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The identification and characterization of cDNA clones related to opioid bovine cellular adhesion molecule (OBCAM)

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The Identification and Characterization of cDNA Clones Related to Opioid Bovine Cellular Adhesion Molecule (OBCAM)

by

David Alan Lippman

DISSERTATION

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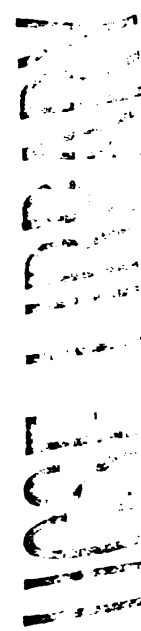
San Francisco



Dedication

To those few

who have nurtured, cajoled
and
accepted me

Acknowledgments

I would like to thank Dr. Loh for accepting me into his lab and allowing me to pursue my degree under his guidance.

I would like to thank my committee members for their constructive criticism and for taking their time to be on my committee.

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To my sisters and friends; thanks for putting up with the long and arduous task of my getting this finished.

This wouldn't have come into being if it were not for the enthusiasm and spark granted upon me by Dr. Daniel Chin who introduced me to Molecular Biology and showed me just how much fun it could be. I also appreciate your introducing me to Macs, back then it could only paint, draw and write.

Thank you Paul and Julie, you are always there, and I appreciate all the favors.

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My most humble admiration and appreciation to: Suganda Phalakornkul, Jason Gilster, Dave Andrus, Kristine Yang Zhang and Stephanie Aynsley Pence. Thanks to Peter, Sam, Luc, Tiffany and Kimberly "B".

A parting thought...

"There is really nothing you must be.
And there is nothing you must do.
There is really nothing you must have.
And there is nothing you must know.
There is really nothing you must become.
However. It helps to understand that fire burns
and when it rains, the earth gets wet...

Whatever, there are consequences.
Nobody is exempt,"

(Robert Fulgam, It was on Fire..., 1990)

The Identification and Characterization of cDNA Clones
Related to Opioid Bovine Cellular Adhesion Molecule (OBCAM)

David Alan Lippman

ABSTRACT

The purpose of this project was to identify and characterize cDNA clones related to OBCAM, an opioid binding molecule cloned previously in our lab. The initial experiments were designed to seek out OBCAM variants possibly containing longer reading frames that would span the cell membrane, since in order to function as a cell surface receptor and transduce a signal a transmembrane region appears to be required. As a result of screening several rat cDNA libraries a number of new OBCAM like clones were discovered which shared a nearly identical 3' coding region and a non conserved 5' coding region. Thus all clones failed to contain a region which could span the cell membrane; however, our results provided evidence for a family of OBCAM like molecules different in their N-terminus. This common feature led us to investigate the cellular effects of these clones using antisense technology. By constructing a unique region of a single clone into a vector in the reverse orientation we could assess OBCAM's activity in both a cell line and mice by negatively attenuating its expression. In both cases it was shown that antisense-DUZ1 could alter opioid sensitivity. Lastly it was of interest to define whether the clones discovered came from alternate splicing of a single gene or were the products of redundant genes. In order to determine this we have screened a somatic cell line and ultimately have undertaken the project of obtaining the genomic clones and sequencing the entire gene.

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INTRODUCTION

Opiates are one of the oldest class of pharmaceutical agents currently in use today, as opium and preparations of the opium poppy (papaver somniferum) have been used for nearly 6 millennia. In this century, purification and characterization of numerous active components have revealed over 20 alkaloids, including morphine and codeine. Scores of new drugs and several new drug classes have been derived from poppy alkaloids.

Exhaustive research into the structure-activity relationships of opiates has revealed many intricate details about opiate ligands, but overall the ligands can be grouped by structure into three categories:

1) Poppy Alkaloids and derivatives

(1a.) Phenanthrenes

Morphine, Codeine, Nalbuphine, Naloxone and Etorphine

(1b.) Morphinans

Levorphanol, Butorphanol, Levallorphan

(1c.) Benzomorphans

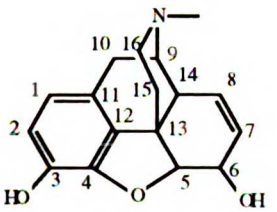
Pentazocine

(2) Phenylheptylamines: Methadone and Propoxyphene

(3) Phenylpiperidines: Fentanyl, Meperidine, and α-Prodine

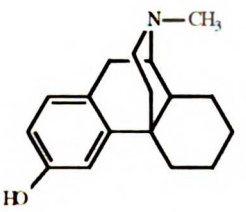
Figure 1

Poppy Alkoloids and derivatives



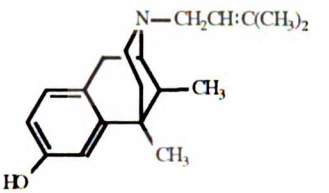
Morphine

1a



Levorphanol

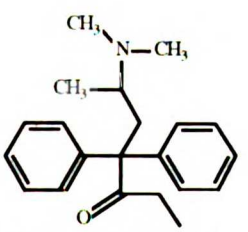
1b



Pentazocine

1c

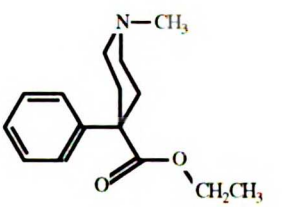
Phenylheptylamines



Methadone

2

Phenylperidines



Meperidine

3

Thousands of structural modifications and chemical analogs of these ligands have been prepared in the hope of separating the main pharmacological actions of opiates--analgesia, antitussive, antidiarrheal--from their deleterious side effects: respiratory depression, tolerance and dependence. There has been little success, however, and in recent years most drug companies have discontinued further research in this area.

A more fundamental approach to understanding and perhaps dissociating the desirable from the undesirable actions of opiates is to investigate their site or sites of action. Drugs are thought to bind to specific receptors, and specific opiate binding sites in neural tissues were independently and simultaneously reported by several investigators; Simon, Snyder, Terenius and their co-workers (1973). Since endogenous receptors existed for these exogenous compounds, it was reasoned that there must also be endogenous ligands for these drug receptors. The first endogenous ligands were reported for "opioid" receptors in 1975 and the class was renamed opioids, a broader term to include all ligands that act at "opioid" receptors.

In a study that would set the cornerstone for all future opioid pharmacology, W. R. Martin et al., (1976) provided the first evidence for the existence of multiple opioid receptors. The pharmacological effects *in vivo* of three prototypic opioid ligands, morphine, ketocyclazocine and SKF 10,047, were quantitatively examined in the chronic spinal dog, and from the discrete pharmacological profiles of these compounds the authors postulated the existence of three different opioid receptors: mu(μ), kappa(κ), and sigma(σ). The receptors were named after the different pharmacological profiles of

their respective prototypic ligands, morphine (mu), ketocyclazocine (kappa) and SKF-10,047 [N-allylnormetazocine] (sigma).

Following this groundbreaking work, the concept of multiple opioid receptors was expanded and demonstrated more directly in the work of Lord et al., (1977), using isolated tissue preparations such as the mouse vas deferens (MVD) and guinea pig ileum longitudinal muscle (GPI). Based on the differential sensitivities of these preparations to several opioid agonists as well as the antagonist naloxone (NX), it was concluded that the opioid receptor populations in these preparations were not identical. In addition, Lord et al., (1977), independently postulated the existence of the kappa receptor originally proposed by Martin et al., (1976), based on the sensitivity of ethlyketocyclazocine (EKC) activity to antagonism by NX. They further observed that the enkephalins displayed unique activity in the MVD, and concluded that there must be an additional opioid receptor, which was christened delta (δ). Since then, σ has been relegated to the PCP class and additional receptors such as ε (β-endorphin) and λ (naloxone) have been proposed. However, μ, δ and κ are still considered the major opioid receptor types and each is now thought to have several subtypes (Loew et al. 1986; Iyengar et al. 1986; Nishimura et al. 1984; Portoghese et al. 1990)

Further pharmacological evidence for the presence of different opioid receptors in isolated tissues continues to accumulate with the development of novel experimental approaches. From the assumption that tolerance to a selective agonist would lead to cross tolerance to only similar selective agonists, several groups conducted experiments that successfully distinguished different receptor

populations (Wuster et al., 1981a,b; Schulz et al., 1981; Schulz et al., 1984). Pharmacologically active opioid receptors can also be discriminated from one another by using selective irreversible antagonists. The two commonly used opioid receptors alkylating agents are beta-chlornaltrexamine (beta-CNA) and beta-funaltrexamine (beta-FNA), both derivatives of naltrexone. A typical experiment would be where a selective ligand is pre-bound to the receptor of interest and the alkylating agent is then used to knock-out all other opioid receptors (Takemori et al, 1981; Takemori and Portoghese, 1985a).

Biochemical evidence for opioid receptor multiplicity only became possible with the development of selective ligands for each class of receptor. These ligands allowed thorough characterization of mu, kappa, and delta opioid binding sites in the synaptosomal fraction of homogenates from rat, mouse, guinea pig (Lord et al., 1977; Kosterlitz et al., 1981; Chang et al., 1979) monkey, human (Lord et al., 1982) and toad brains (Simon et al., 1982), as well as in the guinea pig ileum (Creese and Snyder, 1975; Zukin, 1984, Corbett et al., 1985). All opioid binding sites were found to be stereospecific, saturable, associated with the membrane fraction of nervous tissue and to have high affinity for both opioid agonists and antagonists. The ligands used to demonstrate the presence of multiple opioid receptors in brain have proved to be invaluable tools in opioid research although they vary greatly in their degree of selectivity. In general, the order of selectivity of opioids for the receptors are as follows (Chang, 1984; Paterson, 1984):

1) for the mu receptor: morphiceptin (PL 017), DAGO (D-Ala^2,N-Met-Phe^4,gly-ol^5-enkephalin), fentanyl, FK-33824 (D-Ala^2,N-Met-Phe^4,Met(O)ol-enkephalin), syndyphalin, morphine, dihydromorphine, normorphine, naloxone;

2) for the kappa receptor: U-69,593, U-50,488H, trifluadom, dynorphin 1-13 and certain fragments of prodynorphin;

3) for the delta receptor: DPDPE ([cyclic]-D-Pen^2-D-Pen^5-enkephalin), DPLPE ([cyclic]-D-Pen^2-L-Pen^5-enkephalin), DTLET (D-Thr^2-D-Thr^6-Leu-enkephalin), DADLE (D-Ala^2-D-Leu^5-enkephalin), DSLET (D-Ser^2-D-Thr^6-Leu-enkephalin), ICI-154129 and ICI-174864.

Although prototypic kappa agonists such as ketocyclazocine and EKC were used to define this binding site, and may still be used in animal studies, they display a high degree of cross-reactivity with the mu receptor.

Furthermore, the receptors show a discrete regional distribution within the brain and spinal cord. This has been shown both directly with autoradiographic studies, and indirectly with binding assays from different areas of the brain. The neuroanatomical localization of mu, kappa and delta opioid receptors has been well characterized and allows some correlation with pharmacological data.

Mu binding is predominant in the thalamus, hypothalamus, periaqueductal gray, interpeduncular nucleus, inferior colliculus, median raphe nucleus, limbic cortex and in the laminae I and IV of the cerebral cortex.

Delta binding sites occur in the amygdala, nucleus accumbens, olfactory tubercle, pontine nuclei, laminae II, III and V of the cerebral cortex and diffusely in the corpus striatum and hippocampus.

Kappa binding appears to be concentrated in laminae V and VI of the cerebral cortex, caudate-putamen, pyriform cortex, lateral habenulae, bulbus olfactory tubercle, substantia nigra and in the molecular layer of the cerebellum.

In addition, mu, delta and kappa receptors are found in lamina VI of the cerebral cortex, nucleus tractus solitarius, vagal nerve, nucleus ambiguous, substantia gelatinosa of the spinal cord, marginal zone and in laminae I and II of the dorsal horn (Kuhar and Uhl, 1979; Chang, 1984; Akil et al., 1984; Zukin and Zukin, 1981; Paterson et al., 1984). Interestingly, opioid binding sites in the rabbit cerebellum are almost all of the mu type, those in the guinea pig cerebellum are nearly all kappa receptors, and the cerebella of rats and mice are devoid of any opioid binding sites (Paterson et al., 1984; Robson et al., 1985). The physiologic consequences of the differential distribution of various opioid receptors in areas of the central and peripheral nervous system is not well understood. However, an important observation is that these receptors are localized in brain sites active in pain sensation, pain perception and pain modulation.

Despite the plethora of information that has been amassed during the last ten years, the significance and inter-relationship of opioid receptors and their subtypes is still unknown. How many sub-types of opioid receptors are there? Are they distinct protein entities? Bowen and co-workers (1981) suggested that mu and delta receptors may be interconvertible, depending on the conditions of

binding. Rothman and Westfall (1982), on the other hand, proposed that the apparent relationship between mu and delta sites might be an allosteric one between sites on the same binding molecules. These questions, however, cannot be answered without complete structural and functional characterization of individual receptors and characterization requires purification and reconstitution of the receptor in a membrane environment.

For many years, purification of opioid receptors lagged behind that of other cell surface receptors, in large part because opioid receptors proved to be very sensitive to the detergents used to solubilize membranes. This situation has been dramatically altered by the recent successful cloning of the δ opioid receptor from NG108-15 cells, as reported at the end of last year by two independent laboratories (Evans et al. 1992; Kieffer et al. 1992). The two laboratories used a similar approach (expression cloning), preparing a cDNA library from NG108-15 cells, transfecting pools of this cDNA into mammalian cells, then assaying the cells for binding of a radioactive opioid ligand. The cloned, expressed receptor showed typical binding properties expected of an opioid receptor, including high affinity, stereoselectivity and preference for a particular class of opioids, namely δ; in addition, Evans, *et al.* demonstrated that binding to the expressed receptor inhibited adenylyl cyclase, as is the case with native δ-opioid receptors on NG108-15 cells.

In NG108-15 cells (Koski and Klee 1981), as well as in at least some areas of the mammalian CNS (Attali and Vogel 1989; Blume et al. 1979; Childers and Snyder 1978; Law et al. 1981), opioid receptors are coupled to G-proteins, so it was no surprise that the

predicted amino acid sequence of the cloned opioid receptor cDNA was homologous to other members of the G-protein coupled receptor superfamily (Dohlman *et al.* 1987); in particular, the sequence displayed seven characteristic hydrophobic regions which are presumed to span the cell membrane, as well as several particular highly conserved amino acids within some of these regions. This is also true of other opioid receptors subsequently cloned. Chen et al. (1993) isolated from rat brain a cDNA that expressed receptor selective for mu ligands such as DAMGO (DAGO), and which was negatively coupled to adenylyl cyclase, while Yasuda et al. (1993) isolated a clone also from rat brain that appeared to express a receptor selective for κ opioids. Both of these receptors exhibit about 60% amino acid homology with the δ-opioid receptor as well as with each other. The greatest homology, as expected, is in the seven putative transmembrane regions, while the least is in the N-terminal and C-terminal sequences, and in the second and third extracellular loops formed by amino acids between transmembrane regions 4 and 5 and 6 and 7.

While the cloned μ, δ and κ opioid receptors appear to be the major molecules mediating the effects of opioids, there may be others that play a functional or regulatory role. Several years ago, our laboratory reported purification of a 58 kD protein from bovine brain that bound opioid ligands with low affinity in the presence of acidic lipids (Cho et al. 1986). Subsequently, the cDNA for the protein was cloned, and found to have homology to immunoglobulin (Ig)-like proteins, particularly cell adhesion molecules (Schofield et al. 1989). Thus, it was called opioid binding cell adhesion molecule

(OBCAM). OBCAM possesses three characteristic Ig domains, which are known to be oriented extracellularly. It has a single hydrophobic region at its C-terminus, but this is not long enough to traverse the cell membrane. Hence, it appears that it could not act by itself as a signal-transducing molecule, relaying ligand-binding events at the cell surface to functional molecules on the cytoplasmic side of the membrane. Furthermore, now that opioid receptors with homology to other G-protein-coupled receptors have been cloned, the question is raised of the relationship of OBCAM to these receptors. Does OBCAM in fact play any role in opioid receptor function?

We have several lines of evidence that it does. First, we have raised antibodies both to purified OBCAM and to peptides corresponding to portions of its predicted amino acid sequence, and demonstrated that these antibodies inhibit opioid binding to the purified protein as well as to brain membranes (Roy et al. 1988a; Roy et al. 1988b). One of the peptide antibodies, to a region of OBCAM known as MN-3, has been studied particularly intensively. By the use of fluorescent labeling together with confocal-microscopy, our laboratory has shown that OBCAM-like material is not only present on the surface of NG108-15 cells, but is down-regulated by chronic treatment of these cells with opioid agonists, in a fashion parallel to the well-established down-regulation of opioid receptors in these cells (Lane et al. 1992). Thus, down-regulation of the MN-3-material followed a similar time course, dose-effect, and was selective to δ agonists. Other cell surface receptors were not affected, nor was an antibody to neural cell adhesion molecule (N-CAM) effective.

These data indicate that OBCAM plays a role in opioid receptor function, and may be closely associated with the receptor. The antibody data do not establish that OBCAM itself binds opioid ligands *in situ*, since an antibody to the molecule could alter its relationship to another molecule that was acting as receptor. In fact, the most direct way of testing OBCAM's ability to bind opioids would be to express it in a cell line, but this has not been possible. However, the cloning of OBCAM has made it possible to explore its function in another way, by selectively blocking its expression through the use of antisense cDNA. By transfecting NG108-15 cells with antisense cDNA to OBCAM, our laboratory was able to create a stable cell line (ST7-3) in which opioid binding is greatly reduced relative to that of cells transfected with OBCAM sense (ST8-4), as well as non-transfected cells (Ann et al. 1992). The selectivity of this effect is suggested by the observation that binding of ligands to other cell surface receptors in ST7-3 cells was unaffected. Scatchard analysis of the binding indicated that most of the reduction was due to a decrease in receptor number, not affinity. Moreover, the remaining receptors could be further down-regulated by chronic opioid agonist treatment of the cells. Thus, it appears that the OBCAM antisense has greatly reduced the number of opioid receptors on the cell, without affecting their intrinsic response to acute or chronic opioid agonist treatment. This result again is consistent with an association between OBCAM and another molecule functioning as the receptor.

These studies all suggest that OBCAM plays a role in opioid receptor function, but do not really address the question of how it does so. Despite its original isolation on the basis of its opioid

binding properties, the recent identification of G-protein-coupled opioid receptor clones suggests it does not play this role. Alternatively, however, it could regulate the coupling of the receptor to its effector molecule. As evidence for this, we have recently shown that coupling to G-proteins is altered in ST7-3 (OBCAM antisense-transfected) cells (Govitrapong et al. 1993). To demonstrate this, we made use of the fact that cholera toxin (CTX) induces ADP-ribosylation of G-proteins only in the presence of ligand, which promotes coupling. In untransfected cells, or cells transfected with OBCAM sense, CTX was shown to induce, in the presence of opioid agonist, ADP-ribosylation of G-proteins that are identifiable on SDS gels as 39-41 kD material that is reactive to G_{i2} and G_o antibodies. In the ST7-3 cells, this labeling is greatly reduced, as was DADLE stimulation of GTPase, a standard measure of receptor-G-protein interaction, and DADLE inhibition of adenylyl cyclase, to which opioid receptors are coupled in these cells. Guanine nucleotide inhibition of cyclase was also reduced.

It is not clear how OBCAM, an extracellular molecule, could couple a seven transmembrane receptor to G-proteins, which are located on the cytoplasmic face of the cell membrane. In fact, to our knowledge there is no other evidence for the existence of a coupling molecule mediating between these two proteins, which are assumed by most investigators to interact directly. Nevertheless, we believe that these several lines of evidence, indicative of a role for OBCAM in opioid receptor function, warranting further study of its relationship to the μ, δ and κ opioid receptors. In the work presented in this thesis, we have addressed this question by characterizing the OBCAM

gene more thoroughly. In particular, we have asked two questions: 1) do alternative forms of OBCAM exist which include a cytoplasmic portion that might interact with G-proteins or other molecules regulating opioid receptor function; and 2) how does blocking *in vivo* expression of the OBCAM gene alter the sensitivity of animals to opioids? In addition, we have sequenced most of the OBCAM gene.

cDNA Cloning

The development of modern molecular biological techniques has made it possible to obtain or infer a great deal of information about proteins indirectly, from the sequences of the DNA coding for them. The DNA sequence determines the amino acid sequence, and from this information alone, the investigator may be able to gain insights into the conformation of the protein, the way it interacts with the cell membrane, and even the second messengers that mediate its cellular effects. For example, almost all the cell surface receptors known to have their functions mediated by GTP-binding proteins (G-proteins) have seven hydrophobic regions of conserved amino acid sequences that are presumed to span the membrane (Dohlman et al. 1987). Two other large receptor or putative receptor families, effects of which are mediated by tyrosine kinase (Yarden and Ullrich 1988) and tyrosine phosphatase (Streuli et al. 1989), have conserved intracellular domains possessing the catalytic activity as well as conserved extracellular domains that are presumed to be involved in ligand binding.

Having available the receptor's DNA and amino acid sequence also opens up numerous possibilities for further studies. Antibodies to peptides corresponding to portions of the amino acid sequence can be raised, and used to pinpoint functional regions of the receptor, to localize it in biological tissues, and to purify it by affinity chromatography. Oligonucleotide probes consisting of portions of the cDNA sequence can be used to determine the localization and regulation of the receptor's mRNA, and to probe the genomic organization of the receptor code. Antisense cDNA, cDNA

complementary to the coding strand of the receptor cDNA, can be used to inactivate selectively a single mRNA species, confirming its main functions and perhaps illuminating more subtle ones.

Cloning of a protein usually follows one of two general methodologies. In the first approach **(1)**, the protein is first purified and partially sequenced, allowing oligonucleotide probes to be synthesized corresponding to a portion of its code. These probes are then used to screen a cDNA library in order to isolate the entire coding sequence.

In the second approach **(2)**, the cDNA of interest is identified by its ability to express the protein, which is identified by its functional activity, for example; receptor binding, enzymatic activation or inhibition, or alteration of the electrophysiological properties of a cell.

1) **Purification**

Microsequencing Oligo Synthesis Library Screening

2) **Expression**

A) **Antibody-** λvector/ E. coli Host

B) **Sense**

I) **Direct** Expression Vector

II) **Subtraction** Eliminate Numerous messages
Expression Vector

III) **Homology Screening**
Use PCR to obtain clones
use known sequences

C) **Antisense/Knockout**

Reverse Orientation Msg

Direct injection or RNA vector

Homologous Recombination

I) **Subtraction**

Eliminate Numerous messages

Expression Vector

II) **Homology Screening**

Use PCR to obtain clones

Use known sequences

The first approach **(1)** is the easiest and most straightforward if even only small quantities of purified receptor are available (for an unblocked N-terminal protein only 100 ng is necessary). The protein is partially sequenced, providing limited amino acid information from which oligonucleotides can be designed; these probes are used to screen a cDNA library, constructed from all the mRNAs present in the tissue containing the receptor. In theory, the probes should hybridize with a unique sequence (sometimes present in several different cDNA's) corresponding to that coding the protein. An additional benefit of this procedure, though, is that under sufficiently relaxed conditions of hybridization, the probes may also recognize other, highly related sequences, providing evidence (and additional clones) for a family of proteins closely related to the receptor of interest.

If even only a partially purified preparation of the protein is available, antibodies can be prepared and used to screen a lambda

fusion protein expression library (**2A**). If a cDNA clone is obtained, it should share homology with the partial sequence obtained from the purified protein. However, in either case (**1** or **2A**) the clone may not be complete--that is, it may not contain a full-length reading frame--since the screening process selects for only a portion of the sequence of interest. In this case, numerous screenings of the original library or other libraries from the same tissue source may be required, or even construction and screening of a new library, finally allowing construction of the full length clone from a selected number of overlapping partial clones. Moreover, even if the clone does contain a complete coding region, or what appears to be a complete coding region, the product may not be capable of expressing a functional protein.

If purified receptor is not available, it is still possible to isolate its cDNA, if there is a rapid and reliable way to measure receptor activity, such as ligand binding or ligand-mediated alteration of some biochemical process. In this case (**2-B**), the cDNA library constructed in an expression vector, or mRNA itself, is introduced into cells that normally do not express the receptor, and the cells screened for receptor mediated activity. This step may be carried out by introduction of cDNA into mammalian cell lines by chemical treatment of the cells, lipofection, electroporation, or by direct injection of mRNA into large cells such as Xenopus oocytes.

While this approach has now been successfully used for dozens of receptors, and can in principle be applied to any receptor of interest, for several reasons it may present difficulties in individual cases. First, in order for the screening process to work, the receptor

must be expressed in the transfected or injected cells in a form capable of recognizing ligand. For such fully functional expression, however, the successful translation of the appropriate message into a polypeptide may not be sufficient. For one thing, post-translational processing is frequently necessary to convert polypeptides into functional receptors--for example, the addition of specific carbohydrate groups to specific portions of the protein molecule--and any cells that normally do not express these receptors may be incapable of carrying out these additional steps with the required specificity. In addition, a receptor may consist of more than one polypeptide chain, each of which is necessary to form the high affinity binding site; this is the case for the interleukin-2 receptor (Smith, 1987).

A second major disadvantage of approaches based on receptor expression is that, even if cells are capable of synthesizing fully functional molecules, the screening process is quite laborious; typically, one is looking for one or a few receptor-containing clones out of millions. Thus this approach may need to be combined with others that are capable of *a priori* limiting the number of clones that need to be examined. Two such techniques that have been used successfully to clone some receptors and other functional molecules are subtractive hybridization and the use of consensus sequences.

Subtractive hybridization **(2BII)** takes advantage of a situation in which two different library sources, or one source under two different conditions, express nearly identical complements of proteins and their mRNAs but differ in that only one contains the receptor/protein-mRNA of interest. In this case, mRNA is isolated

from both tissues, and cDNA prepared from the mRNA that includes the receptor transcripts. This cDNA is then hybridized with the other population of mRNA, which, in theory, should "subtract" all the common messages between the two sources, leaving a cDNA population highly enriched in receptor-encoding sequences. (In practice, the mRNA from the source deficient in the message of interest is added in excess, to ensure that all the common messages in the other source are hybridized; excess mRNA can then be separated from cDNA by means of prelabeling the mRNA with a recognizable substance such as biotin). This relatively small library can then be transfected into cells and screened for receptor or receptor mediated function.

While the principle of subtractive hybridization is very simple, in practice it can be very difficult to apply and often misleading. One reason for this is that there are relatively few (if, indeed, any) situations in which only a single molecule of interest, and no other, is differentially expressed in two tissues. It is much more likely that differences will be observed with respect to several, perhaps many, molecules. Research in our laboratory with the opioid receptor, for example, based on down-regulation of the receptors in response to chronic exposure to opioid agonist, has identified more than a dozen different, apparently unrelated proteins. While all of these proteins may in some way play a role in the down-regulation process, and thus be of some relevance to our understanding of opioid receptors, their presence greatly complicates the task of identifying the receptor itself.

A second difficulty with subtractive hybridization is that, even in an ideal case, where only a single protein is differentially expressed, this protein may not be detected unless it is relatively abundant in one tissue, and either absent or greatly reduced in the other tissue. This is because even large changes in a relatively rare message would be smaller, in absolute value, than small, statistically insignificant changes in highly abundant messages. Thus, contamination of genuinely altered messages with messages from non-altered but abundant proteins is to be expected.

Finally, as has been shown by Lefkowitz and Benovic et al. (1988) for the β-adrenergic receptor, the mRNA level does not necessarily correlate with the level of expression of the receptor; that is, down-regulation of the receptor was not accompanied by down-regulation of message over the same time period. Of course, a change in message level must occur for subtractive hybridization to work. The level of functional activity is irrelevant for the purposes of this technique.

Despite these difficulties, subtractive hybridization has been used successfully to clone several proteins. Kavathas (1984) cloned the gene coding the human T-lymphocyte differentiation antigen Leu-2 by preparing mRNA from two sets of L-cells, those expressing and not expressing this antigen. Fornace (1986) identified several transcripts involved in the response of Chinese hamster cells to heat shock, by subtraction of sequences from shocked and non-shocked cells. Subtractive hybridization has also been used to enrich transcripts specific for certain brain regions, by subtracting messages from other brain regions (Rhyner et al. 1986). These investigators

estimated that transcripts comprising only 0.0005% of the total cellular mRNA could be identified in this way.

In addition to subtractive hybridization, the search for a specific cDNA can also be greatly accelerated by the use of consensus sequences **(2BIII)**. The rationale underlying this approach is that most cell surface receptors belong to a well-defined family of proteins that share significant homologies in their amino acid sequence at specific regions. By designing oligonucleotide probes that correspond to the most commonly shared or consensus portions of these sequences, one can preferentially screen for members of this family.

The use of oligonucleotide probes based on consensus sequences has been successfully applied to the isolation of new members of the G-protein coupled receptors (Hershey and Krause 1990; Parmentier et al. 1989). This is a family of cell surface receptors that is coupled to second messengers by G-proteins, and members of which each possess seven putative transmembrane sequences. These sequences show a relatively high degree of conservation among members of this family, and so can be used to screen cDNA libraries for new members. This method has been used, intentionally and unintentionally, to clone new receptors related to known ones.

All of the expression systems mentioned so far are based on creating the protein and/ or it's effects in a deficient system. They were therefore grouped into a category of **Sense (2B)** cloning. A newer variation on the expression theme of cloning is listed under the heading of **Antisense/Knockout (2C)**. Suppose a clone has

been obtained that is homologous with the oligonucleotide probes or the antibodies used to isolate it, and which has been thoroughly sequenced and contains a complete coding region, and yet still lacks the ability to express binding or activity. How can it be proven to be the protein of interest? If the functional activity can't be expressed, it may still be possible to block its expression. The cDNA is inserted into an expression vector in the opposite orientation, which means it will synthesize mRNA complementary to the message of the protein of interest. It will thus form a double-stranded complex with the normal message, which the cell usually destroys. Thus the expression of the protein of interest is blocked or reduced. A recent addition to this direct repertoire is referred to as knockout. In this experimental design a large segment of DNA is introduced into an embryonic cell in early stages of initial cellular division. The segment of DNA is required to be highly homologous to the region of interest, usually an endogenous portion of DNA.

In summary, there are two general methodologies for cloning the cDNA of a receptor/protein of interest: **1) Purification**: oligonucleotide probes are designed from a partially sequenced protein, and used to screen a cDNA library prepared from a tissue containing the receptor; and **2) Expression**: either directly via a fusion protein or by introduction of cDNA or mRNA into cells that are subsequently screened for their expression of the receptor or by antisense expression and elimination of the receptor. While purification is simpler and faster, it requires purified protein and, as discussed above, may not produce full length, functional clones of interest. Expression screening, if successful, is much more likely to

identify the correct clone, as several stringent criteria must be fulfilled:

1) the protein must be in an active functional conformation
2) the protein be fully (completely) expressed {including subunits}
3) if functional activity is measured, rather than ligand binding, then the signal transduction system, or a substitute, must be available and functional.

For opioid receptor cloning, an expression approach is required, as it has not been possible to purify a protein definitively shown to be an opioid receptor. The most logical method of screening such a library would be to use opioid binding; however, opioid ligands have not lent themselves well to introduction of an isotopic substituent; the structure activity requirements for receptor specific opiate ligands are very stringent, precluding the addition of any high energy emitters, such as ^{125}I, or ^{32}P. Molecules such as ^{14}C or ^{3}H could easily be incorporated but the receptor density needs to be fairly high in order to follow the signal of such emitters. This same problem plagues the shorter opioid ligands (peptides) such as Met and Leu enkephalin.

MATERIALS AND METHODS

Molecular biology today requires the use of a very large number of protocols for the isolation, manipulation and analysis of DNA. Furthermore, many of these protocols do not work consistently, necessitating the use of alternative procedures. As a convenient reference, I have included at the end of each sub-section a step-by-step description of all the major protocols used in these work; the reader is referred to these for specific experimental details. The remainder of this section provides a general overview of my experimental approach, and a rationale for the use of particular procedures.

Overview

The overall goal of this project was to analyze at the molecular level OBCAM and its gene. Several major approaches were used, including;

1) Isolation and analysis of new cDNAs coding for molecules closely related to OBCAM;
2) Analysis of OBCAM's role in whole animals through transgenic procedures, designed to block specifically the action of the OBCAM gene;
3) Sequencing of the OBCAM genome, and determining its chromosomal location.

The procedures used in this work I have for convenience divided into the following general categories:

A Isolation of DNA/RNA
B Manipulation of DNA/RNA
C Manipulation of Bacteria

A Isolation of DNA/RNA

The basic methods for isolation of DNA have changed little in the last eight years; what has changed is the availability of innumerable kits that package the necessary reagents and cookbook protocols enabling mindless and reproducible production of DNA as fast as if not faster than the original methodologies. The basic premise of DNA isolation, whether genomic, plasmid or phage, is to solubilize the host containing the DNA, precipitate the extraneous garbage (cellular debris, proteins, RNA) and retrieve the DNA. Probably the only recent novel contribution to DNA isolation has been the ongoing research into special matrices that will selectively bind DNA, allowing for rapid and clean extraction of DNA from a solubilized cellular homogenate. The drawbacks to such resins are limited binding capacity, and expense. Promega Magic Mini-preps for example, are extremely fast (up to 30 preps in 30 minutes), and produce DNA of sequencing quality, but a single preparation can dependably produce a maximum of only 10 ug. This is enough for analysis and limited sequencing, and further manipulations in most cases, but the traditional fashioned alkaline lysis Mini-prep is still the method of choice when larger quantities are required. It is capable of producing over 100 ug from a 10 ml culture, also of sequencing quality (with a few modifications of the original protocol), which is enough DNA for any further manipulations. Rarely is it necessary to do a large scale plasmid prep these days, except when large amounts of DNA are required for eukaryotic transfection. Under these conditions, there is no other procedure that will yield the quantity and quality of DNA obtained from a cesium chloride alkaline lysis plasmid prep.

Preparation of lambda phage DNA is slightly different from plasmid as it is necessary to eliminate the host proteins before the destruction of the phage heads and rescuing of the phage DNA. These protocols have not been improved upon.

The preparation of RNA has advanced little from the original procedures using chaotropic reagents such as guanadinium

thiocyanate. Again the major advances have been the production of novel binding resins. In the production of mRNA a number of these resins are now available, all sharing the feature of a poly oligo d(T) tail attached to a linker arm which is affixed to such "tags" as biomagnetic particles, biotin, or a solid support such as Affigel. The logic of these is to bind the mRNA to the oligo d(T) and then hold the tag and rinse away the extraneous garbage. This technology allows the efficient and quality production of mRNA directly from tissues or cells without the intermediate production of total RNA.

1) Preparation of Genomic DNA
2) Preparation of Plasmid DNA
    a) Mini-prep
    b) Large Scale Plasmid Preps
    c) Plate Lysate Phage Preps
3) Preparation of RNA

Genomic DNA Prep

(modified from Methods in Enzymology, vol. 152, 1987))

Four grams of tissue are frozen in liquid Nitrogen and then ground into a fine powder with a mortar and pestle (precooled in liquid nitrogen). This powder is sprinkled onto 40 mls of TEN 9 solution containing 10 ul RNAse cocktail and mixed by swirling the container. Once the entire sample of tissue is added the mixture is transferred to a 50ml conical tube, to which 2 ml of 10% SDS are added and the tube agitated gently for 10 minutes, after which 1 ml of 10 mg/ml proteinase K is added and the tube incubated overnight at 55°C with constant agitation. The following morning another aliquot of proteinase K and RNAse are added and the incubation continued for another hour. Extraction with 20 ml of acidified phenol is accomplished by gentle agitation on a rocking platform for 30 min. and then centrifuged in a GPR centrifuge at 2500 rpm for 15 min at 4°C. Using a large bore plastic pipette the DNA is transferred to a clean tube and the extraction repeated until a clean interface is obtained. Following the phenol extraction is an equivolume extraction with a Chloroform:Isoamyl Alcohol (24:1). The DNA is now transferred into a sterile beaker and mixed with one half volume of 7M NH_4OAc. To this mixture 2.5 volumes of EtOH are mixed in using a fishing loop made with a flamed borosilicate disposable pastuer pipette. By mixing slowly the DNA is spooled out of solution. The DNA is then redissolved into ten mls of TE and reprecipitated.

PLASMID MINI-PREP

1 Grow 10mls of Terrific Broth with antibiotics in a 50ml conical tube overnight. Take 100λ for bacterial stock. Spin the remaining cells in GPR centrifuge at 3000rpm at 4°C for 20-30 min. Discard the supernatant.

2 Freeze cell pellets at -20°C for 10 min (or overnight).

3 Resuspend the pellets in 100λ GTE (glucose, Tris, EDTA, as described in Manniatis) with light vortexing. **Do not leave cell clumps**. If necessary use a Pipetman in order to obtain an even suspension.

4 Add 10λ lysozyme, 10 mg/ml lysozyme solution (30 mg lysozyme in 3 ml cold H_2O). Vortex for 5 seconds, incubate at room temp for 10 min.

OR

Add 20λ frozen lysozyme solution. Vortex for 5 seconds, incubate at room temp for 10 min.

5 Add 400λ of 1%SDS in 0.2 N NaOH. Vortexing gently, add dropwise in 100λ aliquots. Incubate 15 min room temp..

6 Add 200λ of 3M NaOAc, pH 5 (Add as in step 5). Incubate at 4°C for 5 min.

7 Spin 50 ml tubes in GPR centrifuge at 3k rpm for 30 min at 4°C.

8 Transfer the clear supernate (≈720λ) to a 1.5 ml eppie tube. Add 150λ each of acidified phenol and chloroform. Vortex at high speed for 1 min. Spin in μfuge 4°C for 10 min. Transfer supernate (top layer) to a clean eppie, extract with 200λ of chloroform: Isoamyl alcohol, vortex for 1 min at high speed, Spin in μfuge 4°C for 10 min.

9 Transfer the supernate to a clean **1.9**ml eppie, and fill with 100% ethanol. Vortex gently and incubate at room temperature for 15 min.

10 Spin in µfuge for 15 min at 4°C. Remove ethanol, rinse pellet with 70% ethanol, and let air dry for 5 min.

11 Resuspend pellet in 200λ H_2O. Add 10λ RNAse {or 3λ RNAse cocktail} and incubate for 30 min at 37°C.

12 Extract with 200λ of acidified phenol, vortexing at high speed for 1 min. Spin in µfuge at 4°C for 10 min. Transfer supernate to clean eppie. Extract with 100λ of acidified phenol:100λ of chloroform, vortexing at high speed for 1 min. Spin in µfuge at 4°C for 10 min. Transfer supernate to clean eppie. Extract with 200λ of chloroform:Isoamyl alcohol, vortex for 1 min at high speed, Spin in µfuge 4°C for 10 min.

13 Transfer the supernate to a clean eppie, add 100λ of 7M NH_4OAc, and 700λ of 100% ethanol. Place on dry ice for 15 min, µfuge at 4°C for 15 min. Remove ethanol, rinse pellet with 70% ethanol, and let air dry for 5 min. Resuspend in 100λ TE.

Modified Alkaline Lysis Large Scale Plasmid Prep

1. Chill 500 ml culture on ice. Spin in 250 ml polypropylene bottles 6500 RPM for at least 10 minutes at 4°C (GSA). Decant supernate, freeze pellets at least 10 minutes at -20°C.

2. Thaw cells, resuspend pellets (from 500 ml of cells, grown in LB media) in 15 ml of GTE (50 mM glucose, 10 mM EDTA, 10 mM Tris-HCl, pH 8) **BE SURE that there are no clumps.** Add 10λ RNAse cocktail.

3. Add 2 ml of 10 mg/ml lysozyme, freshly dissolved in 10mM Tris-HCl pH 8. Incubate 10 minutes at room temperature.

4. Add 40 ml of 1% SDS, 0.2 N NaOH dropwise with constant agitation while on ice. Incubate 30 minutes on ice stirring occasionally.

5. Add 20 ml of 3 M NaOAc (pH 5.2) and incubate on ice.

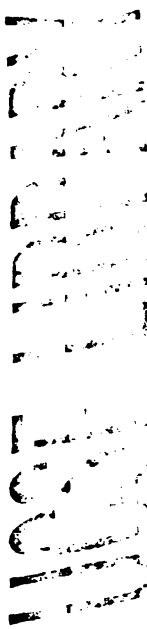
6. Spin 9000 RPM for 20 minutes at 4°C in 250 ml polypropylene bottles

7. Add 1.5 volumes of 7.5 M NH_4OAc (about 105 ml). Incubate 10 minutes at room temperature

8. Spin 6500 RPM for 20 minutes at 4°C. Save supernate, pellet contains RNA.

9. Add 1.5-2 volumes of absolute ethanol (255-340 ml). Do not fill past the shoulder of the bottles. Freeze at -80°C for 20 minutes then thaw at -20°C for ten minutes (or -20°C overnight). Spin at 6500 RPM at -10°C for 15 minutes.

10. Decant ethanol supernate. Rinse pellet with 70% ethanol and allow to dry but not bone dry. Resuspend pellet with 4 ml TE.



11. Pipet DNA solution into 15 ml conical bottom culture tubes containing 8.9 grams CsCl. QS to 10.5 ml with TE. Transfer to 15 ml thick walled round bottom tubes and add 1 ml EtBr cover with Parafilm and mix by inverting the solution ***gently***

12. Place culture tubes in a **room temperature** SM-24 rotor. Spin 8000 RPM for 30 minutes at room temperature.

13. Transfer the supernate into Beckman 13ml UltraClear ultracentrifuge tubes using 5 ml syringe stuffed with a 1 inch square gauze pad fitted with an 18 gauge needle.

14. Balance tubes in pairs to ±0.01 g. Spin 65000 RPM (Ti 80 rotor) for at least 12 hours at 23°C

15. Pierce the top of the tube two times to allow ventilation. Remove the upper DNA band with a 16-18 ga needle (this band contains damaged DNA and is garbage). Slowly remove the lower DNA band with a 16-18 ga needle extract repeatedly with 0.5 M NaCl saturated n-butanol (until the solution is colorless). Extract one time with TE saturated diethyl-ether to remove any residual butanol.

16. Transfer the DNA to a prepared piece of Type 2 10mm SpectroPor dialysis tubing and dialyze for 4-6 hour at room temperature in 6 liters of TE, with constant stirring.

17. Transfer the DNA from the dialysis tubing, into a 15ml tube, rinse the dialysis tubing with 1 ml of ddH_2O and speedvac to dryness.

18. Resuspend the DNA in 2 ml of H_2O, add 1 ml 7.5 M NH_4OAc, aliquot 600 ul into five 1.9 ml tubes and fill with 100% EtOH. Precipitate at room temperature for 10 min and microfuge for 15 min at 4°C for 15 min. Aspirate supernatant and rinse pellet with 70% EtOH, air dry for 5 min then resuspend in 500ul TE.

Rapid, Small-Scale Isolation of Bacteriophage λ DNA

Plate Lysate Method

(make one 150-mm plates for each λ clone to be grown up)

1. Mix 150 λ of the bacteriophage suspension (10^5 pfu) with 100 λ of 2x C600 hfl in 10mM $MgSO_4$. Incubate for 20 minutes at 37°C . Add 6 ml of molten 0.7% top ***agarose*** and spread on the surface of a freshly made, pre-warmed at 37°C, 150-mm plate containing 30-50 ml of ΔNZCYM plus 1.5% ***agarose.***

   NOTE: Do not use **agar** since most batches of **agar** contain potent inhibitors of restriction endonucleases!!!

2. Leave the plates 5 min at room temperature, then invert and incubate at 37°C for 6-8 hours, until the plaques cover the entire surface of the plate.

3. Add 13 ml of SM directly onto the plate and allow the bacteriophage to elute by agitating for at least 2 hours at room temperature .

4. Transfer the SM from the plate (approx. 12 ml) to a 12-ml polypropylene (blue cap tube), and remove the bacterial debris by centrifugation at 10k rpm for 10 minutes at 4°C.

5. Recover the supernatant (remove 1ml for high titre phage stock) and add 25 λ RNAse A (10mg/ml) and 1 λ DNAse I (2mg/200λ), each to a final concentration of 1 ug/ml. Incubate for 30 minutes a 37°C.

6. Add an equal volume of PEG solution: For 50ml: 10 g PEG 8000
   6.1 g NaCl
   0.1 g $MgSO_4 \cdot 7H_2O$
   2.5ml 1M Tris pH 8

   Incubate 1 hour at 0°C (ice water) or store overnight in cold room (4°C).

7. Recover the precipitated bacteriophage particles by centrifugation at 10,000 rpm for 20 minutes at 4°C.

8. Remove the supernatant by aspiration, then spin the tubes again briefly, and aspirate off ***all*** the liquid. Allow to air dry for 10 minutes.

9. Add 900λ SM (first solvate the pellet in 500 λ SM by vortexing and/or pipetting and transfer to an eppie, then rinse the original tube with an additional 400 λ SM) .

10. Spin in μ-fuge for 5 minutes at 4°C to remove debris.

11. Transfer the supernatant to a clean eppie tube. Add 9 λ 10% SDS, 9 λ of 0.5M EDTA, and 3 λ Proteinase K (20mg/ml). Incubate at 65°C for 1 hour.

12. Extract with phenol, phenol/chloroform(1:1) and once with chloroform. Transfer the aqueous to a clean eppie tube.

13. To the final aqueous phase add an equal volume of isopropanol. Store at -70°C for 20 minutes. Thaw and centrifuge at 4°C for 15 minutes.

14. Wash the pellet with 70% ethanol. Dry the pellet and resuspend it in 50 λ of TE.

15. Use 1/5-1/10 of the total DNA for a digest, digest in 200 λ total. Use at least 40 units of enzyme, and 1 λ of RNAse.

RNA Preparation

Total RNA preparation

Total RNA is prepared by the acid guanidinium-phenol-chloroform method (Chomczynski and Sacchi, 1987). Briefly, 1-2 gm of tissue are homogenized in 4ml of denaturing buffer (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7, 0.5% sarcosyl, and 0.1M 2-mercaptoethanol). Sequentially, 100 ul of 2M sodium acetate, 500 ul phenol, and 200 ul chloroform-isoamyl alcohol (49:1) are added, this solution is vortexed for 20 seconds. After centrifugation, the RNA is present in the aqueous phase, whereas the DNA and proteins were in the interface and phenol phase. The RNA is precipitated with isopropanol at -20°C for 30 min and then microfuged at 4°C for 20 min, the pellet is washed with 80% EtOH. The final RNA pellet is air dried and dissolved in DEPC treated water.

B Manipulation of DNA/RNA

Manipulation of nucleic acids involves one or more of several major steps, including 1) digestion with enzymes in order to cleave them, remove or add to one or both strands at their ends, or modify one or more bases; 2) separation of the digested piece(s) from unwanted products enzymes and other materials, using gel electrophoresis; 3) purification of the modified DNA from the gel; 4) subcloning of the DNA into vectors and the subsequent insertion into bacteria, allowing the DNA to be synthesized in relatively large quantities; and 5) sequencing of the DNA after isolation from the bacteria. In addition, other methods of amplifying DNA, such a polymerase chain reaction (PCR), were used in these studies.

Restriction endonuclease digestions of DNA has not changed since its discovery. The number of known restriction endonucleases is always increasing, yet relatively few new restriction enzymes (RE), which recognize new and unique nucleotide sequences, are now discovered. A vast majority of the recently discovered RE are isoschizomers of known RE and are brought to market because of a quality or cost difference. Currently all suppliers of RE provide their 10x incubation buffer, thereby decreasing the end user error. Furthermore, thorough investigation of RE and their subsequent cloning has provided the end user with a dependable product whose nuances are clearly defined.

Gel electrophoresis has changed little at the gel end of the procedure although the equipment has improved a bit and the power supplies are much more reliable and smaller than ever before. The visualization and documentation is still accomplished with ethidium bromide and a photograph (Polaroid), although computer digitalization is definitely breaking ground in this area.

Immobilization of both RNA and DNA has seen numerous "gadgets" come to market that promise better transfer than the capillary method of E.M. Southern, but his method has withstood the test of time and is still used as the mainstay of most labs. Of the various methods electrical transfer, pressure blotting and vacuum

transfer equipment available, the comparative advantage of any one method over another, if any, would be the speed of transfer at the cost of yield. One of the problems of transfer apparatuses is the need for masks to block the areas that aren't to be transferred. If the lab is doing numerous different size gels, then the number of masks necessary can be daunting and cumbersome. Capillary blotting/transferring is still preferred for most procedures.

1) Restriction Site Analysis

    a) Incubation Conditions
    b) Agarose Gels
        1) Preparation
        2) Running
        3) Staining
    c) Transferring
        1) Southerns
        2) Northerns

2) Fragment isolation/purification

    a) Gene-Clean
    b) Phenol-Chloroform extraction
    c) EtOH precipitation

3) Ligation/Transformation

4) Probe generation & purification

    a) Random labeling
    b) End labeling
    c) Ribo-probes

5) PCR

a) General Protocol
b) Product analysis

6) Sequencing

a) Sequenase
    1) Single Stranded
    2) Double Stranded
b) PCR Sequencing
    1) Vent
c) Acrylamide Gel conditions

7) Antisense Construction

a) Vector / Insert construction
b) DNA preparation for Stable Transfection
c) DNA preparation for Transgenic Mice
    1)Preparation of Tail Genomic DNA
        a)PCR analysis of Tail DNA

8) P1 Subcloning

a) TN 1000

Analytical restriction enzyme analysis is usually carried out in a small volume, such as 25 ul. The digestion typically contains:

2.5 ul of the vendor supplied 10x Buffer
1.0 ul of the restriction enzyme (usually 10 u / ul)
1.0 ug of DNA
x ul of H_20 (enough to bring the volume to 25 ul)

The components are mixed together by finger flicking, briefly spun down in a microfuge then placed into a 37°C incubator for 1 hour. Certain enzymes like Acc-1 or Sma-1 are much more efficient at temperatures other than 37°C, as stated in the enzyme description and the temperature is adjusted accordingly.

Preparative restriction enzyme is done on whatever scale is necessary to produce enough of the desired band - back calculating from after isolation from the gel. For example, if I need 5ug of purified DNA from a certain fragment, and I allow for a 50% yield for the procedure of isolating the DNA from the gel, then I should load enough digested DNA onto the preparative gel to electrophoresis 10ug of that specific band. Depending upon what percentage the desired fragment accounts for within the source DNA (construct or genomic) it may be necessary to digest 10-20 times the amount of the desired product, in an appropriate volume (not to exceed 1ug DNA/ul) and then precipitate the DNA before running the gel.

Agarose concentrations are altered in order to obtain maximum resolution of the DNA being electrophoresed. The chart below is taken from Sambrook et al (1989) and is followed accordingly. The buffer conditions for DNA gels are generally 0.5-1x TBE.

% Agarose	Size separated (Kb)
0.5	1 to 30
0.7	0.8 to 12
1.0	0.5 to 10
1.5	0.2 to 3
3	0.01 to 1

Staining of the Gels is accomplished by soaking in deionized water with trace amounts of Ethidium Bromide. Best results are obtained by staining overnight at 4° C. The stained bands are then visualized in UV light with a wave length of 300nM.

Southern Blots

1) EtBr stained gels were irradiated for 3-5 minutes on the UV light box in order to introduce nicks into the DNA

2) the gels were depurinated 15 minutes in 6 N HCl

3) Denaturation in 0.5 N NaOH/1.0 M NaCl solution (2 x 15 minutes)

4) Neutralization 0.5 M Tris pH 7.6/1.5 M NaCl solution (2 x 15 minutes)

5) 10x SSC (2 x 10 minutes) prior to transferring.

6) Transferring of the DNA is via capillary transfer using a 10xSSC solution and allowing the transfer to proceed at least 10 hours.

7) The membrane containing the DNA is then UV irradiated (Stratalinker, Stratagene autocrosslink)

HYBRIDIZATION PROTOCOL

Hybridization solution (100ml)
10ml 50x Denhardts
30ml 20x SSC
5ml 10% SDS
1ml 10% Sodium Pyrophosphate
2ml 0.5M EDTA
52ml dH_20

Add 100µg/ml (final concentration) Salmon sperm DNA (ssDNA).

100µl of 10mg/ml ssDNA for each 10ml of solution used for hybridization.

Prepare the hybridization solution and warm it to the temperature to be used for hybridization.

Prewet the membrane with ddH_2O for five minutes then squeegee out the H_20

Add the appropriate amount of hybridization solution to a seal-a-meal bag.

Aliquot the appropriate amount of ssDNA into a screw cap tube. Denature by heating at 95°C for 10min and snap cool on ice.

Add the denatured ssDNA to the bag with the hybridization solution and seal twice.

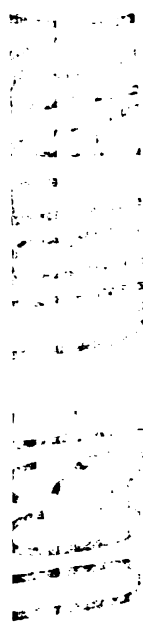
Prehybridize for at least two hours. In a constant temperature water bath with constant agitation.

After prehybridization, remove the solution from the bag and add fresh hybridization solution, same as above.

Denature the appropriate amount of probe (10 million cpm) by heating at 95°C for 10min and snap cool on ice for a few minutes.

Add the probe to the bag with the hybridization solution and ssDNA and seal twice.

Hybridize for at least eight hours or overnight. In a constant temperature water bath with constant agitation.



GeneClean

Know the approximate quantity of DNA that is being loaded on the gel

Run and stain the gel

Cut out the band with the least excess agarose

1) **Weigh the gel slice** (WEIGHT in gm = equivalent Volume in ml)

2) **Add the corresponding amount of TBE modifier and Sodium Iodide (0.5 equivalent volume of TBE modifier and 4.5 equivalent volumes of NaI.**

3) **Solubilize the gel slice thoroughly.** Vortex and heat to 65°C as necessary, but do not heat excessively.

4) **Add the Glass Milk.** For 1ug or less DNA loaded use 25λ, for other quantities of DNA use 5υl /μg of DNA in addition to the 25λ.

5) **Allow the solution to sit in ice for 15 minutes,** agitating at least three times during the 15 minutes.

6) **Centrifuge the solution for 10 seconds,** aspirate off the liquid, avoid the pellet.

7) **Resuspend the pellet in 500λ of NEW WASH, centrifuge the solution for 10 seconds**, aspirate off the liquid, avoid the pellet. Repeat two more times.

8) **After the third wash, resuspend the pellet in 100λ H_2O.** Heat at 50°C for 10 minutes.

9) **Centrifuge for 3 minutes, carefully remove the aqueous** avoid the pellet and loose glass milk.

10) **Speedvac to dryness and resuspend in 20λ TE or H_2O.**

Phenol-Chloroform-Extraction Ethanol-Precipitation

add 1/2 vol. Phenol. (acidified phenol saturated with 0.4M NaOAc)
add 1/2 vol. Chloroform

vortex thoroughly (at least 1 min)
microfuge 10-15 min at 4°C
remove the upper phase and transfer to a clean 1.5ml microcentrifuge tube

add 1/2 vol. Chloroform: IsoAmyl Alcohol (24:1)

vortex thoroughly (at least 1 min)
microfuge 10-15min at 4°C
remove the upper phase and transfer to a clean 1.5ml microcentrifuge tube (if interface is not clean repeat the entire procedure now)

add 1/2 vol. 7.5 NH_4OAc,
add 2.25x vol. 100% EtOH

Vortex, then place at:

fastest:	15 minutes on dry ice
fast:	2h at -70°C
normal:	overnight at -20°C

microfuge 15min at 4°C, aspirate off supernatant and wash pellet with 70% EtOH, aspirate off all liquid and allow to air dry for 10 minutes.

Ligation Reaction

Subcloning Fragments

10x Ligation Buffer (500mM TRIS pH 7.5 and 100mM $MgCl_2$)
20x DTT - BSA (200mM DTT and 100µg/ml BSA)
10mM ATP (1.1mg in 180λ H_2O)

”””

1 λ Ligation Buffer
0.5 λ 20x DTT-BSA

[It is wise to double these volumes-make a premix to decrease pipette errors]

1.0 λ 10mM ATP
2.3 λ H_2O
2.0 λ Isolated precut fragments-(1-2 times equimolar amount)

2 λ DNA - Isolated precut Vector-(About 25-50ng)

1 λ T-4 ligase

”””””””””””””””””””””””””””””””””””””””

mix-let sit overnight at 10°C

Transformation:

-Thaw cells on ice (or use fresh cells)

-Aliquot 200ul into a Prechilled 17 x 100 sterile glass tube containing 3ul of 0.3M 2-mercaptoethanol (freshly made). Allow to sit on ice for 5 minutes.

-Add DNA ligation mixture, mix gently (finger flick) then put on ice for 30 min.

-Heat shock at 42°C for 90 seconds

-Put tubes back on ice for 2 min.

-Add 800 ul for SOB + 10mM $MgCl_2$/10 mM $MgSO_4$ + 20 mM Glucose (can be prewarmed to 37°C)

-Incubate in a 37°C shaking water bath for 30 to 60 min. (60 is better)*

-Transfer to eppie tubes and μ-fuge for 30 seconds

-Aspirate off the supernatant and resuspend in 1ml of LB/amp

-100 λ of incubation mixture (on LB + amp plates)

*- Alternately take 1 λ of the incubation mixture and add to a 100 λ drop of LB/amp on the center of a plate -Spin the remainder of the cells down and resuspend in 200 λ of LB and plate 25 and 50 λ -Add 850 λ LB + amp, refrigerate.

Random Labeling

Take 2λ of DNA

put in 4.5 λ H_2O

put into 95° water bath for 5 min.

cool in ice water for 3 min,

then add:

3 λ H_2O

11.5 λ of LS

1.0 λ BSA

3 λ of ^{32}P-d CTP

----------VORTEX

Remove 1 λ to a GFC filter paper

Add 1-1.5 λ of Klenow

----------Vortex

Incubate at 37° for 1 hour

{or leave at room temperature overnight}

then remove 1λ to another filter paper,

wash filter papers with 5% TCA solution

at least three times

---

if incorporation is high enough

Preare Boehringer Mannheim Quick Spin Column (GF-50) by centrifuging in GPR for 3 min at 1000 k, repeat spin again with a 1 ml wash of TE

ADD to Labeling Reaction:

25 λ TE

Load onto a Quick Spin Column

Add additional 50 ul TE to the column

centrifuge in GPR tabletop for 5 min at 1200 rpm

take aliquot and determine activity in scintillation counter

End-Labeling Primers

Place the following ingredients in a 500ul screw top tube:

8.0 λ dd H_2O

3.5 λ 10X kinase buffer (must be same company as enzyme)

10.0 λ Primer (100 ng)

12.5 λ γ^{33}P-ATP

1.0 λ T-4 polynucleotide kinase
(diluted 1:10 with 50 mM Tris-HCl pH = 8.0)

-light vortex

-spin briefly in micro centrifuge

-incubate at 37°C for 60-90 minutes

-heat to 100°C for 10 minutes

-spin briefly in micro centrifuge

-store at -20°C

Riboprobe Protocol

1. Linearize template DNA to completion using appropriate enzyme.
2. Check digest completion by running an agarose gel.
3. Extract and precipitate to remove inactivated enzyme and other garbage.
4. Add the following components at room temperature in the order listed:

a.	Transcription 5X buffer	4λ
b.	100mM DTT	2λ
c.	RNAsin ribonuclease inhibitor	0.5λ (20 units)
d.	2.5mM ATP, GTP, CTP	4λ

(made by mixing 1 volume water with 1 volume each of the 10mM stocks)

e.	100μM UTP (diluted from stock)	2.4λ
f.	linearized DNA (1-2 mg/ml)	1λ
g.	[α-^{33}P]UTP (100μCi at 20mCi/ml)	5λ
h.	RNA polymerase (SP6, T3, T7)	1λ (15-20u/μl)

Incubate for 60 minutes at 37-40°C

Polymerase Chain Reaction (PCR)

PCR is a theoretically simple, though often practically difficult, procedure for amplifying DNA. It is particularly useful when the DNA sequence of interest comprises a very small proportion of a mixture of other DNAs, as is the case, for example, in a cDNA library. A pair of primers corresponding to the sense and antisense sequence of a portion of the sequence of interest are used to initiate synthesis of opposite strands of the DNA sequence between them. Since each primer synthesizes sequence that the other can anneal to, the result is a two-fold amplification of DNA per cycle of denaturing, annealing and extending. In theory, therefore, 20-30 cycles can amplify the DNA of interest a million or more fold, though in practice factors such as nucleotide triphosphate depletion and product inhibition set limitations in the microgram range.

The main trick underlying successful PCR is optimizing conditions: finding the most suitable temperatures and times for each of these steps, as well as the best ionic conditions. As a general rule, the denaturation step is carried out at temperatures exceeding 90°C, the annealing step at a temperature slightly above the melting temperature of the primer/DNA hybrid (40-70°C); and the extension step at 70-75°C. The first two steps of the cycle normally do not require more than 1-2 minutes, while the extension step requires approximately 1 minute per 1000 bases of extension. In addition to time and temperature, the Mg^{++} concentration is also often critical, and may have to be varied if PCR using the standard concentration in the available buffer is not successful.

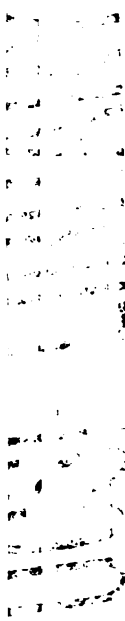
The General PCR Cycle protocol is

Initial Denature	5 min	94°C
Denature	60 seconds	94°C
Anneal	30-60 seconds	58-64°C
Elongate	30-60 seconds	72°C

cycle 35 times (one cycle is contained within above box)

the reaction volume varies between 25 and 100 ul. The buffer conditions remain relatively constant, and are supplied with the particular Thermal DNA polymerase being used.

Because of the sensitivity of the DNA polymerase to changes in ion concentration - some method is employed to seal the reaction environment. Most commonly a drop of mineral oil is placed directly upon the reaction mixture. Variations of this are use of wax or petroleum jelly or heated bonnets on the thermal cyclers themselves.



Analysis of PCR Products

Because many of the PCR products obtained are often between 60 and 1000 bp, and it is necessary to accurately discriminate their size, so a high percentage agarose gel is necessary in order to resolve the experimental products. The best results are obtained from a 2% NuSieve (FMC Products) 1% agarose (Gibco BRL) with a 1%TBE buffer, although a 2-3% agarose will suffice much clearer results will be consistently obtained with NuSieve. Furthermore TBE gels definitely produce better results than TAE. Due to the low molecular weight a different DNA ladder is used, a low molecular weight ladder from BioVentures which not only sizes the small fragments better then the 1Kb ladder(Gibco-BRL) but is also calibrated to stain (using ethidium bromide and UV detection) with a specific DNA density of 50 ng per band.

Occasionally the band(s) of interest might need to be reamplified. This is easily accomplished by plugging out a small tube of the desired band with a sterile Pasteur pipette, and equilibrating the plug in 1 ml of sterile H_2O in order to eliminate as much EDTA is possible. The equilibrated plug is then cut and a portion of it added to a new PCR reaction. As previously stated these DNA polymerases are extremely sensitive to variations in the Mg^{++} concentrations, and even small amounts of chelating agents can inhibit the reaction.

Sequencing

Sequencing of Nucleic acids has advanced from the original methods set forth by Sanger in his invention of dideoxy chain termination sequencing. Although at the same time Maxim and Gilbert had also devised a means of sequencing DNA by means of chemical treatment their methodology never caught on, and although some scientists persist in sequencing with this methodology there have been no improvements in it since its inception. The premise of Sanger dideoxy chain termination sequencing has not changed since it original description; the idea is remarkably simple. Initiate chain elongation on a single stranded template with a synthesized primer which is complimentary to a known sequence on the template. Supply a polymerase and all the nucleotides necessary to allow for chain elongation, and then after some period of time allow random introduction of a modified nucleotide that will terminate elongation. By knowing which modified nucleotide was introduced the identity of that nucleotide can be deduced, and by electrophoresing the products of this reaction on a high resolution denaturing electrophoresis apparatus the exact size of the terminated fragments can be determined. The variabilities of this reaction initially were few, for the radioactive nucleotide only ^{32}-P was available, and for the template, single-stranded DNA was acquired by use of an M-13 based cloning vector which produced a filamentous phage as part of its replication cycle. Therefore, in order to sequence a segment of DNA it needed to be cloned into a vector and grown up in order to generate the phage DNA, which in turn needed to be isolated and purified before sequencing could be started. Additionally, sequencing with a high energy emitter such as ^{32}-P is possibly not too healthy for the user, based upon the amount of radioactivity needed, the constant exposure required in the handling and manipulation of the reactions and the need for special areas and shielding in order to use it. ^{35}-S was soon introduced as a labeled nucleotide and provided a much safer radioactive tracer. Double stranded sequencing was developed by denaturing the DNA to be sequenced with sodium

hydroxide and then neutralizing the reaction and precipitating the DNA. About this time, RNA sequencing was introduced in which an RNA polymerase such as T-7 or SP-6 was used both as the polymerase and the initiating primer. One immediate advantage of RNA sequencing was the amplification of template that would occur. RNA polymerases synthesize the complementary strand and simultaneously peel it off, so when they come to a termination, they can go right back to the beginning and start again, in essence amplifying sequence obtained from a small amount of starting material. Another advantage to RNA sequencing was the ability to sequence double stranded DNA without preparing single stranded DNA. The major disadvantage which prevented most scientists from embracing this slick technology was the inherent instability of RNA. The precautions necessary in order to produce and handle RNA are incredibly stringent and very intolerant. Any contamination of any solution, pipettor, tip, tube, enzyme even loading buffer with RNAse will instantly destroy the sample.

Problems which plagued Sanger sequencing are constantly being met with new solutions. Initially the DNA polymerase used for sequencing was Klenow, and because of the low temperatures that Klenow functions at, one of the most common problems was secondary structure of the template, interfering with the sequencing reaction. Modified nucleotides were developed, such as 7-deaza-GTP, d-ITP and a few others. These were very successful at eliminating many of the structural problems, and now it seemed as though the enzyme was the limiting factor. A scientist experimenting in the modification of Klenow developed an exonuclease-deficient enzyme that was highly processive and produced consistent results. This discovery provided the scientific community with an enzyme that has cornered the market in DNA sequencing, known as Sequenase.

With the advent of PCR a wonderful new venue for sequencing evolved. It combined all the advantages of RNA sequencing, no need to single strand the template, amplification of the sequence generated and it added one new advantage that couldn't be previously addressed. Increased temperature was known to

eliminate secondary structure, but was toxic to the enzyme. The thermal stable enzymes used for PCR were very promising and numerous companies have invested in this competitive market. Recently a few kits have become available that function as well as could be hoped for. The DNA used for sequencing can come from nearly anywhere, even genomic DNA, no less Magic Mini Preps.

In a continuing effort to decrease the personal exposure to radioactive materials and increase the quality of the results obtained the newest addition to sequencing is ^{33}-P nucleotides. They have the relatively safety of ^{35}-S with an increased signal similar to ^{32}-P. Ideally, one wants a non radioactive nucleotide that would be easily detectable without excessive manipulations of the electrophoresis procedure or the sequencing reactions, but this has not yet come to market. This theory is the basis of automated sequencers, using a fluorescent chromophorically modified nucleotide, that is incorporated into the sequenced DNA, but in order to detect the chromophores a special computerized detector is necessary, and is quite expensive for the average lab. Another variation that has recently surfaced in the direct incorporation of a modified nucleotide that is detected by a luminescent compound. Though the theory is promising the results have been disappointing.

Initially all sequencing done in this project was with Sequenase. The methodology of obtaining single stranded DNA progressed from base denaturation, neutralization, and precipitation to a heat denaturation in the presence of the sequencing primers, and finally the sole method of sequencing became PCR, commonly referred to as cycle sequencing. The methodology currently used is to end label the sequencing primer with ^{33}-P and then incorporate additional radioactivity by supplying ^{35}-S into the elongation reactions. This was arrived upon by trial and error and tends to produce consistent results on any DNA template supplied.

Sequencing Gel Recipes

Sequagel™

	4%	5%	6%	8%
Concentrate	20.8 ml	26.0 ml	31.2 ml	41.6 ml
Buffer*	13.0 ml	13.0 ml	13.0 ml	13.0 ml
Diluent	96.2 ml	91.0 ml	85.8 ml	75.4 ml
Ammonium Persulfate	50 mg	50 mg	50 mg	50 mg

* can use 10X Taurine buffer (Taurine is the preferred buffer as it seems to be tolerant to glycerol in the sample loading buffer) in place of supplied buffer

	5 %	6%	8%
Urea	52.8 g	50.4 g	50.4 g
Ammonium Persulfate	50 mg	50 mg	50 mg
40 % Acrylamide(19:1)	15 ml	18 ml	24 ml
10X Buffer*	12 ml	12 ml	12 ml
dd H_2O	72 ml	48 ml	42 ml

* can use 10X TBE or 10X Taurine buffers

Dissolve, filter through a GF/B filter, de-gas, and add 60 λ TEMED before pouring

Sequencing Gel Set-up

**Assuming gel was already poured and gel is polymerized (takes about an hour)

1. remove clamps from plates
2. carefully remove comb and bottom spacer
3. rinse under running water outside of plates to remove dried gel, dry inner plate
4. clamp gel into gel apparatus pushing top rubber spacers tightly into place
5. add 1X TBE or Taurine buffer (same as in the gel) to buffer tanks, check for buffer leaks along rubber spacers.
6. remove air bubbles from between the top and bottom areas of the plates with a syringe filled with buffer
7. hook up to power supply and start run at 65 watts(for a single gel)
8. pre-run gel until gel temperature reaches 40-45°C (about 40 min.)
9. denature samples in 76°C for 5 minutes, place on ice
10. load 2.5-4.5 λ of each sample into wells, at the bottom of the wells, load in groups of 4
11. after loading 4-12 samples run samples into the gel before loading next set of samples
12. skip a lane to give orientation to gel

Double Stranded DNA Sequencing

Template Preparation

Use stopwatch to time intervals!

8-12μg DNA QS to 40λ with H_20 (10μg recommended)
Warm to room temp (RT).

Add 4λ 2M NaOH/ 2mM EDTA

(this solution should not age more than 2-3 weeks)

Finger Flick

RT 8 min : Exactly!

(space out samples by 1min if doing multiple samples)

QUICKLY Add in order :

14λ ice cold H_20 (Pipette once up and down)
11λ 3M NaOAc (pH 4.7)
150λ ice cold EtOH

VORTEX

Put on Dry Ice, for 15 min.

Take 1λ, check pH on paper, want pH ≤ 7

If Necessary: Add 1λ 3M NaOAc until pH ≤ 7

μ-fuge 4°C for 15 min.
70% EtOH wash (0.5ml)
Vac Dry, store -20°C

Sequenase II Protocol

Dissolve prepared template DNA pellet in 28λ H_2O: Take 7λ for annealing step

I) Annealing 37°C for 20 min

DNA	7λ
Reaction Buffer	2λ
Primer or Reverse Primer	1λ

During the annealing reaction:

1} Dilute labeling mixture - 1:4
(1λ mix plus 3λ H_2O) Make whatever is needed

2} Add 2.5λ of the ddNTP's termination mixes to their respective tubes on ice.(ddGTP to G, etc.)

3} Dilute Sequenase enzyme with buffer.
Just Before Use. {1.5λ Enzyme, 10.5λ buffer}

After annealing, place tubes on ice -

II) Labeling Reaction 2-5 min, RT (< 20°C)

To each tube on ice,

Add:

1.	DTT	1.0λ
2.	diluted labeling mix	2.0λ
3.	^{35}S-d-ATP	1.0λ

Place 2 sets G A C T tubes in 37°C H_2O bath

Tube	Add 2λ enz. (RT 3-5 min)	Portion out 3.5λ to G A C T tubes at 37°C µfuge, then incubate 37°C 5 min	4λ STOP
1	0:00	5:00	10:00
2	2:30	7:30	12:30

Vent Sequencing

1. label planning sheet, calculate master mix (over-estimate) for one reaction:

a. PCR water	5 λ
b. Circumvent buffer	1.5 λ
c. Triton	1 λ
d. Primer	2 λ
e. ^{35}S-dATP	1.5 λ
f. Vent Polymerase	1 λ

2. lay down white diaper in ^{32}P area
3. thaw out ^{35}S, PCR kit, previously end-labeled primers, and DNA
4. place PCR tubes in rack following planning sheet (use racks specified for sequencing)
5. set up one 500ul tube for each sample of DNA and each primer
6. add 3 λ G mix to G tubes in rack, A mix to A tubes, etc..... cover with tape and set aside

Radioactive Precautions Must Be Used From This Point On - Do Not Leave Area Without Removing Gloves

7. make master mix- must wear gloves and lab coat, use filter tips cap tubes and set aside
8. distribute 12 λ master mix to 500ul tubes (radioactive!)
9. add 3 λ DNA to above 500ul tubes following planning sheet, mix with gentle pipetting
10. immediately distribute 3.2 λ of above mix to tubes into PCR rack
11. add 1 drop Nujol oil to each tube, cap tubes, quick spin in table-top centrifuge
12. place tubes in PCR machine, run 20 cycles of: 94°C 30 seconds, 62°C for 45 seconds and 72°C for 45 seconds (for a PE 9600)
13. at end of PCR program add 4 λ stop/loading dye to each tube
14. store samples at -20°C
15. check area and equipment for radioactive contamination, clean up if necessary, dispose of waste in short half-life waste

C.) Bacterial Manipulation

1) Plating
    a) plasmid plates
    b) phage plates
2) Transformation
    a) Competent cells
3) Libraries
    a) Construction
    b) Screening

Competent Cells:

-Make a fresh overnight in **SOB** + 10mM $MgCl_2$/10 mM $MgSO_4$ (from a single colony)

-Inoculate 100ml of SOB + 10mM $MgCl_2$/10 mM $MgSO_4$ with 1ml of the overnight (use a 1L flask)

-Grow until A_{550} approx. 0.55 (for MH1 this takes about 1.5 hours)

-Pour into two 50 ml conical tubes and ice for 10 minutes

-Centrifuge at 4°C (approx. 2500rpm in Table top Beckman) for 10 min.

-Decant supernatant quickly and resuspend both pellets in 50 ml (total) ice cold 100mM $CaCl_2$ (use a 10ml pipette to resuspend cells and keep on ice at all times)

-Incubate on ice for 30 min.

-Centrifuge at 4°C, 2500 rpm for 10 min.

Decant supernatant, resuspend in 7ml (total) of freshly made 100mM $CaCl_2$ (for fresh cells) or 100mM $CaCl_2$ + 15% Glycerol for cells to be frozen.‡.

-To freeze, place screw to 1.5ml tubes screw top tubes (i.e. Sarstedt) in a dry ice bath (or liquid N_2 bath), aliquot 600 ul directly into tubes.

Place immediately into -80°C freezer

‡ Alternately the cell pellet can be resuspended in RF2 buffer for freezing (Hannah DNA Cloning)

LIFTING COLONIES/ PHAGE FROM PLATES

1. place membrane on precooled plates (label membrane 1st w/S&S Pen)

2. Place face down, allow the filter to wet evenly

3. mark orientation by poking through the membrane with an 18gauge needle dipped in Indian ink. Mark asymmetrically in three different locations.

4. carefully lift off the membrane with forceps and float on top of the following solutions without immersing, with the side of filter which has been exposed to bacteria/ phage on top (sunny side up). Allow the membrane to remain on the solution for at least 3 minutes before moving it onto the next solution.

5. 1st solution: 0.2M NaOH + 1.5M NaCl

6. 2nd solution: 0.2M Tris pH 7.0 + 1.5M NaCl

7. 3rd solution 6xSSC

8. place on filter paper < DNA side up> (Whatman 3mm) allow to capillary dry (only to a damp state)

9. UV crosslink (Stratalinker, on AutoCrosslink Setting)

Direct Colony Screening

1 Pick one colony from the plate with a pipette tip, touch down lightly onto a clean grid plate and then place the pipette into a µfuge tube containing 12λ of lysis solution.

LYSIS SOLUTION
10mM Tris pH 7.5
1 mM EDTA
50µg/ml Proteinase K
5 u RNAse

2 Resuspend colony by pipetting up and down.

3 Heat at 50°C for 15 minutes

4 Heat at 95°C for 10 minutes

5 add 10 λ H_2O vortex, microfuge sample for 3 minutes

6 Remove 10λ for PCR

7 PCR rxn is 50λ volume:

10λ	lysed colony
2λ	primers (1λ forward :1l reverse)
5λ	10x Buffer
4λ	dNTPs
0.5λ	Taq
28.5λ	H_2O

PCR Program: 95°C 5 min

95°C	45 sec	
42°C	30 sec	
72°C	45 sec	1 cycle

35 cycles

Library Construction

Initially I wanted to create my own library, isolating and purifying the mRNA from rat and then constructing the cDNA into a lambda GT-10 bacteriophage vector. The kit chosen for library construction was the Boehringer Mannheim lambda GT-10 kit. Unfortunately neither attempt using this protocol resulted in a library with a high enough titer, therefore, postponing the screening.

So in an effort to make progress, a preconstructed library was acquired from Stratagene. A major reason for choosing this library was that it would prepare me for a later attempt at constructing my own library, using a new, not yet released Stratagene Vector Kit called Uni-Zap. This kit was a modified version of their well proven Lambda Zap Phagemid. The advantage of a phagemid over a phage as a host vector is that subcloning of the cDNA contained within the positive phage obtained from library screening is circumvented. Through the mechanism of helper phage added into a growth of bacteria containing the positive phage, the replication of lambda phage is interrupted and the insert cDNA contained within the plasmid pBluescript is replicated as a single stranded phage which was then isolated and used to infect new host cells; these circularize and fill in the second strand, thereby creating a stable plasmid subclone. The modification of the Lambda Zap library construction kit is asymmetrical restriction cuts of the vector arms and asymmetrical adapters attached to the cDNA, thereby allowing the direct generation of riboprobes in sense and antisense orientations, and providing a predetermined orientation of the reading frame.

UNI-Zap

Simultaneously with the screening of the Stratagene library, another library was constructed using a prerelease version of the Uni-Zap library construction kit. The mRNA used was from Clontech (#6712-1 Lot #0320). The instructions in the kit were followed to the letter. All the steps proceeded as stated and the yields were near perfect. A small amount of the double stranded cDNA was analyzed by electrophoresing on a 1% agarose gel and then drying the gel and exposing to film. The results indicate the range of cDNA sizes, from approx. 800 bp to 3000 bp with the highest density around 2200 bp. The procedure for packaging and titering the library was to work with 20% of the ligated cDNA product at a time. So as per the instructions 1/5 of the library was packaged in Giga-Pak Gold and titered as per the instructions; the final titre was within 10% of that stated in the instructions. Library screening was carried out as detailed in the following section.

Screening a cDNA library

The library obtained from Stratagene was amplified one time, as are most commercially available libraries. The problem with amplified libraries is that some clones will lag behind in their growth rate, compared to the major population of clones; these can be easily lost in a single amplification step. So with this inherent flaw in mind I screened the library. The initial step of library screening entails the titering of the library obtained. This step was necessary in order to quantitate and regulate the number and density of phage that were to be plated out. The original host strain of bacteria was PLK-F', a proprietary bacterial strain of Stratagenes' that was promoted as producing more definitively clear plaques. Although that may be true, it was also a rather sickly strain that necessitated constant reselection in order to keep the genotype. Often the host bacteria growth failed to grow or did not provide reproducible phage titer. In spite of this, thirty 105mm plates of NZcym bottom agar were plated with 7ml top agarose with a phage density of approximately 50,000 plaque forming units (pfu) per plate. The phage particles were allowed to preinfect 200 ul of the host bacteria for 20 min. at 37°C, the latter were prepared by growing a 25ml overnight growth in NZcym at 30°C, spinning down the bacteria at 4°C the following morning and resuspending the bacterial pellet on ice with 10 ml ice cold 2M $MgSO_4$. After infection, plates were incubated inverted at 37°C for 7-10 hours, until discreet pin head sized plaques formed across the surface of the plate. They were then placed at 4°C for at least 2 hours before lifts were done. In order to provide a certain degree of confidence that radioactive spots represent genuine phage with the DNA of interest, the plaque lifts were done in duplicate. The first nitrocellulose filter (Schleicher & Schuell) was laid down for 1 min. on the phage and the second filter laid down for 4 min. (see Lift Protocol above). The filters were hybridized with the appropriate probes. The prehybridization, hybridization and washes were all carried out in high stringency conditions in HY buffer at 65°C, 13 ml for the first blot and 7 ml for each additional blot (see Hybridization Protocol above). The filters were put up on film in a sandwich

format and exposed at -80°C for 1 week. The autorads were aligned to the corresponding plates and the possible positives marked on the plate; the plaques which appeared positive with each of the probes were selected for a second round of screening. The blunt end of a P1000 blue tip was used to excise the regions which contained positive plaques from the primary plates, and the plug soaked in 1 ml of SM containing 20 ul of $CHCl_3$, for 1 hour at 4°C. The second and third rounds of screening were done in 85mm petri dishes, using NZcym as before, but only 100 ul of host bacteria and 3ml of top agarose. The hybridization, washing and exposure were carried out as before. Those plaques which appeared positive at the end of a second round of screening were plugged from the plate using the blunt end of a P200 yellow tip, eluted as before, and used for the final round of screening. The plaques which appeared positive at the end of a third round of screening were removed from the plate with the sharp end of a sterile Pasteur pipette. Before any analysis of the plaques was undertaken, the in vitro excision of the plasmid contained within the phage was done as per the protocol accompanying the library.

Antisense Construction

In order to prepare the cDNA for injection into the mouse embryo a suitable initiation and termination signal needs to be attached to the fragment of DNA used for the antisense. The vector chosen was Pharmacia pSVL. It contains the SV 40 promoter and SV 40 polyadenylation signal and the transcriptional region was flanked by two unique restriction enzyme sites, allowing easy removal of the constructed transcriptional unit. The fragment of cDNA chosen for the construct was the upstream (5') untranslated region of DUZ1. In order to subclone this fragment of cDNA any existing Eco R1 sites had to be destroyed, as this enzyme would to be used to excise the entire transcriptional unit for the mouse embryo injections. So 50 ug of DUZ1 plasmid was digested with EcoR1 and the DNA extracted and precipitated (See M & M). The semi-dried pellet was resuspended in 1x Mung Bean Nuclease buffer and the 5' overhanging ends of the

fragments cleaved off with Mung Bean Nuclease. The reaction mixture was electrophoresed on a 1% agarose TBE gel and the 500 bp band cut out with a razor blade, the DNA isolated with Geneclean (See M & M). A portion of the isolated fragment was then run on another 1% agarose gel to confirm purity and concentration. The vector pSVL was prepared by digesting with Sma-1, and then extracting and precipitating. See Figure 12 Pg. 113. The ligation was performed (See M & M) and the transformed cells XL1-Blue (See M & M) spread on NZcym+Ampicillin plates (See Recipes). Because the insert contained identical blunt ends, it could be oriented in either the sense or antisense direction within the vector, so ten colonies were picked off the plate and grown up in 10ml Terrific Broth + Ampicillin overnight. The DNA was prepared in a Mini-prep format (See M & M) and analyzed by restriction digestion and PCR. About 60% contained inserts, of these four clones were in the sense orientation and the remaining two in antisense orientation; for a blunt end ligation this was very good efficiency. Primers were designed against the regions immediately flanking the insert in order to allowing sequencing of the clones, and confirm their integrity.

Preparation of DNA for Stable Transfection

An antisense clone to DUZ1, pSVL3, was chosen to be the construct used for both the stable transfection and transgenic mice experiments. So it is grown in a large scale prep (See M & M) and the DNA cesium chloride purified, the DNA band removed from the first cesium chloride gradient and directly loaded onto another cesium chloride gradient. After the second gradient the DNA is processed as stated in M & M. The DNA is resuspended and spectrophotometer readings are taken. This DNA is now ready for transfection.

Preparation of DNA for Transgenic Mice

From the double banded cesium chloride preparation mentioned above, 50 ug of pSVL3 were cut with EcoR1 in a 75 ul volume, precipitated, resuspended and cut with Sal-1 in the same volume. This DNA is then precipitated and resuspended in 25 ul of TAE. This DNA was then electrophoresed on a 1% TAE low melt gel and the two bands of 2.3 and 3.0 kb cut out with a razor and the DNA isolated with GeneClean (See M & M). The 3.0 kb fragment was the transcriptional unit, and after isolation from the gel, the DNA was speed-vacuumed to dryness and resuspended in filter sterilized solution of 10mM Tris (pH 7.6) / 0.25mM EDTA. The resuspended solution was then microcentrifuged for 1 hour at 4°C in order to pellet any particulate matter. The concentration was checked by A_{260}/A_{280} and adjusted to 2.2ug/ul with the aforementioned Tris/EDTA solution. The initial injections were done on 8-28-90. The first set of injections was a complete loss due to technical difficulties, while the second set of injections produced 20 pups out of two litters, 12 of which were female, & 8 male. After weaning the pups, their tails were snipped in order to screen them for the transgene.

Preparation of Tail Snip Genomic DNA

1 Cut 1.3 cm of tail using scissors into a 1.5 screw top microfuge tube containing 750 ul of 50mM Tris pH 8, 100mM EDTA, 100mM NaCl, 1% SDS. Some bleeding will occur, but no special treatment is necessary.

2 Add 10 ul of a 50mg/ml solution of Proteinase K dissolved in H_2O.

3 Incubate at 55°C for 8-12 hours (overnight) with constant agitation.

4 Remove from 55°C, allow to cool to room temperature and quick spin before adding 5 ul RNAse cocktail (Stratagene). Incubate at 37°C for 1 hour.

5 Add 750 ul phenol and gently agitate for 20 minutes (Do not Vortex).

6 Separate phases by microfuging for 3 min.

7 Transfer aqueous phase with a large bore blue tip into a clean tube containing 750 ul phenol:chloroform (1:1). Using a rotary invertor mix for 5 minutes.

8 Separate phases by microfuging for 3 min.

9 Transfer aqueous phase with a large bore blue tip into a clean tube containing 750 ul chloroform:isoamyl alcohol (24:1). Using a rotary invertor mix for 5 minutes.

10 Separate phases by microfuging for 3 min.

11 Transfer aqueous phase with a large bore blue tip into a clean 1.5 ml tube avoiding any interface. Fill tube with isopropanol. Invert several times, until snotty precipitate forms.

12 Make fish hook with a flamed off micro capillary tube and fish out clump of DNA.

13 Wash carefully with 70% ethanol

14 Wash carefully with 100% ethanol

15 Allow DNA to air dry by inserting microcapillary into a styrofoam block.

16 Using a glass scribe, score the end of the capillary containing the DNA and carefully break off into a clean 1.5 ml tube containing 500 ul TE.

17 Place tubes on a rotary invertor overnight at 4°C.

18 Remove an aliquot to determine the DNA concentration. DNA is now ready for further analysis.

PCR analysis of Tail Genomic DNA

The primers used in the PCR reaction were:

5'-GGATGTTGCCTTTACTTCTA-3' (TSVL-upr)
5'-CTGCATTCTAGTTGTGGTTTGTCC-3' (TSVL-lwr)

(nucleotides 1442-1461 and 1565-1589 of plasmid pSVL SV40 respectively). All tail DNA samples were tested with PCR positive control primers:

5'-CTACCCACGGCAAGTTCAATGGCA (GAPDH-1)
5'-TCCAGGCGGCATGTCAGATCCACA-3' (GAPDH-2)

from the mouse GAPDH gene (glyceraldehyde 3-phosphate dehydrogenase). The PCR cycle program utilized was:

95°C for 4 min;

95°C for 1 min,	
55°C for 2 min,	
72°C for 3 min	(25 cycles)

72°C for 7 minutes.

All reagents and enzyme were from Perkin Elmer.

Because non-transgenic mice don't contain the pSVL vector sequence only those mice with the transgene should produce a 520 bp band. Because a greater degree of resolution was needed a NuSieve Gel (FMC) was used (See Recipe) and the ladder was a 100 bp ladder (BioVentures). PCR of genomic DNA prepared on this scale was very difficult. Because of the small amount of DNA prepared any contamination can strongly affect the TAQ polymerases ability to amplify the sequence of interest. Initially a number of different protocols were assayed to see which one provided reproducible results. Often Southern blots of the gels were done in order to reconfirm the identity of very faint bands.

Transposon Subcloning

Prepare the following media:

LB-agar plate with ampicillin at 50ug/ml
LB-agar plate with both kanamycin and carbenicillin at 50ug/ml (Don't replace carbenicillin with ampicillin)

All constructs are made with pMob (1.8Kb).
Transform constructs into DH5α competent cells.
Perform miniprep to obtain DNA and analyze inserts.

Day 1: Transformation of Donor Host DPWC

1. Defrost a tube of DPWC competent cells on ice for 30 min. At the same time chill two sterile 13 x 100mm glass tubes on ice.
2. Add 50ul bacteria to each of the glass tubes.
3. Add 2ul of undiluted miniprep DNA from the above to one tube and 2ul of a 1/25 dilution of miniprep DNA to the other tube and incubate on ice for 30min.
4. Heat shock for 90 second at 42°C then incubate on ice for 2min.
5. Add 900ul of SOC to the transformation mixture and incubate at 37°C with shaking (225rpm) for 1hr.
6. Spread 50-100ul of transformation mixture on LB ampicillin (50ug/ml) plates and incubate overnight at 37°C.

Day 2: Grow Cultures of DPWC and BW23

Pick 2 to 3 colonies of transformed DPWC from the plate and grow 2ml overnight culture containing ampicillin at 50ug/ml.
At the same time start a 2ml overnight culture of BW23 containing kanamycin at 50ug/ml.

Day 3: Conjugation

1. Add 2ml of LB media to a 15ml disposable centrifuge tube. Prewarmed to 37°C.

2. Add 100ul of DPWC and 100ul of BW26 to the media, cap the tube, place in on its side on a rotary shaker and rotate at a very slow speed(30 rpm) at 37°C. Conjugation begins.
3. At every hour after the conjugation starts (at 1, 2, 3, 4 hrs) dilute 2 and 5ul of the mating mixture into 300ul of TE and spread 100ul of the mixture onto LB-agar plates containing carbenicillin and kanamycin and incubate overnight at 37°C.

So for each DNA sample there will be 8 plates.

Day 4: Picking Colonies

For each sample, pick 20 individual colonies from one plate out eight that has the most number of colonies, and grow 2ml overnight cultures containing ampicillin.

Day 5: Prepare Miniprep

Prepare miniprep DNA and digest with restriction enzyme.

Day 3: Conjugation

1. Add 2ml of LB media to a 15ml disposable centrifuge tube. Prewarmed to 37°C.
2. Add 100ul of DPWC and 100ul of BW26 to the media, cap the tube, place in on its side on a rotary shaker and rotate at a very slow speed(30 rpm) at 37°C. Conjugation begins.
3. At every hour after the conjugation starts (at 1, 2, 3, 4 hrs) dilute 2 and 5ul of the mating mixture into 300ul of TE and spread 100ul of the mixture onto LB-agar plates containing carbenicillin and kanamycin and incubate overnight at 37°C.

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Prepare miniprep DNA and digest with restriction enzyme.

RESULTS

cDNA Cloning of Alternative Forms of OBCAM

As discussed in the Introduction, our laboratory has purified and cloned an opioid binding protein (OBCAM) from bovine brain. Though several lines of evidence indicate this protein has a role in opioid receptor function, it lacks the characteristic structure of G-protein coupled receptors, including seven transmembrane regions. Furthermore, the recent cloning of opioid receptors, accomplished after this work was begun, indicates that OBCAM cannot be the opioid receptor.

Nevertheless, it might play a supporting role of some sort. The most recent studies from our laboratory, as discussed in the Introduction, suggest that OBCAM can promote coupling of the opioid receptor to G-proteins. However, G-proteins are located intracellularly, while OBCAM's structure indicates that it is an extracellular molecule. It seemed likely that if OBCAM were indeed to promote coupling, it should possess a transmembrane region and an intracellular region. One possibility is that an alternate form of OBCAM exists with such a structure. In fact, several receptors that do have this structure, are known to possess alternate forms in which only the extracellular portion exists. These include the receptors for growth hormone (Baumbach et al. 1989), insulin-like growth factor-I (Czech 1989), polio virus (Koike et al. 1990) and interleukin-4 (Mosley et al. 1989) It has been speculated that the extracellular forms, which presumably bind ligand without

transducing a signal, may serve to scavenge excess amounts of ligand, regulating its concentration in the vicinity of the authentic receptors.

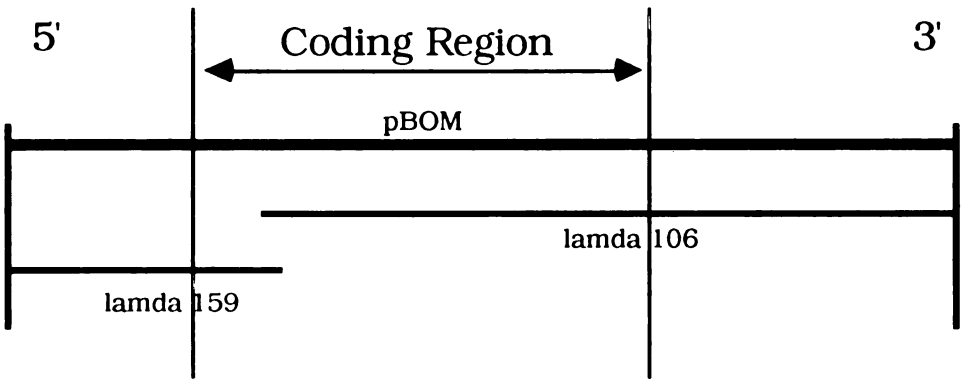
When we began our work, we had no direct evidence for alternate forms of OBCAM. However, in the original paper by Schofield, et. al. (1989), it was noted that multiple bands were observed when Northern blots were probed with pBOM (the cDNA for bovine OBCAM), which was consistent with this possibility. The original pBOM clone was in fact a construction of two clones, neither of which spanned the entire coding region.

Accordingly, we decided to search for alternative forms of OBCAM that might include an intracellular region. We were also open to the possibility of alternative forms differing at the 5' terminus, since extensions in this direction are somewhat easier to examine. Initially we attempted to extend the clone in the 5' direction, following the procedure of Chirala (1989). The strategy was to open the clone at its 5' end and remove a small segment of the cDNA to be used as a probe later on. The remaining clone was then digested using Exo-III in order to create a single strand cDNA which would be used to hybridize with mRNA. The cDNA acts as primer allowing an elongation of the first strand to be synthesized with reverse transcriptase directly attached to the original clone, thereby producing an extension of the cDNA clone This idea, although used successfully in other systems, did not produce positive results.

Next, preliminary experiments were conducted where unique regions of the two original bovine clones (lambda Bom 106 and 159) were isotopically labeled and used to hybridize with mRNA from rat.

Figure 2

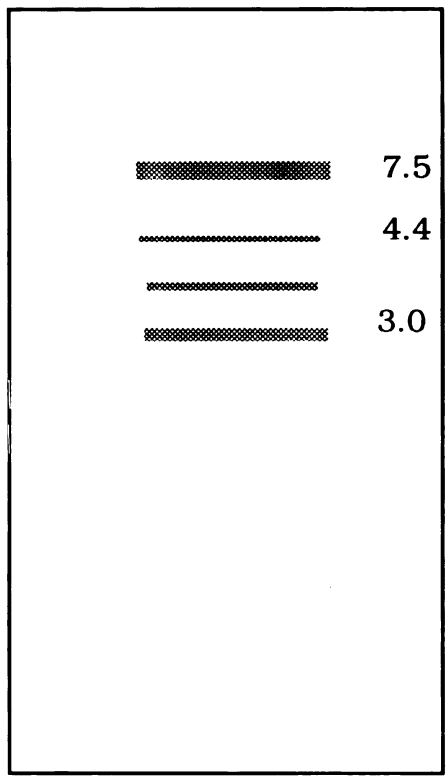
Alignment of Lambda clones which were assembled into pBOM



The purpose of these experiments was to verify the idea that other mRNA species existed that were longer than pBOM, bovine cDNA for OBCAM. The results of these experiments not only confirmed that hypothesis, but revealed that the most predominant mRNA species in the rat was over twice the length of pBOM Figure 3. This result was reproducible, with the 7.5kb transcript always being predominant in every case. In addition, other discrete bands were seen at 4.3kb, 3.7kb, and 2.9kb. In light of these findings I set out to obtain additional pBOM-like clones from the rat.

From the results of Schofield et. al., it could be expected that very few clones should be obtained when screening a library. In the original publication Schofield mentioned that there was a high degree of homology between the bovine and rat cDNAs in the areas that they compared. Consequently I acquired from him the partial rat cDNA clone (pROM). In order to achieve some degree of specificity during the screening process, it was decided that two near contiguous non-overlapping fragments of the coding region of pBOM/pROM would be used as probes Figure 4.

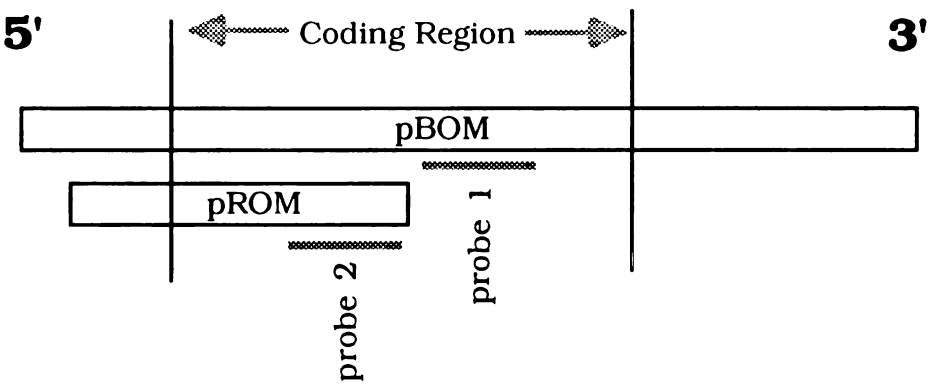
Figure 3



Northern of Rat mRNA Hybridized with ^{32}P labeled λ 106 cRNA

Figure 4

Alignment of Rat and Bovine OBCAM cDNA Clones



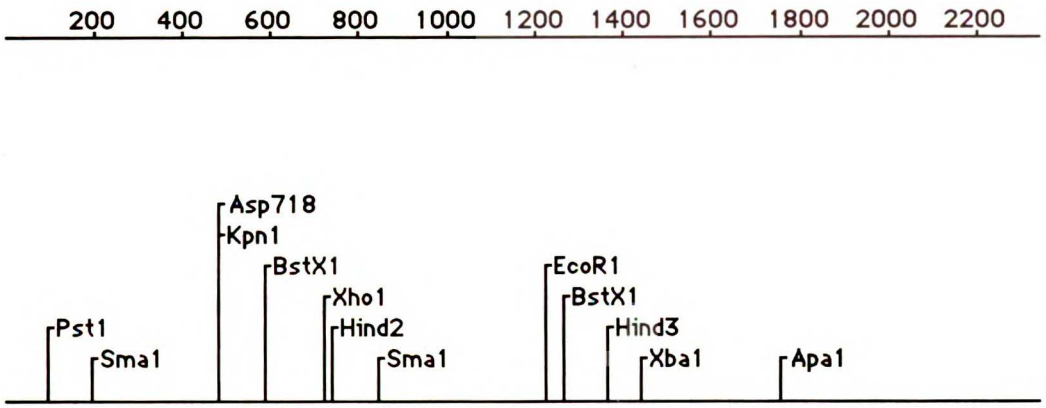
The bovine probe was near the 3' end of the coding region of pBOM, extending from a *Hinc*II to a *Xba*I site (nt 661-976). After digestion with these enzymes, a 315 bp fragment was purified by gel electrophoresis and GeneClean. The rat probe was from a more 5' portion of the pROM coding region, extending from a *Xho*I to an *Eco*RI site (nt 255-755), and generating a 500 bp fragment that was prepared in similar fashion. The probes were labeled with [α-^{32}P]-dCTP (800 Ci/mmol) using Boehringer Mannheim random labeling kit, and purified by passage through a Boehringer Mannheim Quick-Spin column (G-50).

Using these probes, we screened a cDNA library obtained from Stratagene, as described in Methods. Eleven clones greater than 1 kb (the minimum length of cDNA that could contain the complete reading frame of OBCAM) were obtained, and analyzed by restriction digestion and sequencing. Most of these clones appeared to be identical to pROM (the rat cDNA) where they overlapped with the latter. Many contained the C-terminal portion of the reading frame missing in the truncated pROM, but this was highly homologous with the corresponding region of pBOM, which contained a complete reading frame. In particular, these clones contained a stop codon in the same position as found in pBOM. The most promising two clones were SG-13 and SG-8, initial analysis indicated they were full length, and not identical. They were extensively restriction mapped to assist in subcloning their fragments for complete sequencing.

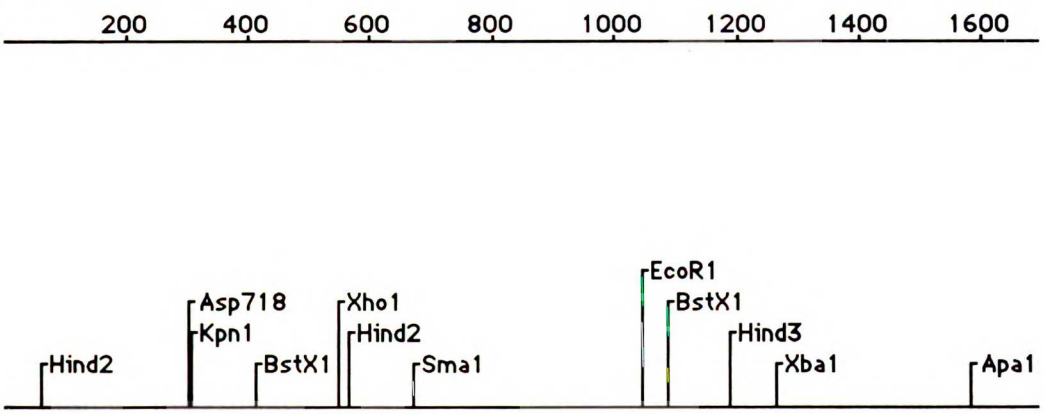
Figure 5

Restriction Maps of OBCAM Like Clones

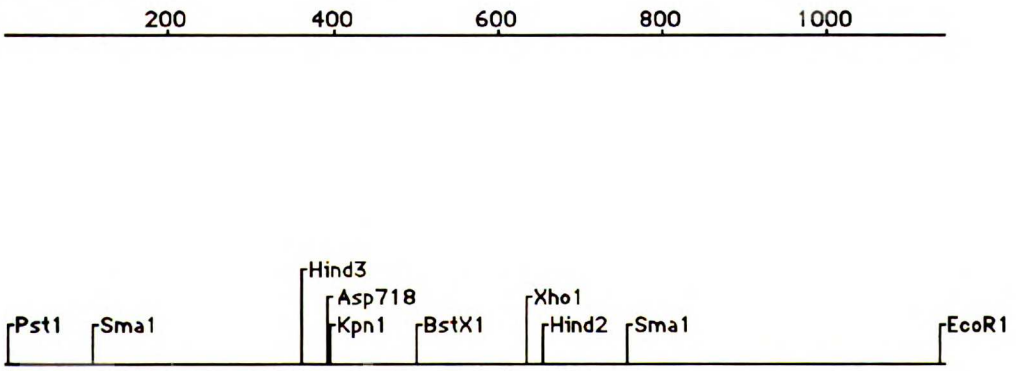
SG-13



SG-8



pROM



SG13 and SG8 were then sequenced in pieces, subcloning various restriction fragments, and the pieces put together using computer software. This sequencing revealed that SG13 was identical in its 3' portion to a clone obtained from another researcher in the lab (J. Hasegawa, unpublished data), the largest of all the clones at 3.5 Kb, though it lacked a complete reading frame. Thus SG13 effectively added 5' sequence to this clone to complete the reading frame, while the longer clone added extensive 3' non-coding region. Putting these clones together, over 4 kb of sequence was obtained.

SG8 was very similar to SG13, but differed in its 5' non-coding region, beginning approximately 150 bp upstream of the start codon (See Figure 6). Thus SG8 and SG13 had identical reading frame, but possibly differed in their upstream control elements. This was our first evidence for different forms of OBCAM messages.

Simultaneously with the screening of the Stratagene library, another library was constructed using a prerelease version of the Uni-Zap library construction kit. Library screening was carried out as stated previously. After the first round of screening only two positive signals were found, but both positives were retained through all three rounds of selection screening. They were excised in vivo and restriction mapped. Both clones were nearly 3200 bp in length and displayed the same restriction map.

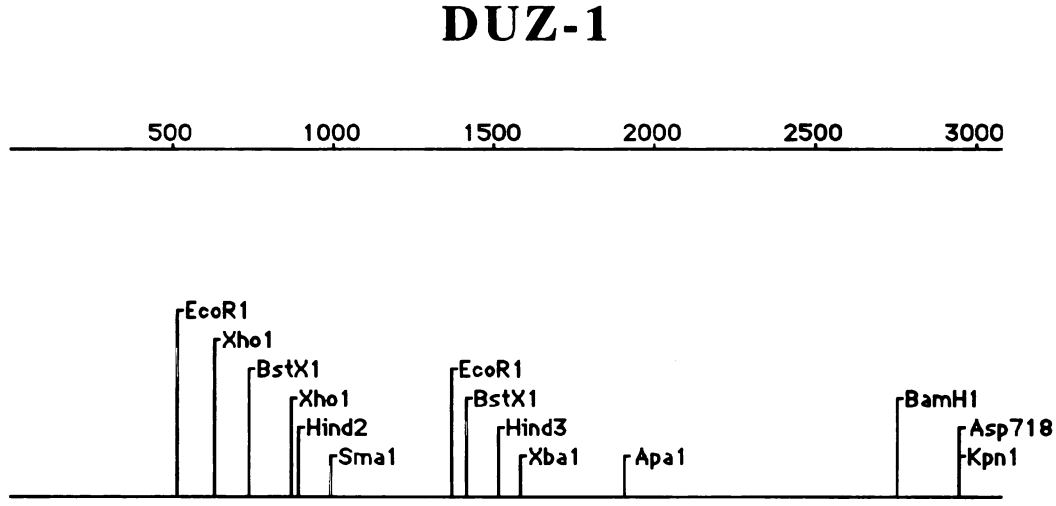
Figure 6

Alignment of SG13 and SG8 Nucleotide Sequence

```
          .         .       -450        .         .         .         .       -400        .         .
13        CTTTTTTTTATTTTTTATTTTTTTTACGACATCTCCTTTCTGTCAGAGACACTTGCCTTCTTTGGGTTATAAACTTTTGATGCCAGACCTCTGCAGAATCG

          .         .       -350        .         .         .         .       -300        .         .         .
 8                                                                                  CGTGTGATCAATGGTCTTAGAAGGCTTGTT
13        CCCAACTGAATCTTAAAGTAGCTTCACCGAGAGAGGCAGAGAACGCACAATCTGTGACCTTCGTCCCTGCT.C.T.TT.GTG.T..G..CTCCCT.C.CCC

          .         .       -250        .         .         .         .       -200        .         .         .
 8        AGACAGCTGTGGGGCTCAGTCTTCTCGCAGAATGTTAACAGTGGGAGCAGG-GGCTGTCTCTGACTCTGTTTGCCTGCCGGC-AG-TGAGCAATGGTCTTA-
13        G.G TC.CTCC.CC.CAG.GAGCG.T.AGA..GC.CTTTTT...AT.....A..GG.CA....GT.....--C.AG..T..AA..C....GCTG.AG.AG.G

          .         .       -150        .         .         .         .       -100        .         .         .
8/13      GAAGACTTATTAGACTCCGGAGAGCCTGGACTCAGCTTGCCTTCTCCCGCTCCCAGCTCGTGGTTTGCTCTCTGTGTGCTTTCGTCCCTCAACATTCCGG

          .         .       -50         .         .         .         .         1         .         .         .
8/13      CTATTCTGAGAGGGCAGGGGACAAGGACCGTGCAGCTGCAAGAGTTCTAGGAAGTTGTGGCTGTCCAGAATGGGGGTCTGTGGGTACCTGTTCCTGCCCTG

          .         .       50          .         .       90
8/13      GAAGTGCCTCGTGGTCGTGTCTCTGAGGCTGCTGTTCCTTGTACCCACA#*

          #sequence continues from nt 61 in DUG-1 Sequence

          * Clone 13 is 2339 nt
          * Clone 8 is 2000 nt
```

Figure 7

Restriction Map of DUZ1



From terminal sequencing with the T-3 and T-7 primers it was determined that these clones were identical; they were named DUZ-1 and DUZ-2. Complete sequencing of this clone proceeded analogously to that of SG-13, cutting the clone with restriction enzymes into pieces small enough that could be completely sequenced. The following contig map illustrates the approach used (Figure 8).

This sequencing revealed that DUZ1 was identical to SG13 in its 3' non-coding region and most of the reading frame. However, beginning at a point about 80 bp 3' of the putative start codon of SG13, and moving upstream, the two sequences differed. Thus SG13 and DUZ1 proved to be identical in their most C-terminal 318 amino acids, differing completely in the amino acids upstream of this point (Figure 9). These amino acids number 27 in SG13 (total length 345) and 21 in DUZ1 (total length - 339). Further analysis of this portion of the reading frame in the two clones revealed that the region was highly hydrophobic in both sequences, and thus most likely represented a signal sequence that would be cleaved off during OBCAM synthesis.

Figure 8

Contig Map of DUZ1 Used to Assemble Entire cDNA Sequence

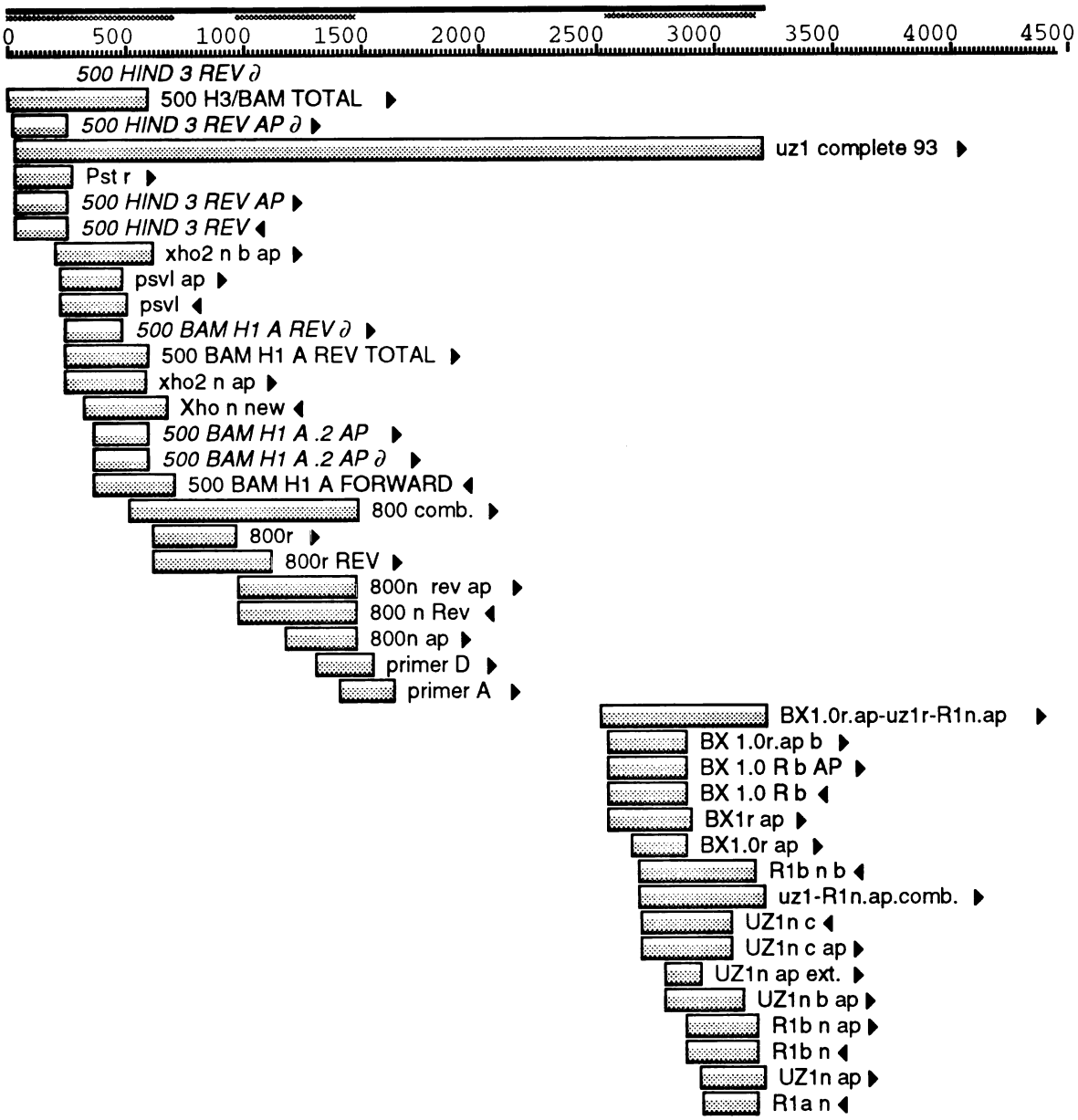
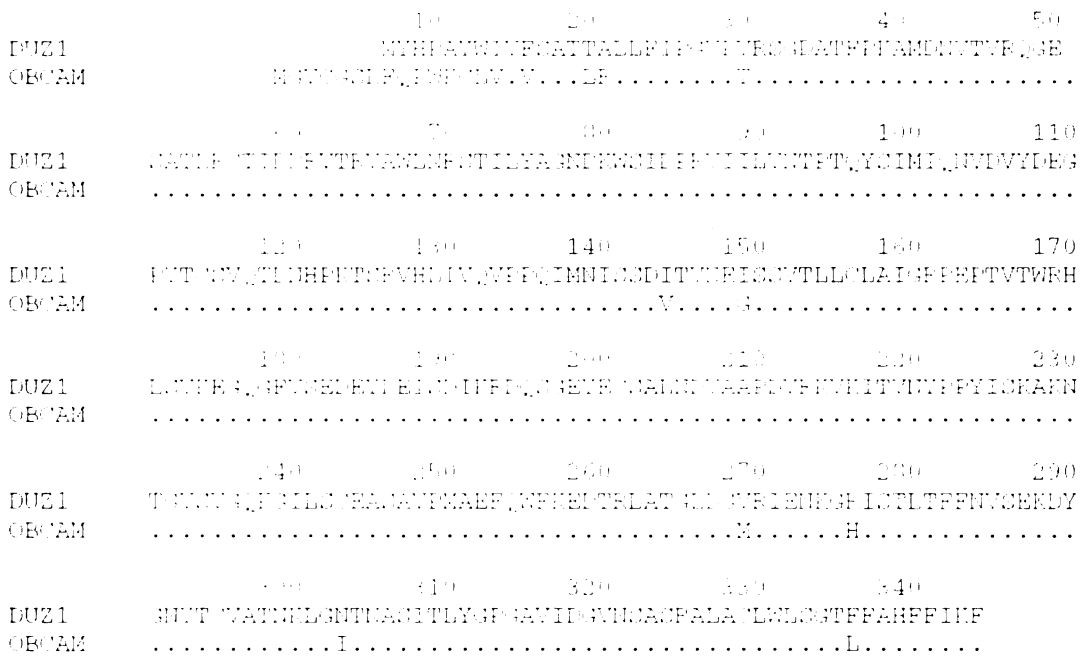


Figure 9

Amino Acid Alignment of DUZ1 with OBCAM



In summary, our cDNA screening, employing both a commercially available library and one which I prepared, revealed three OBCAM like clones, one of which was apparently identical to the original pBOM (minor differences being accounted for by species distinctions), while the other two differed in their 5' region. No evidence was obtained for the existence of clones differing in their 3' region, which might give rise to an intracellular region. However, the presence of two types of N-terminal signal sequences, and three types of 5' noncoding regions, suggests different types of processing for OBCAM.

Transfection of Cells with Antisense DUZ1

As discussed in the Background section, our laboratory has not been able to provide direct evidence that OBCAM is involved in opioid receptor function, in particular, demonstrate that the cDNA, upon transfection into a mammalian cell line, expresses material that either binds opioids or modulates opioid receptors. Several lines of indirect evidence, including antibody studies and antisense transfections, have been obtained. The antisense approach is particularly effective, as in theory this is capable of specifically blocking production of OBCAM in cells. As our laboratory had some success with this approach, using a portion of bovine OBCAM cDNA, it was decided to apply it to the DUZ1 clone.

Restriction digestion was used to isolate a 5'-terminal portion of DUZ1 of approximately 500 bp in length. This was subcloned into the pSVL vector and a total of six different transfections were screened with PCR. Of those screened, four contained the transfected DNA, three in the antisense orientation and one in the sense orientation. One sense clone UZS-2/14 and one antisense clone UZAS-3/7 were assayed for opioid binding of diprenorphine compared to non-transfected NG108-15 cells. Opioid binding was seen to decrease significantly with the stable transfection of DUZ1 antisense (clone UZAS-3/7) (Arany, I., personal communication).

Figure 10

Construct Design of DUZ1 Antisense into pSVL Vector

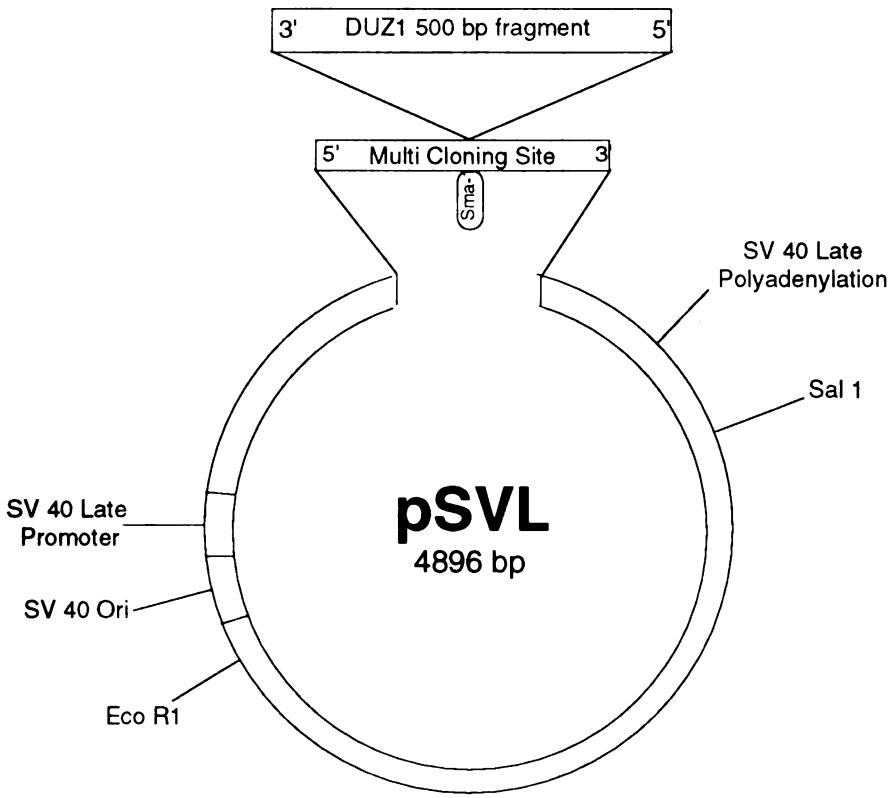


Table1

Binding of Different Ligands in Transfected and Nontransfected NG108-15 (expressed as % of cpm in non-transfected control).

Ligand	**Clone**		
	UZS-2/14	UZAS-3/7	Nontransfected
Opioid	91±4	31±4	100
α_2 Adrenergic	87±5	94±5	100
Muscarinic	91±4	101±6	100

*ligand concentrations used was 2nM ^{3}H-diprenorphine

These results paralleled those of an earlier study employing antisense to pBOM, the originally isolated OBCAM clone (Ann et al. 1992). These results thus provide evidence for a role of the DUZ1 product in opioid receptor function.

Creation of Mice Transgenic for DUZ1 Antisense

We also decided to extend the antisense approach by attempting to create a line of transgenic mice containing the DUZ1 antisense cDNA. The general procedures, including injection of oocytes and implantation into pseudopregnant females, mating, PCR screening of mouse tail genomic DNA to confirm the presence of the transgene are described in the Methods section. The first mouse pup proven to be transgenic positive was #1355, a male. It was interesting that this pup was very sickly and thought not to survive after weaning. Furthermore, #1355 also had a deformed front right paw, where the center toes had fused together, so it only had three toes. Special food and housing was provided and #1355 did survive. In addition four of the other eight litter mates were identified as containing the transgene, two males, (#1354, #1348) and two females (#1344, #1345). One male (#1355) and one female (#1344) were chosen for breeding. The N1 generation from #1344 produced 14 pups of which 3 contained the transgene, and the N1 generation from #1355 produced 32 pups of which 12 contained the transgene. After the first litter was born and screened for the transgene, the N1 generation and some of the F1 generation (founders), using nontransgenic litter mates as control mice were characterized

pharmacologically in our lab. The complete results of the pharmacology will be reported elsewhere, but I will present some of the preliminary findings.

The transgenic mice F1 and N1 generations showed a decreased sensitivity to morphine analgesia, relative to controls. Thus it appears that the presence of the transgene altered opioid receptor function, providing further evidence for a role of DUZ1 in this system.

Table 2

Antinociceptive Activity of Morphine Sulfate in Transgenic Mice

	Non-transgenic	Transgenic	
Normal	7.05 ± 1.18	----	
Founders	7.07 ± 1.54	11.22 ± 1.32	$p<0.02$
1355 N1	7.07 ± 1.18	22.44 ± 1.22	$p<0.001$
1355 N2	7.07 ± 1.13	14.14 ± 1.12	$p<0.001$
1344 N1	7.05 ± 1.12	17.80 ± 1.26	$p<0.001$

Antinociceptive activity of morphine sulphate was determined by the tail flick method (D'Amour and Smith 1941). Morphine sulphate was injected s.c. Because of the small number of animals available (6 in each group matched for sex and age), the up-and-down method (Dixon,1965) was used to determine AD_{50}. Statistical analysis was performed using the mixed model ANOVA test (Myers 1972), consisting of one between-subject factor (non-transgenic and transgenic) [S. Nair, MS Submitted]

Chromosomal Localization of OBCAM like clones

Since DUZ1, SG13 and SG8 share such a high degree of homology, it is possible they arise from a single gene, through alternate splicing, for example. Determining their chromosomal location is one approach to answering this question, since locations on different chromosomes would rule out the possibility of a common gene, though their presence on the same chromosome would not, conversely, confirm it. In order to do chromosomal localization a somatic cell hybrid cell line was necessary, so we collaborated with Dr. Christine Kozak, an authority in this field with many cell lines available. We did the initial screening of mouse and hamster genomic DNA in addition to the first round of somatic cell genomic DNA. In order to screen a somatic cell hybrid there needs to be distinct identification of the host cell DNA (hamster) and the exogenous DNA (mouse). Dr. Kozak provided the genomic DNA from mouse and hamster, which was digested with several restriction enzymes, followed by Southern analysis using our labeled probes. The following tables summarize our results.

Table 3

Southern Analysis of Mouse & Hamster Restriction Endonuclease Digested Genomic DNA

(DUZ1 R1-500)

Enzyme	Bam H1	Eco R1	Hind III	Xba-1
Mouse	6300	850	5800	4400
Hamster	4000	1750	4050	6200

(SG13 Kpn1)

Enzyme	Bam H1	Eco R1	Hind III	Xba-1
Mouse	M 3100 m 3750	M 4500 m 5200	M 3300 m 3650	M 6100 m7500/5000
Hamster	2950	4400	2050	4650

*Major band
*minor band

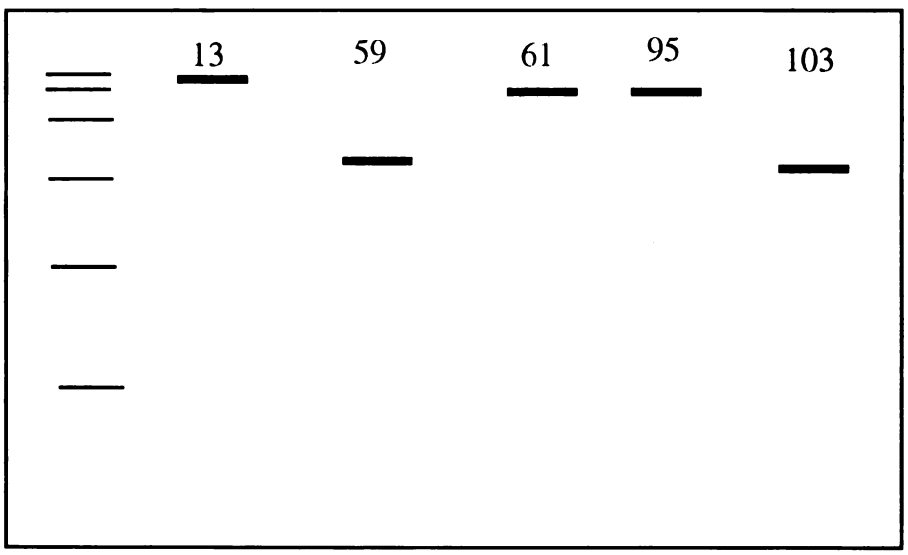
(pROM)

Enzyme	Bam H1	Eco R1	Hind III	Xba-1
Mouse	M 540	M 2300 m 4400/1950	M1600 m 4300	M 5000 m 4650/3000
Hamster	M5700 m 4600	M 5050	M 3500 m 1550	M 5000 m 4050

On the basis of the very distinct profiles of Eco R1 fragments produced from hamster and mouse, as probed using DUZ1-R1500, it was decided that this enzyme would be used for the panel screening of the hybrid cell DNA. That is, the patterns of Eco R1 digestion between hamster and mouse genomic DNA are different enough to allow determination of which species an unknown sample of DNA origin when somatic cells containing a species mixture are probed. Twelve samples of genomic DNA from different somatic cell hybrids were digested with Eco-R1 (as before) and transferred to a solid support, and hybridized with the DUZ1-R1500 probe. The results suggested two possible positive somatic cell hybrids, #59 and #103. This information and the purified fragment of cDNA to be used as probe were supplied to Dr. Kozaks' lab who screened additional somatic cell DNA, localizing all of the clones to the short arm of chromosome 9, sandwiched between Icam-1 (intercellular adhesion molecule-1) and Fli-1 (Friend leukemia virus integration site-1) albeit ± 3 centimorgans (Chakraborti, et. al. 1993). From these results, we cannot conclude anything about the relationship of the 3 OBCAM genes. Further studies will be necessary in order to address this question, in particular sequencing of the complete genomic region. This was the subject of our next study.

Figure 11

Genomic Blot of Somatic Cell (Hamster/Mouse) DNA Probed with DUZ1-R1500



Cloning of Rat Genomic DNA Containing OBCAM Sequences

In order to determine the relationship among these different sequences--for example, whether they result from alternate splicing or comprise wholly different genes--I have begun to isolate these sequences from the rat genome.

Initial screening of the rat genomic DNA was by hybridization screening with different segments from the three cDNA clones discussed previously (DUZ1, SG13 and SG8). The regions chosen for probes were either unique to each clone or common among all of them. The genomic DNA was subjected to restriction digestion using 6 base pair restriction endonucleases, separated on an agarose gel, transferred to nylon membrane and then UV cross linked to fix the DNA. The probes were made by random labeling cDNA fragments with ^{32}P-d-CTP. The hybridization was carried out at high stringency (65°C). Because there was no interest in closely related genes (no degeneracy) both hybridization and washing conditions were kept at high stringency. The existence of positive results using each of the probes indicated that the regions selected for use as probes were in fact present in continuous or largely continuous form in genomic DNA. These results are presented in the Tables 4 and 5.

Table 4

Summary of Rat Genomic Southerns

Probe	Enzyme	Cuts (kb)
DUZ1 R1500	Eco R1	4700 700
	Bam H1	7000 4300 3200
	Hind III	4500 4300 320 1300
	Kpn-1	9300 2700
	Pst-1	5400 3400
	Xba-1	6500 5000
pROM	Eco R1	8400 5400 1200 700
	Bam H1	9400 6800 5700 5200 4300
	Hind III	7500 5700 1300 1000
	Kpn-1	2600 2100 1700
	Pst-1	5300 5000 2100
	Xba-1	5900 5400 5000 4000
SG13 Kpn-1	Eco R1	7000 1000
	Bam H1	5800 2300
	Hind III	2100, 1300
	Kpn-1	4700
	Pst-1	2300 1200
	Xba-1	8700 4900 3800 2400

Table 5

Summary of Mouse Genomic Southerns

Probe	Enzyme	Cuts (kb)
DUZ1 R1500	Eco R1	3000 1600 700
	Bam H1	7500 4500
	Hind III	6800 4800 1500
	Kpn-1	7700 2500
	Pst-1	6700 5700
	Xba-1	5700 5000
pROM	Eco R1	9000 3000 2400 1200 700
	Bam H1	12000 10000 7000 6000
	Hind III	8000 1600 1400
	Kpn-1	2500 6800 6100 5300 4300 2300 2100
	Pst-1	6000 2000 1600 7500
	Xba-1	11000 8000 6000 5000 3500
SG13 Kpn-1	Eco R1	7000 1000
	Bam H1	4000 2100
	Hind III	4300
	Kpn-1	---
	Pst-1	2400
	Xba-1	5000

From the genomic Southern data it was not clear whether the DNA containing the different probed sequences represents a single gene, with introns separating the portions of the DNA unique for each region, or multiple genes. That is, each probe hybridized with a different constellation of fragment sizes, which could have come resulted from cutting different portion of one gene, or entirely different genes.

In addition to the information obtained from genomic digestion and genomic mapping, we have also begun screening a rat genomic library for individual clones containing the sequences we have been hybridizing with, DUZ-1, SG-13 Kpn and pROM. We were unable to obtain unambiguous data with the latter two of these probes, but DUZ-1 yielded three clones through three rounds of hybridizations. The following tables summarizes the patterns obtained with various restriction enzyme digestions of the three Rat Genomic Lamdba DASH clones isolated.

Table 6

Rat Genomic Digestion Patterns of DUZ1 Lambda DASH Clones

Clone 2

Enzyme	Bam H1	Hind III	Xho-1	EcoR1	Xba-1	Sac-1
Bands	9000+		9000+	3850	5800	2900
	2500			3800		2750
	2400	1850	4600	3300	3800	2700
	600	800	2850	1000		1450
				800	1300	
				650		

Clone 1

Enzyme	Bam H1	Hind III	Xho-1	EcoR1	Xba-1	Sac-1
Bands		2750	15000	6900	4850	4300
	7000	1750	8500	4850	4000	2850
	4200	1500	2900	3600	3900	1300
	2200	800	1000	3100	3300	
	700			2850	2500	
				1450	2000	
				800	1100	
				650	600	
				270		

Clone 3

Enzyme	Bam H1	Hind III	Xho-1	EcoR1	Xba-1	Sac-1
Bands	6000	7800	8800	4600	5900	4350
	2200	1850	4400	3850	3800	2900
			2800	3700	2525	2800
				3050	1300	1400
				700		
				600		

From these experiments it was determined that there were three unique Lambda Phage clones that contained a DUZ-1 complementary sequence. These three clones ranged in size from 12-24 kb. These clones were then extensively mapped with all of the unique restriction enzyme sites of the multicloning region of the vector. From each of these clones lambda DNA was prepared using the plate lysate method. Each of the DNA samples were digested with a number of enzymes. Those inserts were further mapped with other enzymes. in order to undertake subcloning of the lambda clones, large scale liquid lysate preparations were carried out. Using this method, we sequenced ca. 25 kb assembled into over 40 contigs ranging in size from 200 to 1500 bp. All of this sequence proved to be unique when compared to the GenBank-EMBL nucleotide database (except for some repeat regions), and all of it differs from the cDNA sequence except for the 5' noncoding region. Thus an additional ca. 3.5 kb of sequence corresponding to the coding region and 3' noncoding of the cDNA is known, though at this point we had no information on existence, size or location of introns. In addition, as there was surprisingly little overlap of sequences it was not possible to estimate the total length of the gene.

There has been much less progress using the other two probes. After a number of attempts at classical screening, and obtaining negative results, a modified method of library screening was devised. The library was plated out and a portion of the phage DNA was used for PCR experiments, where it was evaluated whether or not a specific library fraction appeared positive for the primers which represented the region from which the probes were generated. The portions of the library which appeared positive were then diluted and plated out again. PCR screening

was repeated a total of four times. The selectively diluted fractions of the library were then plated out and screened again with the remaining two probes. The results from this were not very successful either.

P1 Genomic Cloning

Therefore, we began screening of a rat genomic P1 library which contains clones on the order of 100 kb in size. As discussed in Materials and Methods, the company supplying the clones (Gold BioTechnology) is given two primers flanking the region of interest, and uses these primers in PCR to screen their library. Three clones were identified and sent to me. The purified DNA of each clone was digested with both Bam H1 and Sac-1 restriction endonucleases, these two enzymes being chosen because Bam-H1 is the site of insertion and the vector lacks the recognition sequence for Sac-1. From gel patterns of the digested DNA it was determined that of the three P1 clones obtained 278, 279, and 280 - clones 278 and 280 were identical, so that in fact there were only two unique clones. A rough estimate of the P1 clone sizes from the gels put 278 at about 70 kb and 279 at about 85 kb. Accurate sizing of these clones was not possible without pulsed field gel electrophoresis, because a number of the fragments were greater that 12 kb and do not separate adequately on a normal submarine gel. Information of immediate interest was to locate the coding sequences if possible, at least the Bam H1 and Sac-1 bands in which they were contained. Southern blots were prepared from this DNA, and the blots hybridized with different cDNA probes (Results not shown).

Because of the vast amount of DNA that would need to be sequenced, and the daunting process of cutting and subcloning thousand of fragments,

a new technology was employed at the onset of the P1 sequencing. This new process entails subcloning a fragment of DNA into a small plasmid vector (pMOB 1.8kb [Gold BioTechnology]), followed by the mating of transformed cells containing the new construct with another E.coli cell line containing a transposon. During the conjugal mating of these two cell lines a transposon was inserted into random sites of the pMOB construct. The conjugal mix was then spread on an Lb/amp plates. From these plates a number of colonies were picked, grown overnight in tb/amp and DNA prepared (See M & M). The approximate location of the transposon can be determined by restriction enzyme analysis and a series of conjugal offspring be sequenced in ascending or descending order based upon the transposonal insertion site. This methodology allows for reasonably rapid sequencing of DNA up to 3 kb and using it, we have been able to sequence an additional 25 kb of DNA, in a total of over 50 new contigs. Thus we now have over 50 Kb of DNA sequence and 100 contigs. The large number of contigs, indicates that very little overlap of sequence has occurred, and therefore, the gene must be very large.

However, we have been able to obtain sequence in the reading frame portion of the gene, revealing the presence of several introns, as well as the 5' noncoding region, where control elements presumably exist. The locations, and in some cases, the sizes, of the introns were determined by comparing genomic sequence with cDNA sequence. The presence of an intron is indicated by a region in the genomic DNA not present in the cDNA, flanked by the consensus dinucleotides GT....AG. In some cases, the presence of introns was further confirmed by analysis of products of 5' RACE (rapid amplification of cDNA ends) produced by another member of our group (L. Augustin). These products, which correspond to just the

5' terminus of several hundred bp of cDNA, revealed cDNAs different from SG-13, SG-8 and DUZ1 and the points of difference corresponded exactly to the location of introns.

Figure 12 shows the relationship of SG-13, SG-8 and DUZ1 and the 5' RACE clones, and Figure 13 shows the resultant map of the OBCAM gene derived from these comparisons.

Figure 12

Intron-Exon Map of OBCAM Gene

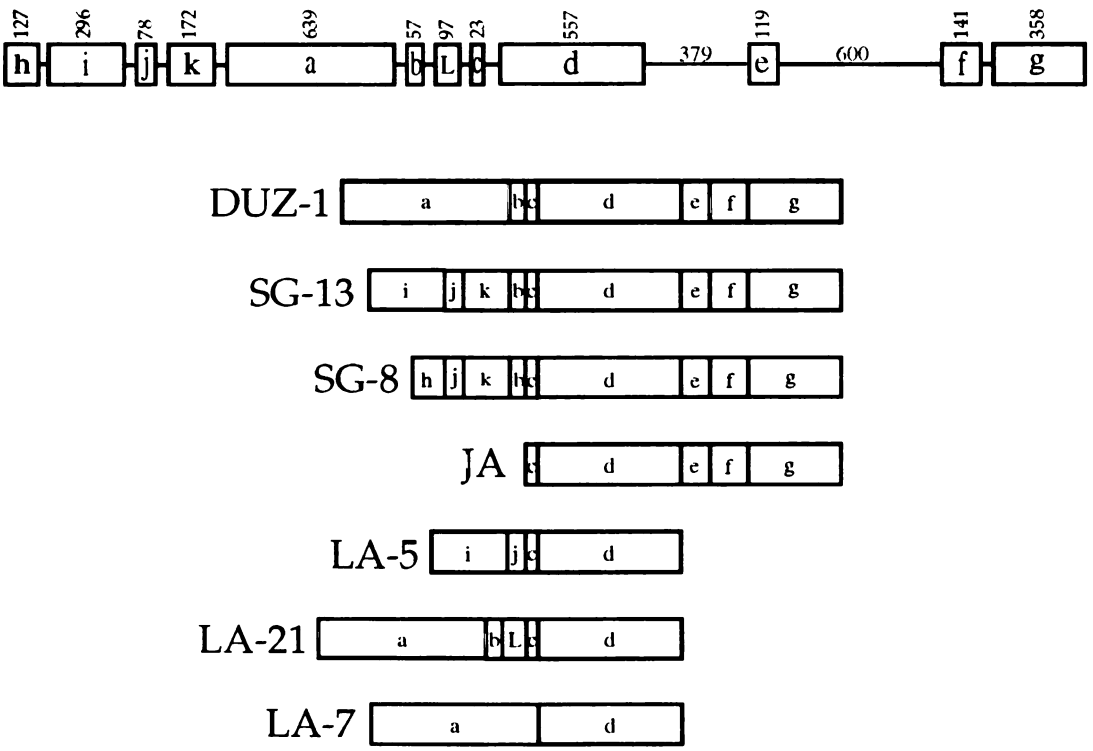
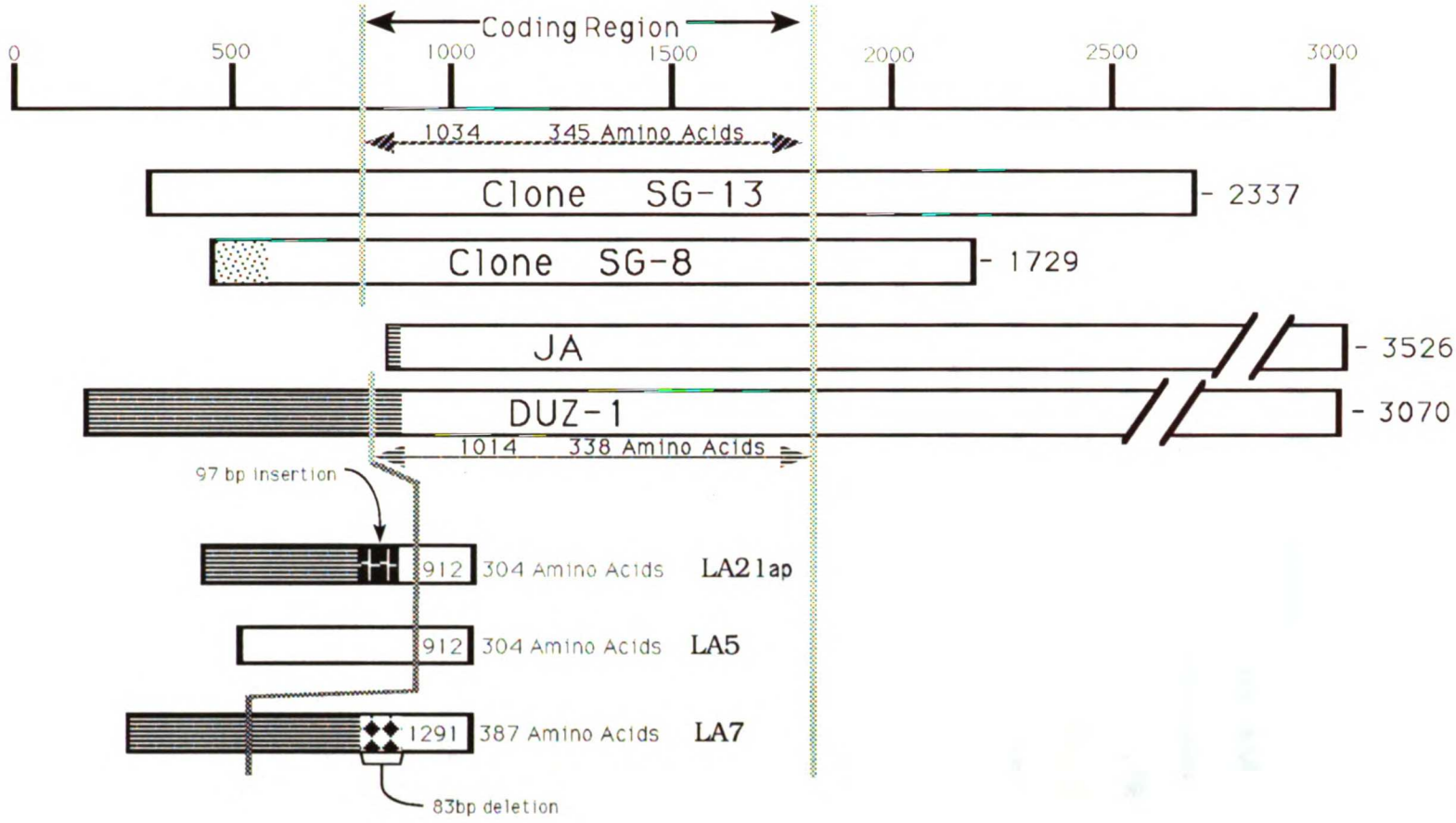


Figure 13

Coding Regions of OBCAM Family



DISCUSSION

The Relationship of OBCAM to Opioid Receptor Function

The purpose of this project was to characterize OBCAM, an opioid binding protein purified in our laboratory by a combination of opioid ligand and lectin affinity chromatography (Cho et al. 1985), then subsequently cDNA cloned (Schofield et al. 1989). At the time the project was begun, several different lines of evidence pointed to a role of OBCAM in opioid receptor function. These included: 1) both monoclonal and polyclonal antibodies to OBCAM, as well as polyclonals to peptides corresponding to portions of its predicted reading frame, inhibited binding to purified OBCAM as well as to opioid receptors in brain membranes (Roy et al. 1988); 2) one of the peptide antibodies, anti-MN-3, was shown by a combination of immunofluorescence and confocal microscopy to bind to the surface of NG108-15 neuroblastoma x glioma cells and to be specifically down-regulated by treatment of these cells with opioid agonist; the time course and δ-opioid specificity of this down-regulation corresponded quite closely to the down-regulation of opioid receptors on these cells observed during chronic opioid agonist treatment (Lane et al. 1992); and 3) transfection of NG108-15 cells with an antisense portion of OBCAM cDNA resulted in an 80% decrease in opioid binding to these cells (Ann et al. 1992), and uptake by cells of short, antisense oligomers to OBCAM cDNA partially inhibited recovery of opioid binding following down-regulation induced by chronic opioid agonist treatment (J.L. Ko, unpublished data).

Despite these suggestive lines of evidence, direct proof of OBCAM's role in opioid receptor function, such as would be offered by expression of OBCAM and opioid binding in a eukaryotic cell line, could not be obtained. In addition, there were theoretical reasons, based on the predicted amino acid sequence of OBCAM, for doubting that it could be the opioid receptor. Opioid receptors, like many other cell surface receptors, have been shown to be coupled to G-proteins, which mediate their biochemical effects, including inhibition of adenylyl cyclase (Griffin et al. 1985), effects on ion channels (North et al. 1987), and more recently shown, stimulation of phosphodiesterase (Law and Loh 1993). Thus opioid binding in all regions of the central nervous system examined is sensitive to guanine nucleotides (Abood et al. 1985; Blume 1978) and in intensively studied systems such as NG108-15 neuroblastoma x glioma hybrid cells, other criteria, such as opioid stimulation of low Km GTPase (Harada et al. 1989) are fulfilled. With very rare exceptions, G-protein coupled receptors have a very characteristic type of primary structure, featuring seven regions of hydrophobicity thought to pass through the cell membrane (Bourne et al. 1991). OBCAM is very different from this structure. Instead, it is similar to another class of cell surface receptors, those activating tyrosine kinase, which consist of an extracellular region with repeating motifs such as immunoglobulin, fibronectin or cytokine domains; a single transmembrane region; and an intracellular domain containing the tyrosine kinase. Though recent evidence suggests that some tyrosine kinase receptors can in fact interact with G-proteins (Bauer et al. 1992), this interaction is thought to be more indirect, perhaps being

mediated by a phosphorylated product of the kinase, rather than by direct association of the receptor with a G-protein. Furthermore, OBCAM does not even appear to belong to this class of receptor, since it lacks a putative transmembrane region and intracellular portion.

Well after this project was under way, the first reports of successful cloning of opioid receptors appeared (Yasuda et al. 1993, Evans et al., 1992, Li et al., 1993), which confirmed that these receptors are indeed members of the G-protein coupled superfamily. The cloned sequences, upon transfection into eukaryotic cells, expressed receptors that not only bound opioid ligands in a manner fulfilling classical pharmacological criteria such as stereoselectivity, high affinity, and rank order, but could inhibit adenylyl cyclase, a second messenger system coupled to opioid receptors in parts of the mammalian central nervous system (Law et al. 1981, Attali et al. 1989; Cooper et al. 1982) and in some cell lines (Klee et al. 1975, Puttfarcken and Cox 1989; Yu et al. 1990). These results have established the identity of opioid receptors, and out-dated reports by numerous other laboratories of purifying these receptors.

Nevertheless, the substantial evidence indicating that OBCAM plays a role in opioid receptor function was felt by our laboratory to warrant further pursuit. Indeed, further evidence for this was obtained subsequent to the cloning of opioid receptors, for in an extension of the immunofluorescence studies, it was observed that anti-MN-3 reacted with cells that had been transfected with opioid receptor cDNA, but not with cells transfected with vector alone, or with cells transfected with cDNA for another, non-opioid receptor (C. M. Lane, et al., manuscript in preparation). Though it might not

actually bind opioids, it might still play a role in signal transduction, perhaps by regulating opioid receptor interaction with G-proteins. A relatively recent publication from our laboratory provided support for this conclusion, by demonstrating that the OBCAM antisense-transfected cells previously shown to have reduced opioid binding (Ann et al. 1992) also exhibited reduced affinity for G-proteins, and reduced ability to inhibit adenylyl cyclase (Govitrapong et al. 1993). Even this role, however, seemed difficult to reconcile with a purely extracellular molecule. The α subunit of G-proteins, which is thought to associate directly with receptors (Bourne et al. 1991), is completely intracellular, as are second messengers such as adenylyl cyclase.

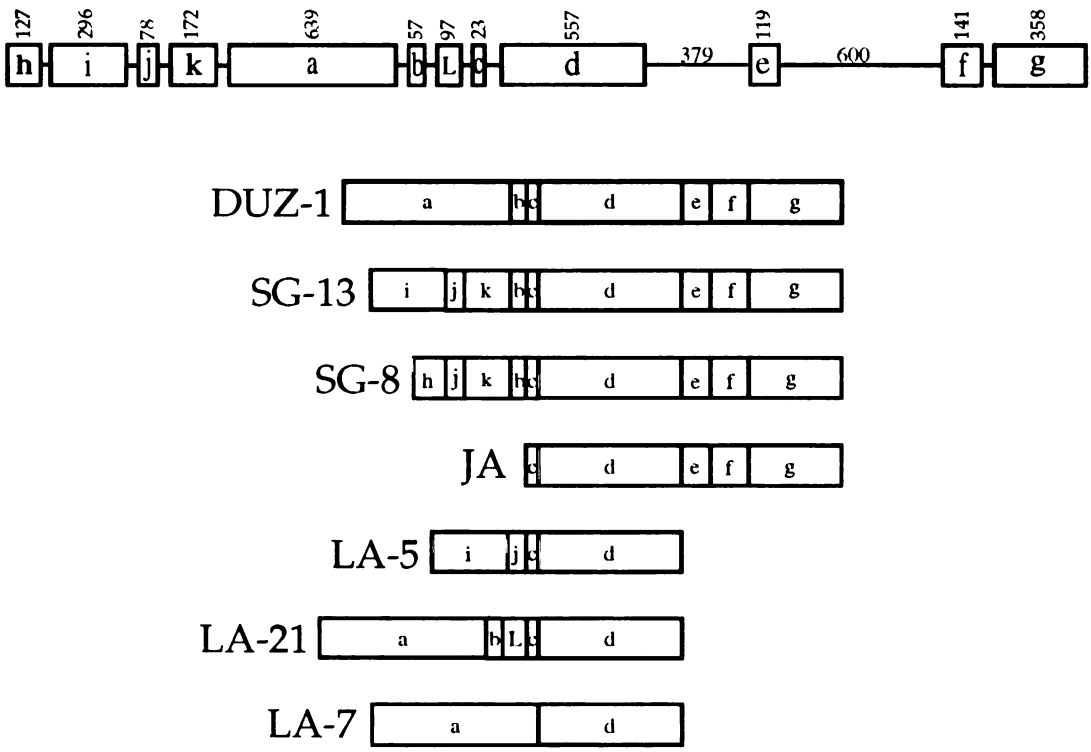
One possibility, which formed the rationale for the first portion of research in this project, was that another version of OBCAM existed which possessed a transmembrane region and cytoplasmic domain. There is precedent for this notion, because other cell surface receptors with a transmembrane region and cytoplasmic portion, including several consisting of Ig domains in their extracellular domains, have been reported to exist in alternative, truncated forms in which only the extracellular region remains (Cunningham et al. 1987). Though the function of these truncated receptors is not clear, it has been suggested that they may play a scavenger role, removing excess ligand from the vicinity of the receptor (Mosley et al. 1989). With this possibility in mind, we began by systematically screening cDNA libraries for other versions of OBCAM.

A Family of OBCAM-like Proteins

As discussed in the Results section, we found no evidence for OBCAM-like clones with an extended C-terminus. We were able to identify and sequence, however, several new OBCAM-like clones that differed from the original sequence at the 5' end, including one, DUZ-1, with a different sequence of amino acids at the N-terminus. This result suggests that our search was thorough, and that we can probably safely conclude that no clones extended at the C-terminus exist, at least not in the libraries that we screened. However, the existence of clones differing at the 5' end is interesting in itself. Furthermore, while this work was being carried out, another researcher in our group, using 5'-RACE, a method which identifies messages of a particular sequence that are extended at the 5' end, also isolated several new OBCAM-like clones (L. Augustin, unpublished results). Two of these clones contained a gap in the unique region--that is, the region in which our clones differ from each other--while a third contains an insertion. It thus appears that there is an intron in this region, with alternate splicing accounting for the existence of these sequence variations.

Figure 12

Intron-Exon Map of OBCAM Gene



Function of OBCAM-like Clones

The functional roles of these different clones remain to be elucidated. In some cases, such as is illustrated by comparison of DUZ-1 vs. SG13, the difference in amino acids is confined to a short hydrophobic sequence at the N-terminus, and is therefore likely to reflect simply a signal sequence. This difference could imply a difference in processing or insertion into the membrane of the respective products, though the final product itself would be the same. In the case of the clones with gaps or insertions present, they may reflect differences in function of the OBCAM molecule.

As a preliminary attempt to gain insights into the function of these clones, and in particular to determine whether they have a direct role in opioid actions, we decided to create a line of mice transgenic for an antisense portion of DUZ-1. In theory, such mice should have expression of the DUZ-1 product specifically blocked, thus providing a clue to the function of that product. As discussed in the Results, pharmacological studies of the founders as well as the N1 and N2 generations of these transgenic mice suggested a correlation between the presence of the transgene and a reduced sensitivity to opioid antinociception. Thus, use of two different tests to determine opioid sensitivity strengthened the conclusion of a correlation between the presence of a transgene and reduction in the sensitivity to opioids. Ideally, we would have liked to test these animals for their sensitivities to other drugs, but because the preparation of transgenic animals requires relatively long periods of time to obtain

even a few animals, and because pharmacological assays require fairly large numbers of animals, this has not been possible. For the same reason, we have not even been able to test the animal's sensitivity to different opioids, or to do more extensive studies on their development of tolerance and dependence. When more mice are available in later generations, this may be possible.

More recently, further evidence for the role of these clones in opioid binding was provided by another member of our research group, Dr. A. Kalyuzhny. An antibody was prepared to a portion of the unique N-terminal amino acid sequence of DUZ-1 and shown to map the entire brain of ICR mice. The uniform distribution of DUZ-1 immunoreactivity in brain resembled that of one common region of OBCAM. However, expression of DUZ-1 antigen in dorsal root and trigeminal ganglia was different: small neurons in sensory ganglia, shown to function in pain transmission, had higher optical density of staining in comparison with large ones. Preliminary experiments revealed the coexpression and overlapping of DUZ-1 and DOR (delta opioid receptor clone) immunoreactivity in neonatal rat DRG neurons cultured in vitro: DOR immunoreactivity was evenly distributed on cell surface while DUZ-1 displayed patch-like pattern. Both DOR and DUZ-1 immunoreactivity seemed to be reduced after chronic administration of morphine. However, further experiments are necessary to figure out the profile of morphine activity on DOR and DUZ-1 immunoreactivity.

In summary, these studies provide further evidence for the role of OBCAM-like proteins in opioid receptor function, and of DUZ-1 in particular. Further work will be necessary to understand exactly

how OBCAM may interact with opioid receptors, but the availability of both OBCAM cDNA probes and OBCAM antibodies provides the tools for doing this. Moreover, studies in our lab of the effect of OBCAM antisense on opioid receptor-G-protein coupling point to a promising system to explore further.

Chromosome Mapping and Genomic Sequencing

Chromosome mapping of the OBCAM clones was carried out as a matter of basic interest, but also in the hopes that it might clarify the relationships of these different clones. Should they have proved to be located on different chromosomes, we could conclude that each OBCAM variety was encoded by a different gene.

As it turned out, these genes were all localized to a single region of a single chromosome, 9. This result is unhelpful, except that it is consistent with the conclusion that all of the OBCAM-like sequences that we and others have identified derive from a single gene, and are created by alternative splicing. To settle this issue firmly, as well as to obtain further characterization of these genes, and perhaps find still others, it was necessary to sequence the entire OBCAM genome.

Genomic sequencing is a daunting, time- and labor-consuming task, and our preliminary results indicated it would be especially challenging with OBCAM, for we initially obtained by screening three clones, covering a total of nearly 36 kb. Through systematic restriction digestion and subcloning of these clones, we were able to isolate and sequence large portions of these three original clones, and

to put some of these sequences together. However, it soon became clear, by comparison of the available sequences with contig-construction software, that very large gaps remained, even after nearly 25 kb had been sequenced. It was at this point that we turned to the transposon-based method of sequencing, described in the Methods section.

Using this approach, we have sequenced an additional 25 kb of DNA. Though we still have not defined the entire OBCAM gene, as shown in the Results, we have identified most of the introns. There are clearly a great many, resulting in an enormous amount of work to sequence through the genome. Figure 14 shows the position of the introns within the amino acid sequence of OBCAM. The most N-terminus of these corresponds to the point at which DUZ1 and SG8/SG13 differ, while the most C-terminus one is located between the 3rd Ig domain and the C-terminus tail of the receptor. The N-terminus intron therefore allows for splicing of different signal sequences, while the C-terminus may possibly allow splicing of longer, cytoplasmic regions, even though we were unable to find evidence of these. Another intron is located between Ig domains 2 and 3 and suggests that there maybe OBCAM molecules with either only 2 such domains, or with a 3rd domain different from the domain 3 in the known OBCAM molecules. Introns between Ig domains are well established in other Ig molecules.

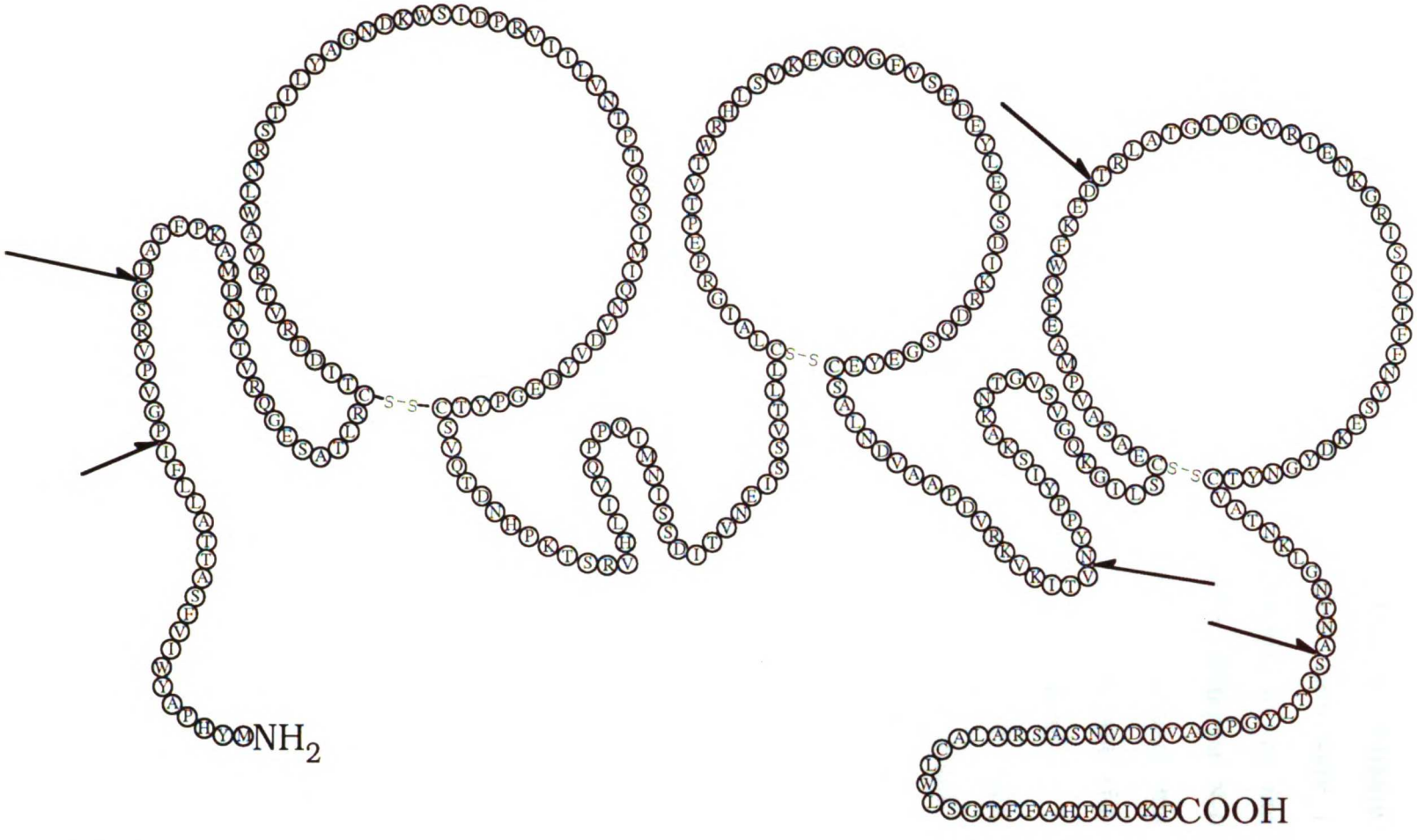


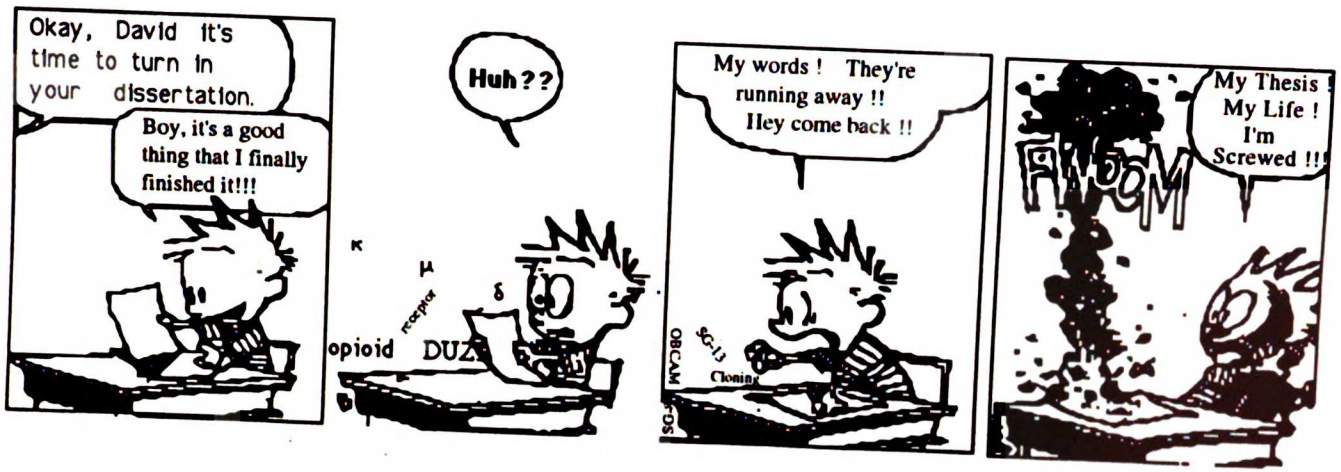
Figure 14

Exon Splice Sites In DUZ1 Protein Sequence

The location of the other 2 introns is more surprising. One of them would splice out half of the 3rd Ig domain, making it functionally useless unless a 2nd equivalent half were to be spliced in. As for the other, an intron just C-terminal to the most N-terminal intron could be a splice site for an entirely different N-terminus.

In addition to these introns, there are several more 5' to the reading frame (see Fig. 12, Results). These do not affect the amino acids sequence, but may be important in regulation.

Though we sequenced approximately 1 Kb of DNA upstream of the cDNA, we could find only 2 possible promoter sides, an AP-2 site in position 155 and a NF-IL6 at position 137. Because these sites are relatively short, they would be expected to occur by chance every couple thousand nucleotides or so, and they not represent real promoters. Their function would have to be confirmed by transferring them to a reporter vector and measuring their ability to promote a reporter gene.


Okay, David it's time to turn in your dissertation.
Boy, it's a good thing that I finally finished it!!!
Huh??
κ
μ
δ
opioid
DUZ
My words ! They're running away !!
Hey come back !!
SC-13
Cloning
OBCAM
FDS
KWOOM
My Thesis ! My Life ! I'm Screwed !!!

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APPENDIX

1) Reagent List
2) Solution Recipes

Reagents	Company	Grade	Item #
2-Mercaptoethanol	Fisher	Reagent	BP176-100
Acetic Acid	EM	Reagent	AX0073-9
Acrylamide	Bio-Rad	Reagent	161-0144
Agarose	Gibco-BRL	Molec. Bio.	5510UB
Ammonium Persulfate	Bio-Rad	Reagent	161-0700
Ammonium Acetate	Fisher	Enzyme	BP326-1
Bisacrylamide	Bio-Rad	Electrophoresis	161-0201
Boric Acid	Fisher	Reagent	BP168-500
Bromophenol Blue	Bio-Red	Electrophoresis	M6309
Butanol	Fisher	ACS	A399-1
Calcium Chloride	Sigma	Reagent	C-1016
Cesium Chloride	Fisher	Ultr Centrofuge	BP210-5--
Chloroform	Mallinckrodt	Reagent	4440
Diethyl Ether	Fisher	Reagent	E136-1
EDTA (di sodium)	Sigma	Sigma Grade	ED4SS
Ethanol	Storehouse	Reagent	
Ethidium Bromide	Sigma	Reagent	E7-637
Ficoll	Pharmacia	Reagent	7-0400-01
Formamide	BRL	Reagent	5515UB
Glycerol	Sigma	Reagent	G-9012
HEPES	USB	Reagent	16926
Hydrochloric Acid	EM	GR	HX0603-1
IPTG	Gold Biotechnology (isopropyl-b-thiogalactopyranoside)	Molec. Bio.	12481C
Isoamyl Alcohol	Fisher	Reagent	BP1150-500
Isopropyl	Storehouse	Reagent	
Low Melt Agarose	BRL	Reagent	5517UB
Magnesium Chloride	Sigma	Reagent	104-20
Magnesium Sulfate	Fisher	ACS	M63-500
Methanol	EM	GR	MX0485-7
MOPS	Sigma	Molec. Bio	M-8899
N,N-Dimethyl Formamide	Fisher	ACS	D119-1
Nusieve Agarose	FMC	Molec. Bio.	50082
Phenol (saturated/acidified)	Amresco	Reagent	0981
Polyethylene Glycol	USB	Molec. Bio.	19959
Potassium Acetate	Fisher	Enzyme	BP364-500
Rubidium Chloride	Sigma	Reagent	R-2252
Sodium Chloride	Fisher	Enzyme	BP358-212

Sodium Citrate	Fisher	Enzyme	BP327-1
Sodium Hydroxide	MCB	Reagent	SX0590-3
Sodium Dodecyl Sulfate	Fisher	Electrophoresis	BP166-500
Sodium Acetate	Sigma	Reagent	S-8750
Sucrose	Fisher	ACS	S5-3
TEMED	Bio-Rad	Reagent	161-0601
	(N,N,N',N',-tetramethylethylenediamine)		
Triton X-100	Sigma	Reagent	T-6878
Trizma Base	Sigma	Reagent	T-4253
Urea	Fisher	Electrophoresis	BP169-212
X-Gal	Gold Biotechnology	Molec. Bio.	X4281C
	(5-Bromo-4-Chloro-3-Indolyl-ß-D-Galactoside)		
Xylene Cyanol	Sigma	Reagent	X2751
Sucrose	Fisher	Reagent	

Other than Restriction Endonucleases:

1 Kb Ladder	Gibco-BRL	Reagent	15615-016
100 bp Ladder	Bio Ventures*	Reagent	
Bal 31 Nuclease	Uniter States Biochemical	Reagent	70011
Calf Intest.Alk.Phosphatase	USB	Reagent	70034
DNAse	Promega	Reagent	1000U
Exo III Nuclease	Strategene	Reagent	
Klenow	Uniter States Biochemical	Reagent	70240
Mung Bean Nuclease	Stratagene	Reagent	
Polynucleotide Kinase	Uniter States Biochemical	Reagent	
Proteinase K	Boehringer Mannheim	Reagent	745723
RNAse	Strategene	Reagent	
RNAse Inhibitor	Fisher	Reagent	
S1 Nuclease	Boehringer Mannheim	Reagent	
Shrimp Alkaline Phosphatase	USB	Bio. Tech.	70092
T-4 Ligase	Stratagene	Reagent	600012
Taq DNA Polymerase	Perkin-Elmer	Reagent	

*Bio Venture, Inc.
848 Scott St.
Murfree sboro TN 37129

Media Reagents

Agar	Diffico	Reagent	0140-01
Casamino Acids	Diffico	Reagent	0230-01-1
Circle Grow	Bio 101	Bio-Tech	CGB-1
Gelatin	Fisher	Reagent	
NZ Amine	Diffico	Reagent	1012901
Tryptone	Diffco	Reagent	0123-01-1
Yeast Extract	Diffico	Reagent	0127-01-7
Gelatin	Fisher	Reagent	

Misc.

Membranes

Nitrocellulose 85cm Circles	S & S	Reagent	20600
Nitrocellulose 105cm Circles	S & S	Reagent	
Hybond N^+	Amersham	Reagent	RPN20208

Radionucleotides

a^{32}-P dCTP	Amersham	Reagent	PB10205
a^{35}-S dATP	Amersham	Reagent	SJ1304
a^{32}-P UTP	Amersham	Reagent	PB10163
g^{33}-P dATP	Amersham	Reagent	BK1000
a^{33}-P dCTP	NEN	Reagent	

Whatman 3mm Paper	Whatman	Reagent	3030917
GlassMilk	Bio 101	Reagent	3113

SOB

For 1 Liter

AUTOCLAVE 25 min

Tryptone	20 g
Yeast extract	5 g
NaCl	600 mg
KCl	186 mg

AUTOCLAVE

when cooled to room temperature;

Make a stock solution of 1M $MgCl_2$ PLUS 1M $MgSO_4$; add 10 ml

Make a 2M Glucose solution (filter sterilize) ; add 10 ml

LB

For 1 Liter

AUTOCLAVE 25 min

Yeast extract	5.0g
Tryptone	10.0g
NaCl	2.5g

QS to 1 liter
adjust pH to 7.4 with about 8 drops of 10N NaOH
Autoclave

NZcym

For 1 Liter

AUTOCLAVE 25 min

10 g	NZ media
5 g	yeast extract.
8.0 g	casamino acid
5.0g	NaCl

QS to 1L
Autoclave
When cool add 2.0 g $MgSO_4$

RF2

For 1 Liter

MOPS	20ml of a 0.5M Stock pH 6.0
$RbCl_2$	1.2 g
$CaCl_2$-$2H_20$	11 g
Glycerol	150 g

Bring volume to 900 ml adjust pH to 6.8 with NaOH -
QS to 1 L Filter sterilize through a 0.22 uM filter

Terrific Broth

For 1 Liter

AUTOCLAVE 25 min

Bacto-tryptone	12 g
Bacto-yeast extract	24 g

Glycerol	4 ml
Gelatin	5 g

Bring volume up to 900 ml ,autoclave .

Add 100 ml 0.17M KH_2PO_4/0.72M K_2HPO_4 Just before using.

0.17 M KH_2PO_4 /0.72 M K_2HPO_4

For 1 Liter

AUTOCLAVE 25 min

KH_2PO_4	23.1 g
K_2HPO_4	125.4 g

QS to 1000 ml Autoclave

HY Hybridization Buffer

For 1 Liter

H_2O	520ml
0.5M EDTA	20 ml
10% Sodium Pyrophosphate	10 ml
50x Denhardts reagent	100 ml
20x SSC	300ml
10% SDS	50ml

Top Agarose

For 1 Liter

AUTOCLAVE 25 min

Hi melt agarose (5510 uB)	3 g
Low melt agarose (5517 uB)	4 g
NZ broth	22 g
Cas Amino acids	5 g

autoclave in 100 ml volumes -20 min. ,then
To each bottle after autoclaving add 0.2 g $MgSO_4$

PEG solution

For 500ml

PEG 8000	100 g
NaCl	61 g
$MgSO_4.7\ H_2O$	1 g
1 M Tris, pH 8.0	25 ml

QS to 500 ml with H_2O

20X SSC

For 20 Liter

NaCl	3506 g
Sodium Citrate	1764 g

adjust volume to 20 liters

1 Kb Ladder (BRL)

TE	75 µl
Tracking dye	35 µl
Ladder	15 µl

Use 3λ for average gel

10% SDS

For 1 Liter

SDS 100 g
Adjust volume to 1 L with H_2O
Filter sterilize if necessary

TE

For 1 Liter
AUTOCLAVE 25 min

1 M Tris pH 7.6	10 ml
0.5 M EDTA pH 8.0	2 ml

0.2 N NaOH / 1 % SDS

For 100ml

NaOH	0.8 g
H_2O	80 ml
Dissolve NaOH first, then add	
10 % SDS	10 ml

Adjust volume to 100 ml

3 M NaOAc pH 5.2

For 1 Liter
AUTOCLAVE 25 min

NaOAc . 3 H_2O	408.1 g
H_2O	600 ml

Adjust pH to 5.2 with Glacial Acetic Acid
QS to 1 liter with H_2O

50X TAE

For 1 Liter

Trizma Base	242 g
Glacial Acetic Acid	57.1 ml
0.5 M EDTA, pH 8.0	100 ml

QS to 1 liter

Sequencing Gel

Urea	57.8 g
Ammonium persulfate	50 mg
40 % acrylamide	15 ml
5x TBE	24 ml
H_2O	60 ml

Dissolve, Degas, filter through a Whatman GF/B filter, add 60 λ TEMED, pour immediately using a silinized 50ml glass pipette

7.5 M NH_4OAc

For 500ml

FILTER STERILIZE IF NECESSARY !

NH_4OAc	500 g
H_2O	417 ml

OR Add 417ml H_2O to a new 500 g bottle of NH_4OAc

5x PEG

For 600ml

PEG 8000	207 g
Dextran Sulfate	6 g
NaCl	49.5 g
H_2O	350 ml

SS DNA (10 mg/ml)

For 30ml

AUTOCLAVE 25 min

H_2O	30 ml
Salmon Sperm DNA	300 mg

Pass numerous times through a 20 gauge needle syringe
Autoclave, pass through a 20ga needle again and aliquot in 1 ml
Sarstedt tubes, then store at -20 C

Ten 9

For 100ml

AUTOCLAVE 25 min

0.1 M Tris, pH 9.0	50 ml
0.5 M EDTA	20 ml
NaCl	0.116 mg

QS to 100ml and autoclave

Tracking Dye

For 15ml

Bromophenol blue	25 mg
Xylene cyanol FF	25 mg
Ficoll	1.5 g
H_2O	7.5 ml
Glycerol	1.0ml

Aliquot into 1.5ml tubes (1 ml dye/100 µl EtBr) AUTOCLAVE 25 min

GTE

For 500ml

Filter Sterilize

Glucose (Dextrose)	8 g
H_2O	250 ml
1 M Tris, pH 8.0	12.5 ml
0.5 M EDTA	10 ml

QS to 500 ml with H_2O

40 % Acrylamide

For 500ml

Acrylamide	190 g
Bis- Acrylamide	10 g
H_2O	300 ml

Mixed bed resin Bio Rad AG501x8 10 g

After adding H_2O, dissolve and adjust volume to 500 ml
Add Resin and mix for 30 min filter, store in brown glass bottle at 4°C

10% Sodium Pyrophosphate

For 100ml

Filter Sterilize

Sodium Pyrophosphate 10 g

QS to 100 ml with H_2O.

Denhardt's Reagent

For 500ml

Ficoll (type 400)	5 g
Polyvinyl pyrrolidone	5 g
Bovine serum albumin	5 g

QS to 500 ml with H_2O

TBE

For 20 Liter

Trizma Base	216 g
Boric acid	110 g
$(Na)_2$ EDTA 1	8.6 g

Mix into 3L H_2O, then QS to 20 liters

SM

For 1 Liter

AUTOCLAVE 25 min

NaCl	5.8 g
$MgSO_4$. 7 H_2O	2 g
1 M Tris, pH 7.6	50 ml
2 % gelatin (0.2 g/10 ml H_2O)	5 ml

Autoclave in 100 ml volumes

Indicating Plates

For 1 Liter

IPTG	460mg in 1.5 ml H_2O
X-Gal	80 mg in 1ml of DMF

Add this to any plate media at the same time antibiotics are added.