstream Wnt ligand, although this remains controversial

(Moon and Kimelman, 1998). The simplest explanation for the cell autonomous nuclear entry of β-catenin is that a maternally derived mechanism promotes entry into vegetal nuclei while preventing nuclear entry in animal cells. Although the mechanism is not yet known, it is possible that the graded activation or inhibition of GSK-3β, which exists as a maternal mRNA and protein, modulates β-catenin levels in the vegetal portion of the early embryo. For instance, fertilization might trigger a cortical rotation process that causes a wave of activation of GSK-3β starting at the animal pole and destabilizing β-catenin as it moves vegetally. In *Xenopus,* Dishevelled (Dsh), which acts downstream of the Frizzled receptor in the Wnt signaling pathway, is associated with vesicle organelles that are enriched on the prospective dorsal side of the embryo after cortical rotation (Miller *et al.,* 1999).

3. Micromeres and β-Catenin

Micromeres are powerful inducers of mesendoderm when transplanted to ectopic sites within the animal half of the embryo. It is thus of interest to ask whether their inductive properties require the vegetal nuclear β-catenin signal and whether the responding animal cells have enhanced levels of nuclear β-catenin, thereby providing an explanation for their acquired mesendoderm fate. Logan *et al.* (1999) demonstrated that micromeres overexpressing truncated C-cadherin, which reduces nuclear β-catenin pools, are unable to induce mesendoderm when transplanted to naive animal halves. These results indicate that nuclear β-catenin is required for the micromere's inductive properties. In contrast, when micromeres from normal embryos were transplanted to animal halves, ectopic archentera were formed as expected; surprisingly, however, micromere signaling did not lead to appreciable levels of nuclear β-catenin in the animal cells. These results indicate that the micromere inductive signal acts independently of the vegetal nuclear β-catenin signaling system. It thus appears that two distinct pathways lead to mesendoderm specification—one that requires nuclear β-catenin and one that does not. The unidentified inductive signal(s) emanating from the micromeres is the source of a major gap in our understanding of mesendoderm specification in the sea urchin embryo.

Because removing micromeres from embryos attenuates but does not eliminate the ability of the embryos to produce gut and secondary mesenchyme, vegetal nuclear β-catenin is likely to be the primary signaling pathway for mesendoderm specification, whereas the signal sent by the micromeres appears to reinforce the specification process. Primitive sea urchin species do not have micromeres, supporting the view that the vegetal nuclear β-catenin signaling system is primal and the micromere signal has been co-opted as a mesendoderm inducer in recent sea urchin clades. Early ingression of skeletogenic mesenchyme is also likely to be a recently derived feature of echinoid development. Ophiuroid (brittle star) embryos do not produce micromeres but have early ingressing skeletogenic mesenchyme

and form a pluteus larva. Asteroid (starfish) embryos also lack micromeres and skeletogenic mesenchyme, but the eggs have a well-defined A–V axis. Depletion and injection experiments indicate that cytoplasm that can confer the capacity to form an archenteron is localized to the vegetal pole even before the germinal vesicle breakdown that provides a marker of the animal pole via polar body formation (Kiyomoto and Shirai, 1993; Kuraishi and Osanai, 1994). Similar experiments have not been performed on echinoid eggs because of the difficulty in identifying the animal pole. Although the distribution of β-catenin has not been investigated for the other embryos of other echinoderms, we predict that it will appear in nuclei of vegetal blastomeres during early cleavage of all echinoderm embryos. In some instances, there may be a maternal mRNA encoding β-catenin localized to the vegetal cytoplasm of the egg.

4. Autonomous Specification of Micromeres

The large micromeres are a classic embryological example of an autonomously specified and determined lineage. Whether cultured in isolation or transplanted to ectopic sites on donor embryos, micromeres will invariably divide several times and initiate differentiation into skeletogenic mesenchyme cells exhibiting well-described developmental and molecular features, although complete differentiation (formation of spicules) requires a permissive signal present in serum or the extracellular matrix (Okazaki, 1975; Harkey and Whiteley, 1985; McCarthy and Spiegel, 1983; Benson and Chuppa, 1990). It is usually anticipated that micromeres must inherit maternal determinants that are responsible for their autonomy. As discussed previously, nuclear β-catenin first appears in micromeres (Logan *et al.,* 1999) and is required for skeletogenic mesenchyme differentiation to occur. However, other vegetal cells also express nuclear β-catenin shortly thereafter, making it unlikely that the presence of β-catenin is the sole arbitrator of the autonomous behavior of micromeres. For instance, overexpressing nuclear β-catenin in animal blastomeres leads mostly to conversion into secondary mesenchyme cells and endoderm. Extensive searches have failed to yield vegetal pole determinants responsible for micromere autonomy, suggesting that the simple localization of a maternal RNA or protein is unlikely to be the explanation. Micromeres acquire a much smaller pool (about 15 times smaller) of SpSoxB1 than macromeres as a result of the unequal fourth cleavage and other regulatory events that remain to be elucidated (Kenny *et al.,* 1999). Thus, micromeres have the highest nuclear β-catenin/SpSoxB1 ratio and this may be sufficient for the irreversible determination of large micromeres as skeletogenic mesenchyme.

Cytoskeletal rearrangements at the vegetal pole might have a role in initiating the autonomous determination program of large micromeres (Dan *et al.,* 1983; Schroeder 1980a, 1982; Tanaka, 1981). During the fourth cleavage the vegetal zone that is segregated into micromeres becomes devoid of surface microvilli. Moreover, pigment granules are actively excluded from the vegetal cytoplasm prior to

formation of micromeres by an active cytoskeleton-based system. Thus, micromeres are born without an actin-based cytoskeletal cortex. Many proteins bind to the actin cortex, and in its absence the environment of the cell would be necessarily altered. In particular, Chuang *et al.* (1996) suggested that nuclear proteins actively retained by the actin cytoskeleton in blastomeres other than micromeres might be free to enter nuclei in micromeres. Indeed, a homeobox transcription factor, SpOtx, appears transiently in nuclei of micromeres at the 16-cell stage but remains in the cytoplasm of macromeres and mesomeres. Only later in development does SpOtx enter the nuclei of other cells. A domain of SpOtx was found to interact with the actin-binding protein α-actinin, thereby suggesting a mechanism that would bind SpOtx to the actin cytoskeleton in all cells but micromeres (Chuang *et al.,* 1996). In combination with nuclear β-catenin–Tcf/Lef, it is possible that a set of uniformly distributed maternal transcription factors enter micromere nuclei at the 16-cell stage due to cytoskeletal changes and subsequently establish the program the events required for skeletogenic mesenchyme differentiation.

Attempts have been made to identify transcription factors involved in primary mesenchyme cell-specific expression. Several genes have been identified that are expressed exclusively in differentiating primary mesenchyme cells; many of these have a role in the formation of the embryonic spicules and larval skeleton. The best studied gene is *SM50*. The transcriptional control region of *SM50* has been characterized in detail and a positively acting 260-bp region surrounding the transcriptional start site was found to be necessary and sufficient for primary mesenchyme cell expression, provided it was supplemented with general enhancer elements (Makabe *et al.,* 1995). Although the factors binding to this region have not been identified, they are likely to be maternal factors and their distribution at the 16-cell stage should produce insights into the early events of primary mesenchyme cell differentiation.

Perhaps the most intriguing recent discovery concerning primary mesenchyme cell differentiation was made by Akasaka and coworkers, who identified and characterized an Ets-related transcription factor in *Hemicentrotus pulcherrimus* (Kurokawa *et al.,* 1999). These workers identified a sea urchin ortholog of Ets1 + 2 called HpEts, which is uniformly expressed in the egg and early embryo but whose expression becomes restricted to the primary mesenchyme cell lineage after the hatching blastula stage. Overexpression of HpEts by mRNA injection dramatically transforms the fate of most cells of the embryo into migratory primary mesenchyme and induces the expression of *SM50* while suppressing the expression of endoderm and aboral ectoderm markers. A dominant-negative form of HpEts represses *SM50* expression and skeletogenesis. In the *H. pulcherrimus* genome, an Ets binding site exists upstream of the *SM50* gene that functions as a positive *cis*-regulatory element. These results are the first to establish that a single transcription factor can convert cells that are not micromeres to a skeletogenic mesenchyme cell fate. Because the HpEts mRNA is uniformly distributed in the egg and early embryo, it will be of great interest to determine whether the HpETs

protein (or its active form) is asymmetrically distributed, perhaps by a cytoskeletal retention mechanism such as that described for SpOtx. Alternatively, HpEts activity may be repressed directly or indirectly by animalizing transcription factors such as SpSoxB1, thus restricting its activity to micromeres.

5. A Possible Animalizing Role for Bep Proteins

Members of the Bep (butanol extractable proteins) family of surface proteins are enriched in the animal zone of unfertilized eggs and early embryos of *P. lividus,* as are Bep maternal mRNAs (Di Carlo *et al.,* 1994, 1996; Romancino *et al.,* 1992, 1998, 2001). Beps are similar in sequence to some extracellular matrix proteins of the hyaline layer and basal lamina cellular matrix. Fab fragments of antibodies raised against Bep1 or Bep4 inhibit reaggregation of dissociated embryos, suggesting a role of Beps in cellular interactions and/or binding (Romancino *et al.,* 1992). Incubation of embryos following fertilization in Fabs against maternal Bep4 or Bep1 caused exogastrulation (Romancino *et al.,* 1992; Di Carlo *et al.,* 1996). Incubation of zygotes in different concentrations of Fabs caused a range of vegetalized phenotypes similar to those produced by treatment with a range of concentrations of lithium, including exogastrulation (Romancino *et al.,* 2001). Treatment of zygotes with Fabs to Bep4 also caused a retraction of the zone of hatching enzyme expression similar to that observed for treatment with lithium ions Ghiglione *et al.,* 1993), indicating a connection to β-catenin signaling; indeed, this treatment was reported to expand the vegetal domain of nuclear β-catenin in early embryos. Bep4 protein concentrates at the adherens junctions in the animal cells where it is enriched, as does β-catenin. Moreover, immunoprecipitation experiments suggest that Bep4 is associated with cadherin. Romancino *et al.* (2001) suggested that the association of Bep4 with cadherin, a transmembrane-binding partner of β-catenin, may restrict β-catenin to adherens junctions in animal cells while allowing it to enter nuclei in vegetal cells. Fabs against Bep1 may disrupt the association of Bep1 with cadherin, allowing β-catenin to enter nuclei in some animal cells. It is thus possible that Bep4 plays a role in establishing the graded distribution of nuclear β-catenin.

6. Events Downstream of Vegetal Nuclear β-Catenin

Mesendoderm must be further specified into separate mesoderm and endoderm domains. This allocation requires the Notch signaling system. Notch signaling is required to produce secondary mesenchyme, and without the Notch signal cells with sufficient levels of nuclear β-catenin become endoderm. Recently, two sea urchin orthologs belonging to the Krox–Krüpple family of zinc-finger transcription factors have been identified that are strong candidates for immediate downstream targets of β-catenin signaling. Angerer and coworkers (Howard *et al.,* 2000) identified *SpKrl,* a gene that is expressed with a spatiotemporal pattern that correlates

closely with entry of β-catenin into vegetal nuclei. *SpKrl* is upregulated when nuclear β-catenin activity is increased by treatment with LiC1 and downregulated in embryos depleted of β-catenin. LiC1-mediated activation of *SpKrl* is independent of protein synthesis, implying that *SpKrl* is a direct target of the β-catenin–Tcf/Lef transcription factor complex, although confirmation of this awaits identification of Tcf/Lef binding sites within the *SpKrl* transcriptional control region. Most notably, blocking *SpKrl* expression with morpholino antisense oligonucleotides inhibits endoderm formation, whereas vegetal structures are restored by forced expression of *SpKrl* in β-catenin-depleted embryos. Because *SpKrl* negatively regulates the expression of the animalizing transcription factor SpSoxB1, Howard *et al.* (2001) proposed that SpKrl functions in patterning the vegetal domain by suppressing animalizing transcription factor activities.

A second Krox–Krüpple gene called *SpKrox-1* is expressed in vegetal blastomeres with a pattern very similar to that of *SpKrl* (Wang *et al.,* 1996; E. Davidson, personal communication). As for *SpKrl, SpKrox-1* expression depends on nuclear β-catenin. Blocking *SpKrox-1* expression with morpholino antisense oligonucleotides results in an expansion of aboral ectoderm and a corresponding reduction in size and alteration in position of oral ectoderm and mesendoderm territories (T. Kiyama and W. Klein, unpublished observations). Interestingly, mesendoderm and oral ectoderm differentiation per se is not affected in these embryos since they have tripartite guts, stomodea, and ciliary bands. It is possible that SpKrox-1 acts to repress aboral ectoderm genes in the vegetal domain where it is normally expressed. Krox factors often act directly as transcriptional repressors in other systems. The forced derepression of the aboral ectoderm gene program by the loss of functional SpKrox-1 in vegetal cells might result in an overproduction of aboral ectoderm cells, with a corresponding reduction in mesendoderm and oral ectoderm cells.

The discovery of two potential repressors of ectoderm gene expression in vegetal cells raises an interesting point. Nuclear β-catenin–Tcf/Lef is thought to activate genes associated with the early events of mesendoderm specification. One of these events could be the activation of a class of genes encoding transcriptional repressors that selectively repress nonmesoendoderm genes in the emerging vegetal plate territory. *SpKrl* and *SpKrox-1* could represent members of the class. This model is appealing because it would directly link the primary mesendoderm specification pathway, namely the maternally derived entrance of β-catenin into vegetal nuclei, with the repression of ectoderm-specific genes in the vegetal plate territory.

C. Opposing Animalizing and Vegetalizing Transcription Factors

The idea of opposing animalizing and vegetalizing morphogen gradients for A–V axis patterning in the sea urchin embryo had been out of vogue for many years. However, recent evidence indicates that opposing animalization and vegetalization

specification systems do indeed exist, supporting, in a general sense, the thinking of classical experimental embryologists such as Boveri, Runnström, and Hörstadius. Nonetheless, it is now clear that the vegetal specification system is not in the form of a diffusible vegetal extracellular morphogen but rather a cell autonomous, maternally inherited mechanism that leads to a graded wave of nuclear localization of β-catenin in vegetal cells. Moreover, in a series of insightful experiments performed largely by the Angerer laboratory, an animalizing specification system was shown to exist, again not in the form of a diffusion gradient but rather as a set of maternally inherited animalizing transcription factors. The discovery of the animalizing transcription factor system was based on an analysis of the *cis*-acting elements regulating the spatial expression of a class of genes termed the "very early blastula" (VEB) genes (Reynolds *et al.,* 1992). The VEB genes include those encoding the hatching enzyme [*HE* in *P. lividus* (Lepage *et al.,* 1992b) and *SpHE* in *S. purpuratus* (Reynolds *et al.,* 1992)] and an astacin protease related to Tolloid and BMP1 [*BP10* in *P. lividus* (Lepage *et al.* 1992a) and *SpAN* in *S. purpuratus* (Reynolds *et al.,* 1992)]. VEB genes are defined by their zygotic expression, which begins at the eight-cell stage and is extinguished by the blastula stage. VEB gene expression is excluded from the vegetalmost regions of the embryo and a pattern of graded expression is found in the vegetal half of each embryo such that embryos express varying levels in their macromere lineages (Reynolds *et al.,* 1992; Nasir *et al.,* 1995). Embryo dissociation experiments show that the activation of VEB expression is a cell autonomous process (Reynolds *et al.,* 1992; Ghiglione *et al.,* 1993). These facts indicate that initial activation of the VEB genes is under maternal control. The VEB genes thus provide an opportunity to identify maternal transcription factors that are responsible for their spatially restricted pattern of expression.

Characterization of the transcriptional regulatory regions of the *SpHE* and *SpAN* genes revealed a cohort of multiple positively acting transcription factors that regulated nonvegetal VEB gene expression (Kozlowski *et al.,* 1996; Wei *et al.,* 1995, 1997). Two of these factors, SpEts4 and SpSoxB1, have been investigated in detail. SpEts4 binding sites are required for normal activation of the *SpHE* gene in the nonvegetal region of the embryo (Wei *et al.,* 1999a,b). The pattern of SpEts4 expression is consistent with its role in regulating *SpHE* (Wei *et al.,* 1999b). Although maternal transcripts are uniformly distributed in the egg and early cleaving embryo, they rapidly disappear and are replaced by zygotic transcripts that accumulate in much the same fashion as VEB gene expression. The uniform maternal expression of *SpEts4* implies that the mechanisms operating initially to confer nonvegetal expression to the VEB class must operate downstream of mRNA localization.

Similarly, SpSoxB1 interacts with a DNA element within the *SpAN* transcriptional control region, which is required for *SpAN* transcription (Kenny *et al.,* 1999). As with SpEts4, SpSoxB1 mRNAs are uniformly distributed in eggs and early cleaving embryos but zygotic transcripts are expressed identically to other VEB genes. However, at the 16-cell stage, the micromeres have very reduced levels of

SpSoxB1 protein, whereas high levels are found in the overlying macromeres and mesomeres. These data support a model whereby SpSoxB1 regulates the nonvegetal transcription of *SpAN* and perhaps other VEB genes by virtue of its distribution at the fourth cleavage and subsequent asymmetric expression pattern. The basis for the nonvegetal distribution of SpSoxB1 can be partially attributed to the smaller reservoir of SpSoxB1 in the micromere cytoplasm, although this is unlikely to be the complete explanation. Regardless of the maternal mechanisms, the essential point is that maternal transcription factors known to regulate nonvegetal gene expression are rapidly excluded from the vegetalmost regions of the early embryo. These transcription factors provide the embryo with the ability to express genes in a manner that opposes the vegetal β-catenin signaling system. It has been proposed that this cohort of transcription factors, in parallel to specification of mesendoderm by β-catenin–Tcf/Lef, conditionally specifies the primitive ectoderm state (Angerer and Angerer, 2000). As discussed later, signals from the vegetal half of the embryo, dependent on the β-catenin signal, are required for primitive ectoderm to become patterned and differentiated along the O–Ab axis.

Further evidence that specification involves the animalizing transcription factor system in the sea urchin embryo derives from gain-of-function and loss-of-function experiments on SpSoxB1 and to a lessor extent on the related factor SpSoxB2. Embryos overexpressing SpSoxB1 resemble dauerblastulae, implying that expansion of the SpSoxB1 domain counteracts the vegetal β-catenin signal that specifies mesendoderm, much like that seen in β-catenin-depleted embryos (R. C. Angerer, personal communication). Overexpression of SpSoxB1 also inhibits the formation of the skeletogenic primary mesenchyme cells, suggesting that vegetal misexpression of SpSoxB1 can affect even the most vegetal specification events. Conversely, expressing a dominant repressor form of SpSoxB1 or injecting morpholino antisense oligonucleotides causes most cells in the embryo to express the 6c10 epitope, a marker for skeletogenic mesenchyme (R. C. Angerer, personal communication). Similar results have been obtained by perturbing SpEts4 and SpSoxB2 function.

The emerging model of cell-fate specification along the A–V axis posits three zones of transcription factor activity. The micromere zone, in which nuclear β-catenin is found at the highest levels, requires β-catenin signaling for the autonomous specification of skeletogenic mesenchyme and for the mesendoderm-inducing activity of micromeres. The mesomere zone relies on a cohort of animalizing transcription factors, three of which (SpEts4, SpSoxB1, and SpSoxB2) have been identified; others are likely to be found. The function of the animalizing transcription factors in the mesomere zone is to specify primitive ectoderm. The macromere zone has overlapping and opposing vegetalizing and animalizing transcription factors. It is likely that within this zone, the ratio of vegetalizing to animalizing factors is critical for the decision to become mesendoderm or ectoderm. This ratio decreases along the vegetal to animal axis. As discussed later, the boundary between secondary mesenchyme and endoderm is established early by a

Notch signaling system, whereas the endoderm–ectoderm boundary is refined and sharpened later in development by the action of a BMP2/4 signaling mechanism also involving Notch.

IV. Extracellular Signals Reinforce and Refine Specification along the A–V Axis

A. Notch Signaling and the Secondary Mesenchyme–Endoderm Border

As discussed previously, macromeres can be distinguished from mesomeres and micromeres of the 16-cell embryo by their autonomous nuclear localization of both β-catenin and animalizing transcription factors such as SpEts4 and SpSoxB1. Both endoderm and secondary mesenchyme cells, the principal mesoderm of the embryo, are derived from the veg2 tier, whereas the veg1 tier produces endoderm and ectoderm. Fate mapping of the mesenchyme blastula-stage embryo indicates that following the ingression of primary mesenchyme cells, the eight small micromeres are at the center of the vegetal plate, surrounded by a ring of prospective secondary mesenchyme cells that in turn is surrounded by a ring of prospective endoderm (Ruffins and Ettensohn, 1996). The vegetal plate is surrounded by prospective hindgut endoderm derived from the veg1 tier, which in turn is surrounded by prospective ectoderm derived from veg1 cells. During gastrulation, the vegetal plate folds inward and the archenteron extends toward the animal pole, led by the secondary mesenchyme cells at its tip. The secondary mesenchyme cells are distinguished by their filopodial activity and delaminate into the blastocoel. Prior to gastrulation, the vegetal plate cells express several markers such as *endo16* (Ransick *et al.,* 1993) and an ortholog of a winged-helix class gene (David *et al.,* 1999) throughout the prospective mesendoderm territory. Prospective endoderm and secondary mesenchyme cells become clonally distinct after the seventh cleavage but prior to late blastula stage (Ruffins and Ettensohn, 1996; Logan and McClay, 1997). If the presumptive secondary mesenchyme cells are microsurgically removed from the center of the vegetal plate prior to or during gastrulation, most differentiated larval cell types derived from secondary mesenchyme cells are replaced by cells whose normal fate is endoderm (McClay and Logan, 1996). Various treatments, such as LiCl, that enlarge the mesendoderm domain do so at the expense of veg2 derivatives normally fated to become ectoderm. These results indicate that the distinction between endoderm and ectoderm fates is allocated during the blastula stage, but that cell–cell interactions continue to have a role in maintaining the specification of endoderm and secondary mesenchyme cells. It appears that prospective secondary mesenchyme represses the potential of endoderm to form mesoderm.

Recent experiments indicate that signaling mediated by Notch is required for the differential specification of derivatives of the veg2 tier as endoderm or secondary mesenchyme cells, and that signals emanating from micromeres have an important role in this distinction. Notch mRNA is present in *Lytechinus variegatus* eggs and throughout embryonic development. LvNotch protein shows a dynamic pattern of localization during early development (Sherwood and McClay, 1997, 1999). Initially, it is expressed on the surface of all blastomeres, with a bias toward basal surfaces of animal cells. In blastulae (between the 8th and 10th cleavage), apical Notch is internalized into vesicles in a ring of cells at the vegetal plate that become primary and secondary mesenchyme cells. This leaves a disk of cells devoid of apical Notch, surrounded by a ring of prospective endoderm cells that have increasing levels of apical LvNotch. As gastrulation progresses, the secondary mesenchyme cells at the tip of the archenteron lack apical LvNotch, whereas the endodermal cells express it strongly. Shortly after experimental removal of prospective secondary mesenchyme cells during gastrulation, apical Notch is endocytosed in the prospective foregut cells now lying at the tip of the archenteron that convert to secondary mesenchyme cell fate (Sherwood and McClay, 1997). Thus, the differential specification of endoderm and secondary mesenchyme cells is associated with internalization of apical Notch, and suppressive signaling from secondary mesenchyme cells prevents endodermal cells from enodcytosing Notch and assuming secondary mesenchyme cell fate.

That Notch mediates specification of secondary mesenchyme cells has been demonstrated by a series of elegant experiments performed by Sherwood and McClay (1999). Overexpression of LvNotch, or a constitutively activated form of it corresponding to its intracellular domain, increased the number of secondary mesenchyme cells while reducing the number of endoderm cells. These secondary mesenchyme cells formed at the vegetal pole, and in some cases so many secondary mesenchyme cells formed that they were extruded from the embryo. Overexpression of a dominant-negative form of LvNotch reduced or eliminated secondary mesenchyme cells in gastrulae and allowed the zone of prospective endoderm cells to expand toward the vegetal pole. It is interesting that most cell types derived from secondary mesenchyme cells (with the frequent exception of pigment cells) eventually appeared in the embryos expressing dominant-negative LvNotch. This is presumably the result of a regulative conversion of endodermal cells, perhaps allowed by a decline in dominant-negative LvNotch resulting from decay of its injected mRNA. Mosaic analyses, in which LvNotch mRNA constructs were injected into one blastomere after first cleavage, suggest that LvNotch acts autonomously in prospective secondary mesenchyme cells to promote the differential specification of secondary mesenchyme cells and endoderm.

Notch signaling is involved in segregation of cell fates mediated by cell–cell interactions in embryos of many animals (Artavanis-Tsakonas *et al.,* 1999). Notch is a large, single-pass transmembrane protein that has several functional domains, including more than 30 extracellular epidermal growth factor repeats and

6 intracellular ankyrin repeats. Following ligand binding the intracellular domain of Notch is cleaved and translocates to the nucleus in which, in combination with other proteins, it regulates gene expression (Kadesch, 2000). In *Drosophila,* mutations of the gene *shibire* phenocopy Notch mutations. *Shibire* encodes the dynamin protein that has a role in the formation of endocytic vesicles. Thus, Notch signaling may involve endocytosis, but the details of the signaling events are not well understood. Removal of apical LvNotch by endocytosis may be part of the signal transduction process resulting in secondary mesenchyme cell specification. Alternatively, endocytosis may occur in response to Notch-mediated signaling that specifies secondary mesenchyme cells and serves to downregulate LvNotch receptor, limiting the domain of cells that respond to the ligand involved in secondary mesenchyme cell specification. Overexpression of activated LvNotch expands the area lacking apical Notch, whereas overexpression of dominant-negative LvNotch contracts this area: These observations are consistent with either of these mechanisms having a possible role in Notch internalization. In summary, overexpression of activated LvNotch expands the area of cells lacking apical LvNotch, whereas the dominant-negative form has the opposite effect. These results indicate that Notch signaling results in endocytosis of apical LvNotch and secondary mesenchyme cell specification. Moreover, as discussed later, the timing of micromere signaling required for secondary mesenchyme cell specification suggests that removal of apical LvNotch is a response to Notch-mediated specification of secondary mesenchyme cells and serves as the earliest known marker of secondary mesenchyme cell specification.

As discussed in Section III, there is considerable evidence that micromeres have a role in specification of the mesendoderm of the vegetal plate. When micromeres are removed just after forming in 16-cell *S. purpuratus* embryos, the domain of expression of *endo16,* which is initially specific for the mesendoderm of the vegetal plate and subsequently restricted to prospective endoderm, is reduced in size and gastrulation is delayed (Ransick and Davidson, 1995). Transplantation of micromeres to the animal pole induces formation of an ectopic archenteron, although sensitivity to this signaling declines between 8- and 32-cell stages (Hörstadius, 1973; Ransick and Davidson, 1993). Micromeres can also induce isolated mesomere derivatives to form pigment cells, a distinctive derivative of secondary mesenchyme cells (Khaner and Wilt, 1991). Thus, micromeres produce signals that can induce prospective ectoderm cells to form endoderm or mesoderm, suggesting a role in vegetal signaling in the intact embryo. The multipotent secondary mesenchyme cells normally form blastocoelar cells, pigment cells, circumesophageal muscle cells, and coelomic pouches. In addition, they can convert to skeletogenic cells in the absence of primary mesenchyme cells (Ettensohn and McClay, 1988); it is primarily the precursors of pigment cells that are converted to skeletogenic mesenchyme (Ettensohn and Ruffins, 1993).

Sweet *et al.* (1999) found that the removal of micromeres from *L. variegatus* embryos almost immediately after their formation delayed gastrulation and the formation of all mesodermal cell types. There was a marked reduction in the number

of cells expressing an antigenic marker specific for most prospective secondary mesenchyme cells and in the number of pigment and blastocoelar cells that eventually formed in larvae. Although normal numbers of skeletogenic cells eventually formed, the reduction in pigment and blastocoelar cells was not the result of the conversion of their precursors to skeletogenic fate. Removal of micromeres prevented the internalization of apical LvNotch that normally occurs in the Notch signaling involved in specification of secondary mesenchyme cells. Transplantation of micromeres to animal-half embryos induced the formation of secondary mesenchyme cell types, although in fewer numbers than when mock transplanted to the vegetal pole after micromere removal, indicating that animal cells are less responsive to micromere signaling. Although providing compelling evidence for a role of micromeres in secondary mesenchyme cell specification, these experiments were complicated by the regulative capacity of the embryos to replace secondary mesenchyme cells even after the normal specification events were prevented. This probably involves conversion of cells from endodermal to secondary mesenchyme cell fate, and this is associated with internalization of apical Notch (Sherwood and McClay, 1999; Sweet *et al.,* 1999).

In a series of technically challenging experiments, McClay *et al.* (2000) elucidated the specification of secondary mesenchyme cells by micromeres. As noted in Section III, *L. variegatus* embryos lacking nuclear β-catenin fail to produce endoderm or secondary mesenchyme cells (Logan *et al.,* 1999). Excess secondary mesenchyme cells form in embryos vegetalized by overexpression of β-catenin, but only in the presence of micromeres. Micromeres can induce the endocytosis of apical LvNotch and secondary mesenchyme cell formation when placed anywhere on these vegetalized embryos. Micromeres taken from embryos injected with truncated C-cadherin and thus lacking nuclear β-catenin were unable to induce secondary mesenchyme cell formation when transplanted to the vegetal plate of normal embryos from which micromeres had been removed, and they failed to ingress as primary mesenchyme cells. Thus, micromeres must have nuclear β-catenin to produce the signal that induces secondary mesenchyme cell specification of veg2 cells as well as to differentiate as skeletogenic mesenchyme. Micromeres were unable to rescue formation of secondary mesenchyme cells or endoderm by macromere derivatives lacking nuclear β-catenin, indicating that it is required for these cells to respond to the micromere signal.

The following evidence indicates that the response to micromeres for secondary mesenchyme cell specification involves Notch (McClay *et al.,* 2000). Embryos expressing activated LvNotch showed a vegetal zone lacking apical LvNotch even when micromeres were removed, indicating that activated Notch replaced the need for the micromere signal (and that activated Notch downregulates apical Notch). Micromeres could not induce secondary mesenchyme cell specification (as detected by lack of apical LvNotch) in embryos expressing dominant-negative LvNotch. Micromeres expressing dominant-negative LvNotch were able to induce apical LvNotch when transplanted to the vegetal poles of control hosts from which

micromeres had been removed. Thus, micromeres do not require Notch function to induce secondary mesenchyme cell specification, but the response to micromere signaling by derivatives of the macromeres involves Notch signaling and requires nuclear β-catenin.

McClay *et al.* (2000) defined some properties of the induction of secondary mesenchyme cell specification by micromeres. A micromere placed laterally to the vegetal pole of embryos lacking signaling micromeres at the vegetal pole induced loss of apical Notch (secondary mesenchyme cell specification) in adjacent cells that normally retain apical Notch and become endoderm. The area of cells specified as secondary mesenchyme was proportional to the number of signaling micromeres. Although these experiments would benefit from the use of other markers for secondary mesenchyme cell specification, they nonetheless indicate that the micromere signal is short range (no more than about one cell diameter), possibly requiring direct cell–cell contact, and quantitative. To define the timing of signaling, micromeres were transplanted from 16-cell embryos to older embryos from which micromeres had been removed after the 4th cleavage. Secondary mesenchyme cell specification occurred when micromeres were transplanted after 1–4 additional cleavages but not after 5. Thus, micromere signaling is not required immediately after their formation, but it must occur prior to the 10th cleavage. Moreover, micromeres retain the capacity to signal for at least 4 cleavages after their formation, but exactly when they gained the capacity to signal was not defined. Apical Notch disappears from prospective secondary mesenchyme cells between the 8th and 10th cleavages (Sherwood and McClay, 1997, 1999) coincident with, or slightly later than, the time when the Notch-mediated inductive signal from the micromeres is required. Thus, endocytosis of apical Notch is either involved in the activation of Notch in prospective secondary mesenchyme cells or follows shortly thereafter.

Photoablation of micromeres and their derivatives from embryos also indicated a peak in micromere signaling activity that promotes formation of pigment and blastocoelar cells coinciding with the time when apical Notch disappears from prospective secondary mesenchyme cells—the hatching blastula stage (H. Sweet, unpublished results). Amemiya (1996) reported that recombination of micromeres with animal halves of 16- or 32-cell stage embryos induced archenteron formation and normal development. Minokawa and Amemiya (1999) used this experimental system to define the time when the micromere signal is produced and necessary in *Scaphechinus mirablis*. Quartets of micromeres and their cultured descendents were recombined with animal halves and then again removed at various times. These experiments indicated that the signal for archenteron induction was produced by micromere descendents beginning around the time of hatching and then continued to be produced. The transmission of the signal was completed within 2 h of recombination, and this allowed for the development of plutei that could complete metamorphosis. Thus, micromere derivatives acquire a capacity to induce mesomeres to form secondary mesenchyme and endoderm only after a period of development, and this capacity can be acquired by cultured micromeres. It may

be that this is the same signal required to induce specification of veg2 cells and secondary mesenchyme cells, but that its production at higher levels at later stages is required to induce vegetalization of animal cells. Micromere deletion experiments reported by Ransick and Davidson (1995) indicated that micromeres are required between the fourth and sixth cleavages to expand the domain of expression of the mesendoderm marker *endo16*. This suggests that another, earlier signal from the micromeres may be involved in expanding the zone of mesendoderm specification, which is initially dependent on the autonomous nuclear localization of β-catenin (see Section III). Removal of micromeres delayed gastrulation, but eventually a complete gut formed. Although the delay may result from the initially incomplete specification of endoderm, the temporary loss of secondary mesenchyme cells might be directly responsible (McClay *et al.,* 2000). Bottle cells formed in the vegetal plate have a facilitative role in the initial buckling in the vegetal plate as gastrulation begins (Nakajima and Burke, 1996; Kimberly and Hardin, 1998). The bottle cells are likely to be derivatives of secondary mesenchyme cells, which are replaced by regulation during the delay in initiation of gastrulation following removal of micromeres (Sweet *et al.,* 1999).

The Notch ligand provided by the micromeres to induce secondary mesenchyme cell specification has not been identified, but homologs of Delta, Serrate, or Jagged are obvious candidates. These Notch ligands are transmembrane proteins involved in juxtacrine signaling, consistent with the limited range of the micromere signal. An *S. purpuratus* ortholog of Delta has recently been cloned, and the mRNA appears to be expressed in micromeres and primary mesenchyme cells but declines as they ingress (C. Ettensohn and H. Sweet, unpublished results). Cells expressing Notch and Delta are frequently involved in lateral inhibitory interactions that distinguish the fates of equipotent neighboring cells, but micromeres and macromeres are not equipotent. It is likely that activation of Notch in prospective secondary mesenchyme cells results in signaling that inhibits the potential of neighboring prospective endoderm cells to form secondary mesenchyme. Loss of this inhibitory signal would explain the rapid conversion from endoderm to secondary mesenchyme cell fate by cells adjacent to extirpated secondary mesenchyme cells. Paradoxically, endoderm specification appears to be the default state for veg2 cells, and in the absence of the micromere signal early secondary mesenchyme cell specification fails but some mesendoderm cells later become spontaneously specified as secondary mesenchyme cells and skeletogenic mesenchyme. The possible basis of such regulative responses is discussed in Section VIII.

In summary, the mesendoderm territory appears to be autonomously specified by localization of β-catenin to macromere nuclei (and subsequently to veg2 nuclei) beginning at the fifth cleavage. Early signaling from micromeres appears to expand this zone toward the animal pole. Signaling from derivatives of micromeres between the 8th and 10th cleavages involving the Notch receptor and requiring nuclear β-catenin specifies secondary mesenchyme cells as a distinct subdomain

of the mesendoderm territory. Even when the micromere signaling systems are disrupted, the embryo regulates to replace, at least in part, endoderm and secondary mesenchyme.

B. Wnt8 Signaling and Reinforcement of Mesendoderm Specification

The canonical Wnt signaling pathway diagrammed in Fig. 1D results in the nuclear localization of β-catenin. In a effort to identify a Wnt ligand that might be involved in the nuclear localization of β-catenin in vegetal cells, A. Wikramanayake and W. Klein (unpublished results) discovered that *Spwnt8* mRNA begins to appear at the late 16-cell stage, first in micromeres, then in veg2 cells, and finally in a band of vegetal blastomeres that does not include those at the vegetal pole. Although other Wnt genes are expressed later in development (Ferkowicz and Raff, 2001), *Spwnt8* is the only *wnt* family gene identified in sea urchins that is expressed early enough to be associated with initial A–V axis patterning. Overexpression of *Spwnt8* in whole embryos or animal halves isolated at the 8-cell stage resulted in formation of multiple small archentera at the expense of some of the ectoderm. Ectoderm was also converted to skeletogenic mesenchyme in the animal halves; the spicules that formed lacked the normal triradiate character and were reminiscent of spicules produced by cultured primary mesenchyme cells that lack the influence of the ectodermal epithelium of the embryo. Little, if any, secondary mesenchyme such as pigment cells formed in the vegetalized animal halves having ectopic guts. Injection into eggs of a dominant-negative form of SpWnt8 prevented the formation of endoderm and secondary mesenchyme cells except for a few pigment cells, whereas primary mesenchyme cells ingressed but failed to form spicules.

Although these results indicate an important role for SpWnt8 in vegetal patterning, as noted in Section III.B.2, it is unlikely to act as a ligand in the signaling pathway responsible for the initial nuclear localization of β-catenin in vegetal cells. Rather, the timing of its appearance suggests that the gene encoding SpWnt8 is activated by the autonomous nuclear localization of β-catenin in vegetal cells in combination with Tcf/Lef. The effects of interfering with SpWnt8 and β-catenin localization are similar but distinct. In embryos vegetalized by expression of truncated C-cadherin, which depletes pools of nuclear β-catenin, endoderm and secondary mesenchyme cells also fail to differentiate, and the descendents of large micromeres are converted to epithelial cells that never ingress (Wikramanayake *et al.,* 1998; Li *et al.,* 1999; Logan *et al.,* 1999). β-Catenin-depleted micromeres cannot induce a secondary vegetal plate when transplanted to the animal pole of a normal embryo. In contrast, micromeres from embryos expressing dominant-negative SpWnt8 are able to induce secondary mesenchyme cell specification at the vegetal pole (D. McClay and A. Wikramanayake, unpublished results) and can ingress but do not differentiate. These results suggest that these

SpWnt8-deficient micromeres produce a Notch ligand and indicate that SpWnt8 is not required for induction of secondary mesenchyme cell specification by micromeres. When dominant-negative SpWnt8 is expressed in mesomeres, they become unresponsive to induction of ectopic vegetal plate formation by ectopic micromeres. This suggests a possible role for SpWnt8 in patterning of the ectoderm.

SpWnt8 activity can mediate at least some of the vegetalizing effects of nuclear β-catenin. Wnt signaling commonly increases β-catenin stability and nuclear localization via signal transduction involving the Frizzled receptor, Dsh, and GSK-3β (Fig. 1D). It is likely that *Spwnt8* expression is activated by nuclear β-catenin–Tcf/Lef in micromeres, and that it in turn acts as a positive feedback regulator to reinforce the nuclear localization of β-catenin in vegetal cells, particularly in the veg2 and veg1 tiers. SpWnt8 may be the paracrine signal produced by vegetal cells that specifies the full mesendoderm territory that is reduced in size after removal of the micromeres (based on the expression of the vegetal plate marker *endo16*). The failure of embryos to form secondary mesenchyme cells when *Spwnt8* expression is greatly reduced, even though their micromeres can still induce secondary mesenchyme cell formation, suggests that SpWnt8 is required, in addition to Notch, by veg2 cells to respond to micromeres, just as β-catenin is required for the competence of veg2 cells to respond to micromere induction of secondary mesenchyme cell specification.

The failure of secondary mesenchyme cells to form when *Spwnt8* is overexpressed in animal halves is puzzling. A higher level of expression of hyperstable β-catenin in animal halves is required to induce mesoderm formation in addition to endoderm (Wikramanayake *et al.,* 1998), but the more vegetal skeletogenic mesenchyme did form when *Spwnt8* was overexpressed. It is possible that expression of the Notch ligand was not activated. Alternatively, secondary mesenchyme cells may have been specified but were converted to the skeletogenic mesenchyme. Mesomere pairs expressing hyperstable β-catenin are able to induce animal half embryos to form a pluteus-like larva, including gut, skeleton, and secondary mesenchyme cells (A. Wikramanayake, unpublished results). Mesomere pairs overexpressing *Spwnt8* are only able to induce animal halves to form a partial gut (hind- and midgut, but lacking foregut) and no mesoderm. This suggests that β-catenin is a more potent vegetalizing agent than is SpWnt8. It also suggests that SpWnt8 is not the only extracellular vegetalizing agent produced by the micromeres in addition to the Notch ligand. However, it is also possible that in these experiments the level of *Spwnt8* overexpression was insufficient to have fully vegetalizing effects, including complete activation of β-catenin in the mesomeres. This might reflect the possible presence of natural antagonists of Wnt signaling in animal blastomeres that are resistant to downregulation by SpWnt8.

Overexpression of *Spwnt8* in animal halves leads to the formation of multiple, scattered archentera, whereas expression of hyperstable β-catenin expands the endoderm but results in a single archenteron. This suggests that animal half embryos overexpressing *Spwnt8* lose their A–V polarity, whereas those overexpressing

β-catenin retain it. This distinction may reflect the inherent autoregulating balance of animalizing and vegetalizing transcription factors along the A–V axis: Overexpression of β-catenin (but not *Spwnt8*) may be counterbalanced by high levels of animalizing transcription factors at the animal pole, thereby maintaining A–V polarity. In normal circumstances, the pattern of *Spwnt8* expression probably responds to and enhances nuclear β-catenin activity, but when overexpressed in the absence of nuclear β-catenin in animal cells, SpWnt8 may overwhelm the maternal A–V polarity while converting some cells to endoderm. During normal development, SpWnt8 appears to act as a vegetalizing signal that may be counteracted by animalizing signals, some of which may be homologs of known Wnt antagonists, such as Cerberus, Frzb, or Dickkopf. It is possible that SpWnt8 acts via an alternative signaling pathway that does not involve β-catenin.

Cells of embryos dissociated at the two-cell stage express little *SpKrl* mRNA (Howard *et al.,* 2001). Treatment of the dissociated embryos with LiC1 restores *SpKrl* expression to nearly normal levels. Since LiC1 promotes nuclear localization of β-catenin, SpWnt8 may promote *SpKrl* expression by enhancing the autonomous nuclear localization of β-catenin in vegetal cells that may be insufficient to fully activate *SpKrl* expression. Although embryo dissociation experiments indicated that β-catenin enters nuclei of vegetal cells autonomously, and high levels of nuclear β- catenin can commit cells to mesendoderm fates, specification of the vegetal plate (mesendoderm) requires ongoing cellular interactions. Expression of the vegetal plate marker *endo16* is inhibited by dissociation of 4- to 16-cell stage or later embryos but can be restored if embryos are reassociated shortly after the 16-cell stage (Godin *et al.,* 1997). *Endo16* expression in dissociated embryos was not rescued by treatment with LiC1, which indicates that specification of mesendoderm requires cellular interactions after the fourth cleavage in addition to nuclear β-catenin. Dissociation of embryos at various stages and monitoring for the endoderm-specific differentiation markers Endo1 and LvN1.2 indicated that cells become committed to endoderm differentiation after hatching at the mesenchyme blastula stage, prior to gastrulation (Chen and Wessel, 1996). Thus, cellular interactions are required throughout early development for specification and then commitment of endoderm cell fate. These interactions presumably depend on signals from large micromeres and may also depend on signaling between vegetal plate cells, similar to the community effect reported by Gurdon *et al.* (1993) for *Xenopus* embryos.

C. BMP2/4 Signaling and the Endoderm–Ectoderm Border

Early fate mapping suggested that the border between endoderm and ectoderm is established upon the sixth cleavage and corresponds to the cleavage plane that separates the veg2 and veg1 tiers (Hörstadius, 1973). Using more sensitive labeling of single cells for fate mapping, Logan and McClay (1997) found that a veg1 cell of

L. variegatus can contribute to both ectoderm and endoderm even after the eighth cleavage (the next horizontal cleavage). Single mesomeres labeled at the 32-cell stage can also sometimes contribute descendents to the archenterons (hindgut). The contribution of veg1 or veg2 cells to distinct parts of the gut is also variable. Similar experiments on *S. purpuratus* also indicated that veg1 cells contribute variably to the hindgut and midgut as well as anal ectoderm (Ransick and Davidson, 1998). The endoderm–ectoderm border of *L. variegatus* embryos is shifted toward the animal pole when the vegetal plate or invaginating archenterons are removed (McClay and Logan, 1996). Since this border does not correspond to cleavage planes or cell lineage and can shift in response to perturbations even during gastrulation, its establishment must depend on cell–cell signaling after the sixth cleavage. These cell fate studies indicate a previously unsuspected plasticity of fates and position within the embryo of cell descendents. Thus, cellular interactions must refine the autonomous specification of cells depending on their position until they become committed to a fate.

Recombination experiments indicate that veg2 cells can induce mesomeres to form endodermal tissues (Logan and McClay, 1999). It therefore seems likely that veg2 derivatives instruct some overlying veg1 derivatives to form endoderm in the intact embryo. When a veg1 tier is recombined with mesomeres, its progeny form a partial gut, lacking the foregut and the constrictions that form between parts of the gut. Inclusion of a single veg2 cell in the recombinant results in formation of a complete gut in which veg1 cells are included in all parts (Logan and McClay, 1999). Thus, veg2 cells can pattern the endoderm derived from veg1 cells. Therefore, it is likely that a signal(s) from veg2 cells is involved in establishing the endoderm–ectoderm border and promoting regionalization of veg1 cells recruited into the endoderm. Cells are allocated to endoderm or ectoderm in the late blastula after the 9th or 10th cleavages (Ruffins and Ettensohn, 1996; Logan and McClay, 1997). Dissociated embryos become capable of autonomous expression of endoderm marker genes at a similar stage (Chen and Wessel, 1996), indicating that specification has occurred.

Isolated animal halves generally form no endoderm (Wikramanayake *et al.,* 1995). However, when the third cleavage plane is subequatorial, animal halves are much more likely to form endoderm (Kitajima and Okazaki, 1980; Henry *et al.,* 1989). This suggests that maternal determinants distributed along the A–V axis have a role in setting the endoderm–ectoderm boundary. Treatment of embryos with the vegetalizing agent LiC1 converts prospective ectoderm to endomesoderm and shifts the endoderm–ectoderm border toward the animal pole. This effect is distinct from, and synergistic with, the vegetalizing effects of extracellular signals derived from micromeres (Livingston and Wilt, 1990a). As noted in Section III.B.1, lithium ions perturb the distribution of nuclear β-catenin by inhibiting GSK-3β activity. Thus, the establishment of the endoderm–ectoderm border is influenced by nuclear β-catenin and the genes that are under its control. Upon the sixth cleavage, β-catenin remains high in nuclei of veg2 cells but is low in nuclei of

veg1 cells; it then disappears from veg1 cells (Logan *et al.,* 1999). After hatching, but before gastrulation begins, β-catenin disappears from nuclei derived from the large micromeres and veg2 cells but then accumulates in nuclei of a ring of cells that correspond to the veg1 cells that become endoderm. Thus, the endoderm–ectoderm border corresponds to the border of cells showing late nuclear localization of β-catenin. Wnt8 or other Wnt proteins may have a role in this process by enhancing the stability of β-catenin, thereby increasing expression of genes such as *SpKrl* necessary for endoderm formation. In addition, Wnt may act indirectly as a vegetalizing signal involved in setting the endoderm–ectoderm border.

The sea urchin homolog of BMP2/4 appears to counteract the vegetalizing signal to help set the endoderm–ectoderm border. BMP2/4 mRNA first appears in blastula embryos with about 200 cells and becomes restricted to presumptive ectoderm cells, predominantly those on the oral side (Angerer *et al.,* 2000). Injection of BMP2/4 mRNA into zygotes expands ectoderm at the expense of endoderm, in addition to radializing the embryo (Angerer *et al.,* 2000). The remaining endoderm shows proper patterning of the reduced archenteron, indicating that overexpression of BMP2/4 resets the endoderm–ectoderm border closer to the vegetal pole rather than altering the patterning of endoderm. In *Xenopus* embryos, dorsal–ventral patterning of the mesoderm and the distinction between epidermal and neural fate of the ectoderm depend on the interaction of BMP4 with antagonists such as Chordin and Noggin secreted by cells of the organizer (De Robertis *et al.,* 2000). Chordin activity, in turn, is counteracted by the activity of the protease Xolloid (Tolloid/BMP1) (Goodman *et al.,* 1998). This signaling system produces a long-range gradient of BMP4 ventralizing activity that patterns the frog embryo. A similar system operates in patterning the ectoderm of *Drosophila* (De Robertis and Sasai, 1996; Podos and Ferguson, 1999). Chordin or Noggin can induce expression of endoderm markers in *Xenopus* animal cap cells normally fated to form ectoderm, implying that BMP4 signaling has a repressive role in endoderm differentiation (Sasai *et al.,* 1996). Injection of mRNA encoding *Xenopus* Noggin into sea urchin zygotes also seems to antagonize BMP2/4, producing effects mostly reciprocal to those of overexpressed BMP2/4 (Angerer *et al.,* 2000). Genes encoding BMP antagonists have not been identified in sea urchins. However, *SpAN*, a *S. purpuratus* ortholog of Xolloid/Tolloid/BMP1, is the product of very early zygotic gene expression and its mRNA is present in animal cells in a concentration gradient declining toward the vegetal pole (Reynolds *et al.,* 1992). Based on injection of synthetic *SpAn* mRNA into *Xenopus* embryos, this astacin protease can modulate BMP-mediated signaling involved in dorsal–ventral patterning of vertebrates (Wardle *et al.,* 1999). SpSoxB1 is an essential regulator of transcription of the *SpAN* gene. Overexpression of SpSoxB1 or SpBMP2/4 produces similar animalized phenotypes (Kenny *et al.,* 1999), suggesting a regulatory connection. An appealing model is that SpSoxB1, and probably other animalizing transcription factors, regulates secreted signals, including BMP2/4 and SpAN, that promote ectoderm differentiation and patterning. In turn, these animalizing agents

are counteracted by unidentified antagonists produced by vegetal cells as a result of the Wnt–β-catenin signaling pathway. The endoderm–ectoderm border would thus form in response to an appropriate threshold of BMP2/4 activity. As described in Section III.B.6, SpKrl is necessary but not sufficient for endoderm formation, and its mRNA appears shortly before endoderm becomes determined (Howard *et al.,* 2001). The synthesis of *SpKrl* mRNA is dependent on the activity of nuclear β-catenin–TCF/Lef. Extracellular signaling may modulate SpKrl and/or SpSoxB1 activities to establish the ratio necessary for veg1 cells to become determined as endoderm. Thus, the establishment of the endoderm–ectoderm border appears to be another example of the role of extracellular signaling in reinforcing and refining patterning established by the intracellular ratios of opposing animalizing and vegetalizing transcription factors.

Sherwood and McClay (2001) found evidence for important roles of Notch signaling in establishment of the endoderm–ectoderm border. Overexpression of LvNotch shifts this boundary toward the animal pole, whereas expression of a dominant-negative form of LvNotch shifts this border toward the vegetal pole. Mosaic experiments in which synthetic mRNAs are injected into single animal or vegetal blastomeres at the eight-cell stage indicate that Notch signaling operates in both the animal and the vegetal hemispheres but via different mechanisms. Overexpression of LvNotch in vegetal cells nonautonomously promotes endoderm formation and an associated endoderm–ectoderm boundary in overlying animal cells. LvNotch also activates the transient localization of β-catenin in these endoderm border cells. Overexpression of LvNotch in animal cells autonomously converts some descendents from ectoderm to endoderm fate; this can occur even in isolated animal halves and is not associated with nuclear localization of β-catenin. As described in Section IV.A, Notch signaling specifies secondary mesenchyme as distinct from endoderm of the vegetal plate. It is likely that this signaling event in turn activates or enhances signaling by veg2 derivatives involved in forming the endoderm–ectoderm border. As noted previously, this may be mediated by Wnt–β-catenin signaling. Derivatives of mesomeres express LvNotch and this may confer the competence to form endoderm when exposed to appropriate signals, such as those provided by ectopic micromeres. Ectopic activation of Notch signaling in mesomeres autonomously converts some derivatives from ectodermal to endodermal fate. This may help explain how normal development is restored by recombination of animal halves with micromeres or veg2 tiers. It is interesting that even though the endoderm–ectoderm border can be shifted, there is little consequence for the gastrula that forms: It regulates to adapt to this shift. It is noteworthy that not all animal cells are converted to endoderm by expression of an activated form of LvNotch, indicating that other factors are involved in the distinction between endoderm and ectoderm fates. As noted previously, extracellular signaling mediated by BMPs from the animal cells also has an important role in setting the endoderm–ectoderm border, indicating that opposing influences operate.

V. Oral–Aboral Axis and Ectoderm Patterning

A. Initial Specification Mechanisms

The O–Ab axis arises in indirect developing sea urchin embryos following fertilization by mechanisms that are unknown. In *S. purpuratus,* there is a fixed relationship between the first cleavage plane and the O–Ab axis (Cameron *et al.,* 1989), but this is not the case for some other echinoid species (Kominami, 1988; Henry, 1998). At least for *S. purpuratus,* this implies that the O–Ab axis is initially specified between fertilization and the first cleavage, but because isolated blastomeres at the two- and four-cell stage give rise to seemingly normal plutei, the initial O–Ab axis specification must be reversible. Unlike the A–V axis, the O–Ab axis is not irreversibly fixed (determined) until the blastula stage when bilateral regions on opposite sides of the embryo become cuboidal and squamous epithelia. For instance, ectoderm can be radialized by treatment with Ni ions shortly before gastrulation (Hardin *et al.,* 1992). Despite this plasticity, there are morphological and molecular signs that indicate that maternally derived mechanisms set the axis specification process in motion. For instance, *SpCOUP-TF* and COLL1α transcripts are asymmetrically distributed in the unfertilized egg perpendicular to the A–V axis (Vlahou *et al.,* 1996; Gambino *et al.,* 1997), as are zygotic *PlHbox12* transcripts, which are expressed during early cleavage (Di Bernardo *et al.,* 1995). A particularly interesting phenomenon is the respiration (or redox) gradient found as early as the eight-cell stage: A higher rate of respiration is exhibited on the future oral side of the embryo than on the aboral side. The redox differences are due to a gradient of cytochrome c oxidase activity. Coffman and Davidson (2001) investigated the features of the redox gradient with respect to O–Ab axis specification. They found that the redox asymmetry can be manipulated within embryos by immobilizing them in tight clusters of four. Within these clusters, a redox gradient is established from the outside to the inside of the cluster, and vital staining shows that the side of the embryo facing the outside tends to become the oral side, whereas the inside face tends to become the aboral side. The relevance of the induced O–Ab axis was tested by expressing a dominant active form of P3A2, a transcriptional regulatory protein whose activity is spatially modulated along the O–Ab axis, together with a *GFP* reporter gene driven by P3A2 binding sites. *GFP* expression occurred predominantly on the outside of the clusters, suggesting that P3A2 activity was spatially regulated by the redox asymmetry established by clustering the embryos.

Based on these experiments and earlier work, Coffman and Davidson (2001) suggested a model for initial O–Ab axis specification. The oral pole may be initially specified by cytoskeletal alterations immediately after fertilization, which could then lead to a redox gradient. The redox gradient could also arise by other stochastic mechanisms, which might account for the dissociation between cleavage and O–Ab

axis specification in some species. During cleavage, the redox asymmetry would be partitioned into different blastomeres and thus the potential to modulate maternal transcription factor activity along the O–Ab axis would arise. Subsequent events would reinforce the asymmetry and set up the secondary responses required for fixing the O–Ab axis and patterning the ectodermal cell types as discussed in the following two sections.

B. Dependence on Vegetal β-Catenin Signaling

As noted in Section II, when mesomeres are isolated from 16-cell stage embryos and cultured as animal halves, they give rise to dauerblastulae. The ball of epithelial cells comprising the dauerblastula is inherently polarized, with a cuboidal epithelium at one pole tapering to a thin squamous epithelium at the other. Wikramanayake and Klein (1997) suggested that this polarization reflects the initial specification of the O–Ab axis, with the cuboidal epithelium being the presumptive oral side and the squamous epithelium being the presumptive aboral side. Consistent with this view, the cuboidal epithelium emits neuronal-like processes characteristically associated with the oral side of the normal embryo. Nevertheless, the oral and aboral ectoderm territories are not definitively polarized in animalized embryos and the ectoderm appears to be in a primitive or preectoderm state. For example, the stomodeum does not form at the oral surface and the ciliary band, which is inductively specified at the O–Ab interface, is absent. In addition, the oral ectoderm marker EctoV is uniformly distributed, and in cultured animal halves of *L. variegatus* or *L. pictus,* aboral ectoderm markers are not expressed. In *S. purpuratus,* aboral markers are expressed, but this is probably due to a premature signal produced artificially during the isolation of the animal halves.

Recombination of animal halves with vegetal blastomeres or exposure to LiCl leads to the restoration of aboral ectoderm gene activity in *L. pictus,* suggesting a requirement for vegetal induction in the differentiation of aboral ectoderm cells (Wikramanayake *et al.,* 1995). This finding was confirmed and extended to demonstrate an overall requirement of vegetal signaling for ectoderm patterning and oral and aboral ectoderm differentiation (Wikramanayake *et al.,* 1998). Embryos depleted of β-catenin by overexpressing truncated C-cadherin or GSK-3β appear virtually identical to cultured animal halves. Wikramanayake *et al.* (1998) showed that overexpressing the hyperstable form of β-catenin could affect ectoderm patterning in animal halves without first inducing endoderm. Animal halves containing hyperstable β-catenin at levels too low to detect expression of endoderm markers expressed aboral ectoderm markers at normal levels. Remarkably, the morphological features of the cultured animal halves were dramatically altered with low levels of β-catenin. A stomodeum formed on the presumptive oral side, and a ciliary band demarcated a sharp O–Ab boundary. In addition, the EctoV marker became restricted to the oral side of the embryo. These results clearly

established that nuclear β-catenin was sufficient for the proper polarization of ectoderm territories and for the activation of aboral ectoderm-specific gene expression.

What is perhaps most interesting about β-catenin's role in ectoderm signaling is that β-catenin is not detectable in nuclei of mesomeres or their descendents in the normal embryo. Thus, it is likely that in normal development vegetal cells having nuclear β-catenin produce a second signal that allows completion of the polarization of the primitive ectoderm and activates the program of aboral ectoderm differentiation. In animal halves overexpressing hyperstable β-catenin, the vegetalmost cells are probably converted to veg2 cells by forced nuclear entry of β-catenin and the A–V axis is reestablished. Because animal halves exhibit an inherent polarization along their O–Ab axis, a maternally derived mechanism must inhibit the ability of the β-catenin-induced second signal to activate aboral ectoderm-specific genes in the oral field.

C. Ectodermal Territories Specified by Opposing Goosecoid and Otx Transcription Factors

Recently, an ortholog of the homeobox-containing transcription factor goosecoid (gsc) was identified in *S. purpuratus* and shown to regulate cell fates along both the A–V and O–Ab axes (Angerer *et al.,* 2001). *SpGsc* is expressed transiently in primary mesenchyme cells at the blastula stage and beginning about that time in oral ectoderm cells through the pluteus stage. SpGsc is a key downstream effector of vegetal nuclear β-catenin function as shown by downregulation of *SpGsc* expression in C-cadherin-treated embryos and by a series of loss-of-function and gain-of-function experiments. Eliminating SpGsc function by injection of morpholino antisense oligonucleotides showed that SpGsc is required for gastrulation and for maintaining the expression of the vegetal plate marker gene *endo16*. Based on its transient expression in primary mesenchyme cells at the blastula stage, it is likely that SpGsc functions non-cell autonomously in producing a signal that, together with vegetal nuclear β-catenin, further elaborates the differentiation of endoderm. Interestingly, SpGsc is also required for differentiation of oral ectoderm, promoting oral fate by repressing aboral ectoderm-specific gene expression. Overexpression of SpGsc results in an expansion of oral ectoderm at the expense of aboral ectoderm. Conversely, loss of SpGsc function in presumptive oral ectoderm or overexpression of a transcriptional activating counterpart, SpGsc–VP16, converts presumptive oral ectoderm to an aboral fate. SpGsc is a K_{50} class homeobox protein that has a strong transcriptional repressor domain. In contrast, SpOtx, which is expressed uniformly in the early embryo, is a related K_{50} class homeobox protein with a strong transcriptional activation domain. Overexpression of SpOtx results in an expanded aboral ectoderm and overexpression of a dominant repressor form of SpOtx, SpOtx–Engrailed, results in a block in aboral ectoderm differentiation and an embryo that has dauerblastula characteristics. These results are the

opposite of what is found with SpGsc. Angerer *et al.* (2001) showed that SpGsc binds selectively to the same DNA elements as SpOtx and that the two factors can compete for binding at this site. SpOtx activates the aboral ectoderm *spec2a* gene and is required for aboral ectoderm differentiation. Furthermore, a transgene promoter from *spec2a* whose activity depends entirely on the SpOtx DNA elements is silenced *in vivo* by coexpression of SpGsc. These results indicate that SpGsc may function, at least in part, by antagonizing SpOtx in presumptive oral ectoderm cells.

A model thus emerges whereby the vegetal nuclear β-catenin signaling system produces a second signal that induces the patterning of the ectoderm in animal cells along an already specified O–Ab axis. The vegetal β-catenin-inducing signal activates *SpGsc* expression in presumptive oral ectoderm, thereby suppressing aboral ectoderm differentiation in this territory. In addition, the vegetally transmitted signal also activates aboral ectoderm-specific gene expression in the aboral ectoderm territory. The proper polarization of the two ectoderm territories leads to the formation of the ciliary band by an inductive interaction between oral and aboral ectoderm. However, this model does not explain why *SpGsc* expression is not activated on the presumptive aboral side of the embryo. There is little evidence to indicate that vegetal nuclear β-catenin is distributed asymmetrically with respect to the O–Ab axis. This suggests that maternally derived factors must be present on the aboral side of the embryo that repress *SpGsc* activation or alternatively, the oral side may contain positive factors for *SpGsc* activation that are absent on the aboral side. Clearly, the mechanism by which *SpGsc* is selectively activated on the oral side of the embryo must be elucidated before the events of O–Ab axis patterning are fully understood.

VI. Transcriptional Regulation of Genes Downstream of the A–V Axis Specification Pathway

A. *Cis*-Regulatory Systems and Regulation of Gene Expression

Considerable effort has been made in attempting to link the initial events of axis specification and the initiation of territorial-specific gene expression. These mechanisms have been revealed in part by analysis of individual *cis*-regulatory regions of genes expressed in spatially restricted domains and the *trans* factors associated with the regulatory regions. In this review, two well-studied examples are described that are particularly relevant to A–V and O–Ab axis patterning—*endo16* in the vegetal plate and *spec2a* in the aboral ectoderm. Not discussed here are numerous other gene transcriptional regulatory regions responsible for spatially restricted expression patterns that have been analyzed in sea urchins, particularly by Davidson

and colleagues, which have also shed light on *cis*-regulatory systems (Kirchhamer and Davidson, 1996; Arnone and Davidson, 1997). Two general emerge from these investigations. First, the expression of genes that are activated in one or more of the five embryonic territories toward the end of the cleavage stage is usually controlled by complex, interacting *cis*-regulatory systems that involve numerous DNA elements and associated *trans*-acting factors. Second, spatially restricted expression is generally imposed by negative regulatory DNA elements that are binding sites for transcriptional repressors whose own activities are spatially restricted by maternally derived mechanisms.

B. The *endo 16 Cis*-Regulatory Region

The most thoroughly studied transcription regulatory region in sea urchin development is that of the vegetal plate and endoderm expressing *endo16* gene. *Endo16* encodes a large secreted protein that is activated at the end of the cleavage stage exclusively in cells of the emerging vegetal plate. By the prism stage, *endo16* expression is restricted to cells of the midgut. A DNA sequence extending about 2.3 kb from the transcriptional start site is necessary and sufficient to express a reporter gene in the same temporal and spatial pattern as displayed by the endogenous *endo16* gene. The *endo16* regulatory system is modular in organization, containing six modules (approximately equivalent to transcriptional enhancers) plus a basal promoter (Fig. 2A). Module A, whose length is 185 bp, is closest to the basal promoter and is followed by modules B, DC, E, F, and G. The *endo16* modules each contain multiple DNA-regulatory elements, some unique and others redundant. A critical feature of *endo16*'s modular organization is the interactive nature of the modules. This type of organization has been found to be associated with the transcriptional regulatory regions of several other territorial-specific genes dependent on conditional specification (Kirchhamer and Davidson, 1996). Module A is responsible for activating *endo16* expression in the vegetal plate during its initial specification. The essential early transcriptional activator of *endo16* is SpOtx, and a single Otx site within module A is absolutely required for the initial activation of *endo16* in the mesendoderm. As discussed in Section V, SpOtx is uniformly distributed in nuclei of all cells at this time in development and plays a critical role in *spec2a* gene activation in aboral ectoderm cells. Thus, SpOtx is a general transcriptional activator in sea urchin embryos that is not sufficient for either vegetal plate- or aboral ectoderm-specific expression. Rather, vegetal plate-specific expression is conferred to *endo16* by repressors that bind to sites within the DC module acting to repress *endo16* expression in primary mesenchyme cells and also by repressors binding to sites within the E and F modules functioning to repress *endo16* expression in the overlying ectoderm cells (Fig. 2A). For the repressor sites within the DC, E, and F modules to work, they require an interaction with a site near the Otx site within module A termed the Z site. Thus, module A is

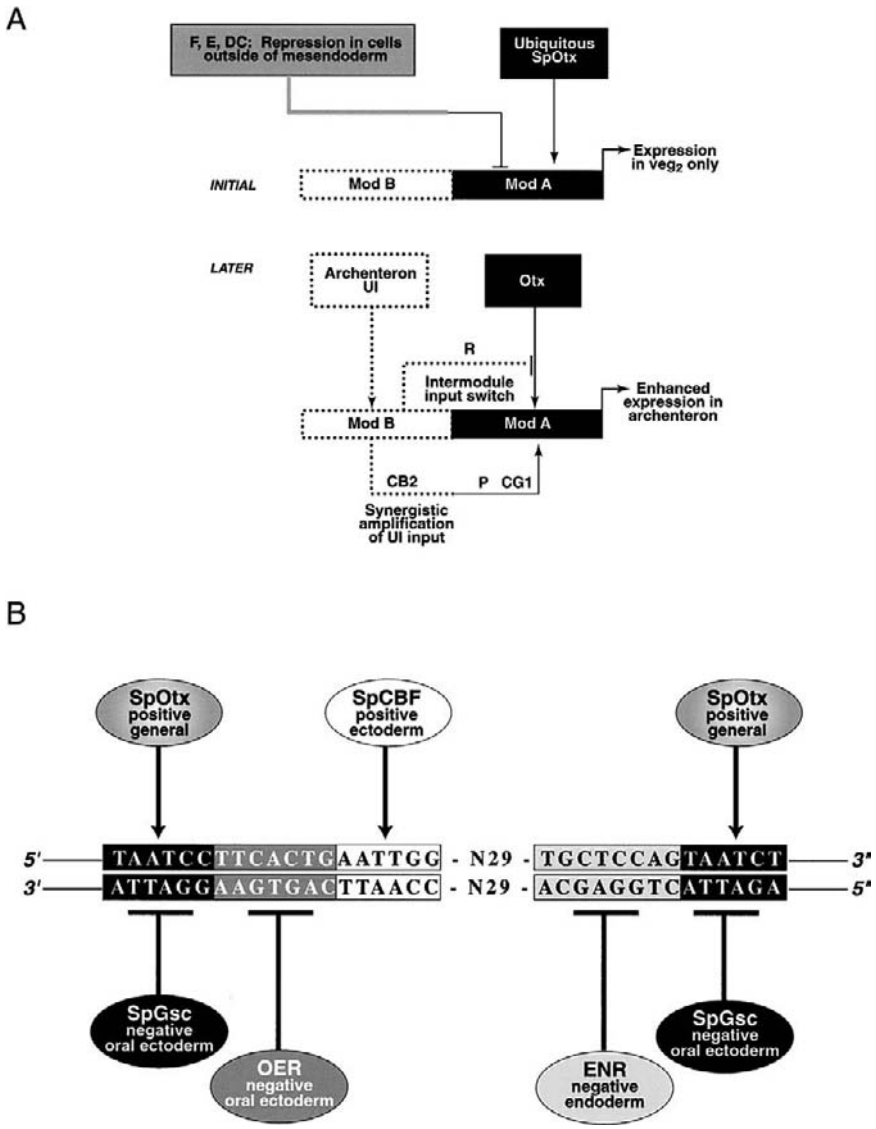

FIG. 2 Transcriptional regulatory regions from the *endo16* and *spec2a* genes. (A) Spatial and temporal control within the *endo16 cis*-regulatory system (adapted from Yuh *et al.,* 2001a). Module A functions are shown by thick lines and module B functions by thin lines. Initial *endo16* activation is restricted to the veg2 lineage, and enhanced expression is found later in mid- and hindgut. (B) Sequence from the *spec2a* enhancer conferring aboral ectoderm (and mesenchyme) expression. A region outside the enhancer is responsible for repressing *spec2a* in mesenchyme cells. The positive factors SpOtx and SpCBF are shown above their respective *cis*-regulatory elements. The negative factors, SpGsc, OER, and ENR, are shown below their elements. Spatial specificity is conferred largely by negative regulation.

not only required for the initial activation of *endo16* but also serves to "process" information from more upstream modules.

Endo16 expression is upregulated four- or fivefold after the gut forms. This late expression depends on a positive DNA element within the B module called UI, and like the repressor sites in modules DC, E, and F, it requires module A to process its function. Two specific binding sites in module A are required to link module B to module A and to mediate the amplification of module B input. These module A sites are termed P and CG1, and they must interact with a site called CB2 on module B for the UI site to function as an activator. Yuh *et al.* (2001a) identified sites within the B module that mediate what is termed the "BA intermodular input switch." The function of the switch is to inactivate module A's Otx site through a repressor site within module B called R. The intermodular switch shifts control from the initial vegetal plate specification system using SpOtx and maternally derived spatial repressors to gut-specific expression requiring UI (Fig. 2A).

With the exception of SpOtx, the proteins binding to the previously mentioned sites within the *endo16* transcriptional regulatory region have not been identified. Clearly, their cloning and characterization will provide major insights into the relationship between A–V axial patterning and vegetal plate-specific gene expression and also into the mechanisms that switch transcriptional control from a specification to differentiation mode. For instance, the gene encoding the R factor, which presumably inhibits SpOtx activity in endoderm cells, may be a downstream effector for the β-catenin–Tcf/Lef pathway.

Although the ectoderm repressors that bind to sites within *endo16* modules E and F and the primary mesenchyme cell repressor that binds to sites within module DC have not been identified, these proteins are likely to be direct mediators of the vegetal nuclear β-catenin signaling system. Consistent with this view is the finding that the repressor sites within modules DC, E, and F are all converted into positively acting elements that increase expression by LiC1 treatment of embryos (Yuh and Davidson, 1996). By inhibiting GSK-3β, LiC1 treatment expands the zone of *endo16* expression into veg1 descendents normally destined to become ectoderm. Because the LiC1 response, like the repression response in untreated embryos, requires the processing function of module A, the presence of nuclear β-catenin in ectopic locations must be interfering with module A's normal interactions with modules E and F, perhaps by suppressing repressor activity in these cells.

C. The *spec2a Cis*-Regulatory Region

The *spec2a* gene also requires Otx sites for its activation at the end of the cleavage stage, although *spec2a* encodes a small intracellular calcium-binding protein that is expressed exclusively in aboral ectoderm cells. Unlike *endo16, spec2a* expression is largely extinguished after the late blastula stage, making the initial specification

events along the A–V and O–Ab axes the sole and ultimate source for *spec2a* spatial regulation. Four redundant Otx sites (from 5′ to 3′, Otx sites 1–4) constitute the major positive *cis*-regulatory elements within the *spec2a* enhancer, and these sites are essential for the transcriptional activity of this gene (Mao *et al.,* 1994; Yuh *et al.,* 2001b). The enhancer is approximately 300 bp in length and is located about 400 bp from the *spec2a* transcriptional start site. It drives reporter gene expression in aboral ectoderm and mesenchyme cells, and a mesenchyme cell repressor element lying further upstream of the enhancer is required for full aboral ectoderm-specific expression.

Five factors in addition to SpOtx interact with *cis*-regulatory elements within the *spec2a* enhancer (Fig. 2B). SpGsc, discussed in Section V, antagonizes SpOtx at Otx binding sites to repress expression in the oral ectoderm at the blastula stage and later (Angerer *et al.,* 2001). A CAAT element that binds to a sea urchin ortholog of mammalian CBF/NFY is located 7 bp from Otx site No. 3 and synergizes with Otx site No. 3 to enhance expression in the ectoderm. A site for an oral ectoderm repressor called OER is situated between Otx site No. 3 and the CAAT element and is thought to function in oral ectoderm by blocking the synergistic interaction between SpOtx and CBF/NFY (Yuh *et al.,* 2001b). It is also possible that the OER confers its repression function by interacting with SpGsc. Finally, an endoderm repressor element, ENR, overlapping Otx site No. 4, is required to repress *spec2a* expression in the vegetal plate territory (Fig. 2B). Remarkably, mutating the ENR site within the full context of the 1.5-kb *spec2a* transcriptional regulatory region leads to ectopic gut expression of a *GFP* reporter gene (Yuh *et al.,* 2001b). These results indicate that a single *cis*-regulatory element within the *spec2a* enhancer is largely responsible for repressing the *spec2a* gene in vegetal plate cells. Unlike the interactions of distant modules in *endo16,* in the *spec2a* enhancer Otx sites lie within only a few base pairs of the OER and ENR repressor sites, and it is likely that SpOtx and these repressor proteins directly interact. However, *spec2a* also requires a more distant repressor element, upstream of the enhancer, to prevent expression in mesenchyme cells.

Analysis of the *spec2a* enhancer leads to a model in which the restricted expression of *spec2a* in aboral ectoderm cells is mediated by negative *cis*-regulatory elements and the factors binding to these elements are under the control of vegetal nuclear β-catenin signaling. This model predicts that nuclear β-catenin–Tcf/Lef in vegetal cells, as part of its role in mesendoderm specification, activates the gene encoding ENR, which in turn represses *spec2a* in the vegetal plate territory. Thus, ENR may belong to the same set of vegetal repressors as SpKrl and SpKrox-1. As discussed in Section V, vegetal cells having nuclear β-catenin produce a second signal that polarizes the ectoderm into definitive oral and aboral ectoderm. This second signal leads to the selective activation of genes encoding OER and SpGsc in the presumptive oral ectoderm. SpOtx and SpCBF are the essential positive transcription factors that activate *spec2a* in the aboral ectoderm. SpOtx is uniformly

expressed in embryos at the time of *spec2a* activation but it is possible that SpCBF has restricted expression in ectodermal lineages. Indeed, SpCBF may belong to the cohort of animalizing transcription factors that oppose vegetal nuclear β-catenin along the A–V axis.

VII. Embryonic Regulation: A Proposed Mechanism

A. A Simple Model for Specification along the A–V Axis

We have reviewed evidence that a simple system for patterning the sea urchin embryo along the A–V axis is initiated following fertilization by a few maternal products that act autonomously and locally within the early embryo. This system is presented in Fig. 3. Initially, translation of maternal mRNAs produces animalizing transcription factors (AFs) such as SpSoxB1 that accumulate in all nuclei. Upon the fourth cleavage, β-catenin begins to accumulate in micromere nuclei and subsequently in other vegetal nuclei. SpSoxB1 and presumably other AFs

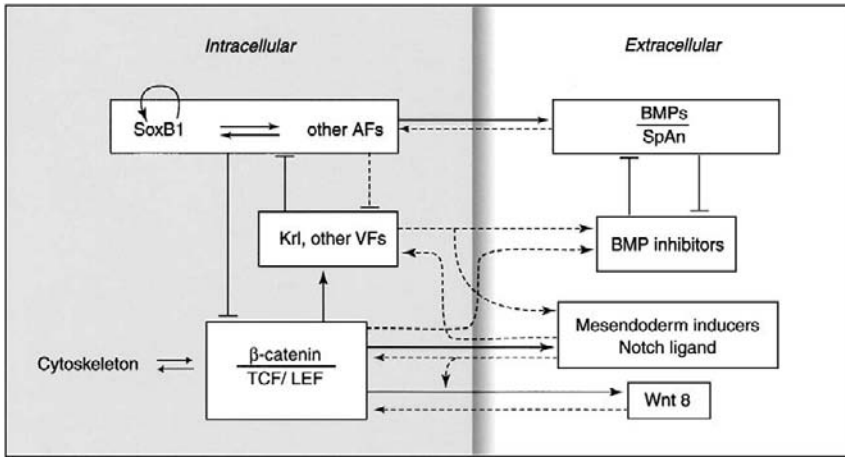

FIG. 3 A model of interacting animalizing and vegetalizing systems operating within cells and as extracellular signals. Within cells, transcription factors localized to nuclei are shown in boxes. Activating interactions are shown with arrows, and inhibitory interactions are shown with bars. Extracellular signals are transduced via a cellular signaling system; in many instances, the signal transduction pathway is complex but ultimately influences the transcription of genes. Interactions for which there is substantial experimental evidence in sea urchin embryos are shown as solid lines and are discussed in the text. Interactions for which there is little or no direct evidence for sea urchins (but there may be for other systems) are shown with dashed lines.

decline as nuclear β-catenin appears in these vegetal nuclei. The ratio of nuclear β-catenin (acting via TCF/Lef) to AFs (the BC/AF ratio) declines in blastomeres along the A–V axis. We have reviewed the considerable evidence indicating that this ratio serves as the initial conditional patterning address for axial specification. This process creates micromeres that have a very high BC/AF ratio, veg1 and veg2 cells that have a range of moderate ratios declining toward the animal pole (resulting in activation of zygotic genes encoding vegetalizing transcription factors such as SpKrl), and cells of the animal half that have very low ratios approaching zero. The AFs are likely to regulate the expression of one another, perhaps reinforcing and fine-tuning patterning events. In response to this autonomous coding, animal cells express genes regulated by the AFs, including extracellular patterning molecules such as BMP2/4 and SpAN, whereas vegetal cells produce SpWnt8 and probably other extracellular molecules that counteract BMP2/4 activity. These extracellular factors enhance and refine the patterning along the A–V axis and are required for endomesoderm specification. It is likely that the inherently unstable, counterinhibitory system of intracellular transcription factor activities is stabilized by autoregulatory systems. There is evidence that SpSoxB1 positively autoregulates: Expression of dominant-negative SpSoxB1 or a fusion of the SpSoxB1 DNA binding domain to an engrailed repressor element inhibit expression of the endogenous SpSoxB1 gene (Kenny *et al.,* 1999). Members of the transforming growth factor-β/BMP family of proteins show a range of complex autoregulatory interactions and are sometimes involved in positive feedback loops. It may be that BMP2/4 feedback promotes expression of the animalizing transcription factor system such as SpSoxB1. As discussed previously, SpWnt8 enhances nuclear β-catenin activity, and its expression is probably driven by β-catenin–Tcf/Lef activity. Notch signaling is also involved in promoting secondary mesenchyme specification as well as the endoderm–ectoderm border, and it may interact with other signaling systems. The extracellular signaling systems form an activity gradient that modulates the activities of (early and late acting) animalizing and vegetalizing transcription systems.

B. A Model for Embryonic Regulation

The remarkable regulative capacity of sea urchin embryos summarized in Section II.B and by Hörstadius (1973) results from the conditional specification of cell fates via intercellular interactions (Davidson *et al.,* 1989). Regulation to replace deleted territories until late in embryonic development depends on the late commitment of multipotent cells that are able to participate in regulatory responses. As noted previously, except for micromeres, explanted and recombined fragments of embryos usually show a greater developmental potential than their fate within the undisturbed embryo (Hörstadius, 1973). Thus, suppressive interactions among cells have an important role in the specification of cell fates

(Brandhorst and Klein, 1992). The information summarized here on the role of maternal transcriptional regulatory factors and territorial gene expression offers new insight into how echinoid embryos regulate to replace missing parts.

A simple diagram of known or suspected interactions of transcriptional regulatory systems is shown in Fig. 3. A notable feature of this system is the mutually inhibitory nature of the animalizing and vegetalizing transcriptional regulatory systems. Patterning of the embryo along the A–V axis depends on the intracellular ratio of these opposing activities. This gene regulatory system is not stable. For example, within a blastomere a decline in nuclear β-catenin would result in an increase in animalizing transcription factors such as SpSoxB1, which in turn would further inhibit β-catenin–Tcf/Lef activity. Conversely, an increase in activity of the β-catenin–Tcf/Lef system would inhibit the activity of the animalizing transcription factor system in that cell, increasing its own activity. As mentioned in Section VII.A, positive autoregulatory mechanisms probably buffer these changes to some extent. It is likely that in the intact embryo, the ratios of animalizing and vegetalizing transcriptional regulatory systems are stabilized by cellular interactions involving secreted signals produced by neighboring cells in response to their own combinations of transcriptional regulators. When these intercellular signals are disrupted by the extirpation of parts of the embryo, the anticipated response would be to establish a more extreme range of transcription factor activities in responding blastomeres. For instance, when vegetal halves are cultured in isolation, an increased number of cells on the more animal side, relieved of suppression from the animal half, might activate animalizing transcription factors, thereby regulating to replace ectoderm. When animal halves are recombined with micromeres, they regulate to form endoderm and secondary mesenchyme, sometimes producing a fully normal pluteus (Hörstadius, 1973; Amemiya, 1996). As discussed in Sections IV.A and IV.B, this clearly involves evocative inductions by micromeres that presumably activate the vegetalizing transcription factors in some derivatives of mesomeres. As noted in Section V.C, this induction may be mediated by Notch signaling and possibly Wnt signaling.

When mesomeres are recombined with veg1 cells, the veg1 cells sometimes regulate to produce skeletogenic and secondary mesenchyme (pigment cells) in addition to their normally fated endoderm (not always) and ectoderm (Horstadius, 1973; Khaner and Wilt, 1991). Isolated veg1 tiers often form apical tuft cilia and then ciliated blastulae similar to the dauerblastulae characteristic of isolated animal halves, but sometimes cultured veg1 tiers fail to form apical tuft cilia and later form small archenterons; in either instance, they do not form skeletogenic mesenchyme (Hörstadius, 1973). Vegetal blastomeres thus seem to suppress the potential of veg1 cells to differentiate as mesenchyme in the intact embryo, whereas recombination with mesomeres seems to enhance it. It is possible that vegetal blastomeres produce secreted signals that repress the β-catenin–Tcf/Lef transcription system in veg1 cells, maintaining a moderate ratio of animalizing to vegetalizing transcriptional regulators, whereas mesomeres suppress the tendency to form apical tuft

cilia and ectoderm by suppressing the animalizing transcription factor system. In isolated veg1 tiers, the ratio of animalizing to vegetalizing factors would tend to be maintained, becoming randomly skewed toward animal or vegetal. However, when combined with mesomeres, the animalizing factors might be suppressed, resulting in an enhanced ratio of nuclear β-catenin–Tcf/Lef (or downstream vegetalizing transcription factors) to SpSoxB1 (or other animalizing transcription factors) in some descendents of veg1, thereby causing their respecification as mesenchymal precursors. These premesenchyme cells would, in turn, begin to signal neighboring cells, suppressing their potential to form mesenchyme but enhancing endoderm specification. Moreover, veg1 cells suppress the potential of isolated mesomeres cultured for long periods of time to form endoderm and mesenchyme (Henry *et al.,* 1989; Khaner and Wilt, 1990). The veg1 cells may thus suppress the tendency of some mesomere derivatives to activate vegetalizing transcription factors. These are testable predictions, at least in part.

Another important consideration concerning regulation is the complexity of transcriptional regulation in conditionally specified blastomeres prior to commitment (Yuh *et al.,* 2001a). As summarized in this review, and as noted for other genes such as *cyIIIa* actin (Cameron and Coffman, 1999), the spatially restricted regulation of genes expressed in conditionally specified cells is complex, depending on multiple positive and negative inputs including those of intercellular signals that must be processed by the *cis*-acting regulatory region of the gene. After cellular commitment to a fate, regulation of these cell-type-specific genes becomes simpler, using regulatory DNA modules dependent only on positively acting, spatially regulated zygotic transcription factors. If a suppressive cellular interaction is disrupted by extirpation of part of the embryo prior to the determination of adjacent cells, those cells will process the change in input signals and may respond by altering their gene expression and state of specification. It is noteworthy that for several genes, early (conditional) spatial regulation is predominantly the result of spatially restricted negative transcriptional regulators acting to modify the activities of broadly distributed transcriptional activators. If the activities of these negative regulators depend on signals from neighboring cells, disruption of these cellular interactions should activate expression of genes in inappropriate lineages. This would initiate a regulative response that could ultimately reestablish a spatially graded pattern of gene regulation that results in appropriate organization of determined cells.

VIII. Some Unresolved Issues and Future Directions

One problem with the model described in Section VII.A is that it fails to explain the distinction in fate between large and small micromeres. Both have a very high ratio of nuclear β-catenin to animalizing transcription factors (BC/AF).

The nondividing small micromeres maintain the high concentration of nuclear β-catenin, whereas it declines during cleavage in the large micromere lineages. This difference, as well as their distinctive fates, must be the result of differences autonomously established by the asymmetric cleavage that creates large and small micromeres. It is possible that the fates of large and small micromeres are distinguished by a difference in ratios of transcription factors resulting from the unequal cleavage: Small micromeres might have an even higher BC/AF ratio than large micromeres. This difference, in turn, might result in different threshold responses in the micromere daughters that form at the fifth cleavage. However, this explanation seems unlikely. There is no indication that small micromeres have an essential role in development and no evidence that they are committed to differentiate as coelomic pouch (Khaner and Wilt, 1991). In addition to being committed to skeletogenesis, cells of the large micromere lineages have at least three roles in patterning: production of a signal (possibly SpWnt8) that enlarges the zone of autonomous endoderm specification, production of the Notch ligand involved in secondary mesenchyme specification, and production of a signal that inhibits the differentiation of secondary mesenchyme cells as primary mesenchyme. It should be possible to definitively identify and characterize these signals. The virtually normal but delayed development of larvae from embryos lacking micromeres indicates that micromeres are not essential for development, another testament to the regulative capacity of the embryo. Although in many respects micromeres behave after transplantation as an organizing center and probably do so in the intact embryo, this capacity is not confined to micromeres but is shared with other vegetal cells that also initially have high levels of nuclear β-catenin.

The mechanism by which β-catenin initially appears in the most vegetal nuclei, and subsequently in other vegetal nuclei but not in animal cell nuclei, is poorly understood. It is presumably the result of localized inhibition of GSK-3β activity, resulting in stabilization of β-catenin. Based on the emerging understanding of the establishment of dorsality in *Xenopus,* Dsh may be localized or preferentially activated in the vegetal cytoplasm, probably as a part of a vesicle (Miller *et al.,* 1999). In principle, any component of the Wnt signaling pathway could be involved in local stabilization of β-catenin so that it accumulates in nuclei. As noted by Romancino *et al.* (2001), binding of β-catenin to cadherin at adherens junctions in animal cells but not in vegetal cells may have a role in the differential distribution of nuclear β-catenin along the A–V axis. The entry of β-catenin into the nuclei of micromeres, and the attendant decline in animalizing transcription factors such as SoxB1, may be sufficient for the early autonomous determination of micromeres as skeletogenic mesenchyme.

It is likely that other animalizing and vegetalizing transcription factors will be identified that are involved in patterning along the A–V axis. Extracellular signaling systems are likely to be produced by vegetal cells that counteract the animalizing signals such as BMP2/4. In addition, there is much evidence for intercellular signals that suppress the developmental potential of neighboring cells in the intact embryo,

possibly by activating transcription factors that act as negative spatial regulators. Such extracellular signals and their targets need to be identified and characterized. The symmetry-breaking events that specify the O–Ab axis remain elusive, as does the role of vegetal signaling in the differentiation of ectoderm. The mechanisms that allow for resetting of O–Ab axial specification in response to experimental manipulations remain unknown.

Organisms whose eggs have a defined polarity, such as *Drosophila* and *Xenopus,* depend on localized maternal mRNAs for patterning the early embryo along the axis. It is striking that no localized maternal mRNA or protein have been identified that are associated with the vegetal pole in the sea urchin species discussed in this review. Although a few maternal mRNAs show localization in the egg and/or early embryo, there is little experimental evidence that they are associated with axial patterning. Maternal mRNAs encoding the Bep family of surface proteins, and the Bep proteins, are enriched in the animal cytoplasm of the egg and early embryo (Di Carlo *et al.,* 1994, 1996; Romancino *et al.,* 1999, 2001). This localization is dependent on a protein factor that appears to associate the mRNAs with the cytoskeleton (Montana *et al.,* 1997; Romancino *et al.,* 1998; Costa *et al.,* 1999). Recent evidence summarized in Section III suggests that they may have a role in early A–V axial specification (Romancino *et al.,* 2001). The distribution of Bep mRNAs in the egg suggests that there are distinctive features of the cytoskeleton along the A–V of the unfertilized egg that may have a role in establishing and maintaining the A–V polarity.

In eggs of the direct developing sea urchin *Heliocidaris erythrogramma,* mRNA encoding a Wnt is restricted to the vegetal half of the egg in a gradient declining toward the vegetal pole (R. Raff, personal communication). Following fertilization, it becomes more strictly localized to the vegetal pole. Thus, sea urchin eggs have a mechanism for localizing and redistributing maternal mRNAs with reference to the vegetal pole, presumably using a cytoskeletal mechanism. The purpose of this localization is unknown, but it should be amenable to experimental analysis. It may be that in the very large, lecithotrophic eggs of *H. erythrogramma* the localization of β-catenin mRNA is required to ensure the proper vegetal localization of nuclear β-catenin. In indirect developing urchins with relatively small, oligolecithal eggs, the autonomously polarized initial distributions of transcription factors resulting in axial patterning may not require any localization of mRNAs. As noted previously, there may be nothing required other than a high ratio of nuclear β-catenin to animalizing transcription factors to commit large micromeres to differentiate as skeletogenic mesenchyme, the result of regulating the local activities of transcription factors such as HpEts, which appears to promote determination of skeletogenic mesenchyme. A slightly lower concentration would result in formation of the mesendoderm of the vegetal plate, with ectoderm the resulting state in animal cells expressing the animalizing transcription factors. Cellular interactions then maintain the states of specification established by the autonomously differential

but transient activities of animalizing and vegetalizing transcription factors. Cellular interactions also define the mesoderm–endoderm and endoderm–ectoderm boundaries.

IX. Concluding Remarks

We have reviewed the considerable recent progress in understanding axial patterning in sea urchin embryos. A striking observation is the very dynamic expression patterns of proteins such as β-catenin, Wnt8, and Notch that have important roles in the patterning of embryos. This may account, in part, for the complex regulation of genes that respond to patterning events. Although many important issues remain to be resolved, an elegantly simple model is emerging. The powerful experimental methods now available provide an opportunity for much more progress to be made. Of particular interest is the emerging area of sea urchin genomics and expressed sequence tag (EST) analysis (Cameron *et al.,* 2000). Large-scale genomic sequencing projects are under way, and a variety of arrayed cDNA libraries from various species, embryonic stages, and cell types have been or are being generated. More than 12,000 ESTs from *S. purpuratus* have already been deposited into GenBank. These libraries and EST databases will serve as valuable platforms for gene expression profiling experiments using high-density macroarray filters (Rast *et al.,* 2000) and microarray slides in differential hybridization screens. In the near future, it will be possible to identify large numbers of genes whose temporal and spatial expression patterns along the A–V and O–Ab axes depend on the initial maternal mechanisms that define the A–V axis.

Acknowledgments

We acknowledge Eric Davidson and David McClay for providing recent references and Athula Wikramanayake, Rudy Raff, Charles Ettensohn, Robert Angerer, and Lynne Angerer for communicating unpublished results. We also thank Shonan Amemiya, Eric Davidson, Jim Coffman, Koji Akasaka, Charles Ettensohn, Andy Cameron, Dave McClay, Athula Wikramanayake, Judith Venuti, Fred Wilt, Greg Wray, Catriona Logan, Hyla Sweet and Lynne and Robert Angerer for stimulating and influencing our thinking about sea urchin development. WHK acknowledges the NICHD and Robert A. Welch Foundation for continued support for investigating gene expression during sea urchin embryogenesis.

Note added in proof. Delta was tentatively proposed in Section IV.A to be the ligand for Notch involved in secondary mesenchyme specification. H. Sweet and C. Ettensohn (unpublished results) now have strong evidence for this. They found that knockdown of Delta in *L. variegatus* embryos copies the phenotype of embryos expressing dominant negative Notch: a smooth archenteron and loss of pigment cells. Delta function is required only in micromere derivatives. Overexpression of Delta results in overproduction of secondary mesenchyme (pigment) cells.

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Cell and Molecular Cell Biology of Melanin-Concentrating Hormone

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Recent advances in the study of melanin-concentrating hormone (MCH) have depended largely on molecular biological techniques. In mammals, which have attracted the most attention, novel findings concern (i) the MCH gene, which can yield several peptides by either posttranslational cleavage or alternative splicing, as well as bidirectional transcription; (ii) the identification of two G protein-coupled MCH receptors in the brain and peripheral tissues; and (iii) the evidence for subpopulations of MCH neurons in the central nervous system, characterized by their chemical phenotypes, connections, and individual physiological responses to different physiological paradigms. The involvement of central MCH in various functions, including feeding, reproduction, stress, and behavior patterns, is reviewed. The stage during evolution at which MCH may have acquired hypophysiotrophic and hormonal functions in lower vertebrates is considered in light of morphological data. Evidence that MCH also has peripheral paracrine/autocrine effects in mammals is provided.

KEY WORDS: Melanin-concentrating hormone, MCH gene, MCH receptors, Neuronal subpopulations, Central neurotransmitter.

I. Introduction

The melanin-concentrating hormone (MCH) is an example of a peptide that plays numerous roles: It serves as a neurotransmitter/neuromodulator in the brain of all vertebrates, as a circulating hormone in fish, or as a putative paracrine/autocrine agent on peripheral tissues. Its original identification as a neurohypophysial

hormone in fish arose from its striking effect on their skin pigment cells and overall color, but MCH-immunoreactive neurons were soon identified in the brain, principally in the hypothalamus, of all groups of vertebrates from lampreys to humans. Early research focused on the structure and pigmentary role of MCH in fishes, but evidence that the peptide could also modulate the hypothalamo-pituitary stress axis aroused interest in the mammalian molecule, leading to the determination of its amino acid and cDNA sequences in rats, mice, and humans. The overall picture that emerged led researchers to suggest that MCH could be implicated in general arousal, goal-orientated behavior, and sensorimotor integration. Detailed reviews of these seminal studies already exist (Baker, 1991, 1994; Nahon, 1994) and this information will not be repeated here except briefly where relevant.

Recent research on MCH has concentrated on mammals and provided new data. Some relate to potential functions of the peptides, such as effects on various behaviors, appetite, or reproduction. Others concern the connectivity of the MCH neurons and possible routes through which they are controlled. Recently, the long-awaited MCH receptors have been identified together with the second messenger systems to which they couple—information which may help explain earlier anomalous results about MCH's biological effects. Although the majority of studies have concentrated on the central nervous system, it is apparent that the peptide and its receptors are also expressed in the periphery—gut, gonads, adipose tissue, pancreas, skin, and immune system. Another intriguing line of enquiry revealed the existence of additional MCH-related genes—the human MCH variant, an alternatively spliced MCH gene (MGOP) in rats, and an antisense MCH gene, all of which potentially serve novel functions. Lastly, a few recent morphological studies of the MCH system in lower vertebrates have contributed to our understanding of the expanding roles of this intriguing peptide during early evolution.

II. Structure of MCH and Related Molecules

A. MCH and Copeptides

The structures of MCH and its cDNA sequence for mammals and fish have previously been described in detail (Baker, 1991; Groneveld *et al.,* 1993; Nahon, 1994; Baker *et al.,* 1995). Only minimal information is given here as a reference point.

MCH is a small cyclic peptide derived by posttranslational cleavage from the C terminus of a larger precursor molecule, pre-proMCH (ppMCH) (Fig. 1). In addition to MCH, the precursor can be cleaved further to yield a 13-amino acid sequence termed Mgrp (MCH gene-related peptide) in fish or an amidated peptide in rodents termed NEI [neuropeptide glutamic acid (N), isoleucine (I)], a process in which the pro-hormone convertase, PC2, plays a key role (Seidah *et al.,* 1993; Viale *et al.,* 1999b). Further potential cleavage at a single basic amino acid might

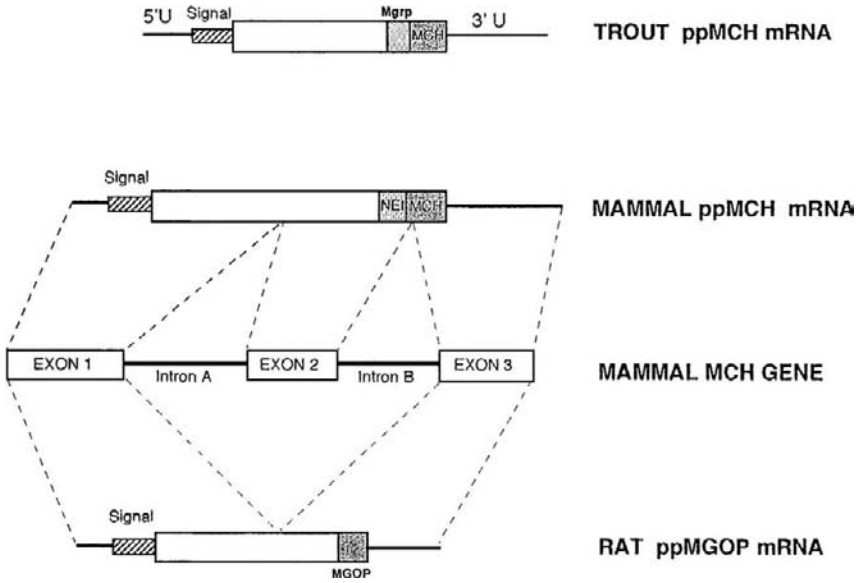

FIG. 1 Comparison of trout and rodent ppMCH mRNA and ppMGOP mRNA. Trout mature ppMCH mRNA is derived from a primary RNA transcript lacking introns. The rodent primary transcript has two introns and can be differentially spliced to give either ppMCH mRNA if all three exons are used or ppMGOP mRNA when exon 1 and exon 3 are spliced together.

release a third peptide, NGE, in mammals but this remains unproven. MCH exhibits considerable structural conservation, being identical in most fish examined (salmonids, tilapia, and bonito); the only variation is in the eel, which has Asp^1 instead of Asn^1 (Kawauchi, 1989). Compared with fish, the mammalian molecule has two additional and four mutated amino acids (Fig. 2), but its structure is identical

Organism & MCH or related peptide	Amino Acid Sequence
Salmonids/bonito tilapia	Asp.Thr.Met.Arg.Cys.Met.Val.Gly.Arg.Val.Tyr.Arg.Pro.Cys.Trp.Glu.Val
Eel	**Asn**.Thr.Met.Arg.Cys.Met.Val.Gly.Arg.Val.Tyr.Arg.Pro.Cys.Trp.Glu.Val
Rat/mouse/ Human	**Asp** **Phe**.Asp.**Met**.**Leu**.Arg.Cys.Met.**Leu**.Gly.Arg.Val.Tyr.Arg.Pro.Cys.Trp.**Gln**.Val
H.var.	**Asp**.**Phe**.Asp.Thr.**Leu**.**Ser**.Cys.Met.**Leu**.Gly.Arg.Val.Tyr.**Gln**.**Ser**.Cys.Trp.**Gln**.Val
Rat MGOP	Thr.**Ile**.**His**.Cys.**Lys**.**Trp**.**Arg**.**Glu**.**Lys**.**Pro**.**Leu**.**Met**.**Leu**.**Met**.

FIG. 2 Comparison of amino acid sequences of fish and mammals. The two cysteine residues are linked (line) to form a cyclic structure. Residues which differ from fish sequence are in bold and underlined. H. var., human variant MCH; MGOP, MCH gene-overprinted polypeptide 14.

among mammals (rat, mouse, and human). Other parts of the ppMCH molecule are less conserved; the Mgrp sequence is longer in tilapia than in salmonids (22 vs 13 residues) and 8 of these amino acids are different. Similarly, mammalian NEI is only 30% identical to salmonid Mgrp. There is even less similarity between the remaining ppMCH sequences of fish and mammals (Baker, 1991), suggesting a lack of selective pressure during evolution to conserve this region.

Recent studies have investigated the relative importance of different amino acids within the MCH sequence for binding to the MCH type 1 receptor (SLC-1) and for inhibiting forskolin-stimulated cAMP production, an indicator of efficient coupling to the G_i/G_o protein (Audinot *et al.,* 2001). Tests using shortened peptides show that the sequence MCH^{6-17}, which includes the ring structure Cys^7–Cys^{16} together with the flanking amino acids Arg^6 and Trp^{17}, is essential for full binding efficacy and G protein coupling. By substituting Ala for individual amino acids in this dodecapeptide, the authors showed that only three of the residues within the ring (Met^8, Arg^{11}, and Tyr^{13}) are essential for full agonistic activity. These amino acids are identical in fish MCH that, although showing a slightly lower receptor binding (perhaps reflecting its different and shorter N-terminal sequence), shows a comparable potency on cAMP suppression in HEK293 cells transfected with the MCH receptor. As already shown for fish (Baker *et al.,* 1985b), the cyclic configuration is important for biological activity.

B. Other Molecules Derived from the MCH Gene

In contrast to the salmonid MCH gene which is intronless (Takayama *et al.,* 1989), the mammalian gene has two introns and three exons (Nahon, 1994; Fig. 1). An additional pre-pro-peptide has been discovered in the rat which is derived by splicing together MCH exons 1 and 3, giving rise to a novel peptide (Toumaniantz *et al.,* 1996). The signal and following sequence derived from exon 1 are identical to the N-terminal region of ppMCH but the peptide translated from exon 3 is completely different because Met^4 in the MCH peptide is coded for by one nucleotide derived from exon 2 and two nucleotides derived from exon 3; omitting exon 2 and using only exon 3 gives rise to a frameshift and consequently different amino acids. The C-terminal region of this novel molecule, termed ppMGOP (MCH gene-overprinted peptide), contains a potential cleavage site (Lys–Lys) which could liberate a 14-amino acid peptide. Immunoreactive ppMGOP has been identified in the lateral hypothalamus and zona incerta in neurons that also express ppMCH; it has also been identified in other areas of the brain (cortex, amygdala, lateral septal nucleus, and caudate putamen) in which ppMCH is not expressed (Toumaniantz *et al.,* 2000). Toumaniantz *et al.* propose that MGOP peptide may be secreted and have roles quite independent of MCH.

Primates, including humans but in apparent contrast to other mammals, possess two variant forms of the MCH gene on chromosome 5 at positions 5p14 and 5q13

(Viale *et al.,* 1997). These lack exon 1 and are thus truncated forms of authentic MCH which is found on another chromosome, 12q23. Only one of these variants, MCH-L1, can be detected by RACE-PCR (Rapid amplification of cDNA ends-polymerase chain reaction) (Viale *et al.,* 2000) and seems to be expressed only in the brain. Its MCH sequence differs from that of authentic MCH in four positions (Fig. 2), and although it possesses Met^{8}, Arg^{11}, and Tyr^{13}—the intracyclic residues crucial for binding and biological activity—it lacks Arg^{6}, which normally enhances binding and biological activity (Audinot *et al.,* 2001). Perhaps as a consequence, it shows poor displacing activity in competitive binding studies using transfected MCH receptors (see Section III).

A final twist to the MCH gene story is the existence in the rat of large mRNA structures that are complementary to the 3′ flanking end of the MCH mRNA gene and which are transcribed in an antisense direction from the opposite strand of the MCH gene (Borsu *et al.,* 2000). Two classes of antisense RNA have been described: a noncoding, unspliced transcript with multiple introns that overlap the coding part of the MCH gene and several spliced RNAs that encode putative proteins exhibiting DNA/RNA binding domains. The gene has been named the AROM gene (antisense RNA overlapping MCH). Borsu *et al.* hypothesize that the unspliced AROM transcripts might be involved in regulating the processing or degradation of authentic MCH RNA, and they cite examples in which AROM and MCH RNA are inversely abundant.

III. The MCH Receptors

A. Identification and Intracellular Signaling of MCH Receptors

Initial attempts to demonstrate MCH binding sites in brain sections or on cells encountered problems partly because MCH is very lipophilic and shows exceptionally high nonspecific binding, partly because the molecule is readily degraded enzymatically, and partly because iodination of authentic MCH, in which the tyrosine residue is contained within the cyclic region of the molecule, distorts its shape, reducing bioactivity 500- to 1000-fold when tested on fish melanophores, the only bioassay that was initially available (Baker *et al.,* 1985b; Kawazoe *et al.,* 1987; Baker, 1991). Therefore, the first binding studies, from Eberle's lab in Switzerland, used an MCH analog in which Tyr^{13} was replaced by Phe and the C-terminal Val^{19} was replaced by Tyr to permit iodination (Drozdz *et al.,* 1995). This analog, [Phe^{13},Tyr^{19}]-MCH, exhibits full biological potency in the fish melanophore bioassay (Drozdz *et al.,* 1995) and also stimulates feeding behavior in rodents in a manner similar to that of native MCH (Kokkotou *et al.,* 2000). Recent work has found, surprisingly, that in apparent contrast to fish receptors, the mammalian receptor will bind iodinated authentic r/hMCH satisfactorily (Table I). The binding

TABLE I
Binding Characteristics of MCH and Related Peptides to the MCH-R1

Cell line/tissue	Peptide	K_d/K_i (nM)	Method[a]	Reference
Studies using ^{125}I-[Phe^{13},Tyr^{19}]-MCH				
G4F-7 melanoma	[FY]MCH	0.1	Saturation binding	Drozdz *et al.* (1995)
	[FY]MCH	6.3	Competitive binding	Drozdz *et al.* (1995)
	r/hMCH	12	Competitive binding	Drozdz *et al.* (1995)
	rANF(1–25)	116	Competitive binding	Drozdz *et al.* (1995)
G4F melanoma	r/hMCH	120	Competitive binding	Drozdz *et al.* (1995)
B16-F1 melanoma	r/hMCH	14	Competitive binding	Drozdz *et al.* (1995)
PC12 phaeochromocytoma	r/hMCH	120	Competitive binding	Drozdz *et al.* (1995)
Keratinocytes SVK-14	^{125}I-[FY]MCH	0.7	Saturation binding	Burgaud *et al.* (1997)
	NaI[FY]MCH	3–3.7	Competitive binding	Burgaud *et al.* (1997)
	[FY]MCH	20–25	Competitive binding	Burgaud *et al.* (1997)
	r/hMCH and sMCH	65–93 161	Competitive binding Competitive binding	Burgaud *et al.* (1997) Burgaud *et al.* (1997)
	h. var. MCH			
	rANF(1–25)	210–365	Competitive binding	Burgaud *et al.* (1997)
Studies using ^{125}I-MCH				
HEK293 + SCL-1	^{125}I-MCH	0.2	Saturation binding	Chambers *et al.* (1999)
	[FY]MCH	0.12	Competitive binding	Chambers *et al.* (1999)
	r/hMCH	0.043	Competitive binding	Chambers *et al.* (1999)
	sMCH	0.39	Competitive binding	Chambers *et al.* (1999)
	h. var. MCH	294.7	Competitive binding	Chambers *et al.* (1999)
HEK293 + SLC-1	^{125}I-MCH	0.46	Saturation binding	Audinot *et al.* (2001)
CHO + SCL-1	^{125}I-MCH	1.3	Saturation binding	Hawes *et al.* (2000)
	r/hMCH	1.0	Competitive binding	Hawes *et al.* (2000)
Human brain	^{125}I-MCH	0.2	Saturation binding	Sone *et al.* (2000)

[a]Saturation binding analysis or competitive binding studies were done using either ^{125}I[Phe^{13},Tyr^{19}]-MCH (Drozdz *et al.,* 1995; Burgaud *et al.,* 1997) or ^{125}I-MCH (Chambers *et al.,* 1999; Hawes *et al.,* 2000; Sone *et al.,* 2000; Audinot *et al.,* 2001). ^{125}I-MCH, iodinated r/hMCH; ^{125}I-[FY]MCH, iodinated [Phe^{13}, Tyr^{19}]-MCH analog; NaI [FY]MCH, analog iodinated with nonradioactive sodium iodide; h. var. MCH, human variant MCH.

properties of iodinated MCH and iodinated [Phe^{13},Tyr^{19}]-MCH differ and yield slightly different results in competitive binding studies.

Specific binding sites on intact cells were first demonstrated using various melanoma and other cell lines (Drozdz *et al.,* 1995) and later using a human SVK-14 keratinocyte cell line (Burgaud *et al.,* 1997). Scatchard plots indicated a single receptor type and receptor numbers between 1000 (melanoma) to 10,000 (keratinocytes) per cell. Estimated K_d and K_i values for MCH binding to whole cells or membranes are shown in Table I. Values varied even within one laboratory, depending on the cell type under investigation. The use of different radioligands and

methodologies renders comparison between laboratories difficult but the approximate K_d for binding to G4F-7 melanoma cells, keratinocytes, and cells transfected with the recently cloned MCH receptor SLC-1 is between 0.2 and 1.3 n*M*. Some authors, however, have found that the radioligand becomes internalized in intact cells and they have questioned the reliability of the values using the MCH analog for small receptor numbers (Kokkotou *et al.,* 2000).

1. MCH Receptor 1

The first MCH receptor (here called MCH-R1) was identified more or less simultaneously in several laboratories during a search for the natural ligand of an orphan receptor called SLC-1 because of a sequence similarity to the somatostatin receptor. The nucleotide sequence for SLC-1 has been determined for human (Kolakowski *et al.,* 1996), rat (Lakaye *et al.,* 1998), and mouse (Kokkatou *et al.,* 2001), and the three show striking homology at the amino acid level, with 98% identity between rat and mouse and 91% between rat and human. The protein is coded for by two exons (Fig. 3). Although exon I is small, its translated product appears to be essential for ligand binding and for bioactivity (Saito *et al.,* 2000). SLC-1 is a G protein-coupled receptor that binds mammalian MCH specifically when transfected into a variety of cells, including *Xenopus* oocytes (Bachner *et al.,* 1999), the HEK293 cell line (Chambers *et al.,* 1999; Lembo *et al.,* 1999; Audinot *et al.,* 2001), and the Chinese hamster ovary (CHO) cell line (Saito *et al.,* 1999; Shimomura *et al.,* 1999). Salmonid MCH is 10–50% less potent than mammalian MCH in competitive binding tests and in bioassays, depending on the radioligand or bioassay used (Burgaud *et al.,* 1997; Chambers *et al.,* 1999; Saito *et al.,* 1999; Audinot *et al.,* 2001). None of the MCH gene-related peptides (NEI, NGE, human variant MCH, or MGOP) or somatostatin 1–14 show a significant ability to displace the radioligand, or agonistic or antagonistic activity in calcium mobilization bioassays. The atrial natriuretic peptide (ANP) and α-MSH appear to be weak competitors in some binding assays (Table I; Burgaud *et al.,* 1997; Sone *et al.,* 2000), a difference that might be attributable to the type of radioligand used.

These studies, together with recent work (Hawes *et al.,* 2000), show that in the presence of MCH, SLC-1 will activate several signal transduction pathways, indicating it can couple to more than one G protein. The consequences of MCH receptor binding include inhibition of forskolin-induced increase in cAMP (G_i and G_o proteins), opening of inwardly-rectifying K channels (G_i proteins), a transient increase in intracellular Ca^{2+} (G_i, G_o, and G_q proteins), activation of phospholipase C and production of IP3 (G_o and G_q proteins), and stimulation of MAP kinase, an enzyme regulating the production of several transcription factors (G_i, G_o, and G_q proteins). The use of various inhibitors (e.g., pertussis toxin) to differentiate between various G proteins indicates that SLC-1 can couple to G_i, G_o, and G_q, although it may preferentially activate one system (Hawes *et al.,* 2000). When dose–response curves are generated to determine the EC_{50} for MCH stimulation of different responses,

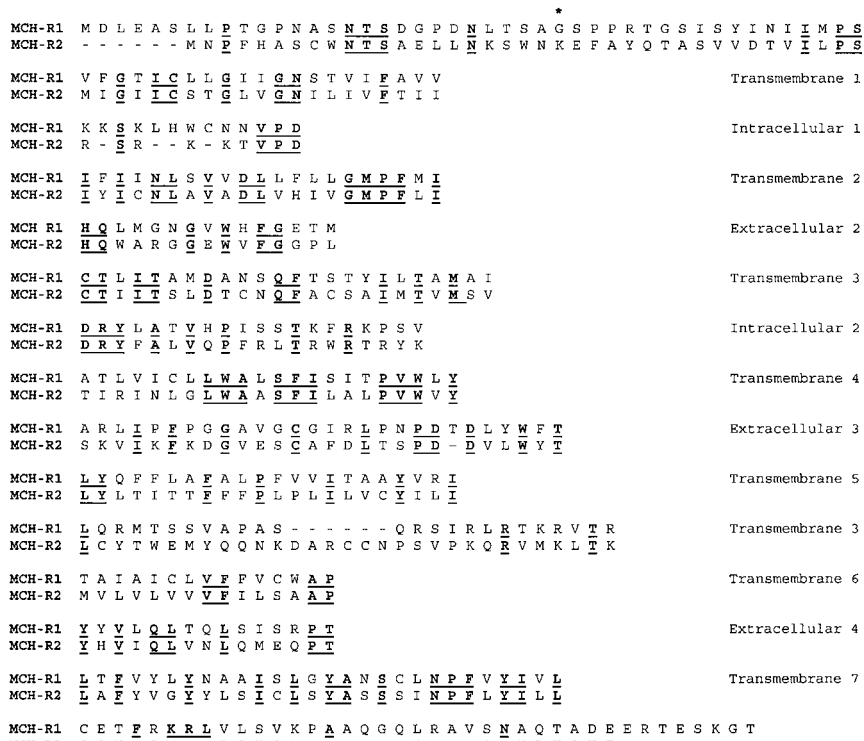

FIG. 3 Sequence comparison of MCH receptor types R1 and R2. The sequences are arranged to show the seven putative transmembrane domains and extracellular and intracellular regions. Amino acids common to both receptors are in bold and underlined. The site of the intron in the MCH-R1 gene is shown by an asterisk [based on Kolakowski *et al.* (1996) and Hill *et al.* (2001)].

the concentration of MCH required to oppose forskolin-stimulated cAMP formation is lower (suggesting a higher affinity for the G_i protein) than the concentration required to elicit a G_q-type response, at least in transfected CHO and HEK293 cell lines (Table II). Whether this preference for the G_i protein can change, depending on the intracellular/physiological conditions which regulate the abundance of particular G proteins or the activity of their signaling pathways, seems possible but remains to be shown. The ability of one receptor to activate more than one signaling pathway has been reported for other G-coupled receptors, e.g., receptors for the parathyroid hormone/parathyroid hormone-related protein; (Smith *et al.,* 1996) and for growth hormone-releasing factor (Ramirez *et al.,* 1999). Similarly, $\alpha_2\beta$-adrenoceptors can couple to both G_s and $G_{i/o}$-type proteins, either enhancing or antagonizing cAMP formation (Pohjansoksa *et al.,* 1997; Rudling *et al.,* 2000), with the outcome apparently depending on the activity of adenylyl cyclase when the receptor is activated.

TABLE II
Concentration of rMCH (nmol/liter) Required to Elicit Intracellular Responses in SLC-1 Transfected Cells

	Response			
Cell type	Inhibition of Forskolin-elevated cAMP	Ca^{2+} mobilization	IP3 production	Reference
CHO-SLC-1	1.0	10.0	50.0	Hawes *et al.* (2000)
CHO-SLC-1	0.2	—	—	Shimomura *et al.* (1999)
CHO-SLC-1	4.1	18.2	—	Saito *et al.* (1999)
HEK293-SLC-1	0.28	7.9	—	Chambers *et al.* (1999)
HEK293-SLC-1	0.28	6.0	—	Audinot *et al.* (2001)

Little is known about the interaction between specific residues in the receptor and MCH apart from the observation that the N-terminal sequence derived from exon I is essential for binding (Saito *et al.,* 2000). Macdonald *et al.* (2000) provided evidence that an important ligand-binding site is Asp^{123} in the third transmembrane domain. Mutations at this residue annul both MCH binding and Ca^{2+} fluxes in SLC-1 transfected cells. By using various MCH analogs, the authors propose that this site may interact with Arg^{11} in the cyclic portion of MCH.

2. MCH Receptor 2

Until recently, SLC-1 was the only known MCH receptor. The existence of a second receptor genetically similar to SLC-1 was questioned in view of the failure to identify such a gene using the SLC-1 coding region to probe genomic DNA from many mammalian species under low stringency conditions (Saito *et al.,* 2000). However, a second MCH receptor, here called MCH-R2, was recently identified by scrutinizing the human gene databank (Hill *et al.,* 2001). At the amino acid level, MCH-R2 shows only 38% identity (59% similarity) with the MCH-R1 (SLC-1) receptor (Fig. 3). When transfected into HEK293 cells it binds Cy5-labeled r/hMCH with a K_d of 6.0 $\pm$ 0.46 n*M*. Such cells respond to MCH by proton extrusion (EC_{50} 1.43 $\pm$ 0.44 n*M*) and a transient increase in intracellular calcium, with sMCH and the analog [Phe^{13},Tyr^{19}]-MCH being equipotent to mammalian MCH (Ca^{2+} mobilization EC_{50} values: r/hMCH, 5.65 $\pm$ 1.8 n*M*; sMCH, 7.14 $\pm$ 3.1 n*M*; [Phe^{13},Tyr^{19}]-MCH, 4.29 $\pm$ 0.48 n*M*). Pretreatment of the cells with pertussis toxin failed to influence calcium mobilization, suggesting that MCH-R2 couples to a G_q rather than G_i protein. Variant MCH was only a very weak agonist (EC_{50} > 3000 n*M*) and no other peptides tested (e.g., NEI, NGE, MGOP, and α-MSH) showed any agonistic or antagonistic activity at concentrations up to

10 μM. The sites of MCH-R1 and MCH-R2 expression are very similar (Hill *et al.,* 2001) but exceptions occur, with MCH-R1 being the predominant receptor in the pituitary and certain brain areas and MCH-R2 predominating in some peripheral sites.

Although much remains to be discovered about MCH-R2, its identification explains some previously puzzling observations, such as the ability of MCH to antagonize cAMP-induced effects in some cells or to potentiate these effects in others. Thus, in melanocytes, α-MSH induces an increase in cAMP, thus stimulating melanogenesis which is antagonized by MCH, with MCH-R1 in this case presumably coupling to G_i/G_o proteins (Hoogduijn *et al.,* 2001). In pancreatic β cells, on the other hand, MCH enhances the insulinotropic effect of forskolin-stimulated cAMP (Tadayyon *et al.,* 2000) suggesting G_q coupling (possibly MCH-R2) in this particular cell. Either variable G protein coupling by MCH-R1 or activation of different MCH receptors might also explain the finding that MCH opposes α-MCH in some tests but mimics it in others. Thus, although α-MSH and MCH exert opposite effects on melanogenesis, they both induce nitric oxide production in melanocytes (A. J. Thody, personal communication), the two responses presumably involving different signaling pathways.

There is preliminary evidence that MCH might achieve some of its biological effects by interacting with one of the melanocortin (MC) receptors for which the MSHs or ACTH are the normal ligands. As indicated previously, MCH can antagonize the effect of α-MSH when the two peptides are administered together. Examples of this, apart from the early demonstration of antagonistic effects on melanophores (Baker, 1988), include opposite effects on the passive avoidance response (McBride *et al.,* 1994), grooming and locomotor behavior (Sanchez *et al.,* 1997, 1999), aggression and anxiety (Gonzalez *et al.,* 1996), the response to auditory stimuli (Miller *et al.,* 1993), and effects on appetite and on ACTH release (Ludwig *et al.,* 1998). The obvious way in which such antagonism could occur is by MCH and α-MSH binding to their specific receptors on the same cell and activating opposing intracellular pathways (e.g., by coupling to G_s and G_i proteins). This seems to be the case in melanocytes (Hoogduijn *et al.,* 2001). On the other hand, some effects of MCH (e.g., its ability to stimulate GnRH release in suitably primed rats) can be prevented by MC receptor antagonists that bind to the MC5 type of receptor (Murray *et al.,* 2000a). The effect of the drug seems difficult to explain in terms of MSH blockade and suggests either that the antagonist can bind to MCH-R as well as to MC5 receptors, which remains to be proven, or that MCH shows some affinity for MC5. Although MCH shows no competitive inhibition of α-MSH binding to cells transfected with MC3–MC5 (Ludwig *et al.,* 1998; Murray *et al.,* 2000a), high concentrations of MCH (1–10 μM) will nevertheless stimulate IP3 production in HEK293 cells expressing MC5 (Murray *et al.,* 2000a). Murray *et al.* also showed that MCH will mimic α-MSH in stimulating growth of the rat preputial gland, a tissue expressing the MC5 form of the receptor (Chen *et al.,*

1997). Clearly, more studies are required to confirm the postulated action of MCH on MC5 and other MC receptors, but the findings are suggestive. MC5 receptors are widely distributed outside the brain (Chhajlani, 1996) and are weakly expressed in the central nervous system.

Many questions regarding the MCH receptors arise. The following are two of the most immediate: Where are they expressed and what regulates their expression?

B. Localization of MCH Receptors

In situ hybridization, immunohistochemical studies, and Northern blots for SLC-1 all show that MCH-R1 is highly expressed in many regions of the rat brain and spinal cord (Chambers *et al.,* 1999; Saito *et al.,* 1999; Hervieu *et al.,* 2000; Sone *et al.,* 2000) and that there is good general agreement between the distribution of receptors and irMCH fibers (Bittencourt *et al.,* 1992). The abundance of receptors in the olfactory telencephalon, hippocampus, and those hypothalamic areas associated with feeding (the lateral hypothalamus, arcuate nucleus, and dorsomedial and ventromedial hypothalamic nuclei) supports the postulated involvement of MCH in taste and olfactory learning and in appetite control. MCH-R1 distribution in the mouse brain has similarly led Kokkotou and coworkers (2001) to suggest that MCH is implicated in the regulation of feeding, body adiposity, and the integration of sensory inputs for smell and taste (see Section V.B). The involvement of MCH in feeding is further emphasized by the finding that MCH-R1 mRNA, like ppMCH mRNA, is much increased by leptin deficiency in genetic *ob/ob* mice or in 48-h fasted normal mice, and that these increased values are restored to control levels by leptin administration (Kokkotou *et al.,* 2001). These authors also showed that MCH-R1 mRNA values in MCH knockout mice are similar to those of control littermates, indicating that receptor expression is not primarily regulated by MCH levels.

MCH has functions in the brain other than appetite control, however. The expression of receptors in the substantia nigra, ventral tegmental area, amygdala, and locus coeruleus suggests a possible involvement in modulating the dopaminergic and adrenergic systems. Other localizations of the receptor associate MCH with a range of motor, sensory, and reward systems, as previously suggested by the distribution of irMCH and by experimental studies. Readers are directed to Hervieu *et al.* (2000) for a detailed semiquantitative evaluation of MCH-R1 abundance throughout the brain.

Not surprisingly, in view of what is known of other neuropeptides, the MCH peptide and MCH receptors are also expressed outside the central nervous system by cells in the pituitary, the eye, various muscles, and adipocytes, pancreatic cells, and skin cells. These are discussed in Section V.C.

The localization of MCH-R2 receptors closely resembles that of MCH-R1, with a few subtle differences: MCH-R1 predominates in the pituitary and certain brain sites, such as the hypothalamus, locus coeruleus, and hindbrain, whereas MCH-R2 appeared more abundant in certain peripheral sites (lymphocytes and adipocytes; Hill *et al.,* 2001). Currently, no specific effect can yet be restricted to only one receptor.

IV. MCH in Nonmammalian Vertebrates

The distribution of immunoreactive MCH perikarya and their major fiber tracts has been mapped in the brain of many lower vertebrates that are placed on the line of evolution leading to teleost fish (Fig. 4). Comparing the distribution of MCH perikarya and fibers between primitive fish helps clarify the stages at which MCH may have acquired its function as a hypophysiotrophic peptide or a neurohypophysial hormone concerned with adaptive color change.

In lampreys, representative of the earliest vertebrates, a few MCH perikarya have been detected in the telencephalon of sexually mature specimens of *Lampetra fluviatilis,* but the vast majority of MCH perikarya are grouped in the dorsal

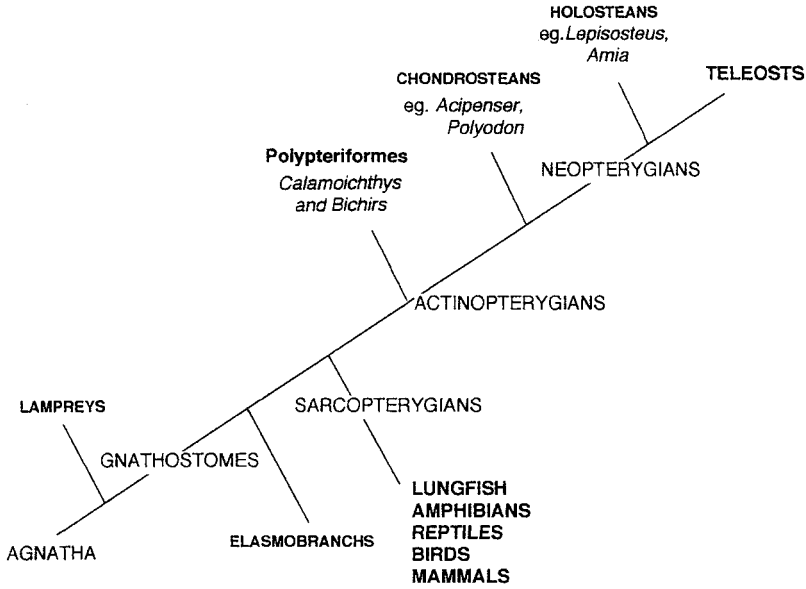

FIG. 4 Phylogenetic relationship between living fish groups.

hypothalamic nucleus in a region containing the periventricular organ, a site rich in monoaminergic and peptidergic neurons that contact the cerebrospinal fluid (Bird *et al.,* 2001). MCH fibers from this region project widely throughout the brain—into the olfactory lobes, the preoptic region, the lateral and medial hypothalamus, and thalamic areas. No irMCH fibers are discernible in the posterior pituitary, but such fibers are found in the neural tissue overlying the pituitary pars distalis, where other neurohypophysiotrophic peptides such as GnRH also accumulate prior to diffusing toward the adenohypophysis. This places MCH in an appropriate location to serve as a hypophysial regulatory peptide.

The association of irMCH with the adenohypophysis is more apparent in the Actinopterygians, in all of which (polypteriformes, chondrosteans, and holosteans) irMCH fibers are abundant either in the median eminence (Fig. 5a) or, in the case of teleosts in which a median eminence is lacking, in the pituitary (Batten *et al.,* 1987, 1999; Powell and Baker, 1987; Baker and Bird, 2001). A hypophysiotrophic role is thus implied, although it has been investigated and demonstrated only in euteleosts, in which it modulates the release of ACTH, MSH, and somatolactin (Baker *et al.,* 1985a; Barber *et al.,* 1987; Groneveld *et al.,* 1995a; Balm and Groneveld, 1998).

The role of MCH as a neurohypophysial hormone developed later in evolution and its involvement in color change occurred later still. In the polypteriform fish *Calamoichthys calabaricus,* neurohypophysial irMCH fibers are not apparent in the pituitary neural lobe and are first detectable in the Chondrostean, *Acipensor stellatus,* in which they encircle the capillary sinuses in the posterior pituitary, suggesting MCH release into the circulation. Neurohypophysial MCH is even more abundant in the holostean fishes *Lepisosteus osseus* (Fig. 5b) and *Amia calva* (Batten *et al.,* 1987; Baker and Bird, 2001), and it is in this latter group that the peptide can first be shown to cause melanin concentration when added to isolated skin fragments (Sherbrooke and Hadley, 1988). Thus, although the ability of the MCH neurons to respond to changes in environmental color has not been investigated in holosteans, the data suggest that MCH might have acquired a role in adaptive color change in a group ancestral to both holosteans and teleosts. Evidence for the pigmentary role of MCH has been validated in euteleosts and interested readers are directed to previous reviews (Baker, 1991, 1993) as well as to recent studies which have monitored MCH gene expression during color adaptation (Groneveld *et al.,* 1995b; Suzuki *et al.,* 1996, 1997).

Along the sarcopterygian line of evolution, MCH distribution has been examined in the brains of the lungfish *Protopterus annectens* (Vallarino *et al.,* 1998); several amphibian anuran species—*Rana ridibunda* (Andersen *et al.,* 1992), *Rana temporaria,* and *Xenopus laevis* (Francis and Baker, 1995; Francis, 1996); and reptiles and birds (Cardot *et al.,* 1994, 1999). In all species, MCH is expressed in several locations in the brain, some of which (e.g., those in the telencephalon of lampreys and amphibians) are detectable only in reproductively mature animals (Bird *et al.,* 2001; Francis, 1996). Little is known about MCH physiology

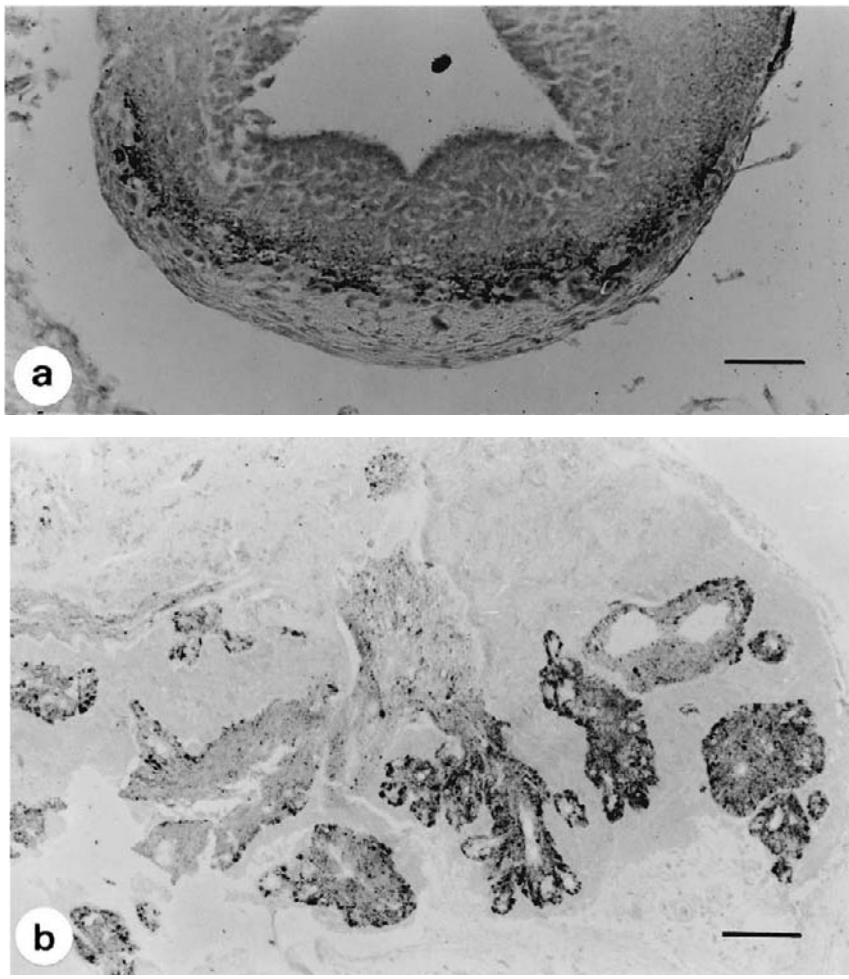

FIG. 5 MCH fibers in median eminence and neurointermediate lobe of primitive fish. (a) Basal hypothalamus of *Calamoichthys* (Polypteriformes) showing darkly immunostained MCH fibers associated with blood capillaries in the median eminence, located between the brain and surrounding connective tissue. Counterstained with hematoxylin. Scale bar = 50 μm. (b) Neurointermediate lobe of *Lepisosteus* showing darkly immunostained MCH fibers in the neurohypophysis, interdigitating between intermediate lobe cells. Scale bar = 100 μm.

in these tetrapods, however. Only amphibians have been studied, and in these the hypothalamic MCH neurons show increased secretory activity during the postmetamorphic exodus from water (Francis and Baker, 1995) and in response to osmotic challenge (Francis, 1996). A role in either osmoregulation or stress is implied.

V. MCH in Mammals

A. Evidence for Subpopulations of MCH Neurons

Although most MCH neurons are concentrated in the hypothalamus, their anatomical distribution and neuronal connections, their different phenotypes in terms of receptors and colocalized molecules, and also variations in their physiological responses all suggest they do not form a homogeneous population.

1. Anatomical Distribution

Anatomical investigations of MCH neurons have been performed on many species, including human (Pelletier *et al.,* 1987; Bresson *et al.,* 1989; Mouri *et al.,* 1993; Knigge *et al.,* 1996; Takahashi *et al.,* 1997), monkeys (Bittencourt *et al.,* 1998), cats (Knigge and Wagner, 1997b), and sheep (Tillet *et al.,* 1996), but most studies have concentrated on their distribution in rodents (Skofitsch *et al.,* 1985; Naito *et al.,* 1986; Zamir *et al.,* 1986; Fellmann *et al.,* 1987; Bittencourt *et al.,* 1992; Presse *et al.,* 1992; Knigge *et al.,* 1996). The following discussion therefore focuses on the rat and mouse, although the topography of the MCH system appears similar in all species.

MCH perikarya are almost exclusively located in the tuberal and posterior diencephalic areas. In a rostrocaudal direction, they extend from the posterior level of the hypothalamic paraventricular nucleus (PVN) to the anterior level of the mammillary region. Their distribution does not respect cytoarchitectonic boundaries, however. In rats that have not been pretreated with colchicine, at least three topographical groups can be recognized after immunocytochemical staining, although there are no clear limits between them. The first group consists of neurons located in the zona incerta (ZI) (i.e., in the ventral part of the thalamus). In the rostral ZI, they occupy a medial position, whereas more caudally they progressively acquire a more lateral distribution; they usually display a fusiform shape and are of medium size (about 15×25 μm). In coronal sections, the two other groups begin posterior to the ZI group and their neurons are larger (20–25 μm in diameter) and multipolar. Perikarya of the second group surround the fornix, whereas those of the third, the most prominent and most caudal group, are scattered throughout the lateral hypothalamic area (LHA) within the fibers of the medial forebrain bundle (MFB) and bounded laterally by the cerebral peduncle. In addition to these relatively organized clusters, some neurons are dispersed, for example, in the anterior, dorsomedial, and posterior nuclei and, very caudally, in the periventricular area. Colchicine treatment reveals two additional small and extradiencephalic populations, one centered in the olfactory tubercle and the other in the pontine tegmentum (Bittencourt *et al.,* 1992). Moreover, in lactating female rats (between 8 and 21 days of lactation), MCH expression has been reported in the medial preoptic area and rostral aspects of the PVN (Knollema *et al.,* 1992).

These separate localizations suggest the existence of subsets of MCH neurons projecting to different areas, receiving afferences from different origins, and thus involved in different circuits.

2. Neuronal Connections

a. Efferent Projections MCH fibers are distributed throughout the brain, from the olfactory bulb to the spinal cord, and are particularly abundant in some territories such as the LHA or the medial septum/diagonal band complex (MS/DB) (Skofitsch *et al.,* 1985; Bittencourt *et al.,* 1992). MCH neurons also project to the median eminence and the pituitary. In the median eminence, the density of fibers is moderate in the internal zone and sparse in the external one, although it is reported that there may be a rapid and remarkable accumulation of MCH in fibers and terminals of both the internal and external median eminence under a variety of stressful conditions (Knigge *et al.,* 1996). The use of anterograde and retrograde tracers reveals that not only do groups of MCH neurons project to more than one specific site but also recipient territories may be targeted by MCH fibers from different sources. Thus, the anterior levels of the dorsal periaqueductal gray (PAG) are innervated by MCH neurons located in the rostromedial ZI, whereas more caudal and ventral levels of the PAG are innervated by neurons from the tuberal LHA (Elias and Bittencourt, 1997). MCH neurons from the rostromedial ZI also project to the MS/DB and the cortical mantle, as do MCH neurons from the tuberal, posterior, and perifornical hypothalamic regions, whereas the spinal cord receives fibers originating from the LHA groups but not from the rostromedial ZI one (Bittencourt and Elias, 1998). A small number of neurons from the LHA contribute both ascending and descending projections.

b. Afferent Projections Little is known about the origin and chemical nature of the afferents impinging specifically on the MCH neurons. Some data suggest a differential innervation of subsets of MCH neurons. For instance, inputs to MCH neurons originating in the arcuate nucleus have been reported in rats in which a close relationship occurs between MCH perikarya and their processes, on the one hand, and neuropeptide Y (NPY), agouti-related peptides (AGRPs), and α-MSH immunoreactive fibers, on the other hand (Broberger *et al.,* 1998; Elias *et al.,* 1998). This innervation by fibers from the arcuate nucleus is particularly dense in the perifornical region and less intense in more lateral regions (Elias *et al.,* 1998). Fibers containing neurokinin B (NKB), the major ligand of the NK3 receptor carried by a number of MCH neurons, exhibit a diffuse pattern in the MCH area, being denser in the LHA, especially near the cerebral peduncle, and sparser in the ZI. Although not conspicuous, they are often very close to MCH neurons (B. Griffond, unpublished observations), but ultrastructural studies are needed to confirm possible contacts. Their origin has not been elucidated since NKB-positive cells are found in many brain structures (Lucas *et al.,* 1992; Marksteiner *et al.,*

1992) including the hypothalamus, in which they are well represented in the arcuate nucleus, and they can also be found in the dorsomedial region of the tuberal LHA. MCH neurons, particularly those of the rostromedial ZI and the perifornical area, are also the targets of extrahypothalamic cholinergic afferents. The main sources of these projections are two pontomesencephalic nuclei, the pedunculopontine and the laterodorsal tegmental nuclei (Bayer *et al.,* 1999a). The MFB, which passes through the lateral hypothalamic distribution of the MCH population, contains descending and ascending fibers, including those of the dopamine meso- and nigrostriatal tracts. Immunocytochemical and retrograde tracing studies suggest that MCH neurons in the LHA receive dopaminergic afferents which originate in the ventral tegmental area (A10 nucleus), whereas the more medial MCH neurons do not (Bayer *et al.,* manuscript in preparation).

In the guinea pig, Knigge *et al.* (1996) observed a tendency for the different groups of MCH neurons to be surrounded by different neuropeptidergic systems, with the rostral dorsomedial MCH neurons embedded in a dense network of enkephalinergic fibers and terminals and the perifornical area enmeshed in POMC fibers from the arcuate nucleus, whereas NPY fibers are particularly dense in the MFB, near which MCH cells are found.

3. Chemical Phenotypes

a. Colocalization of MCH with Other Peptides In contrast to peptides derived from the MCH gene (MCH, NEI, and MGOP) which are expressed by the whole MCH population, others have been identified in rats whose expression is restricted to subsets of MCH neurons. This is the case for the cocaine–amphetamine-regulated transcript (CART) which is coexpressed in almost all MCH neurons of the rostromedial ZI, whereas only about half of the more lateral and caudal ones display CART mRNA (Broberger, 1999, Vrang *et al.,* 1999). Substance P is also colocalized in a small subset of MCH neurons preferentially distributed in the LHA, in proximity to the cerebral peduncle; this subpopulation is not clearly delineated but is scattered between other MCH neurons (V. Cvetkovic, personal communication).

Further studies may show that peptide colocalizations in the MCH neurons differ in other species. In the cat, for instance, MCH appears to be expressed in many of the oxytocinergic neurons of the paraventricular and supraoptic nuclei (Knigge and Wagner, 1997b), although this is not seen in the rat (Knollema *et al.,* 1992) or the human (Takahashi *et al.,* 1997).

b. Receptors on MCH Neurons The first receptor demonstrated on MCH neurons was NK3, the preferential receptor for NKB (Griffond *et al.,* 1997), which is expressed on both MCH cell bodies and their processes. About 60% of all MCH neurons exhibit this receptor. Rostrally, all neurons occupying a medial position are NK3 immunoreactive; in the perifornical group, only a few are not, whereas

in the LHA, NK3-positive and -negative MCH neurons are strongly intermingled. More caudally, MCH neurons lacking NK3 can be found in all localizations including the medial one. This topography of MCH/NK3 neurons is clearly reminiscent of that of MCH/CART neurons. Recent unpublished observations by our group confirm that most of the MCH neurons possessing NK3 also express CART, which helps to define one of the MCH subpopulations.

MCH neurons also carry a splice variant of the Ob receptor for leptin (Ob-R) (Hakansson *et al.,* 1998). Despite the detection of the functional Ob-Rb form in the LHA (Mercer *et al.,* 1996; Fei *et al.,* 1997; Elmquist *et al.,* 1998), additional observations are needed to determine if MCH neurons express this form and could be directly targeted by leptin. It would be useful, for example, to investigate their immunoreactivity for SOCS-3, an intracellular sensitive marker for direct leptin action, or for STAT3, a transcription factor activated by leptin and present only in Ob-Rb-containing neurons (Hakansson and Meister, 1998).

4. Physiological Responses

A final basis for differentiating subpopulations of MCH neurons depends on their response to experimental paradigms. Thus, in salt-loaded rats, MCH mRNA abundance is decreased in neurons in the anterior LHA, increased in clusters of cells located in the ZI, around the fornix, and near the internal capsule, and only slightly affected in neurons in other regions of the posterior LHA (Presse and Nahon, 1993). In ovariectomized rats, estrogen reduces the intensity of the MCH hybridization signal in cells of the medial ZI but not in those of the lateral ZI or the LHA (Murray *et al.,* 2000b) (see Section V.B). Finally, a group of MCH neurons that are normally undetectable by either immunohistochemistry or *in situ* hybridization become detectable in the anterior periventricular region of postpartum mothers during the second week of lactation, abruptly disappearing again upon weaning (Knollema *et al.,* 1992).

All these data support the concept of several subpopulations of MCH neurons distinguished by their topography, by the copeptides they express, their physiological responses, or their connectivity. However, these subpopulations are usually strongly overlapping and intermingled and it is not possible to draw clear boundaries between them. Identifying the functional circuitry in which they are involved will no doubt shed further light on their organization.

B. Central Functions and Regulation of MCH

Before discussing its functions, it is helpful to recall that in mammals, as in fish, MCH follows a diurnal pattern of gene expression and probably peptide release (Lyon and Baker, 1993; Presse and Nahon, 1993; Nahon, 1994; Suzuki *et al.,* 1996). MCH neurons are possibly controlled by direct inputs from the suprachiasmatic

circadian pacemaker (Abrahamson *et al.,* 2001). In rats, MCH mRNA levels reach a maximum at approximately 22 h and basal levels between 10 and 15 h. These variations are significant (up to fourfold) and closely follow those of plasma corticoids (Presse *et al.,* 1992). They should be taken into consideration when interpreting experimental results. In addition, one should note that most of the data on changes in gene expression or peptide content relate to global MCH responses, without discriminating between neuronal subpopulations, which may explain the frequent contradictions between the results.

1. MCH and Neuroendocrine Activity

a. The Hypothalamo–Pituitary–Adrenal Axis Based on the inhibitory activity of MCH on the hypothalamo–pituitary–adrenal (HPA) axis in teleosts (Baker, 1991), the possibility that MCH plays a similar role in mammals has been investigated by several laboratories. The studies have yielded conflicting results, with MCH either having no effect *in vitro* (Navarra *et al.,* 1990) or stimulating ACTH release when injected *in vivo* (Jezova *et al.,* 1992). The study by Bluet-Pajot *et al.* (1995) shed new light on these paradoxical observations. These authors examined the effects of MCH when given icv to adult rats under resting conditions or after a mild ether stress, in the light period as well as at night. They showed that MCH acts centrally to regulate ACTH secretion but that its effects vary depending on the time of injection. Thus, administration of MCH at the end of the light period (i.e., at the peak of ACTH circadian secretion) decreased ACTH release but it had no effect during the dark period when ACTH levels were already low. Following a moderate ether-induced stress, ACTH release was stimulated by ether stress only during the dark phase and this was prevented by MCH. MCH's copeptide, NEI, had no effect under any conditions when given alone, but when coadministered with MCH it negated MCH's effect. Therefore, as in teleosts, MCH may depress ACTH by a central action, perhaps contributing to its circadian rhythmicity, and it can be functionally antagonized by its copeptide, NEI. This ability of MCH to inhibit ACTH secretion following mild stress in the dark period has been confirmed by Ludwig *et al.* (1998).

The pathways controlling MCH secretion and the physiological conditions under which the peptide comes into play to regulate ACTH release are still not well established. To summarize the situation in fish (the trout), which have been more fully studied, cortisol and ACTH secretion after stress are restrained when endogenous MCH secretion is high (Baker and Rance, 1981; Baker *et al.,* 1985a), although MCH seems to restrain the response to only modest, not intense, stress (Gilchriest *et al.,* 2001). Reciprocally, a mild stress enhances MCH release and usually enhances MCH synthesis, as judged by MCH mRNA values or MCH translational activity (Green and Baker, 1991; Baker and Bird, 1992; Suzuki *et al.,* 1996, 1997). Repeated or more severe stress, however, will suppress MCH synthesis (Baker and Bird, 1992; Gilchriest *et al.,* 1999). To what extent MCH is regulated

by glucocorticoid feedback is not entirely clear. The MCH neurons are believed to possess cortisol receptors (O. Kah, unpublished observations) and the increase in stress-induced MCH release is rapidly depressed by glucocorticoids (Green and Baker, 1991). The effect of glucocorticoids on MCH synthesis in fish has not been determined, however. When plasma cortisol concentration was increased in trout subjected to 80% seawater stress, MCH mRNA values were significantly reduced but gradually returned to control levels despite persistently high cortisol levels. The overall picture for fish suggests that the MCH system is deployed in moderating HPA activity during modest stresses but is restrained during intense stresses.

The findings in rats are similar to the results in fish. Nothing is known about the effect of mild, acute stress on the MCH system in mammals, but chronic foot shock stress more than halves the overall abundance of MCH mRNA in the hypothalamus (Presse *et al.,* 1992), whereas a variety of stresses, such as osmotic or immobilization stress or food deprivation, increase MCH immunoreactivity in the hypothalamus (Zamir *et al.,* 1986; Fellmann *et al.,* 1993) and in the external and internal median eminence (Knigge *et al.,* 1996). These changes could be interpreted as inhibition of MCH release. In contrast to fish, in rats MCH gene expression seems to be positively controlled by glucocorticoids: Adrenalectomy reduces the MCH mRNA content to 40% of controls, and the effect is reversed by dexamethasone administration (Presse *et al.,* 1992). This could be a direct stimulatory action of glucocorticoids on the MCH neurons since, when tested on dispersed hypothalamic cells *in vitro,* dexamethasone increased MCH cell content and release (Parkes and Vale, 1992). Additionally, changes in CRH release might also modulate MCH secretion: CRH is reported to suppress MCH release *in vitro* (Parkes and Vale, 1992) and its release is increased during stress but suppressed by glucocorticoids. From these data, it is still not clear how and when the MCH system adopts a role in restraining CRH/ACTH release. It is possible that, as in fish, it is involved only during modest stress, a situation not yet examined in mammals.

b. MCH and Reproduction MCH influences the release of luteinizing hormone (LH) by a central effect on the gonadotrophin-releasing hormone (GnRH), but results have been contradictory. In ovariectomized rats given 5 μg replacement estradiol, LH release is low but can be triggered within 15–60 min after 100 ng MCH is injected directly into the medial preoptic area (MPOA) (Gonzalez *et al.,* 1997a; Murray *et al.,* 2000b). The time of day when the peptide is administered was immaterial. Progesterone injections given ip also trigger LH release in such estrogen-primed rats and this effect is prevented by MCH antiserum, again administered into the MPOA. The antiserum similarly antagonized the increase in LH induced by an injection of leptin into the medial ZI (Murray *et al.,* 2000c). Thus, an unidentified subpopulation of MCH neurons that projects to the MPOA seems to be involved with GnRH release. In contrast to these results, however, 1 μg MCH inhibited pulses of LH secretion when injected directly into the ventricle

(icv) of ovariectomized, estrogen-replaced rats, although a 10-μg dose was less potent (Tsukamura *et al.,* 2000). These results might be attributable to the different sites of injection (icv or MPOA), to different regimes of estrogen replacement, or to the fact that the rats used by Gonzalez and by Murray and coworkers were anesthetized, whereas those used by Tsukamura were not.

MCH also stimulates sexual receptivity and the lordosis response when injected into either the MPOA or the ventromedial nucleus (VMN) of ovariectomized, estrogen-primed rats (Gonzalez *et al.,* 1996). NEI exerts the same effect when injected into the MPOA after; it had no effect alone but opposed the effect of MCH in the VMN (Gonzalez *et al.,* 1998). These actions might be mediated by monoaminergic systems, possibly by 5-HT for MCH (Gonzalez *et al.,* 1997b) and dopamine for NEI (Gonzalez *et al.,* 1998).

The response of at least some MCH neurons is estrogen sensitive, although the effect could be an indirect one since the neurons have not been demonstrated to possess estrogen receptors. In ovariectomized monkeys injected with estrogen, a transient increase in MCH peptide abundance was observed, synchronous with the induced LH surge (Viale *et al.,* 1999a). Whether this reflects increased synthesis or decreased release of the peptide is uncertain. In rats, in which estrogen exerts the opposite effect, inhibiting LH release, the steroid depressed MCH mRNA levels in a subset of MCH neurons located in the medial ZI (Murray *et al.,* 2000a), with the effect becoming apparent 24–52 h after estrogen injection. No significant variation in MCH mRNA was observed in these neurons during the estrous cycle, however (Knollema *et al.,* 1992; Murray *et al.,* 2000b), and it should be emphasized that it has not been confirmed that the MCH neurons projecting to the MPOA are those in the ZI. Another report also suggests that additional MCH neurons are affected by estrogens: Thus, the increased MCH mRNA, normally expected in response to restricted food intake and weight loss, is selectively and completely suppressed in two models of anorexia characterized by supraphysiological estrogen levels in male rats (Mystkowski *et al.,* 2000).

The induction of ppMCH expression in novel, preoptic sites during lactation (Knollema *et al.,* 1992) and the stimulatory effect of MCH and NEI on oxytocin secretion from isolated neurointermediate lobes *in vitro* (Parkes and Vale, 1993) indicate that both peptides may be involved in some aspects of lactation, including the release of oxytocin. Since the MCH fibers projecting to the neurointermediate lobe are believed to arise from the posterior hypothalamus and not the preoptic area (Knollema *et al.,* 1992), several sites of MCH action during lactation may be envisaged.

In summary, the data suggest that MCH plays a role in the regulation of multiple aspects of female reproduction—LH release, sexual behavior, and lactation, acting at different central sites and also in the pituitary neural lobe. Several subpopulations of MCH neurons appear to be involved in these activities and probably more than one of them are estrogen sensitive. Furthermore, the experimental results suggest that not only MCH but also its copeptide NEI could be involved in these

activities. This is not to suggest, however, that the MCH system is crucial for reproduction to occur adequately. MCH knockout mice are fertile (Shimada *et al.,* 1998), suggesting that the role of MCH in reproduction is only neuromodulatory.

c. MCH Influence on Other Pituitary Hormones In experiments investigating the role of icv MCH on ACTH release, Bluet-Pajot *et al.* (1995) concomitantly measured changes in plasma prolactin and growth hormone (GH) concentrations. They found that neither MCH nor NEI modified plasma levels of these two hormones when measured up to 40 min after peptide administration. Earlier experiments by Kawauchi *et al.* (1986), however, found that ip injections of very high doses of MCH (400 μg/kg body weight) into 10-week-old Wistar rats stimulated the release of GH. The response began only 1 h after the injection and lasted for several hours. It is likely that the effect is not a direct one on the pituitary but that, at these doses, MCH may penetrate the blood–brain barrier and act centrally. The time difference perhaps explains the discrepancy between the two studies.

2. Influence on Appetite

Among the putative roles of MCH neurons, its involvement in feeding behavior, energy homeostasis, and body weight control are by far the best documented. MCH perikarya are most abundant in the LHA where lesions result in aphagia and weight loss, indicating it is a site of orexigenic neurons. The ability of MCH to trigger food intake was initially questioned, however, because early studies on rats gave divergent results: icv injections of MCH were reported to have no effect (10 ng to 1 μg/Long–Evans rats; Max *et al.,* 1993), to decrease food intake within 2 h of injection (1–100 ng/Wistar rats; Presse *et al.,* 1996), or to stimulate a rapid increase in consumption which persisted over a 6-h period (5 and 30 μg/Long–Evans rats; Qu *et al.,* 1996). The reason for these divergent findings is still not clear. In reexamining the influence of MCH in Wistar rats, Rossi *et al.* (1997) tested a wide range of doses and found that at no dose did MCH inhibit feeding. A stimulatory effect was observed in response to 1.5–5 μg, given icv in both light and dark phases; it was most significant during the 2-h period after injection and was most readily demonstrated at the beginning of the light phase, when both feeding activity and MCH synthesis are normally low. Its sister peptide, NEI, had no effect on food intake and did not antagonize MCH-induced feeding. When investigating the time course and site of MCH action, the same authors observed that MCH initiated feeding very rapidly (mean onset 13 min) after direct injection into the PVN and had a short duration, ending within 30 min (Rossi *et al.,* 1999). When MCH was administered chronically (5 μg icv twice daily over an 8-day period), food intake was doubled during the first 2 h after injection, and this response persisted up to Day 5, although food intake over a 24-h period was not altered and body weight did not increase (Rossi *et al.,* 1997). The stimulatory effect of MCH disappeared after 5 or 6 days of chronic treatment, indicating tolerance to a

chronic challenge. These results show that MCH will stimulate short-term feeding but may not be so important for long-term maintenance of body weight. The dose-dependent, short-term increase in appetite induced by MCH has been confirmed by other studies (Ludwig *et al.,* 1998; Sahu, 1998b), but its effect is less potent than that of NPY (Edwards *et al.,* 1999).

Despite the failure of chronic MCH administration to maintain high levels of food consumption over the long term, additional observations support a role for MCH in the circuitry that integrates various aspects of feeding behavior. First, transgenic mice overexpressing MCH tend to overeat and are obese (Ludwig *et al.,* 2001). Second, in contrast, mice carrying a deletion of the entire coding region of the ppMCH gene are hypophagic, small, and lean, with a reduced triglyceride content (Shimada *et al.,* 1998). Third, ppMCH gene expression in mice more than doubles after a 1- or 2-day fast (Qu *et al.,* 1996). This also occurs in rats and is normalized by refeeding (Presse *et al.,* 1996; Hervé and Fellmann, 1997). Expression of the MCH receptor is stimulated to an even greater extent during starvation, increasing sevenfold after a 2-day fast (Kokkotou *et al.,* 2001). Finally, MCH gene expression is also increased in several models of genetic obesity in which the animals exhibit unappeasable eating: It is overexpressed in the leptin-deficient *ob/ob* mouse (Qu *et al.,* 1996), in the leptin receptor-deficient *fa/fa* rat (Rossi *et al.,* 1999), and in the yellow agouti mice, in which the anorexic effects of α-MSH are suppressed by the oversecretion of AGRP, a melanocortin receptor blocker (Hanada *et al.,* 2000).

In determining which factors could be responsible for regulating MCH secretion, researchers have examined some of the metabolites and hormones commonly associated with changes in feeding—glucose and fatty acid levels, insulin and leptin, as well as other central neuropeptides.

Glucose has long been regarded as a rapid, short-term regulator of appetite (Mayer, 1955). Its role in MCH control has been examined by inducing hypo- or hyperglycemia or by administering glucose antimetabolites. Addition of glucose to primary cultures of fetal neurons caused a dose-dependent release of MCH (Fellmann *et al.,* 1993), but since saccharose and mannitol had similar effects, this response was probably related to increased osmolarity rather than to glucose sensitivity. The effects of experimental glucopenia induced by insulin injections or by gold thioglucose (GTG), a toxic form of glucose, or by 2-deoxyglucose (2DG), a nonmetabolizable glucose analog, are summarized in Table III. The results suggest that glucose deficiency may increase MCH mRNA expression, but the results are transient and inconsistent. When administered *in vivo,* GTG causes major damage in the arcuate nucleus, the site of other appetite-regulating neuropeptides which project to the lateral hypothalamus. Thus, any effect of glucose on the MCH neurons is most likely to be indirect, for example, via the arcuate nucleus. Other studies examining the role of fatty acids show that a single dose of 2-mercaptoacetate, an antimetabolite which blocks fatty acid oxidation, also increases MCH gene transcripts (Sergeyev and Akmaev, 2000; Sergeyev *et al.,* 2000).

TABLE III
Effects of Changes in Glucose and Fatty Acid Availability on MCH mRNA and MCH Peptide[a]

		Response		
Rat strain	Treatment	Gene expression	MCH peptide abundance	Reference
SD[a]	Insulin, ip 20 IU/kg, AM	Increase	Increase	Bahjaoui-Bouhaddi *et al.* (1994)
Wistar	Insulin, ip 30 IU/kg	Increase	—	Presse *et al.* (1996)
SD	GTG, ip 100 mg/kg, AM	No effect	No effect	Grillon *et al.* (1997)
Wistar	2DG, ip 250 mg/kg	Transient increase at 1 h	—	Presse *et al.* (1996)
SD	2DG, *in vitro*	No effect at 2, 5, or 10 h	—	Bayer *et al.* (1999b)
SD	2DG, ip 400 mg/kg, AM	No effect at 1, 3, or 5 h	—	Bayer *et al.* (2000)
SD	2DG, ip 600 mg/kg, noon	Increase at 2 h	—	Sergeyev *et al.* (2000)
SD	MA, ip 600 μmol/kg, AM	Increase at 3 h	—	Sergeyev and Akmaev (2000)
SD	MA, ip 600 μmol/kg, noon	Increase at 2 h	—	Sergeyev *et al.* (2000)

[a]Abbreviations used: SD, Sprague–Dawley strain; 2DG, 2-deoxyglucose; GTG, gold thioglucose; MA, mercaptoacetate. Where available, the time of treatment is indicated (AM indicates the treatment was given during the light phase).

An inverse correlation is often apparent between MCH mRNA levels and leptin, the adipocyte hormone that is actively secreted in well-nourished and recently fed animals but secreted less during starvation or during drug-induced anorexia (Mantzores *et al.,* 1996). As shown in Table IV, leptin administration has been reported to depress MCH gene expression in several studies. Additional studies show the same inverse correlation even when appetite does not respond as anticipated. For instance, mice treated with a toxin to cause brown adipose tissue damage exhibit low MCH mRNA and high circulating leptin but their appetite is abnormally high (Tritos *et al.,* 1998a). Inversely, mice treated with a β_3-adrenergic agonist have high MCH mRNA expression and depressed circulating leptin but low appetite (Mantzoros *et al.,* 1996); both cases indicate a dissociation between leptin levels and appetite. Only one example in which MCH gene expression fails to respond to leptin concentrations has been reported: Huang *et al.* (1999) found that longer term (7 days) administration of small doses of leptin to mice

TABLE IV

Influence of Leptin, Neuropeptides, and MC3/MC4 Receptor Antagonists on ppMCH mRNA or MCH-Induced Appetite[a]

		Response		
Animals	Treatment	ppMCH mRNA	MCH-induced appetite	Reference
SD rat	Leptin, *in vitro*	Decreased	—	Bayer *et al.* (1999c)
SD rat Fed	Leptin, icv 5 μg for 3 days, PM	Decreased	—	Sahu (1998a)
SD rat Fed	Leptin, icv 4 μg, AM	—	Depressed	Sahu (1998b)
Mice 60-h fast	Leptin, ip 2 × 1 μg/day	Decreased	—	Tritos *et al.* (2001)
ob/*ob* fed	Leptin, ip 2 × 1 μg/day	Decreased	—	Tritos *et al.* (2001)
Mice, fed Lean	Leptin, sc minip 100 μg/kg for 7 days	Increased	—	Huang *et al.* (1999)
ob/*ob*	Leptin, sc minip 100 μg/kg for 7 days	Increased	—	Huang *et al.* (1999)
LE rat Fed	α-MSH, icv 10 μg, PM	—	Depressed	Ludwig *et al.* (1998)
SD rat 12-h fast	α-MSH, icv 10 μg, PM	—	Depressed	Tritos *et al.* (1998b)
Wistar rat Fed	α-MSH, icv 1 nmol	No effect	—	Hanada *et al.* (2000)
Wistar rat Fed/12-h fast	AGRP, icv 1 or 3 nmol	Increased	—	Hanada *et al.* (2000)
Wistar rat Fed	SHU 9119, icv 0.5 or 1 nmol	Increased	—	Hanada *et al.* (2000)
SD rat Fed	GLP-1, icv 10 μg, PM	—	Depressed	Tritos *et al.* (1998b)
SD rat 12-h fast	NT, icv 10 μg, PM	—	Depressed	Tritos *et al.* (1998b)

[a]Abbreviations used: SD, Sprague–Dawley strain; LE, Long–Evans strain; AGRP, agouti gene-related peptide; SHU 9119, MC3/MC4 receptor antagonist, GLP-1, gastrin-like peptide; sc minip, subcutaneous minipump.

(2.5–4 μg/mouse/day delivered subcutaneously by minipump) reduced food intake as expected but enhanced MCH mRNA levels, a result seen in both lean and *ob*/*ob* mice. Given the orexigenic properties of MCH, this result is initially difficult to reconcile with the appetite-reducing effect of leptin. It is interpreted by the authors as a mechanism to compensate for the weight-reducing effects of leptin. Their

findings seem to indicate that low food intake or weight loss can increase MCH gene expression through some other agency, despite the presence of increased leptin.

The low expression of the leptin receptor Ob-Rb in the LHA, together with failure to find significant amounts of SOCS-3 in neurons of this area in fed or fasted animals, suggests that any influence of leptin on the MCH neurons is probably indirect (Elias *et al.,* 1999; Baskin *et al.,* 2000). Leptin receptors are abundant in several hypothalamic sites including the arcuate nucleus, a region which contains neurons cosecreting the appetite-suppressing neuropeptides α-MSH (derived from POMC) and CART (Elias *et al.,* 1998; Vrang *et al.,* 1999) and other neurons cosecreting the orexigenic peptides NPY and AGRP, a MC3/4 receptor antagonist (Broberger *et al.,* 1998; Hahn *et al.,* 1998). Axons from these neurons project to the lateral hypothalamus and terminate on or near MCH and ORX perikarya (see Section V.A.2); together with the presence in the LHA of MC4 receptors (Mountjoy *et al.,* 1994) and NPY (Y5) receptors (Gerald *et al.,* 1996), this has encouraged the proposal that MCH neuronal activity may be regulated via the opposing actions of these (and possibly other) anorexic and orexigenic neuropeptides, and that they mediate the effects of leptin on the LHA (Elmquist *et al.,* 1999; Lawrence *et al.,* 1999; Tritos and Maratos-Flier, 1999; Gura, 2000; Halford and Blundell, 2000; Schwartz *et al.,* 2000; Woods and Seeley, 2000). Some relevant experiments testing this proposal are listed in Table IV. Neurones cosecreting α-MSH and CART express leptin receptors (Cheung *et al.,* 1997); they are positively controlled by leptin and may restrain MCH secretion. Although icv α-MSH injection did not depress MCH mRNA levels, administration of MSH receptor antagonists AGRP and SHU 9119 enhanced MCH gene transcripts, suggesting that MSH normally exerts a tonic inhibition (Table IV; Hanada *et al.,* 2000). One should note, however, that since icv-administered α-MSH could oppose the increase in feeding induced by injected MCH, antagonism must also occur at a site distal to MCH release in addition to an effect on the MCH neurons. There are no data showing whether CART can influence MCH neuronal activity.

The neurons cosecreting NPY and AGRP also express leptin receptors (Mercer *et al.,* 1996) and their gene expression is restrained by leptin. Therefore, under low leptin conditions (e.g., starvation), AGRP might promote MCH neuronal activity. Whether NPY influences the MCH neurons has not been investigated but NPY receptors occur in the LHA. Finally, MCH-R1 receptors (SLC-1) occur in the arcuate nucleus (Kokkotou *et al.,* 2001), suggesting the possibility of a feedback loop.

Neuropeptides outside the arcuate nucleus may also influence MCH neuronal activity. For instance, communication between the MCH and ORX neurons is possible. ORX receptors are expressed in the LHA (Trivedi *et al.,* 1998) and the addition of 10^{-6} *M* ORX to hypothalamic slice cultures (Fig. 6, see color insert) increased MCH mRNA values when applied after 24-h incubation; it had the opposite effect when applied to long-term cultures after which time intermediate axonal

pathways could have degenerated (L. Bayer *et al.,* unpublished observations). Two other neuropeptides, glucagon-like peptide-1 and neurotensin, which are known to have appetite-suppressing properties (Hawkins, 1986; Turton *et al.,* 1996), also counteract MCH-induced appetite when injected (Tritos *et al.,* 1998b). This experimentally induced effect must occur downstream of the MCH neurons, but there are no data indicating whether these neuropeptides also influence MCH secretion.

Many neuropeptides and transmitters are known to be involved in feeding activity and their specific functions, and how they all interact, has yet to be clarified. From what is known of the circuitry of the lateral hypothalamus and zona incerta (Bittencourt *et al.,* 1992; Sawchenko, 1998), the location of the MCH neurons places them in a favorable position to receive and integrate incoming sensory, somatic, and visceral information. The distribution of their fibers and receptors is compatible with their involvement in arousal and reward. For instance, studies by Bayer *et al.* (1999a) show that MCH neurons receive a cholinergic input from two pontomesencephalic nuclei associated with arousal; furthermore, their diffuse projections to the cortex are thought to provide a basis for generalized cortical arousal (Bittencourt *et al.,* 1992). MCH neurons are on the path of the MFB which contains dopaminergic fibers connecting the ventral tegmental area and the accumbens nucleus, two major components of the reward system (Liebman and Cooper, 1989; Pothos *et al.,* 1995). The surrounding MCH neurons could be contacted by these dopaminergic fibers (see Section IV.2) while they project in both territories, which express moderate to high levels of MCH receptor (Saito *et al.,* 1999). Other studies suggest a role for MCH in gustation: MCH fibers project to the gustatory zone of the parabrachial area (Touzani *et al.,* 1993), whereas MCH receptors are expressed in the nucleus of the solitary tract suggesting a role for MCH in gating gustatory information (Kokkotou *et al.,* 2001). MCH neurons also project into olfactory centers in the cortex (Gura, 2000) and MCH receptor transcripts are present in neurons of the olfactory pathway (Kokkotou *et al.,* 2001). In addition to providing a drive to eat, MCH neurons are thus positioned within the circuitry that responds to and modulates the sensory inputs of odor, palatability, and other hedonic qualities of food.

3. Influence on Fluid and Electrolyte Balance

MCH neuron activity is modified by various paradigms that alter plasma osmolality and/or volemia (Zamir *et al.,* 1986; Fellmann *et al.,* 1993). In experiments involving water deprivation and salt loading (Presse *et al.,* 1993), some neuronal groups responded by gene activation and others by depression, emphasizing the heterogeneity of the MCH population. It also appears that rat gender or strain may affect the response (Presse *et al.,* 1993). Two additional experiments indicate that MCH neurons are sensitive to both osmotic changes and hypovolemia: (i) Increased osmolarity caused by the addition of sucrose to the medium of primary

fetal hypothalamic cultures stimulated MCH release (Fellmann *et al.,* 1993), and (ii) subcutaneous injections of polyethylene glycol, a colloid that induces hypovolemia, significantly increased ppMCH mRNA values within 5 h (Hervé *et al.,* 1998).

Which ppMCH-derived peptide, MCH or NEI, is involved in fluid or electrolyte balance is not clear. When added to the culture medium of incubated rat posterior pituitaries, only NEI significantly reduced the release of arginine vasopressin (AVP) (Parkes and Vale, 1993). When applied icv to sheep, however, both MCH and NEI increased the excretion of water, sodium, and potassium, whereas water consumption, plasma AVP, and plasma osmotic pressure remained unchanged during the 24-h infusion (Parkes, 1996). The mechanisms underlying the effects on diuresis and natriuresis are not clear and could be indirect. Although plasma AVT was unchanged by the infused peptides, plasma aldosterone decreased (Parkes, 1996).

These experimental data, together with the observation that neurons of the LHA modify their electrical activity in response to osmotic changes (Tanaka and Seto, 1988), argue in favor of a role of MCH neurons in the regulation of water and electrolyte balance, even if the mechanisms of their action remain to be elucidated.

4. Modulation of Other Functions

The putative influences of MCH/NEI on a variety of behaviors have been investigated in rats. Since MCH and α-MSH display opposite effects on the skin pigment cells of teleosts (Baker, 1988), MCH, NEI, and α-MSH were tested independently and in combination. When injected directly into either the VMN or the MPOA, MCH alone had no effect on grooming, rearing, aggressive or exploratory behaviors, but it antagonized the ability of α-MSH to increase aggression (VMN) and to reduce exploration (MPOA) when the peptides were injected together (Gonzalez *et al.,* 1996). When administered icv, MCH reduces α-MSH-induced grooming score by about 50% (Sanchez *et al.,* 1997). NEI resembles α-MSH in stimulating grooming, rearing, and locomotion, and its effects are also annulled by MCH (Sanchez *et al.,* 1997). When administered into the VMN, NEI also stimulated exploratory behavior (Gonzalez *et al.,* 1998), showing that it has specific behavioral effects in its own right. Thus, although MCH alone had no effect on any of the investigated behaviors, it has proved to be a potent antagonist of NEI and α-MSH effects.

The multiple behavioral effects induced by MCH, α-MSH, and possibly NEI are probably interrelated in many behavioral repertoires. For example, at mealtimes, MCH may stimulate feeding behavior while discouraging grooming and exploratory activities, but following a meal the presumed decrease in MCH release and increase in α-MSH may then encourage grooming (Sanchez *et al.,* 1997; Ludwig *et al.,* 1998). The same argument could also apply to potential interactive effects of α-MSH and MCH during grooming when displayed as a displacement activity under certain stressful conditions.

The topography of MCH neurons and the fact that icv administration of MCH abolished or attenuated motor phenomena that characterize convulsions in a model of experimentally induced seizures in rats suggest that MCH neurons may be involved in the circuitry and activity of extrapyramidal motor pathways, from striatal centers to the thalamus and cerebral cortex and to the midbrain and spinal cord (Knigge and Wagner, 1997a; Knigge *et al.,* 1997).

The occurrence of MCH fibers in brain areas associated with learning and memory such as hippocampal formation and septum, together with the modifications registered in learned responses to different behavioral tests after subcutaneous or central MCH treatments, suggests that MCH may play a role in modulating memory formation (McBride *et al.,* 1994; Monzon and de Barioglio, 1999; Monzon *et al.,* 2000). Finally, the detection of abundant MCH receptors in the hippocampus and olfactory regions also reveals a possible role for MCH in olfactory learning, which is fundamental in the regulation of food selection (Saito *et al.,* 1999; Hill *et al.,* 2001).

C. MCH and Its Receptors in Peripheral Tissues

Although the cytological picture suggests that negligible amounts of the peptide are released from the neurohypophysis in higher vertebrates, MCH or its coderivative NEI may nevertheless play peripheral roles in mammals and other vertebrates. Early studies on fish failed to find evidence of MCH gene expression outside the brain (Ono *et al.,* 1988; Minth *et al.,* 1989), but in mammals evidence from Northern blots and reverse transcriptase-polymerase chain reaction (RT-PCR) shows that the mRNAs for both ppMCH and the MCH receptors are expressed in a variety of peripheral tissues in which the peptide presumably plays a paracrine/autocrine role. Some of these sites are indicated in Table V. Although its amount per gram may be small, the overall amount of MCH produced by some tissues such as the gut could be considerable, and if the peptide were released into the general circulation its concentration might reach potentially physiologically significant levels. Some authors report the existence of MCH in rat plasma (Bradley *et al.,* 2000) although its origin is unknown. This aspect of MCH has received no attention and nothing is known about the conditions regulating the levels of either the MCH receptor or MCH in peripheral sites. At present, only a few studies hint at possible biological effects of MCH in these tissues, making the importance of its influence difficult to assess.

1. Pituitary

In teleost and probably holostean fishes, MCH is a circulating hormone released from abundant stores in the neurohypophysis (Kishida *et al.,* 1989). In contrast, in

TABLE V
MCH in Peripheral Tissues[a]

Tissue	ppMCH mRNA	MCH peptide	SLG-1 mRNA	Biological effect	Reference
Pituitary	NHp	+	+	+	Bittencourt *et al.* (1992), Knigge *et al.* (1996), Saito *et al.* (1999), Parkes and Vale (1993), Hill *et al.* (2001)
Skin	+	+	+	+	Thody *et al.* (2001), Drozdz *et al.* (1995)
Gut	+	+	+	+	Hervieu and Nahon (1995), Hervieu *et al.* (1995), Hill *et al.* (2001)
Pancreas	+	?	+	+	Hervieu and Nahon (1995), Tadayyon *et al.* (2000), Hill *et al.* (2001)
Adipocyte	?	?	+	+	Bradley *et al.* (2000), Hill *et al.* (2001)
Testis	+	+	ND	+	Saito *et al.* (1999), Hervieu *et al.* (1996)
Adrenal	+	?	+	+	Saito *et al.* (1999), Hervieu and Nahon (1995), Baker *et al.* (1985b), Takahashi *et al.* (2001)
Immune system	+	?	+	+	Hervieu and Nahon (1995), Harris and Bird (1998), Hill *et al.* (2001)

[a]Abbreviations used: +, Shown to occur; ?, not investigated; ND, not detected; NHp, mRNA in hypothalamo-neurohypophysial perikarya.

other vertebrates MCH peptide is sparse in the posterior pituitary and the modest amounts found here are probably involved only with paracrine effects. Northern blots and RT-PCR demonstrate the presence of small amounts of MCH-R1 (SLC-1) mRNA in the rat pituitary (Saito *et al.,* 1999; Hill *et al.,* 2001), where MCH and/or NEI have been shown to influence the release of oxytocin and vasopressin from the neural lobe (Parkes and Vale, 1993). In teleosts, MCH also affects adenohypophysial hormones, depressing the release of α-MSH from the intermediate lobe (Barber *et al.,* 1987; Groneveld *et al.,* 1995a) and ACTH from the pars distalis (Baker *et al.,* 1985a) and stimulating the release of somatolactin (Balm and Groneveld, 1998). Similar regulatory effects have not been demonstrated in tetrapods, but the presence of irMCH in the median eminence (Knigge *et al.,* 1996) suggests that some modulation of pituitary cell activity probably occurs.

2. Skin

Pre-proMCH mRNA and immunoreactive MCH are found in the mammalian skin, in which they are located in keratinocytes and possibly other cell types (Hoogduijn *et al.,* 2001). The melanocytes express MCH-R1, and MCH increases their intracellular calcium and IP3 concentration *in vitro* and opposes α-MSH-induced cAMP and melanogenesis (Hoogduijn *et al.,* 2001), in line with results from earlier studies (Drozdz *et al.,* 1995; Burgaud *et al.,* 1997). The rat skin thus forms a self-contained paracrine system, responding to locally produced melanotropic peptides. Other skin derivatives, such as the preputial gland, show a significant increase in weight in response to either MCH or α-MSH (Murray *et al.,* 2000a). The melanocortin MC5 receptors have been demonstrated in this tissue (Chen *et al.,* 1997) but whether MCH-R1 or MCH-R2 are present has not been examined.

Comparison with the effect of MCH on fish melanophores is of interest. Although MCH will oppose the melanin dispersing effect of α-MSH or forskolin in the grass carp (Baker, 1988, 1993), it is reported that melanin concentration in eel melanophores is not prevented by treatment with pertussis toxin, implying that G_i/G_o proteins (which oppose cAMP production) are not involved (Suply *et al.,* 1999). Other studies show that protein kinase C and IP3 will induce melanin concentration in fish melanophores (Abrao *et al.,* 1991; Fujii *et al.,* 1991). The fish MCH receptor has not been characterized.

3. Digestive Tract

Pre-proMCH mRNA and irMCH peptide can be detected in various regions of the rat gut (Hervieu and Nahon, 1995; Hervieu *et al.,* 1995), together with low levels of MCH receptor mRNA (Hill *et al.,* 2001). The precise nature of the irMCH molecules has not been established. High-performance liquid chromatography analysis indicates that only about 10% of the total immunoreactivity corresponds to mature MCH (Hervieu *et al.,* 1995), but the nature of the remaining MCH was not determined. Using Northern blots and RT-PCR, Hervieu and Nahon found multiple forms of ppMCH mRNA within the stomach, duodenum, and colon of Wistar and Sprague–Dawley rats. One form was mature ppMCH mRNA (0.95 kb). Other forms include large-sized mRNA ($\sim$1.29 kb), which might be attributable to unspliced RNA transcript. The smallest form (0.8 kb) appeared to be ppMCH with a shortened poly-A tail. Subsequent studies (Hervieu *et al.,* 1995) located both mRNA and irMCH and also NEI within enterochromaffin cells in the lamina propria of the intestinal villi. No signals were found in the overlying epithelial cell layer. Bioassays done on anesthetized rats with ligated gut segments indicate that intravenous injections of MCH can enhance the net secretion of water, sodium, and potassium into the gut cavity (Hervieu *et al.,* 1995). In this respect, MCH mimicked some of the effects of ANF, which is found in cells with a similar gut distribution

to MCH. The findings suggest that locally produced MCH has a paracrine effect on water and ion within the gut. Nothing is known about the physiological conditions that regulate MCH secretion by these cells.

4. Pancreas

Northern blots reveal the presence of ppMCH mRNA in the rat pancreas (Hervieu and Nahon, 1995), although the specific cell type in which MCH is produced is not known. Both MCH receptor types, R1 and R2, are expressed in the islets of Langerhans and the receptor has been demonstrated by immunofluorescence on the cell surface of insulin-secreting cell lines (Tadayyon *et al.,* 2000). MCH stimulates insulin secretion by these cell lines and, in contrast to its action on melanocytes, this effect of MCH is significantly enhanced rather than opposed by forskolin. Tadayyon and coworkers (2000) conclude that MCH may amplify the insulinotropic effect of other cAMP-inducing stimuli.

5. Adipocytes

Adipocytes secrete the hormone leptin, which has a marked effect on various brain peptides, including MCH, that regulate feeding behavior. The ultimate results of leptin release are reduced food intake, reduced plasma insulin levels, and increased metabolic rate. One of the factors that enhances leptin secretion by adipocytes is insulin, which, as indicated previously, appears to be modulated by MCH. Recently, Emilsson *et al.* (1997) and Bradley *et al.* (2000) showed that MCH can act directly on adipocytes to stimulate leptin secretion. Adipocytes express predominantly MCH-R2 type receptors (Hill *et al.,* 2001) and respond to the addition of 1 μM MCH *in vitro* by a two-fold increase in leptin release (Bradley *et al.,* 2000). This response was seen within 4 h but was preceded by a transient but significant increase in leptin mRNA 1 h after the addition of MCH. The source of the MCH that might influence adipocytes *in vivo* is not known. Bradley and coworkers (2000) asserted that MCH circulates in rat plasma but they gave no details. In contrast to its appetite-enhancing effects within the brain (see Section V.B), MCH might modulate appetite indirectly by stimulating the secretion of insulin and leptin, both appetite-suppressing hormones.

6. Testis

Both mature ppMCH mRNA with a short poly-A tail (0.8 kb) and a large form of ppMCH mRNA (1.3 kb) are found within the testis, as are immunoreactive MCH and NEI molecules (Hervieu *et al.,* 1996). Although an earlier report suggested these were Sertoli cell products (Hervieu and Nahon, 1995), a later study located the immunoreactive peptide within the nuclei of spermatogonia and less extensively in early spermatocyte nuclei of rats, mice, and humans. Peptide

immunoreactivity was apparent from an early age, being present already in the immature rat testis 10 days postpartum. Its abundance within the germ cells seemed to vary with the spermatogenic cycle. *In situ* hybridization identified ppMCH mRNA in the same peritubular region and its abundance varied between seminiferous tubules, suggesting that gene expression fluctuates during the spermatogenic cycle. Expression also seemed to be hormonally regulated since the abundance of mature (0.8 kb) mRNA increased in testicular tissue incubated with follicle-stimulating hormone for 24 h (Hervieu *et al.,* 1996). The role of MCH or NEI in the testis is not known. The presence of a peptide within the nucleus rather than in the cytoplasm suggests some intranuclear role, such as that of a transcription factor. MCH receptors would not be required for a nuclear role. Only one study (Saito *et al.,* 2000) has searched for MCH receptors in the rat testis but SLC-1 mRNA was undetectable by Northern blot analysis, although it is possible that RT-PCR might reveal low concentrations.

7. Adrenal Gland

Early bioassays on the rat adrenal gland suggested that MCH might oppose the stimulatory effect of α-MSH on corticosterone but not aldosterone production by the glomerulosa cells (Baker *et al.,* 1985b). ACTH and α-MSH are believed to influence adrenal tissue via the MC2 (ACTH) and MC5 (MSH) receptor types (Chhajlani, 1996). The MCH-R1 receptor SLC-1 is also present in the adrenal; although undetectable by Northern blot analysis, it has been demonstrated after amplification by RT-PCR, suggesting the receptors are present in low abundance (Saito *et al.,* 2000; Takahashi *et al.,* 2001). Since ppMCH mRNA can also be detected in the adrenal gland by Northern blot analysis (Hervieu and Nahon, 1995), it seems likely that the peptide is produced locally and exerts a paracrine/autocrine effect; however, nothing is known about the regulation of peptide or receptor expression.

8. Immune System

Strong signals for ppMCH mRNA have been observed in spleen and thymus extracts from Wistar and Sprague–Dawley rats. Northern blotting showed that the mature form was shorter (0.8 kb) than ppMCH mRNA extracted from the hypothalamus (approximately 1.0 kb) (Hervieu and Nahon, 1995), a difference probably attributable to a reduced poly-A tail length, as in the gut. As in other peripheral tissues, the abundance of mRNA was variable. This could be due to problems with extraction efficiency during poly-A fraction purification because of the short tail. It is also probable that peptide expression in immune tissues is strongly affected by the immunological status of the animal. MCH receptors have not been searched for in immune tissues. Also, the effect of MCH on the immune response has not been studied in mammals, but in fish MCH (5–500 n*M*) was found to enhance phagocytic

activity in leukocytes isolated from the head kidney (Harris *et al.,* 1998). α-MSH, known to be produced by mammalian leukocytes, similarly enhanced phagocytic activity in fish, but when added together the two peptides were not additive and were sometimes mutually antagonistic (Harris and Bird, 1998). Harris and Bird (2000) proposed that the peptides influence phagocytes indirectly by stimulating another immune cell to produce some macrophage-activating factor.

Other tissues in addition to those discussed previously express ppMCH mRNA or the MCH receptor. MCH mRNA has been detected in the ovary, heart, and lung but not in kidney or liver (Hervieu and Nahon, 1996). MCH receptor (SLC-1) mRNA has been detected in the tongue, skeletal muscle, and eye (ciliary epithelium) but not in kidney, lung, or liver (Saito *et al.,* 1999; Hintermann *et al.,* 2001). Thus, in many cases both the peptide and its receptor are present in the same tissue. When this does not seem to be the case, one should bear in mind that the signal may be detectable only under specific physiological conditions.

VI. Concluding Remarks

In contrast to early studies on MCH in fish, which were done using long-established methods such as bioassays, radioimmunoassays, and physiological experimentation, recent advances in the study of MCH in mammals have depended largely on the application of gene technology and molecular biology techniques. The surprising resemblance of mammalian MCH RNA to the 7SL riboprotein made it necessary to characterize rat MCH by conventional peptide purification (Nahon, 1994), but subsequent studies—identification of the MCH genes, the creation of MCH "knockout" mice or transgenics that overexpress MCH, and the eventual identification of the MCH receptors and their second messenger systems—have all depended on modern technology.

Particularly intriguing findings are the diversity of the MCH gene products, the ability of the MCH gene to give rise to a range of peptides by posttranscriptional cleavage or by alternative splicing, the evolution of variant forms of MCH in primates, and the expression of an "antisense" gene that may be involved in transcriptional control. The significance of these findings and the extent to which they are the result of recent mutations and replications, found only in mammals, will doubtless be the topic of further study in the near future.

Progress regarding the physiology of MCH has been slower. Many of the advances in this area have depended on monitoring gene expression following particular challenges, such as stress, dehydration, or starvation, but the full range of effects elicited by MCH during such conditions are still poorly understood. Moreover, such studies have frequently depended on measuring global changes in MCH mRNA by Northern blot, and thus have not pinpointed the neurons involved. A particularly intriguing finding is the large response that such treatments elicit,

suggesting that a large percentage of neurons may be involved in each case. This has been confirmed by Presse and Nahon (1993) for the response to osmotic challenge, and their *in situ* hybridization study also revealed that some MCH neurons respond in the opposite direction to the majority, underlining the heterogeneity of the neurons and the complexity of MCH's influence. In cases in which transcriptional changes are associated with specific MCH neuronal groups, for instance, in the preoptic area during lactation (Knollema *et al.,* 1992), the physiological effects of MCH are again a matter of speculation. These effects have been studied by central injection of the peptide, but future advances will probably be made by the development and local administration of MCH receptor antagonists.

Improved understanding of the brain regions to which particular MCH neurons project has been achieved by retrograde and anterograde neuronal tracing combined with immunostaining, a painstaking task which has received attention by disappointingly few laboratories. Nevertheless, such studies are the foundation for gaining an understanding of MCH circuitry. It has long been clear from the widespread distribution of MCH fibers, and recently by the location of the MCH receptors, that MCH potentially affects a broad spectrum of sensory, motor, and emotional responses. The integration of this MCH system with other neurons, particularly with the POMC system which MCH often seems to oppose, will be a long-term but worthwhile undertaking.

Finally, it is perhaps no surprise that MCH and its receptors, as with so many neuropeptides, is expressed in many peripheral sites in which it is presumed to exert paracrine/autocrine effects that still await discovery. Although not yet proven, peripheral MCH peptide and receptor expression is likely to occur in all vertebrates; for example, the presence of receptors in the skin is a prerequisite for the adoption of MCH as a fish pigmentary hormone early in evolution. It is hoped that it will not be too long before these MCH receptors are characterized in nonmammalian vertebrates so that comparative endocrinology may again prove its value in illuminating potential functions of the MCH system in mammals.

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