2 Molecular Approaches to Behavioral Neuroendocrinology

Margaret M. McCarthy and David Crews

There has been an explosion of information about the way changes in gene expression impact brain and behavior. Since hormones produce many of their effects on the body and on behavior by altering gene expression in their target cells, the modern behavioral endocrinologist must become familiar with the techniques of molecular biology.

How do you know if a hormone has turned on a gene in its target cells? What do you do if you want to clone a gene? What is a transgenic animal? This chapter was designed to help you become more familiar with new techniques and the exciting questions that can now be answered with them.

Introduction

Now that you have reviewed some of the basic concepts of behavioral neuroendocrinology, you are prepared to review some of the more sophisticated approaches that are becoming increasingly important in the field. Of particular note is the increased emphasis on understanding the molecular or genetic basis for hormonal modulation of behavior. Nowadays behavioral neuroendocrinologists can choose from a wide range of techniques and can customize a particular method or combine several approaches to suit their needs.

By and large, hormones exert their effects by influencing the expression of particular genes. As we saw in chapter 1, this is accomplished via different pathways for peptide versus steroid hormones. In the case of steroid hormones, this is generally achieved by a direct interaction with the DNA, whereas peptide hormones usually bind to membrane receptors and activate a signal transduction cascade that may ultimately activate gene expression. As a result of their different sites and mechanisms of action, steroids and peptides can generally be distinguished by their effects being relatively slow (hours to days) versus extremely fast (milliseconds to minutes), respectively. Changes in gene expression alter the protein composition of a cell, thereby altering its function and perhaps changing its influence on other cells as well. We will begin with a more detailed review of the receptors. This will be followed by a discussion of some of the techniques used to measure and characterize messenger RNA (mRNA), DNA, and proteins. Then we will look at some of the new techniques for altering gene expression that are being used by behavioral neuroendocrinologists.

Mechanisms of Hormone Receptor Action

Peptide Hormone Receptors

As you saw in chapter 1, peptide hormone receptors are found on the surface of cells. In order for them to influence the expression of genes, the signal must be transduced to the nucleus. This is referred to as the **signal transduction pathway** (Albers et al. 1994). The peptide or protein hormone binds to its receptor and this results in changes in intracellular molecules and, in some cells, the production of **second messengers.** A number of second messengers are produced (depending on the cell and the receptor), including cAMP and the inositol phospholipids, better known as IP3. Recent evidence suggests that calcium ions also function as important second messengers.

METABOTROPIC RECEPTORS

Probably the most broadly distributed type of receptor are the **G-protein coupled receptors**, sometimes called **metabotropic receptors.** These are large proteins with a structure that spans the extracellular membrane 7 times (referred to as 7-transmembrane domains) with a piece of the protein that extends into the extracellular space and binds to its ligand. Intracellularly, these receptors are associated with the G-proteins. Binding of the receptor to the ligand extracellularly activates the G-protein complex inside the neuron and, as a consequence, enzymes are activated that result in the production of second-messenger molecules such as cAMP and IP3. These types of receptors are utilized by many peptide hormones such as oxytocin, gonadotropin releasing hormone (**GnRH**), **corticotropic releasing hormone** (**CRH**), and the glycoprotein hormones luteininzing hormone (LH) and follicle stimulating hormone (FSH).

LIGAND-GATED ION CHANNELS

A second type of extracellular receptor are the **ligand-gated ion channels.** This type of receptor tends to be the exclusive domain of acetylcholine and the amino acid transmitters GABA, glutamate, and glycine. Interestingly, progesterone and some progesterone metabolites are known to bind to a subunit of the GABA-A receptor, which enhances the receptor response to GABA. So here we have a receptor that looks like it should respond to peptide hormones but responds to steroid hormones.

RECEPTOR KINASES

Finally, the **receptor kinases** are receptors that function as enzymes and phosphorylate other proteins to regulate their activity. Growth factors such as nerve-growth factor (NGF) or brain-derived neurotrophic factor (BDNF) use receptors of this type.

Thus, the majority of peptide hormones we are concerned with act through G-protein-coupled receptors. Different hormones will activate receptors that selectively increase either cAMP or IP3. For instance, GnRH increases IP3, and

ultimately the level of free calcium in a cell, whereas the factors it releases from the pituitary, LH and FSH, both bind to G-protein coupled receptors that increase cAMP. Ultimately, the increased calcium released by IP3 and cAMP can influence gene expression by activating a protein called CREB (cAMP-response-element-binding protein) which finds a site on the DNA and binds to it. This is analogous to the hormone-response elements we discuss below for steroid receptors. A brief summary of these different signal transduction pathways is schematically illustrated in figure 2.1.

Steroid Hormone Receptors

ACTIVATION OF SELECTIVE GENE EXPRESSION

Because of their ability to interact directly with the DNA, steroid hormone receptors are referred to as **transcription factors**; in other words, they either increase or decrease the transcription of particular genes. As we learned in chapter 1, the first step in steroid-hormone action involves the steroid passing through a cell's exterior plasma membrane. Inside the cell, the steroid molecule encounters unoccupied (or unactivated) receptors. These receptors are present as individual proteins (monomers) that are part of a complex of multiple chaperone proteins. These chaperone proteins (that include inhibitory heat shock proteins) serve to lock the receptor in a high affinity state for ligand binding. After the steroid ligand binds to its receptor, the inhibitory heat shock proteins dissociate and the receptor is activated. Activated receptors bound to their ligand dimerize (two receptor-ligand units bind together) and then interact with the DNA.

Before interacting with the DNA, the steroid receptors may bind to other proteins, forming part of a **transcriptional complex**. This complex consists of multiple proteins, many of which are enzymes that will unwind the DNA and help transcribe it into mRNA. Some of these additional proteins are referred to as **co-activators** and **co-repressors**. Their presence in the transcriptional complex can be essential for steroid responsiveness. For instance, the first of these co-activators to be discovered is called steroid receptor co-activator 1 (SRC-1). If levels of this co-activator are reduced, animals do not show normal development of secondary sex characteristics or expression of sexual behavior (Auger et al. 2000; Xu et al. 2000).

One important function of steroid hormone receptors in this transcriptional complex is to confer specificity. It would not be very useful to have the complex randomly settling down on the DNA and turning on genes. Instead, the steroid hormone receptor will only interact with a specific sequence of nucleotides that must be present in the upstream or **promoter** region of a gene. The promoter is just as it sounds, a part of the gene that promotes its expression by specific factors as a result of its specific nucleotide sequence (keep in mind that not all portions of a gene are transcribed into mRNA). For steroid receptors, this sequence is referred to as a **hormone response element or HRE**.

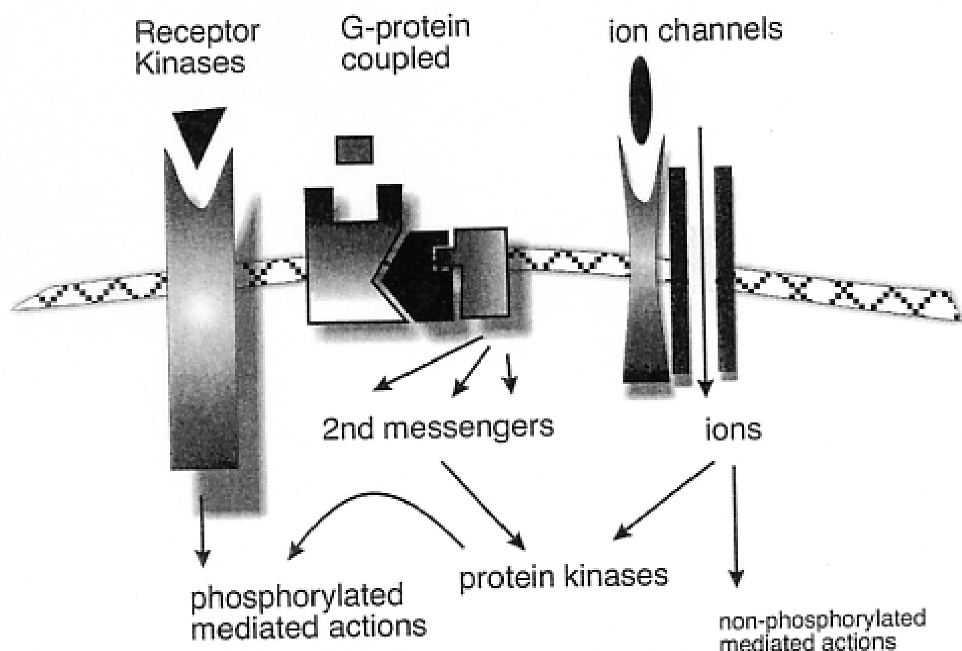


Figure 2.1 Signal transduction following membrane receptor binding. There are three general types of membrane receptors: (1) the kinase receptors, (2) G-protein-coupled receptors, and (3) ligand-gated ion channels. Kinase receptors bind growth factors, thereby activating their intrinsic enzymatic activity to phosphorylate various substrates, inducing a cascade of cellular changes. The G-protein-coupled receptors may bind peptides, neurotransmitters, or prostaglandins. They can be coupled to the production of cAMP or to the phosphotidylinositol pathway that can release internal calcium from the endoplasmic reticuluum. The ligand-gated channels are ionophores, which are permeable to various ions (Cl^-, K^+, and Ca^{++}) and which bind neurotransmitters, in particular, the amino acids GABA and glutamate.

There is a different HRE for the estrogen receptor than for the progesterone receptor and so on. The sequence is about 6 to 15 nucleotides long and is usually a palindrome (something that is the same when read forward or backward, like the name HANNAH). For instance, the HRE for the estrogen receptor is AGGTCANNNTGACCT / TCCAGTNNNACTGGA where the N refers to any nucleotide. The DNA binding site on the estrogen receptor will specifically recognize and bind to this sequence on the DNA and either activate or suppress the associated gene. The estrogen receptor also contains a binding site for its ligand, estrogen, and several other domains that are highly conserved across species (Rollerova and Urbancikova 2000).

The androgen, progesterone, estrogen, and glucocorticoid receptors, along with thyroid receptors and an even larger group of proteins termed orphan receptors (meaning that their ligands and/or function are as yet undetermined), are members of a nuclear receptor superfamily. Almost all members of this family act as transcription factors that exert broad influences on gene expression. For some receptors, such as the androgen receptor, both testosterone (T) and dihydrotestosterone (DHT) operate through a single receptor, but their effect on gene expression can be quite distinct. For example, as will be discussed in more detail in chapter 3, T is required for the development and differentiation of the internal accessory sexual organs, including the transformation of the Wolffian ducts into the epididymis, vas deferens, and seminal vesicles. On the other hand, DHT is required for the development of the penis, scrotum, and prostate.

To further complicate things, steroid hormone receptors exist in multiple forms. For example, in most avian and mammalian species, the progesterone receptor is expressed as two major DNA-binding forms, PR-A and PR-B. Another example is estrogen receptor alpha (α), beta (β), and now gamma (γ) (Hawkins et al. 2000). It seems likely that additional forms of steroid receptors will continue to be discovered in the near future. Having multiple forms of a receptor is another mechanism by which the cell can selectively regulate its response to a steroid.

NONTRADITIONAL STEROID EFFECTS

When a steroid binds to its receptor and induces gene transcription, this is referred to as a genomic action and has long been considered the classic or traditional form of steroid action. This distinction is important because for all the steroid hormones, actions have been reported that occur very rapidly in cells devoid of the classical intracellular receptors (see chapters 4 and 13); these rapid effects are referred to as "nontraditional" steroid effects. In the past they were referred to as "nongenomic" steroid actions, but it is now clear that this terminology should be avoided because it can easily be confused with rapid effects that result in genomic activation or with transgenerational effects such as the influence of maternal behavior, handling, intrauterine position, temperature, and so on.

Exactly how the rapid effects of steroid hormones are exerted in the ce
still under investigation. Many reports have implicated a metabotropic re
tor in the rapid effects of estrogen in the striatum (for example, Mermels
et al. 1996; chapter 13). Recently, however, it was found that the traditi
nuclear receptor can incorporate into a cell's lipid bilayer, with the ster
binding pocket of the receptor actually extruding through the memb
(Razandi et al. 1999) and possibly picking up passing molecules of ste
This was a surprising finding and may help explain many anomalies re
ing the rapid versus traditional effects of steroids. It also highlights the
that steroid effects cannot be easily pigeonholed into one type or anothe

LIGAND-INDEPENDENT ACTIVATION

Another surprising discovery was that the traditional nuclear steroid rec
also moonlights as a signal transduction protein and interacts with va
signaling pathways in the cytoplasm of a cell. Estrogen receptors, for
ple, can associate with specific cytoplasmic proteins to activate ki
enzymes that phosphorylate and thereby activate other proteins. This w
in motion a signaling cascade that eventually ends in the nucleus wi
induction of gene transcription (Singh et al. 2000). The spark that ignit
pathway may or may not be a steroid hormone molecule. In another ex
dopamine binding to the D_1 dopamine receptor can result in activatior
progesterone receptor in certain cells (see chapter 4 for more details
results in what is known as ligand-independent activation of steroid
tors. Such discoveries of novel and unexpected ways in which ster
ceptors can be activated and interact with other cellular proteins has u
the traditional view and greatly expanded our concept of steroid h
action. As a result, the terms nongenomic and genomic actions of
steroids have gone the way of the dial phone and become obsolete.

Techniques for Measuring Gene Expression

What do we mean when we say "gene expression"? How does a gene
itself? A diamond nose ring and purple hair dye? No, much more mu
unfortunately. As we discussed in chapter 1, gene expression refers
crease (or decrease) in the transcription rate of a gene to produce
less) mRNA, which may (or may not) ultimately result in more (or l
tein. To measure gene expression, we usually resort to measuring
uct: mRNA or the protein produced. We have several technical appr
doing this. *Molecular Cloning* by Sambrook, Fritsch, and Maniatis (
done for the laboratory what the *Joy of Cooking* did for the home kit

A number of the techniques currently in use to measure gene e
will be described below (so don't worry about what the names me
moment). First, it is important to clarify what question you are a
which technique will best answer it. For instance, if you need
whether a gene expressed ubiquitously and in abundance is chang
a tissue, then you will want to take advantage of the quantitative

Northern Blots. Alternatively, if the gene is widely distributed but rare, you will probably want to exploit the sensitivity of RNase protection assay or quantitative PCR. Often, however, a particular gene may be modulated only in a subset of cells, such as those that express androgen receptors; then you may want to retain the cellular resolution afforded by in situ hybridization. Lastly, it may be that your gene of interest has not even been cloned yet, in which case you must begin by fishing your gene out of the pool and sequencing it.

All of this may sound rather daunting, but in fact the development of techniques for research in molecular genetics has been transformed into a growth industry with simplified kits and reagents that allow virtually any laboratory, no matter how small, to study gene expression. The two most important criteria are attention to detail and excellent laboratory procedure. For example, in all RNA-based work, the investigator must be aware that the enzyme RNase will instantly chew up RNA, so it is important that all glassware and other items be free of this enzyme. Unfortunately, one of the major sources of this enzyme is the oil on your hands, making the use of latex or rubber gloves a must.

Measuring mRNA

Measuring the mRNA level for a particular gene requires having a probe specific to that gene. This allows us to distinguish our needle (i.e., gene) in the haystack (the hundreds of thousands of other mRNAs). The probes come in a variety of types, distinguished mostly by their length and whether they are made of RNA or DNA. If you are lucky, the sequence for your gene of interest is already known and you simply design a probe complementary to the mRNA sequence. What does complementary sequence mean? Remember that DNA is double-stranded, but only one strand of DNA codes for mRNA to synthesize the protein your gene codes for. That strand of DNA is complementary to its mRNA and will bind or hybridize with its mRNA under the right conditions. So you can order a short synthetic stretch of DNA complementary to your mRNA, known as an **oligonucleotide**, from any number of companies.

Sometimes, however, either your gene has not been cloned and sequenced because you are breaking new ground by studying the gastric-brooding frog, or you desire a probe of a different sort which requires you to make it yourself. The most common approach is to use primers selective for a portion of a particular gene to extract a chunk of that gene from the RNA with the use of polymerase chain reaction (PCR). We will discuss these steps in more detail below.

NORTHERN BLOTS

The most direct way to determine how much of a species of mRNA, DNA, or protein you have is to do electrophoresis to isolate and characterize your molecule of interest. If you are doing an analysis of mRNA on your tissue, you would do what is called a **Northern blot**. This odd-sounding procedure is

named in response to another technique we will discuss below, the Southern blot, which gets its name from its inventor, E. M. Southern (Southern 1975). Rather than measuring the mRNA as it exists in the cell, the Northern blot technique requires breaking open the cell, extracting the mRNA with chemicals, and then separating it by size using electrophoresis. Electrophoresis refers to passing an electric current through a gel or matrix, which results in molecules separating along a gradient by size. After this step is achieved, the gradient of mRNAs is transferred to a membrane and then this membrane is probed using a radioactively labeled antisense probe for the mRNA (an antisense probe is the complementary sequence of RNA which will bind or hybridize only to the mRNA that mirrors its sequence.) After various washings to eliminate nonspecific hybridizations, the membrane is apposed to X-ray film, and if all has gone well, a band will appear on the film at the location that corresponds to the original place that the mRNA was separated in the gradient on the gel by electrophoresis.

To determine the molecular weight of your particular band, you will use a molecular weight marker that appears as a ladder on the gel and consists of different-sized bands of RNA. If the size of the band corresponds to the known size of your mRNA, then you are in business. The darkness of the band is directly proportional to the amount of mRNA that was in the original sample. For example, say you have the idea that estradiol will stimulate transcription of the gene coding for oxytocin in a particular part of the brain (we will discuss how to isolate parts of the brain in a moment). To test this hypothesis, you will use female rats whose ovaries have been removed. You create two groups, one in which you inject a small amount of estradiol and the other in which you inject the same volume of vehicle, but lacking the hormone (sometimes called the vehicle control). If the hypothesis is supported, you will find a much darker band in the mRNA prepared from the estradiol-treated females than from the vehicle-control females.

In such studies, it is important to have a way of determining that your measure of gene expression is specific. For this you will use a "housekeeping gene," a gene found ubiquitously in all cells and presumably not regulated by estradiol. Actin is a commonly used housekeeping gene that can be probed on the same membrane as the control. An advantage of Northern blot hybridization, as well as of in situ hybridization, is that you can often use the probe from another species if that particular sequence has not been cloned in your species. But unlike in situ hybridization, substantially more tissue is required with Northern blots. This can be a potential drawback when dealing with rare species (like our frog) or endangered species.

It is important to keep in mind that you can never be sure that what you are measuring is newly synthesized mRNA, since changes in mRNA stability or half-life could also result in changes in mRNA levels between conditions. In fact, estrogen has been found to increase the stability of vitellogenin (an egg yolk protein) and, in some cases, GnRH, independent of any effects on transcription. So this caveat must always be borne in mind when using quantita-

tive assays for mRNA levels. Nonetheless, these tools are very powerful and are an important part of the technical arsenal of any neuroendocrinologist.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

The development of the **reverse transcriptase polymerase chain reaction** (RT-PCR) resulted in a Nobel prize for Kary Mullis in 1993 and depended in large part upon the Nobel prize–winning discovery of an enzyme that would transcribe RNA into DNA awarded to David Baltimore and Howard Temin in 1975. The name of the technique actually describes it quite effectively. We begin by extracting the mRNA from cells, in exactly the same manner as we would do for a Northern blot. The difference in this case is that we add an enzyme called reverse transcriptase, which will transcribe RNA into DNA. In other words, it is the *reverse* of the normal process (DNA to RNA). Once that is accomplished, you have what is known as cDNA or copyDNA. This can then undergo the polymerase chain reaction (Erlich 1991).

The word "polymerase" refers to the enzyme DNA polymerase, an essential enzyme used to copy DNA during cell replication or mitosis. In this case, however, the DNA polymerase is of a special form in that it can tolerate very high temperatures, close to boiling. These special polymerases were discovered in bacteria living in the deep ocean next to vents that release steaming hot water warmed by the earth's core, and in the bubbly cauldrons familiar to any visitor to Yellowstone National Park. The enzymes are called *Vent* polymerase or *Taq* (from *Thermus aquaticus*) polymerase, depending on where they were discovered. The function of these enzymes is to recognize the end of a short stretch of double-stranded DNA formed by the primer and the cDNA and then elongate it so that the entire length of that piece of DNA becomes double stranded. The initial stretch of double-stranded DNA is generated by you, the investigator, by adding the primers discussed below along with free nucleotides and the DNA polymerase.

The key step is that you design the primers so that they are specific only to your cDNA of interest. In other words, with hundreds of thousands of mRNAs present in a cell, this method lets you pick out and amplify just one. These primers are oriented so that their growing ends are directed toward each other. If two such primers flank the target DNA sequence, over a million-fold amplification of a single starting target sequence can be achieved. This is because the amplification comes about as part of a chain reaction, or exponential process. This is achieved by successively heating and cooling the sample so that the double strands of DNA will fall apart (when heated) and then the primers will reanneal (when cooled) and be extended again by the DNA polymerase. Since there is an excess of single-stranded DNA available, each cycle will increase the number of copies, so it is like a chain reaction (figure 2.2). After the reaction is completed, usually allowing for 30 to 35 cycles of amplification, melting, reannealing and amplification, the resulting DNA must be separated by gel electrophoresis and visualized to assure that a band of the appropriate molecular weight has been achieved. In this case, the band is visualized

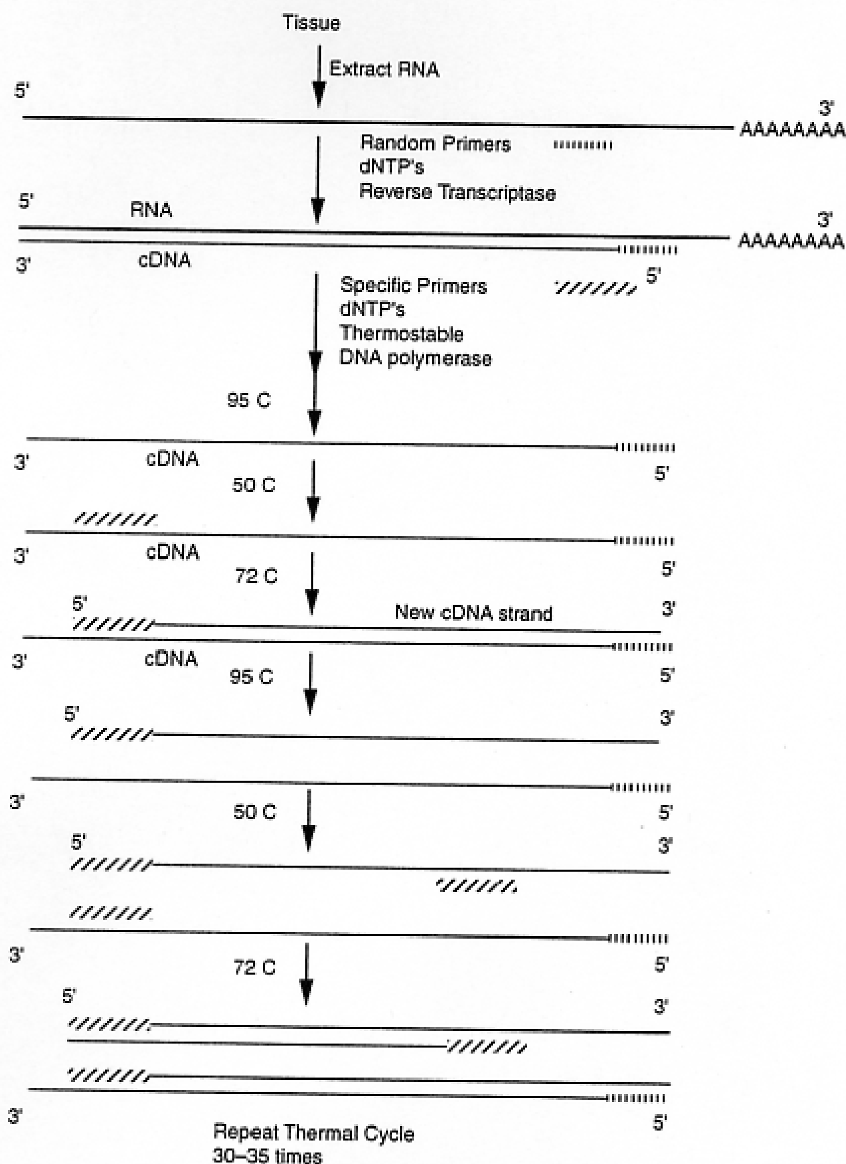


Figure 2.2 Reverse transcriptase polymerase chain reaction: RT-PCR begins with the extraction of RNA from a tissue known to contain an mRNA of interest. This is then converted into DNA using the enzyme reverse transcriptase. The addition of specific primers would then allow for the amplification of only the original mRNA of interest so that it could be selectively amplified from all the others. Repeated cycles of heating and cooling allow for annealing of the primers, primer extension (amplification of the cDNA), and denaturation (separation of the DNA strands so that they can be amplified again). The result is an exponential increase in the rate of synthesis of a particular stretch of cDNA. As indicated in the text, a nested primer strategy will increase the likelihood of obtaining the correct mRNA.

directly in the gel used for electrophoresis by soaking it in ethidium bromide, a chemical that naturally intercalates into double-stranded DNA and fluoresces when exposed to ultraviolet light.

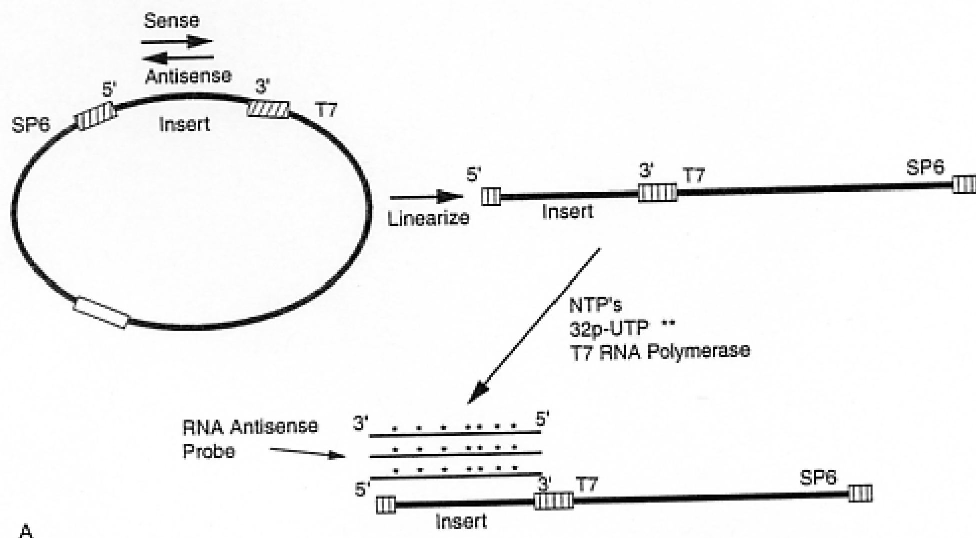
The RT-PCR technique has many uses and, as you can imagine, is extremely sensitive. With this method you are able to detect a single copy of mRNA. Under very carefully controlled conditions, the amount of PCR product produced can be related to the amount of mRNA originally found in the sample and can therefore give a quantitative estimate.

IN SITU HYBRIDIZATION

A frequently asked question in behavioral neuroendocrinology is how do steroids or other stimuli influence the expression, or mRNA levels, of a particular gene. There are a variety of techniques available for quantifying mRNA. We now have the ability to measure the mRNA in an individual cell maintained in its natural place in the brain. *In situ* means "being in place." This is accomplished by first cutting the brain area of interest into very thin slices, usually while the brain is kept frozen. The sections are placed on slides and then a solution that contains a probe for a particular mRNA is pooled on top of the section. This solution optimizes conditions so that the probe can hybridize with the mRNA. Hybridization takes place in airtight plastic containers such as Tupperware lined with moistened paper towels to keep the incubation chamber humid. The container is then placed in an oven and the reaction allowed to proceed overnight at temperatures of 37 to 50 °C. The reason the probe can hybridize to a specific mRNA is that it is a piece of either synthetic DNA or RNA that consists of a nucleotide sequence that is complementary to a portion of the mRNA of interest. In other words, the probe is an antisense sequence to the sense sequence of the mRNA.

A commonly used control is a synthetic sense sequence that is now identical to, not complementary to, the mRNA and therefore will not hybridize. Instead, when the tissue undergoes a series of washes, this sense probe will be washed away. Of course, hybridization kinetics are not perfect and some of the antisense and sense probes will stick to the tissue. As much as possible of this nonspecific hybridization is removed by the washing procedure, which increases by a property known as stringency. By lowering the salt concentration and raising the temperature, i.e., increasing the stringency, it is made increasingly difficult for the nonspecific hybridization to be maintained. When completed, the overwhelming majority of the remaining probe should only be hybridized to the mRNA of interest.

All that is required now is to visualize the remaining probe. In most cases, the probe has been radiolabeled with an isotope attached to one or more of the nucleotides. The probe can then be detected in one of two ways. One way is to appose the slides to X-ray film in the dark for a period of time. This will give an **autoradiogram**, or a film image of the entire section (figure 2.3). Areas where there is a lot of probe will appear dark on the film and areas where



B

C

Figure 2.3 In panel (*A*) you see a schematic representation of how a riboprobe used for in situ hybridization detection of an mRNA is generated from a plasmid insert. The circular plasmid is linearized using a restriction enzyme that cuts only one place. Then enzymes specific to the SP6 or T7 promoters will transcribe the insert in either the antisense or sense direction. Radioactive UTP is used in the reaction and incorporated into the probe, which can now be detected by autoradiography or on photographic emulsion. The images in panel (*B*) are autoradiograms made on film by a radioactive probe for the enzyme glutamic acid decarboxylase (GAD), which synthesizes GABA. These are coronal sections, one through the mediobasal hypothalamus and the hippocampus and the other through the preoptic area. Darker areas indicate regions of high mRNA expression, and light areas indicate that little to no mRNA is present. Note how there is a heterogenous distribution of the mRNA for this enzyme throughout the brain. The image in panel (*C*) is a high magnification of cells lining the third ventricle in the turkey brain, which are expressing GnRH. The dark grains are in the emulsion that coats the slide and are generated by the radioactivity released from the probe used to detect the GnRH mRNA in the brain section.

there is little probe will appear light. However, this makes resolution of individual cells impossible.

A second way to detect the probe, which will yield resolution of individual cells, is to dip the slides in photographic emulsion, which is essentially liquid film. This process must be done in the dark. The emulsion is allowed to dry on the slides, which are then stored in light-protected boxes for a period of days to weeks after which the emulsion is "developed" just like a photograph. Only in this case, the photograph consists of small black dots or silver grains that form as the radioactive particles being released by the probe on the tissue pass through the emulsion. The cells themselves are visualized by counterstaining the tissue with a Nissl stain such as cresyl violet. Cells that contain a high level of probe will have lots of silver grains over their surface whereas cells that do not have probe should have few to no grains over their surface. In fact, the number of grains per cell can actually be counted and this can be used as an index of how much mRNA there was in that particular neuron. A comparison can then be made between brain sections from two different conditions, such as male versus female, estrus versus proestrus, and young versus old.

As with all techniques, in situ hybridization histochemistry has its drawbacks. One is that it is considered "semiquantitative," meaning that it is often difficult to obtain highly reliable quantitative data because there are numerous sources of variation in the protocol. A second drawback is that the technique uses radioactivity to interact with the photographic emulsion, which is of concern to many scientists. Methods for conducting in situ hybridization without radioactivity have been developed and are likely to become more common as their resolution is improved. Radioactive in situ hybridization is also a procedure that can take weeks to months to complete (Eberwine et al. 1994). Since you are afraid that the gastric-brooding frog is on the verge of extinction, time is of the essence and it would be advisable to seek a faster technique for quantifying your estrogen receptor mRNA.

RNASE PROTECTION ASSAY

The RNase protection assay is a quantitative method used to measure RNA levels in homogenized tissues or cells which is relatively quick compared to in situ hybridization. The procedure is sometimes called solution hybridization because the hybridization reaction occurs in a solution in the tubes containing samples or standards. This is in contrast to the application of probe to tissues as in in situ hybridization, or hybridization of probe to RNA that has been immobilized on a filter, as in the case of Northern Blot.

In the RNase protection assay, an RNA probe is allowed to hybridize with RNA in extractions of dissected tissues or cell cultures. Additionally, a standard curve is produced using increasing amounts of known sense, or reference, RNA. The antisense riboprobe is allowed to hybridize with RNA in these standards. This standard curve is later used as a basis of comparison for

quantification of the absolute amount of RNA in the unknown samples. You may have noticed that this is similar to the technique employed in radioimmunoassay (see chapter 1) in which known amounts of hormones are used to produce a standard curve. One advantage of the RNase protection assay is that it allows absolute as opposed to relative amounts of the RNA to be quantified.

The methodology of the RNase protection assay is as follows: First, a plasmid containing the cDNA sequence of interest is linearized. Often the cDNA insert is cloned into a plasmid containing two RNA promoter sites, one at the 5′ end and one at the 3′ end, which can be used to make both sense and antisense RNAs. For the antisense RNA, a radiolabelled ribonucleotide (e.g., alpha^{32}P UTP) is used in the transcription of the probe. This radioactivity is later used for quantitation of how much the probe hybridizes with the sample or standards for analysis (McCarthy 1998). Once the probe is made, it is added in excess to the standard curve dilution tubes as well as to the homogenized and extracted RNA samples containing unknown amounts of the specific RNA, such as oxytocin receptor in the rat or estrogen receptor in the whiptail lizard.

The samples hybridize overnight and then a buffer containing a mixture of two RNases is added to the reaction. These RNases are enzymes that specifically digest single-stranded RNA. Thus, any double-stranded hybrids formed by the binding of the probe to the sample or standard are spared from the digestion. However, excess unbound probe and any unbound RNA not complementary to the probe (which is the majority of the RNA in the unknown sample tubes) will be digested by this RNase mixture. It is this step that gives the procedure the name RNase protection assay, or RPA; that is, the double-stranded hybrids are protected from RNase digestion.

After RNase digestion, samples are processed to purify and concentrate the RNA hybrids. The molecules are then separated by molecular size using electrophoresis and the gels exposed to X-ray film to produce an autoradiogram. The amount of radioactivity in the standard curve increases linearly with amount of RNA in the standard, and a regression analysis is performed to relate the amount of radioactivity to the absolute amounts of RNA in the standard curve. This regression analysis is used to calculate how much RNA is in each unknown sample, since the amount of radioactivity in each sample can be quantified, and the amount of RNA extrapolated from the regression curve can be calculated from the standard curve.

Ribonuclease protection assays are quite sensitive and allow detection of RNA amounts as low as picogram (10^{-12}) quantities. Another advantage is that many different RNA transcripts (up to 12 at last count) can be measured in the same sample. This is accomplished using riboprobes of differing molecular sizes. Each probe will hybridize specifically to its complement, thereby enabling multiple RNAs to be quantified without interfering with the hybridization of other probes. During electrophoresis, multiple hybridized RNA bands can be resolved on the gel as long as the probes are different lengths.

Therefore, if we wanted to and we planned correctly, we could simultaneously measure the receptors for estradiol, progesterone, and oxytocin in the same sample of rat brain.

But the brain is a highly heterogeneous structure and not all neurons in an estrogen-concentrating area will have receptors for estradiol, progesterone, and oxytocin; that is, a particular gene might be regulated by estradiol in only a few select cells. Pooling together large chunks of tissue could dilute or wash out any specific effects of the estradiol on individual neurons. This lack of anatomical specificity can be overcome in part, however, if specific brain areas are examined. The most common method is to place thick (~300 μM) frozen sections of the brain on microscope slides (making sure they are RNase-free). Then, using a dissection microscope and hollow tubing, usually a spinal tap needle (also RNase-free), you can remove or "punch" those areas of the brain of particular interest (Palkovits 1988). You might think of this as the cookie-cutter method. These punches, which may be a circumscribed brain nucleus like the VMH, can then be assayed. Still, even these procedures do not replace the cellular level of specificity afforded by in situ hybridization.

QUANTITATIVE PCR

We have already discussed the technique of PCR at some length as a tool in cloning. However, given its sensitivity and selectivity, this technique also lends itself to quantification. The procedure is essentially the same as the RNase protection assay with two notable changes. One is that great care is taken to use the exact same amount of starting mRNA for each reaction that is being compared in a particular experiment. The second is that an internal control is included in the reaction. This control can be one of two types. It can be a standard template with its own primers that you include in the reaction and use to compare across reactions. This will control for differences in the rate of the reaction or slight differences in the amount of enzyme, template, nucleotides, and so on that were added to the reaction tube.

Given that PCR involves exponential amplification of mRNA, it is not hard to imagine that small variances in any of these parameters can have big effects on the amount of final PCR product. This approach allows for relative comparison between samples. The second type of control involves creating a competitive template that your primers will compete for. The competitive template is identical to your PCR product of interest but has been mutated so that a large piece is missing. This allows you to distinguish the endogenous PCR product and the template when separated by electrophoresis. This approach has the additional advantage of allowing for the construction of a standard curve (similar in principle to that described for the RNase protection assay) so that the absolute amount of the endogenous mRNA can be calculated. Again, the lack of cellular resolution that comes from homogenizing chunks of tissue can be lessened if micropunches are taken of the target and control brain areas.

Isolating and Cloning a Gene

SOUTHERN BLOTS

A **Southern blot** is essentially the same as a Northern blot with the exception that instead of extracting mRNA from the cell, the genomic DNA is extracted. In general, for behavioral neuroendocrinologists, this technique is used to confirm that a particular gene or allelic variant of a gene is or is not present in one or two copies in a particular animal. The genotyping of transgenic animals uses this technique to determine if an animal is a homozygote, heterozygote, or wild-type.

In fact, unless you limit your studies to a species where the entire genome has been cloned and mapped, at some time in your illustrious career you are likely to need to clone a gene. Let's say you want to know whether an estrogen-receptor gene's expression is controlled by day length, diet, or behavioral interactions. To your dismay, the gene has not yet been cloned in the gastric-brooding frog. This will require you to get out your toolbox of molecular biology tricks, roll up your sleeves, and subclone it yourself.

TISSUE CHOICE AND RNA ISOLATION

In order to isolate your gene, you start with cells that make the product of your gene. First, you choose a tissue that contains abundant levels of the target RNA transcript needed, and then you can use the mRNA to pull out the DNA you are looking for. Let's use a steroid hormone receptor as an example. Since the mRNA of steroid hormone receptors is rare in the brain, you'll want to use another tissue that is rich in the hormone receptor you are interested in—the uterus, for instance, which contains abundant estrogen receptor mRNA that can be enriched further with an injection of exogenous estradiol into the animal. You harvest the tissue and immediately freeze it in liquid nitrogen until enough has been accumulated. Usually several grams are collected, but methods have been developed recently that enable you to suck up the contents of a single cell and apply some of the techniques described below. In most instances, a total RNA preparation is then performed from which the mRNA is isolated, usually comprising only 1 to 2% of the total RNA. This isolation is done by selecting the mRNA from the total RNA using reagents that bind the mRNA at the poly-A tail. Quantification of pure mRNA is then confirmed using an ultraviolet spectrophotometer. With this material in hand you are now ready to do some molecular genetic analyses.

PRIMER DESIGN

Your next step in the cloning process is an intellectual one, the designing of the primers to be used for the PCR which you are going to use again, this time to pull out the DNA you are interested in isolating. If you pick the right primers, your job will be easier. As before, primers are short stretches of synthetic DNA, usually 18 to 22 bases long, that are complementary to a portion of our gene of interest. Remember that all DNA is double stranded, but

only one of the strands codes for our gene; the other strand is said to be *complementary*. So, one of our complementary primers is "upstream" on the gene (toward the 5′ end) and the other is "downstream" (toward the 3′ end). When we do PCR with these two primers, we get a product that is the complementary sequence from the first primer through the end of the second primer. The distance by which they are separated will dictate the size of our PCR product. For instance, if the primers are separated by ~300 base pairs, the PCR reaction will produce a product that is ~300 base pairs long.

The nucleic acid sequence for steroid receptors was first elaborated for conventional animal species such as the mouse, rat, guinea pig, monkey and human. In many instances this work has been extended to domesticated species such as the chicken, dog, pig, cow, and horse, and in some instances, wild species of bird, fish, amphibian and reptile. With this diverse group of species, we find there is substantial overlap at the amino acid level in the DNA-binding and ligand-binding domains; this is called serial homology and usually is expressed as the percentage of amino acid residues identical to the sequence of the mouse, rat, or human (figure 2.4).

Conservation at the amino acid level doesn't necessarily guarantee conservation at the nucleotide level due to the **degeneracy** of the genetic code (some amino acids are coded by as many as six different codons). Even with this caveat, however, the amino acid–sequence information can be used to create degenerate oligonucleotide primers, which usually consist of 10 to 20 nucleotides. These primers will amplify the "sense" or "+" strand and are used in conjunction with an "antisense" or "−" primer, which is the reverse complement of the receptor sequence. When working with steroid receptors, these primer sequences are usually located in the Hormone- or ligand-Binding Domain (HBD) because it is a highly conserved region.

In cloning and sequencing by RT-PCR, sometimes it is advisable to use a "nested primer" approach. The advantage of this strategy becomes apparent when cloning a member of a family of genes, such as steroid hormone receptors. A characteristic of these families is that they share a number of conserved regions or domains that are very similar (e.g., portions of the HBD and the hinge region, the intervening domain between the HBD and the DNA binding domain). In the nested approach there are two rounds of 25 cycles of PCR amplification. The primers in the first round of PCR amplification may amplify not only the desired gene, but also related members of the gene family. This leads to multiple products and more extensive screening to identify the desired target. Using a second pair of primers that lie between, or are internal to, the original pair of primers during the second round of PCR frequently leads to the selective amplification of the desired target.

So, for the case of the gastric-brooding frog, you would design nested PCR primers based on the sequence from the most closely related species you could find. Next, you would isolate some RNA from a collection of steroid hormone–concentrating tissues such as gonads, oviducts, vas deferens, and liver, conduct the PCR under highly permissive conditions, and *voila*! You've got it.

DNA Binding Domain | Hinge Region | Hormone Binding Domain

A/B | C | D | E | F

primer 1 | primer 2

Lycuer 1 920 bp

RPA probe — Lycuer 1a 380 bp

Lycuer 1b 540 bp — in situ hybridization probe

	C	D	E
Turtle	100%	62%	91%
Chicken	100%	55%	92%
Rat	100%	53%	92%
Human	100%	53%	91%

Consensus – – – K – K R Q R – – – – – R – – – – – – – – – – R – – – – – –
Lizard r i l K h K R Q R e e h d n R n a g n i v e r R s p n l w p
Turtle r m l K h K R Q R s s q d a R t a g p s t e m R s p h p l t
Chicken e m m K q K R Q R e e q d s R n g e a s s t e l R a p t l w t
Rat r m l K h K R Q R d d l e g R n e n g t s g d m R a a n l w p
Human r m l K h K R Q R d d g e g R g e v g s a g d m R a a n l w p

Consensus S P L – – – – – K K N S – A L S L T A – Q – V S A L L – A E P P
Lizard S P L m i t h n K K N S p A L S L T A d Q i V S A L L e A E P P
Turtle S P L v i k h s K K N S p A L S L R A e Q m V S A L L e A E P P
Chicken S P L v v k h n K K N S p A L S L T A e Q m V S A L L e A E P P
Rat S P L v i k h t K K N S p A L S L T A d Q m V S A L L d A E P P
Human S P L m i k r s K K N S l A L S L T A d Q m V S A L L d A E P P

Figure 2.4 Cloning of the estrogen receptor from the whiptail lizard. At the top is a schematic diagram of functional domains of the estrogen receptor cDNA. Below that is a depiction of the clones and subclones generated to characterize the estrogen receptor of the whiptail lizard, *Cnemidophorus uniparens*. The 920 bp PCR clone of the whiptail lizard estrogen receptor spans from the DNA-binding domain to the end of the hormone-binding domain. Subclones of the fragment can be used as probes for RNase protection assay and in situ hybridization, as indicated. The remainder of the figure is a comparison of the deduced amino acid sequence of the whiptail lizard estrogen receptor to published sequences of estrogen receptor of the turtle, chicken, rat, and human. The sequences shown are comparisons of the relatively nonconserved hinge region (boxed) and a portion of the hormone-binding domain. Percentages indicate the degree of serial homology among the different vertebrate species listed. Dashes indicate nonconserved amino acid in the consensus sequence; space indicates an amino acid deletion. (From Young et al. 1994.)

LIGATION OF THE PCR PRODUCT INTO THE PLASMID VECTOR

The next step is to clone the DNA that has just been isolated. To do this, the PCR product must be ligated or joined to a vector for subsequent transformation into a bacterial host. A **plasmid** is a special form of chromosome found in bacteria which is circular and exists outside the nucleus. This unusual feature gives the unique advantage of letting us make lots of plasmid by growing up large numbers of bacteria. The PCR product is inserted into a vector (or plasmid) using enzymes that cut open the circular DNA and then insert the PCR product, creating a recombinant hybrid molecule (figure 2.5). Once transformed into bacteria, these hybrids replicate in the same manner as the original plasmid would. Therefore, as the bacteria multiply, so do the recombinant plasmids within them. The ultimate outcome is the production of large amounts of the clone from the bacterial hosts. Different types of plasmids have been engineered by molecular biologists and can be chosen to fit with your particular experimental needs. You need only thumb through the catalog and go shopping for the right size, style, and color

TRANSFORMATION OF LIGATED VECTOR INTO E. COLI

Once the PCR product has been ligated into the vector, it is introduced into a bacterial host. This process, called **transformation** or transfection, involves the uptake of the exogenous hybrid DNA by the bacteria through its plasma membrane. Bacterial cells that are treated to perform this task are referred to as competent cells. The transformed cells are grown in media and then plated on ampicillin-containing agar plates. Ampicillin, you might recall, is an antibiotic. Since the transformed cells have a gene that confers resistance to ampicillin, only bacteria that have been successfully transformed with the insert will grow. The next thing is to make sure that your cells are producing the gene that you have isolated.

A colorometric assay is often used to provide a second level of selection. For example, a vector may contain a portion of the *lac*Z gene, which normally codes for the enzyme beta-galactosidase. When your cells are grown under the right conditions, white colonies are produced when a recombinant vector containing the *lac*Z gene has been transformed into the host. While the appearance of white cells tentatively indicates that the vector possesses an insert of some sort, it does not guarantee that the PCR insert contained the desired target product (i.e., both the *lac*Z gene and your PCR product). Thus, a number of white colonies must be selected for further analysis by restriction mapping and DNA sequencing to establish the clone that has the desired target gene. Each colony is then digested with restriction endonuclease enzymes that recognize short DNA sequences and catalyze the cleavage of double stranded DNA (dsDNA) at specific sites at or near the recognition sequence. A specific pattern of digestion products is predicted from a known sequence. If the pattern generated experimentally matches that predicted, then you have subcloned what you think you have. A second replica of the original colonies

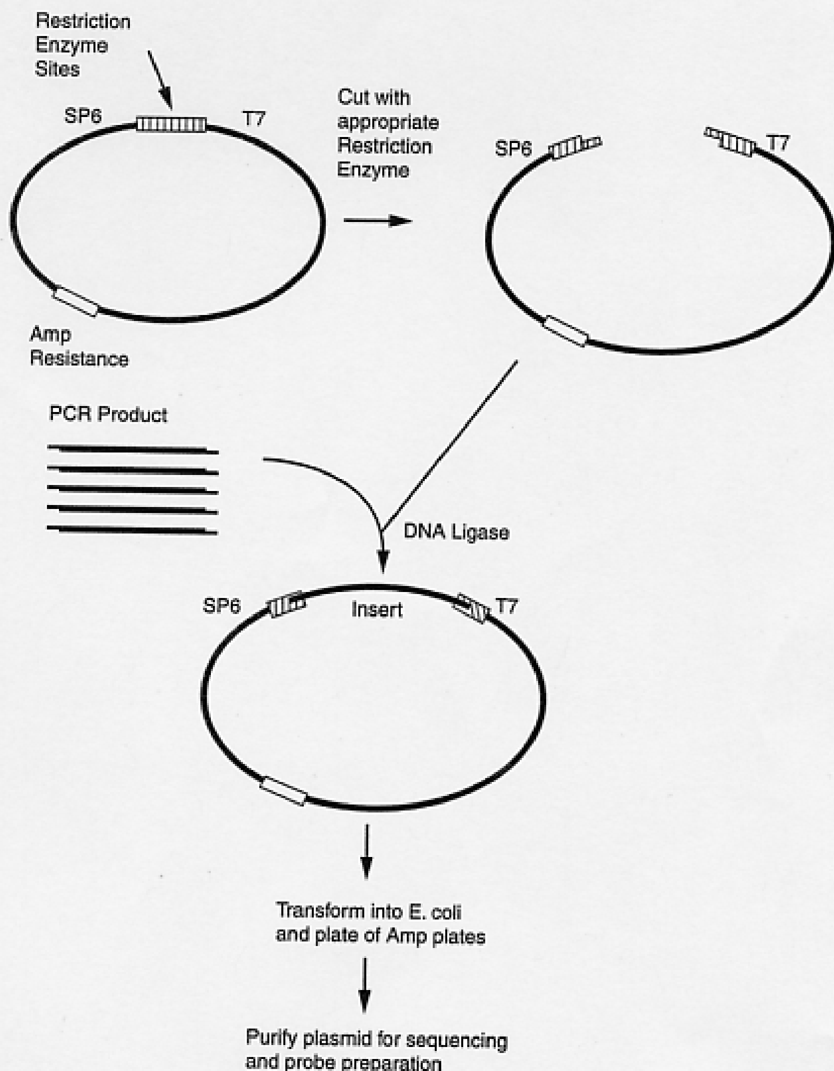


Figure 2.5 Insertion of a PCR product into a plasmid vector. Plasmids are naturally occurring circular pieces of DNA found in bacteria. They contain DNA sequences which can be cut by specific enzymes, called restriction enzymes. The plasmid DNA can be cut at a single site, opening up the circle, and under the appropriate conditions, a piece of DNA that has been generated by PCR can be inserted into this spot and the circle closed again with an enzyme called DNA ligase. The plasmid is then reinserted into its bacterial host and grown in large numbers. This will allow for the subsequent use of the plasmid to generate probes for in situ hybridization, RNase protection assay, and Northern blots.

that does not undergo restriction endonuclease digestion is kept and can be used for additional purposes.

SEQUENCING THE PRESUMPTIVE CLONES

The presumptive clones are sequenced today using kits. For example, a kit may employ the dideoxy-DNA sequencing method from a single-stranded DNA template. In this procedure, the plasmid template is denatured to separate the dsDNA. Oligonucleotide primers added to the reaction tube are allowed to anneal to its target sequence on the clones. A sequencing buffer solution containing a mixture of deoxynucleotides to serve as the reaction substrate is also added to the reaction tube. One of these deoxynucleotides is radioactively labeled with Sulfur-35 to facilitate detection and visualization of the reaction products on an autoradiographic film. The sequencing reaction is initiated by adding the sequencing enzyme to the reaction tube.

The sequencing reaction contents are given a quick spin in a microcentrifuge, which allows the dideoxynucleotides to participate in the sequencing reaction. The sequencing reaction generates a variety of nucleic acid fragments of varying lengths with a fixed 5′ end and variable 3′ ends that depend on when the dideoxynucleotide is incorporated into the nucleic acid. These fragments are then separated on a denaturing polyacrylamide gel, which can resolve single nucleotide bases. Lastly, voltage is applied to the sequencing gel apparatus to separate the nucleic acid fragments. The DNA sequence is then read directly from the autoradiographic film, starting at the bottom of the film and working to the top. This corresponds to the 5′ to 3′ direction, which is the convention for reporting nucleic acid sequences.

If all has gone well, you will be able to read the sequence of your subclone and compare it to the published sequences for large numbers of genes, which are available on the Internet at a site called the Genbank. Using very simple commands, you can compare your sequence to all those already existing and see if there is any match that would indicate significant serial homology. As you can see in figure 2.4, the estrogen receptor cloned from the whiptail lizard is highly homologous to the estrogen receptor of other vertebrates.

Although it is important to understand the basic principles used for nucleotide sequencing, it is unlikely that you will find yourself in the position of ever actually using this technique. The advent of automated sequencers that can efficiently sequence large numbers of nucleotides at a reasonable cost is rapidly making manual sequencing seem as quaint as a black and white television. It is now much more common to send your DNA sample to a sequencing facility which will then e-mail you results that you can quickly check against the databases available at Genbank. All too often you may find you have actually cloned a contaminant from a neighboring lab bench instead of the estrogen receptor of the gastric-brooding frog; however, with greater attention to working on clean surfaces with pure reagents, you will succeed. Once you do clone the desired gene and a probe has been successfully manufactured, you can move on to the process of using it to ask questions about how

gene expression changes under various experimental conditions. Several different approaches can be taken at this point, each with its own advantages and disadvantages.

cDNA ARRAYS

We noted previously that the RNase protection assay is sometimes referred to as "solution hybridization" because the assay is carried out in liquid. A variant of this assay involves attaching a template for a particular gene to a substrate, such as a nylon filter, and then allowing your probe to hybridize to this immobilized substrate. This is referred to as "slot blot" hybridization since probes are sometimes administered through a slot in an apparatus.

Recently biotech companies have carried this approach to another level by manufacturing arrays of multiple genes immobilized on a suitable substrate such as glass (DNA sticks to glass). These arrays may contain hundreds of identified genes. The investigator prepares a unique set of probes from each experimental condition, only in this case, rather than being a probe for a single gene of interest, all of the mRNAs represented in a sample are labeled and available to hybridize to the immobilized cDNA array. In this way, if several genes exhibit altered expression, in a particular experimental condition, either down or up, they can be detected simultaneously. The most advanced form of this technology, called "DNA chip technology," involves tens of thousands of genes immobilized on a glass microchip smaller than a postage stamp. These displays look like New York City on a clear night viewed from an airplane. This exciting new technology is likely to change the way we investigate gene expression in the near future.

Techniques for Measuring Proteins

In addition to knowing the levels of gene expression, it is often of equal if not greater importance to assess the level of protein for a particular receptor or gene product. In fact, levels of mRNA do not necessarily translate into equivalent levels of protein. Differences in the rate of translation, stability, and half-life of the mRNA can all contribute to variance between levels of mRNA and protein. Since the protein is one step closer to the physiological outcome, it is often more important to quantify protein levels. Unfortunately, our techniques for measuring proteins are more restricted than those for measuring gene expression.

WESTERN BLOTS

The **Western blot** is analagous to the Northern and Southern blot techniques. The advent of the Western blot heralded a whole new direction in this type of analysis in that it involves the measurement of the actual protein rather than the mRNA or DNA. Most of the steps are essentially the same as for the Northern or Southern blots, except that the cellular content extracted is protein instead of nucleic acids and the probe consists of an antibody to the protein rather than a synthetic antisense RNA or DNA.

The procedure requires the generation of an antibody to a particular protein, which is accomplished by injecting a pure form of the protein into another species, which will recognize it as foreign (i.e., an antigen) and make antibodies to it. A commonly used species for antibody production is the rabbit, which will be injected with rat or human estrogen receptor or whatever other protein is of interest, such as oxytocin receptor. This would be referred to as a rabbit-anti-rat antibody and is the **primary antibody**. Fortunately, this primary antibody can often be used in a variety of other species but that has to be determined on an experimental basis. An antibody made for estrogen receptor in the whiptail lizard might very well work in the gastric-brooding frog, but it just as well might not.

The primary antibody is labeled with a substrate for an enzyme that when reacted will release light and can therefore be detected by X-ray film in the same manner as radioactivity. Again a molecular weight ladder of proteins of various sizes must be used to confirm that your band appears at the appropriate place, and more problematically, that there is only one band. The latter criterion is particularly important since antibodies are notorious for their ability to crossreact with more than one protein. Nonetheless, this can be an extremely powerful approach. For instance, although you may see a 50-fold increase in the mRNA levels of the progesterone receptor following estradiol treatment, this does not mean there has been a commensurate change in the receptor protein levels. By using a Western blot you can confirm or deny that there is a similar level of change between mRNA and protein.

IMMUNOCYTOCHEMISTRY

We have just discussed the use of Western blot to measure protein in chunks of whole tissue. This is an antibody-based procedure and, if you are lucky, the same antibody used in a Western blot can also be used for immunocytochemistry. As its name implies, immunocytochemistry is an antibody-based procedure; however, it involves maintaining the integrity of the cell so that proteins can be visualized in their appropriate intracellular location. It also allows for an assessment of cellular heterogeneity within the brain where one neuron may express a particular protein, such as the estrogen receptor, but the neuron immediately next door may not.

Immunocytochemistry requires two antibodies. First, the primary antibody, which is generated to the protein you want to visualize. Then it is necessary to have a secondary antibody, which is made in another species. The secondary antibody serves the purpose of recognizing all antibodies made by the first species. So, for instance, a goat will be immunized against a rabbit, and this is referred to as goat-anti-rabbit and is the **secondary antibody**. The secondary antibody is conjugated to some sort of signal marker, such as a biotin-strepavidin, or more commonly, to an enzyme such as alkaline phosphatase, which due to its catalytic nature will amplify the signal. In the case of alkaline phosphatase, the substrate is diaminobenzadine (DAB) which when reacted makes a brown reaction product that allows us to visualize the antibody.

Sometimes secondary antibodies are conjugated to fluorescent markers and can be visualized directly using a fluorescence microscope.

In either case, it is the secondary antibody we actually see, not the primary, but we can see it only if we have conjugated some signal marker to the secondary. The careful reader might now ask, but why use the secondary antibody at all? Why not just visualize the primary antibody? There are two reasons. One is that conjugating a signal molecule to the primary antibody could easily compromise its ability to bind to the target antigen, such as the estrogen receptor. The second is that the chemical procedure of labeling the antibody is wasteful, because up to half the antibody can be lost in the procedure. Antibodies generated against an entire species, such as goat-anti-rabbit, can be obtained by the proverbial bucket load, whereas a highly selective antibody to estrogen receptor in the gastric-brooding frog might only be generated by the thimbleful. So we take advantage of the ease of generating secondary antibodies to help us in visualizing our precious primary antibody.

The good news is that many primary and secondary antibodies are commercially available and kits containing all the ingredients you need for immunocytochemistry make these procedures fairly straightforward these days. If all goes well, only those cells containing our protein of interest will bind the primary antibody, and therefore the secondary as well, and we will be able to see which cells selectively have the protein. However, it is difficult to make quantitative statements about proteins visualized in this manner and we usually have to resort to an "all or none" or "high versus low" statement. Sometimes under very carefully controlled conditions, comparisons can be made between treatments so that one animal can be said to have relatively more protein/cell than another does. The development of new quantitative ("stereological") techniques has enabled a more reliable quantitative analysis of amounts of protein visualized by immunocytochemistry.

CIRCUIT MAPPING WITH THE USE OF IMMEDIATE EARLY GENES

One avenue in which immunocytochemistry has proven to be of great benefit is in the detection of what are known as **immediate early genes.** This refers to a family of genes that are some of the first turned on in response to a stimulus. These genes are often not detected until the cell is stimulated; in other words they are "inducible." The most famous and frequently used of these is *fos*, and its protein product Fos. Also of frequent interest are the gene *jun* and its protein product Jun. Just like steroid receptors, Fos and Jun are transcription factors, and they can interact (dimerize) with each other and, in the case of Jun, with themselves. Fos/Jun and Jun/Jun dimers then interact with the DNA at a particular place on a gene called an AP-1 site, where AP stands for "activator protein". Steroid receptors, such as the estrogen receptor and the glucocorticoid receptor, can also interact with Fos/Jun complexes and thereby indirectly influence transcription at AP-1 sites.

Because they are transcription factors, Fos and Jun are nuclear proteins and they are readily visualized by immunocytochemistry. In the nervous system,

when a neuron is activated or "depolarized" it will frequently express the *fos* gene as a consequence. The usual time course is an increase in Fos protein by 30 minutes, with levels diminishing within 2 to 4 hrs (there is a rapid negative feedback loop between Fos protein and *fos* gene expression). This attribute has proven of great benefit to behavioral neuroendocrinologists since it can be used to essentially map which neurons are activated in response to the expression of a particular behavior or exposure to a particular stimulus. For instance, when female rats display the sexually receptive posture of lordosis, neurons in the ventromedial hypothalamus and preoptic area express high levels of Fos (Flanagan-Cato and McEwen 1995; Auger et al. 1996; Pfaus et al. 1996). Similarly, male rats exposed to the odors of a sexually receptive female exhibit high levels of Fos in the amygdala (Coolen et al. 1997).

A further advantage of Fos immunocytochemistry as a marker for neuronal activation is that in many cases it can be combined with assays for a cytoplasmic protein, allowing the investigator to establish the neurochemical identity of a particular cell. For instance, you may wish to know whether oxytocin neurons are particularly activated following mating behavior. The picture in figure 2.6 demonstrates the activation of GnRH neurons occurring at the time of the LH-surge in a female rat (Hoffman et al. 1990). Many examples of the use of Fos as a marker will be apparent throughout the text.

IN VITRO RECEPTOR AUTORADIOGRAPHY

There is one other way to visualize proteins in the brain that can be very useful and is quantitative, but it is restricted to the detection of receptors. Most commonly, this technique is used for peptide receptors, such as those for neurotransmitters. The approach is quite simple. Very thin slices of the brain mounted on glass slides and maintained at a cold temperature are incubated in a buffer containing the ligand of interest and binding is allowed to occur. The ligand is tagged with a radioisotope such as tritium (^{3}H) or iodine 125 (^{125}I). After a series of washes to remove the unbound ligand, the slides containing the tissue sections are apposed to isotope-sensitive film, which is placed in the dark and allowed to expose for a number of days to weeks (short for iodine, long for tritium). When the film is developed, there is an image of where in the brain the ligand is bound to the receptor. For example, the GABA-A receptor is distributed widely throughout the brain, whereas the oxytocin receptor is found more selectively in the amygdala and ventromedial hypothalamus of the female rat (figure 2.7). If a set of standards with which to generate a standard curve is included in the assay, absolute amounts of the protein can be quantified using a technique called densitometery, which simply refers to measuring the density of signal of a particular area on the film. One drawback of this technique with peptide receptors is that cellular resolution is lost because the image of the brain slice obtained is not of sufficient resolution to allow the detection of individual cells.

Steroid receptors can also be visualized by receptor autoradiography but with some challenging twists. The incubation and washing procedures

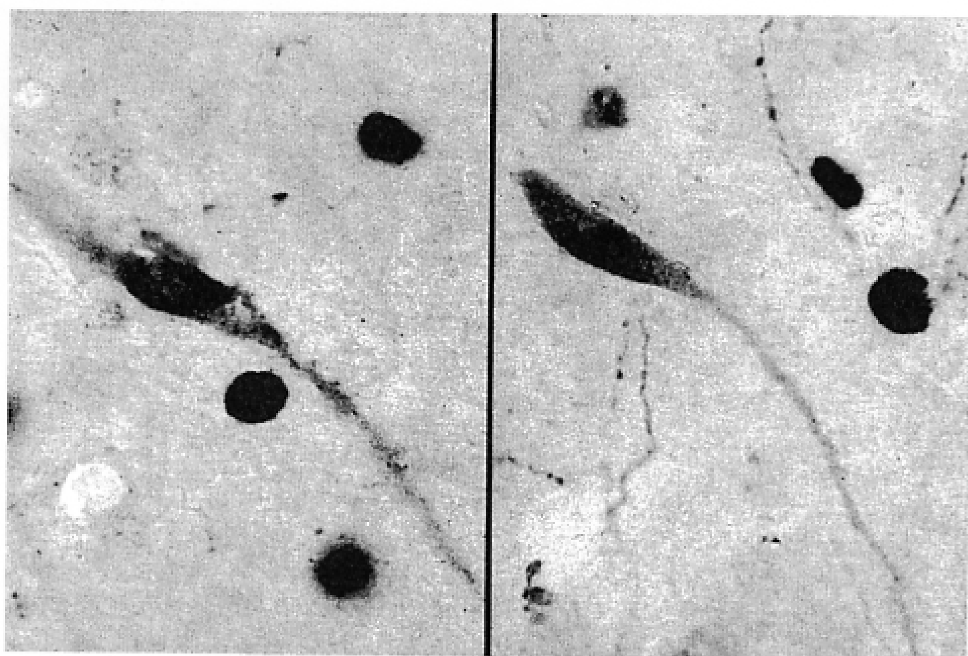


Figure 2.6 Combined immunocytochemistry for Fos and GnRH. The immediate early gene Fos is a nuclear protein and appears as a dark black circle. It is detected by a conventional immunocytochemical technique but enhanced with nickel sulfate to appear black. The lighter-stained material in the cell cytoplasm is the neuropeptide GnRH, also detected by conventional immunocytochemistry and having a grayish caste. These are cells from the preoptic area. The panel on left is from a brain just before the LH-surge, and you can see there is no Fos protein in the nucleus. The panel on the right is from a brain at the time of the LH-surge, when GnRH neurons are activated, as evidenced by the Fos signal in this neuron. Note that other cell types are also activated, as indicated by the presence of a dark nucleus, but the neurochemical identity of those cells remains unknown. (Courtesy of G. E. Hoffman.)

routinely used as a part of this technique will uncouple the steroid ligand from its receptor and all resolution will be lost. Therefore, the steroid ligand must be injected into the animal and allowed to bind in vivo. Remember that the ligand is radioactive, which makes it expensive (mostly because of the costs of disposal, not synthesis), so when using mammals such as rats or monkeys, it is usually given to the animal via an indwelling catheter into the jugular vein; with relatively small animals weighing 20 grams or less, the radioactive hormone can be injected in the peritoneal cavity. After an hour or so, the animal is killed and the brain removed and sliced very thin (10–20 μM thick), a process called "sectioning", which is also used for in situ hybridization and immunocytochemistry. However, in this case, the sections must be placed onto slides that are already coated with emulsion, the liquid film we referred to above in the section on in situ hybridization. It is important not to expose the emulsion to light, which would defeat the whole purpose. Thus, the brains must be sliced and placed on the emulsion-coated slides in the dark.

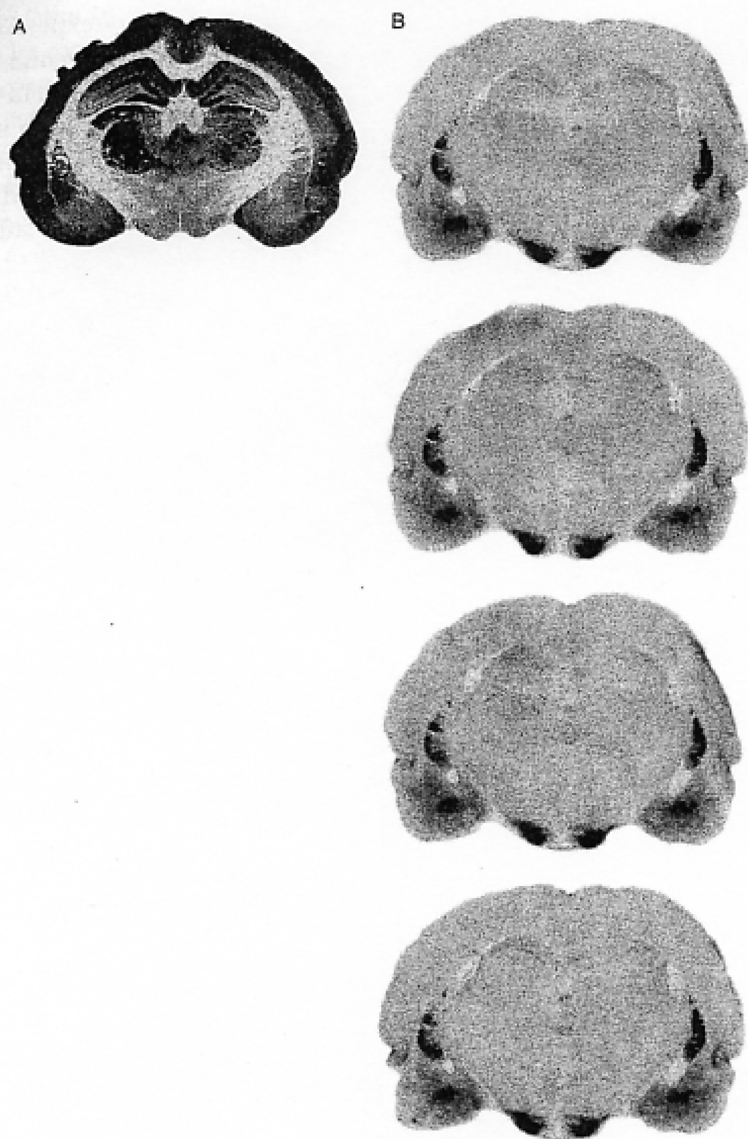


Figure 2.7 In vitro receptor autoradiography. The detection of membrane receptors for neurotransmitters in the brain can be accomplished with the use of a radioactive ligand. Brain slices placed on glass slides are incubated in a buffer containing the ligand and the excess removed by washing. The slides are then exposed to X-ray film for a period of time and an image of where the ligand has bound is obtained. Panel (*A*), on the left, shows the distribution of the GABA-A receptor, which is broadly distributed throughout the brain, and panel (*B*), on the right, is for the oxytocin receptor, which exhibits a relatively restricted distribution, being mostly expressed in the ventromedial nucleus of the hypothalamus.

Fortunately, a dim red light will not excessively expose the emulsion so the investigator has some assistance, avoiding an epidemic of fingerless behavioral neuroendocrinologists. After a period of exposure in the dark (sometimes for months), the slides are developed the same way as for in situ hybridization, and those cells which contain steroid receptors will have a dense collection of silver grains over their surface. This technique was used to generate the first atlas of estrogen-concentrating cells in the brain (Pfaff and Keiner 1973), which remains a valuable resource to this day.

Techniques for Altering Gene Expression

The origin of changes in behavior, physiology, and morphology in many cases stems from changes in gene expression. Understanding the underlying genetic mechanisms has put us in the position where we can alter the patterns of gene expression. Here we describe some of the most commonly used methods.

So far we have been talking exclusively about the ability to measure differences in mRNA or gene expression under varying conditions. Throughout this book, points will be made about the important contribution of expression of a particular gene to the manifestation of a hormonally influenced behavior. Traditionally, these types of inferences could only be made by comparing genetically distinct strains of animals or by correlating changes in gene expression with changes in behavioral states. The last decade or so has presented an array of new tools to the behavioral neuroendocrinologist, which allow the investigator to selectively manipulate the genome or gene expression of individual animals. Some manipulations result in permanent alterations in the genome of an animal, whereas others involve only temporary and restricted changes.

Transgenics

What is a **transgenic animal**? In Webster's New Collegiate Dictionary, one of the many definitions attributed to the word "trans" is "beyond, across, so as to change." In that light, the term transgenic could not be more apt since it refers to an animal that has received a gene from beyond itself or across species, which changes its phenotype. Transgenic sheep, cows, goats, chickens and fish have all been generated. But by far the most popular and most successful has been the transgenic mouse. The making of a transgenic animal requires equal parts of molecular biology, reproductive endocrinology, and animal husbandry.

The process of making a transgenic animal is relatively straightforward. Step number one is the creation of the desired DNA "construct," or, more simply, gene. This gene may be a mutated form of a naturally occurring gene that will be over-expressed, and thereby swamp the effect of the normal gene (often called a "dominant negative"), or it may be a normal allelic variant of a gene. Alternatively, it might be a gene that codes for cytotoxic molecules or even a bacterial gene used as a marker for other cellular processes. What genes can be used to generate transgenics is limited only by the imagination

of the investigator (Fukamizu 1993). However, in order for the **transgene** to be expressed, it must contain all the necessary accoutrements that accompany the coding sequence of any gene. These include the promoter region, which may or may not be tissue specific, the 5′ and 3′ untranslated regions, and the polyadenylation site that codes for the poly-A tail necessary for stability of the mRNA. Creating this DNA construct may sound like an overwhelming task best left to the molecular jocks, but in fact with the increasingly available technology, it is essentially a cut-and-paste operation accessible to all life scientists.

The second major step is the preparation of the mouse. This actually requires two female mice, one to be the donor of the fertilized eggs into which the transgene, or DNA construct, will be injected, and one to serve as the incubator for the injected eggs. The first female is induced to superovulate (produce more than the usual number of eggs) by treatment with the gonadotropins FSH and LH, allowed to mate normally with a fertile male, and then her fertilized eggs are harvested before the first cleavage. This results in the demise of the original female, so another female must be tricked into thinking she is pregnant (a physiological state called pseudopregnancy) to receive the transgenic embryos.

The third critical step is the difficult business of injecting the transgene into the fertilized eggs. The site of injection is the male pronucleus formed after the sperm fuses with the egg. Usually about 100 to 300 copies of the transgene are microinjected using a microscope at 200× magnification and a micromanipulator. As you might imagine, this is not a trivial process and is usually performed by highly trained experts skilled in this particular technique.

The last step is taken by nature. During the normal processes of recombination and meiosis, the transgene is randomly inserted into the genome of the embryo. The number of copies of the gene inserted may vary from 1 to 200, although when multiple copies are inserted it is usually in a head-to-tail orientation at the same site. Since the integration of the transgene occurs before the first cell division, all the cells, including the germ line, will be heterozygous for the transgene. The ultimate level of expression of the transgene can vary greatly between animals and depends on such factors as the number of copies, the site of integration, and the intrinsic efficiency of the transgene promoter.

Only a small percentage of the injected embryos will ever become live mouse pups and actually express the transgene. Those select few that do reach this status are termed "founders." The next step is to mate founders with wild-type mice to generate some heterozygote offspring. These, as well as the founders, can be identified by extracting DNA from a small piece of the mouse's tail and probing for the presence of the transgene by PCR or Southern blot. Once the F1 heterozygotes have been identified, they are mated to each other to generate homozygotes and the arduous process of assessing the phenotype can begin. The protocol for generating a transgenic mouse is illustrated in figure 2.8.

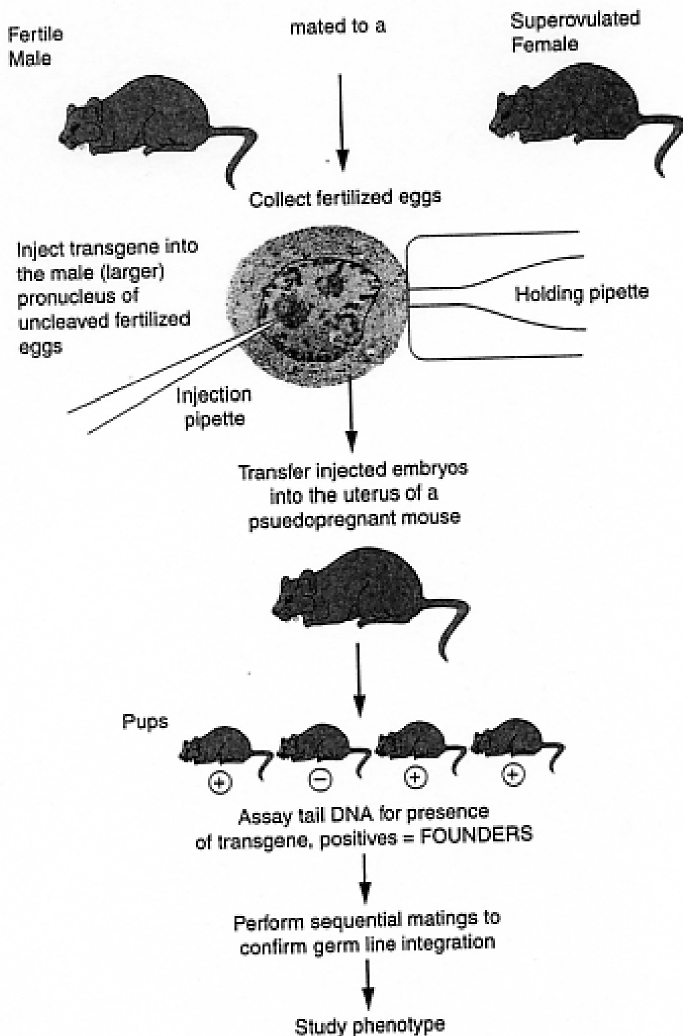


Figure 2.8 Generation of a transgenic mouse. A female mouse is superovulated to maximize the harvesting of fertilized embryos following mating to a fertile male. These eggs are then directly injected with multiple copies of the transgene and then inserted in the uterus of a pseudopregnant female. Once the pups are delivered, they must be genotyped to test for the presence of the transgene by extracting DNA from a small piece of the tail. The transgene is detected by PCR or Southern blot. If positive, the animals are grown to adulthood, mated to wild-type mice, and heterozygous offspring obtained. These heterozygotes can then be bred together to generate homozygotes and the phenotype characterized. (Adapted from Shuldiner 1996.)

Knockouts

What is a **knockout mouse**? Basically, a knockout mouse is a specific type of transgenic mouse in which a naturally occurring gene is disrupted, rather than allowing a pre-constructed gene to be inserted randomly into the genome (Galli-Tallidoros et al. 1995). The terms "gene disruption," "gene targeting," and "targeted gene replacement" all refer to the process by which a specific gene is manipulated via homologous recombination. This refers to a naturally occurring process in which identical sequences (homologs) will align and be recombined. The experimenter constructs a gene in which portions are homologous to the targeted gene but other portions are severely mutated or missing. When this construct lines up with the naturally occurring gene on the chromosome, in a small number of cases it will be substituted for that gene.

Unlike the generation of transgenic animals, knockouts do not begin with a fertilized egg but start with embryonic stem cells (ES cells). Stem cells are the precursors to the germ cells of the mature gonad. The DNA construct that will be used for homologous recombination is referred to as the "targeting vector." In addition to containing the mutated or disrupted gene, the targeting vector includes markers that will allow for the selection of the ES cells that have incorporated the gene into the correct site and thereby disrupted the naturally occurring gene of choice. This selection procedure is usually based on resistance to antibiotics. So, for instance, the disrupted gene might contain what is called a "neomycin-resistance cassette," with neomycin being a commonly used antibiotic. When the stem cells are treated with the neomycin, only those that contain the cassette, and hence the transgene, will survive. This is not because stem cells are bacteria, but rather because most antibiotics act by blocking gene transcription or protein translation and the cells will quickly die without these cellular processes.

After this selection procedure, the ES cells that meet criteria are inserted into a developing mouse embryo at the blastocyst stage (this is when the embryo has formed a fluid-filled ball with a knot of cells at one end that eventually will become the developing fetus). This recipient embryo is of a different strain than the mouse strain that the ES cells came from. It is important to be able to distinguish the strains, so strains having different coat colors are used; if working with fish, the investigator will use strains with different pigmentation patterns. The embryos are implanted into a host female and, when they grow to the point of developing hair, the mice that have the ES cells incorporated can be detected by their coat color. The chimeric (multicolored) males are mated to wild-type females to generate heterozygotes, as before, which in turn are mated. Mendelian genetics insures that one in four offspring will carry the disrupted gene in both alleles of the gene, i.e., a homozygous knockout.

The nomenclature for these animals is often an acronym for the gene, such as ER for estrogen receptor, followed by the traditional genomic nomenclature for dominant versus negative genes, only in this case they refer to wild

type versus knockout. So ER−/− is a homozygous knockout, ER−/+ is a heterozygote, and ER+/+ is a wild-type littermate. If heterozygotes having different null mutations are mated, it is possible to obtain "double knockouts," or offspring that will carry disruptions in both genes. To continue with the estrogen receptor example, recently mice have been generated with both the estrogen receptor α and the estrogen receptor β knocked out (Ogawa et al. 2000).

To date, the technology for generating knockout vertebrate animals has been limited to mice and zebra fish and in invertebrates to fruitflies and worms; and this situation may remain for some time. However, major progress is being made in overcoming one of the frequent criticisms of this technology, namely, that the organism lacks the gene throughout its lifespan. For example, an ER α−/− mouse develops and grows up without a functional estrogen receptor α. Thus, estrogen-dependent changes in behavior may be due to the pleiotropic consequences of missing that gene rather than due to the gene's function during adulthood. Recently the development of tissue-specific and temporally constrained knockouts has enabled the researcher to control where and when a particular gene can be turned on or off. These preparations are sometimes called "conditional knockouts" or "tissue-specific knockouts" and, while difficult to produce at this time, will no doubt be a valuable and frequently used tool in the very near future.

ANTISENSE OLIGONUCLEOTIDES

Elegant in its simplicity, **antisense technology** exploits the fact that single-stranded mRNA will hybridize with a complementary sequence of synthetic DNA. You can use antisense sequences of DNA with in situ hybridization to visualize where mRNA has been made, or you can use antisense sequences to look at the functional consequences of preventing gene expression. The formation of a duplex between the antisense sequence and mRNA in the animal will disrupt the ability of the mRNA to be translated into protein. Because the formation of the duplex is dependent on the sequence specificity of the genetic code, it is theoretically possible that one and only one gene product will be disrupted (figure 2.9). The requirements for the investigator are relatively few. If the sequence of the targeted mRNA is known, then a short piece (15 to 20 bps) of synthetic DNA can be ordered from any number of companies or university facilities. So after subcloning and sequencing the estrogen receptor from the gastric-brooding frog, you can order up your antisense. This antisense **oligonucleotide** (a generic term for a short synthetic stretch of DNA) is injected directly into the brain and a behavioral endpoint is monitored.

There are a number of advantages to antisense technology in behavioral neuroendocrinology (McCarthy 1998; Stein and Krieg 1998; McCarthy et al. 2000). For instance, a gene product can be selectively reduced in only one brain region and only for a short period of time. However, the gene product can only be reduced, not knocked out, and sometimes it can only be reduced

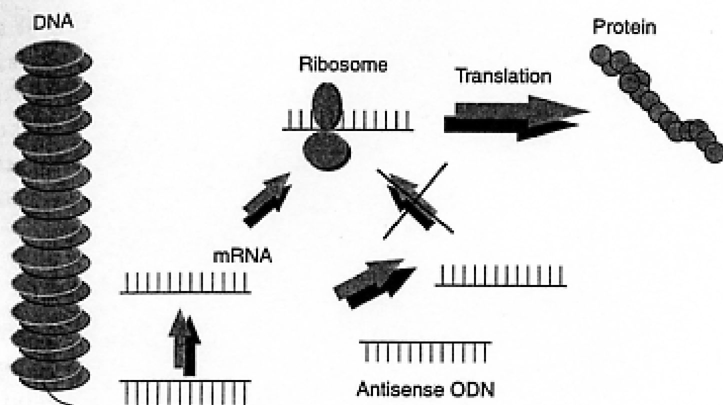


Figure 2.9 Antisense technology. A short (15–22 mer) piece of synthetic DNA that is complementary to mRNA is known as an antisense oligonucleotide and can be used to selectively disrupt gene expression. The antisense oligo is administered directly into the brain where it gains access to cells. There it will hybridize with its complementary mRNA, and only that mRNA, and will prevent it from being translated into protein. This process may involve a variety of mechanisms, including the prevention of proper splicing of the mRNA, blocking of the ribosomal complex that is needed to transcribe the mRNA and inducing the degradation of the mRNA by selective enzymes. (Drawing by A. P. Auger.)

by 20 to 30%. In addition, the investigator must conduct numerous controls to demonstrate a lack of general toxicity before a conclusion can be reached regarding the role of a particular gene product in a behavioral response. These would include making sure that general locomotion has not been impaired and that animals continue to eat well and lack any signs of overt illness, which have been witnessed following some antisense treatments (Spanagel et al. 1998). Nonetheless, because the primary action of steroid hormone receptors is inducing gene expression, this technique has been very informative in the field of behavioral neuroendocrinology.

VIRAL VECTORS

In some circumstances it would be useful if an antisense construct could be continuously expressed, rather than administered intermittently as is most commonly done. This has been achieved by actually infecting neurons with viruses that then express at high levels an antisense that is mRNA (rather than DNA). The most commonly used viruses are the adenovirus and the herpes simplex virus, both of which have been inactivated so that they cannot replicate and wreak the havoc that viruses so often do. The generation of additional virus is accomplished by growing them in special cell lines that possess the replication proteins that the virus is missing. The use of these viruses is similar to that of the plasmids we discussed earlier in that they are vectors and we can insert our gene construct of choice. This can be an antisense construct or some other variant of a naturally occurring gene; it could even be an unmodified gene that we simply want to try and express in

a different part of the brain (Kaplitt et al. 1993; Akli et al. 1993). The virus carrying our gene construct is then injected into the brain where it is taken up by neurons in a process called transfection, just like transforming bacteria with plasmid. Not all cells will take up the virus so we make sure that it also expresses a marker protein that we can detect, such as green fluorescent protein or beta-galactasidase. This approach is just beginning to be used by behavioral neuroendocrinologists.

Summary

In this chapter we have reviewed some of the basic principles of hormone action and the techniques commonly used for measuring gene expression in behavioral neuroendocrinology. We have also discussed recently developed techniques that allow for the control of gene expression, either by knocking out or overexpressing specific genes, or by temporarily dampening translation of mRNA into protein. These points can be summarized as follows:

1. Peptide hormones bind to receptors on the cell membrane. There are three basic types of these receptors: kinases, G-protein coupled, and ligand-gated ion channels. Different receptor types are linked to specific signal transduction pathways, but there is overlap and crosstalk between these pathways.
2. Steroid hormones combine with nuclear receptors and together they are integral components of a transcription complex that induces gene expression.
3. Steroids and their receptors can interact with proteins in the membrane and in the cytoplasm and modulate gene expression indirectly.
4. In order to study the expression of a gene it is necessary to know its nucleotide sequence. This may require subcloning the gene and sequencing it yourself.
5. Once the nucleotide sequence is known, levels of mRNA can be measured by in situ hybridization, Northern blot, RNase protection assay, or quantitative PCR. Each approach has distinct advantages and disadvantages.
6. Specific proteins can be measured by Western blot, immunocytochemistry, or in the case of receptors, autoradiography. Again, each approach has distinct advantages and disadvantages.
7. Transgenic mice are animals in which a novel gene has been inserted and is expressed in addition to, or instead of, a naturally occurring gene.
8. Antisense technology is a tool for temporarily dampening the translation into protein of a particular mRNA.

Study Questions

1. What is the difference between the mode of action of steroid and peptide hormones?
2. What techniques are available for studying gene expression and why would you choose a particular approach?
3. What techniques are available for measuring protein levels and why would you choose a particular approach?

4. How are transgenic animals generated and what advantages do they offer to a behavioral neuroendocrinologist?

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